Functional and Genetic Analysis of Viral Receptor ACE2 Orthologs Reveals Broad Potential Host Range of SARS-CoV-2

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Abstract

The pandemic of a newly emerging coronavirus (SARS-CoV-2), the causative agent of severe pneumonia disease (COVID-19), is a major global health threat. Epidemiological studies suggest that bats are the natural zoonotic reservoir for SARS-CoV-2, however, the host range of SARS-CoV-2 and the identity of intermediate hosts that may facilitate the transmission to humans remains unknown. Coronavirus–receptor interaction is a key genetic determinant of the host range, cross-species transmission, and tissue tropism. SARS-CoV-2 uses Angiotensin-converting enzyme II (ACE2) as the receptor to enter its host cells in a species-dependent manner. It has been shown that human, palm civet, pig and bat ACE2 can support virus entry, while the murine ortholog cannot. In this study, we aimed to characterize ACE2 from diverse species for its ability to support viral entry. We found that ACE2 is expressed in a wide range of host species, with high conservation especially in mammals. By analyzing critical amino acid residues in ACE2 for virus entry, based on the well-characterized SARS-CoV spike protein interaction with ACE2 (human, bat, palm civet, pig and ferret ACE2), we identified approximately eighty ACE2 proteins from mammals could potentially function as the receptor to mediate SARS-CoV-2 entry. Functional assays showed that 44 of these mammalian ACE2 orthologs, including domestic animals, pet animals, livestock animals and even animals in the zoos or aquaria, could bind viral spike protein and support SARS-CoV-2 entry. In summary, our study demonstrates that ACE2 from a remarkably broad range of species support SARS-CoV-2 entry. These findings highlight a potentially broad host tropism and suggest that SARS-CoV-2 might be distributed much more widely than previously recognized, emphasizing the necessity to monitor the susceptible hosts, especially their potential of cross-species, which could prevent the future outbreaks.

Key words: COVID-19, SARS-CoV-2, ACE2, Host range, Intermediate host

Introduction

Coronaviruses are a group of positive-stranded, enveloped RNA viruses that circulate broadly among humans, other mammals, and birds, causing respiratory, enteric, or hepatic diseases (Perlman and Netland, 2009). In the last two decades, coronaviruses have caused two major outbreaks: severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) (Graham et al., 2013). As of April 24, 2020, the recent outbreak of a new severe pneumonia disease (COVID-19) has already caused 2.7 million infections, leading to 192,000 deaths. The pathogen responsible is a novel coronavirus, SARS-CoV-2 (Wu et al., 2020; Zhou et al., 2020). Phylogenetic analyses suggest that SARS-CoV, MERS-CoV and SARS-CoV-2 likely originated from bats, with SARS-CoV spreading from bats to palm civets to humans, and MERS-CoV spreading from bats to camel to humans (Cui et al., 2019). However, the intermediate host of SARS-CoV-2, fueling spillover to humans, remains unknown.

The SARS-CoV-2 genome encodes a spike (S) protein, the receptor-binding domain of which binds the cellular receptor angiotensin-converting enzyme II (ACE2) to mediate viral entry (Hoffmann et al., 2020; Zhou et al., 2020). Following binding of ACE2, the S protein is subsequently cleaved by the host transmembrane serine protease 2 (TMPRSS2) to release the spike fusion peptide, promoting virus entry into target cells (Hoffmann et al., 2020; Wan et al., 2020). It has been repeatedly demonstrated that the interaction of a virus with (a) species-specific receptor(s) is a primary determinant of host tropism and therefore constitutes a major interspecies barrier at the level of viral entry (Douam et al., 2015). For example, murine ACE2 does not efficiently bind the SARS-CoV or SARS-CoV-2 S protein, hindering viral entry into murine cells; consequently, a human ACE2 transgenic mouse was developed as an in vivo model to study the infection and pathogenesis of these two viruses (Bao et al., 2020; Yang et al., 2007).

ACE2 is expressed in a diverse range of species throughout the subphylum *Vertebrata*. Several recent studies demonstrated that ferrets, cats, dogs and some non-human primates are susceptible to SARS-CoV-2 (Kim et al., 2020; Lu et al., 2020; Rockx et al., 2020; Shi et al., 2020; Zhang et al., 2020a). However, the exact host tropism of SARS-CoV-2 remains unknown and is an urgent area to explore for identifying other putative zoonotic reservoirs. In this study, we surveyed ACE2 orthologs from a broad range of species for their ability to support SARS-CoV-2 entry. Our data demonstrate that an evolutionarily diverse set of ACE2 species variants can mediate SARS-CoV-2 glycoprotein-dependent uptake, suggesting that SARS-CoV-2 has a broad host range at the level of virus entry that may contribute to cross-species transmission and viral evolution.

