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Review

ACE2-using merbecoviruses: Further evidence of convergent evolution of ACE2 recognition by NeoCoV and other MERS-CoV related viruses

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ABSTRACT

Angiotensin-converting enzyme 2 (ACE2) was recognized as an entry receptor shared by coronaviruses from *Sarbecovirus* and *Setracovirus* subgenera, including three human coronaviruses: SARS-CoV, SARS-CoV-2, and NL63. We recently disclosed that NeoCoV and three other merbecoviruses (PDF-2180, MOW15-22, PnNL 2018B), which are MERS-CoV relatives found in African and European bats, also utilize ACE2 as their functional receptors through unique receptor binding mechanisms. This unexpected receptor usage assumes significance, particularly in light of the prior recognition of Dipeptidyl peptidase-4 (DPP4) as the only known protein receptor for merbecoviruses. In contrast to other ACE2-using coronaviruses, NeoCoV and PDF-2180 engage a distinct and relatively compact binding surface on ACE2, facilitated by protein-glycan interactions, which is demonstrated by the Cryo-EM structures of the receptor binding domains (RBDs) of these viruses in complex with a bat ACE2 orthologue. These findings further support the hypothesis that phylogenetically distant coronaviruses, characterized by distinct RBD structures, can independently evolve to acquire ACE2 affinity during inter-species transmission and adaptive evolution. To date, these viruses have exhibited limited efficiency in entering human cells, although single mutations like T510F in NeoCoV can overcome the incompatibility with human ACE2. In this review, we present a comprehensive overview of ACE2-using merbecoviruses, summarize our current knowledge regarding receptor usage and host tropism determination, and deliberate on potential strategies for prevention and intervention, with the goal of mitigating potential future outbreaks caused by spillover of these viruses.

Coronaviruses, members of the Coronaviridae family, are enveloped, positive-sense, single-stranded RNA viruses capable of infecting a wide range of vertebrates, leading to various respiratory, enteric, or other diseases (V'Kovski et al., 2021; Chen, Zhao, & Zhang, 2022). They can be classified into four genera: α-, β-, γ- and δ-coronaviruses (Zhou et al., 2021). α- and β-coronaviruses primarily infect humans and other mammals, most of which have been found in bat species. γ- and δ-coronaviruses primarily infect birds, some infect mammals, and occasionally humans (Cui et al., 2019; Lednicky et al., 2021; Li et al., 2018). To date, seven coronaviruses have been identified that can transmit between humans, comprising two α-coronavirus and five β-coronaviruses (V'Kovski et al., 2021). Among them, three highly pathogenic β-coronaviruses have caused outbreaks or pandemics in the past two decades (Ksiazek et al., 2003; Zaki et al., 2012; Zhou et al., 2020). Severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 belong to the *sarbecovirus* subgenus, while Middle East respiratory syndrome coronavirus (MERS-CoV) is classified under the *merbecovirus* subgenus (Cui et al., 2019). The remaining three subgenera in the β-coronaviruses are embecoviruses, nobecoviruses, and hibecoviruses (Gupta & Khadka, 2022). Thus far, specific receptor remains to be identified for most of these viruses.

In 2012, the MERS-CoV emerged in Saudi Arabia, causing severe respiratory symptoms with a case fatality rate of about 36% (Shishido & Letizia, 2015; Zaki et al., 2012). MERS-CoV infection has resulted in approximately 2700 fatalities and sustained low-level local transmission in Saudi Arabia (WHO, 2012). Relatives of MERS-CoV have been found in several animal species, including bats, camels, hedgehogs and pangolins (Chen et al., 2023; Chu et al., 2014; Hemida et al., 2014; Lau et al., 2019; Luo et al., 2018; Mols et al., 2023; Shishido & Letizia, 2015; Speranskaya et al., 2023). Dromedary camels have been well-documented as

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intermediate hosts of MERS-CoV, and subsequent research has uncovered many relatives of MERS-CoV in bats, supporting the hypothesis that the origins and evolution of MERS-CoV trace back to bats (Anthony et al., 2017; Chu et al., 2014; Cui et al., 2019; Lau et al., 2013; Memish et al., 2013). These bat merbecoviruses exhibit varying sequences in their RBDs, resulting in different receptor usage. DPP4 was initially identified as an entry receptor for the MERS-CoV and long remained the only known receptor for merbecoviruses (Chen et al., 2023; Raj et al., 2013; Tse et al., 2023; Yang et al., 2014). However, we have recently identified four merbecoviruses that use bat ACE2 as their functional receptors infecting African or European bats, which are NeoCoV, PDF-2180, MOW15-22, and PnNL 2018B (Ma et al., 2023a; Xiong et al., 2022). Here, we provide a comprehensive overview of our current knowledge regarding these ACE2-using merbecoviruses and elaborate on the molecular mechanism of how this newly identified virus-receptor interaction determines the potential host range and inter-species transmission of these viruses, thereby highlighting their zoonotic risks in potential future outbreaks.

1. Introduction of ACE2-using merbecoviruses

The discovery of ACE2-using merbecoviruses has significantly expanded our understanding of the presence and distribution of these viruses (Fig. 1a). NeoCoV was reported sampled in *Neoromicia capensis* (Cape serotine, reclassified as *Laephotis capensis*) in South Africa in 2013, whose name likely derived from the host species of the virus (Corman et al., 2014; Ithete et al., 2013). PDF-2180, a close relative of NeoCoV, was identified in samples from *Pipisrellus hesperidus* bats in Southwest Uganda, and its discovery further supports the hypothesis that bat coronaviruses contribute to the evolutionary origin of MERS-CoV (Anthony et al., 2017; Menachery et al., 2020). Another two closely related bat merbecoviruses, MOW15-22 and PnNL 2018B (also known as PN-βCoV), are both identified in *Pipistrellus nathusii*, and with complete genome sequences released in 2023 (Mols et al., 2023; Speranskaya et al., 2023). Notably, *P. nathusii* is a bat species commonly found in Europe and is known for its seasonal long-distance migration across the continent (Paunović & Juste, 2023; UNEP/EUROBATS, 2023). MOW15-22 was discovered in bat fecal viromes with samples collected in 2015 in the Moscow region, while PnNL 2018B can be regularly detected in *P. nathusii* bats with a potential intestinal tropism (Speranskaya et al., 2023). It's worth noting that the earliest report of a virus with high sequence identity with PnNL 2018B, VM314, can be traced back to a 2010 study involving fecal samples from *P. pipistrellus* in the Netherlands, but its spike protein sequence remains unavailable (Reusken et al., 2010).

