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Soluble ACE2-mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-angiotensin system

Graphical abstract

Highlights
- Human kidney cell line, HK-2, is highly susceptible to SARS-CoV-2
- SARS-CoV-2 infection depends on soluble ACE2 (sACE2)
- sACE2-spike ± vasopressin complex enables cell entry by receptor-mediated endocytosis

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In brief
Using an infection-permissive human kidney cell line, Yeung et al. show that a soluble form of ACE2 that is cleaved and liberated from the host cell surface mediates SARS-CoV-2 binding and uptake by receptors involved in renin-angiotensin system signaling.
Article

Soluble ACE2-mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-angiotensin system

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https://doi.org/10.1016/j.cell.2021.02.053

SUMMARY

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can cause acute respiratory disease and multiorgan failure. Finding human host factors that are essential for SARS-CoV-2 infection could facilitate the formulation of treatment strategies. Using a human kidney cell line—HK-2—that is highly susceptible to SARS-CoV-2, we performed a genome-wide RNAi screen and identified virus dependency factors (VDFs), which play regulatory roles in biological pathways linked to clinical manifestations of SARS-CoV-2 infection. We found a role for a secretory form of SARS-CoV-2 receptor, soluble angiotensin converting enzyme 2 (sACE2), in SARS-CoV-2 infection. Further investigation revealed that SARS-CoV-2 exploits receptor-mediated endocytosis through interaction between its spike with sACE2 or sACE2-vasopressin via AT1 or AVPRT1B, respectively. Our identification of VDFs and the regulatory effect of sACE2 on SARS-CoV-2 infection shed insight into pathogenesis and cell entry mechanisms of SARS-CoV-2 as well as potential treatment strategies for COVID-19.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a pandemic with >109 million confirmed cases and approximately 2.4 million fatalities (https://covid19.who.int/). SARS-CoV-2 can cause substantial pulmonary diseases. Besides, numerous extrapulmonary manifestations such as cardiac and renal complications have been reported to be associated with an increased risk of death in patients with COVID-19 (Gupta et al., 2020; Li et al., 2020).

Host factors required for SARS-CoV-2 infection are largely unknown due to the lack of highly susceptible human cell lines for studying SARS-CoV-2 infection. As of now, a few studies have attempted to identify host factors required for SARS-CoV-2 infection either using non-human cells or modified human cells—that may not truly reflect the authentic infection process. These include studies using the CRISPR screening approach...
to identify host factors in SARS-CoV-2-infected Vero-E6 cells, which are of non-human origin (Wei et al., 2020), or in modified human cell lines—A549 and Huh7.5.1—transduced with SARS-CoV-2 entry factors to become susceptible to SARS-CoV-2 infection (Daniloski et al., 2020; Heaton et al., 2020; Schneider et al., 2021; Wang et al., 2021). In addition, another study reported the use of a focused CRISPR screening to target a small number of host factors, which were recently identified to be involved in SARS-CoV-2 infection (Hoffmann et al., 2021). Therefore, there remains a pressing need to identify host factors essential for SARS-CoV-2 infection, which may uncover potential therapeutic targets for SARS-CoV-2 and provide insights into COVID-19 pathogenesis.

SARS-CoV-2 utilizes a cellular receptor, angiotensin converting enzyme II (ACE2), for cell entry (Zhou et al., 2020). Besides being a virus receptor, ACE2 functions as an important regulator of the renin-angiotensin system (RAS) (Gheblawi et al., 2020). To serve its regulatory role in the RAS, cellular ACE2 (cACE2) first needs to be transported to the cell surface, where it is cleaved by host proteases such as disintegrin and metalloproteinase 17 (ADAM17), to release an enzymatically active soluble form of ACE2 (sACE2) into the plasma (Lambert et al., 2005). The sACE2 retains an intact SARS-CoV-2 interaction site, suggesting its ability to bind to SARS-CoV-2. Interestingly, a recent study has observed that ACE2 shedding may be induced by regulatory pathways influenced by COVID-19 infection and that the concentrations of sACE2 may correlate with the level of systemic inflammation that occurs (Kornilov et al., 2020). However, most of the current research focus on studying the function of cACE2 in SARS-CoV-2 pathogenesis; the impact of circulating sACE2 on viral entry is largely unknown.

Tissue tropism of SARS-CoV-2 cannot be fully explained only by the expression pattern of cACE2. While a majority of cell susceptibility studies are based solely on the mRNA level of cACE2 (Sungnak et al., 2020), studies on its protein expression pattern is limited (Bertram et al., 2012; Hamming et al., 2004). Recently, Wang et al. (2020) comprehensively investigated the tropism of SARS-CoV-2 in human tissues and discovered discordance between mRNA and protein expression levels of ACE2 in many tissues (Wang et al., 2020). Indeed, studies of cACE2 mRNA and protein expression levels showed that only a small population of the lung cells has a detectable expression level of cACE2—that is in contrast to the common knowledge that the lungs are the primary site of infection (Wang et al., 2020; Zou et al., 2020). Potentially, circulating sACE2, which retains the interaction site for binding to SARS-CoV-2, could interact with SARS-CoV-2 in the extracellular compartment forming complexes, which may affect the infectivity.

In this study, we report the identification of a human renal cell line—HK-2—which is highly susceptible to SARS-CoV-2. Using HK-2 cells, we performed a genome-wide RNAi screening and successfully identified virus dependency factors (VDFs), the expression of which is required for SARS-CoV-2 infection. Detailed analysis of these VDFs suggested that perturbation of their functions could be linked to clinical symptoms and complications of COVID-19. Finally, we elucidated the important but overlooked role of sACE2 in SARS-CoV-2 infection using multiple methods, thus advancing our understanding of pathogenesis and treatment strategies for COVID-19.

RESULTS

Identification of a human cell line highly susceptible to SARS-CoV-2 infection

To find an optimal human cell line that is highly susceptible to SARS-CoV-2 infection, we examined 11 human cell lines of different organs, including HK-2, Caco-2, A549, Calu3, Huh7, HepG2, PLC/PRF/5, RD, HeLa, NT2, and 293T, along with Vero-E6 by inoculating the virus at a multiplicity of infection (MOI) of 1. At 72 h post-infection, median tissue culture infective dose (TCID₅₀) results showed that Vero-E6 cells strongly supported SARS-CoV-2 replication with high viral titer of $5.7 \times 10^8$ TCID₅₀/mL (Figure 1A). Much lower viral titers, ranging from $4.2 \times 10^3$ to $7.9 \times 10^4$ TCID₅₀/mL, were detected in most human cell lines (Figure 1A). Intriguingly, SARS-CoV-2 can replicate efficiently in human renal proximal tubule cells (HK-2) with high viral titers of up to $3.8 \times 10^6$ TCID₅₀/mL (Figure 1A). Among these cell lines are Vero-E6 and Caco-2, representing unmodified cell lines of non-human and human origins, respectively, which are known to be susceptible to SARS-CoV-2 (Bojkova et al., 2020). Therefore, effective replication of SARS-CoV-2 in HK-2 and Vero-E6 cells was further confirmed by western blotting (WB) and immunofluorescence assay (IFA), which detected strong viral protein expression in the infected cells (Figures 1B and 1C). In comparison, WB analysis was unable to detect nucleocapsid protein (NP) expression in Caco-2 cells (Figure 1B), which was consistent with the IFA results showing a few positive-stained cells (Figure 1C). Consistent with viral load and the protein expression data (Figures 1A–1C), strong and moderate cytopathic effects (CPEs) were observed in HK-2 and Vero-E6 cells, respectively, but not in Caco-2 cells upon SARS-CoV-2 infection (Figure 1D).

SARS-CoV-2 exhibited similar replication kinetics in HK-2 and Vero-E6, whereas in Caco-2 cells, it replicated less efficiently (Figure 1E). Taken together, HK-2 strongly supports the lytic infection of SARS-CoV-2, representing a suitable cell line model of human origin for studying the molecular interactions between SARS-CoV-2 and human host.

