

1 The *Rhinolophus affinis* bat ACE2 and multiple animal orthologs are functional
2 receptors for bat coronavirus RaTG13 and SARS-CoV-2
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4 Pei Li^{1#}, Ruixuan Guo^{1#}, Yan Liu^{1#}, Yintgao Zhang^{2#}, Jiaxin Hu¹, Xiuyuan Ou¹, Dan
5 Mi¹, Ting Chen¹, Zhixia Mu¹, Yelin Han¹, Zhewei Cui¹, Leiliang Zhang³, Xinquan
6 Wang⁴, Zhiqiang Wu^{1*}, Jianwei Wang^{1*}, Qi Jin^{1*}, Zhaohui Qian^{1*}
7 NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen
8 Biology, Chinese Academy of Medical Sciences and Peking Union Medical College¹,
9 Beijing, 100176, China; School of Pharmaceutical Sciences, Peking University²,
10 Beijing, China; Institute of Basic Medicine³, Shandong First Medical University &
11 Shandong Academy of Medical Sciences, Jinan 250062, Shandong, China; The
12 Ministry of Education Key Laboratory of Protein Science, Beijing Advanced
13 Innovation Center for Structural Biology, Beijing Frontier Research Center for
14 Biological Structure, Collaborative Innovation Center for Biotherapy, School of Life
15 Sciences, Tsinghua University⁴, Beijing, China;
16
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20 #These authors contributed equally to this work.
21 *To whom correspondence should be addressed: zqian2013@sina.com,
22 jinqi@ipbcams.ac.cn, wangjw28@163.com, wuzq2009@ipbcams.ac.cn
23

24 **Abstract**

25 Bat coronavirus (CoV) RaTG13 shares the highest genome sequence identity with
26 SARS-CoV-2 among all known coronaviruses, and also uses human angiotensin
27 converting enzyme 2 (hACE2) for virus entry. Thus, SARS-CoV-2 is thought to have
28 originated from bat. However, whether SARS-CoV-2 emerged from bats directly or
29 through an intermediate host remains elusive. Here, we found that *Rhinolophus affinis*
30 bat ACE2 (RaACE2) is an entry receptor for both SARS-CoV-2 and RaTG13,
31 although RaACE2 binding to the receptor binding domain (RBD) of SARS-CoV-2 is
32 markedly weaker than that of hACE2. We further evaluated the receptor activities of
33 ACE2s from additional 16 diverse animal species for RaTG13, SARS-CoV, and
34 SARS-CoV-2 in terms of S protein binding, membrane fusion, and pseudovirus entry.
35 We found that the RaTG13 spike (S) protein is significantly less fusogenic than
36 SARS-CoV and SARS-CoV-2, and seven out of sixteen different ACE2s function as
37 entry receptors for all three viruses, indicating that all three viruses might have broad
38 host ranges. Of note, RaTG13 S pseudovirions can use mouse, but not pangolin ACE2,
39 for virus entry, whereas SARS-CoV-2 S pseudovirions can use pangolin, but limited
40 for mouse, ACE2s enter cells. Mutagenesis analysis revealed that residues 484 and
41 498 in RaTG13 and SARS-CoV-2 S proteins play critical roles in recognition of
42 mouse and human ACE2. Finally, two polymorphous *Rhinolophous sinicus* bat
43 ACE2s showed different susceptibilities to virus entry by RaTG13 and SARS-CoV-2
44 S pseudovirions, suggesting possible coevolution. Our results offer better
45 understanding of the mechanism of coronavirus entry, host range, and virus-host
46 coevolution.

47

48 Introduction

49 Coronavirus disease 2019 (COVID-19) is caused by a newly emerged
50 coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first
51 identified in late 2019 in Wuhan, China¹⁻⁴, and currently it has spread to over 200
52 countries. On March 11, the World Health Organization declared a global pandemic of
53 COVID-19. As of August 23rd, there are more than 23 million confirmed cases and
54 over 800,000 deaths caused by SARS-CoV-2 worldwide⁵.

55 Phylogenetically, coronaviruses (CoVs) are classified into four genera, alpha,
56 beta, gamma, and delta, and beta-CoVs are further divided into four lineages, A, B, C,
57 and D. SARS-CoV-2 is a lineage B beta-CoV, including SARS-CoV and bat SARS-
58 like CoVs (SL-CoV)^{6,7}. The genome of SARS-CoV-2 shares approximately 80% and
59 96.2% nucleotide sequence identity with SARS-CoV and bat SL-CoV RaTG13,
60 respectively³. The high sequence homology between SARS-CoV-2 and bat SL-CoVs
61 suggests that SARS-CoV-2 might originate from bats^{3,8,9}. However, whether zoonotic
62 transmission from bats to humans is direct or through an intermediate animal host
63 remains to be determined.

64 CoVs use their trimeric spike (S) glycoproteins to bind the receptors and mediate
65 virus entry, and the interaction between the S protein and its cognate receptor largely
66 determines the virus host range and tissue tropism. The S protein contains two
67 subunits, S1 and S2. While S1 binds to the receptor, S2 contains the membrane fusion
68 machinery. Recently we and others showed that SARS-CoV-2 uses human angiotensin
69 converting enzyme 2 (hACE2) as the entry receptor^{3,10,11}. The structure of hACE2 and

70 the SARS-CoV-2 S protein or receptor binding domain (RBD) complex was also
71 solved recently¹²⁻¹⁵, and there are extensive interactions between the SARS-CoV-2 S
72 protein and hACE2, including 17 residues in the S protein and 20 residues in hACE2
73 (Table 1). Several critical residues, such as K31 and K353 in hACE2 and F486 and
74 Q498 in the S protein, were also identified. Many animals, including cats, ferrets,
75 minks, tigers, hamsters, dogs in lesser degree, are susceptible to SARS-CoV-2
76 infection¹⁶⁻²², indicating the potential broad host range of SARS-CoV-2.

77 RaTG13 was first discovered in the *Rhinolophus affinis* bat³, and it can use
78 hACE2 for virus entry^{13,23}. CryoEM structure of its S protein in prefusion
79 conformation was also solved, and all three monomers in trimeric S proteins are in
80 “down” position²⁴, revealing more stable in native conformation and significantly
81 lower affinity to hACE2 than SARS-CoV-2 S protein. Recently, Li *et al* reported that
82 SARS-CoV-2 and RaTG13 can use several domesticated animal orthologs of hACE2
83 for virus entry²³. However, whether RaACE2 is a functional receptor for RaTG13 and
84 SARS-CoV-2 remains unknown. In this study, we determined the susceptibility of 17
85 diverse animal species including *Rhinolophus affinis* to SARS-CoV-2 and RaTG13
86 viruses by using their S pseudovirions, and found that RaACE2 and several other
87 ACE2s could efficiently mediate the entry of SARS-CoV-2, SARS-CoV, and RaTG13
88 virus. We further identified two residues, 484 and 498, that are critical for recognition
89 of mouse and human ACE2s

90 **Results**

91 To investigate the potential intermediate host for SARS-CoV-2, we determined

92 the receptor usage and host range of RaTG13 using a pseudotype system. We also
93 included the S protein of ZC45 in our study, sharing approximately 88% of genome
94 nucleotide sequence identity with that of SARS-CoV-2^{2,25}. Previously we found that
95 removal of a conserved ER-retention motif, KxHxx, increased the level of S protein
96 present on cell surface and incorporation into lentiviral pseudovirions^{10,26}. Sequence
97 alignment of the S proteins of SARS-CoV, SARS-CoV-2, RaTG13, and ZC45
98 revealed that KxHxx motif was also present on the S proteins of RaTG13 and ZC45
99 (Fig 1A). The last 19 amino acids of the S proteins of RaTG13 and ZC45 were
100 removed and a 3xFLAG tag was also added to C-terminus of S proteins for detection.
101 The plasmids encoding the S proteins of RaTG13 and ZC45 were transfected into
102 293T cells, and the levels of S protein expression were evaluated by western blot
103 using various antibodies. The S proteins of RaTG13 and ZC45 were expressed at
104 levels similar to those of SARS-CoV and SARS-CoV-2, and they were readily
105 detected by monoclonal anti-FLAG M2 antibody (Fig 1B) and polyclonal anti-SARS-
106 CoV-2 S2 antibodies (Supplementary Fig 1A), suggesting that the immunoepitope(s)
107 for anti-SARS-CoV-2 S2 antibodies were also conserved among all four CoVs. The S
108 proteins of SARS-CoV-2 and RaTG13 were also detected by anti-SARS-CoV-2 RBD
109 antibodies, but weakly bound to rabbit polyclonal anti-SARS S1 antibody T62 and
110 mouse monoclonal anti-SARS S1 antibody MM02 (Supplementary Figs 1B, 1C, and
111 1D).

112 The level of S protein incorporation on pseudovirions was also evaluated. The S
113 proteins of RaTG13 and ZC45 were efficiently incorporated into pseudovirions (Fig

114 1B and Supplementary Fig 1E). Next, we determined whether the RaTG13 and ZC45
115 S proteins can use any known coronavirus receptors for viral entry. The pseudovirions
116 were used to transduce HEK293 cells stably expressing hACE2 (293/hACE2),
117 HEK293 cells stably expressing hDPP4 (293/hDPP4), BHK cells stably expressing
118 human aminopeptidase N (BHK/hAPN), or HEK293 cells stably expressing mouse
119 carcinoembryonic antigen related cell adhesion molecule 1a (293/mCEACAM1a).
120 SARS-CoV and SARS-CoV-2 S pseudovirions were used as controls. As expected,
121 SARS-CoV and SARS-CoV-2 S pseudovirions utilized only hACE2, not
122 mCEACAM1a, hDPP4, or hAPN, for virus entry (Fig 1C). While 293/hDPP4,
123 BHK/hAPN, and 293/mCEACAM1a cells only showed background level of
124 transduction with RaTG13 S pseudovirions, 293/hACE2 cells gave approximately
125 850-fold increase in luciferase activities over the HEK293 control when transduced
126 by pseudovirions with RaTG13 S proteins, indicating that SL-CoV RaTG13 could use
127 hACE2 as the entry receptor, in agreement with previous report^{13,23}. In contrast,
128 pseudovirions with ZC45 S protein did not transduce any cells effectively, indicating
129 that SL-CoV ZC45 could not use any of them for virus entry.