RESULTS

Evolutionary and phylogenetic analysis of ACE2 orthologs from a diversity of species

ACE2, the cellular receptor for SARS-CoV-2 and SARS-CoV, is expressed in a diverse range of vertebrate animals. We analyzed 294 ACE2 orthologs in the NCBI database, including from birds (75 species), alligators (4 species), turtles (4 species), lizards (9 species), mammals (129 species), amphibians (4 species), coelacanths (1 species), bone fish (67 species) and cartilaginous fish (1 species) (Fig. S1). These ACE2 orthologs range from 344 to 861 amino acid residues in length and are characterized by an N-terminal leucine-rich repeat (LRR) domain and a C-terminal low-complexity acidic region (LCAR). Structures of SARS-CoV S protein complexed with human ACE2 or other orthologs have been solved, and five critical, highly conserved amino acid residues of ACE2 that are indispensable for interaction with S protein and viral entry have been identified (Li, 2008, 2015; Li et al., 2005; Wan et al., 2020). Based on this structural information and conservation of these 5 critical residues, we carried out primary sequence alignment across the 294 ACE2 proteins (Fig. S1). Our analysis found ACE2 orthologs from 80 species that could potentially function as the receptors of SARS-CoV-2 (Fig. S1, and Fig 1). All of the 80 ACE2 orthologs were derived from mammals, including wild animals, domestic animals, pets, livestock and even animals in the zoo or aquaria, with protein size ranging from 790 to 811 amino acids (Table S1). Other ACE2 orthologs from mammals (49/129 species) that were predicted as non-functional receptors of SARS-CoV and SARS-CoV-2, including mouse and rat, are summarized in Table S2 and were not included in the experiments described in the present study.

We performed phylogenetic analysis of these 80 ACE2 orthologs to explore their potential function in mediating virus infection and to gain insights into the evolution of the ACE2 protein. Additionally, we aligned the twenty residues of ACE2 located at the interface with the SARS-CoV-2 S protein (Lan et al., 2020; Shang et al., 2020; Yan et al., 2020) (Fig. 1). The ACE2 protein sequences were highly conserved across the species we analyzed. Of note, the twenty residues at the ACE2-S protein interface were identical across the Catarrhini, which includes great apes and Old World monkeys. However, these residues in the ACE2 orthologs of New World monkeys were less conserved. For example, Y41 and Q42 in human ACE2 are responsible for the formation of hydrogen bonds with S protein and are highly conserved across all other species but in New World monkeys are substituted by H and E, respectively. In non-primate mammals, an increasing number of substitutions are evident, even in the residues such as Q24, D30, D38, and Y83 that form hydrogen bonds or salt-bridges with S protein (Fig. 1).

Collectively, our analysis suggests that ACE2 orthologs are highly conserved across a wide range of mammals, and many of these ACE2 orthologs might function as an entry receptor for SARS-CoV-2.

Interaction of ACE2 proteins with SARS-CoV-2 spike protein

Based on our evolutionary analysis, we chose 48 representative ACE2 orthologs (Table S1 and Fig. 1) from *Primates*, *Rodentia*, *Cetartiodactyla*, *Chiroptera*, *Diprotodontia*, *Perissodactyla*, *Carnivora* and *Pholidota* and assessed whether they support SARS-CoV-2 entry by ectopically expressing each ortholog in HeLa cells, which have limited endogenous ACE2 expression (Zhou et al., 2020). These 48 species include wild animals; animals in the zoo and aquaria; pets and livestock frequently in close contact with humans; some model animals used in biomedical research; and endangered species (Fig. 1).

Binding of SARS-CoV-2 S protein to ACE2 is a prerequisite for viral entry. To examine this interaction, we employed a cell-based assay that used flow cytometry to assess binding. We cloned the cDNA of 49 ACE2 orthologs (mouse ACE2 was included as a negative control), each with a C-terminal FLAG tag, into a bicistronic lentiviral vector (pLVX-IRES-zsGreen1) that expresses the fluorescent protein zsGreen1 via an IRES element to monitor transduction efficiency. Next, a purified fusion protein consisting of the S1 domain of SARS-CoV-2 S protein and an Fc domain (S1-Fc) was incubated with HeLa cells transduced to express the ACE2 orthologs. Binding of S1-Fc to ACE2 was then quantified by flow cytometry, with the binding efficiency defined as the percentage of cells positive for S1-Fc binding within zsGreen1+ cells. As expected, the binding of mouse ACE2 to S1-Fc was very low and comparable to the empty vector control, whereas human ACE2 efficiently bound S1-Fc protein, consistent with previous findings (Wan et al., 2020; Zhou et al., 2020). Surprisingly, we found that ACE2 from 44/49 species could bind the S1-Fc protein, albeit slightly less efficient than human ACE2 (Fig. 2A). In contrast, ACE2 from *Callithrix jacchus* (marmoset, #11), *Sapajus apella* (tufted capuchin, #12), *Saimiri boliviensis boliviensis* (squirrel monkey, #13), –all New World monkeys– did not bind S1-Fc and ACE2 from *Phascolarctos cinereus* (koala, #34) and *Mustela ermine* (stoat, #44) bound only poorly to the S1-Fc fusion (Fig. 2A).