Phylogenetic analysis based on complete genome sequences reveals that the NeoCoV and PDF-2180 form a sister clade to MERS-CoV. By contrast, MOW15-22 and PnNL 2018B constitute a distinct clade separate from NeoCoV and MERS-CoV (Fig. 1b). NeoCoV and PDF-2180 exhibit high phylogenetic similarity, sharing 91% amino acid homology on the S1 subunit. Notably, NeoCoV represents a bat merbecovirus with the highest genome-wide homology (85%) to MERS-CoV (Corman et al., 2014; Xiong et al., 2022). However, phylogenetic analyses based on RBD protein sequences indicated a relatively closer relationship between NeoCoV, PDF-2180, and hedgehog coronaviruses (EriCoVs, such as HKU31) compared to MERS-CoV (Fig. 1c). MOW15-22 and PnNL 2018B form a distinct clade, exhibiting a relatively distant genetic distance from NeoCoV, PDF-2180 and MERS-CoV. Further similarity plot analyses (Simplot) underscore that the S1 subunits of these ACE2-using merbecoviruses exhibit even greater divergence from MERS-CoV than observed in HKU4 (Ma et al., 2023a; Xiong et al., 2022). To date, ACE2-using merbecoviruses have been identified only in Africa and Europe. However, it is very likely that these viruses exhibit greater diversity and wider distribution than we currently know.

2. An unexpected ACE2 usage

The spike proteins of coronaviruses undergo further processing into S1 and S2 subunits, playing a crucial role in receptor engagement and membrane fusion, respectively (Huang et al., 2020). The RBD, typically located in the CTD or B domain of the S1 subunit, plays a critical role in receptor recognition (Ou et al., 2017). DPP4 was previously the only known protein receptor for MERS-CoV and related merbecoviruses, including bat coronavirus HKU4, HKU25, and pangolin coronavirus MjHKU4r (Chen et al., 2023; Lau et al., 2018; Lu et al., 2013; Raj et al., 2013; Wang et al., 2014; Yang et al., 2014) (Fig. 2). However, the entry receptor for several other merbecoviruses, such as HKU5 and HKU31, remains unknown (Han et al., 2017; Lau et al., 2019; Xiong et al., 2022). The receptor for NeoCoV also remained undisclosed for a decade after its discovery. It had been speculated that PDF-2180 does not use DPP4 as its receptors due to variations in putative key residues for receptor recognition (Anthony et al., 2017; Menachery et al., 2020). Nevertheless, the initial reports on MOW15-22 and PnNL 2018B both predicted DPP4 as the entry receptor for these viruses based on *in silico* molecular docking analysis (Mols et al., 2023; Speranskaya et al., 2023).

To identify the entry receptor of these merbecoviruses, we first tested several known coronavirus receptors from humans using vesicular stomatitis virus (VSV) based pseudoviruses carrying spike proteins from NeoCoV and PDF-2180 (Xiong et al., 2022). As expected, these two viruses failed to use DPP4 for cellular entry due to their low RBD sequence identity compared to MERS-CoV. However, these pseudoviruses unexpectedly showed reproducible entry into BHK-21 cells expressing human ACE2 rather than human DPP4, albeit with relatively low efficiency. This result was totally unexpected, as the RBD folding of NeoCoV and PDF-2180 resembled MERS-CoV more than other known ACE2-using

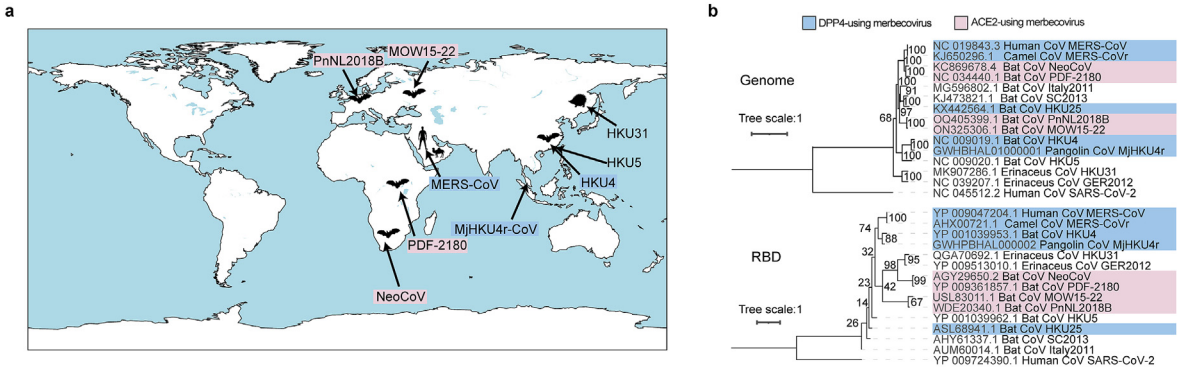

Fig. 1. Geographic origins and phylogenetic relationships of representative merbecoviruses. (a) The discovery locations of representative merbecoviruses and their host species are depicted. (b) Phylogenetic trees illustrating the evolutionary relationships among representative merbecoviruses based on their genomic sequences (upper tree) or RBD protein sequences (lower tree). GeneBank or NGDC-GWH accession numbers are provided. Blue background: DPP4-using; pink background: ACE2-using.

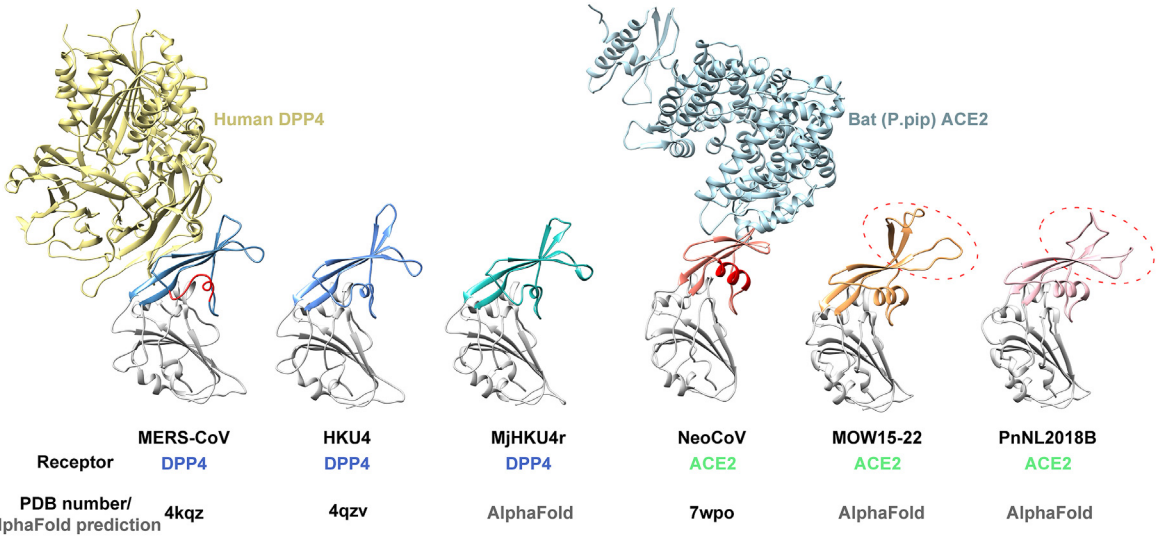

Fig. 2. Comparison of the receptor binding modes of representative ACE2-using and DPP4-using merbecoviruses. The core domain of RBDs are colored in light gray, while the RBMs and receptors are marked in different colors. The α-helix or equivalent sequences critical for receptor determinantion are highlighted in red. Dashed-line ellipses indicate the two featured RBM loop extensions in MOW15-22 and PnNL 2018B.

