A mouse model for MERS coronavirus-induced acute respiratory distress syndrome

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Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel virus that emerged in 2012, causing acute respiratory distress syndrome (ARDS), severe pneumonia-like symptoms and multi-organ failure, with a case fatality rate of ∼36%. Limited clinical studies indicate that humans infected with MERS-CoV exhibit pathology consistent with the late stages of ARDS, which is reminiscent of the disease observed in patients infected with severe acute respiratory syndrome coronavirus. Models of MERS-CoV-induced severe respiratory disease have been difficult to achieve, and small-animal models traditionally used to investigate viral pathogenesis (mouse, hamster, guinea-pig and ferret) are naturally resistant to MERS-CoV. Therefore, we used CRISPR-Cas9 gene editing to modify the mouse genome to encode two amino acids (positions 288 and 330) that match the human sequence in the dipeptidyl peptidase 4 receptor, making mice susceptible to MERS-CoV infection and replication. Serial MERS-CoV passage in these engineered mice was then used to generate a mouse-adapted virus that replicated efficiently within the lungs and evoked symptoms indicative of severe ARDS, including decreased survival, extreme weight loss, decreased pulmonary function, pulmonary haemorrhage and pathological signs indicative of end-stage lung disease. Importantly, therapeutic countermeasures comprising MERS-CoV neutralizing antibody treatment or a MERS-CoV spike protein vaccine protected the engineered mice against MERS-CoV-induced ARDS.

The severity of respiratory illness caused by Middle East respiratory syndrome coronavirus (MERS-CoV), its pandemic potential through human-to-human respiratory transmission and a dearth of effective treatments necessitate the development of new MERS-CoV therapies and vaccines. Effective vaccine and therapeutic development require preclinical animal models that resemble the pathogenesis of human MERS-CoV infection. Additionally, these models should: (1) include a measure of mortality associated with severe respiratory disease; (2) not be confounded by neurological complications due to high viral loads in the brain; (3) exhibit sustained, high-level virus replication within the lungs of infected animals; (4) exhibit lung pathology associated with human acute respiratory distress syndrome (ARDS); (5) maintain innate expression of the MERS-CoV host receptor, dipeptidyl peptidase 4 (DPP4), to prevent perturbation of immunological homeostasis; (6) be genetically tractable to study host genes that regulate responses to MERS-CoV vaccines and therapeutics; and (7) exhibit reproducibility.

Conventional non-human primate (NHP) models have been established for MERS-CoV in both the rhesus macaque and common marmoset1–4. NHPs are instrumental for the preclinical development of therapeutics; however, these models are cost-prohibitive for initial screening of large numbers of vaccine and therapeutic candidates, challenging to work with for routine pathogenesis studies, limited in availability and typically require high virus challenge doses into multiple sites. Furthermore, two recent studies have contradicted the initial studies in NHPs, which may complicate use of the rhesus macaque5 or the common marmoset6 model for routine vaccine or therapeutic testing.

MERS-CoV fails to replicate in traditional small-animal models (mouse, hamster, guinea-pig and ferret) due to the inability of the receptor-binding domain in the MERS-CoV spike protein to interact with the respective DPP4 receptor7–10. In addition to acting as the MERS-CoV receptor, DPP4 regulates T-cell activation, cytokine function and trans-endothelial migration to sites of inflammation11. Therefore, overexpression of DPP4 may result in immune dysregulation. Effective models would therefore ideally promote functional MERS-CoV/DPP4 interactions, with minimal perturbations of innate DPP4 expression, signalling activity or tissue distribution. Classical strategies to overcome receptor incompatibilities to generate susceptible mice have relied on generalized or tissue-specific transgenic overexpression approaches to drive expression of the human receptor (hDPP4) in the mouse12–15. Although MERS-CoV can elicit respiratory disease in hDPP4 overexpression models, these models exhibit a fatal central nervous system (CNS) and systemic multi-organ disease12–14, probably due to non-specific overexpression of the receptor throughout the animal, which complicates the study of MERS-CoV-induced respiratory pathogenesis in these models.

In this study, we used our knowledge of which determinants allow mouse DPP4 to act as a functional MERS-CoV receptor7 by using CRISPR–Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated gene 9) genome editing technology to insert codons that match the human sequence at positions 288 and 330 in the mouse Dpp4 gene. This strategy resulted in a mouse that is permissive for MERS-CoV infection, while maximally preserving the species-specific interaction networks critical for DPP4 immune function. Generation of mice

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carrying a chimaeric mouse DPP4 (mDPP4) molecule (A288L/T330R), combined with a mouse-adapted strain of MERS-CoV, allowed us to generate a mouse model that resembles severe MERS-CoV-induced respiratory disease without bystander neurological disease. In parallel, we demonstrated that this model system can be used for the development and testing of MERS-CoV vaccines and therapeutics.