Identification of host factors essential for SARS-CoV-2 replication using a genome-wide RNAi screening

To identify the VDFs essential for SARS-CoV-2 replication, we used a puromycin-marked feline immunodeficiency virus (FIV)-based lenti-vector-short hairpin RNA (shRNA) library to target human transcripts in HK-2 cells followed by the challenge of SARS-CoV-2 (Figures 2A and S1). We anticipated that HK-2 cells that were knocked down for mRNA—which were dispensable for SARS-CoV-2 replication—would succumb rapidly upon infection. In contrast, a shRNA clone silenced an mRNA essential for lytic SARS-CoV-2 replication would survive. The identity of shRNA in the survival cells was determined by high-throughput sequencing (Figure 2A). Principal component analysis (PCA) plot and heatmap based on the abundance of all identified shRNA sequences mapped against the human reference genome revealed good spatial-temporal separation between SARS-CoV-2-infected and mock-infected samples (Figures 2B...
Figure 1. Susceptibilities of human cell lines of different organs to infection by SARS-CoV-2
(A) Human cell lines (HK-2 [red], Caco-2, A549, Calu3, Huh7, HepG2, PLC/PRF/5, RD, HeLa, NT2, and 293T) and Vero-E6 (deep red) were subjected to infection by SARS-CoV-2. Cell lysates were collected and assayed for infectious viral titer.
(B) Western blot (WB) analysis detecting viral antigens using antinucleocapsid protein (NP) antibody in SARS-CoV-2-infected Vero-E6, HK-2, and Caco-2 cells.
(C) Immunofluorescence assay (IFA) of Vero-E6, HK-2, and Caco-2 cells infected with SARS-CoV-2 using rabbit antisera against NP. Scale bars represent 50 \( \mu \text{m} \).
(D) SARS-CoV-2-induced cytopathic effects (CPEs) were monitored in infected Vero-E6, HK-2, and Caco-2 cells. Arrows point to the cells showing CPEs with rounding up of cells progressively detaching from the monolayer.
(E) Virus replication kinetics in infected Vero-E6, HK-2, and Caco-2 were monitored. Cell lysates were harvested from respective time points and viral titers were determined.

The TCID\textsubscript{50}/mL results from (A) and (E) were derived from three independent experiments. Each data point and error bar depict the mean value and SEM, respectively. Statistical analyses were carried out using Student’s t test. Statistical significance is indicated by the asterisks (****p < 0.001). Images from (B–D) are representatives of three independent experiments.
and 2C). Pathway analysis of VDFs with ≥3-fold enrichment in SARS-CoV-2-infected HK-2 cells revealed 16 significantly affected biological pathways (p < 0.05) related to cardiac (pink), pulmonary (blue), liver (green), and renal (brown) diseases (Figure 2D). Notably, 11 of these 16 biological pathways were related to cardiac diseases (pink; Figure 2D). In addition, PANTHER pathway analysis of the identified VDFs was performed using a cut-off p value of 0.001, and eight major affected molecular pathways were discovered (Figure 2E). Among these, the top-affected molecular pathways “Vasopressin-like receptors (R-HSA-388479),” and “Defective AVP does not bind AV-PR1A,B and causes neurohypophyseal diabetes insipidus (NDI) (R-HSA-5619099)” were also involved in the regulation of the cardiovascular system (pink; Figure 2E). Knockdown of individual VDFs involved in the vasopressin-related pathway using corresponding small interfering RNAs (siRNAs) resulted in significant inhibition of SARS-CoV-2 infection (p < 0.01; 45.6%–90.7% of inhibition; Figure 2F). The strong inhibitory effects warranted further study of the VDFs that are functionally related to the vasopressin pathway on SARS-CoV-2 infection.

**VDFs of cytokinesis, vesicle trafficking, and endosomal/lysosomal system play important roles in SARS-CoV-2 infection**

Vasopressin can enter cells via receptor-mediated endocytosis through membrane remodeling and vesicle trafficking (Innamorati et al., 2001). Indeed, our gene ontology (GO) enrichment analysis revealed a close relationship of the VDFs to plasma membrane (79%), membrane raft (12%), as well as intracellular trafficking and secretion, and vesicle (9%), which are known to be important in regulating cytokinesis and vesicle trafficking (Figure 3A; left). Similarly, we also performed GO enrichment analysis using gene candidates that were previously reported to be important for the replication of other coronaviruses (Ashburner et al., 2000; Bairoch et al., 2005; McKusick, 2007; Oughtred et al., 2019; Pruitt et al., 2005). Consistently, gene candidates related to plasma membrane (20%) as well as intracellular trafficking and secretion, and vesicle (18%) also contributed substantially to the biogenesis of coronaviruses (Figure 3A; right).

Next, we performed a volcano plot analysis to identify VDFs that could be involved in SARS-CoV-2 replication (Figure 3B). Manual gene annotation revealed that certain enriched VDFs have functional roles related to the regulation of the cardiovascular system, the cytokinesis and vesicle trafficking pathway, and the renal-related disease (Figure 3B), which was consistent with the results generated from our pathway and GO enrichment analyses (Figures 2D, 2E, and 3A). The differential gene enrichment of the VDFs with a false positive rate (FDR) of <5% is displayed in the heatmap (Figure 3C). Of these, 22 VDFs were selected according to their biological roles for further validation. Consistent with our RNAi screening results, knockdown of individual VDFs using corresponding siRNAs resulted in significant inhibition of SARS-CoV-2 infection (p < 0.01; 46.5%–95.4% of inhibition; Figure 3D). Particularly, potent inhibition (>80%) was observed in some VDFs with biological functions related to the control of cytokinesis and vesicle trafficking pathway (GPR176, CAPNS1, IL18RAP, ARL4D, and CXCR1; red boxed; Figure 3D) and the endosomal/lysosomal system (ANXA8 and ANXA1; green boxed; Figure 3D).

Together, our results evidenced that cytokinesis, vesicle trafficking, and the endosomal/lysosomal system are important biological pathways for the replication of SARS-CoV-2.

**Vasopressin interacts with sACE2 and spike of SARS-CoV-2**

In view of the importance of vasopressin-related VDFs in SARS-CoV-2 infection (Figure 2F), we further tested if SARS-CoV-2 infection depends on vasopressin. Compared to the mock-treated control, HK-2 cells pretreated with increasing doses of vasopressin showed a significant increase in the SARS-CoV-2 infectivity as determined by the TCID50 assays (Figure 4A). It is known that circulating sACE2 is an important regulator of the RAS, while vasopressin production could also be regulated by the RAS (Reid et al., 1983). Given that sACE2 retains an intact interaction site for binding to SARS-CoV-2, we hypothesized that there may be interaction between vasopressin and sACE2, which could modulate the SARS-CoV-2 infectivity. To examine their interactions, we spiked recombinant spike (S) of SARS-CoV-2 into culture medium of 293T cells, which were doubly transfected with FLAG-tagged vasopressin and V5-tagged ACE2. Secretions of sACE2 and vasopressin were confirmed by WB analyses of the conditioned medium of transfected cells (Figure 4B; lane 1). As expected, pull-down of sACE2 could effectively co-immunoprecipitate the S (Figure 4B; lane 2). Intriguingly, vasopressin was detected in the same co-immunoprecipitation, suggesting that sACE2 could form a complex with S and vasopressin (Figure 4B; lane 2). To further examine if there was any interaction between S and vasopressin, we removed vasopressin from the co-immunoprecipitation experiment.
Figure 3. VDFs related to cytokinesis, vesicle trafficking, and endosomal/lysosomal system are important for SARS-CoV-2 infection
(A) Gene ontology (GO) enrichment analysis of VDFs identified in our RNAi screening (left) and that reported by other coronavirus studies (right) using a threshold p value of 0.05. The percentage of each GO term is shown in pie charts.
(B) Volcano plot analysis of VDFs identified in our RNAi screening. Gene annotation revealed that some enriched VDFs ≥3-fold enrichment and false positive rates (FDRs) <5% have functional roles related to the regulation of the cardiovascular system (filled red), cytokinesis and vesicle trafficking pathway (filled green), and renal-related disease (filled brown).
(C) Heatmap analysis of the ≥3-fold enriched VDFs with FDRs < 0.05. S1–3 and M1–3 are biological replicates of SARS-CoV-2- and mock-infected samples, respectively.
(D) siRNA knockdown of selected VDFs in HK-2 cells leads to an inhibition of SARS-CoV-2 replication as assessed by qRT-PCR. Transfection of non-targeting siRNA was included as negative control (mock). Data points below the dotted line indicate siRNA inhibitory effects on SARS-CoV-2 infection to be >80%. The results were derived from three independent experiments. Each data point and error bar depict the mean value and SEM, respectively. Statistical analyses were carried out using Student’s t test. Statistical significance is indicated by the asterisks (**p < 0.01; ***p < 0.001).
Figure 4. Formation of a protein complex containing sACE2, S of SARS-CoV-2, and/or vasopressin facilitates SARS-CoV-2 cell entry via Dyn2-dependent endocytosis

(A) Effect of vasopressin on SARS-CoV-2 infectivity. The HK-2 cells were pretreated with increasing doses of vasopressin followed by SARS-CoV-2 infection. Mock-treated HK-2 cells were included as a control.

(B) Co-immunoprecipitation of sACE2, vasopressin, and S. WB analysis of conditioned supernatants of FLAG-tagged vasopressin- and V5-tagged ACE2-doubly transfected 293T cells with spike-in recombinant S (lane 1). Co-immunoprecipitation of V5-tagged ACE2 (lanes 2–4) and FLAG-tagged vasopressin (lane 5) from conditioned supernatants of transfected 293T cells using anti-V5 and anti-FLAG antibodies, respectively. Co-immunoprecipitation of FLAG-tagged vasopressin from conditioned supernatants of transfected HK-2 cells using anti-FLAG antibodies (lane 6–8). Detection of S, ACE2, and vasopressin was performed using specific antibodies.

