

Supplementary Information

Unveiling Alternate Pathways for SARS-CoV-2 Infection via Extracellular Vesicle-Mediated Transfer of ACE2 and TMPRSS2

Martha Rea-Moreno^{1,2#}, Lu Tian^{1#}, Tara N. Tavakol¹, Min-Chi Yang¹, Nicole M. Pek^{3,4,5}, Shubham Gulati⁶, Helena Bugacov⁶, Cristina Cusmai⁶, Gbalekan Dawodu⁷, Remi V. Klotz^{8,9}, Irving M. Garcia^{10,11}, Hsu-Yu Chen¹², Chennan C. Zhang¹³, Heng Pan^{14,15}, Xisheng Li¹⁶, Andrea S. Wolf⁴, Huachao Huang^{17,18,19}, Diana H. Yu²⁰, Justin K. Ichida²¹, Susmita Sahoo¹⁶, Paula M. Cannon^{12,21}, Jianwen Que^{17,18,19}, Scott A. Scheinin⁷, Daniel Laskey⁷, Alfin G. Vicencio^{22,23}, Megan N. Januska^{22,24}, Brandon A. Guenthart²⁵, Jeffrey Johnson^{14,15}, Min Yu^{8,9}, Mingxia Gu^{3,4,5,26,27}, Caterina Tiozzo^{28,29}, Ya-Wen Chen^{1,29,30*}

¹Department of Otolaryngology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

²Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

³Division of Pulmonary Biology, Perinatal Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

⁴Center for Stem Cell and Organoid Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

⁵Department of Pediatrics, University of Cincinnati, College of Medicine, Cincinnati, OH, USA

⁶Icahn School of Medicine at Mount Sinai, New York, NY, USA

⁷Department of Thoracic Surgery, Icahn School of Medicine at Mount Sinai, New York, NY, USA

⁸Department of Pharmacology and Physiology, University of Maryland School of Medicine, Baltimore, MD, USA

⁹Marlene and Stewart Greenebaum NCI Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA

¹⁰Hastings Center for Pulmonary Research, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

¹¹Division of Pulmonary, Critical Care and Sleep Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

¹²Department of Immunology and Immune Therapeutics, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

¹³PhD Programs in Biomedical and Biological Sciences, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

¹⁴Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

¹⁵Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

¹⁶Cardiovascular Research Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

¹⁷Columbia Center for Human Development, Columbia University Irving Medical Center, New York, NY, USA

¹⁸Division of Digestive and Liver Diseases, Columbia University Irving Medical Center, New York, NY, USA

¹⁹Department of Medicine, Columbia University Irving Medical Center, New York, NY, USA

²⁰Division of Pulmonary, Critical Care, Allergy, and Sleep Medicine, University of California, San Francisco, San Francisco, CA, USA

²¹Department of Stem Cell Biology and Regenerative Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

²²Division of Pediatric Pulmonology, Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY, USA

²³Department of Pediatrics, Joseph M. Sanzari Children's Hospital and Hackensack Meridian School of Medicine, Hackensack, NJ, USA

²⁴Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

²⁵Department of Cardiothoracic Surgery, Stanford University, CA, USA

²⁶Department of Anesthesiology & Perioperative Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, USA

²⁷Broad Stem Cell Research Center, University of California, Los Angeles, Los Angeles, CA, USA

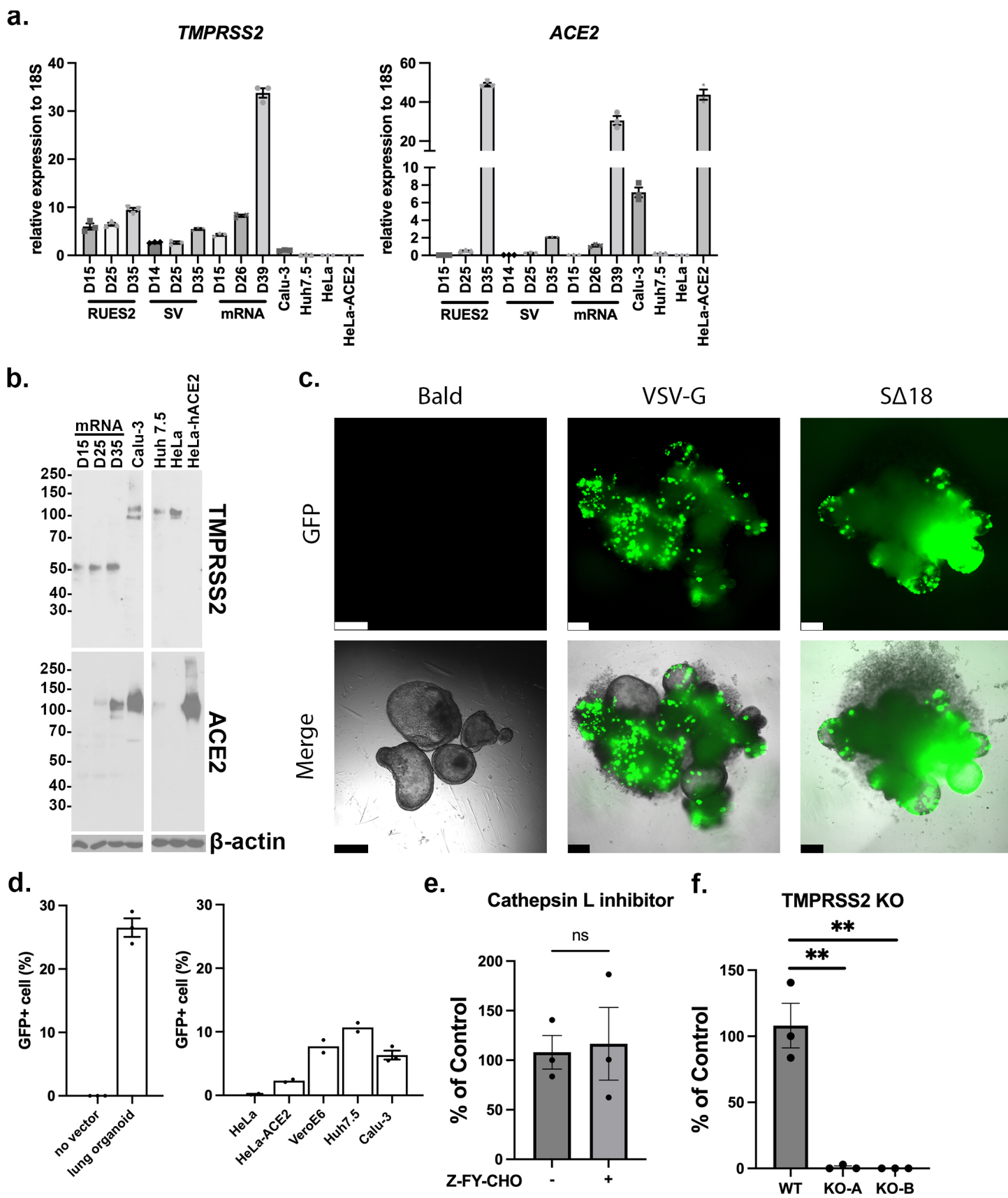
²⁸Division of Neonatology, Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY, USA

²⁹Department of Stem Cell Biology and Regenerative Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA

³⁰Institute for Airway Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

These authors contribute equally.

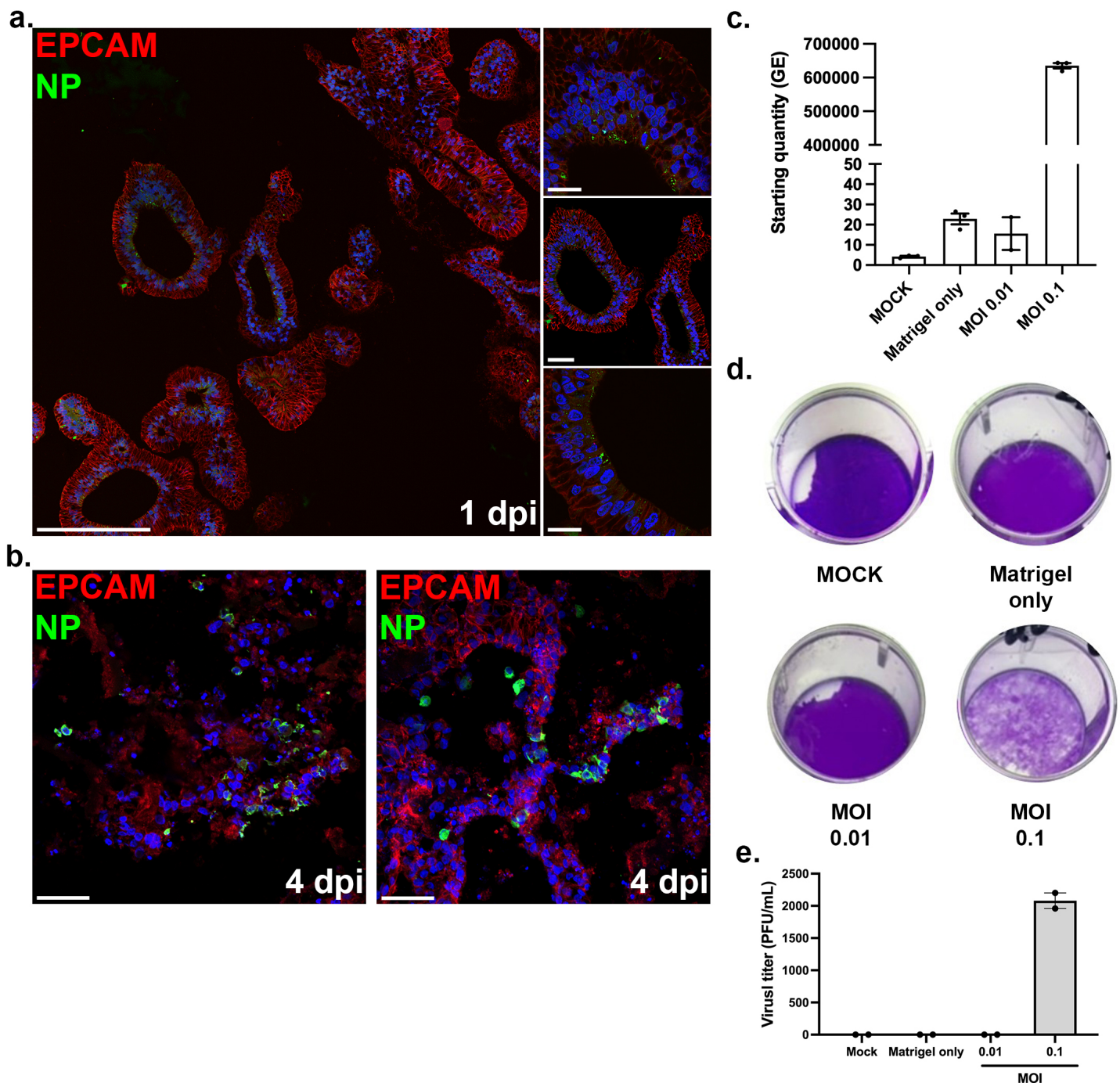
* Correspondence: yawen.chen@mssm.edu



Supplementary Figure 1. hPSC-derived lung organoids are permissive to SARS-CoV-2 infection.