Results

A CRISPR–Cas9-generated mouse model for MERS-CoV infection. We have demonstrated previously that the introduction of two amino acids that match the human sequence at positions 288 and 330 in the mDPP4 receptor can support MERS-CoV docking, entry and replication in cell culture. These determinants are located within exons 10 and 11 of mDPP4 on chromosome 2 (Fig. 1a and Supplementary Fig. 1). Therefore, we used CRISPR–Cas9 genome editing to introduce these determinants (A288L and T330R) into the mDPP4 receptor (Fig. 1a and Supplementary Fig. 1). Two lines of C57BL/6J-derived mice were generated that were either homozygous (288/330+/+) or heterozygous (288/330+/−) for the chimaeric mDPP4 alleles (Fig. 1a). The 288/330+/− mice were genotyped as either homozygous (288/330+/+) or heterozygous (288/330+/−) for the chimaeric mDPP4 alleles (Fig. 1a). The 288/330+/− mice were genotyped as either homozygous (288/330+/+) or heterozygous (288/330+/−) for the chimaeric mDPP4 alleles (Fig. 1a). The 288/330+/− mice were genotyped as either homozygous (288/330+/+) or heterozygous (288/330+/−) for the chimaeric mDPP4 alleles (Fig. 1a). The 288/330+/− mice were genotyped as either homozygous (288/330+/+) or heterozygous (288/330+/−) for the chimaeric mDPP4 alleles (Fig. 1a).

Overall expression levels, expression patterns, biological function and therapeutic potential of the 288 and 330 alleles does not alter basal T-cell activation status. This is consistent with the lack of detectable differences in basal CD4+ T-cell expression patterns in the lungs, kidneys and brains of 288/330+/+ and 288/330+/− mice (Supplementary Fig. 3). Notwithstanding functional T-cell assessment, these results suggested that minimal alteration of the 288 and 330 alleles.

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single amino acid change (S885L) in the S2 region of the spike gene. This recombinantly derived virus, MERS-0, encoding the RMR and S885L S2 mutations, demonstrated significantly enhanced replication both in cell culture (Supplementary Fig. 4) and in the lungs of 288/330+/+ and 288/330+/- mice (Fig. 1d; P<0.05). Despite replicating to significantly higher virus titres in vivo than the other isolates, MERS-0 exhibited no evidence of severe clinical disease symptoms (Supplementary Fig. 4). Lung histology demonstrated that nucleocapsid antigen from MERS-0 (Fig. 1e), and from the other strains (not shown), was readily detected in the lungs of infected mice by immunohistochemistry, but infected lungs exhibited only moderate signs of respiratory pathology and inflammation.

These results demonstrated that we had developed a MERS-CoV model that could support high levels of virus replication up to day 3 post-infection (p.i.), but that further in vivo adaptation was required to achieve the respiratory symptoms characteristic of MERS-CoV infection in humans.

Mouse adaptation of MERS-CoV induces severe ARDS-like disease. The recombinantly derived MERS-0 virus was mouse adapted by serial passage for 15 rounds through the lungs in 288/330+/- mice at 3-day intervals, resulting in the MERS-15 strain. Infection of 288/330+/+ mice via the intranasal route with MERS-15 resulted in ∼70% mortality (genuine mortality, rather than mice meeting the typical 20% weight loss cut-off associated with humane euthanasia criteria), while 100% of infected 288/330+/- mice survived (Fig. 2a). However, both lines exhibited 20–25% weight loss by day 6 p.i., in contrast to MERS-0-infected 288/330+/- mice or MERS-15-infected C57BL/6J wild-type mice, which exhibited no weight loss (Fig. 2b). Significantly higher levels of MERS-15 replication were detectable in the lungs of both 288/330+/- and 288/330+/- mice at days 3 and 6 p.i., while MERS-0 was mostly cleared from the lungs by day 6 p.i. (Fig. 2c,d). Similar to MERS-0, MERS-15 did not replicate in C57BL/6 wild-type mice. Importantly, the observed decreases in survival and weight loss
induced by MERS-15 were not confounded by neurological complications from brain infection, as plaque assays for replication-competent virus and PCR with reverse transcription (RT-PCR) at days 3 and 6 p.i. were negative (Supplementary Fig. 5). Moreover, quantitative RT-PCR on the same samples demonstrated an increase of $>10^6$ detectable viral transcripts in infected lungs compared with similarly infected C57BL/6J mice, with no detectable viral transcripts in the brains of these mice (Supplementary Fig. 5c).