(C) IFA of SARS-CoV-2 S, sACE2, and AVPR1B-transfected 293T cells. The 293T cells were transfected with a plasmid encoding AVPR1B prior to inoculation with HK-2 conditioned supernatant containing sACE2, recombinant S, and vasopressin. The cells were fixed and immunostained with anti-S (magenta), anti-ACE2 (red), and anti-AVPR1B (green) antibodies. Scale bars represent 20 μm.

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down of sACE2 was able to co-immunoprecipitate the S (Figure 4B; lane 3), but the amount was lower compared to the reaction containing vasopressin (Figure 4B; lane 1). Remarkably, when S was removed from the co-immunoprecipitation reaction, vasopressin was no longer able to be co-immunoprecipitated by sACE2, suggesting that the S was required for the formation of sACE2-S-vasopressin complex (Figure 4B; lane 4). The interaction between vasopressin and S was supported by successful co-immunoprecipitation of S by pulling down of vasopressin in the absence of ACE2 (Figure 4B; lane 5). The interactions between vasopressin, sACE2, and S of SARS-CoV-2 were further confirmed by reciprocal co-immunoprecipitation using HK-2 cells. It is expected that the HK-2 cells, which are highly susceptible to SARS-CoV-2, could have sufficient endogenous sACE2 for the formation of the sACE2-S-vasopressin complex. Consistent with the co-immunoprecipitation results using 293T cells, pull-down of the vasopressin using supernatants of FLAG-tagged vasopressin transfected HK-2 cells—in the presence of S—was able to co-immunoprecipitate endogenous sACE2 and S (Figure 4B; lane 6). In contrast, sACE2 was unable to be co-immunoprecipitated by vasopressin in the absence of S (Figure 4B; lane 8). Overall, our results supported the possible formation of sACE2-S-vasopressin complex, in which the strength of the sACE2-S interaction could be enhanced in the presence of vasopressin.

Vasopressin functions by binding to its membrane receptors, which could trigger a process called receptor-mediated endocytosis. Consistent with our interaction assay results, which showed that vasopressin could form a complex with sACE2 and S (Figure 4B), knockdown of a vasopressin receptor—AVPR1B—potently inhibited SARS-CoV-2 infection (89.66% ± 0.14 inhibition; Figures 2F and S2). Therefore, we hypothesized that SARS-CoV-2 may enter cells via the receptor-mediated endocytosis through AVPR1B. To test this hypothesis, non-permissive 293T cells were first transfected with a plasmid overexpressing vasopressin, followed by inoculating the conditioned supernatant containing sACE2-S-vasopressin complex—like AVPR1B—could also be internalized into cells via AT1, we performed overexpression experiments using the same experimental conditions as described above. Over the infection time course, IFA results detecting the YFP-tagged AT1 and recombinant S showed that 293T cells transfected with the AT1 also displayed increasing puncta staining pattern in the cytosol after inoculation with conditioned supernatant containing sACE2-S-vasopressin complex (Figure 4D). As our interaction assay showed that the sACE2 could interact with S in the absence of vasopressin (Figure 4B), we tested if the sACE2-S complex could also enter cells by utilizing the same cell entry mechanism. Under the same experimental conditions, similar puncta staining pattern could be observed in the YFP-tagged AT1-transfected 293T cells following inoculation of the sACE2-S complex (Figure 4E). Together, our results suggested that the sACE2-S complex and sACE2-S-vasopressin complex could enter cells via receptor-mediated endocytosis through AVPR1B and/or AT1 receptors.

**Dynamic 2-dependent endocytosis is important for SARS-CoV-2 to establish an effective infection**

Receptor-mediated endocytosis is known to be regulated by cytokinesis, vesicle trafficking, and the endosomal/lysosomal system (Simonetti and Cullen, 2019). To further examine the role of cytokinesis and vesicle trafficking in SARS-CoV-2 infection, we treated the HK-2 cells with two cytoskeletal drugs—Jasplakinolide and Cytochalasin D (CytD)—prior to SARS-CoV-2 infection. Compared to the mock-treated control cells, HK-2 cells pretreated with Jasplakinolide and CytD showed 2.15-log (p < 0.001) and 1.33-log (p < 0.001) reductions in viral load, respectively (Figure 4F). WB and IFA results confirmed undetectable and minimal SARS-CoV-2 NP expression in pretreated cells (Figures 4G and 4H). Besides a substantial reduction in the number of positively stained cells in the CytD-treated HK-2 cells, we observed an altered staining pattern with the viral antigen expression primarily restricted to the plasma membrane forming a “ring-like” structure (Figure 4H). These results suggested that perturbation of the cytokinesl organization may hamper the entry of SARS-CoV-2 into the cytosol.

Besides, CytD is known to be able to modulate the function of the endosomal-lysosomal system. Indeed, our RNAi screening also identified several VDFs that are related to this system, of which four (ANXA8, ANXA1, ANXA8L1, and ALS2) have been
Figure 5. ACE2 shedding modulates the SARS-CoV-2 infectivity

(A) WB analysis showing expression levels of cellular ACE2 (cACE2) and secretory ACE2 (sACE2) in HK-2 cells pretreated with different doses of ACE2 sheddase inhibitor prior to SARS-CoV-2 infection (lanes 1 and 2). Untreated (lane 3) and mock-infected (lane 4) controls were included. SARS-CoV-2 NP was detected using specific antibodies.

(B) Cell lysates of samples described in (A) were assayed for viral titer. Untreated and mock-infected cells were included as controls.

(C) IFA of SARS-CoV-2 NP (green) in pretreated HK-2 cells described in (A) using specific antibodies. Cells were counterstained with DAPI to label nuclei (blue). Scale bars represent 100 μm.

(D) WB analysis showing expression levels of cACE2 and sACE2 in HK-2 cells transfected with different amount of siADAM17 (lanes 2 and 3). siControl-transfected (lane 1) and mock-transfected (lane 4) controls were included. The SARS-CoV-2 NP was detected using specific antibodies.

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validates (Figure 3D). Given that endosomal acidification plays important roles in receptor-mediated cell entry in some viruses (Glebov, 2020), we examined if the change of endosomal pH would affect SARS-CoV-2 replication by impairing the function of the proton pump—vacuolar H+–ATPase (V-ATPase)—which is responsible for controlling the endosomal pH via a specific inhibitor, Bafilomycin A1 (BafA1) (Cotter et al., 2015). Pretreatment of HK-2 cells using the BafA1 at a concentration of 25 nM showed a significant inhibition (>4-log) of SARS-CoV-2 infection when compared with the mock-treated control cells (p < 0.001; Figure 4F). Pretreatment with a 10-fold lower dose (2.5 nM) of BafA1 also achieved a similar level of inhibition (>4-log) (p < 0.001; Figure 4F). Consistently, viral protein expression was not detected by WB and IFA in the BafA1-treated cells (Figures 4G and 4H).

Thus far, our results supported a potential cell entry mechanism by which SARS-CoV-2 exploits the endocytosis pathway (Figure 4C–4H). Receptor-mediated endocytosis is known to involve membrane scission of nascent vesicles from plasma membrane by dynamin 2 (Dyn2) (González-Jamett et al., 2013). To further test the requirement of Dyn2, we targeted its function by transfecting a plasmid overexpressing the dominant-negative Dyn2 (Dyn2-K44A) prior to SARS-CoV-2 infection. In contrast to the GFP-wild-type Dyn2-transfected cells showing strong colocalization between the GFP-tagged Dyn2 and SARS-CoV-2 NP, no co-localization was detected in the GFP-Dyn2-K44A-transfected cells (Figure 4I), suggesting entry of SARS-CoV-2 into the cells through the Dyn2-dependent endocytosis pathway.

ACE2 shedding modulates the SARS-CoV-2 infectivity
Dyn2-dependent endocytosis involves a vesicular transport event to facilitate the internalization and recycling of receptors (Cullen and Steinberg, 2018). In addition to being a receptor for SARS-CoV-2, ACE2 controls blood pressure homeostasis, which requires complex regulatory events involving protein trafficking as well as post-translational modifications. First, surface trafficking and sorting are required for plasma membrane expression of the cACE2. On the surface, cACE2 requires proteolytic cleavage by proteases such as disintegrin and ADAM17 to release circulating sACE2 to the extracellular space. It is conceivable that the shedding of cACE2 could modulate SARS-CoV-2 infectivity. To test this possibility, prior to SARS-CoV-2 infection, we pretreated HK-2 cells with GW280264X, which inhibits the enzymatic activity of ADAM17. Following the treatment, successful inhibition of sACE2 but not cACE2 expression was confirmed by WB (Figure 5A). Notably, pretreatment of GW280264X potently inhibited SARS-CoV-2 infection in a dose-dependent manner as shown by WB, TCID$_{50}$, and IFA (Figures 5A–5C).