The limited or undetectable interaction of certain ACE2 orthologs with the S1-Fc protein was not due to low expression of ACE2. The expression of ACE2 orthologs in HeLa cells following transduction was assessed by Western blot using an anti-FLAG antibody. All 49 ACE2 proteins were readily detected at the expected size of 100-130 kDa. The differences in molecular weights are likely attributable to their varying degrees of glycosylation (Fig. 2B). Our findings are consistent with the recent report that experimental SARS-CoV-2 infection could be established in Old World monkeys (*Macaca mulatta* and *Macaca fascicularis*) but not in New World monkeys (*Callithrix jacchus*, marmoset) (Lu et al., 2020).

In summary, these results demonstrate that ACE2 proteins from a broad range of diverse species can bind the SARS-CoV-2 S protein, suggesting that these species may indeed be capable of mediating viral uptake.

Functional assessment of ACE2 orthologs in SARS-CoV-2 entry

It has been shown that HeLa cells lacking expression of endogenous ACE2 were not permissive to SARS-CoV-2 infection (Zhou et al., 2020). To test directly whether different ACE2 orthologs can indeed mediate viral entry, we performed genetic complementation experiments in HeLa cells.

HeLa cells ectopically expressing individual ACE2 orthologs were infected with SARS-CoV-2 (MOI=1). At 48 h post-infection, the complemented HeLa cells were fixed and underwent immunofluorescent staining for intracellular viral nucleocapsid protein, an indicator of virus replication. As expected, HeLa cells expressing mouse ACE2 were not permissive to SARS-CoV-2 infection while those expressing human ACE2 were permissive. Consistent with our binding data, HeLa cells expressing ACE2 orthologs from marmoset (#11), tufted capuchin (#12), squirrel monkey (#13) or koala (#34) were non-permissive to SARS-CoV-2 infection. HeLa cells expressing ACE2 from stoat (#44) were permissive, albeit with low efficiency; the remaining 44 ACE2 orthologs supported SARS-CoV-2 infection, as evidenced by nucleocapsid protein readily detectable within ACE2-expressing (zsGreen1+) cells (Fig. 3).

Collectively, our results demonstrate that SARS-CoV-2 can utilize ACE2 from evolutionarily diverse species of mammals as a cellular receptor for viral entry, suggesting that SARS-CoV-2 may have a broad host range.

The potential genetic determinants of ACE2 from New World monkeys that restrict SARS-CoV-2 entry

Although the overall protein sequences of ACE2 were largely conserved across all tested species (71%–100% identity compared with human ACE2) (Fig.4A), this does not necessarily correlate with its function to support virus entry. For example, as shown in Fig. 3 and Fig. 4, ACE2 orthologs from the New World monkeys marmoset (#11), tufted capuchin (#12), and squirrel monkey (#13) had limited or undetectable ability to mediate SARS-CoV-2 entry despite sharing 92-93% identity with human ACE2. In contrast, the ACE2 proteins from *Bos taurus* (cattle, #28) or *Sus scrofa* (pig, #20) efficiently facilitated virus entry, with 78% or 81% identity, respectively, to human ACE2 (Fig. 4A). Thus, we hypothesized that changes in critical residues in ACE2 proteins from New World monkeys may restrict viral entry.

New World monkeys are widely used in biomedical research. Our results showed that their ACE2 proteins do not bind SARS-CoV-2 S protein and do not promote virus entry, which is in line with a recent finding that *Callithrix jacchus* (marmoset) is resistant to SARS-CoV-2 infection (Lu et al., 2020). To identify the genetic determinants within ACE2 orthologs from New World monkeys that restrict viral entry, we analyzed the ACE2 protein residues that contact the S protein, especially those that form hydrogen bonds or salt bridges with S protein, such as Q24, D30, E35, E37, D38, Y41, Q42, Y83, K353 and R393 (Lan et al., 2020; Shang et al., 2020; Yan et al., 2020). When aligned with orthologs that support SARS-CoV-2 entry, we found that residues at the

ACE2-S interface in New World monkeys only differed from humans at H41Y and E42Q (Fig.1). The hydroxyl group of the Tyr at human ACE2 position 41 forms hydrogen bonds with the side chain oxygen atom of T500 and side chain nitrogen atom of N501 in the SARS-CoV-2 S protein. The side chain nitrogen atom of Q42 of human ACE2 forms hydrogen bonds with the main chain oxygen atom of G446 and side chain hydroxyl group of Y449 of the SARS-CoV-2 S protein. Changes at these two consecutive residues, 41 and 42, may disrupt critical hydrogen-bonding interactions and thus impair the binding of New World monkey ACE2 with SARS-CoV-2 S protein (Fig. 4B).

Thus, our analysis identifies the potential genetic determinants of ACE2 function as the SARS-CoV-2 cellular receptor and provides greater insight into the species-specific restriction of viral entry, which can inform the development of animal models.