Although mortality and weight loss provide important measures of MERS-CoV-induced disease, these parameters do not directly assess the impact of virus replication on respiratory function. Therefore, to directly assess the impact of MERS-15 infection on respiratory function in 288/330+/+ and 288/330+/− mice, we measured respiratory function using unrestrained plethysmography, as demonstrated previously for respiratory pathogenesis in mouse models of severe acute respiratory syndrome (SARS) and influenza. MERS-15 elicited severe lung disease as quantified by enhanced pause (Penh), a unitless measure that reflects airway obstruction/restriction due to debris in the airway, and midtidal expiratory flow (EF50), which represents the flow rate at which 50% of the tidal volume has been expelled in a single breath. MERS-15 infection led to significant increases in both Penh and EF50 in 288/330+/+ and 288/330+/− mice up to day 6 p.i. compared with 288/330+/− mice infected with MERS-0 and C57BL/6J wild-type mice infected with MERS-15 (Fig. 3a,b), demonstrating that MERS-15 elicited severe respiratory distress in mice carrying the chimaeric DPP4 receptor. This was further supported by our observation of severe haemorrhage in the lungs of both 288/330+/+ and 288/330+/− mice infected with MERS-15 at days 3 and 6 (Supplementary Fig. 6), inflammatory infiltrates by day 3 (Fig. 3c) and respiratory pathology associated with severe acute respiratory distress, including hyaline membrane formation, intra-alveolar oedema, perivascular cuffing and severe inflammation, at day 6 p.i. (Fig. 3d). Quantitative comparison of the lung pathology in 288/330+/+ mice infected with MERS-15 and MERS-0 demonstrated that MERS-15 induced significant levels of pathology commensurate with ARDS by day 6 p.i. (Supplementary Fig. 7). Although we did not conduct an exhaustive assessment of all extrapulmonary tissues in the 288/330+/+ mice, we could not detect virus replication in the brain, even at doses of $5 \times 10^8$ plaque-forming units (p.f.u.) up to day 6 when the humane euthanasia end points were reached, and the pathology observed in our model was consistent with the severe respiratory pathology associated with fatal ARDS in the only published case study of a human MERS-CoV infection.

Identification of MERS-CoV-adapted mutations associated with severe respiratory disease. We anticipated that the MERS-CoV genome would acquire mutations due to immunological pressure and/or enhanced virus fitness during mouse adaptation. Two viral
Clonal isolates of mouse-adapted MERS-CoV exhibit severe respiratory disease. Mice were inoculated intranasally with 5 × 10^6 p.f.u. a. The mortality of 288/330+/+ mice infected with MERS-15 C1 (n = 7) or MERS-15 C2 (n = 11) was monitored daily up to day 6 p.i. Data reflect the percentage of surviving mice. b. Mouse weights were monitored daily for 288/330+/+ mice infected with MERS-15 C1 (n = 7), MERS-15 C2 (n = 11) or MERS-0 (n = 6), and C57BL/6J wild-type (WT) mice infected with MERS-15 C2 (n = 6). Data are daily means ± s.d. c. Viral lung titres were determined at day 3 p.i. (288/330+/+ + MERS-15 C1, n = 4; 288/330+/+ + MERS-15 C2, n = 3; 288/330+/+ + MERS-0, n = 3; C57BL/6J WT + MERS-15 C2, n = 3) and day 6 p.i. (288/330+/+ + MERS-15 C1, n = 4; 288/330+/+ + MERS-15 C2, n = 6; 288/330+/+ + MERS-0, n = 3; C57BL/6J WT + MERS-15 C2, n = 3). The limit of detection (LOD) is indicated. Bars are means ± s.d. d. IHC of lung sections at 3 days p.i. from 288/330+/+ mice infected with MERS-15 C1 (i) or MERS-15 C2 (ii) and stained for inflammation (iii), oedema (iv), hyaline membrane formation (v) and perivascular cuffing (vi). All H&E images are representative of at least three samples. Scale bar (d), 1 mm.

Human monoclonal antibody 3B11 protects from MERS-CoV-elicited severe respiratory disease. Human monoclonal antibodies provide a robust strategy for the treatment of newly emerged viruses...
in humans. 3B11 is a human monoclonal antibody that targets the receptor-binding domain of the MERS-CoV spike protein\(^{19}\), and is effective in NHPs\(^5\). As the MERS-15 C2 adapted virus acquired no receptor-binding domain mutations (Supplementary Fig. 9), we reasoned that 3B11 should protect 288/330+/+ mice from MER-15 challenge. Pretreating mice for 12 h with 3B11 provided 100% protection against MERS-15 C2 challenge (Fig. 5a,b). Moreover, 3B11 treatment reduced viral loads in the lungs of infected mice to undetectable levels (Fig. 5c and Supplementary Fig. 11) and protected from loss of respiratory function (Fig. 5d) (Supplementary Fig. 11), pulmonary haemorrhage (Supplementary Fig. 11) and severe pathological changes (Supplementary Fig. 12). In contrast, pretreatment with isotype control antibody provided no protective effect. Therefore, these data convincingly demonstrated that our preclinical mouse model of severe respiratory disease and mortality can serve as a platform for assessing MERS-CoV therapeutics.

Spike protein vaccines derived from Venezuelan equine encephalitis virus replicon particles protect from lethal infection.