In addition, we independently performed knockdown experiments to confirm the regulatory effect of ADAM17 on SARS-CoV-2 infectivity. Consistent with the results of the GW280264X treatment experiment, a reduction of sACE2 was also observed in the siADAM17-transfected cells (Figure 5D). Dose-dependent reductions in viral loads (p < 0.001) and NP expression were observed in siADAM17-transfected cells (Figure 5D–5F). Overall, our results suggested that targeting ACE2 shedding could modulate SARS-CoV-2 infectivity.

SARS-CoV-2 infection depends on sACE2
Our results showed that sACE2 could facilitate virus cell entry through receptor-mediated endocytosis, suggesting its potentially important role on SARS-CoV-2 infection. To further substantiate its role, we tested the effect of sACE2 on SARS-CoV-2 infectivity in HK-2 cells at a low MOI of 0.01. Under this infection condition, ~10% of cells could be infected (Figure 6A). With increasing doses of recombinant ACE2 (rACE2) on HK-2 cells, SARS-CoV-2 infectivity increased to 30% and 50%, respectively, as evidenced by stronger CPEs and higher expression of SARS-CoV-2 NP observed in the infected cells (Figure 6A). The results suggested that the addition of rACE2, mimicking the sACE2, could enhance SARS-CoV-2 infectivity. This effect, however, was not observed when performing the experiment using Middle East respiratory syndrome (MERS)-CoV under the same conditions (Figure S3). To further confirm the role of sACE2 in SARS-CoV-2 infection, we repeated the same experiment using two human lung cell lines—Calu3 and A549. Consistently, WT and TCID$_{50}$ results showed a dose-dependent augmentation of SARS-CoV-2 infectivity in both cell lines treated with an increasing dose of rACE2 (Figure 6B). Intriguingly, cell lines—including RD, NT2, HepG2, and Huh7—derived from organs other than the lung also showed increase in SARS-CoV-2 susceptibility when treated with an increasing dose of rACE2 (Figure 6B).

To investigate the role of sACE2 under physiologically relevant conditions, we selected a panel of human cell lines from different organs and examined their relationship between the susceptibility to SARS-CoV-2 and sACE2 and cACE2 expressions. Consistent with previous studies on the rare expression of ACE2 in many cell types (Lukassen et al., 2020; Wang et al., 2020), WB results demonstrated that the protein expression of ACE2 could only be detected in a few cell lines (Figure 6C). Expectedly, high expression of sACE2 and cACE2 was found in the conditioned supernatants and cell lysates of HK-2 cells (Figure 6C; lane 11), respectively, and the results were in line with the high susceptibility of HK-2 to SARS-CoV-2 (Figure 1). As shown by the detection of NP in the infected cell lysates, relatively high expression level of sACE2 but undetectable level of cACE2 in Calu3 cells seemed to support SARS-CoV-2 infection (Figure 6C; lane 10). We also noted the expression of sACE2 but not cACE2 in Caco-2 cells (Figure 6C; lane 3). Although WB analysis was unable to detect the NP expression (Figures 1B and 6C; lane 3), IFA and TCID$_{50}$ assays consistently detected viral antigens...
and RNAs, though at a much lower levels compared to HK-2 cells, in the infected CaCo-2 cells (Figures 1C and 1E). Notably, HepG2 cells were unable to support SARS-CoV-2 infection despite a strong expression of ACE2 (but not sACE2) detected in this cell line (Figure 6C; lane 6).

To monitor sACE2 during the SARS-CoV-2 infection process, we utilized a GFP-tagged ACE2 mutant lacking the transmembrane domain—GFP-ACE2ΔTM—to mimic sACE2 (Procko, 2020) for infection. We first validated the subcellular localization of GFP-ACE2ΔTM by transfecting it into 293T cells. In contrast to the cells transfected with the wild-type ACE2, where the ACE2 proteins were readily detected in both cell lysates and conditioned supernatants (Figure S4A, lane 1), cells transfected with the GFP-ACE2ΔTM could only be detected in the conditioned supernatants (Figure S4A, lane 2), suggesting that most ACE2 (i.e., GFP-ACE2ΔTM) was exported out into the extracellular space and not retained inside the cells. These results were consistent with the IFA results where the GFP signal could be detected in cells transfected with the wild-type ACE2 but not the GFP-ACE2ΔTM (Figure S4B). We then performed confocal microscopy analysis of the GFP-ACE2ΔTM-transfected cells upon SARS-CoV-2 infection. Remarkably, orthogonal projections of confocal sections revealed strong co-localization of SARS-CoV-2 NP and GFP-ACE2ΔTM (Figure 6D). Three-dimensional (3D) reconstruction of the confocal images is available in Video S1. Profiles of fluorescent intensity measured at different focal planes also revealed a high degree of overlapping signals between the SARS-CoV-2 NP and GFP-ACE2ΔTM (Figure 6E; Video S1). Further optical sectioning of the infected cells revealed that similar to the formation of vesicles during the endocytosis, a punctate pattern of viral antigen predominantly localized on the cell surface (Figure 6F). Together, these results support that sACE2 could interact with SARS-CoV-2 during the infection process and that sACE2 has a potentially determining role in SARS-CoV-2 infection.

**DISCUSSION**

We have successfully identified VDFs that are important for SARS-CoV-2 to establish a productive virus replication (Figure 7), and their annotated functions were correlated with clinical manifestation and complications of SARS-CoV-2 infection. Increasing evidence support the association between COVID-19 and cardiovascular and renal-related diseases (Camm and Camm, 2020; Yang et al., 2020). These observations were also in line with the RNAi screening results, in which the functional annotation of some enriched VDFs and their biological pathways were also linked to the regulation of cardiac and renal functions. In fact, pathway analysis of VDFs showed ~70% of significantly affected biological pathways are related to cardiac diseases (pink; Figure 2D). The most affected biological pathway is related to cardiac arrhythmias, which is often observed in SARS-CoV-2-infected patients, especially in critically ill cases (Figure 2D) (Babapoor-Farrokhran et al., 2020; Karamchandani et al., 2020; Rav-Acha et al., 2020). Besides, we also identified three significantly affected renal disease-related pathways (brown; Figure 2D), which is in line with the high incidence of acute kidney injury reported in SARS-CoV-2-infected cases (Yang et al., 2020). A previous study also reported the presence of coronavirus-like particles in >75% kidney biopsies of deceased SARS-CoV-2 patients (Su et al., 2020). Furthermore, PANTHER pathway analysis of the enriched VDFs identified vasopressin-related pathways as the top-affected molecular pathways (Figure 2E). The important roles of these vasopressin-related VDFs in SARS-CoV-2 infection were further validated by performing knockdown experiment (Figure 2F). Previous studies showed that vasopressin can induce differentiation of stem cells into cardiomyocytes and promote heart muscle homeostasis (Yasin et al., 1994). It can also control toxicity of body fluids by converting to arginine vasopressin (AVP) that can further regulate the arterial blood pressure (Demiselle et al., 2020). Consistently, due to their regulatory roles in maintaining homeostasis of kidney and heart, dysregulated expressions of vasopressin and AVP were also directly linked to the development of renal failure and cardiovascular system diseases (Czarzasta et al., 2018). Therefore, further investigation of the identified VDFs involved in cardiac and renal disease development may provide mechanistic insights into SARS-CoV-2-induced extrapulmonary diseases. sACE2 and vasopressin play important roles in SARS-CoV-2 infection. ACE2, being a receptor for SARS-CoV-2 (Wan et al., 2020), is also known to serve as one of the key regulators controlling the release of vasopressin into the plasma for the maintenance of blood pressure homeostasis via the RAS
To serve its regulatory role in the RAS, the tissue-bound form of ACE2 (i.e., cACE2) is shed by proteases to produce the sACE2, which can then enter the circulatory system. As sACE2 preserves the binding site for SARS-CoV-2, it is possible that sequestration of SARS-CoV-2 by sACE2 may enable cell entry of tissues where cACE2 is poorly expressed. Indeed, our in vitro data showed that endogenous sACE2 could interact with the S of SARS-CoV-2 in the extracellular compartment (Figure 4B). The resulting sACE2-S complex could then enter cells through receptor-mediated endocytosis via the AT1 surface receptor (Figures 4D and 4E). Additionally, we found that the S of SARS-CoV-2 could interact with vasopressin forming an sACE2-S-vasopressin complex, which facilitated cell entry via another vasopressin receptor, AVPR1B (Figures 4B and 4C). This new cell entry mechanism may explain our data showing that cells from various organs could be sensitized to SARS-CoV-2 upon administration of rACE2 (Figures 6A and 6B).

sACE2 expression contributes to the cell line susceptibility to SARS-CoV-2. Little or low infectivity of SARS-CoV-2 was detected in all tested human cell lines, except for the HK-2 cells (Figure 6C). In contrast to HK-2 cells, SARS-CoV-2 is unable to replicate efficiently in 293T, although both cell lines were derived from human kidney. We speculate that the differential susceptibility may be linked to their differences in sACE2 level. We also noted that while highly susceptible HK-2 cells exhibited very strong expressions of both cACE2 and sACE2 (Figure 6C; lane 11), expression of cACE2 alone does not render the cells susceptible to SARS-CoV-2 as exemplified in HepG2 and 293T cells where cACE2, but not sACE2, was detected (Figure 6C; lanes 6 and 9). In contrast, expression of sACE2 alone in Caco-2 and Calu3 cells was able to support SARS-CoV-2 infection (Figure 6C; lanes 3 and 4).