130 Because RaTG13 virus was initially and only discovered in specimens from a
131 single *Rhinolophus affinis* bat, we then investigated whether RaACE2 could also be
132 the entry receptor for RaTG13 virus or not. The binding of RaACE2 to the S protein
133 of RaTG13 was first evaluated. HEK293 cells transiently expressing RaACE2
134 proteins (Fig 1D) were incubated with soluble RaTG13 receptor binding domain
135 (RBD) and their affinities were measured by flow cytometry. The RaTG13 RBD

136 bound to RaACE2 proteins efficiently, at a level similar to that of hACE2 (Fig 1E,
137 bottom panel). Of note, RaACE2 also bound to the SARS-CoV-2 RBD, but the
138 affinity was significantly weaker than that of the hACE2/SARS-CoV-2 RBD (Fig 1E,
139 top panel). The mean fluorescence intensity (MFI) of RaACE2/SARS-CoV-2 RBD
140 interaction was less than 1/3 of that of the hACE/SARS-CoV-2 RBD (Fig 1F).
141 RaTG13 RBD also demonstrated slightly weaker binding to hACE2 than SARS-CoV-
142 2 RBD. Next, we determined whether RaACE2 could mediate the entry of RaTG13
143 and SARS-CoV-2 viruses. RaTG13 S pseudovirions entered 293/RaACE2 cells at a
144 level similar to hACE2, whereas SARS-CoV-2 S pseudovirions also transduced
145 293/RaACE2 cells efficiently, at slightly lower levels than hACE2 (Fig 1G). RaACE2
146 is a functional entry receptor for both RaTG13 and SARS-CoV-2 viruses.

147 Recently cats, civets, ferrets, minks, tigers, hamsters, dogs, and monkeys were
148 reported to be susceptible to SARS-CoV-2 infection¹⁶⁻²¹, and in silico analysis also
149 showed that ACE2 from other animals might be able to mediate SARS-CoV-2 entry²².
150 We next investigated which other animal ACE2 could confer susceptibility to RaTG13
151 virus entry. Sixteen different animal species (Table 1) were chosen, most of which are
152 commonly found in wild animal meat markets in China, and we also included
153 pangolins and two horseshoe bats (*Rhinolophus sinicus*), one from Yunnan (RS-YN
154 bat), and the other from Hubei (RS-HB bat) in this study²⁷, due to the discovery of
155 some CoVs that are highly homologous to SARS-CoV and SARS-CoV-2 in them^{9,28-}
156 ³⁰. Among the 20 residues in hACE2 making direct contact with SARS-CoV-2 S
157 proteins (Table 1), deer ACE2 differs in three positions with hACE2, squirrel has four

158 residues that are different, ACE2s of fox, camel, pig, and RS-HB bat each have five
159 residues that are different, RS-YN bat ACE2 has six residues that are different, ACE2s
160 of pangolin, ferret, and guinea pig each have seven different, both rat and mouse
161 ACE2s have eight residues different, and ACE2s of the remaining animals have nine
162 or more residues different with hACE2 (Table 1). The plasmids encoding individual
163 ACE2 proteins from these 16 different animal species (total 17, two horseshoe bats)
164 were transfected into 293 cells, and the levels of their expression in 293 cells were
165 determined by western blot (Fig 2A). While all ACE2 proteins were expressed in 293
166 cells (Fig 2A), expression levels varied among different ACE2 proteins, with the
167 lowest for deer and snake ACE2s and the largest for hedgehog ACE2. The size for
168 different ACE2 proteins also varied. While the deer ACE2 was the smallest, turtle
169 ACE2 was the biggest. The deer ACE2 sequence we obtained from Genbank seems to
170 lack the transmembrane domain (TMD) of ACE2, indicating that there might be
171 different splicing variants of ACE2 in deer. We then investigated whether all different
172 ACE2 proteins were present on the cell surface using a surface biotinylation assay.
173 Except for the deer ACE2 protein, which lacked a TMD, most ACE2 proteins were
174 present on the cell surface (Fig 2B). However, the levels of ACE2 proteins from
175 guinea pigs and snakes were significantly lower on the cell surface than on the other
176 surfaces. Integrin- β 1 was used as the positive control for cell surface membrane
177 proteins (Fig 2B). Because of the lack of TMD, the deer ACE2 was then removed
178 from the rest of the analysis.

179 Next we determined whether these different ACE2 proteins could bind to the S

180 proteins of RaTG13. For comparison purposes, we also included SARS-CoV and
181 SARS-CoV-2 in the rest of the experiments. HEK293 cells transiently expressing
182 different ACE2 proteins were incubated with soluble RaTG13, SARS-CoV, and
183 SARS-CoV-2 receptor binding domains (RBDs), and the percentage of cells that
184 bound the RBD and the level of RBD bound to different ACE2 proteins were
185 quantitated by flow cytometry (Fig 3 and Supplementary Fig 2). All RBDs of
186 RaTG13, SARS-CoV-2, and SARS-CoV bound to HEK293 cells transiently
187 expressing hACE2 protein, with RaTG13 RBD showing slightly lower levels of
188 binding than SARS-CoV and SARS-CoV-2 RBDs (Supplementary Fig 2), consistent
189 with the slightly lower transduction on 293/hACE2 by RaTG13 S pseudovirion than
190 SARS-CoV and SARS-CoV-2 S pseudovirions (Fig 1C). Fox, camel, and pig ACE2
191 proteins also gave strong binding to RBDs of RaTG13, SARS-CoV, and SARS-CoV-2
192 at levels similar to hACE2 (Fig 3). In contrast, rat ACE2 proteins also bound to all
193 three RBDs, but only at modest levels, ranging from 16% to 28% of hACE2. While
194 squirrel and mouse ACE2 proteins bound strongly to RBDs of RaTG13 and SARS-
195 CoV, they only bound to SARS-CoV-2 RBD at levels that were 36% and 12% of
196 hACE2, respectively. In contrast, pangolin ACE2 proteins showed high affinity for
197 both SARS-CoV and SARS-CoV-2 RBDs, but only weakly bound to RaTG13 RBD.
198 SARS-CoV RBD bound civet and ferret ACE2 proteins at levels similar to hACE2,
199 whereas SARS-CoV-2 RBD only showed binding to these ACE2 proteins at levels
200 that were 24% and 15% of hACE2, respectively, and RaTG13 RBD only showed
201 background level of binding. Of note, the RaTG13 and SARS-CoV-2 RBDs showed

202 modest and strong binding to the ACE2 proteins of the RS-YN bat, respectively, but
203 neither bound to the ACE2 proteins of the RS-HB bat (Fig 3A and 3B), in which there
204 were seven S protein-interacting residues differing from those of RS-YN bat (Table
205 1). In contrast, SARS-CoV RBD showed modest but consistent binding to the RS-HB
206 bat, not RS-YN bat (Fig 3C), reflecting the differences of receptor-contacting residues
207 in RBDs among the three CoVs. None of ACE2 proteins from the other animal
208 species showed any significant binding to either one of three RBDs. Overall, the
209 fewer the number of critical binding residues that differ from hACE2 (Table 1), the
210 higher the levels of binding detected. Both the RaTG13 and SARS-CoV-2 RBDs
211 showed high affinity to ACE2 proteins of five different animals at levels of 60% or
212 above that of hACE2, where SARS-CoV RBD bound to ACE2 proteins of seven
213 different animals at 60% or above that of hACE2, indicating their potential broad
214 range of hosts.

215 Membrane fusion is a prerequisite step for virus entry. We next evaluated the
216 effect of different animal ACE2 proteins on the S protein of RaTG13 mediated
217 membrane fusion by cell-cell fusion assay. SARS-CoV and SARS-CoV-2 S proteins
218 were also used for comparisons. In agreement with our previous report¹⁰, HEK293
219 cells transiently expressing hACE2 proteins showed extensive syncytium formation
220 when coincubated with 293T cells overexpressing eGFP and SARS-CoV or SARS-
221 CoV-2 S proteins in the presence of trypsin (Fig 4A). Syncytia were also induced
222 when RaTG13 S protein expressing cells were overlaid on HEK293 cells transiently
223 expressing hACE2 with trypsin. However, the frequency and size of the syncytium

224 were much lower and smaller than the S proteins of SARS-CoV and SARS-CoV-2
225 (8.7% for RaTG13, 37.3% for SARS-CoV-2, and 29.1% for SARS-CoV) (Fig 4A, 4B,
226 4C, 4D and Supplementary Figure 3). Of note, HEK293 cells expressing fox and rat
227 ACE2 proteins, and to a lesser extent, squirrel and mouse ACE2 proteins showed
228 significantly higher amount of syncytium formation than hACE2 when mixed with
229 RaTG13 S protein expressing cells and trypsin (Fig 4B), although all were present on
230 the cell surface at similar level (Fig 2B). Camel ACE2 also induced syncytia at a level
231 similar to hACE2, whereas civet, ferret and pig ACE2s showed syncytia at 65%, 49%
232 and 61% of hACE2, respectively. None of the other animal ACE2s, including ACE2s
233 from two horseshoe bats, induced marked syncytium formation by RaTG13 S
234 proteins. Overall, the cell-cell fusion results were largely in agreement with the ability
235 of ACE2 proteins binding RaTG13 RBD.

236 The S protein of SARS-CoV-2 induced extensive syncytia on HEK293 cells
237 transiently expressing squirrel, pangolin, fox, civet, camel, ferret, rat, mouse, pig, and
238 RS-YN bat ACE2 proteins (Fig 4C), although ferret, rat, and mouse ACE2 protein
239 only showed binding to the SARS-CoV-2 RBD slightly above background level (Fig
240 3B). Because several recent studies reported that mouse ACE2 is not susceptible to
241 SARS-CoV-2 infection^{3,31}, we repeated the cell-cell fusion experiments multiple times
242 with mouse ACE2 plasmids, prepared with extra caution and verified by sequencing,
243 and significant amount of syncytia were still detected (Supplementary Figure 4). All
244 other animal ACE2 proteins gave background levels of syncytia, consistent with their
245 inability to bind to the SARS-CoV-2 RBD. The overall pattern of SARS-CoV S

246 protein mediated syncytium formation on different animal ACE2 expressing 293 cells
247 was similar to that of SARS-CoV-2 S protein. Of note, although RS-YN bat ACE2 did
248 not show any marked binding to SARS-CoV RBD, it induced SARS-CoV S protein
249 mediated syncytium formation at a level of 62% of hACE2. HEK293 cells expressing
250 RS-HB bat and guinea pig ACE2 also induced noticeable syncytium formation upon
251 addition of SARS-CoV S expressing 293T cells and trypsin (Fig 4D). Overall, the S
252 proteins of SARS-CoV and SARS-CoV-2 showed much higher fusogenicity than the
253 RaTG13 S proteins.