viruses. Given both viruses were discovered in bats, the functionality of 46 bat ACE2 orthologues was evaluated by RBD binding and viral entry assays. Intriguingly, NeoCoV and PDF-2180 displayed distinct species-specific ACE2 preferences among bat species. Most Yangochiroptera bat ACE2 orthologues efficiently supported the entry of NeoCoV and PDF-2180, whereas most ACE2 orthologues from Yinpterochiroptera bats demonstrated poor entry-supporting ability, even less efficient than human ACE2 (Ma et al., 2023b). Flow cytometry binding assays and spike-mediated fusion assays further confirmed the ACE2 usage. These assays highlighted that the *Pipistrellus* (Bat37 in the initial report, abbreviated as P. pip). This ACE2 orthologue exhibited the highest avidity for both NeoCoV and PDF-2180 RBDs, characterized by an exceptionally low k_{off} rate, rendering it a suitable candidate for viral entry competition assays and Cryo-EM structure determination. Furthermore, the possibility of both viruses using bat DPP4 orthologues for cellular entry was almost ruled out following testing of bat DPP4 orthologues from species with highly functional ACE2 orthologues for NeoCoV entry (Xiong et al., 2022).

Since the sequence of the ACE2 orthologue from the NeoCoV host species (*Laephotis capensis*) remains unknown, the structure of NeoCoV/PDF-2180 in complex with *P. pipistrellus* ACE2 was resolved to explore the molecular basis of this novel ACE2 binding mode. A comparison of these structures with MERS-CoV in complex with human DPP4 revealed that although the receptor binding motif of NeoCoV/PDF-2180 and MERS-CoV both consist of similar four-stranded antiparallel β-sheets, while a conformational shift in the η3 and β8-sheets, along with shorter β6–7 and β8–9 loops in NeoCoV/PDF-2180 RBMs, allowed them to recognize ACE2 but not DPP4 (Xiong et al., 2022).

The investigation into the usage of ACE2 as a functional receptor by merbecoviruses extended to other members of this group, especially MOW15-22, PnNL 2018B, and HKU31 with unknown receptor identity. Structural modeling of the RBD structures indicated that the receptor binding loops of the three mentioned merbecoviruses are characterized by conformational changes in RBM compared with MERS-CoV, particularly the presence of an α-helix structure that disrupted the four-stranded antiparallel β sheets, which is important for DPP4 engagement by MERS-CoV or HKU4 RBMs (Ma et al., 2023a; Xiong et al., 2022) (Fig. 2). Subsequent experiments demonstrated that both MOW15-22 and PnNL 2018B could efficiently use some bat ACE2 orthologues but not DPP4 for cellular entry, although ACE2 from their natural host (*P. nathusii*) was not tested due to the sequence unavailability. Intriguingly, while the hedgehog coronavirus HKU31 displays high RBD sequence and structural homology with NeoCoV, it is unable to recognize any tested bat or hedgehog ACE2 or DPP4, leaving uncertainty regarding their receptor usage (Fig. 2)[26]. These findings underscore the promiscuity of coronavirus receptor usage and emphasize the importance of conducting functional experiments to validate *in silico* predictions of coronavirus receptor usage (Speranskaya et al., 2023).

3. Evolutionary convergent ACE2 recognition modes

ACE2 functions as a type I transmembrane carboxypeptidase responsible for cleaving a C-terminal residue from Angiotensin-I (Ang I, or Ang 1–10) to produce Ang1-9, and hydrolyzing AngII (or Ang 1–8) to generate Ang1-7 (Donoghue et al., 2000; Imai et al., 2010; Tipnis et al., 2000). ACE2 is highly expressed in various tissues, including the duodenum, small intestine, kidney, heart, thyroid, gallbladder, and adipose tissue, and relatively weakly expressed in blood, spleen, bone marrow, brain, blood vessels, muscle, lungs, liver, and bladder (Li et al., 2020). Interestingly, ACE2, but not its homolog angiotensin-converting enzyme ACE1, has been identified as the functional entry receptor for a range of phylogenetically-distant coronaviruses, including the α-coronavirus NL63 (Setracovirus) (Hofmann et al., 2005), lineage B β-coronaviruses (sarbecoviruses) SARS-CoV (Li et al., 2003), SARS-CoV-2 (Zhou et al., 2020), and lineage C β-coronaviruses (merbecoviruses) described in our studies (Ma et al., 2023a; Xiong et al., 2022). These viruses exhibit marked differences in their receptor binding domain (RBD), particularly in the structures and sequences of their receptor-binding motif (RBM), suggesting the presence of convergent ACE2 usage acquisition events during the evolution of different coronaviruses (Fig. 3).

As an uncommon case of the ACE2-using virus of *alphacoronavirus*, NL63-CoV displays a complete different RBD structure compared to other viruses in the β-coronavirus group. NL63-CoV RBD comprises a galectin-like β-sandwich core formed by two layers of 3-stranded β-sheets, stabilized through extensive hydrophobic interactions (Wu et al., 2009). By contrast, the core subdomain structure of RBDs from β-coronaviruses, including NeoCoV, PDF-2180, SARS-CoV and SARS-CoV-2, share common features characterized by a 5-stranded antiparallel β-sheet connected by α-helices and loops. Sarbecoviruses SARS-CoV, and SARS-CoV-2 share similar receptor binding subdomains, comprising approximately 70-residue loop extensions between β4 and β7 of the RBD, along with a few spatially adjacent residues beyond this loop extension