Figure 7. A coronavirus life cycle map
The function and subcellular localization of VDFs were determined based on the use of multiple databases (please see STAR Methods) and the literature. VDFs that were discovered only in this study are indicated by red-filled dots, and the gene symbols are written in red. VDFs discovered in this study and previously reported in other studies are indicated by red-filled dots, and the gene symbols are written in black. VDFs identified in other studies are indicated by open dots, and the gene symbols are written in black. The information of the VDFs identified in this study is listed in Table S1.
10). Although WB results failed to detect SARS-CoV-2 NP expression in Caco-2 cells, our qRT-PCR and IFA results confirmed the presence of the SARS-CoV-2 RNA and protein in the infected Caco-2 cells (Figures 1A, 1C and 1E). The low expression level of sACE2 in Caco-2 cells may weakly support SARS-CoV-2 infection. This observation coincides with IFA and WB results, which showed a dose-dependent augmentation of SARS-CoV-2 infectivity in cells administered with an increasing dose of rACE2 (Figures 6A and 6B). Together, our in vitro infection data using human cell lines that originated from different organs support the important role of sACE2 in SARS-CoV-2 infection.

We discovered the dual role of sACE2 in SARS-CoV-2 infection. Modulating the SARS-CoV-2 infectivity using recombinant sACE2 has been previously suggested as a treatment strategy for COVID-19. Attempts have been made to utilize recombinant soluble human ACE2 to inhibit SARS-CoV-2 infection using in vitro model (Cocozza et al., 2020; Monteil et al., 2020). In these studies, very high concentrations of rACE2 (10−10–200 μg/mL of ACE2, concentrations are much higher than its physiological range in plasma, i.e., 1 ng/mL; Ridwan et al., 2019; Sama et al., 2020) were required to achieve inhibitory effects. Indeed, our results were also in line with their findings, where 25 and 100 μg/mL of rACE2 could inhibit SARS-CoV-2 infection (Figure S3). We speculate that the addition of excessive amounts (i.e., μg/mL level) of recombinant ACE2 may saturate endocytic recycling of the ACE2 receptor, competing with the SARS-CoV-2-ACE2 complex for cell entry, therefore resulting in the reduction of SARS-CoV-2 infectivity. In contrast, rACE2 concentrations close to physiological range (i.e., ng/mL level) could enhance SARS-CoV-2 infection (Figures 6A, 6B, and S3). Interestingly, a similar phenomenon was recently reported in a study that provided an important clue on the in vivo effect of the rACE2 in SARS-CoV-2-infected patient (Zoufaly et al., 2020). The study showed that substantial and steady increases in viral loads were detected in tracheal aspirates (from ~10^4 copies/mL at day 0 to ~10^6 copies/mL at day 2) and nasopharyngeal swabs (from ~10^4 copies/mL at day 0 to ~10^6 copies/mL at day 5), respectively, after the administration of rACE2. Although the patient eventually recovered after the appearance of neutralizing antibodies, this in vivo data and our findings suggested that treatment that may alter sACE2 level in patients with COVID-19 should be carefully considered.

The discovery of a highly permissive human renal cell line—HK-2—to SARS-CoV-2 is useful for studying the biogenesis of SARS-CoV-2 and the possible therapeutic options for COVID-19. The physiological and pathological relevance of using this HK-2 cell line in studying SARS-CoV-2 biogenesis is supported by recent ultrastructural studies that detected SARS-CoV-2 in renal autopsy of patients with COVID-19 (Farkash et al., 2020). The versatility of HK-2 is further supported by our RNAi screening results, in which many successfully identified VDFs were not only linked to renal-related diseases but also other clinical manifestations and complications of COVID-19 (Figures 2D and 2E). Furthermore, most current studies on SARS-CoV-2 infection were based on the use of pseudovirus-expressing viral surface S protein or non-human cell lines, which may not truly reflect an authentic infection process (Heaton et al., 2020; Shang et al., 2020). Our findings using authentic virus to infect physiologically relevant HK-2 cells revealed that SARS-CoV-2 entered the cells mainly through Dyn2-dependent endocytosis (Figures 4F–4I). The involvement of cytokinesis, vesicle trafficking, and the endosomal/lysosomal system in SARS-CoV-2 cell entry was further confirmed through the use of inhibitors (Jasplakinolide, CytD, and BafA1) and a dominant-negative Dyn2 construct (Figures 4F–4I). Of note, our results showed that a potent inhibition (>4-log) of SARS-CoV-2 replication could be achieved even when cells were pretreated with a low dose of BafA1 (i.e., 2.5 nM) (Figures 4F–4H), which could inhibit receptor-mediated endocytosis (Harada et al., 1996). Further investigation of the BafA1 and its analogs as potential therapeutic agents for COVID-19 is warranted.

Modulating the shedding of sACE2 may provide insights into treatment strategies for COVID-19. A previous study suggested that the ADAM17 activity could be correlated with infectivity of SARS-CoV, which also utilized ACE2 as a cellular receptor (Haga et al., 2008). The authors reported that the S of SARS-CoV induced the ADAM17 activity causing an enhanced ACE2 shedding, which was positively correlated with SARS-CoV infectivity. It is possible that cells could be sensitized to both SARS-CoV-2 and SARS-CoV by enhancing the production of sACE2 through the induction of ADAM17 activity. On the flip side, controlling the activity of ADAM17 could be a potential treatment strategy for COVID-19. Indeed, we demonstrated that inhibition of ADAM17 by a sheddase inhibitor, GW280264X, or by a specific siRNA potently suppressed SARS-CoV-2 infection (Figures 5A–5F). The therapeutic potential of targeting the ADAM17 has also been widely investigated in cancer research with minimal side effects (Bandsma et al., 2015; Blaydon et al., 2011), suggesting that it may represent a safe target in controlling SARS-CoV-2 infection. Overall, our identification of inhibitors, including the sheddase inhibitor, the cytoskeletal drugs and/or siRNAs/drugs targeting the VDFs, could provide insights into future development of drug combination therapy, which can be considered as an effective strategy in preventing the generation of drug-resistant mutants, particularly in the case of RNA viruses (Presloid and Novella, 2015), for the treatment of COVID-19.

Limitations of the study
This study has several limitations as the findings are based on HK-2 cells, which are of renal origin. As the primary infection site of SARS-CoV-2 is lung, further examinations will be needed to study the involvement of these factors during pulmonary infection. Besides, during the preparation of our manuscript, several CRISPR/Cas9 screening studies have been published, in which the VDFs identified are largely different in these studies. One reason may be due to the key difference between CRISPR and RNAi, where CRISPR results in true loss-of-function effects, which is in contrast to RNAi that may create hypomorphic phenotypes, thus allowing us to study essential genes whose knockout may otherwise be cell lethal. While both technologies are considered powerful tools to identify VDFs for replication, perhaps combining results from each approach could help fully to understand the underlying biology of SARS-CoV-2.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:
DECLARATION OF INTERESTS

J.F.-W.C. has received travel grants from Pfizer Corporation Hong Kong and Astellas Pharma Hong Kong Corporation Limited and was an invited speaker for Gilead Sciences Hong Kong Limited and Luminex Corporation. The other authors declare no competing interests.