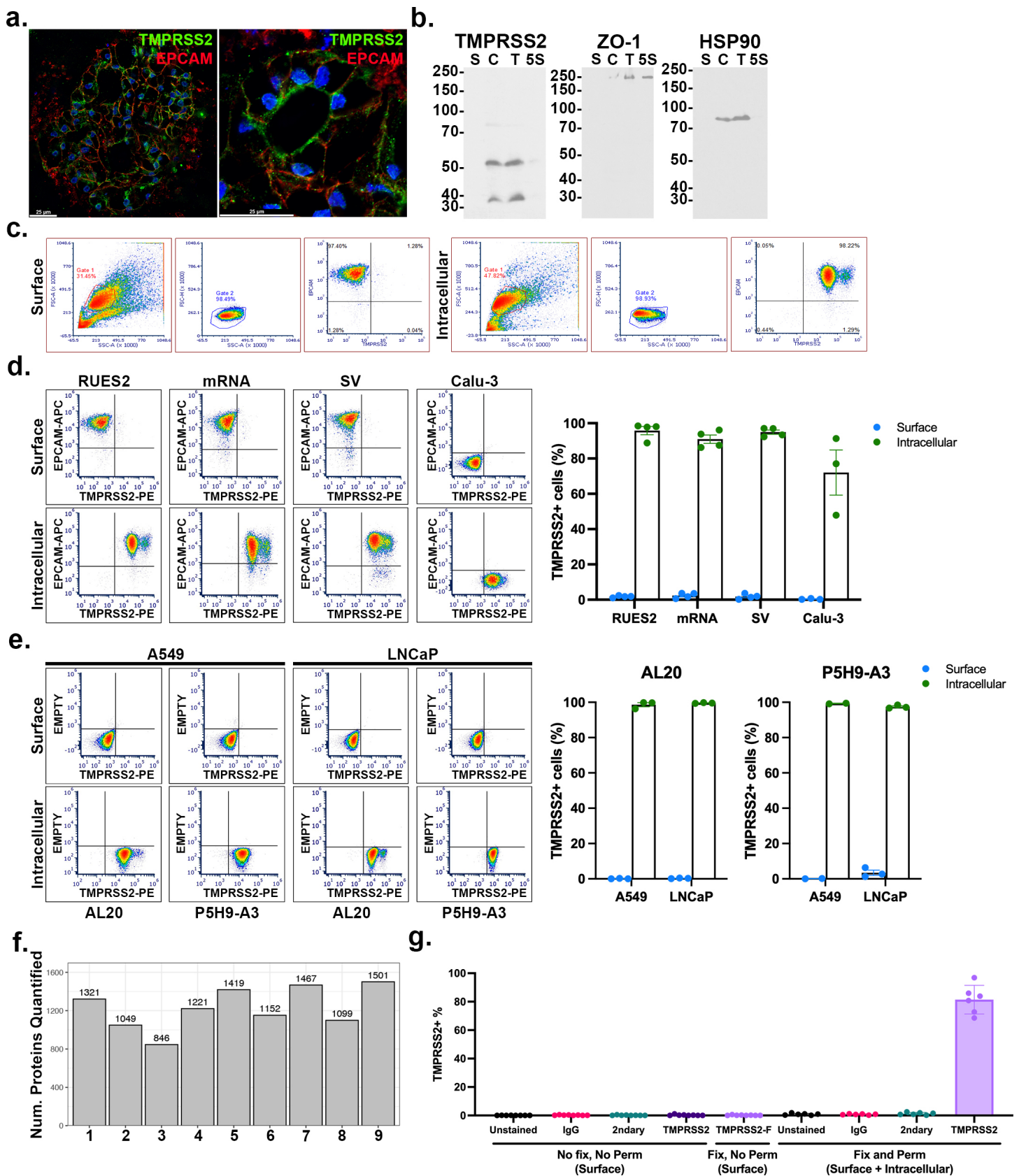
a. Total RNA expression level of *TMPRSS2* and *ACE2* in lung organoids derived from one ESC (RUES2) and two iPSC (mRNA and SV) lines at different differentiation stages and indicated cell lines. $n=3$, biological independent

experiments. D: day. **b.** Total protein expression level of TMPRSS2 and ACE2 in hPSC-derived lung organoids (mRNA line) at different differentiation stages and in indicated cell lines. Samples were prepared under non-reducing and non-boiling conditions to preserve protein complexes. $n=3$, biological repeats. D: day. **c.** Lung organoids were transduced with equal amounts of GFP-encoding pseudoviral particles bearing either SARS-CoV-2 Spike $\Delta 18$ ($S\Delta 18$), VSV-G protein, or no envelope protein (Bald). GFP expression was assessed 24 hours post-transduction. **d.** hPSC-derived lung organoids and cell lines transduced with SARS-CoV-2-GFP pseudovirus. GFP expression was visualized and quantified by flow cytometry 24 hours post transduction. $n=2$ to 3, biological independent experiments. **e.** Lung organoids were pretreated with the cathepsin L inhibitor Z-FY-CHO (+) or left untreated (-) prior to pseudovirus transduction. No significant difference was observed between treated and untreated groups. $n=3$, biological independent experiments. **f.** Lung organoids derived from wild-type (WT) or two independent TMPRSS2 knockout lines (KO-A and KO-B) were transduced with pseudovirus. $n=3$, biological independent experiments. Data represent mean $\pm$ s.e.m. Source data are provided as a Source Data file.



Supplementary Figure 2. SARS-CoV-2 infection in lung organoids.

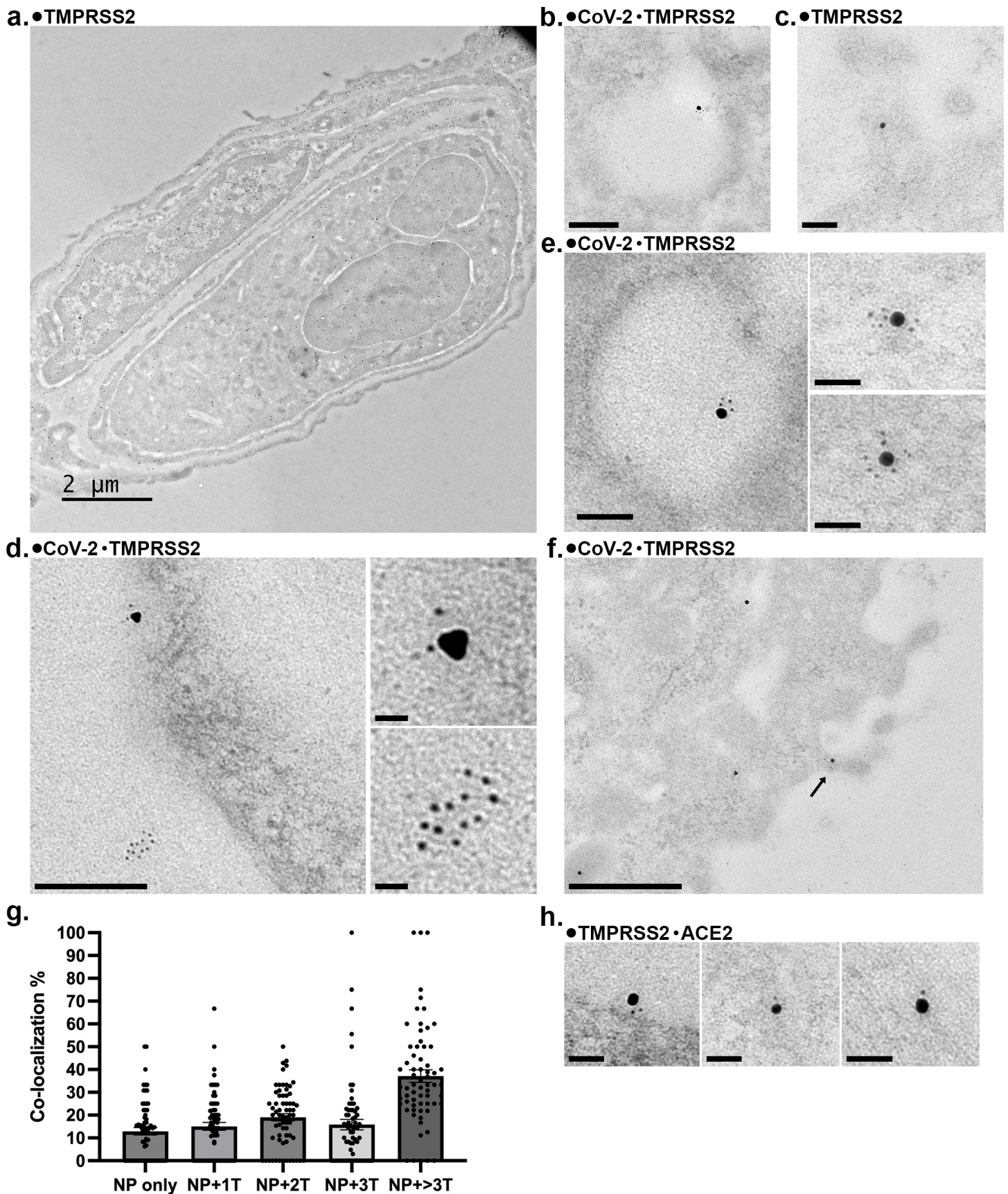
a-b. Immunofluorescent staining of SARS-CoV-2 infected lung organoids at 1 (**a**) and 4 (**b**) day post infection (dpi). Scale bar, a = 200 μ m (left), 20 μ m (right, top), 50 μ m (right, middle), and 20 μ m (right, bottom), b = 50 μ m. **c.** qRT-PCR analysis of viral N sgRNA in SARS-CoV-2 infected hPSC-derived lung organoids at 4 dpi. $n=2$ to 3, biological repeats. MOI: multiplicity of infection. **d-e.** Plaque assay performed on supernatant samples from lung organoids infected with SARS-CoV-2 at 4 dpi. $n=2$, biological independent experiments. Data are represented as mean $\pm$ s.e.m. Source data are provided as a Source Data file.