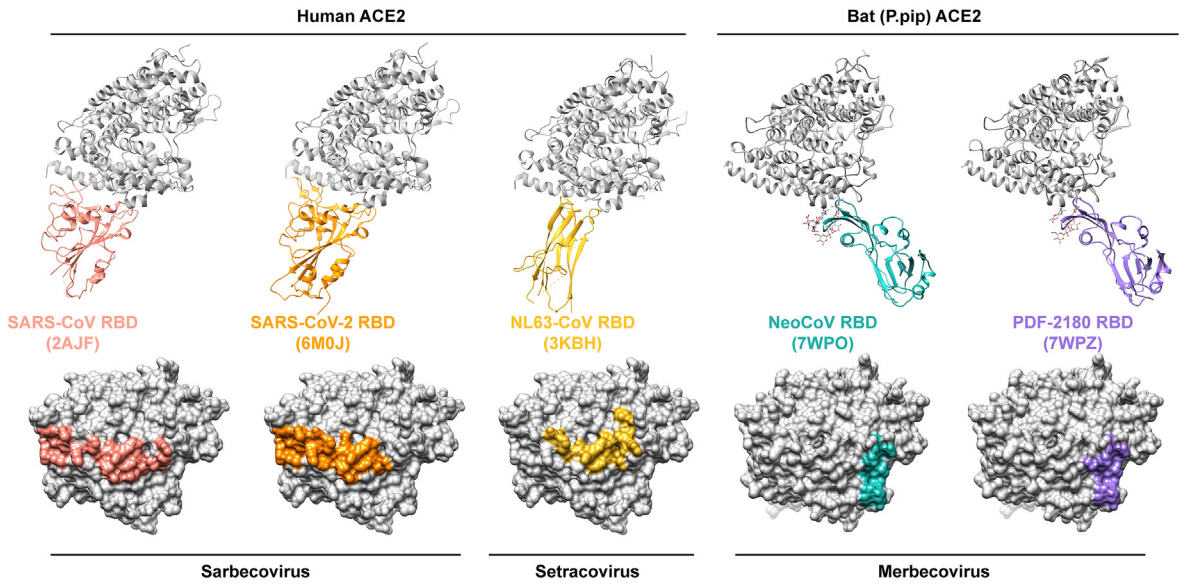

Fig. 3. Structures of ACE2-using viruses' RBD in complex with human or Bat (*P. pipistrellus*) ACE2. The structures of the RBD-ACE2 complexes for SARS-CoV, SARS-CoV-2, NL63-CoV, NeoCoV and PDF-2180 are color-coded. RBD binding footprints on ACE2 orthologues are highlighted in corresponding colors.

(Lan et al., 2020; Li, Li, et al., 2005; Yan et al., 2020). By contrast, NeoCoV/PDF-2180 RBD forms an RBM through four β-sheets (β5 to β8) and a η3-helix situated between β4 and β9 of the core subdomain (Xiong et al., 2022).

Despite the structural differences, SARS-CoV, SARS-CoV-2, and NL63 all bind to a largely overlapping surface on the apex of the ACE2 ectodomain, primarily involving the α1 helix, but with variations between different viruses. For instance, residues 26–45 of human ACE2 form polar or hydrophobic interactions with SARS-CoV, SARS-CoV-2, and NL63-CoV, whereas residues 79–83 on the α2 helix of ACE2 contribute limited interactions with RBDs of SARS-CoV and SARS-CoV-2 but not NL63-CoV. Additionally, the loops between α10-α11 and β3-β4 also reinforce the ACE2 interactions with SARS-CoV, SARS-CoV-2, and NL63-CoV, with the former loop being particularly significant for NL63-CoV and the latter being important for SARS-CoV and SARS-CoV-2. Overall, the ACE2-binding surface area of NL63 is relatively smaller than that of SARS-CoV and SARS-CoV-2 (Lan et al., 2020; Wu et al., 2009; Yan et al., 2020).

By contrast, NeoCoV and PDF-2180 recognize a distinct surface on bat ACE2 through a very different binding mode (Fig. 3). Instead of the bridge-shape surface observed in ACE2 binding by sarbecoviruses, NeoCoV and PDF-2180 target a much more compact area on the apical region of *P. pipistrellus* ACE2, formed by the α9 and α10 helices and a loop connecting α10 and β4 (around residues 304–340). This binding surface barely overlaps with that of NL63 and ACE2-utilizing sarbecoviruses on human ACE2. The interaction consists of a network of electrostatic and hydrophobic interactions, facilitated by extensive interactions mediated by two ACE2 glycans (N54 and N329) (Xiong et al., 2022). Conversely, glycans (N90 and N322) that are geometrically proximate to SARS-CoV and SARS-CoV-2 RBD binding sites on human ACE2 are dispensable for receptor recognition and may even cause steric hindrance (Isobe et al., 2022; Li et al., 2005b). The importance of ACE2 glycans mediated interaction varies among different ACE2-using merbecoviruses. For instance, the N54 glycan is critical for NeoCoV receptor recognition but largely tolerable by PDF-2180, while both N54 and N329 glycosylation sites are dispensable for MOW15-22 receptor recognition (Ma et al., 2023a, 2023b; Xiong et al., 2022).

Together, the three distinct ACE2 binding modes exhibited by *Setracovirus* (Alphacoronavirus NL63), *Sarbecovirus*, and *Merbecoviruses* subgenus represent at least three independent evolutionary events in the acquisition of ACE2 receptor usage by coronaviruses. Interestingly, it raises the question of whether MOW15-22, PnNL 2018B, and other coronaviruses have independently developed additional ACE2 binding modes. The advantage of ACE2 receptor usage remains unclear, but it may associate with certain evolutionary benefits in efficient viral transmission.

4. Potential host tropism of ACE2-using merbecoviruses

The potential host tropism of ACE2-using merbecoviruses remains an open question, as there have been no report of these viruses infecting bats other than their host species or establishing virus reservoirs in non-bat hosts. Given that the receptor recognition by coronaviruses represents a primary barrier for inter-species transmission at the entry-level, a comprehensive understanding of these merbecoviruses' capacity to employ ACE2 receptors from various species is crucial for assessing the potential zoonotic risk associated with these viruses (Li, 2015; Wan et al., 2020; Yan et al., 2021). Besides, it is also important to elucidate how species-specific ACE2 recognition influences the potential host tropism, as well as the ability of these viruses to jump between different species. In a follow-up study investigating potential natural and intermediate hosts for NeoCoV and PDF-2180, we examined ACE2 orthologues from 102 representative species spanning eleven mammalian orders (Ma et al., 2023b). Specifically, among these orthologues, 49 originate from bat species across 11 families, categorized into Yinpterochiroptera (Yin-) and Yangochiroptera (Yang-) suborders. The comprehensive evaluation of bat ACE2 receptors is necessary because bats serve as the natural host for most α- and β-coronavirus and show remarkable ACE2 genetic diversity among different species or even within species (Cui et al., 2019; Guo et al., 2020; Yan et al., 2021).