Discussion

To prevent the zoonotic transmission of SARS-CoV-2 to humans, the identification of animal reservoirs or intermediate hosts of SARS-CoV-2 is of great importance. Recently, a coronavirus was identified in pangolins with 90% sequence identity compared to SARS-CoV-2 (Lam et al., 2020; Zhang et al., 2020b). However, the result of such phylogenetic analysis does not necessarily support the notion that pangolins are indeed an intermediate host of SARS-CoV-2. The host range and animal reservoirs of SARS-CoV-2 remain to be explored.

For the cross-species transmission of SARS-CoV-2 from intermediate hosts to humans, the virus needs to overcome at least two main host genetic barriers: the specificity of viral S protein-ACE2 receptor interactions and the ability to escape the host's antiviral immune response. The interaction of a virus with its host cell receptor is the first step to initiate virus infection and is a critical determinant of host species range and tissue tropism. SARS-CoV-2 uses cellular receptor ACE2 in a species-specific manner: human, palm civet, bat and pig ACE2 can support virus entry whereas mouse ACE2 cannot (Zhou et al., 2020). To explore the possible SARS-CoV-2 animal reservoirs and intermediate hosts, we analyzed the ACE2 genes from hundreds of vertebrates, particularly mammals. Our results suggest that ACE2 orthologs are largely conserved across vertebrate species, indicating the importance of its physiological function. Notably, we also found that ACE2 orthologs from a wide range of mammals could act as a functional receptor to mediate SARS-CoV-2 infection when ectopically expressed in HeLa cells, suggesting that SARS-CoV-2 may have a diverse range of hosts and intermediate hosts.

It is of note that our findings are based on a functional study of ACE2 proteins with authentic virus infection instead of pseudotyped virus. Our results are consistent with recent *in vivo* findings that ferrets, cats, dogs, and Old World monkeys are susceptible to SARS-CoV-2 infection but not marmoset, which is a New World monkey (Lu et al., 2020; Shi et al., 2020; Zhang et al., 2020a). The host range or specificity of a virus is often limited due to several reasons, such as the lack of host factors the virus depends on or the incompatibility of these factors' orthologs in different species. Alternatively, but not necessarily mutually exclusive, the ability to evade the antiviral immune response of a given host can also shape the species tropism of viruses (Ding et al., 2018; Douam et al., 2015).

Development of prophylactic vaccines or effective antivirals are urgently needed to combat SARS-CoV-2 infection (Rome and Avorn, 2020). Establishment of better animal models to evaluate the efficacy of vaccine candidates and antiviral strategies *in vivo* is thus of utmost importance. Additionally, there is a need for suitable, experimentally tractable animal models to dissect mechanistically viral transmission and pathogenesis. Human ACE2 transgenic mice have been used to study SARS-CoV and SARS-CoV-2 *in vivo* (Bao et al., 2020; Yang et al., 2007). However, the unphysiologically high expression level of ACE2 driven by the ubiquitous K14 promoter may not recapitulate the human disease caused by SARS-CoV-2. Recently, a ferret

model of SARS-CoV-2 infection was established that mimics transmission and recapitulates aspects of human disease (Kim et al., 2020). Our study found that ACE2 from multiple species of laboratory animals, including but not limited to ferrets, crab-eating macaques, and Chinese hamsters could be utilized by SARS-CoV-2 to mediate viral infection. Our data provide a rationale to assess the susceptibility of such species whose ACE2 ortholog serves as a functional receptor for SARS-CoV2.

Our results further demonstrate that ACE2 orthologs from marmoset (#11), tufted capuchin (#12), and squirrel monkey (#13), all New World monkeys, do not support SARS-CoV-2 entry. We identified specific residues - H41 and E42 – within ACE2 that likely restrict SARS-CoV2 in these species. It is worthwhile to determine whether swapping these critical amino acids with human residues rescues the entry of SARS-CoV-2.

Our unexpected finding that SARS-CoV-2 uses ACE2 from diverse species highlights the importance of surveilling animals in close contact with humans as potential zoonotic reservoirs. We found that pets such as cats and dogs, livestock such as pigs, cattle, rabbits, sheep, horses, and goats, and even some animals kept frequently in zoos or aquaria may serve as the intermediate hosts for virus transmission. Our study also identified the broad range of wild animals as potential susceptible hosts of SARS-CoV-2, highlighting the importance of banning illegal wildlife trade and consumption.

In summary, ours is the first study to systematically assess the functionality of ACE2 orthologs from nearly 50 mammalian hosts using the authentic SARS-CoV-2 virus, which provides new insight into the potential host range and cross-species transmission of this virus. It also suggests that SARS-CoV-2 might be much more widely distributed than previously thought, underscoring the necessity of monitoring susceptible hosts, especially their potential for causing zoonosis, which could aid in preventing future outbreaks.