Supplementary Figure 3. Cellular localization of TMPRSS2 in hPSC-derived lung organoids and conventional cell lines.

a. Immunofluorescent staining of TMPRSS2 and EPCAM in lung organoids. **b.** TMPRSS2 protein expression in the cytoplasmic and cell surface portion of LNCaP cells. S: surface. C: cytoplasmic. T: total lysates. 5S: 5X surface

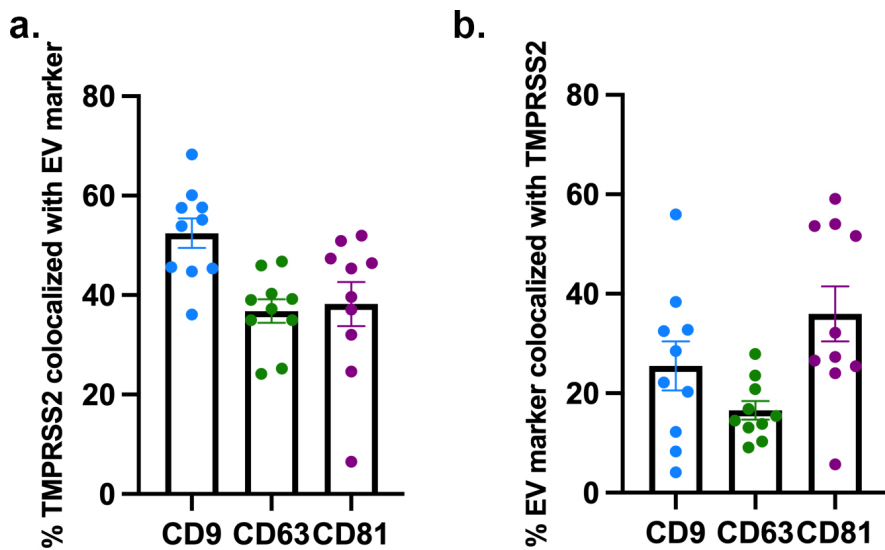
protein loading. HSP90: cytoplasmic marker. ZO-1: cell membrane marker. For TMPRSS2, samples were prepared under non-reducing and non-boiling conditions to preserve protein complexes. For ZO-1 and HSP90, samples were prepared under reducing and boiling conditions as suggested by the antibody manufacture. $n=3$, biological independent experiments. **c.** Flow cytometry gating strategy for analysis of surface and intracellular TMPRSS2 expression. **d. Left:** Flow cytometry analyses of surface and intracellular expression of TMPRSS2 in lung organoids derived from one ESC (RUES2) and two iPSC (mRNA and SV) lines and in Calu-3 line. **Right:** Fraction of surface and intracellular TMPRSS2⁺ cells in the indicated cell lines. $n\geq 3$, biological independent experiments. **e. Left:** Flow cytometry analysis of surface and intracellular expression of TMPRSS2 in A549 and LNCaP cells using two different TMPRSS2 antibodies, AL20 and P5H9-A3. **Right:** Fraction of surface and intracellular TMPRSS2⁺ cells in the indicated cell lines using the indicated antibodies. $n=2$ or 3 , biological independent experiments. **f.** Quantification of proteins detected in the supernatants from antibody-incubated lung organoid cells by mass spectrometry. $n=9$, biological independent experiments. **g.** Surface expression of TMPRSS2 is not detected by flow cytometry in lung organoids. Lung organoid cells were incubated with an antibody targeting the extracellular domain of TMPRSS2 on ice to minimize potential shedding. Cells were then fixed (TMPRSS2-F) or not fixed and analyzed by flow cytometry. Staining was performed under non-permeabilized (surface staining) or permeabilized (intracellular staining) conditions, with or without primary antibody. Robust TMPRSS2 signal was detected only in permeabilized samples, indicating intracellular localization. No surface signal was detected, even after fixation. $n\geq 3$, biological independent experiments. IgG: IgG staining control. 2ndary: secondary antibody only stain. Perm: permeabilization. Data are represented as mean $\pm$ s.e.m. Source data are provided as a Source Data file.



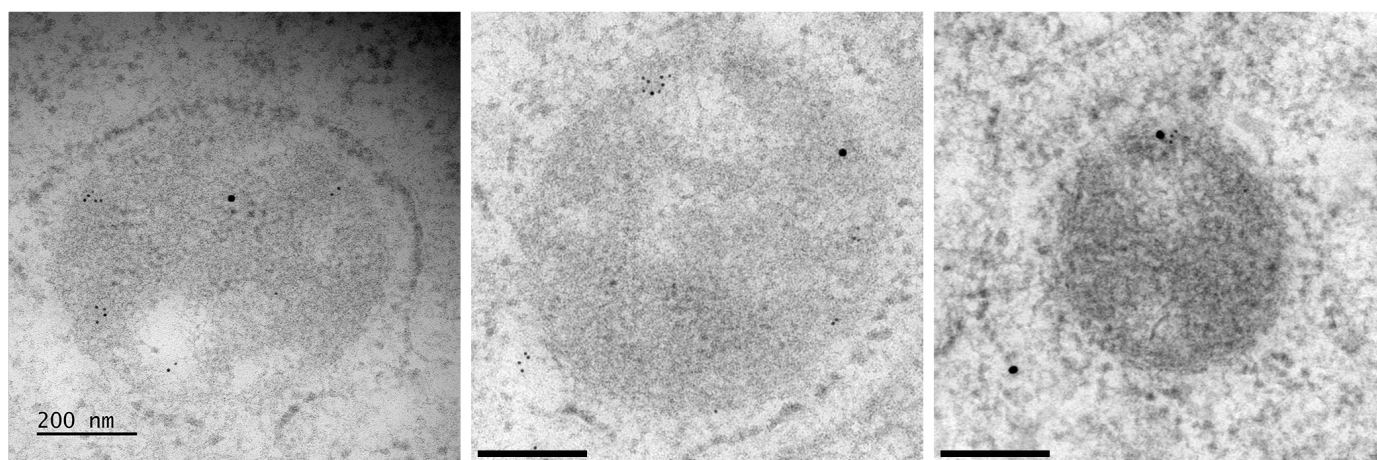
Supplementary Figure 4. Immuno-EM of healthy and COVID-19 lungs.

a and **c**, Immuno-EM of TMPRSS2 in healthy lung. **b** and **d-f**, Immuno-EM of SARS-CoV-2 NP (CoV-2) and TMPRSS2 in COVID-19 lung. The arrow in **f** indicates secretion of TMPRSS2 and SARS-CoV-2 NP together. **g**, Quantification

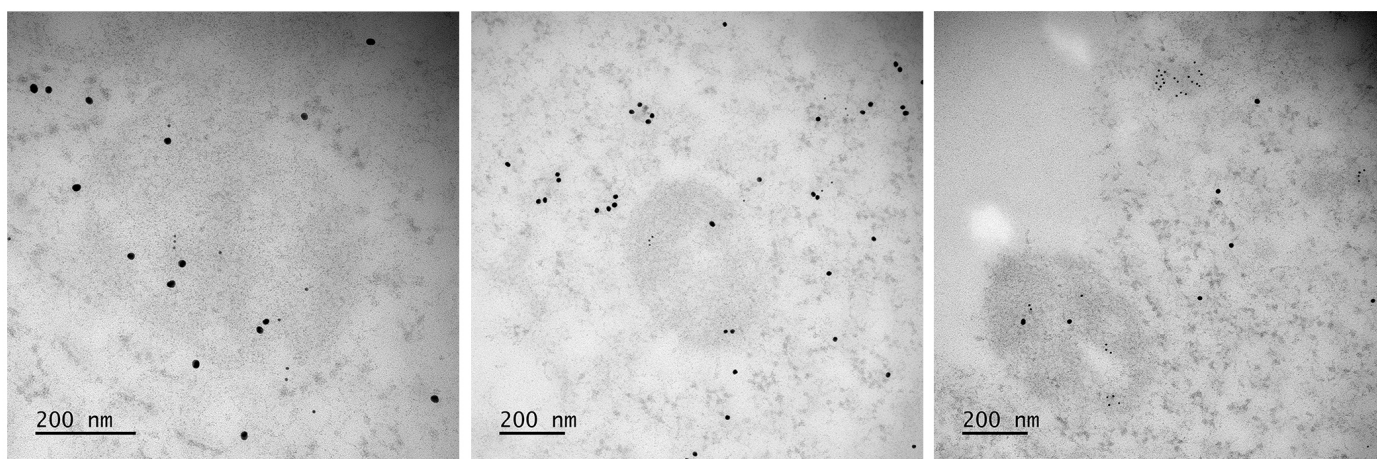
of individual SARS-CoV-2 NP particles surrounded by one, two, three, or more than three TMPRSS2 molecules. Each dot represents one NP particle. $n=69$ independent EM images. NP: nucleocapsid protein. T: TMPRSS2. **h.** Immuno-EM of TMPRSS2 and ACE2 in COVID-19 lung. Scale bar, a = 2 μm , b = 200 nm, c = 100 nm, d = 200 nm (left) and 20 nm (right), e = 100 nm (left) and 50 nm (right), f = 500 nm, g = 50 nm. The sizes of the gold particles used for TMPRSS2, ACE2, and SARS-CoV-2 NP (CoV-2) are as indicated. Data are represented as mean $\pm$ s.e.m. Source data are provided as a Source Data file.