254 Next we investigated whether ACE2 proteins from different animal species could
255 mediate virus entry by the RaTG13, SARS-CoV, and SARS-CoV-2 S proteins.
256 Lentiviral pseudovirions with VSV-G protein were used as a positive control. As
257 expected, all cells were susceptible to VSV-G pseudoviron transduction (Fig 5).
258 Compared to vector control, 293/hACE2 cells showed an over 3500-fold increase in
259 luciferase activities when transduced with RaTG13 S pseudovirions (Fig 5A). Over a
260 1000-fold increase of transduction was also detected in HEK293 cells transiently
261 overexpressing squirrel, fox, camel, and mouse ACE2 proteins (Fig 5A), indicating
262 that they might be susceptible to RaTG13 infection. Rats and pigs also seem to be
263 susceptible to RaTG13 infection, and their ACE2s resulted in over 225- and 630-fold
264 increases in luciferase activities (Fig 5A), respectively, when transduced by RaTG13
265 S pseudovirions, largely in agreement with their ability to bind to RaTG13 RBD. In
266 contrast, although civet and ferret ACE2 only showed minimal binding to the RaTG13
267 RBD, they gave close to 500- and 90-fold increases in luciferase over the vector

268 control (Fig 5A), respectively, when transduced by RaTG13 pseudovirions, indicating
269 that they might also be susceptible to RaTG13 infection. Of note, neither horseshoe
270 bat ACE2s showed high susceptibility to RaTG13 S pseudovirion transduction.
271 While RS-YN bat ACE2 gave an approximately 13-fold increase in transduction over
272 the vector control, RS-HB bat ACE2 only showed a background level of transduction
273 (Fig 5A). None of the other animal ACE2s showed a significant increase in virus
274 entry by the RaTG13 S protein.

275 Overall, SARS-CoV-2 S protein pseudovirions showed similar levels of host
276 ranges to RaTG13 among the different ACE2s we tested (Fig 5B). However, they
277 differed dramatically in susceptibility to pangolins and mice. Pangolin ACE2 was
278 susceptible to SARS-CoV-2 S-mediated transduction (Fig 5B), but not RaTG13 (Fig
279 5A), whereas mouse ACE2 was fully susceptible to RaTG13 transduction (Fig 5A),
280 but limited to SARS-CoV-2 (Fig 5B). SARS-CoV-2 S pseudovirions showed only
281 0.4% of hACE2 transduction in mouse ACE2 (Fig 5C). HEK293 transiently
282 overexpressing squirrel and pig ACE2s gave a level of transduction similar to that of
283 hACE2 (Fig 5B), although squirrel ACE2 bound to the SARS-CoV-2 RBD at a level
284 of less than 40% of that of hACE2 (Fig 3B). Fox, civet, camel, rat, or RS-YN bat
285 ACE2 proteins also gave an over 100-fold increase in luciferase activities (Fig 5C),
286 indicating that these animals might be susceptible to SARS-CoV-2 infection. Ferret
287 ACE2 also showed an approximately 35-fold increase in transduction (Fig 5C), in
288 agreement with recent studies showing that ferrets were susceptible to SARS-CoV-2
289 infection^{32,33}. RS-HB bat and guinea pig ACE2 proteins only gave approximately 15-

290 and 10-fold increases in transduction by SARS-CoV-2 S pseudovirions, in agreement
291 with their low affinity of binding to SARS-CoV-2 S protein. Compared to RaTG13
292 and SARS-CoV-2, SARS-CoV S showed broader host range. HEK293 cells
293 transiently expressing squirrel, pangolin, fox, civet, camel, ferret, rat, mouse, pig, RS-
294 YN bat, RS-HB bat, guinea pig, and koala ACE2 showed marked increases in
295 luciferase activities (Fig 5B), when transduced by SARS-CoV S pseudovirions. Of
296 note, both RS-YN and RS-HB bat ACE2 proteins showed a more than 100-fold
297 increase in transduction (Fig 5C), compared to vector control, although they exhibited
298 substantial differences in binding to the SARS-CoV RBD and syncytium formation
299 (Fig 4D).

300 To identify the residues in the S proteins of RaTG13 and SARS-CoV-2 critical for
301 the interaction and recognition of ACE2 in different animal species, we applied in
302 silico analyses of SARS-CoV-2/RaTG13 RBDs and different ACE2 interactions using
303 HAWKDOCK and PYMOL (Supplementary Fig 5), particularly focusing on mouse
304 and pangolin ACE2. In the SARS-CoV-2 RBD/hACE2 crystal structure, interactions
305 between hACE2 and the SARS-CoV-2 RBD complex consist of extensive network of
306 hydrogen bonding salt bridges and hydrophobic interactions (Supplementary Fig 5A,
307 5B, 5C and 5D)^{12,14}. F28, L79, M82, and Y83 in hACE2 form a hydrophobic pocket
308 interacting with the critical F486 in the SARS-CoV-2 S protein³⁴ (Supplementary Fig
309 5B). L79T, M82S, and Y83F changes in mouse ACE2 might collapse this
310 hydrophobic pocket and weaken the interaction with F486 in S protein
311 (Supplementary Fig 5E). The D30N change in mouse ACE2 likely abrogates the salt

312 bridge with K417 in the S protein of SARS-CoV-2, and K31N and K353H changes in
313 mouse ACE2 also likely disrupt the hydrogen bonding network between ACE2 and
314 SARS-CoV-2 S protein, resulting in mouse ACE2 acting as a poor receptor for SARS-
315 CoV-2. In contrast, K439, Y493 and Y498 in the RaTG13 S protein might make
316 hydrogen bonds with Q325, N31 and Q42 in mouse ACE2(Supplementary Fig 5E),
317 resulting in an increase in the overall affinity between mouse ACE2 and the RaTG13
318 S protein (Fig 3A) and virus entry by the RaTG13 virus (Fig 5A).

319 Pangolin ACE2 differs from human ACE2 at seven critical positions making
320 contact with the RBD (Table 1), of which three (E30, E38, and I79) are homologous
321 and four (E24, S34S, N82, and H354) are different. While these changes do not affect
322 SARS-CoV-2 RBD binding to pangolin ACE2 (Supplementary Fig 5F), they appear to
323 be detrimental to RaTG13 RBD binding (Fig 3A) and virus entry (Fig 5A). In silico
324 analysis showed that Y449F, E484T and Q493Y changes in RaTG13 S protein might
325 disrupt their hydrogen bonding with E38, K31, and E35 of pangolin ACE2,
326 respectively (Supplementary Fig 5F), resulting in weak interaction between RaTG13
327 S protein and pangolin ACE2 and poor transduction efficiency of pangolin ACE2 by
328 RaTG13 S protein (Fig 5A).

329 Based on the results from the in silico analysis, we selected residues 449, 484,
330 493, and 498 in the S proteins for further studies (Fig 6A). Single mutations F449Y,
331 T484E, Y493Q, and Y498Q were introduced into the RaTG13 S protein, and
332 individual Y449F, E484T, Q493Y, and Q498Y mutations were also introduced into the
333 SARS-CoV-2 S protein. All mutant RaTG13 S proteins were expressed as well as WT

334 in HEK293T cells and incorporated into pseudovirion efficiently (Fig 6B), whereas all
335 mutant SARS-CoV-2 S proteins except for Y493Q were expressed and incorporated
336 into pseudovirions at levels similar to WT (Fig 6C). Because Q493Y mutation in
337 SARS-CoV-2 S had significant effect on S protein incorporation into pseudovirions,
338 they were removed from further analysis. We then determined whether any mutations
339 affected virus entry using hACE2. While both the F449Y and Y498Q mutations in the
340 RaTG13 S protein significantly reduced virus entry into 293/hACE2 cells (Fig 6D),
341 indicating that both F449 and Y498 of RaTG13 might be critical for virus entry
342 through hACE2, the Y493Q substitution in the RaTG13 S protein significantly
343 increased transduction into hACE2 cells, suggesting that Q might be advantageous at
344 position 493 for interaction with hACE2. In contrast, only the Y449F mutation in the
345 SARS-CoV-2 protein showed greater than 50% reduction in infectivity in 293/hACE2
346 cells (Fig 6E). Next, we investigated whether any mutations influenced virus entry
347 into mouse and pangolin ACE2s. The overall patterns of mutant RaTG13 S
348 pseudovirion infectivity in mouse ACE2 cells were very similar to those on hACE2
349 cells except for T484E (Fig 6F and 6G). The F449Y, T484E, and Y498Q mutant
350 RaTG13 S proteins showed a significant reduction in infectivity on mouse ACE2,
351 whereas the Y493Q substitution slightly increased transduction on mouse ACE2 by
352 RaTG13 S pseudovirions (Fig 6F). These results suggested that residues 449, 484, and
353 498 of RaTG13 S protein might also be important for interaction with mouse ACE2
354 and that Q might be preferred over Y at position 493 of the RaTG13 S protein to
355 interact mouse ACE2. None of the mutations could significantly rescue the infection

356 of RaTG13 S pseudovirions on pangolin ACE2 expressing cells (Fig 6F). The effects
357 of individual mutations in SARS-CoV-2 S proteins on virus entry through pangolin
358 ACE2 were relatively limited (Fig 6G), and very similar to those in hACE2 cells (Fig
359 6E). Y449F and E484T mutants showed slightly over 50% and 30% reduction in
360 infectivity in pangolin ACE2-expressing cells, respectively, whereas Q498Y
361 mutations in SARS-CoV-2 had no effect on virus entry into pangolin ACE2
362 expressing cells. Strikingly, mutant E484T and Q498Y SARS-CoV-2 S proteins
363 increased transduction on mouse ACE2 expressing cells by more than 16 and 70-fold,
364 respectively, indicating that residues 484 and 498 of SARS-CoV-2 S proteins might
365 play critical roles in determining receptor usage of mouse ACE2.

366

367 **Discussion**

368 Viral entry is the first step for zoonotic transmission, and the interaction between
369 the host receptor and viral S protein determines the host range and viral tropism.

370 Although the origin of SARS-CoV-2 remains unknown, RaTG13 has been speculated
371 to be the possible origin of SARS-CoV-2^{3,8,9,35}, because the genomes of SARS-CoV-2
372 and bat SL-CoV RaTG13 share the highest nucleotide sequence identity. Here we
373 showed that, although both SARS-CoV-2 and RaTG13 could use hACE2 for virus
374 entry, the S proteins of the two CoVs have marked differences in biological
375 properties, in terms of their affinity to ACE2s of different animal species, their
376 fusogenicity in membrane fusion, and virus entry using different ACE2 proteins,
377 especially for pangolin and mouse ACE2s, supporting the hypothesis that SARS-CoV-
378 2 might not arise from RaTG13 virus directly, consistent with previous analysis^{36,37}.