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MATERIALS AND METHODS

Cell cultures and SARS-CoV-2 virus. HEK293T cells (American Tissue Culture Collection, ATCC, Manassas, VA, CRL-3216), Vero E6 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) and HeLa (ATCC #CCL-2) were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, NY, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 10mM HEPES, 1mM sodium pyruvate, 1×non-essential amino acids, and 50 IU/ml penicillin/streptomycin in a humidified 5% (vol/vol) CO2 incubator at 37°C. Cells were tested routinely and found to be free of mycoplasma contamination. The SARS-CoV-2 strain nCoV-SH01 (GenBank accession no. MT121215) was isolated from a COVID-19 patient and propagated in Vero E6 cells for use. All experiments involving virus infections were performed in the biosafety level 3 facility of Fudan University following the regulations.

Plasmids. The cDNAs encoding ACE2 orthologs (Table S1) were synthesized by GenScript and cloned into pLVX-IRES-zsGreen1 vectors (Catalog No. 632187, Clontech Laboratories, Inc) with a C-terminal FLAG tag. All the plasmids were verified by Sanger sequencing.

Lentivirus production. Vesicular stomatitis virus G protein (VSV-G) pseudotyped lentiviruses expressing ACE2 orthologs tagged with FLAG at the C-terminus were produced by transient co-transfection of the third-generation packaging plasmids pMD2G (Addgene #12259) and psPAX2 (Addgene #12260) and the transfer vector with VigoFect DNA transfection reagent (Vigorous) into HEK293T cells. The medium was changed 12 h post transfection. Supernatants were collected at 24 and 48h after transfection, pooled, passed through a 0.45-μm filter, and frozen at -80°C.

Phylogenetic analysis and sequence alignment. The amino acid sequences of ACE2 orthologs for jawed vertebrates (Gnathostomata) were exported from the NCBI nucleotide database. Numbers in each sequence correspond to the GenBank accession number. 81 sequences were collected for the presence of five critical viral spike-contacting residues of ACE2 corresponding to amino acids Lys31, Glu35, Asp38, Met82 and Lys353 in human ACE2 (NM_001371415.1). The protein sequences of different species were then passed into MEGA-X (Version 10.05) software for further analysis. The alignment was conducted using the MUSCLE algorithm (Edgar, 2004). Then the alignment file was used to construct the phylogenetic tree (Neighbor Joining option of the MEGA-X with default parameter).

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting was performed as follows: After trypsinization and cell pelleting at 2,000 × g for 10 min, whole-cell lysates were harvested in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma). Lysates were electrophoresed in 12% polyacrylamide gels and transferred onto nitrocellulose membrane. The blots were blocked at room temperature for 0.5 h using 5% nonfat milk in 1× phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20. The

blots were exposed to primary antibodies anti-β-Tubulin (CW0098, CWBIO), or anti-FLAG (F7425, Sigma) in 5% nonfat milk in 1× PBS containing 0.1% Tween 20 for 2 h. The blots were then washed in 1× PBS containing 0.1% Tween 20. After 1h exposure to HRP-conjugated secondary antibodies, subsequent washes were performed and membranes were visualized using the Luminescent image analyzer (GE).

Surface ACE2 binding assay. HeLa cells were transduced with lentiviruses expressing the ACE2 from different species for 48 h. The cells were collected with TrypLE (Thermo #12605010) and washed twice with cold PBS. Live cells were incubated with the recombinant protein, S1 domain of SARS-CoV-2 spike C-terminally fused with Fc (Sino Biological #40591-V02H, 1μg/ml) at 4 °C for 30 min. After washing, cells were stained with goat anti-human IgG (H + L) conjugated with Alexa Fluor 647 (Thermo #A21445, 2 μg/ml) for 30 min at 4 °C. Cells were then washed twice and subjected to flow cytometry analysis (Thermo, Attune™ NxT).

Immunofluorescence staining of viral nucleocapsids. HeLa cells were transduced with lentiviruses expressing the ACE2 from different species for 48 h. Cells were then infected with nCoV-SH01 at an MOI of 1 for 1 h, washed three times with PBS, and incubated in 2% FBS culture medium for 48 h for viral antigen staining. Cells were fixed with 4% paraformaldehyde in PBS, permeablized with 0.2% Triton X-100, and incubated with the rabbit polyclonal antibody against SARS-CoV nucleocapsid protein (Rockland, 200-401-A50, 1μg/ml) at 4 °C overnight. After three washes, cells were incubated with the secondary goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Thermo #A11034, 2 μg/ml) for 2 h at room temperature, followed by staining with 4',6-diamidino-2-phenylindole (DAPI). Images were collected using an EVOS™ Microscope M5000 Imaging System (Thermo #AMF5000). Images were processed using the ImageJ program (http://rsb.info.nih.gov/ij/).

Statistics analysis. One-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test was used to test for statistical significance of the differences between the different group parameters. *P* values of less than 0.05 were considered statistically significant.