c. •CD63 •TMPRSS2

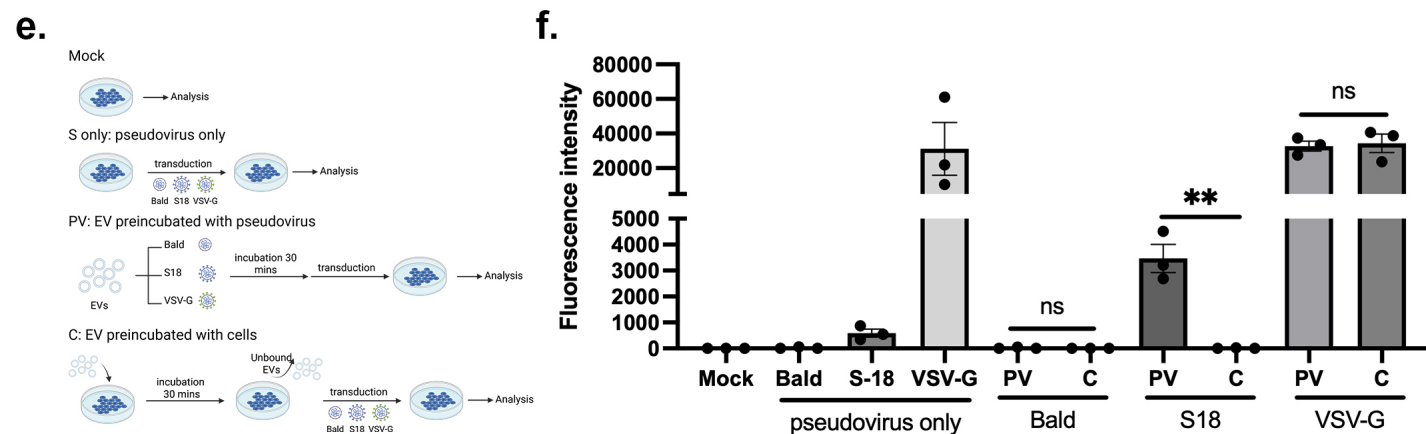
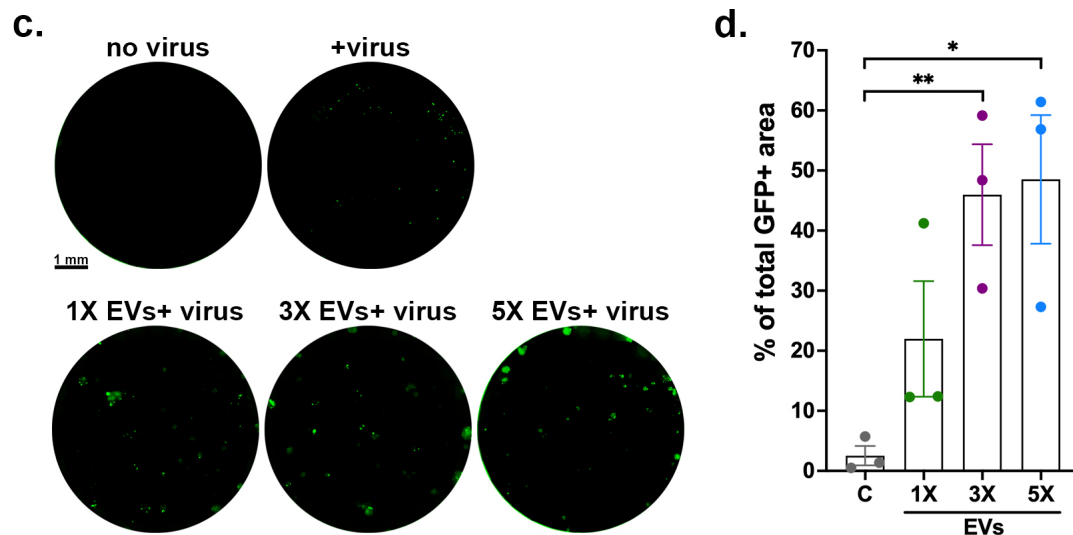
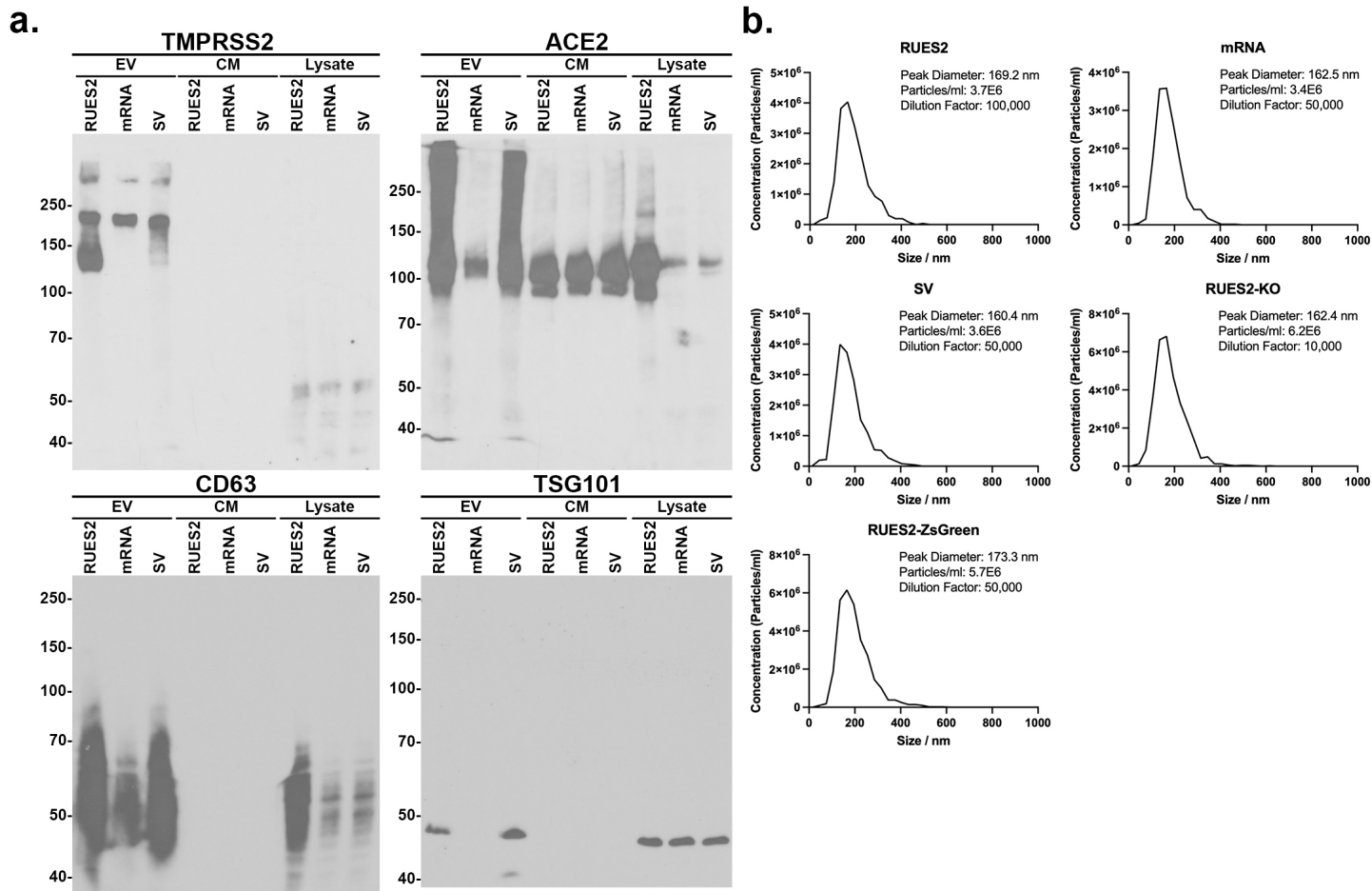


d. •TMPRSS2 •TSG101



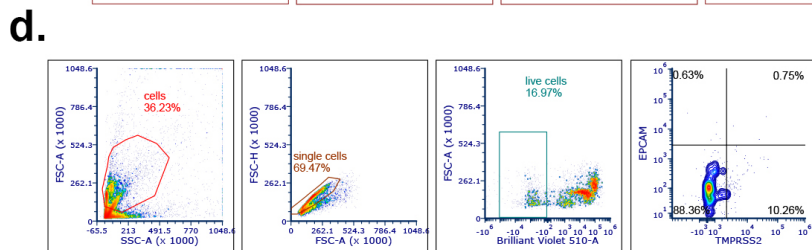
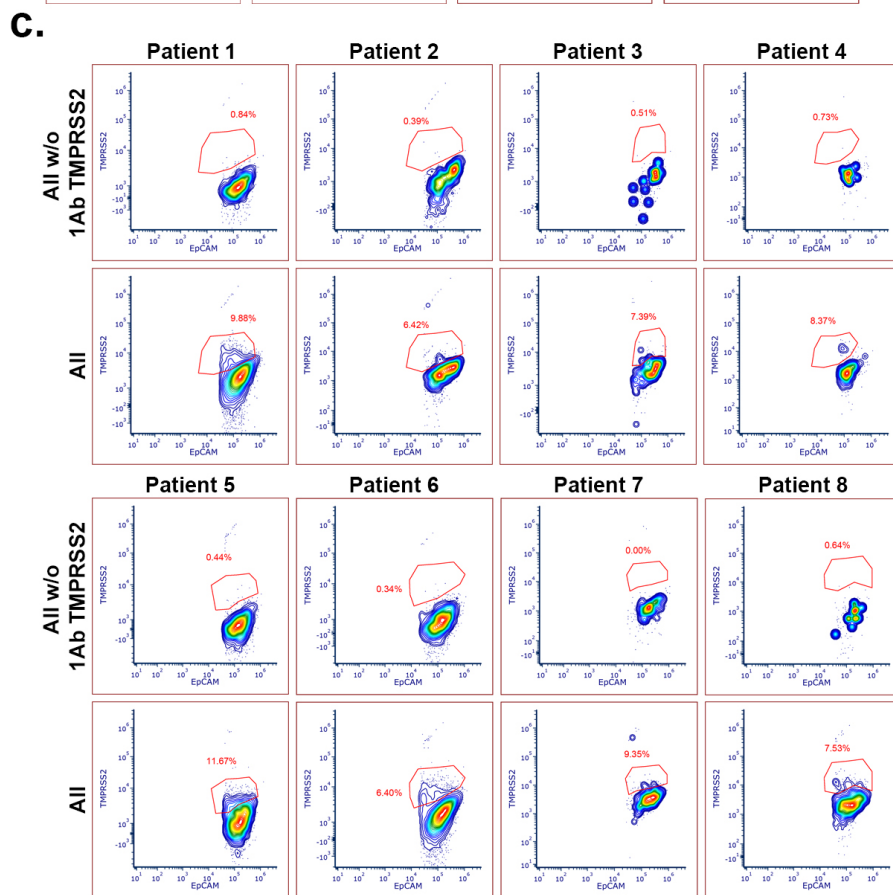
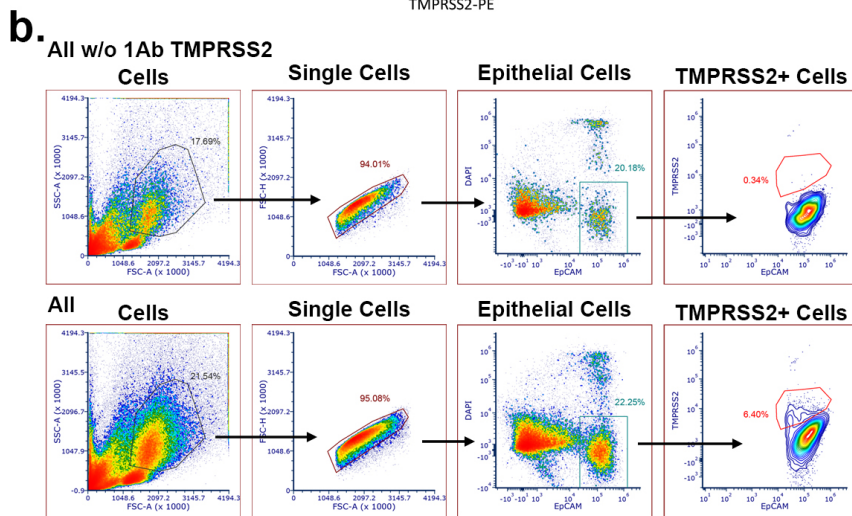
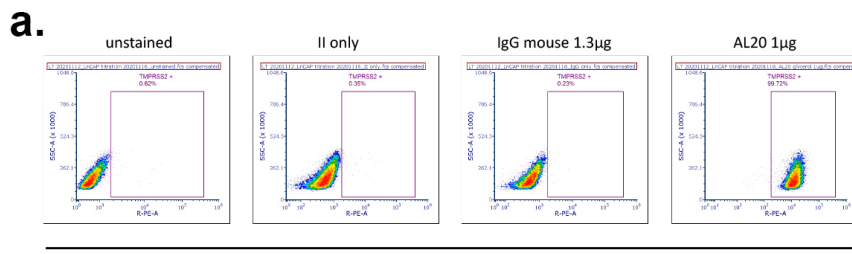
Supplementary Figure 5. TMPRSS2 co-localizes with canonical EV markers in lung organoids.

a. and b. Quantification of confocal images showing the percentage of TMPRSS2⁺ signals that co-localize with CD9, CD63, or CD81. (**a.**), and the percentage of EV marker⁺ signals that co-localize with TMPRSS2 (**b.**) in lung organoids. Bars represent mean $\pm$ s.e.m. $n \geq 3$, biological independent experiments. **c. and d.** ImmunoEM of lung organoids shows TMPRSS2 in close proximity to the canonical EV markers CD63 (**c.**) and TSG101 (**d.**). The sizes of the gold particles used for staining are as indicated. Scale bars: 200 nm. EV: extracellular vesicle. Source data are provided as a Source Data file.