To examine vaccine efficacy in the 288/330+/+ MERS-15 C2 model, mice were vaccinated with Venezuelan equine encephalitis replicon particles (VRPs) expressing MERS-CoV spike protein (spike–VRP) or mock vaccinated with VRPs expressing green fluorescent protein (GFP–VRP), boosted at 4 weeks postprime and challenged with MERS-15 C2 at 4 weeks postboost. All mice receiving spike–VRP survived and exhibited no weight loss following challenge compared with GFP–VRP mock-vaccinated animals (Fig. 6a,b). Spike–VRP vaccination significantly reduced MERS-15 C2 replication in the lungs of infected mice as shown by both plaque titre (Fig. 6c) and viral antigen staining (Supplementary Fig. 14), while also protecting against severe respiratory disease, as assessed by measurement of Penh (Fig. 6d) and EF\(_{50}\) (Supplementary Fig. 13), lung haemorrhage (Supplementary Fig. 13) and pathological indications of severe acute respiratory disease (Supplementary Fig. 14). Neutralization of MERS-15 C2 with prechallenge serum from spike–VRP-vaccinated mice validated the presence of high-titre neutralizing antibodies in the serum of vaccinated 288/330+/+ mice (Supplementary Fig. 13). Our data demonstrated that the spike–VRP vaccine provoked an adaptive immune response capable of protecting mice from a lethal challenge with MERS-CoV, thereby extending the utility of our preclinical mouse model of severe respiratory disease to include vaccine evaluation.
escape mutants, 3B11 has also been tested in NHPs and the only published human autopsy from a MERS-CoV fatality exhibit severe respiratory distress requiring mechanical ventilation, loss of respiratory function and mortality. The MERS-CoV preclinical mouse model described here demonstrates for the first time that the CRISPR–Cas9 system can be used to genetically edit a non-permissive host receptor to generate a susceptible model for an emerging infectious pathogen. The 288/330+/+ MERS-CoV mouse model resembles the severe, and often fatal, respiratory distress syndrome observed in humans, and can be prevented through treatment with the 3B11 neutralizing monoclonal antibody or a VRP-based vaccine directed against the MERS-CoV spike protein. Coupled with an inability to elicit escape mutants, 3B11 has also been tested in NHPs, making it an excellent candidate for downstream human studies. However, existing NHP models rely on quantitative RT-PCR, rather than measures of infectious virus, to quantify viral loads, and these models do not reproducibly result in the severe respiratory disease or mortality observed in human MERS patients. Therefore, the results from our model complement the NHP 3B11 studies by directly demonstrating that 3B11 reduces levels of infectious MERS-CoV in the lungs, while preventing severe virus-induced respiratory pathology, loss of respiratory function and mortality.

In fatal cases of human MERS-CoV infections, individuals exhibit severe respiratory distress requiring mechanical ventilation, and the only published human autopsy from a MERS-CoV fatality reported histopathology that included diffuse alveolar damage with denuding of bronchiolar epithelium, hyaline membrane formation, type II pneumocyte hyperplasia and oedema. Furthermore, MERS-CoV antigen staining localized the virus to pneumocytes and syncytiotial cells. Infection of type I and II pneumocytes can lead to cell death, as observed in autopsies of patients who have died from severe respiratory infections with influenza virus and SARS-CoV. Moreover, pneumocyte cell death has been proposed to cause decreased respiratory function, as measured by whole-body plethysmography in a mouse model of influenza. Commensurate with these previous studies, our MERS-CoV mouse model demonstrated widespread infection of pneumocytes and pathology consistent with diffuse alveolar damage and severe respiratory disease. This was corroborated by decreased pulmonary function in the MERS-CoV model, as measured by plethysmography, which may be associated with widespread infection and possibly the death of pneumocytes and airway epithelial cells. Therefore, this model system provides the field with the opportunity to investigate the mechanisms that lead to MERS-CoV-induced pathology and severe respiratory disease, in the absence of any CNS complications.

Other MERS-CoV mouse models have used the more traditional method of expressing the full-length human DPP4 receptor to facilitate MERS-CoV infection. These models exhibited infection/
replication in the lungs following intranasal administration at low viral doses (10^7–10^9 p.f.u.), which in some cases resulted in pathology indicative of pneumonia-like disease24–26. One limitation of the 288/330+/+ MERS-CoV model described here was the use of high viral loads to achieve severe, and often fatal, respiratory disease. Further adaptation may allow the use of lower infectious doses that would produce a model of mild disease with subsequent recovery at later time points, as has been described with SARS-CoV27. In this context, it is interesting that the deletion of MERS-CoV ORF4b, a phosphodiesterase and antagonist of RNase L activity in human cells, appeared less critical for eliciting severe disease in rodents28–30, suggesting possible species-specific modes of action in vivo. Deletions in some SARS-CoV interferon antagonist genes and accessory open reading frames have also yielded subtle changes in overall virulence in vivo27,28. However, it is important to point out that a balance must be achieved between the virulence of mouse-adapted virus, which enhances the model’s capacity to replicate human disease phenotypes, and the number of mutations required to significantly reduce the dose lethal to 50% of animals tested.

Conventional models using constitutive overexpression of the hDPP4 MERS-CoV receptor have demonstrated widespread infection of extrapulmonary tissues including brain, kidney, liver, spleen and heart21–23, and two of these studies indicated that the mice exhibited multi-organ failure12,13. Furthermore, high viral loads were detected in the brains of mice in the transgenic hDPP4 overexpression models22,24. Importantly, Li et al.31 concluded that “mortality correlated with brain infection, suggesting that infection of this organ was most important for the high mortality observed in K18-hDPP4 mice”. While these lethal models have value for vaccine and immunotherapeutic testing32, small-molecule inhibitors that are effective in the lung may be limited in their efficacy due to an inability to cross the blood–brain barrier20. Similar neurological complications have been observed in model systems for SARS that used overexpression, or tissue-specific constitutive promoters, to express human angiotensin 1-converting enzyme 2 (ACE2) in mice33. While additional human pathology studies are needed to determine the extent of extrapulmonary sites of MERS-CoV replication and their impact on MERS-CoV disease, it is clear that respiratory replication and pathology is an important aspect of human MERS-CoV disease. Therefore, an important attribute of the 288/330+/+ model is that the lack of detectable virus replication in the CNS means this model can be used to study MERS-CoV-induced pulmonary disease without the confounding effects of death due to CNS infection.