Received: December 16, 2020
Revised: February 4, 2021
Accepted: February 25, 2021
Published: March 2, 2021

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| ACE-2               | R&D    | Cat#AF933  |
| ADAM17              | Abcam  | Cat#ab2051 |
| γ-Tubulin           | Sigma-Aldrich | Cat#T6557 |
| AVPR1B              | Abcam  | Cat#ab116246 |
| SARS-CoV-2 NP (Rabbit polyclonal) | This paper | N/A |
| SARS-CoV-2 Spike (Mouse monoclonal) | GeneTex | GTX632604 |
| FLAG                | Sigma-Aldrich | Cat# F7425 |
| EZview™ Red ANTI-FLAG M2 Affinity Gel | Sigma-Aldrich | Cat# F2426 |
| ANTI-V5 agarose affinity gel | Sigma-Aldrich | Cat# A7345 |
| Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific (Invitrogen) | Cat#A-11055 |
| Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific (Invitrogen) | Cat#A-11008 |
| Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 | Thermo Fisher Scientific (Invitrogen) | Cat#A-11012 |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 | Thermo Fisher Scientific (Invitrogen) | Cat#A-21235 |
| Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP | Thermo Fisher Scientific (Invitrogen) | Cat#62-6520 |
| Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP | Thermo Fisher Scientific (Invitrogen) | Cat#31460 |
| Rabbit anti-Goat IgG (H+L) Secondary Antibody, HRP | Thermo Fisher Scientific (Invitrogen) | Cat#81-1620 |
| **Bacterial and Virus Strains** | | |
| MAX Efficiency™ DH5α Competent Cells | Thermo Fisher Scientific | Cat# 18258012 |
| SARS-CoV-2, isolate HKU-001a | Laboratory of Microbiology, HKU | N/A |
| MERS-CoV, EMC/2012 strain | A gift from Ron Fouchier (Erasmus Medical Center) | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Pierce Protease and Phosphatase Inhibitor Mini Tablets, EDTA-free | Thermo Fisher Scientific | Cat# A32961 |
| Fetal bovine serum (FBS) | Thermo Fisher Scientific (GIBCO™) | Cat#10082 |
| PBS, pH 7.4 | Thermo Fisher Scientific (GIBCO™) | Cat#10010023 |
| Trypsin-EDTA (0.25%), phenol red | Thermo Fisher Scientific (GIBCO™) | Cat#25200056 |
| Dulbecco’s Modified Eagle Medium (DMEM) | Thermo Fisher Scientific (GIBCO™) | Cat#11965-092 |
| Minimal Essential Media (MEM) | Thermo Fisher Scientific (GIBCO™) | Cat#11095-080 |
| DMEM/F12 (1:1) (1X) | Thermo Fisher Scientific (GIBCO™) | Cat#11320-033 |
| Paraformaldehyde Aqueous Solution-16% | Electron Microscopy Sciences | Cat#30525-89-4 |
| Triton X-100 | Sigma-Aldrich | Cat# 93443 |
| Immun-Blot PVDF Membrane | Bio-Rad | Cat#1620177 |
| Lipofectamine 2000 | Invitrogen | Cat# 11668027 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Lipofectamine™ Transfection Reagent | Thermo Fisher Scientific (Invitrogen) | Cat#18324020 |
| PLUS™ Reagent | Thermo Fisher Scientific (Invitrogen) | Cat#11514015 |
| VECTASHIELD® Mounting Medium with DAPI | Vector Laboratories | Cat#H-1200 |
| Jasplakinolide, Actin polymerization and stabilization inducer | Abcam | Cat#ab141409 |
| Cytochalasin D | Sigma-Aldrich | Cat#C8273 |
| Bafilomycin A1 | Sigma-Aldrich | Cat#SML1661 |
| Angiotensin Converting Enzyme-2, ACE2 (Human recombinant, expressed in HEK293 cells) | Sigma-Aldrich | Cat#SAE0064 |
| SARS-CoV-2 (2019-nCoV) Spike Protein (S1+S2 ECD, His tag) | Sino Biological | Cat#40589-V08B1 |
| Vasopressin | Origene | Cat#TP316816 |

**Deposited Data**

| RNA-seq data | This paper | GEO accession number: GSE159272 |

**Experimental Models: Cell Lines**

| Cell Line | Source | Cat# |
|-----------|--------|------|
| RD | ATCC | Cat#CCL-136 |
| H1HeLa | ATCC | Cat#CRL-1958 |
| Caco-2 | ATCC | Cat#HTB-37 |
| NT2 | ATCC | Cat#CRL1973 |
| Huh-7 | JCRB cell bank of Okayama University | Cat#JCRB0403 |
| HepG2 | ATCC | Cat#HB-8065 |
| PLC/PRF/5 | ATCC | Cat#CRL-8024 |
| A549 | ATCC | Cat#CCL-185 |
| Calu3 | ATCC | Cat#HTB-55 |
| 293T | ATCC | Cat#CRL-3216 |
| HK-2 | ATCC | Cat#CRL-2190 |
| Vero-E6 | ATCC | Cat#CRL-1586 |

**Oligonucleotides**

| Oligonucleotide | Source | N/A |
|-----------------|--------|-----|
| SARS-CoV-2 E-gene, Forward: | This paper | N/A |
| ACAGGTACGTTAATAGTTAATAATGCT | This paper | N/A |
| SARS-CoV-2 E-gene, Reverse: | This paper | N/A |
| ATATTGCAGCAGTACGCACACACACA | This paper | N/A |
| Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Forward: | This paper | N/A |
| TCACGACCATTGGAGAAGGC | This paper | N/A |
| Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Reverse: | This paper | N/A |
| GCTAAGCAGGTGTTGTGCTGCA | This paper | N/A |
| GNF primer | This paper | N/A |
| ATTTATTGTATCTCTGGAGGACCTC | This paper | N/A |
| Fwd GNF primer | This paper | N/A |
| TGCAAGTGACTATGTCTTGTATG | This paper | N/A |
| Rev GNF primer | This paper | N/A |
| ACAAGCAAGCTTGGGACCTACGAA | This paper | N/A |
| H1 Forward primer | This paper | N/A |
| GTTCTGTTATGAGCACCACCTTGGATCC | This paper | N/A |
| RevPrimer | This paper | N/A |
| AAAGAATGTTATGGAGGACCTAGAA | This paper | N/A |

(Continued on next page)
## REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Novel Coronavirus 2012 Real-Time RT-PCR assay | CDC | Cat#KT0136 |
| ON-TARGETplus Human ADAM17, siRNA- SMARTpool, 5nmol | Horizon Discovery/Dharmacon | Cat#L-003453-00-0005 |
| ON-TARGETplus Human TMEM8B, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-017716-02 |
| ON-TARGETplus Human BASP1, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-019008-00 |
| ON-TARGETplus Human ZNF143, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-013965-00 |
| ON-TARGETplus Human XIST, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#R-188298-00 |
| ON-TARGETplus Human HIST1H2AL, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-011434-01 |
| ON-TARGETplus Human SLC25A46, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-007353-02 |
| ON-TARGETplus Human GGGBP1, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-015703-00 |
| ON-TARGETplus Human ARFGEF2, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-012208-02 |
| ON-TARGETplus Human ZNF548, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-016400-02 |
| ON-TARGETplus Human ALS2, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-014151-00 |
| ON-TARGETplus Human IL18RAP, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-007950-00 |
| ON-TARGETplus Human CXCR1, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-005646-00 |
| ON-TARGETplus Human CACNB4, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-009062-00 |
| ON-TARGETplus Human CAPNS1, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-009979-00 |
| ON-TARGETplus Human ARL4D, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-011583-00 |
| ON-TARGETplus Human GPR176, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-005522-00 |
| ON-TARGETplus Human S1PR5, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-004791-00 |
| ON-TARGETplus Human ANXA8L2, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-011570-01 |
| ON-TARGETplus Human ANXA8, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-183964-00 |
| ON-TARGETplus Human GRB2, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-019220-00 |
| ON-TARGETplus Human ANXA1, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-011161-00 |
| ON-TARGETplus Human AVP, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-010997-00 |
| ON-TARGETplus Human OXT, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-011185-00 |
| ON-TARGETplus Human AVPR1B, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-005431-00 |

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**continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| ON-TARGETplus Human HLF, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-010514-02 |
| ON-TARGETplus Human BTC, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-011597-01 |
| ON-TARGETplus Human CCNE1, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-003213-00 |
| ON-TARGETplus Human HIF1A, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-004018-00 |
| ON-TARGETplus Human KHDRBS3, siRNA- SMARTpool, 0.1nmol | Horizon Discovery/Dharmacon | Cat#L-012748-01 |

**Recombinant DNA**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| K44A dynamin 2-pEGFP | A gift from Sandra Schmid | Addgene #34687 |
| Wt dynamin 2-pEGFP | A gift from Sandra Schmid | Addgene #34686 |
| pcDNA3.1 | Thermo Fisher Scientific (invitrogen) | Cat#V79020 |
| pcDNA3.1-ACE2-GFP | A gift from Utpal Pajvani | Addgene #154962 |
| pcDNA3.1-ACE2ΔTM-GFP | Chan et al., 2020 | Addgene #145171 |
| Vasopressin V1b receptor (AVPR1B) (NM_000707) Human Tagged ORF Clone | OriGene | Cat# RC215919 |
| pCXN2-HA-AT1R-YFP | Inuzuka et al., 2016 | Addgene #101659 |