379 The RaTG13 virus was originally found in specimens from a *Rhinolophus affinis*
380 bat³, indicating that the *Rhinolophus affinis* bat might be a natural host for the
381 RaTG13 virus. Our finding of RaACE2 as a functional entry receptor for RaTG13
382 virus (Fig 1E and 1G) provides the first direct evidence of this. In fact, RaACE2 was
383 almost as efficient as hACE2 in binding to RaTG13 RBD and facilitating entry of
384 RaTG13 S pseudovirions (Fig 1E and 1G). In contrast, SARS-CoV-2 clearly favored
385 hACE over RaACE2 for receptor binding and modestly favor hACE2 over RaACE2
386 for virus entry (Fig 1E, 1F, and 1G), reflecting possible adaptation of SARS-CoV-2 in
387 human beings or an unknown intermediate host, if SARS-CoV-2 evolved from
388 RaTG13 or a RaTG13-like virus. There are four residues (R24, I27, N31, and N82) in

389 RaACE2 that differ from hACE2 (Table 1). In silico analysis revealed that K31N and
390 M82N changes in RaACE2 likely reduce hydrogen and hydrophobic interactions with
391 the SARS-CoV RBD (Supplementary Fig 6), respectively, resulting in a decrease in
392 overall affinity. In contrast, R24 in RaACE2 likely forms an extra hydrogen bond with
393 S477 in the RaTG13 RBD but not S477 in SARS-CoV-2 (distance: 2.8 Å vs 4.1 Å),
394 stabilizing the interaction between RaACE2 and the RaTG13 RBD.

395 Identification of a direct natural animal reservoir and/or zoonotic intermediate
396 host of SARS-CoV-2 is essential to prevent future emergence and re-emergence of
397 SARS-CoV-2 or SARS-CoV-2 like viruses. Recently, several novel pangolin CoVs
398 were discovered in Malayan pangolins rescued during an anti-smuggling campaign in
399 Guangdong, China^{9,28-30}. Among them, one RBD was almost identical to the SARS-
400 CoV-2 RBD in terms of amino acid sequence, except for one single noncritical
401 residue⁹, leading to the hypothesis that SARS-CoV-2 might result from recombination
402 of RaTG13-like CoV and pangolin CoV and pangolin might be the intermediate host
403 for SARS-CoV-2^{9,28,30}. Pangolin ACE2 not only showed strong binding to SARS-
404 CoV-2 S protein (Fig 3B) and triggered large syncytia mediated by SARS-CoV-2 S
405 protein (Fig 4C), but HEK293 cells expressing pangolin ACE2 were also highly
406 susceptible to SARS-CoV-2 S protein mediated virus entry, suggesting that pangolin
407 should be susceptible to SARS-CoV-2 infection. However, RaTG13 RBD only
408 showed very limited binding to pangolin ACE2 (Fig 3B), its S protein only induced
409 background level of syncytia on HEK293 cells transiently expressing pangolin ACE2
410 (Fig 4C), and RaTG13 S pseudovirions also only gave background level of

411 transduction on HEK293 cells transiently expressing pangolin ACE2 (Fig 5A),
412 indicating that RaTG13 virus might not be able to infect pangolin. This raises the
413 question of whether pangolins could be intermediate hosts for SARS-CoV-2 if
414 RaTG13 or RaTG13-like viruses could not infect pangolins. Moreover, pangolins are
415 solitary animals, and infection by these pangolin CoVs is lethal for most pangolins⁹,
416 suggesting that these pangolin CoVs might not be native to pangolins. Recent studies
417 on 334 *Sunda pangolins* did not find any CoVs or other potential zoonotic viruses in
418 these animals³⁸, further supporting that pangolins might not be reservoir hosts for
419 these pangolin CoVs. Where, when, and how these pangolins acquired these CoVs
420 remain elusive.

421 Among the 17 different ACE2s we tested, squirrel and pig ACE2s were highly
422 susceptible to transduction by all SARS-CoV, SARS-CoV-2, and RaTG13 S
423 pseudovirions (Fig 5), although recent studies reported that pigs might not be
424 permissive to SARS-CoV-2 infection^{17,39}, likely resulting from low level of
425 expression of ACE2 proteins on pig respiratory track²². Fox, civet, camel, ferret, and
426 rat were also susceptible to virus entry by all three S pseudovirions (Fig 5), indicating
427 the potential broad host range of all three viruses. Ferret has been used as a SARS-
428 CoV-2 infection and transmission model^{17,32,33,40}. Whether any of these other
429 susceptible animals could be used as animal models for SARS-CoV-2 remains to be
430 determined, especially for rats, which are cheaper and widely available. More
431 importantly, whether any of these susceptible animals might be potential intermediate
432 hosts for SARS-CoV-2 warrants further investigation.

433 Of note, while SARS-CoV-2 could bind and use pangolin ACE2 for virus entry,

434 its ability to use mouse ACE2 was very limited, and conversely, RaTG13 could bind

435 and use mouse ACE2 for virus entry, but not pangolin ACE2. Among the 20 residues

436 making direct contact with SARS-CoV-2 S proteins (Table 1), mouse ACE2 protein

437 differs at eight RBD-interacting residues from human ACE2 (Table 1), whereas

438 pangolin ACE2 has seven critical positions differing from hACE2 (Table 1). In silico

439 analyses showed that multiple amino acid changes in mouse ACE2, including D30N,

440 K31N, and K353H, likely disrupt the salt bridge and hydrogen bonding network

441 between ACE2 and SARS-CoV-2 S protein, whereas several changes in RaTG13 S

442 protein, including N439K, F486L, Q493Y, and Q498Y (N, F, Q, Q from SARS-CoV-2

443 S and K, L, Y, Y from RaTG13 S), might reestablish the interactions with mouse

444 ACE2 (Supplementary Fig 5E). This notion is strongly supported by the results for the

445 Q498Y mutation in the SARS-CoV-2 S protein and the Y498Q mutation in the

446 RaTG13 S protein. Replacement of Q498 with Y increased the infectivity of SARS-

447 CoV-2 S pseudovirions on mouse ACE2 expressing cells by more than 70-fold. In

448 contrast, substitution of Y498 with Q almost abrogated transduction by RaTG13 S

449 pseudovirions on mouse ACE2, indicating the importance of residue 498 of both the

450 RaTG13 and SARS-CoV-2 S proteins in the recognition of the mouse ACE2 protein.

451 Of note, Q498 mutations were found in two recent mouse adapted SARS-CoV-2

452 strains, Q498H in one⁴¹, and Q498T in the other³¹. We did not identify any residue in

453 the S protein essential for interacting with pangolin ACE2. Y449F, E484T, and

454 Q498Y substitutions in the SARS-CoV-2 S protein had moderate effect on virus entry

455 into pangolin ACE2 cells, and none of the mutations in the RaTG13 S protein
456 significantly increased virus infectivity in 293/pangolin ACE2 cells.

457 RS-YN bat ACE2 showed strong binding to SARS-CoV-2, induced syncytium
458 formation effectively, and was susceptible to transduction by SARS-CoV-2 S
459 pseudovirions, consistent with previous reports³. In silico analysis (Supplementary
460 Figure 5) revealed that Y449, E484, and Q493 in SARS-CoV-2 S could form
461 hydrogen bonds with D38, K31, and T34 in RS-YN bat ACE2, resulting in strong
462 binding of SARS-CoV-2 RBD with RS-YN bat ACE2. In contrast, although RS-HB
463 bat ACE2 has only a 5 amino acid difference from hACE2 and one fewer than RS-YN
464 bat ACE2, it only showed a background level of binding to SARS-CoV-2. K31E,
465 T34S, and D38N changes in RS-HB bat ACE2 might disrupt their hydrogen bonding
466 with E484, Q493, and Y449 of SARS-CoV-2 RBD, respectively, critical for SARS-
467 CoV-2 RBD and bat ACE2 interaction (Supplementary Figure 5). Both RS bat ACE2s
468 seem to be poor receptors for RaTG13 virus. Y449F, E484T, and Q493Y changes in
469 RaTG13 might abolish those critical hydrogen bonds, leading to very limited binding
470 to both RS bat ACE2 (Fig 3B), background level of syncytium formation (Fig 4B),
471 and limited virus entry by RaTG13 S pseudovirions (Fig 5A). Whether failure of
472 SARS-CoV-2 and RaTG13 using RS-HB bat ACE2 for virus entry might result from
473 pathogen-driven host revolution remains to be determined. Although SARS-CoV-2
474 likely evolved from bat-CoV RaTG13 or RaTG13-like bat-CoV with or without
475 recombination with other CoVs, the difference in susceptibility of the two RS bat
476 ACE2s between SARS-CoV-2 and RaTG13 raises two important questions: 1. Which

477 bat species other than *Rhinolophus affinis* might harbor RaTG13 or RaTG13-like

478 virus? 2. How does RaTG13 or RaTG13-like CoV evolve to SARS-CoV-2?

479 In summary, we determined the susceptibility of bat-CoV RaTG13 to 17 diverse

480 animal ACE2s and compared them with those of SARS-CoV-2 and SARS-CoV. We

481 found that RaACE2 is an entry receptor for RaTG13 and SARS-CoV-2. All three

482 CoVs likely have a broad host range with SARS-CoV being the broadest, and mice,

483 not pangolins, are susceptible to RaTG13 infection, whereas pangolins, not mice, are

484 susceptible to SARS-CoV-2 infection. Residues 484 and 498 in the S protein play

485 critical roles in the recognition of mouse and human ACE2.

486

487 **Materials and Methods**

488 **Constructs and plasmids.** Codon-optimized cDNA (sequences are shown in
489 Supplementary Table 1) encoding SARS-CoV-2 S protein (QHU36824.1),SARS-CoV
490 S protein (AAP13441.1) and S proteins of SARS-like bat CoV RaTG13
491 (MN996532.1) and ZC45 lacking C-terminal 19 amino acids (aa)were synthesized
492 and cloned into the eukaryotic cell expression vector pCMV14-3×Flag between the
493 *Hind III* and *Xba I* sites. The VSV-G encoding plasmid and lentiviral packaging
494 plasmid psPAX2 were obtained from Addgene (Cambridge, MA). The pLenti-GFP
495 lentiviral reporter plasmid that expresses GFP and luciferase was generously gifted by
496 Fang Li, Duke University. The cDNAs encoding ACE2 orthologs (Table 1) were
497 synthesized by Sango Biotech (Shanghai, China) and cloned into the pCMV14-
498 3×Flag vector between the *Hind III* and *BamH I* sites. All the constructs were verified
499 by sequencing.

500 **Cell lines.** Human embryonic kidney cell lines 293 (#CRL-1573) and 293T
501 expressing the SV40 T-antigen (#CRL-3216) were obtained from ATCC (Manassas,
502 VA, USA), HEK239 cells stably expressing recombinant human ACE2 (293/hACE2),
503 baby hamster kidney fibroblasts stably expressing recombinant human APN
504 (BHK/hAPN), HEK239 cells stably expressing recombinant human DPP4
505 (293/hDPP4), HEK-293 cells stably expressing murine CEACAM1a
506 (293/mCEACAM1a)were established in our lab. All above cells were maintained in
507 Dulbecco's MEM containing 10% fetal bovine serum (FBS) and 100 units of
508 penicillin, 100 µg of streptomycin, and 0.25 µg of fungizone (1% PSF, Gibco) per

509 milliliter.