Our comprehensive analysis of these ACE2 orthologues demonstrated that NeoCoV and PDF-2180 can efficiently use ACE2 across a wide range of species. Notably, NeoCoV and PDF-2180 showed suborder-specific usage among bat species, with the majority of ACE2 orthologues from Yang-bats facilitating NeoCoV and PDF-2180 entry. By contrast, most ACE2 orthologues from Yin-bats, with the exception of several from the Rhinolophidae family, failed to support entry of these viruses (Fig. 4a). By contrast, the majority of the non-bat mammals tested (47 out of 53) exhibited functional ACE2 receptors to mediate relatively efficient entry of NeoCoV and PDF-2180 (Ma et al., 2023b) In contrast to NeoCoV and PDF-2180, which demonstrated a broad ACE2 usage spectrum, MOW15-22 and PnNL 2018B displayed a much narrower receptor

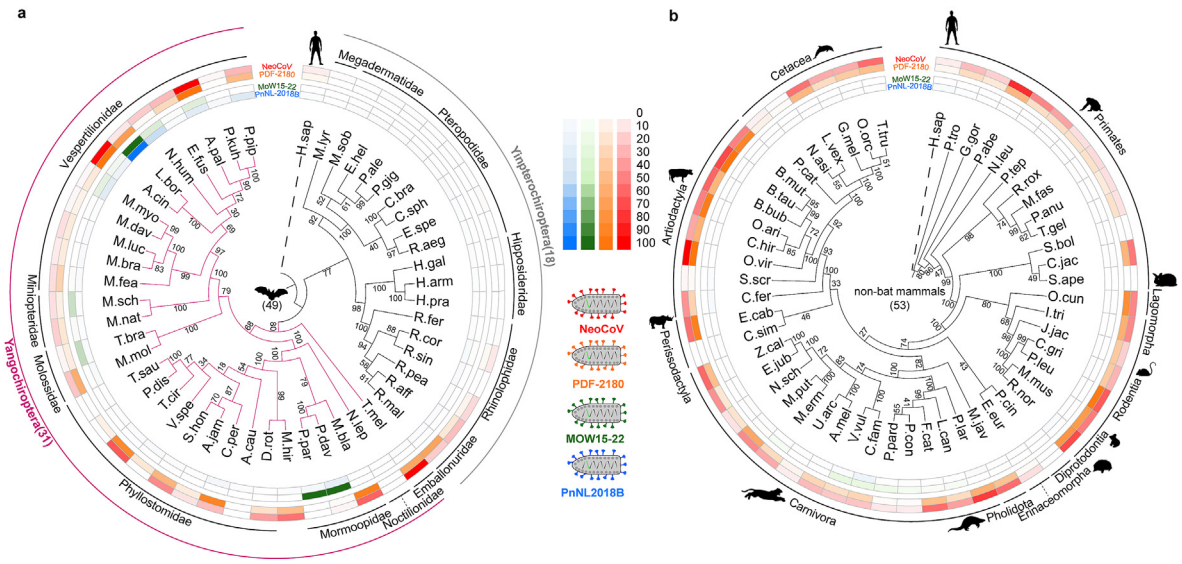

Fig. 4. Potential host tropism of ACE2-using merbecoviruses across 102 different mammalian species. Phylogenic trees of ACE2 protein sequences from 102 mammals, including 49 bats (a) and 53 non-bat mammals (b), generated by IQ-Tree (http://igtree.cibiv.univie.ac.at/) and polished with iTOL (v6) (https://itol.embl.de/) (Letunic & Bork, 2021). Heatmaps illustrate the pseudovirus entry-supporting abilities of ACE2 orthologues for indicated ACE2-using merbecoviruses, normalized based on our previous reports with the entry efficiencies of the most capable ACE2 set as 100%[26,40 27]. Species orders (for non-bat mammals) or families (for bats) are indicated. Complete species names corresponding to the four-letter abbreviations are provided in Table S1.

recognition range. Specifically, efficient entry of PnNL 2018B was only observed with several bat ACE2 receptors, and entry of MOW15-22 can be observed in several bat species as well as several other mammalian species (Fig. 4b). Furthermore, efficient binding was exclusively observed for MOW15-22 RBD in two ACE2 orthologues from bats belonging to the *Pteronotus* genus (Ma et al., 2023a).

Antigenic drift within the receptor-binding interface has the potential to alter the species tropism of coronaviruses (Guo et al., 2020; Letko et al., 2018; Oude Munnink et al., 2021; Saville et al., 2023; Yan et al., 2021). In the case of ACE2-using merbecoviruses, although human ACE2 has been determined to be a less efficient receptor for NeoCoV and PDF-2180, a T510F mutation in the NeoCoV RBM rendered it capable of efficiently utilizing human ACE2, potentially by enhancing hydrophobic interactions with a corresponding hydrophobic pocket on ACE2. It is noteworthy that the corresponding amino acid in PDF-2180 (site 511) is already phenylalanine, suggesting that efficient binding to human ACE2 is achievable for ACE2-using viruses through natural mutations or recombination events. Upon analyzing the ACE2 orthologues sequences from 102 species, we found that hydrophobic residues in this pocket are highly conserved. Consequently, the NeoCoV-T510F variant displayed an expanded potential host range compared to its wild-type counterparts (Ma et al., 2023b). Other changes in spike protein residues may similarly enable NeoCoV and its relatives to cross species boundaries, including humans.

5. Host range determinants

Our previous findings suggest that suborder-specific ACE2 usage does not strictly adhere to the phylogeny, and specific ACE2 residues may play critical roles in host range determination. Through extensive analyses focusing ACE2 orthologues deficient in supporting NeoCoV and PDF-2180 entry, we unveiled several host range determinants on ACE2 by sequence analysis and swap mutagenesis based on different bat ACE2 orthologues. Four major determinants, denoted as A to D, are located within the receptor binding interface, two of which (determinants A and