FIGURE LEGENDS

Figure 1. Phylogenetic analysis of ACE2 orthologs with potential to support SARS-CoV-2 entry and alignment of ACE2 residues at the interface with the viral spike protein. The ACE2 protein sequences (Supplemental Table 1), as well as *Mus musculus* (mouse) and *Rattus norvegicus* (rat) ACE2, were chosen and analyzed by MEGA-X (Version 10.05) software and MUSCLE algorithm. The phylogenetic tree was built using Neighbor Joining method of the MEGA-X. The contacting residues of human ACE2 (distance cutoff of 4 Å) at the SARS-CoV-2 receptor binding domain (RBD)/ACE2 interface are shown. The contacting network involves at least 20 residues in ACE2 and 10 residues in the SARS-CoV-2 RBD, which are listed and connected by a solid line. Black lines indicate hydrogen bonds, and the red line represents a salt-bridge interaction. The tested species are highlighted in purple and the ID number of each species in subsequent experiments is labeled on the right. Only the amino acids different from human are shown.

Figure 2. Binding of the SARS-CoV-2 spike protein to different ACE2 orthologs. (A) HeLa cells were transduced with ACE2 orthologs of the indicated species, incubated with the recombinant S1 domain of SARS-CoV-2 spike C-terminally fused with Fc, and then stained with goat anti-human IgG (H + L) conjugated to Alexa Fluor 647 for flow cytometry analysis. Binding efficiency was defined as the percentage of cells positive for S1-Fc binding within zsGreen1+ cells. Values are means plus standard deviations (SD) (error bars). ns, no significance; ***, $P < 0.001$. Significance assessed by one-way ANOVA. (B) HeLa cells transduced with lentiviruses expressing FLAG-tagged ACE2 orthologs were subjected to immunoblotting. Tubulin served as the loading control.

Figure 3. Functional assessment of ACE2 orthologs mediating SARS-CoV-2 virus entry. HeLa cells transduced with lentiviruses expressing ACE2 orthologs or empty vector were infected with SARS-CoV-2 virus (MOI=1). Expression of the viral nucleocapsid protein was visualized by immunofluorescence microscopy. Viral nucleocapsid (N) protein (red) and nuclei (blue) are shown. Green signal indicates the transduction efficiency of ACE2 orthologs. Marmoset (#11), tufted capuchin (#12), squirrel monkey (#13), and koala (#34) were non-permissive to SARS-CoV-2 infection, highlighted in purple. The images were merged and edited using Image J software.

Figure 4. Identification of the species-specific genetic determinants of ACE2 restriction of SARS-CoV-2 entry. (A) Protein sequence identity matrices of ACE2 from the tested species. The ACE2 sequences from different species were analyzed using SIAS (Sequence Identity And Similarity) tool (http://imed.med.ucm.es/Tools/sias.html) to determine the percent identity of

ACE2 proteins across different species. (B) The binding interface of human ACE2 with SARS-CoV-2 receptor-binding domain (RBD) surrounding ACE2 Y41 and Q42. Residue Y41 forms hydrogen bonds with T500 and N501 of SARS-CoV-2 RBD, and Q42 can also interact with G446 or Y449 by hydrogen bonds. The differences in ACE2 from New World monkeys, especially the Y41H replacement, may disrupt the hydrogen-bonding interactions and impair the binding with SARS-CoV-2 spike. PDB code of the complex of human ACE2 with SARS-CoV-2: 6M0J.

SUPPLEMENTAL INFORMATION

Supplemental Figure 1. **ACE2 orthologs from the jawed vertebrates**. ACE2 orthologs were recorded in the NCBI dataset and further parsed to 80 ACE2 orthologs with potential function for supporting SARS-CoV-2 entry based on conservation of the 5 amino acids required for binding between the host receptor ACE2 and the SARS-CoV spike protein (Li, 2008, 2015; Li et al., 2005; Wan et al., 2020).