**Software and Algorithms**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| DAVID Bioinformatics Resources | Huang et al., 2007 | https://david.ncifcrf.gov/tools.jsp |
| PANTHER (Protein Analysis Through Evolutionary Relationships) | Mi et al., 2019 | http://www.pantherdb.org/ |
| Cutadapt | Martin, 2011 | https://cutadapt.readthedocs.io/en/stable/ |
| Bowtie2 | Langmead and Salzberg, 2012 | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| HTSeq | Anders et al., 2015 | https://htseq.readthedocs.io/en/master/ |
| DESeq2 | Love et al., 2014 | https://bioconductor.org/packages/release/bioc/html/DESeq2.html |
| R | Gu et al., 2016; Ito and Murphy, 2013 | https://www.r-project.org/ |
| UniProt | Bairoch et al., 2005 | https://www.uniprot.org/ |
| Gene Ontology | Ashburner et al., 2000 | http://geneontology.org/ |
| NCBI Reference Sequence | Pruitt et al., 2005 | https://www.ncbi.nlm.nih.gov/refseq/ |
| NCBI OMIM database | McKusick, 2007 | https://www.ncbi.nlm.nih.gov/omim |
| BioGRID | Oughtred et al., 2019 | https://thebiogrid.org/ |
| PubMed | NCBI | https://pubmed.ncbi.nlm.nih.gov/ |
| GraphPad Prism 8 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Man Lung Yeung (pmlyeung@hku.hk).

**Materials availability**

All requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact author. Materials will be made publicly available either through publicly available repositories or via the authors upon execution of a Material Transfer Agreement.

**Data and code availability**

Additional Supplemental Items, including supplementary figures, tables and video, are available from Mendeley Data: https://data.mendeley.com/datasets/gcdpsxd6d5/2. All sequencing data associated with this study have been deposited to NCBI with GEO.
accession number: GSE159272. The sequencing throughput and a summary of the read counts are available in Table S2 (see also Figure S5).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

Eleven human cell lines (HK-2, Caco-2, A549, Calu-3, Huh7, HepG2, PLC/PRF/5, RD, HeLa, NT2, and 293T) derived from different organs were maintained in a humidified atmosphere at 37 °C with 5% CO2, in Dulbecco’s modified Eagle’s medium (DMEM) or minimum essential medium (MEM; Gibco) or DMEM/F12 (Gibco) containing 10% (v/v) fetal bovine serum (FBS, Gibco). Similarly, a type of African green monkey kidney epithelial cells—Vero-E6—were maintained in DMEM containing 10% (v/v) FBS. All cell lines have been authenticated by short tandem repeat (STR) profiling and tested negative for mycoplasma contamination (Pangenia Life-sciences Limited).

Viruses

SARS-CoV-2 (HKU-001a) was isolated from a nasopharyngeal aspirate of a laboratory-confirmed COVID-19 patient in Hong Kong (To et al., 2020). MERS-CoV (EMC/2012 strain) and was a gift from Ron Fouchier (Erasmus Medical Center)(Zaki et al., 2012). The isolates were propagated through Vero-E6 cells (ATCC) in DMEM (Gibco) supplemented with 10% FCS (Gibco) and 100 units/mL penicillin plus 100 μg/mL streptomycin (1% PS). All experiments entailing live SARS-CoV-2 followed the approved standard operating procedures of our Biosafety Level 3 (BSL-3) facility (Yeung et al., 2016).

METHOD DETAILS

Production of shRNA library and stable clones

FIV-based shRNA library was produced as described previously (Yeung et al., 2009; Yeung et al., 2018). Briefly, 2 μg of the shRNA library in lentiviral constructs (SBI) were co-transfected with 10 μg of the pPACK packaging plasmid mix into HEK293T cells using Lipofectamine and Plus reagents according to the manufacturer’s protocol (ThermoFisher). At 48 h after transfection, conditioned culture media were collected to harvest the packaged viruses. In parallel, GFP-packaged viruses were produced. The infectivity of the viruses was established based on the percentage of the GFP-transduced HK-2 cells. Four days after transduction, the cells were selected for 3 weeks in a growth medium containing 3 μg/mL of puromycin. The shRNA-transduced HK-2 cells were challenged with SARS-CoV-2 (MOI = 1). Three days after infection when CPE of the SARS-CoV-2-infected GFP-transduced HK-2 cells reached over 90%, the shRNA-transduced HK-2 cells were recovered by replenishing with DMEM/F12 (Gibco) containing 10% (v/v) of FBS (Gibco). After 24 h, the survived shRNA-transduced HK-2 cells were re-challenged with SARS-CoV-2 using the same infection conditions. At day 3 post-infection, total RNA of the shRNA-transduced HK-2 cells was harvested using TRIzol (ThermoFisher). Seven micrograms of total RNA was reverse transcribed into cDNAs in the presence of 10 μM of GNF primer (5’-ATTATTGTTATCTGTGG-GAGCCTC-3’), 100 mM of dithiothreitol, 10 mM of each dNTP, 1 μM reverse transcriptase buffer, and 200 U of SuperScript® III Reverse Transcriptase (Invitrogen). The reaction mixtures were incubated at 50 °C for 1 h. The reaction was stopped by heat inactivation at 72 °C for 5 min. Half of the reaction mixture was then transferred to a tube containing 1 × PCR reaction buffer, 20 mM of dNTP, 20 μM of Fwd GNF primer (5’-TATCATGTGCTATGTGTCTGGA-3’), 20 μM of Rev GNF primer (5’-CAAAAGCCTGGAAGC-TATCGCT-3’), and 1 μM of H1 Forward primer (5’-ACAAAGCCTGGAAGC-TATCGCT-3’), and iProof (Bio-rad). PCR amplification of the shRNA target region was performed under the following conditions: Step 1: 94 °C for 4 min; Step 2: 94 °C for 30 s and then 68 °C for 1 min; Step 3: Repeat Step 2 for 20 cycles; Step 4: 68 °C for 3 min. Nested PCR reactions using 1 μL of the 1st round PCR products were performed using 100 μM of H1 Forward primer (5’-GTTCTGTATGA-GACCACATTGATCC-3’), 100 μM of Rev Primer (5’-AAAAGATGTCTATGACGCTAGAA-3’), and iProof (Bio-Rad). PCR amplification of the shRNA target region was performed under the following conditions: Step 1: 94 °C for 2 min, 50 °C for 2 min and then 68 °C for 1 min; Step 2: 94 °C for 30 s and then 68 °C for 30 s; Step 3: Repeat Step 2 for 18 cycles; Step 4: 68 °C for 3 min. The PCR products were then gel purified using QIAquick PCR purification kit (Qiagen) as described in the manufacturer’s protocol. The purified PCR products were then submitted to Centre for PanorOmic Sciences at HKU for high-throughput sequencing.

Knockdown of gene expressions by siRNAs

For independent validation, siRNAs (Human ON-TARGETplus) were purchased from ThermoFisher. These siRNAs were transfected into the HK-2 cells using Lipofectamine 2000 (ThermoFisher) according to the manufacturer’s protocol. Forty-eight h after transfection, the cells were challenged with SARS-CoV-2 (MOI = 1). At day 3 post-infection, total RNA of the shRNA-transduced HK-2 cells were harvested as described above.

High-throughput sequencing

All samples for high-throughput sequencing were processed by the Centre for PanorOmics Sciences at HKU. Briefly, libraries of each sample were prepared by KAPA Hyper Prep Kit (KAPA Biosystems) according to the manufacturer’s protocol. Fifty nanograms of each library was then first purified by AMPure XP (Beckman Coulter) before end-repairing. Next, the 3’ end of the libraries were ligated to adaptors using xGen Dual Index UMI Adapters (IDT) according to the manufacturer’s protocol. The adaptor-ligated libraries were
then validated by Agilent Bioanalyzer, Qubit, and qPCR for quality control analysis. Then, the adaptor-ligated libraries were sequenced using NovaSeq 6000 (2 × 150 bp paired-end reads) System (Illumina). The dataset of the shRNA screening results has been deposited in NCBI with GEO accession number: GSE159272. The sequencing throughput and a summary of the read counts are available in Table S2 (see also Figure S5).

**Virus titration by TCID\textsubscript{50} assay**

The median tissue culture infective dose (TCID\textsubscript{50}) per mL was determined for SARS-CoV-2 in Vero-E6 cells as previously described (Yeung et al., 2018). Briefly, cells were seeded in 96-well plates at a density of 5 × 10\textsuperscript{4} cells/well in 150 μL of DMEM. The virus was serially diluted by half-log from 10\textsuperscript{5} to 10\textsuperscript{3} in DMEM. One hundred microliters of each dilution were added per well; and the plates were observed daily for CPE for five consecutive days.