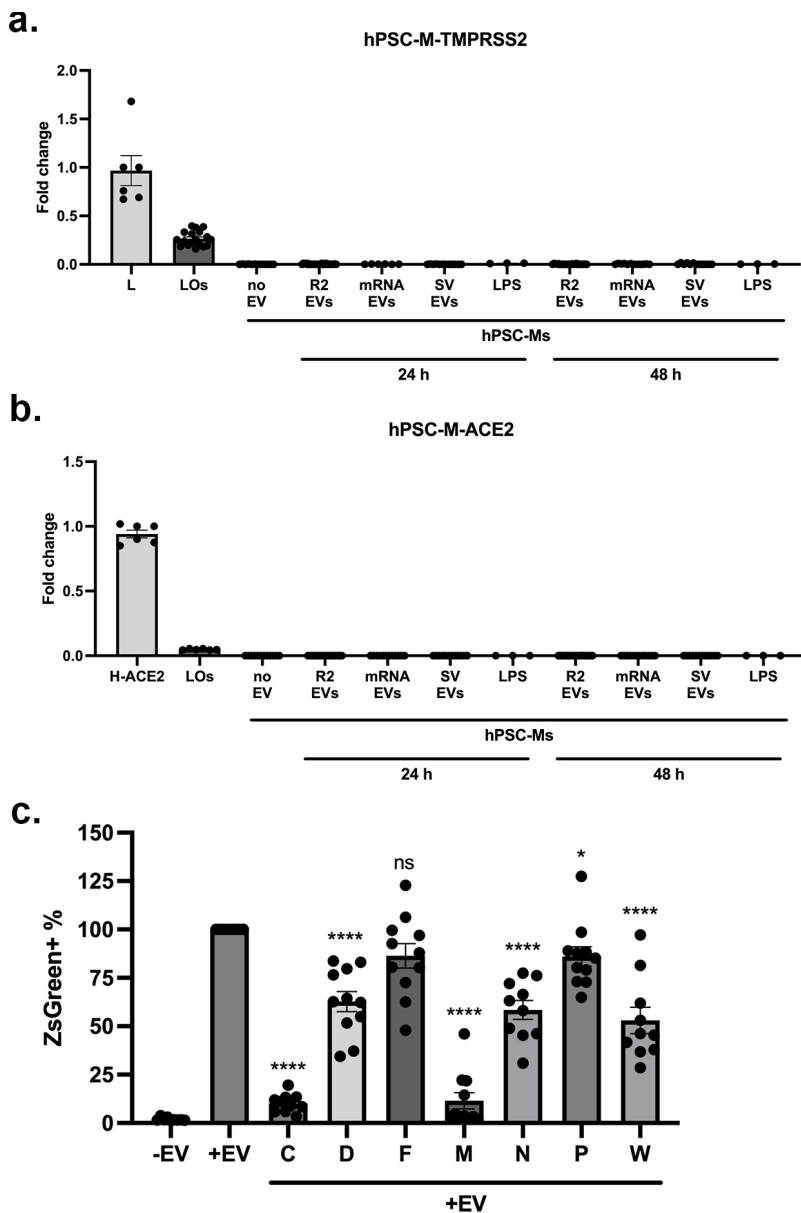


Supplementary Figure 6. TMPRSS2 and ACE2 are secreted in extracellular vesicles of lung organoids.

a. Expression of TMPRSS2 and ACE2 in extracellular vesicles (EV), conditioned media without EVs (CM) and cell lysates (lysate) from lung organoids derived from one hESC (RUES2) and two iPSC (mRNA and SV) lines. CD63 and TSG101: EV markers. For TMPRSS2, ACE2, and CD63, samples were prepared under non-reducing and non-boiling conditions to preserve protein complexes. For TSG101, samples were prepared under reducing and boiling conditions as suggested by the antibody manufacture. $n > 3$, biological independent experiments. **b.** ZetaView NTA analysis of the size of EVs purified from lung organoid derived from different hPSC lines. **c-d.** hPSC-derived lung organoids transduced with SARS-CoV-2-GFP pseudovirus alone, or with increasing amount of EVs. GFP expression was quantified 72 hours post transduction. $*p < 0.05$ and $**p < 0.01$ by two-tailed t -test. Data are represented as mean $\pm$ s.e.m. $n = 3$, biological independent experiments. **e.** Schematic illustration of infection assay design. Created in BioRender. Chen, Y. (2026) <https://BioRender.com/ofh9u35>. **f.** EVs were either preincubated with pseudoviruses (PV) or added to target cells before viral exposure (C). Mock and pseudovirus-only controls were included for all pseudotypes. Preincubation of EVs with S18 pseudovirus significantly increased transduction efficiency. $n = 3$, biological independent experiments. Data represent mean $\pm$ s.e.m.; $** p < 0.01$. PV: EV preincubated with pseudovirus. C: EV preincubated with cells. Source data are provided as a Source Data file.

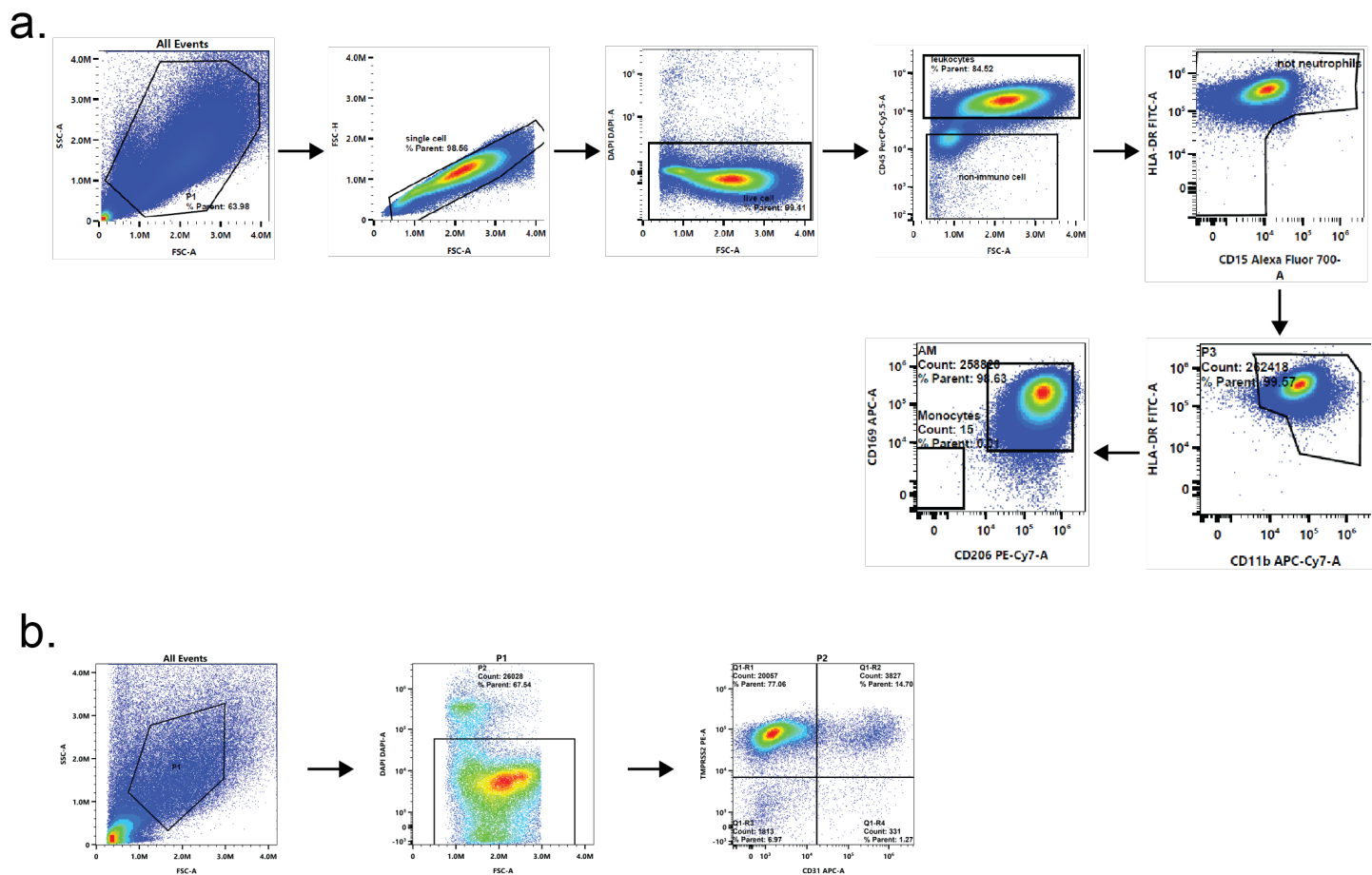


Supplementary Figure 7 Controls for TMPRSS2 FACS analyses. **a.** Secondary-only and isotype control of TMPRSS2 to demonstrate antibody specificity. Cells were fixed and permeabilized and incubated with either secondary antibody alone (II only), isotype mouse IgG (IgG mouse), or TMPRSS2 (AL20) antibody followed by secondary antibody. **b.** Fluorescence minus one control. Cells were stained with all fluorescently labeled antibodies in the panel except for the TMPRSS2 primary antibody, to assess the influence of each fluorophore on the panel. **c.** Representative FACS plots showing the TMPRSS2-positive epithelial cell fraction in diseased lung samples from eight individual patients. Plots compare staining with and without the TMPRSS2 primary antibody to confirm specificity of TMPRSS2 detection. **d.** Gating strategy for flow cytometry analysis of TMPRSS2 surface expression in human lung cells.

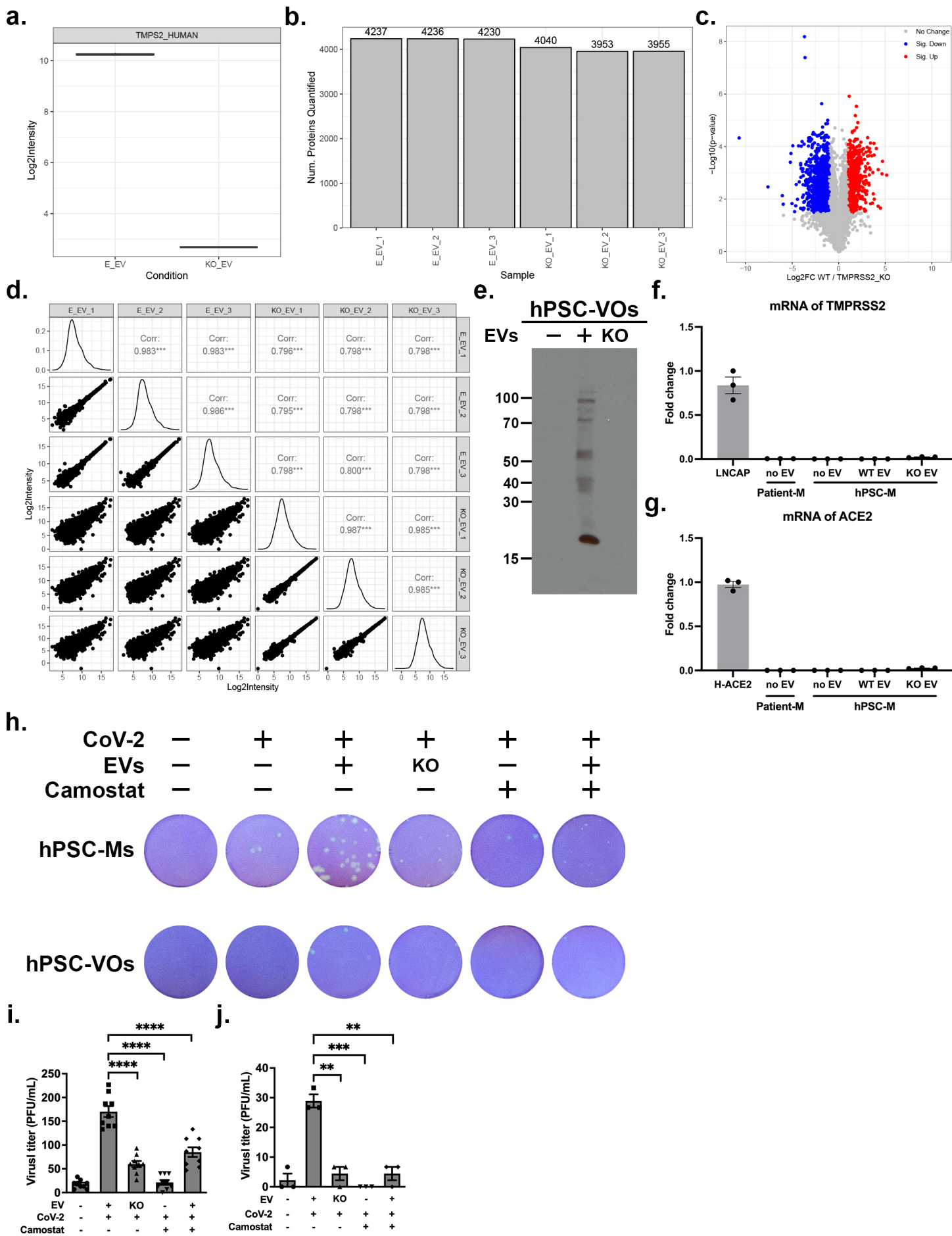


Supplementary Figure 10. mRNA expression of TMPRSS2 and ACE2 is not induced in hPSC-Ms following EV or LPS exposure.