FIGURES

Figure 1

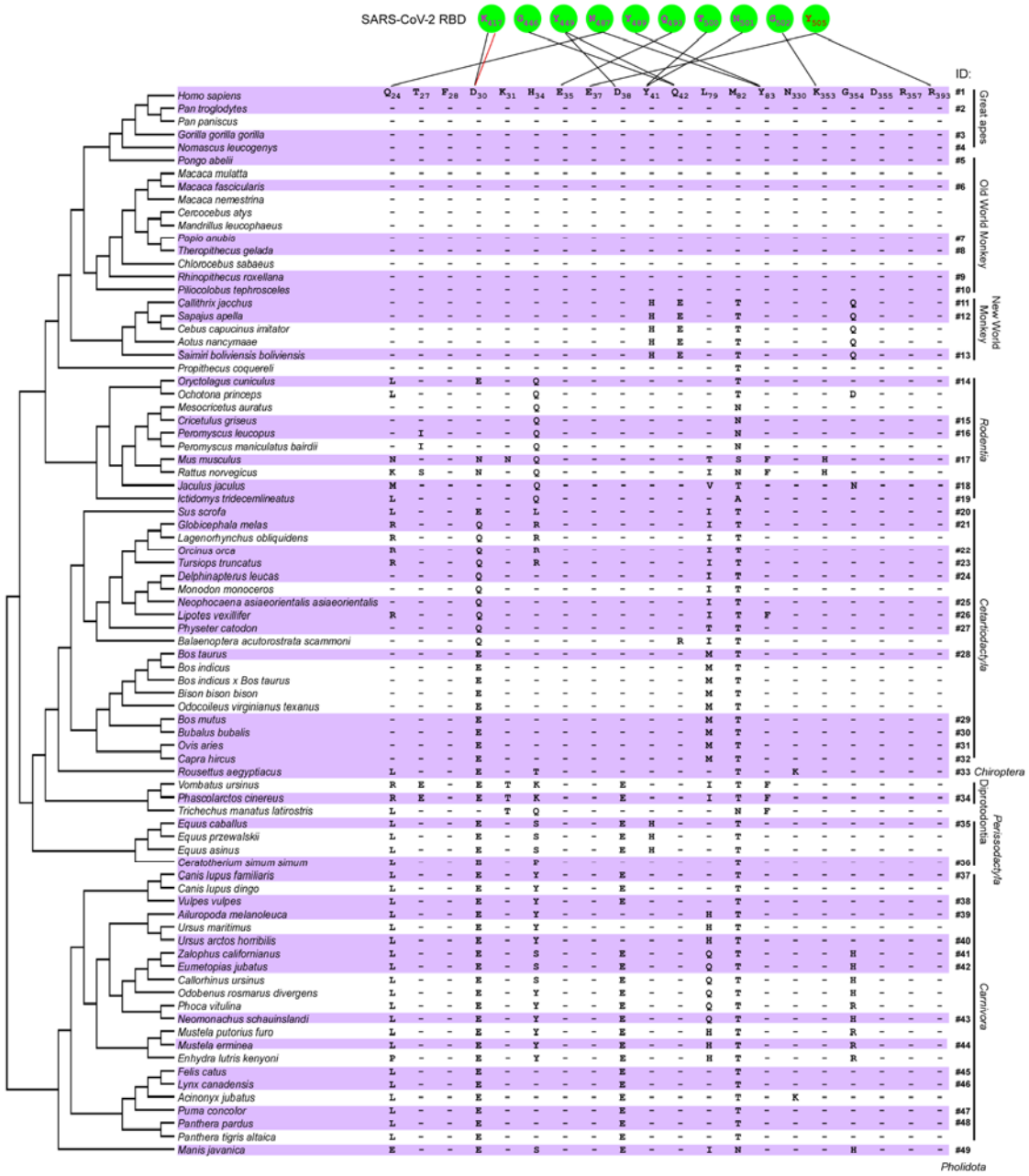


Figure 2

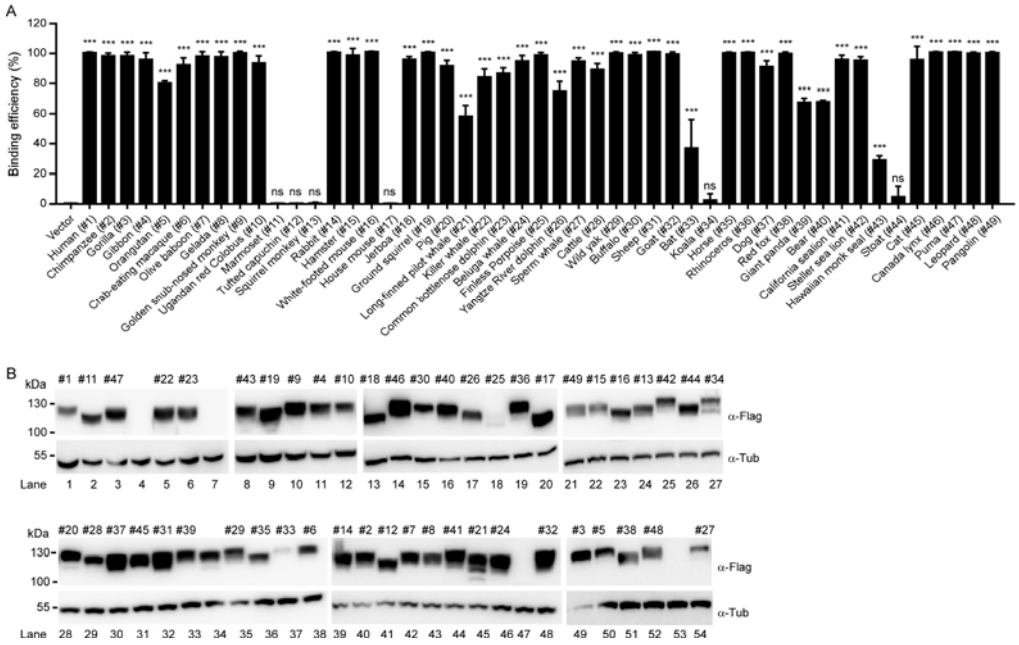
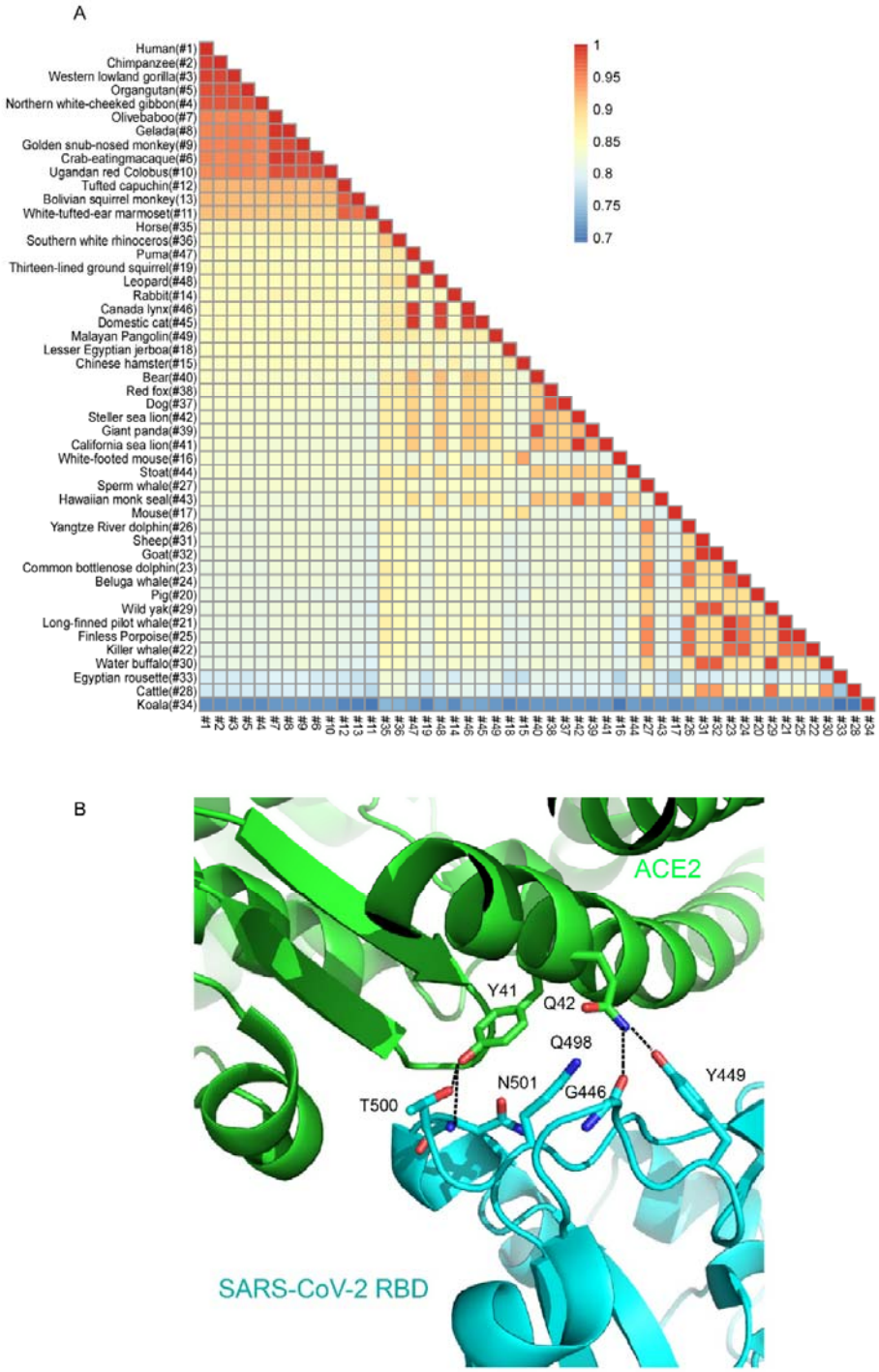