**Confocal and immunofluorescence microscopic analysis**

Vero-E6, HK-2, 293T, and Caco-2 cells were fixed in 4% paraformaldehyde solution in 1 × PBS containing 0.1% Triton X-100. After 1 h blocking with 3% bovine serum albumin (BSA) at room temperature, the cells were stained with anti-SARS-CoV-2 antibodies (developed by our group), anti-FLAG (Sigma) and/or with anti-ACE2 antibodies (R&D systems) for 1 h at room temperature as we previously described (Yeung et al., 2008). Unbound antibodies were washed away six times with 1 × PBS. Positively stained cells were detected by secondary IgG (H+L) antibodies conjugated either with Alexa Fluor 488 or Alexa Fluor 594 (Life Technology) for 30 min at room temperature. Following six 1 × PBS washes, the stained cells were mounted onto glass slides with VECTASHIELD mounting medium with 4',6'-diamidino-2-phenylindole (DAPI) (Vector Lab) and examined with a Leica TCS-NT microscope (Leica Microsystems) or a LSM700 confocal microscope (Zeiss).

**Reverse transcription and quantitative real-time PCR (qRT-PCR)**

Total RNAs and viral RNAs were isolated using TRizol (ThermoFisher) and Viral RNA Mini kit (QIAGEN), respectively, as described in our previous literature (Yeung et al., 2016)). Following RNA quantification, one microgram of RNA was reverse transcribed into cDNA using random hexamers. Detection of SARS-CoV-2 and MERS-CoV was performed using primers 5'-ACAGGTACGTAAATAGTTAATAGCGT-3' and 5'-ATATTGCAGCAGTACACACA-3' targeting E gene of SARS-CoV-2 as described by WHO and by Novel Coronavirus 2012 Real-Time RT-PCR assay (CDC; Catalog # KT0136), respectively. Sample normalization was done by quantifying their respective housekeeping gene expression using primers 5'-TCACCACATGAGAAGGC-3' and 5'-GCTAAGCAGTTGGTGGCA-3' (GAPDH). For each reaction, equal amounts of cDNA were mixed with FS Universal SYBR Green Master Rox (Roche) plus 5 pmol each of forward and reverse primers. Amplification was carried out at 95°C for 15 s and 60°C for 1 min for 55 cycles in a 7900 real-time PCR detection system (ABI).

**Western blot (WB) analysis**

Cell lysates or tissue extracts were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Detection of antigens was performed using anti-SARS-CoV-2 antibodies (developed by our group), anti-FLAG (Sigma) and/or with anti-ACE2 antibodies (R&D systems) as previously described (Yeung et al., 2016)). As loading controls, the membranes were stripped with Restore western blot stripping buffer (Pierce) before reprobing with anti-γ-tubulin (Sigma).

**Co-immunoprecipitation assay**

Recombinant SARS-CoV-2 S proteins (Sino Biological) were incubated with protein lysates expressing V5-tagged or untagged ACE2 and FLAG-tagged vasoressin under previously described conditions (Yeung et al., 2018) with the following modifications: both recombinant S and the protein lysates were mixed in the presence of 0.1% Tween 20 at 4°C overnight with shaking. The next day, the anti-FLAG-M2 affinity agarose gel (Sigma) or anti-V5 agarose affinity gel (Sigma) was added into the mixture and allowed to incubate for an additional 2.5 h. Following six washes with a buffer containing 50 mM of NaH\textsubscript{2}PO\textsubscript{4}, 300 mM of NaCl, and 20 mM of imidazole pH 8.0, agarose-bound proteins were fractionated by SDS-PAGE and detected by WB analyses using anti-S (Abcam), anti-FLAG (Sigma), and anti-ACE2 (Abcam) antibodies.

**Cytopathic effect (CPE) measurement**

The CPEs of unfixed, unstained, infected cells were measured using an optical microscope, with the condenser down and the iris diaphragm partly closed as described in “Cytopathic Effects of Viruses Protocols,” by Sushman E and Blair C (ASM Microbe Library. American Society for Microbiology. Archived from the original on June 2, 2012. Retrieved 20 November 2014). We consider total detachment of the monolayer cell as 100%. To determine the percentage of CPE, Vero-E6, HK-2, and Caco-2 cells were inoculated with SARS-CoV-2 at a virus titer of MOI = 1. The virus induced CPEs were monitored daily.

**Identification of virus-dependency factors (VDFs)**

High-throughput sequencing raw reads were processed with Cutadapt (Martin, 2011) removing constant lentivirus vector and hairpin loop nucleotide sequences. Resulting sequences were then filtered to contain only the anti-sense shRNA arms ranging from 15 to
40 bp, which were representative of the shRNA sequences. The shRNA sequences were then aligned to human reference genome (GENCODE GRCh38) using Bowtie2 (Langmead and Salzberg, 2012). Subsequently, the number of reads mapping to shRNA probes and shRNA target genes were counted using HTSeq (Anders et al., 2015). The shRNA probes and shRNA target genes were generated by retrieving unique and properly paired alignments to non-overlapping genomic locations with a mapping quality over 15, following by target gene annotation (GENCODE GRCh38 annotation v24). Finally, shRNA counts were median-normalized and analyzed with DESeq2 (Love et al., 2014) to infer changes between samples and to evaluate statistical significance. Next, we adjusted the gene counts of raw reads within each treatment group by median normalization. For each treatment group, the gene counts with missing values from individual replicates were removed. The resulting datasets were subjected to clustering analyses, including principal components analysis (PCA), volcano plot, and heatmap hierarchical clustering analysis, using the stat function in R, ggplot2 function in R, and Complex Heatmap, respectively (Gu et al., 2016; Ito and Murphy, 2013).

Pathway analysis
The average of the adjusted gene counts from SARS-CoV-2-infected samples was compared with that of the mock-infected samples. Fold changes of gene counts between the SARS-CoV-2-infected samples and the mock-infected samples were generated with counts from replicates higher than 100. GO enrichment analysis was performed on 3-fold enriched genes using DAVID GO Cellular Component using default parameters with p < 0.05 (Huang et al., 2007). Similarly, molecular pathway analysis was conducted by analyzing the enriched VDFs into PANTHER (Protein Analysis Through Evolutionary Relationships) using the FISHER test type (Mi et al., 2019).

The coronavirus life cycle map
To generate the coronavirus life cycle map, relevant genes reported from different database: UniProt (Bairoch et al., 2005), Gene Ontology (Ashburner et al., 2000), NCBI Reference Sequence (Pruitt et al., 2005), NCBI OMIM database (McKusick, 2007) and BioGRID (Oughtred et al., 2019) were obtained. Text mining of the genes properties, including biological processes, molecular functions, and cellular components, were analyzed using PubMed query via iHop (Hoffmann and Valencia, 2004). The information was summarized and integrated into a database framework to form a schematized coronavirus viral life cycle. Newly discovered VDFs were added manually into this framework according to the cellular functions inferred from literature curation and database review. All SARS-CoV-2 VDFs identified in this study are available in Table S1.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical significance was determined as p < 0.05 using GraphPad Prism 8 unless otherwise indicated. Experiments were analyzed by unpaired two-tailed t tests.
Figure S1. Design of the shRNA expression cassette, related to Figure 2
The HK-2 cells were transfected with different doses of siAVPR1B 24 h before SARS-CoV-2 infection. Transfection of non-targeting siRNA was included as negative control (Mock-transfected). Statistical analyses were carried out using Student’s t test. Statistical significance is indicated by the asterisks (***p < 0.001).

Figure S2. Knockdown effect of AVPR1B on SARS-CoV-2 infectivity, related to Figure 2
Figure S3. Effect of ACE2 on SARS-CoV-2 and MERS-CoV cell entry, related to Figure 6
The HK-2 cells were treated with different doses of recombinant ACE2 (rACE2) protein prior to SARS-CoV-2 (black) or MERS-CoV (purple) infection separately. Cell entry of the SARS-CoV-2- or MERS-CoV-inoculated HK-2 cells was evaluated by measuring viral RNAs using qRT-PCR. Sample with undetectable viral RNA is marked with a red cross.
Figure S4. Characterization of subcellular localization of the wild-type and mutant ACE2, related to Figure 6
(A) WB analysis showing expression levels of cACE2 and sACE2 in 293T cells transfected with a plasmid encoding wild-type ACE2 (lane 1), GFP-tagged mutant ACE2 lacking the transmembrane domain (GFP-ACE2ΔTM) (lane 2), or GFP (lane 3) prior to SARS-CoV-2 infection.
(B) IFA of SARS-CoV-2 NP (green) in 293T cells transfected with wild-type ACE2 (left) and mutant GFP-ACE2ΔTM (right). Cells were counterstained with DAPI to label nuclei (blue). Scale bars represent 20 μm.
Figure S5. Mapping of high-throughput sequencing reads to human genome, related to Figures 2, 3, and 7