510 **Antibodies.** Rabbit polyclonal against SARS S1 antibodies (#40150-T62), mouse

511 monoclonal against SARS S1 antibody (#40150-MM02), rabbit polyclonal against

512 SARS-CoV-2 RBD antibodies (#40592-T62), rabbit polyclonal against SARS-CoV-2

513 S2 antibodies (#40590-T62), rabbit polyclonal against HIV-1 Gag-p24 antibody

514 (11695-RB01) were purchased from Sino Biological Inc. (Beijing, China). Mouse

515 monoclonal anti-FLAG M2 antibody and Mouse monoclonal anti- β -Actin antibody

516 were purchased from Sigma-Aldrich. Integrin β -1 rabbit polyclonal antibody was

517 purchased from Proteintech (Wuhan, China). Alexa flour 488 conjugated rabbit

518 monoclonal His-tag was purchased from Cell Signaling Technology (Danvers, MA,

519 USA). Fluorescein-conjugated goat anti-human IgG (#ZF-0308) was purchased from

520 ZSGB-BIO (Beijing, China). Donkey anti-rabbit IgG (#711-035-152), goat anti-

521 mouse IgG (#115-035-146), rabbit anti-goat IgG (#305-035-003) were purchased

522 from Jackson ImmunoResearch (West Grove, PA, USA).

523 **Expression and purification of SL-CoV RaTG13, SARS-CoV-2 and SARS-CoV**

524 **RBDs.** Receptor-binding domains (RBDs) of SL-CoV RaTG13, SARS-CoV-2 and

525 SARS-CoV were expressed in Hi5 cells using the Bac-to-Bac baculovirus system

526 (Invitrogen). Briefly, the codon optimized DNA sequences encoding the SL-CoV

527 RaTG13 RBD (residues Arg319-Phe541), SARS-CoV-2 RBD (residues Arg319-

528 Phe541), and SARS-CoV RBD (residues Arg306-Phe527) were inserted into

529 pFastBac (Invitrogen) with an N-terminal gp67 signal peptide and a C-terminal 6 \times

530 His tag. The constructs were transformed into DH10Bac competent cells, and the

531 resulting bacmids were transfected into Sf9 cells using Cellfectin II Reagent
532 (Invitrogen) to generate initial virus stock. After amplification, viruses were used to
533 infect Hi5 cells at a density of 2×10^6 cells/ml. The supernatants containing the
534 secreted RBDs were harvested at 60 hrs postinoculation and purified using a Ni-NTA
535 column (GE Healthcare), followed by a Superdex 200 gel filtration column (GE
536 Health care).

537 **Soluble RBD binding assay.** HEK293 cells were transfected with plasmids encoding
538 different ACE2 orthologs (Table S1) by polyetherimide (PEI) (Sigma, St Louis, MO,
539 USA). After 40 hrs incubation, cells were washed with PBS, lifted with PBS
540 containing 1 mM EDTA, and immediately washed twice with PBS with 2% FBS.
541 About 2×10^5 cells were incubated with 5 μ g of soluble RATG13, SARS-CoV-2, or
542 SARS-CoV RBD for 1 hr on ice. After washing three times with PBS with 2% FBS,
543 cells were incubated with rabbit polyclonal anti-6xHis antibody (1:200 dilution)
544 (Shanghai Enzyme-Linked Biotechnology Co., Shanghai, China), followed by
545 incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200). Cells were
546 fixed with 1% paraformaldehyde and analyzed by flow cytometry.

547 **Pseudovirion production and transduction.** For pseudotyped virion production,
548 HEK-293 cells were transfected with psPAX2, pLenti-GFP, and plasmids encoding
549 either SARS-CoV-2 S, SARS-CoV S, RaTG13 S, or ZC45 S protein at equal molar
550 ratios by PEI. After 40 hrs of incubation, viral supernatants were harvested and
551 centrifuged at 800 g for 5 min to remove cell debris. For transduction, receptor-
552 expressing cells were seeded into 24-well plates at 30-40% confluence. The next day,

553 cells were inoculated with 500 μ l viral supernatant, followed by spin-inoculation at
554 800g for 30 min. After overnight incubation, cells were fed with fresh media, and
555 cells were lysed with 120 μ l of lysis buffer (ratio of medium and Steady-glo
556 (Promega) at 1:1) at 48 hrs postinoculation. The luciferase activities were quantified
557 by using a Modulus II microplate reader (Turner Biosystems, Sunnyvale, CA, USA).
558 All experiments were performed in triplicate and repeated at least twice.

559 **Detection of S protein by western blot.** Briefly, HEK293T cells transfected with
560 plasmids encoding either SARS-CoV, SARS-CoV-2, bat SL-CoV RaTG13, or bat SL-
561 CoV ZC45 S proteins were lysed at 40 hrs post transfection by RIPA buffer (20 mM
562 Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP40, 1 \times protease
563 inhibitor cocktail). After 30 min of incubation on ice, cell lysate was centrifuged at
564 12,000g for 10 min at 4°C to remove nuclei. To pellet down pseudovirions, viral
565 supernatants were centrifuged at 25,000 rpm for 2 hrs in a Beckman SW41 rotor at
566 4°C through a 20% sucrose cushion, and virion pellets were resuspended in 30 μ l
567 RIPA buffer. The samples were boiled for 10 min, separated in a 10% SDS-PAGE gel
568 (WB1102, Beijing Biotides Biotechnology, Beijing, China) and transferred to
569 nitrocellulose filter membranes. After blocking with 5% milk, the membranes were
570 blotted with primary antibodies, followed by horseradish peroxidase (HRP)
571 conjugated secondary antibodies (1:5000), and visualized with Chemiluminescent
572 Reagent (Bio-Rad). The primary antibodies used for blotting were polyclonal goat
573 anti-MHV S antibody AO4 (1:2000), polyclonal anti-SARS S1 antibodies T62
574 (1:2000) (Sinobiological Inc, Beijing, China), mouse monoclonal against SARS S1

575 antibody MM02 (1:1000) (Sinobiological Inc, Beijing, China), rabbit polyclonal
576 anti-SARS-CoV-2 RBS antibodies (1:1000) (Sinobiological Inc, Beijing, China),
577 rabbit polyclonal anti-SARS-CoV-2 S2 antibodies (1:1000) (Sinobiological Inc,
578 Beijing, China) and anti-FLAG M2 antibody (1:1000) (Sigma, St. Louis, MO, USA),
579 respectively.

580 **Cell surface protein biotinylation assay.** To determine the level of ACE2s of each
581 species on the cell surface, FLAG-tagged ACE2 expressing cells at 80-90%
582 confluence were incubated with PBS containing 2.5 µg/mL EZ-linked Sulfo-NHS-
583 LC-LC-biotin (Thermo-Pierce, #21388) on ice for 30 min after washing with ice-cold
584 PBS. Then, the reaction was quenched by PBS with 100 mM glycine and cells were
585 lysed with RIPA buffer. To pull-down the proteins labeled with biotin, the lysates were
586 incubated with NeutrAvidin beads (Thermo-Pierce, #53150) overnight at 4°C . After
587 washing 3 times with RIPA buffer, samples were resuspended in 30 µl of loading
588 buffer and boiled for 10 min, and the level of ACE2 expression was determined by
589 western-blotting using an anti-FLAG M2 antibody (1:1000). Integrin- β 1 were serving
590 as a control.

591
592 **Cell-cell fusion assay** HEK293T cells transiently overexpressing the S protein and
593 eGFP were detached by brief trypsin (0.25%) treatment, and overlaid on a 70%
594 confluent monolayer of ACE2 expressing cells at a ratio of approximately one S-
595 expressing cell to three receptor-expressing cells. After 4 hrs of incubation, images of
596 syncytia were captured with a Nikon TE2000 epifluorescence microscope running

597 MetaMorph software (Molecular Devices). All experiments were performed in
598 triplicate and repeated at least three times. Three images for each sample were
599 selected, and the total number of nuclei and the number of nuclei in fused cells for
600 each image were counted. The fusion efficiency was calculated as the number of
601 nuclei in syncytia/total number of nuclei x100.

602 **Structure modeling.** The PDB files of the crystal structures of hACE2/SARS-CoV-2
603 (6m0j) and hACE2/SARS-CoV (2ajf) and the cryo-EM structure of the RaTG13 spike
604 glycoprotein (6zgf) were downloaded from the RCSB PDB website (www.rcsb.org).
605 Homology models of the receptor binding domain (RBD) of different host ACE2s
606 were built with the Structropedia web server (mod.farooq.ac). Hot spot residues were
607 predicted with the Hotpoint web server (prism.ccbb.ku.edu.tr/hotpoint)^{42,43}. The RBD
608 structures of SARS-CoV-2, SARS-CoV, and RaTG13 were extracted from the pdb
609 files and docked into the homology models with the HADDOCK server
610 (wenmr.science.uu.nl)⁴⁴, using conserved active residues on the interfaces as docking
611 restraints. Docking poses were viewed, aligned, and analyzed with PyMOL software.
612
613