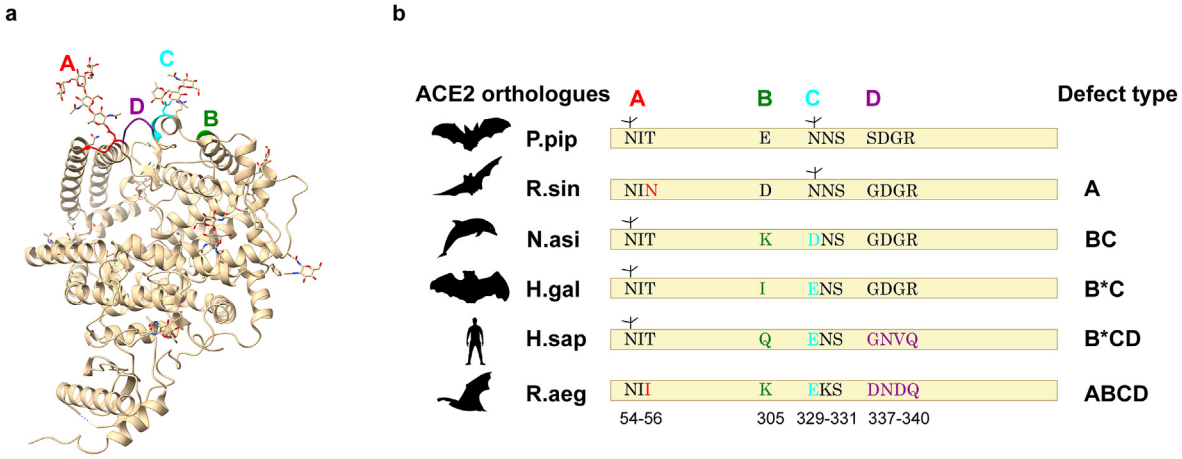

Fig. 5. Host range determinants for NeoCoV and PDF-2180 on different ACE2. (a) Four determinants of *P. pipistrellus* ACE2 are indicated by different colors. (b) A diagram displaying the determinant sequences from *P. pipistrellus* ACE2 and ACE2 orthologues with unfavorable residues in indicated determinants. The glycans and defect types (based on previous reports) are indicated, and the residue numbers are based on *P. pipistrellus* ACE2 sequences.

C) are species-specific glycosylation sites (Fig. 5a). Glycan at determinant A (N54 glycosylation) is relatively distant from the main protein-protein based interaction interface but plays a more significant role than glycosylation at determinant C (N329 glycosylation) in NeoCoV-receptor interactions. By contrast, determinant C (N329 glycan) resides within the protein-protein based interaction interface. Loss of interactions mediated by this glycan be compensated by other residues in the interface, resulting in a relatively minor impact on the overall interaction. Determinants B (site 305) and D (residues 337–340) have the potential to form salt bridges with NeoCoV and PDF-2180 RBD, thereby strongly contributing to the binding affinity (Ma et al., 2023b).

Each deficient Yin-bats' ACE2 orthologue carries one or more unfavorable residues within the determinants, and acquisition of receptor function is achievable in all tested orthologues through substitutions with favorable residues in specific determinants (Fig. 5b). For instance, *Rousettus aegyptiacus* ACE2 can only become functional when all four determinants are updated with favorable residues. Residue in determinant B (position 305) is critical for receptor function for NeoCoV and PDF-2180 as different residues at this site can either form a salt bridge, hydrogen bond, or result in steric hindrance. Consequently, the six deficient ACE2 orthologues from non-bat mammals (*Sapajus apella, Saimiri boliviensis, Sus scrofa, Neophocaena asiaeorientalis, Ceratotherium simum, and Phascolarctos cinereus*) carry unfavorable residues at site 305, and they can obtain varying degrees of entry-supporting capability once their residue 305 is replaced by a glutamine (305 E), a substitution facilitates a salt bridge interaction with NeoCoV RBD K512. Determinant D plays a major role in restricting the receptor function of human ACE2 for NeoCoV and PDF-2180. Thus, N338D substitution in human ACE2 significantly enhances its receptor function by facilitating its interactions with N504/N506 and R550 of NeoCoV RBD (Xiong et al., 2022).

In addition to determinants A to D, other residues beyond the binding interface, such as residue 134 and residues within 337–354, can also indirectly affect binding efficiency, as observed in ACE2 orthologues from two primates (*Sapajus apella, Saimiri boliviensis*) and Koala (*Phascolarctos cinereus*) (Ma et al., 2023b). Our recent study indicates that MOW15-22 and PnNL2018 exhibit a different binding mode compared to NeoCoV, as theoretically unfavorable residues in determinants A-D, based on NeoCoV studies, do not impact the entry efficiency of MOW15-22 (Ma et al., 2023a). Thus, additional determinants are to be identified to elucidate the host tropism determination mechanism of different ACE2-using merbecoviruses.

6. Insights into MERS-CoV origin

The natural host and evolutionary origin of MERS-CoV remains unclear. While the dromedary camel is established as the intermediate host of MERS-CoV, bats have been proposed as potential natural hosts for the virus (Corman et al., 2014; Cui et al., 2019; Han et al., 2016; Lau et al., 2013). Although bat merbecovirus HKU4 and some other viruses were also found to use DPP4 as their entry receptor, these virues are all phylogenetically distant from MERS-CoV (Luo et al., 2018). Previous studies have proposed that MERS-CoV could have emerged through recombination between a NeoCoV-related virus and a DPP4-using virus, such as HKU4-related viruses (Corman et al., 2014; Hassan et al., 2020). Simplot similarity analyses of the whole genome sequences of MERS-CoV and ACE2-using merbecoviruses, especially NeoCoV, strongly indicate that recombination played a significant role in the evolution of merbecoviruses (Forni et al., 2020; Xiong et al., 2022) (Fig. 6). It remains unknown that whether this recombination event occurred in bats or camels, and at what point the host switching happened. Despite the global distribution of bat merbecoviruses, the two MERS-CoV closely related ACE2-using merbecoviruses, NeoCoV and PDF-2180, were identified in Africa. Notably, a significant proportion of camels in the Arabian Peninsula, displaying serological evidence of prior MERS-CoV infection, are imported from the Greater Horn of Africa, a region inhabited by several bat species closely related to the host of NeoCoV and PDF-2180 (Chu et al., 2014; Younan et al., 2016). Given that both viruses currently exhibit limited infectivity in human cells, the acquisition of the hDPP4 binding domain might have been crucial for the emergence of human or camel MERS-CoV. Additionally, the identification of European bat ACE2-using merbecoviruses, MOW15-22 and PnNL 2018B, indicate the human emergence of these could also occur beyond the regions of Africa or the Middle East. Nevertheless, our study does not exclude alternative scenarios, such as NeoCoV and PDF-2180 originating from the recombination between DPP4-using merbecoviruses and other unidentified viruses carrying ACE2-using RBD sequences. Further studies are necessary to shed light on the enigmatic evolutionary trajectory of MERS-CoV.