Recently, a debate has emerged around the safety of performing gain-of-function (GOF) studies with highly pathogenic viruses (such as MERS-CoV, SARS-CoV, influenza virus H5N1) (http://www.griffinscientific.com/gain-of-function/). As demonstrated here, GOF studies were absolutely necessary to develop a mouse model that reflects the ARDS pathology observed previously in humans infected with respiratory pathogens. Importantly, the GOF studies performed here yielded MERS-CoV strains that reflect the complexity of clinical isolates identified recently in humans, where deletions were identified in ORF3 and ORF4A32. Moreover, these GOF studies have allowed us to identify mutations in MERS-CoV proteins that may influence how MERS-CoV interacts with, and possibly circumvents, host immune responses. Nevertheless, future studies to evaluate host factors that contribute to MERS-CoV disease will be constrained in this model, as well as in hDPP4 expression models, by the fact that the mice must be backcrossed to mouse lines harbouring modified endogenous genes, such as knock-out mice. This limitation may be overcome through additional GOF studies that facilitate MERS-CoV adaptation to the innate mDPP4 receptor molecule. The continued threats from novel emerging pathogens, such as Zika virus, will demand the rapid development of physiologically relevant animal models to evaluate therapeutic countermeasures, thereby necessitating virus adaptation to host immunity to achieve effective models. It is critical that the GOF regulatory structure does not impede the development of robust animal models of human disease, which are essential for protecting the public health.

Methods

Viruses, cells and plaque assays. All virus stocks were prepared on Vero CCL81 cells (ATCC). CCL81 cells were maintained routinely in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1x antibiotic/antimycotic (Gibco). All viruses were harvested in Opti-MEM medium (Gibco) supplemented with 3% FBS, 1x antibiotic/antimycotic, 1x non-essential amino acids (Gibco) and 1x l-arginine (Gibco). C57BL/6J mice (Jackson Laboratories) were infected with the 2012 strain of MERS-CoV was used at passage 10 (originally provided by Bart Haagmans (Erasmus Medical Center Rotterdam, The Netherlands) at passage 8), i.e.MERS was generated previously by Scoley et al.32. MERS camel strain Dromedary/Al-Hasa-KFU-HKU/13/2013 was used at passage 5 and was provided at passage 4 by Malik Peiris (University of Hong Kong, China) and MERS-0 was generated in the laboratory of R.S.B. as described below. Virus titres were determined by plaque assays on Vero CCL81 cells33. All viruses were maintained under Biosafety Level (BSL) 3 conditions with redundant fans and personnel powered air-purifying respirators, scrubs, Tyvek suits, Tyvek aprons and double layers of gloves.

Generation of mice with mDPP4 modified at positions 288 (exon 10) and 330 (exon 11). The alleles encoding amino acids 288 and 330 are shown in Supplementary Fig. 1. Genomic engineering of these alleles with the CRISPR–Cas9 genome editing system was performed at the University of North Carolina at Chapel Hill (UNC-CH) Animal Models Core Facility. The messenger RNA (mRNA) encoding Cas9 endonuclease and guide RNAs (gRNAs) were based on the system established by Mali et al.34 and prepared as described below. The gRNAs (Supplementary Table 1) were generated by an in vitro transcription reaction using a T7 High Yield RNA Synthesis kit (NEB), where 1 µg Drosophil-linearized template DNA was used in a 20 µl reaction following the kit guidelines for short RNA transcripts. The reaction was incubated at 37 °C overnight, followed by DNase I (RNase-free) digestion for 15 min at 37 °C. The gRNAs were then purified using an RNase free column (Qiagen) following the guidelines for short RNA purification. Capped and polyadenylated Cas9 mRNA was prepared by an in vitro transcription reaction using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). Capped mRNA was generated with 1 µg Cas9-T7 linearized plasmid DNA in a 20 µl reaction containing 1× T7NTP/ARCA Solution, 1× T7 reaction buffer and 2 µl T7 enzyme. The reaction was incubated at 37 °C for 1 h, followed by addition of 1 µl TURBO DNase and digestion at 37 °C for 15 min. To add a poly(A) tail to the capped mRNA, the mRNA reaction mix from step 1 was mixed with nuclease-free water (36 µl), 5× E-PAP buffer (20 µl), 25 mM MnCl2 (10 µl), ATP solution (10 µl) and E-PAP (4 µl) to a final reaction volume of 100 µl. The reaction was incubated at 37 °C for 30–45 min. The capped and polyadenylated RNA was purified by lithium chloride precipitation and resuspended in microinjection buffer (5 mM Tris/HCl buffer, 0.1 mM EDTA, pH 7.5).