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717

718

719 Table 1 Alignment of critical S protein-interacting residues of different animal ACE2 proteins

Species ^a	24 ^a	27 ^a	28 ^a	30 ^a	31 ^a	34 ^a	35 ^a	37 ^a	38 ^a	41 ^a	42 ^a	79 ^a	82 ^a	83 ^a	330 ^a	353 ^a	354 ^a	355 ^a	357 ^a	393 ^a	Accession ^a
Human ^a	Q ^a	T ^a	F ^a	D ^a	K ^a	H ^a	E ^a	D ^a	Y ^a	Q ^a	L ^a	M ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	R ^a	NP_068576 ^a	
Squirrel ^a	L ^a	T ^a	F ^a	D ^a	K ^a	Q ^a	E ^a	D ^a	H ^a	Q ^a	L ^a	D ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	R ^a	XP_026252505 ^a	
Pangolin ^a	E ^a	T ^a	F ^a	E ^a	K ^a	S ^a	E ^a	E ^a	Y ^a	Q ^a	I ^a	N ^a	Y ^a	N ^a	K ^a	H ^a	D ^a	R ^a	R ^a	XP_017505746 ^a	
Fox ^a	L ^a	T ^a	F ^a	E ^a	K ^a	Y ^a	E ^a	E ^a	Y ^a	Q ^a	L ^a	T ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	R ^a	XP_025842512 ^a	
Civet ^a	L ^a	T ^a	F ^a	E ^a	T ^a	Y ^a	E ^a	Q ^a	E ^a	Y ^a	Q ^a	L ^a	T ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	AAX63775 ^a	
Camel ^a	L ^a	T ^a	F ^a	E ^a	E ^a	H ^a	E ^a	E ^a	D ^a	Y ^a	Q ^a	T ^a	T ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	XP_006194263 ^a	
Ferret ^a	L ^a	T ^a	F ^a	E ^a	K ^a	Y ^a	E ^a	E ^a	E ^a	Y ^a	Q ^a	H ^a	T ^a	Y ^a	N ^a	K ^a	R ^a	D ^a	R ^a	NP_001297119 ^a	
Rat ^a	K ^a	S ^a	F ^a	N ^a	K ^a	Q ^a	E ^a	E ^a	D ^a	Y ^a	Q ^a	I ^a	N ^a	F ^a	N ^a	H ^a	G ^a	D ^a	R ^a	NP_001012006 ^a	
Mouse ^a	N ^a	T ^a	F ^a	N ^a	N ^a	Q ^a	E ^a	E ^a	D ^a	Y ^a	Q ^a	T ^a	S ^a	F ^a	N ^a	H ^a	G ^a	D ^a	R ^a	NP_001123985 ^a	
Pig ^a	L ^a	T ^a	F ^a	E ^a	K ^a	L ^a	E ^a	E ^a	D ^a	Y ^a	Q ^a	I ^a	T ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	NP_001116542 ^a	
RA Bat ^a	R ^a	I ^a	F ^a	D ^a	D ^a	N ^a	H ^a	E ^a	D ^a	Y ^a	Q ^a	L ^a	N ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	QM039244 ^a	
RS-YN Bat ^a	E ^a	M ^a	F ^a	D ^a	K ^a	T ^a	K ^a	E ^a	D ^a	H ^a	Q ^a	L ^a	N ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	AGZ48803 ^a	
RS-HB Bat ^a	R ^a	T ^a	F ^a	D ^a	E ^a	S ^a	E ^a	E ^a	N ^a	Y ^a	Q ^a	L ^a	N ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	ADN93475 ^a	
Guinea pig ^a	L ^a	I ^a	F ^a	D ^a	E ^a	S ^a	E ^a	E ^a	N ^a	Y ^a	Q ^a	L ^a	N ^a	Y ^a	N ^a	K ^a	N ^a	D ^a	R ^a	ACT66270 ^a	
Deer ^a	Q ^a	T ^a	F ^a	E ^a	K ^a	H ^a	E ^a	E ^a	D ^a	Y ^a	Q ^a	M ^a	T ^a	Y ^a	N ^a	K ^a	G ^a	D ^a	R ^a	XP_020768965 ^a	
Hedgehog ^a	Q ^a	S ^a	F ^a	T ^a	T ^a	N ^a	E ^a	E ^a	N ^a	Y ^a	Q ^a	K ^a	F ^a	K ^a	L ^a	N ^a	D ^a	R ^a	XP_004710002 ^a		
Koala ^a	R ^a	E ^a	F ^a	E ^a	T ^a	K ^a	E ^a	E ^a	E ^a	Y ^a	Q ^a	I ^a	T ^a	F ^a	N ^a	K ^a	G ^a	D ^a	R ^a	XP_020863153 ^a	
Turtle ^a	E ^a	N ^a	F ^a	S ^a	E ^a	V ^a	Q ^a	E ^a	D ^a	Y ^a	A ^a	N ^a	K ^a	Y ^a	N ^a	K ^a	K ^a	D ^a	R ^a	XP_006122891 ^a	
Snake ^a	V ^a	K ^a	F ^a	E ^a	Q ^a	A ^a	R ^a	T ^a	D ^a	Y ^a	N ^a	N ^a	M ^a	F ^a	N ^a	K ^a	E ^a	D ^a	R ^a	ETE61880 ^a	

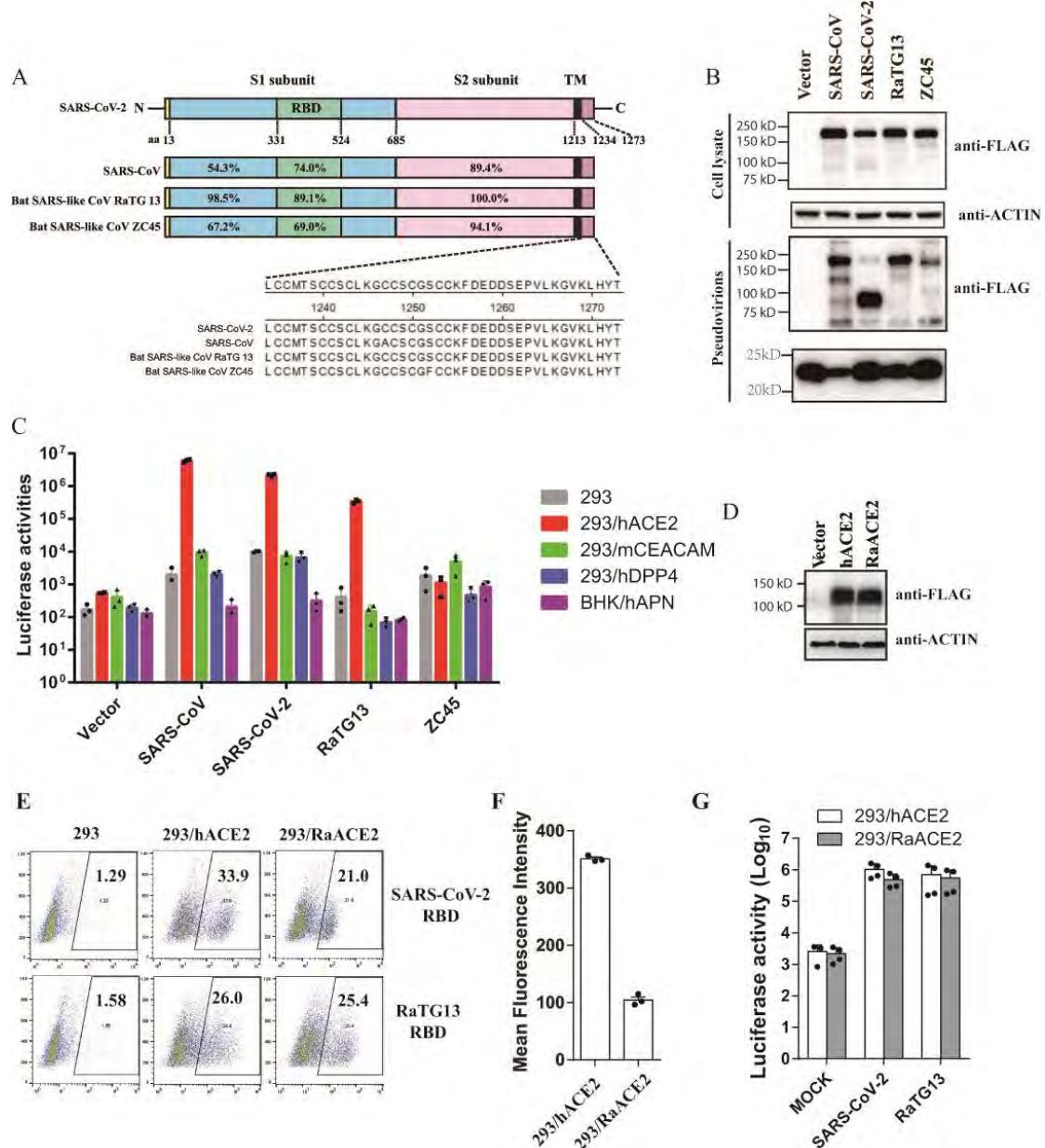
720

721 Green: residues homologous to that of human ACE2

722 Orange: residues different from that of human ACE2

723 Black: residues identical to that of human ACE2

724



725

726 **Figure1. Bat SL-CoV RaTG13 uses hACE2 and RaACE2 for virus entry.** (A)
 727 Schematic diagram of the full length of different CoV S proteins and the amino acid
 728 sequence identities of each region are shown in corresponding places. S1, receptor
 729 binding subunit; S2, membrane fusion subunit; TM, transmembrane domain. (B)
 730 Detection of the S proteins of SARS-CoV, SARS-CoV-2, Bat SL-CoV RaTG13 and
 731 ZC45 in cells lysates and pseudovirions by western blot. HEK293T cells transfected
 732 with either empty vector or plasmids encoding the indicated CoV S proteins were

733 lysed at 40 hrs post transfection. The S proteins in cell lysates and pseudovirions were
734 subjected to WB analysis by blotting with mouse monoclonal anti-FLAG M2
735 antibody. Actin and gag-p24 served as loading controls (cell lysate, top panel,
736 pseudovirions, bottom panel). The full length S protein is about 180 kDa, while
737 cleaved S protein is about 90 kDa. Experiments were done three times and the
738 representative was shown. (C) Entry by RaTG13 S pseudovirions on different CoV
739 receptors. Cells were spin-inoculated with indicated pseudovirions. At 48 hrs post
740 inoculation, transduction efficiency was determined by measurement of luciferase
741 activities. HEK293 cells (grey), HEK293/hACE2 (red), HEK293 cells stably
742 expressing hACE2; 293/mCEACAM (green), HEK293 cells stably expressing
743 mCEACAM, the MHV receptor; 293/hDPP4 (blue), HEK293 cells stably expressing
744 hDPP4, the MERS-CoV receptor. BHK/hAPN(purple), BHK cells stably expressing
745 hAPN, the hCoV-229E receptor; Experiments were done triplicate and repeated at
746 least three times. One representative is shown with error bars indicate SEM. (D)
747 Expression of Rhinolophus affinis ACE2 protein in HEK 293 cells. HEK 293 cells
748 transiently transfected with the plasmids encoding either FLAG-tagged hACE2 or
749 Rhinolophus affinis ACE2 (RaACE2) proteins were lysed at 40 hrs post-transfection.
750 Expression of ACE2 proteins were detected by mouse monoclonal anti-FLAG M2
751 antibody. (E) Binding of hACE2 and RaACE2 by SARS-CoV-2 and RaTG13 RBDs.
752 HEK 293 cells transiently expressing hACE2 or RaACE2 proteins were incubated
753 with either SARS-CoV-2 RBD or RaTG13 RBD on ice, followed by rabbit anti-his
754 tag antibodies and alexa-488 conjugated goat anti rabbit IgG, and analyzed by flow

755 cytometry. The experiments were done three times, and one representative is shown.