7. Entry inhibitors against ACE2-Using mebecoviruses

Apart from recombinant ACE2 or RBD-Fc proteins, so far, there are no entry inhibitors specifically targeting NeoCoV or other ACE2-using merbecoviruses, such as specific antibodies interfering with the

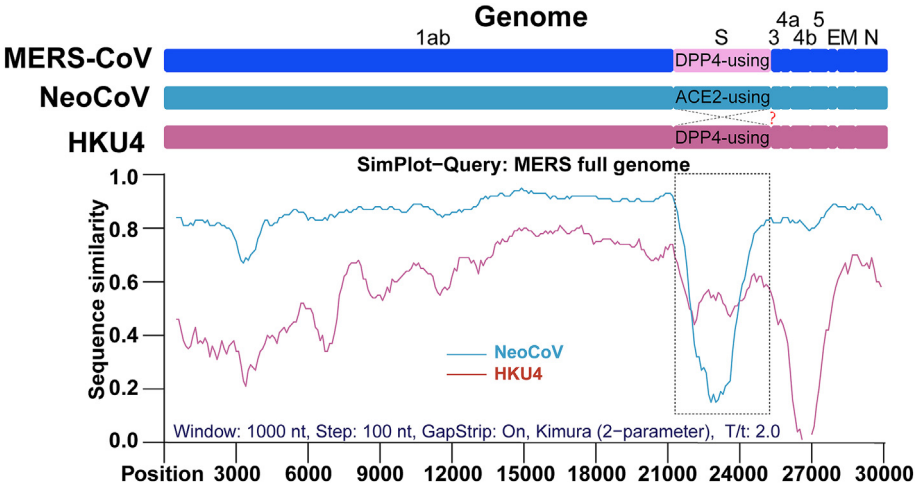

Fig. 6. Hypothesis for the recombination driving the origin of MERS-CoV. Upper: Protein-coding region boundaries for the three merbecoviruses are indicated. Below: Simplot analysis comparing the similarity of complete genome nucleotide sequences of NeoCoV and HKU4 with MERS-CoV. The dashed box outlines regions of the spike protein with notable divergence.

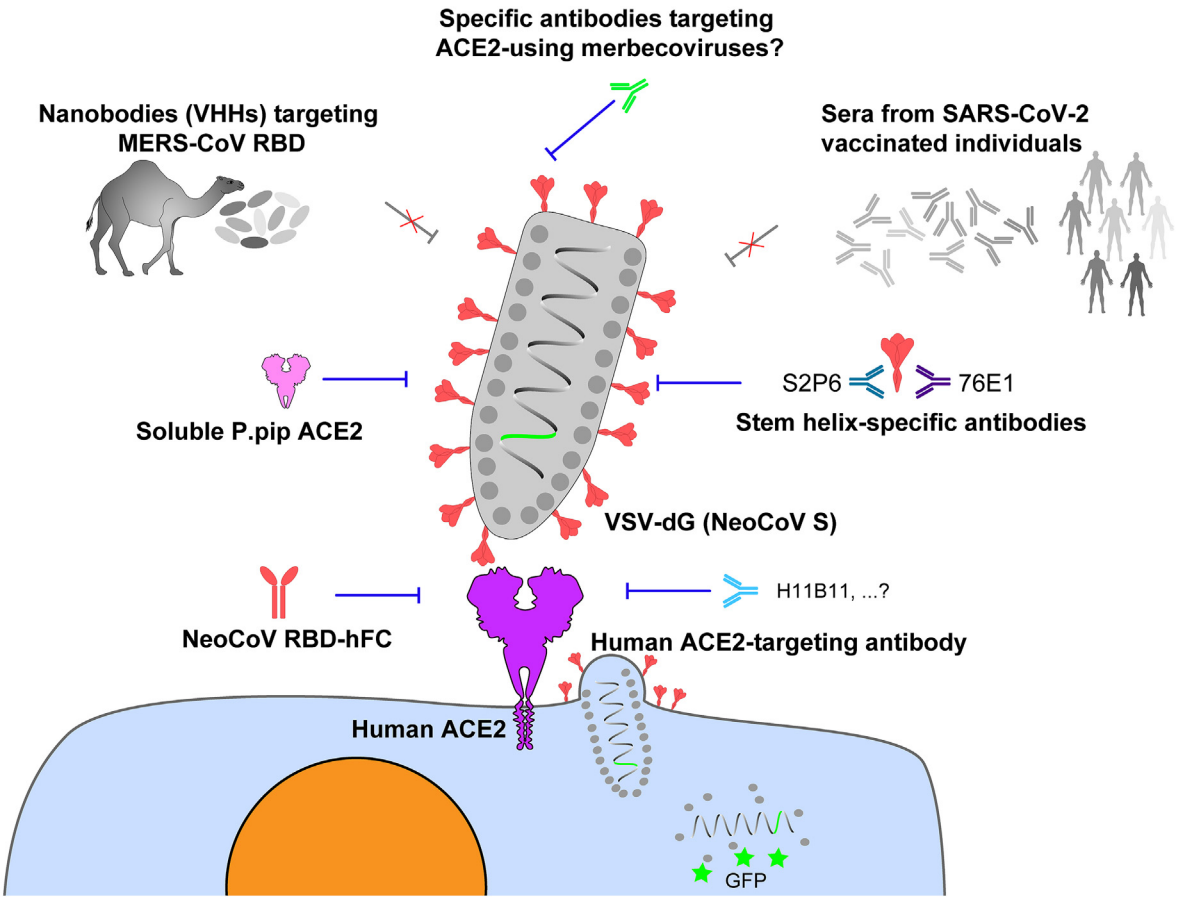

Fig. 7. Entry inhibitors targeting NeoCoV receptor recognition in human cells. Efficient inhibition of NeoCoV pseudovirus entry can be achieved using soluble *P. pipistrellus* ACE2, NeoCoV RBD-hFc recombinant proteins, human ACE2 targeting-antibody (H11B11), Pan-β broadly neutralizing antibodies (S2P6, B6 and 76E1), but not MERS-CoV RBD specific nanobodies and SARS-CoV-2 antisera from vaccinated individuals. Specific antibodies targeting ACE2-using merbecoviruses remains to be developed.