Fertilized zygotes were collected from C57BL/6J females that had been superovulated and mated to C57BL/6J males. Pronuclear microinjection was performed with 50 ng µl⁻¹ Cas9 mRNA, 50 ng µl⁻¹ gRNA Dpp4-5′R, 25 ng µl⁻¹ gRNA Dpp4-5′G and 50 ng µl⁻¹ of each of Dpp4-2A88L-B and Dpp4-5′T330R-B per microinjected blastomere.
donor oligonucleotides (Supplementary Table 1). Injected embryos were implanted into pseudopregnant females, and the resulting pups were screened for alleles encoding changes (Supplementary Fig. 1) at positions 288 and 330. Mutations at the 288 position were detected by amplifying biopsy DNA samples with the following primers: Dpp4-E105S-F1: 5′-GATTCTGAGCAAGAAGACCC-3′, and Dpp4-E105S-R1: 5′-CCACAAAGTATCCACAGGAG-3′. The 752 bp PCR product was sequenced with primer Dpp4-E105S-F1: 5′-GATTCTGAGCAAGAAGACCC-3′. The PCR products were run on a 3% agarose gel and the band was excised, purified with QIAquick gel extraction kit, and then sent for sequencing.

The 330 position were detected by amplifying biopsy DNA samples with the following primers: Dpp4-E111S-F1: 5′-AAGTGTCGATATAGGTGGTCAC-3′, and Dpp4-E111S-R1: 5′-GGTTTATCCATTAATGTGGTTCGT-3′. The 767 bp PCR product was sequenced with primer Dpp4-E111S-F1: 5′-GATTCTGAGCAAGAAGACCC-3′. The PCR products were run on a 3% agarose gel and the band was excised, purified with QIAquick gel extraction kit, and then sent for sequencing.

Mouse infections. Genetically engineered mice with a modified mDPP4 receptor were housed and bred in accordance with guidelines established by the Department of Laboratory Animal Medicine at UNC-CH. As the 288/330+* and 288/330+/− mice are novel mouse lines developed in the laboratory of R.S.B., experiments utilized available animals and male and female mice that ranged from 12 to 20 weeks of age. Based on availability at the time of each experiment, experimental and control animals were age- and sex-matched. No blinding was used in any animal experiments, and animals were not randomized. Sample sizes were determined from preliminary data that was collected and compiled from experiments conducted under animal BSL3 conditions as described previously. Before viral infection, mice were anesthetized by administering 50 µl ketamine/xylazine mixture intraperitoneally and then infected intranasally with 50 µl virus solution containing 5 × 10^5 p.f.u. Incomplete infections due to bubbling of inoculum from the nasal cavity, the inability to inhale the entire dose or inoculum going into the mouth were noted, and these mice were considered failures and were excluded, as described previously. Following sedation and infection, mice were monitored daily for weight loss and survival, as well as for signs that the animals were moribund (including laboured breathing, lack of movement and lack of grooming). Mice that reached ≥20% weight loss were placed under exception and monitored at least twice daily. Mice that approached 30% weight loss were euthanized immediately. Mice deemed moribund were euthanized at the discretion of the researcher. Mice were euthanized with an isoflurane overdose followed by a secondary thoracotomy, at various time points, to collect lung tissues. In the absence of a thoracotomy, cervical dislocation was used as a secondary euthanasia method. All are approved methods of the Institutional Animal Care and Use Committee (IACUC) at the UNC-CH.

Ethics statement. Mouse studies were carried out in accordance with the recommendations for the care and use of animals by the Office of Laboratory Animal Welfare (OLAW, IACUC at UNC-CH) and approved the animal's handling and use (protocol IACUC 13-272), using a weight loss cut-off point of ~30% for humane euthanasia. Syntheticetically reconstructed MERS-CoV were approved by the UNC-CH Institutional Biosafety Committee, which also considered GOF research concerns before execution of these experiments.

Analysis of serum glucose levels. All blood glucose measurements were taken following a 6 h fast. Blood glucose was measured by tail clip sampling using an Analysis of serum glucose levels. performed here (protocol, IACUC 13-272), using a weight loss cut-off point of 5 × 10^5 p.f.u. (number of plaques with spike–VP (1 × 10^5 p.f.u.) or control GFP–VP (1 × 10^5 p.f.u.). After 24 h, the mice were boosted with the same dose of their respective VP strain. At 28 days postboost, all vaccinated mice were challenged with 5 × 10^5 p.f.u. MERS-15 C2. Mice were monitored daily for weight and survival, and killed at days 3 and 6 p.i. for lung lavage, histology, assessment of viral titre by plaque assay and evaluation of lung haemorrhaging. Respiratory function was measured at day 0 to establish a baseline and then again at days 3 and 6 p.i. The isolation and production of the 3B11 and F10 antibodies has been described previously.

Spike–VP vaccine study and virus neutralization assay. The MERS-CoV gene encoding the spike protein and GFP gene were packaged into VRPs with helper constructs from the V5326 attenuated strain of Venezuelan equine encephalitis virus (complexed with serum). Sequentially, MERS-CoV was administered a primary vaccination of 10 µl by footpad injection of spike–VP (1 × 10^5 p.f.u.) or control GFP–VP (1 × 10^5 p.f.u.). After 24 h, we observed the mice to be boosted with the same dose of their respective VP strain. At 28 days postboost, all vaccinated mice were challenged with 5 × 10^5 p.f.u. MERS-15 C2. Mice were monitored daily for weight and survival, and killed at days 3 and 6 p.i. for lung lavage, histology, assessment of viral titre by plaque assay and evaluation of lung hemorrhaging. Respiratory function was measured at day 0 to establish a baseline and then again at days 3 and 6 p.i. The isolation and production of the 3B11 and F10 antibodies has been described previously.