a. qRT-PCR analysis of TMPRSS2 mRNA levels in hPSC-Ms 24 and 48 hours after treatment with extracellular vesicles (EVs) derived from RUES2 organoids (R2 EVs), mRNA organoids (mRNA EVs), and SV organoids (SV EVs). Untreated hPSC-Ms (no EV) and hPSC-Ms treated with lipopolysaccharide (LPS) were included as controls. Lung organoids (LOs) and LNCaP cells (L) served as positive controls. $n \geq 3$, biological independent experiments. **b.** qRT-PCR analysis of ACE2 mRNA levels under the same conditions as in **a**. HeLa ACE2-overexpressing cells (H-ACE2) and LOs were used as positive control. No significant induction of TMPRSS2 or ACE2 mRNA expression was observed in hPSC-Ms across any EV treatment condition or following LPS stimulation, supporting that EV-mediated surface acquisition of these proteins occurs independently of transcriptional activation. $n \geq 3$, biological independent experiments. **c.** hPSC-Ms were pretreated with various inhibitors targeting distinct endocytic pathway prior to EV exposure. Each inhibitor was applied 1 hour before addition of ZsGreen-EVs. After 20 hours, cells were analyzed by flow cytometry for ZsGreen signals. Data represent mean $\pm$ s.e.m.; **** $p < 0.0001$; * $p < 0.05$. ns: not significant. C: Cytochalasin D. D: Dynasore. F: Filipin. M: Methyl- β -cyclodextrin. N: Nystatin. P: Pitstop 2. W: Wortmannin. $n = 9-11$, biological independent experiments. Source data are provided as a Source Data file. h-PSC-M: human pluripotent stem cell-derived macrophage.



Supplementary Figure 11. Gating strategy for flow cytometry analysis of TMPRSS2 surface expression in hPSC-derived macrophages (hPSC-Ms) **(a)** and hPSC-derived vessel organoids (hPSC-VOs) **(b)**.



Supplementary Figure 12. TMPRSS2 is critical in EV-mediated infection.

a. Mass spectrometry analysis of TMPRSS2 protein levels in extracellular vesicles (EVs) derived from wildtype (E_EV) and TMPRSS2-KO (KO_EV) lung organoids. TMPRSS2 was detected in EVs from wildtype but not from knockout organoids, confirming effective depletion in KO-derived EVs. **b.** Quantification of total proteins identified by mass spectrometry in EVs from wildtype (E_EV_1-3) and TMPRSS2-KO (KO_EV_1-3) lung organoids. The number of proteins quantified was comparable across all samples, indicating that TMPRSS2 KO did not broadly alter EV protein composition. $n=3$, biological independent experiments. **c.** Volcano plot comparing proteomic profiles of EVs derived from wild-type (WT) and TMPRSS2-KO (KO) lung organoids. **d.** Correlation plots showing pairwise comparisons of $\log_2$ protein intensities among biological replicates of WT and TMPRSS2-KO EVs. **e.** Protein expression of TMPRSS2 in hPSC-VOs primed with (+), without (-), or TMPRSS2-knockout (KO) EVs. Samples were prepared under non-reducing and non-boiling conditions to preserve protein complexes. $n=2$, biological independent experiments. **f.** qRT-PCR analysis of TMPRSS2 expression in hPSC-Ms, patient macrophages (patient-M), and LNCaP cells (positive control) shows that TMPRSS2 mRNA remains undetectable in hPSC-Ms after incubation with either WT EVs or TMPRSS2-KO EVs. Data are shown as mean $\pm$ s.e.m. $n=3$, biological independent experiments. **g.** Similarly, ACE2 mRNA was not detected in hPSC-Ms following treatment with WT or KO EVs. Data are shown as mean $\pm$ s.e.m. $n=3$, biological independent experiments. **h-j.** Plaque assays performed on supernatant samples from hPSC-Ms (**h** and **i**) and hPSC-VOs (**h** and **j**) primed with or without WT TMPRSS2 EVs, or TMPRSS2-knockout (KO) EVs, and with or without pre-treatment with camostat (a serine protease inhibitor), followed by SARS-CoV-2 (CoV-2) infection. $n \geq 3$, biological independent experiments. * $p < 0.05$ and ** $p < 0.01$ by unpaired two-tailed t -test. Data are represented as mean $\pm$ s.e.m. Source data are provided as a Source Data file. hPSC-M: human pluripotent stem cell-derived macrophage. hPSC-VO: human pluripotent stem cell-derived vessel organoid.

Legends of the supplementary data

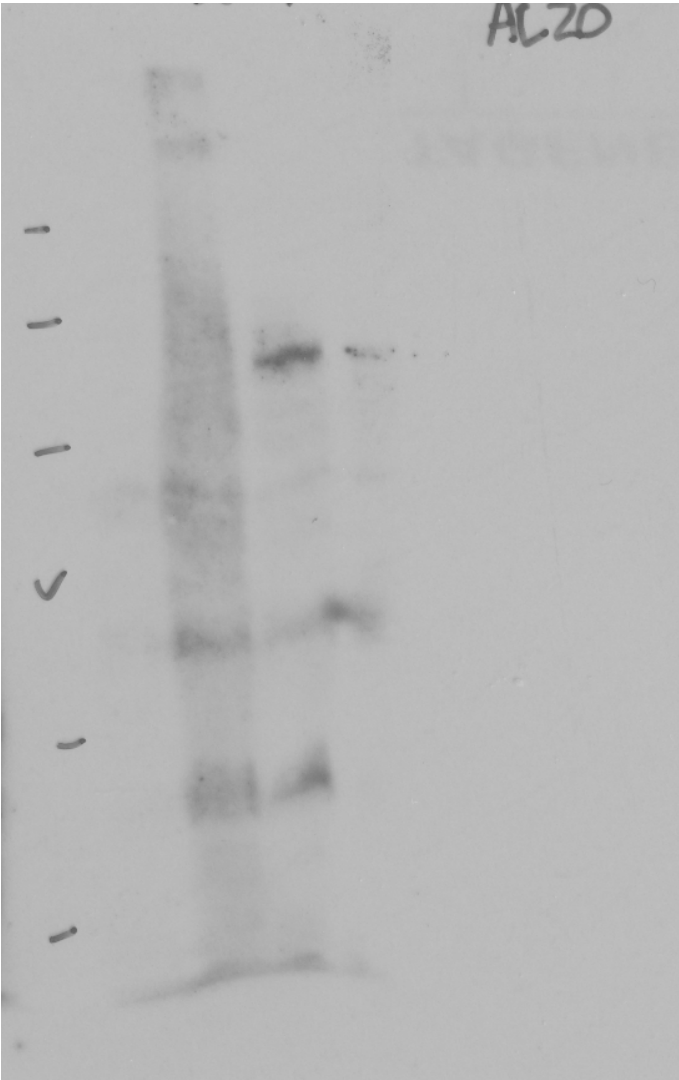
Supplementary Table 1. Nanoparticle tracking analysis of extracellular vesicles derived from hPSC-derived lung organoids. Raw nanoparticle tracking analysis data showing the size distribution and particle concentration of extracellular vesicles purified from lung organoids derived from different hPSC lines.

Evs purified from	Concentration (particles/mL)	Dilution Factor	Original concentration	Average counted particles per fram	Peak analysis (Concentration)					X values (all sizes are given in nm)				
					Number of traced particles	Diameter/nm	Particles/mL	FWHM/nm	Percentage	X10	X50	X90	Mean	SD
RUES2-1	1.90E+07	100000	1.90E+12	56.00	1080.00	169.20	3.70E+06	133.40	100.00	106.10	163.70	274.20	192.00	68.60
mRNA	1.50E+07	50000	7.60E+11	45.00	978.00	162.50	3.40E+06	124.60	100.00	97.90	152.50	2.38E+02	177.10	5.81E+01
SV	1.70E+07	50000	8.70E+11	51.00	869.00	160.40	3.60E+06	128.00	100.00	93.90	152.60	2.54E+02	180.10	6.71E+01
KO	3.10E+07	10000	3.10E+11	92.00	1533.00	162.40	6.20E+06	138.10	100.00	92.90	154.20	2.51E+02	180.70	6.63E+01
RUES2-ZsGreen	2.90E+07	50000	1.50E+12	88.00	1223.00	173.30	5.70E+06	137.90	100.00	109.30	169.60	2.75E+02	198.40	7.22E+01