756 (F) Mean fluorescence intensities of the gated cells positive for SARS-CoV-2 RBD

757 binding to 293/hACE2 and 293/RaACE2 cells in (E). (G) Entry of SARS-CoV,

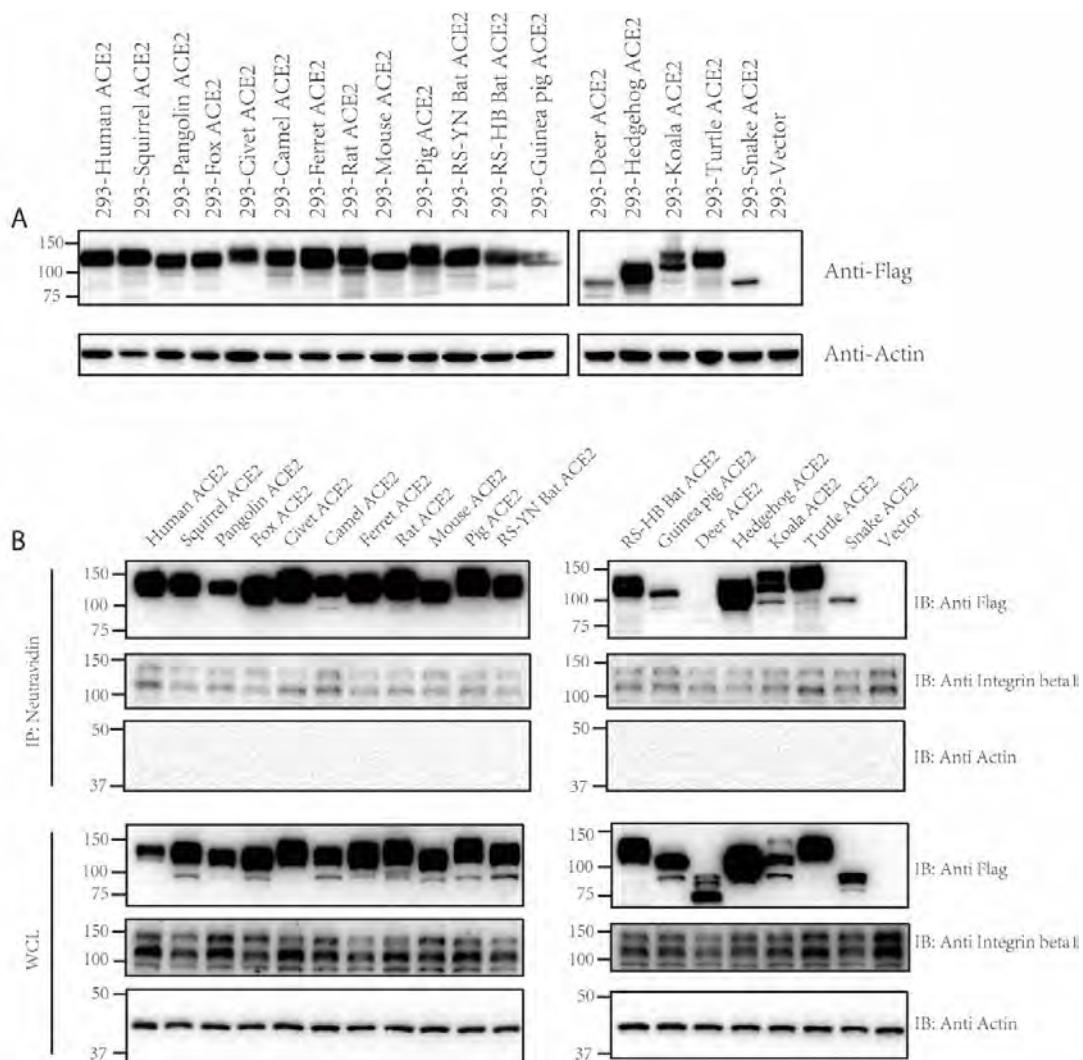
758 SARS-CoV-2, and RaTG13 S protein pseudovirions on 293/RaACE2 cells.

759 Experiments were done three times, and one representative is shown with error bars

760 indicating SEM. *P<0.05; **P<0.001 (compared with control by ANOVA followed

761 by Dunnett's multiple comparisons t test)

762



764 **Figure 2 Expression and cell surface localization of ACE2 orthologs of various**
765 **species. (A) Expression of ACE2s of different species in HEK293 cells. HEK293**
766 **cells were transfected with plasmids encoding FLAG tagged different ACE2s by PEI,**
767 **and lysed at 40 hrs post transfection. The expression of different ACE2 proteins in**
768 **cell lysates was determined by western blotting using anti-FLAG M2 antibodies. The**
769 **accession numbers for each ACE2 orthologs are as follows: human ACE2:**
770 **NP_068576, squirrel ACE2: XP_026252505, pangolin ACE2: XP_017505746, fox**
771 **ACE2: XP_025842512, civet ACE2: AAX63775, camel ACE2: XP_006194263,**
772 **ferret ACE2: NP_001297119, rat ACE2: NP_001012006, mouse ACE2:**

773 NP_001123985, pig ACE2: NP_001116542, RS bat: AGZ48803, RS-HB bat:

774 ADN93475, guinea pig ACE2: ACT66270, deer ACE2: XP_020768965, hedgehog

775 ACE2: XP_004710002, koala ACE2: XP_020863153, turtle ACE2: XP_006122891,

776 snake ACE2: ETE61880. (B) Analysis of different ACE2 proteins on cell surface by

777 cell surface protein biotinylation assay. HEK293 cells transiently overexpressing

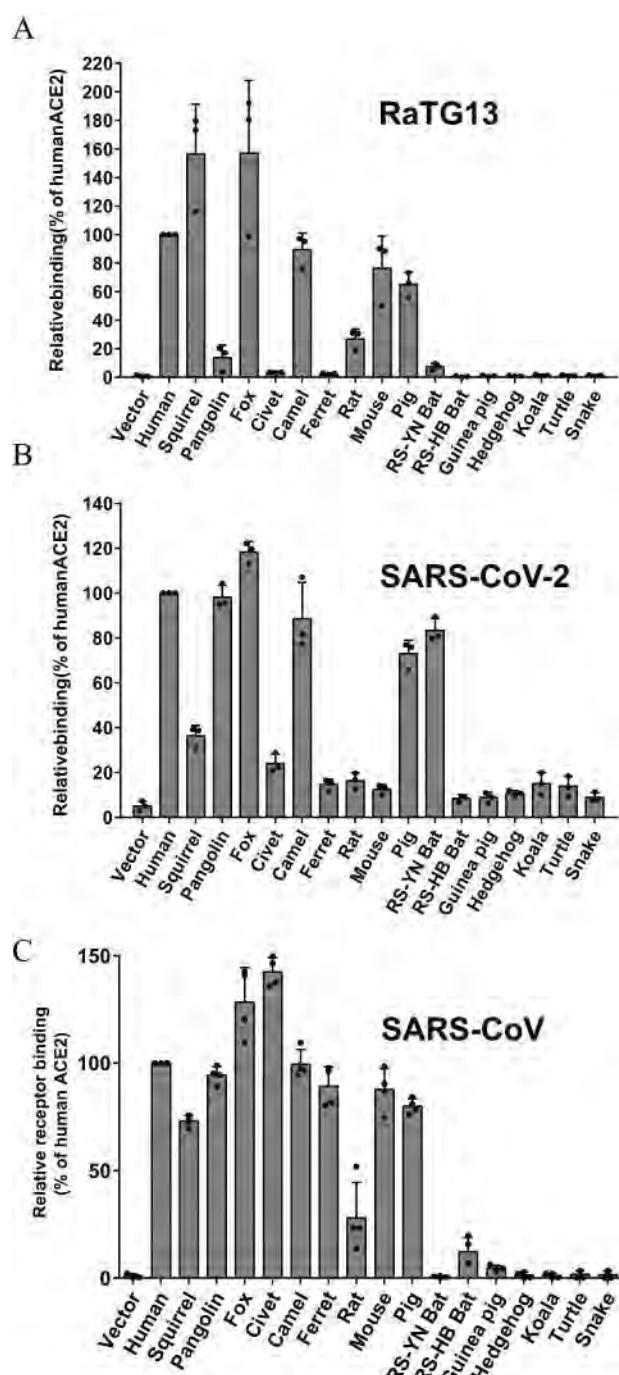
778 different ACE2 proteins were labeled with EZ-link Sulfo-NHS-LC-LC-biotin on ice,

779 and lysed with RIPA buffer. Biotinylated proteins were enriched with NeutrAvidin

780 beads and analyzed by western blot using mouse monoclonal anti-FLAG M2

781 antibody. WCL, whole cell lysate.