Northern blot analysis and RT-PCR. Northern blot analysis was performed for detection of full-length mDPPI in the lungs of 288/330+/−, 288/330+/+ and C57BL/6J wild-type mice. Poly(A) RNA was isolated to eliminate ribosomal RNA (rRNA; Quagen). Equalized amounts of RNA were resolved on 0.8% agarose gels and transferred to nitrocellulose membrane. A biotinylated probe (5′-biotin-gATG-biotin-GCGTGCTGAGC-biotin-3′) was used to detect mDPP4.
To quantify viral and targeted host mRNAs, MERS-CoV, mDP4, and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were measured in brain and lung tissue from MERS-CoV-infected mice. Briefly, tissues were removed and placed into RNALater (Ambion) solution and stored at –80°C until analysis by RT-PCR. Lung tissue was homogenized in TRIzol reagent (Invitrogen) and isolated according to the manufacturer’s instructions. Standard RT-PCR was performed with the following primers for each of the indicated mRNAs: MERS-CoV subgenomic leader sequence: 5′-TTAGGTTCCG-3′; mouse GAPDH: 5′-CAAGGACCCTTATAGTTACC-3′ and 5′-GCAGGATGTGTCTGGG-3′; and mDP4: 5′-ATACCGACAGGATGCG-3′ and 5′-TCTTGTTGTTGTTACGAGG-3′. Equivalent volumes of PCR product were resolved on gels for a non-quantitative answer (presence or absence) of viral subgenomic transcript expression in each brain and lung.

Quantitative RT-PCR was carried out on a Roche LightCycler 480 II, with accompanying software, to analyse MERS-CoV viral RNA and mDP4 mRNA, and 18S rRNA as an endogenous control. All RNAs were reverse transcribed under standard conditions in a 20 µl reaction volume using SuperScript III reverse transcriptase (Invitrogen). MERS-CoV viral RNA was assessed using the following primers at 900 nM in a 20 µl reaction in a SYBR Green assay with 2× Taqman conditions.

The data that support the findings of this study are available from the corresponding author on request.