Western blot Source Data

Figure 1b

TMPRSS2

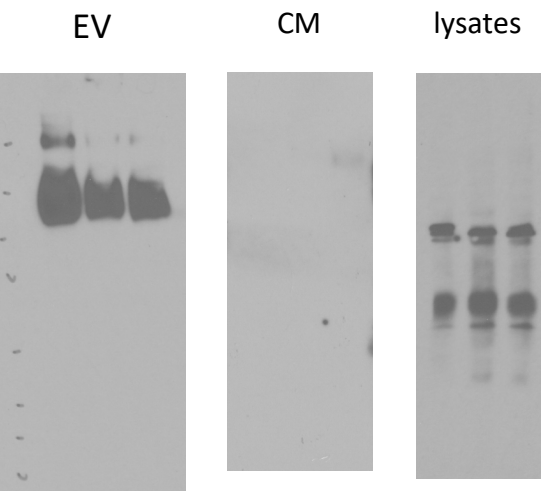


HSP90

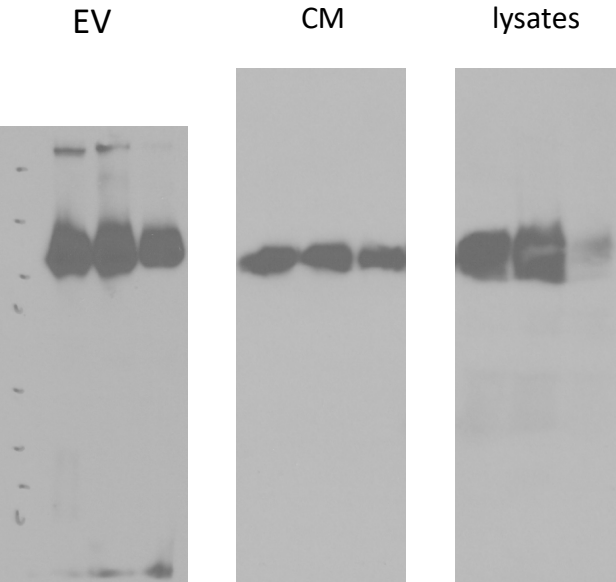


Figure 2b

TMPRSS2



ACE2



TSG101

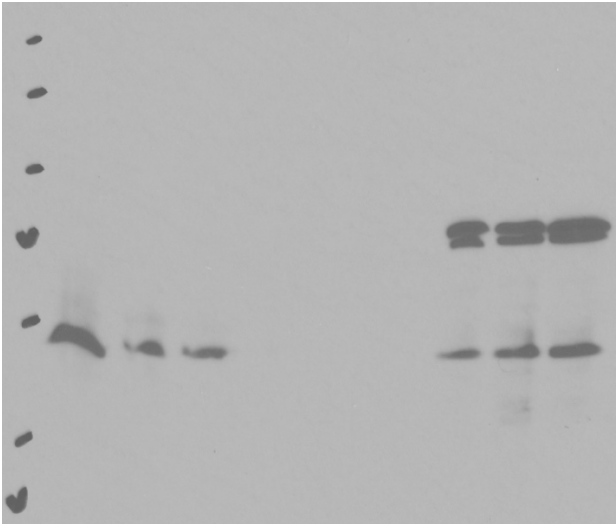
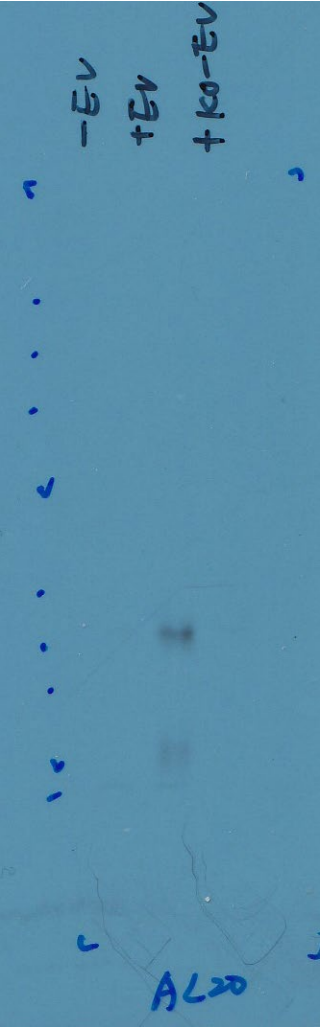
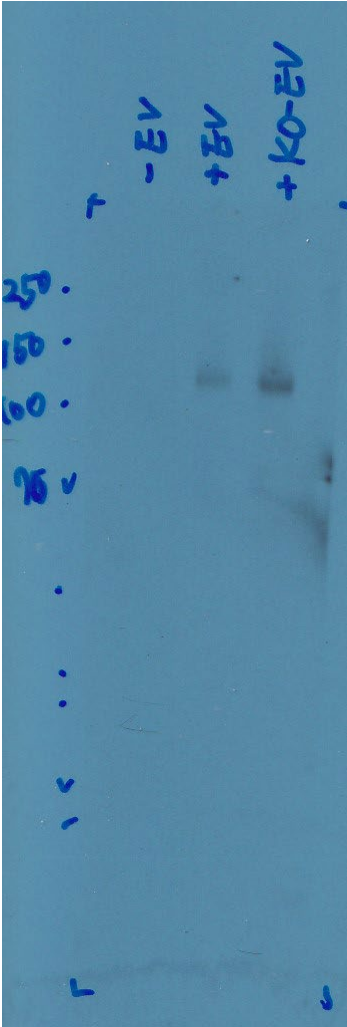


Figure 6b

TMPRSS2



ACE2



b-actin

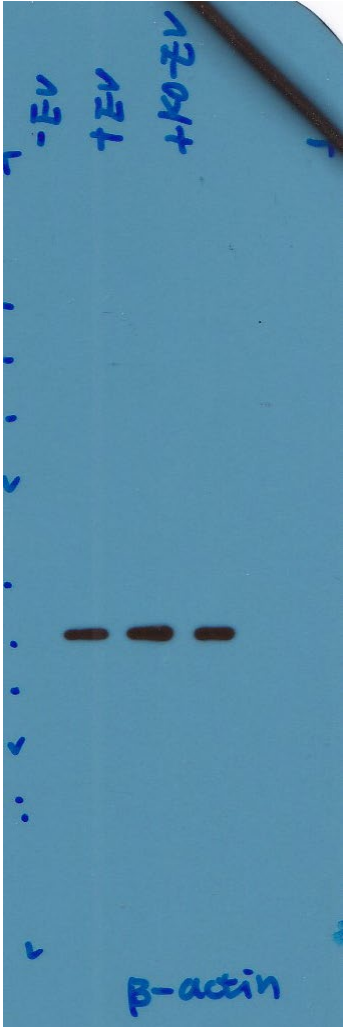
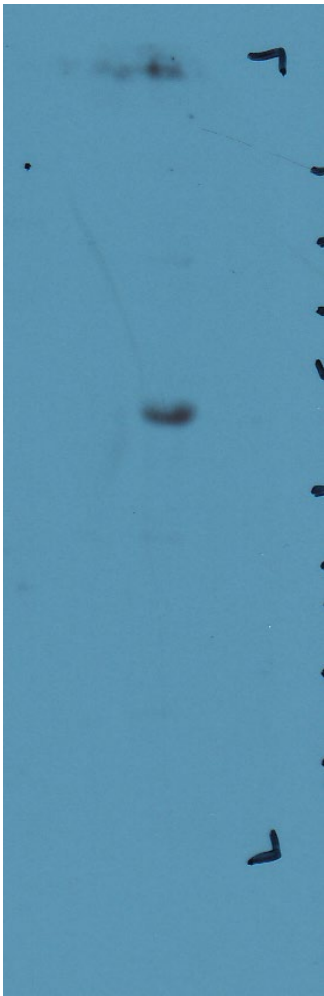
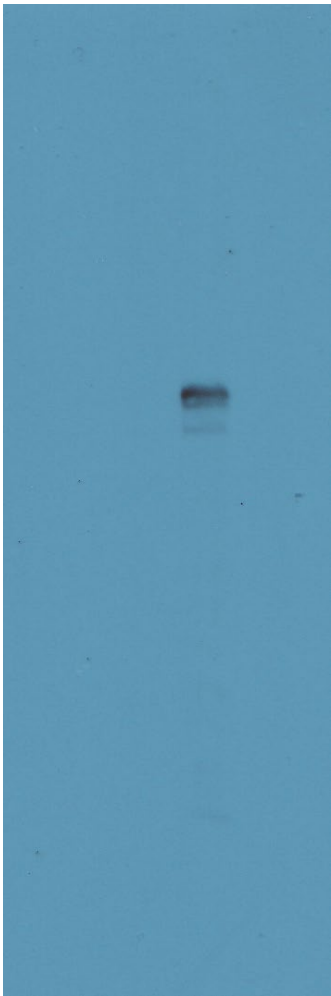


Figure 6d

TMPRSS2



ACE2



b-actin

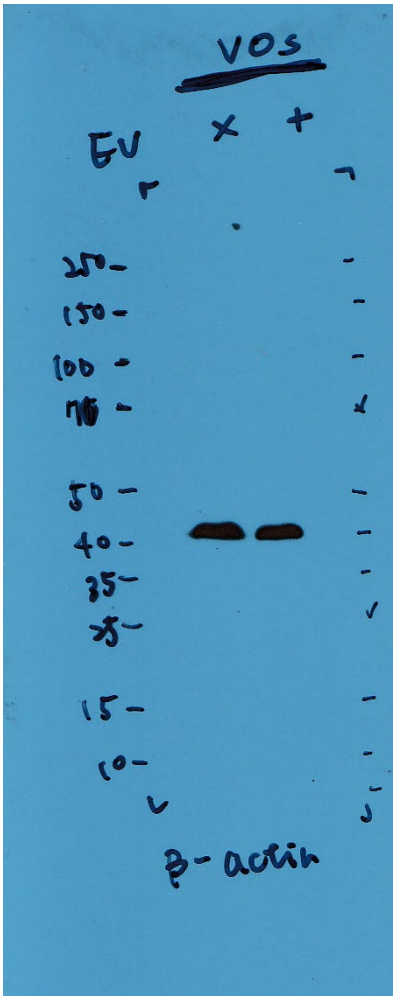
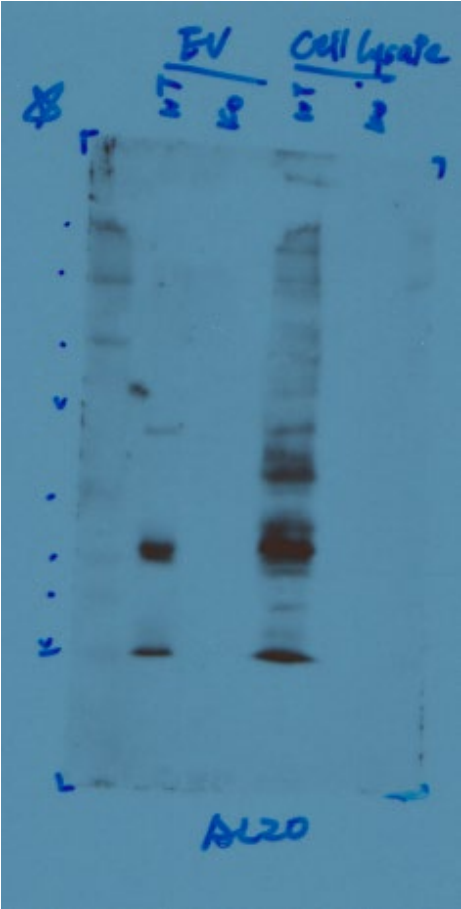
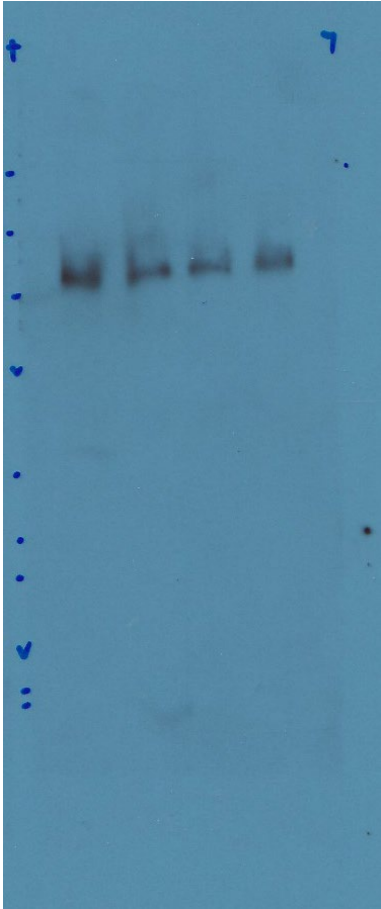