receptor recognition (Fig. 7). Antibodies developed against SARS-CoV-2 and MERS-CoV may not offer enough protection against the ACE2-using merbecoviruses. This is evident from the inefficiency of cross-neutralizing antibodies present in sera from individuals who received inactivated SARS-CoV-2 vaccines, which failed to neutralize NeoCoV and PDF-2180 infections (Xiong et al., 2022). Furthermore, the antiviral efficacy of MERS-CoV RBD-specific nanobodies was also demonstrated to be inadequate in inhibiting ACE2-using merbecoviruses entry (Xiong et al., 2022). These results are reasonable given the significant differences between the RBD sequences from SARS-CoV-2/MERS-CoV RBD and ACE2-using merbecoviruses. Encouragingly, some pan-β coronavirus antibodies targeting the stem-helix (like B6, S2P6) or fusion peptide (like 76E1) within the fusion machinery of the S2 subunit remain effective in blocking the entry of NeoCoV or other ACE2-using merbecoviruses (Pinto et al., 2021; Sauer et al., 2021; Sun et al., 2022). However, their potency is also influenced by the sequence variation in epitopes of these viruses (Ma et al., 2023a; Xiong et al., 2022). As a result, there is a need for the development of NeoCoV/PDF-2180 specific antibodies and vaccines, or pan-β coronavirus vaccines, to mitigate the potential outbreak of ACE2-using merbecoviruses. Moreover, ACE2 itself could be utilized, either as inhibitors or drug target, for preventing infection by ACE2-using merbecoviruses, as demonstrated by the competitive entry inhibition by soluble ACE2-ectodomain proteins and an ACE2-targeting antibody, H11B11 (Du et al., 2021; Ma et al., 2023a; Xiong et al., 2022). It is worth noting that H11B11 was screened against the binding interface of human ACE2 engaged by SARS-CoV-2, so its antiviral efficacy against NeoCoV/PDF-2180 is not as potent as observed for neutralizing SARS-CoV-2 infection. Consequently, the development of ACE2-specific antibodies targeting the more relevant surface recognized by NeoCoV/PDF-2180 or other ACE2-using merbecoviruses could potentially yield greater antiviral potency.

8. Perspectives

The COVID-19 pandemic served as a wake-up call to human awareness regarding the threat of the newly emerging coronaviruses to humans (Li et al., 2023). MERS-CoV, which has the highest reported case fatality rate of 36%, continues to transmit sporadically in the Middle East, raising concerns about the potential for future pandemics caused by MERS-CoV-related viruses (WHO, 2012). Our research has identified a group of ACE2-using merbecoviruses, which infect bats inhabiting in Africa and Europe. Some of the viruses share high genome similarities with MERS-CoV, highlighting the zoonotic potential and biosafety implications associated with these viruses (Xiong et al., 2022).

These studies provided further evidence, in addition to NL63, to support the hypothesis that convergent evolution exists among coronaviruses for achieving ACE2 recognition. Intriguingly, similar instances of receptor promiscuity can also be observed with Aminopeptidase N (APN), another important coronavirus receptor shared by many α-coronaviruses such as human 229 E, porcine transmissible gastroenteritis virus (TGEV), CCoV-HuPn-2018, and δ-coronaviruses like porcine deltacoronavirus (PDCoV) and several avian deltacoronaviruses (Li et al., 2018; Liang et al., 2023; Tortorici et al., 2022; Tresnan et al., 1996; Yeager et al., 1992). Similar to the scenario of ACE2, APN was recognized

by these viruses through different binding modes (Li et al., 2018; Liang et al., 2023). These observations suggest that the convergent receptor recognition strategy, involving structurally distinct RBDs binding to the same receptor, might be common among coronaviruses. On the other hand, viruses with similar RBDs can recognize different receptors through sequence variations in receptor binding motifs, as exemplified by merbecoviruses in the aforementioned studies. The reasons behind the preference for ACE2 and APN as receptors remain unclear. It is conceivable that the tissue-specific expression of these exopeptidases in the respiratory and digestive tract offers certain evolutionary advantages in viral transmission, as is observed in the case of efficient airborne transmission of SARS-CoV-2 Omicron variant (Chen et al., 2022b; Fan et al., 2022). However, recent evidence indicated that PnNL 2018B most likely exhibited an intestinal tropism (Mols et al., 2023). Consequently, it remains an open question whether these ACE2-using merbecoviruses can achieve airborne transmission if they spill over to humans.

Thus far, there is no evidence of ACE2-using merbecoviruses infecting humans. While current NeoCoV and other ACE2-using viruses are inefficiently to use human ACE2 for entry, it should be noted that coronaviruses hold the potential across species boundaries via adaptive mutations or recombinations in the receptor recognition sites, as is demonstrated in the NeoCoV T510F mutation and let alone undiscovered ACE2-using merbecoviruses already adapted to human ACE2 in nature (Gu et al., 2020; Oude Munnink et al., 2021; Xiong et al., 2022). Furthermore, NeoCoV and their relatives with broad potential host tropism could also establish viral reservoirs and evolve in intermediate hosts, and subsequently jump to humans. It is also important to note that successful zoonotic spillovers are influenced by multiple factors beyond receptor recognition, including proteolytic spike activation, replication, tissue tropism and immune response (Plowright et al., 2017). Hence, a comprehensive understanding of the mechanisms governing cross-species transmission of ACE2-using merbecoviruses is crucial for evaluating the zoonotic potential of these ACE2-using merbecoviruses.

Previous studies and our own reports indicate that NeoCoV and PDF-2180 require exogenous proteolytic activity for efficient spike cleavage upon entry into human or other mammalian cells (Menachery et al., 2020; Xiong et al., 2022). Notably, while furin cleavage sites exist within the S1/S2 junction sequences of both MERS-CoV and NeoCoV/PDF-2180, only MERS-CoV undergoes efficient spike cleavage when expressed in HEK293T cells (Xiong et al., 2022). This discrepancy suggests that NeoCoV/PDF-2180 and other ACE2-using viruses may exhibit restricted amplification in human cells in the absence of optimal proteolytic activity, which could act as an additional barrier to human emergence. However, human ACE2 can mediate moderate entry of NeoCoV and PDF-2180 in the presence of exogenous trypsin, despite its relatively low binding affinity with NeoCoV/PDF-2180 RBD (Menachery et al., 2020; Xiong et al., 2022). Further exploration into the proteolytic activation mechanism and the identification of host proteases for ACE2-using merbecoviruses are warranted to assess the zoonotic risk posed by these viruses.

As of now, our knowledge of ACE2-using merbecoviruses has been primarily derived from experiments using VSV-pseudotyped viruses. These non-replicating and non-pathogenic pseudoviruses serve as surrogates for investigating viral entry mechanisms (Letko et al., 2020). However, many key scientific questions regarding the pathogenesis, transmission, tissue tropism, and immune responses of these ACE2-using merbecoviruses have to be addressed using authentic viruses, both *in vitro* and *in vivo*. Nevertheless, it is advisable to seek the assessment of a Biosafety committee before isolation or rescue these viruses due to our limited knowledge of their pathogenicity.

Taken together, since it is nearly impossible to entirely eradicate all ACE2-using merbecoviruses from nature, and it is challenging to avoid any potential contact with these viruses, it is imperative for us to improve surveillance of these viruses in their natural habitat. Furthermore, we should enhance our understanding of these viruses and prepare a repertoire of antiviral strategies to confront potential future spillovers and outbreaks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellin.2023.100145.

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