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References

1. Chan, J. F.-W. et al. Treatment with lopinavir/ritonavir or interferon-β1b improves outcome of MERS-CoV infection in a nonhuman primate model of common marmoset. J. Infect. Dis. 212, 1904–1913 (2015).
2. de Wit, E. et al. Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. Proc. Natl Acad. Sci. USA 110, 16598–16603 (2013).
3. Falzarano, D. et al. Infection with MERS-CoV causes lethal pneumonia in the common marmoset. PLoS Pathogens 10, e1004250 (2014).
4. Munster, V. J., de Wit, E. & Feldmann, H. Pneumonia from human coronavirus in a macaque model. N. Engl. J. Med. 368, 1560–1562 (2013).
5. Johnson, R. F. et al. 3B11-N, a monoclonal antibody against MERS-CoV, reduces lung pathology in rhesus monkeys following intratracheal inoculation of MERS-CoV Jordan-n3/2012. Virology 465, 49–58 (2016).
6. Johnson, R. F. et al. Intratracheal exposure of common marmosets to MERS-CoV Jordan-n3/2012 or MERS-CoV EMC/2012 isolates does not result in lethal disease. Virology 485, 422–430 (2015).
7. Cockrell, A. S. et al. Mouse dipeptidyl peptidase 4 is not a functional receptor for Middle East respiratory syndrome coronavirus infection. J. Virol. 88, 5381–5399 (2014).
8. Coleman, C. M., Matthews, K. L., Goscochea, L. & Frieman, M. B. Wild-type and innately immune-deficient mice are not susceptible to the Middle East respiratory syndrome coronavirus. J. Gen. Virol. 95, 408–412 (2014).
9. de Wit, E. et al. The Middle East respiratory syndrome coronavirus (MERS-CoV) does not replicate in human lung explants. J. Virol. 89, 3402–3411 (2015).
10. Raj, V. S. et al. Adenosine deaminase acts as a natural antagonist for dipeptidyl peptidase 4–mediated entry of the Middle East respiratory coronavirus. J. Virol. 88, 1834–1838 (2014).
11. Ohnuma, K., Deng, N. H. & Morimoto, C. Revisiting an old acquaintance: CD26 and its molecular mechanisms in T cell function. Trends Immunol. 29, 295–301 (2008).
12. Agrawal, A. S. et al. Generation of a transgenic mouse model of Middle East respiratory syndrome coronavirus infection and disease. J. Virol. 89, 3659–3670 (2015).
13. Li, K. et al. Middle East respiratory syndrome coronavirus causes multiple organ damage and lethal disease in mice transgenic for human dipeptidyl peptidase 4. J. Infect. Dis. 213, 712–722 (2016).
14. Zhao, G. et al. Multi-organ damage in human dipeptidyl peptidase 4 transgenic mice infected with Middle East respiratory syndrome coronavirus. PLoS ONE 10, e0145561 (2015).
15. Zhao, J. et al. Rapid generation of a mouse model for Middle East respiratory syndrome coronavirus. Proc. Natl Acad. Sci. USA 111, 4970–4975 (2014).
16. Lambeir, A. M., Durinx, C., Scharpe, S. & de Meester, I. Dipeptidyl-peptidase IV from bovine to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit. Rev. Clin. Lab. Sci. 40, 209–294 (2003).
17. Menachery, V. D., Graiminski, L. E., Baric, R. S. & Ferris, M. T. New metrics for evaluating viral respiratory pathogenicity. PLoS ONE 10, e0131451 (2015).
18. Ng, D. L. et al. Clinicopathological, immunohistochemical, and ultrastructural findings of a fatal case of Middle East respiratory syndrome coronavirus infection in the United Arab Emirates, April 2014. Am. J. Pathol. 186, 652–658 (2016).
19. Tang, T. C. et al. Identification of human neutralizing antibodies against MERS-CoV and their role in virus adaptive evolution. Proc. Natl Acad. Sci. USA 111, E2018–E2026 (2014).
20. Agnlothrom, S. et al. A mouse model for Betacoronavirus subgroup 2c using a bat coronavirus strain HKU5 variant. mBio 5, e0047-14 (2014).
21. Korteweg, C. & Gu, J. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. Am. J. Pathol. 172, 1153–1170 (2008).
22. Ng, W.-F., To, K.-F., Lam, W.-W., Ng, T.-K. & Lee, K.-C. The comparative pathology of severe acute respiratory syndrome and avian influenza A subtype H5N1 – a review. Hum. Pathol. 37, 381–390 (2006).
23. Sanders, C. J. et al. Compromised respiratory function in lethal influenza infection is characterized by the depletion of type I alveolar epithelial cells beyond threshold levels. Am. J. Physiol. Lung Cell. Mol. Physiol. 304, L481–L488 (2013).
24. Pascal, K. E. et al. Pre- and postexposure efficacy of fully human antibodies against Spike protein in a novel humanized mouse model of MERS-CoV infection. Proc. Natl Acad. Sci. USA 112, 8738–8743 (2015).
25. Frieman, M. et al. Molecular determinants of severe acute respiratory syndrome coronavirus pathogenesis and virulence in young and aged mouse models of human disease. J. Virol. 86, 884–897 (2012).
26. Thornbourough, J. M. et al. Middle East respiratory syndrome coronavirus NS4b protein inhibits host RNase L activation. mBio 7, e00258 (2016).
27. Dediego, M. L. et al. Pathobiology of severe acute respiratory syndrome coronavirus deletion mutants in hACE-2 transgenic mice. Virology 376, 379–389 (2008).
28. Sims, A. C. et al. Release of severe acute respiratory syndrome coronavirus nuclear import block enhances host transcription in human lung cells. J. Virol. 87, 3885–3902 (2013).

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29. Agrawal, A. S. et al. Passive transfer of a germline-like neutralizing human monoclonal antibody protects transgenic mice against lethal Middle East respiratory syndrome coronavirus infection. Sci. Rep. 6, 31629 (2016).
30. Laksitorini, M., Prasasty, V. D., Kiptoo, P. K. & Siahaan, T. J. Pathways and progress in improving drug delivery through the intestinal mucosa and blood–brain barriers. Ther. Deliv. 5, 1143–1163 (2014).
31. McCray, P. B. Jr et al. Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. J. Virol. 81, 813–821 (2007).
32. Lamers, M. M. et al. Deletion variants of Middle East respiratory syndrome coronavirus from humans, Jordan, 2015. Emerg. Infect. Dis. 22, 716–719 (2016).
33. Scobey, T. et al. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. Proc. Natl Acad. Sci. USA 110, 16157–16162 (2013).
34. Mali, P. et al. RNA-guided human genome engineering via Cas9. Science 339, 823–826 (2013).
35. Menachery, V. D. et al. A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. Nat. Med. 21, 1508–1513 (2015).
36. Ayala, J. E. et al. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Dis. Model. Mech. 3, 525–534 (2010).
37. US Government Deliberative Process Research Funding Pause on Selected Gain-of-Function Research Involving Influenza, MERS, and SARS Viruses (US Government, 2014); http://www.phe.gov/s3/dualuse/Documents/gain-of-function.pdf

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Author contributions
A.S.C. conceived/designed, coordinated and executed the experiments, analysed the data and wrote the manuscript. B.L.Y. developed and recovered infectious clone viruses. T.S. completed mouse experiments. K.J. designed and completed immunological experiments. M.D. helped establish and maintain the mouse colony and perform molecular analyses. A.B. helped complete the mouse experiments. X.-C.T. and W.A.M. provided critical monoclonal antibody reagents. M.T.H. and R.S.B. conceived/designed the experiments and wrote the manuscript.

Additional information
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Competing interests
W.A.M. has a financial interest in AbViro. The other authors declare no competing financial interests.