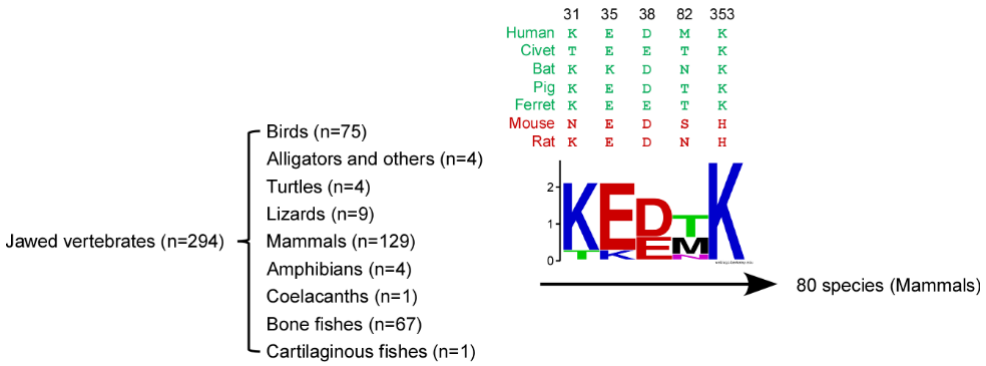


Figure 3

Figure 4



Supplemental Figure 1



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