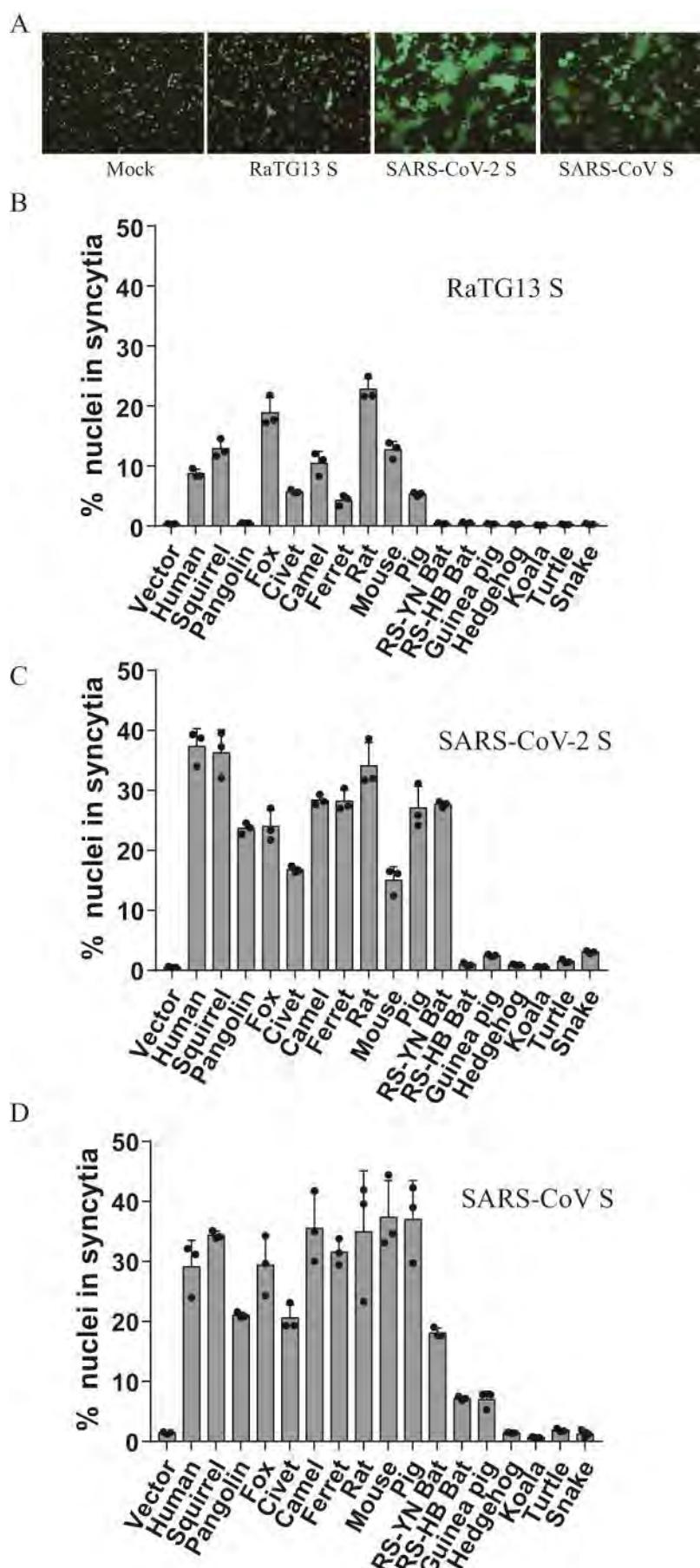
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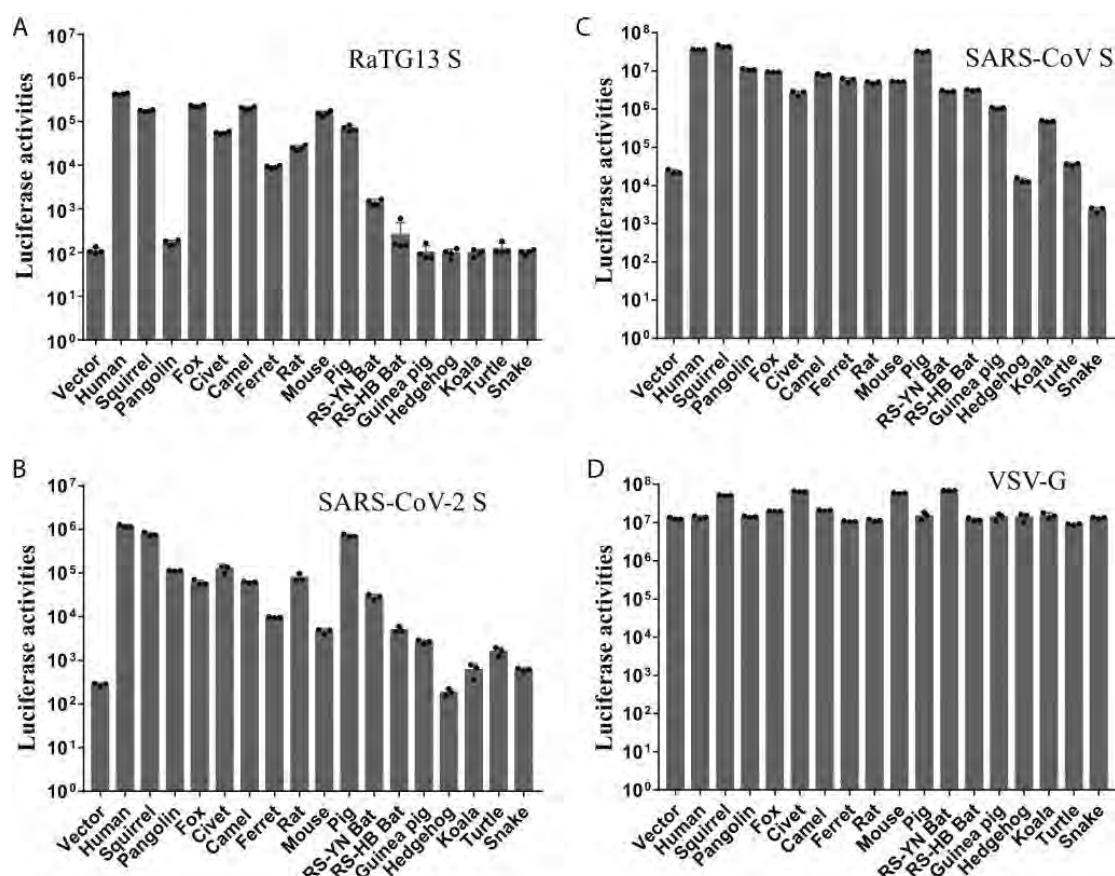
784 **Figure 3. Binding of different ACE2 proteins by RBDs of bat SL-CoV RaTG13,**
785 **SARS-CoV-2, and SARS-CoV.** HEK293 cells transiently expressing different ACE2
786 cells were incubated with either RaTG13 (A), SARS-CoV-2 (B), or SARS-CoV (C)
787 RBDs, followed by rabbit anti-His tag antibodies and Alexa-488 conjugated goat anti
788 rabbit IgG, and analyzed by flow cytometry. The experiments were done at least three

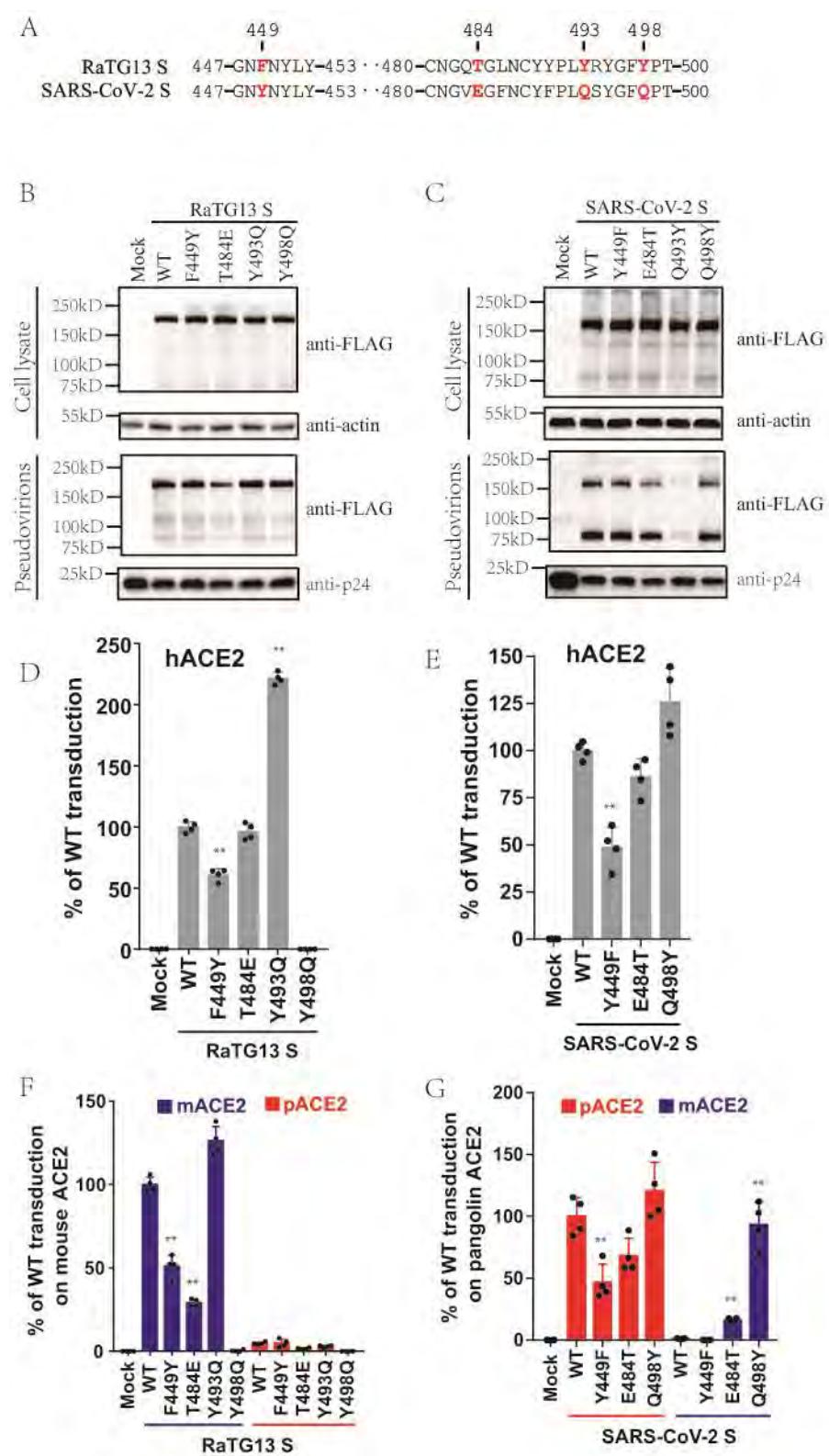
789 times. The results of percentage of positive cells from hACE2 binding were set to
790 100%, the rest was calculated as percentage of hACE2 binding according to results in
791 flow cytometry analysis. Data are shown as the means \pm standard deviations.



793 **Figure 4. Cell–cell fusion mediated by RaTG13, SARS-CoV, and SARS-CoV-2**

794 **spike proteins.** HEK293T cells transiently expressing eGFP and spike proteins of
795 either RaTG13, SARS-CoV, or SARS-CoV-2 were detached with trypsin, and
796 overlaid on different ACE2 expressing HEK293 cells. After 4 hrs of incubation,
797 images were taken. (A) Representative images of syncytia for hACE2; (B-D)
798 Percentage of nuclei in syncytia induced by RaTG13 S (B), SARS-CoV-2 S (C), and
799 SARS-CoV S (D). Syncytium formation for each image was quantified by counting
800 the total nuclei in syncytia and total nuclei in the image and calculated as the
801 percentage of nuclei in syncytia, and three images were selected for each sample.
802 Experiments were done three times, and one representative is shown with error bars
803 indicating SEM. The scale bar indicates 250 μ m.
804





813

814 **Figure 6. Entry of lentiviral pseudovirions with mutant RaTG13 S and SARS-
 815 CoV-2 S proteins on 293/hACE2, 293/mouse ACE2, and 293/pangolin ACE2 cells.**

816 (A) Alignment of partial amino acid sequences of RaTG13 and SARS-CoV-2 S
817 proteins. Residues 449, 484, 493, and 498 are labeled in red. Detection of mutant S
818 proteins in cells lysates and pseudovirions by western blotting using a mouse
819 monoclonal anti-FLAG M2 antibody. (B) RaTG13 S. (C) SARS-CoV-2 S. Top panel,
820 cell lysate; bottom panel, pseudovirions; β -actin and HIV p24 were used as loading
821 controls. (D)(E) Entry of pseudovirions with mutant RaTG13 (D) and SARS-CoV-2
822 (E) S proteins on 293/hACE2 cells. Pseudovirions carrying mutant S proteins were
823 inoculated on 293/hACE2 cells. After 40 hrs incubation, transduction efficiency was
824 determined by measuring the luciferase activities in cell lysate. Transduction from
825 WT pseudovirions was set as 100%. Experiments were done in quadruplicate and
826 repeated at least three times, and one representative was shown with SEM. (F) Entry
827 of pseudovirions with mutant RaTG13 S proteins on 293 cells expressing mouse (blue)
828 and pangolin (red) ACE2 proteins. Transduction from WT pseudovirions on mouse
829 ACE2 cells was set as 100%. (G) Entry of pseudovirions with mutant SARS-CoV-2 S
830 proteins on 293 cells expressing mouse (blue) and pangolin (red) ACE2 proteins.
831 Transduction from WT pseudovirions on pangolin ACE2 cells was set as 100%. The
832 experiments were performed in quadruplicate with at least three replications and the
833 representative data are shown with SEM. *P<0.05; **P<0.001 (compared with
834 respective WT control by ANOVA followed by Dunnett's multiple comparisons t
835 test)
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