Figure 7d

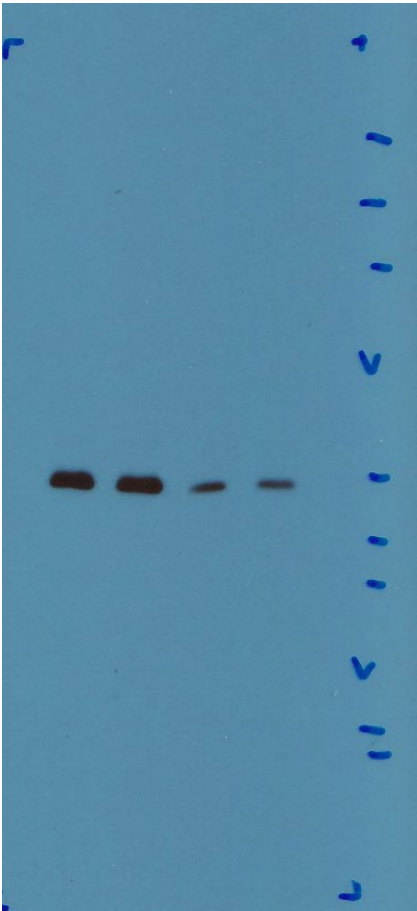
TMPRSS2



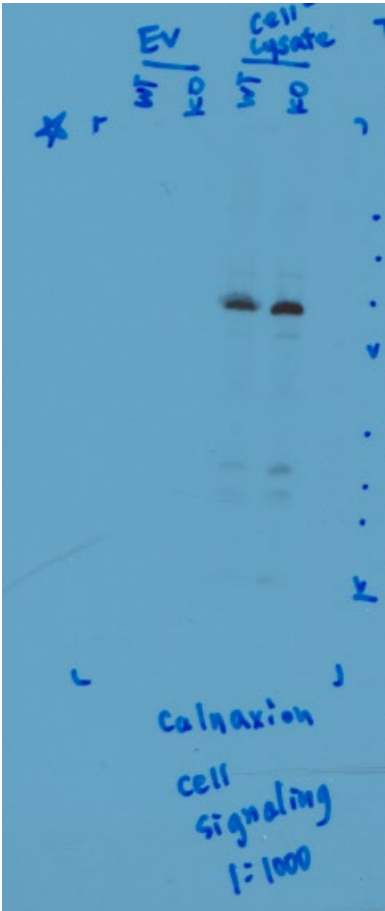
ACE2



TSG101



Calnexin



b-actin

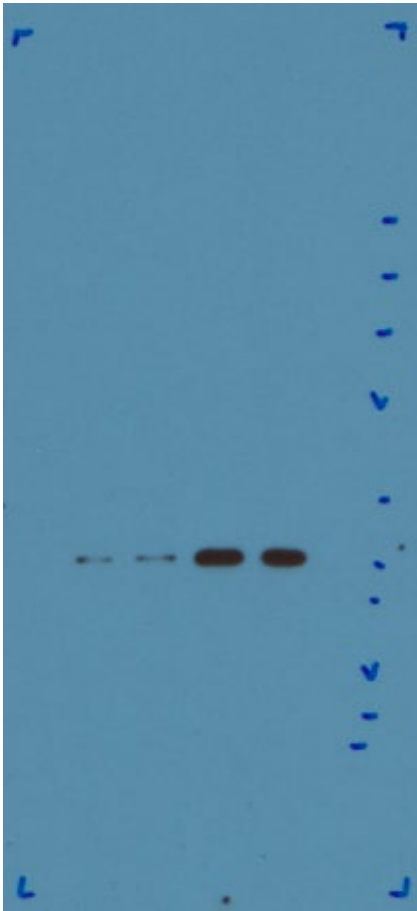
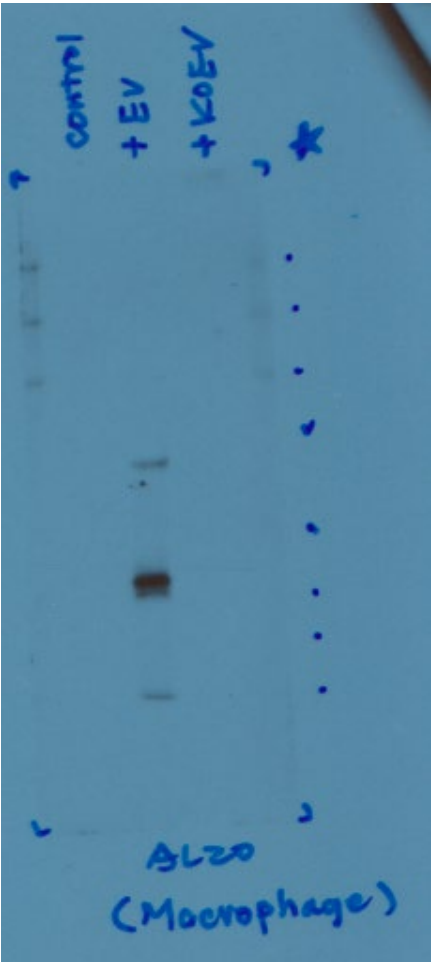
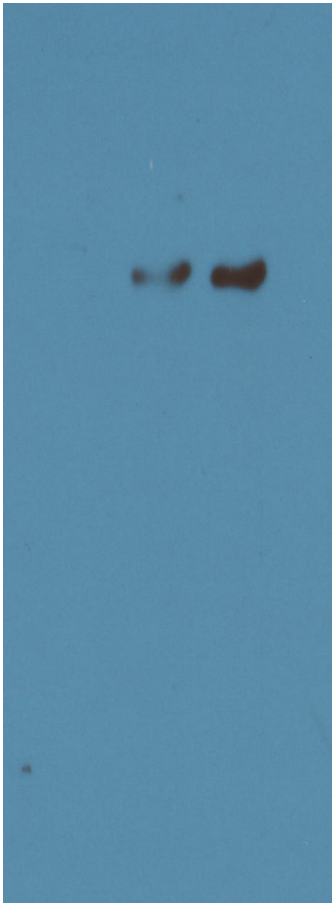


Figure 7e

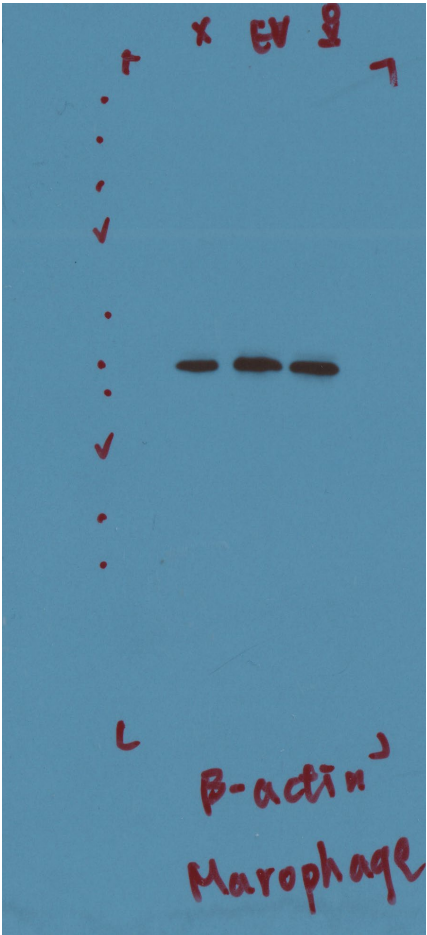
TMPRSS2



ACE2



b-actin



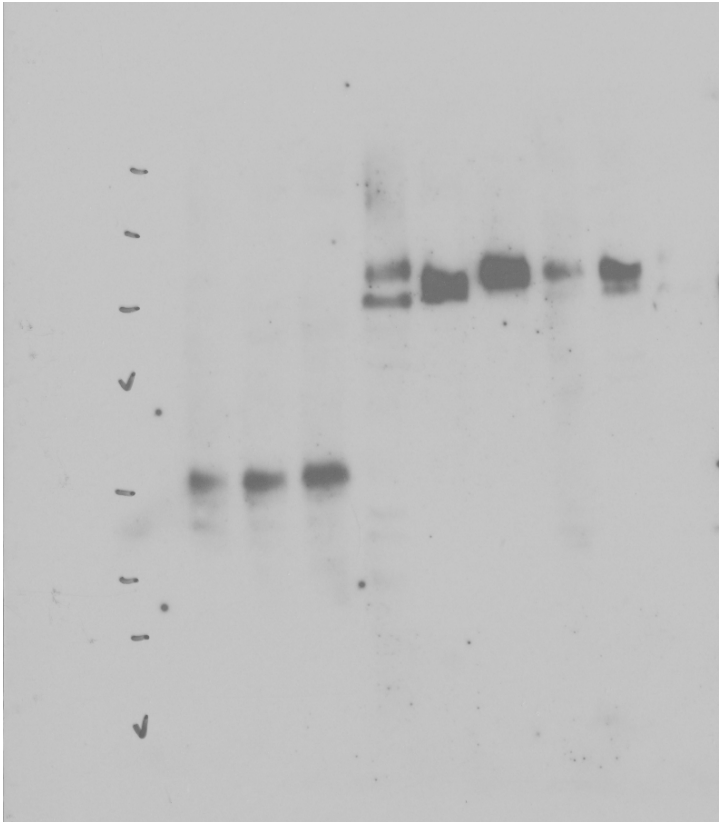
Supplementary Figure

Western blot

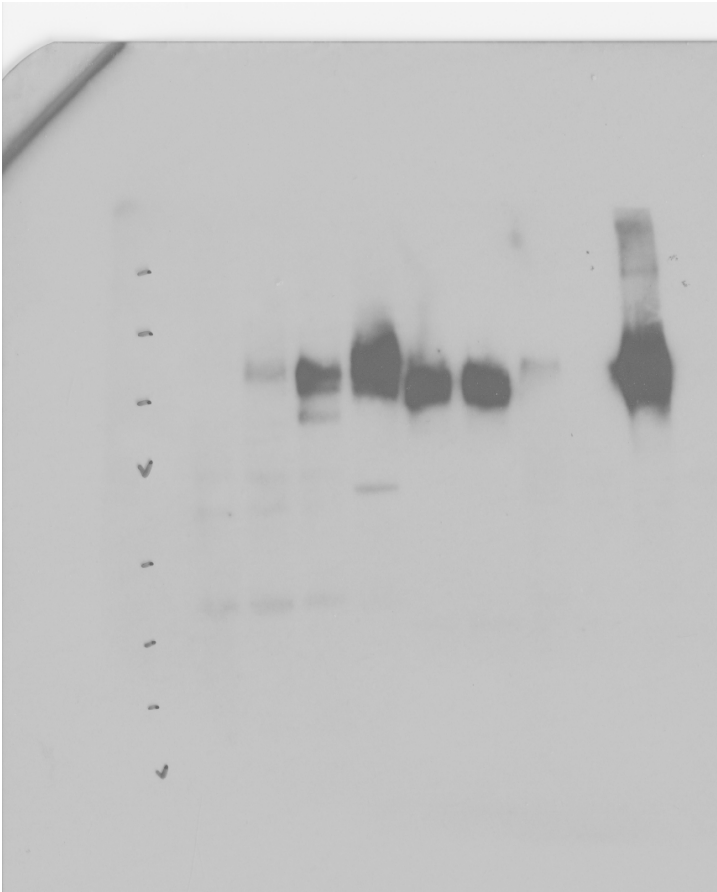
Source Data

Supplementary Figure 1b

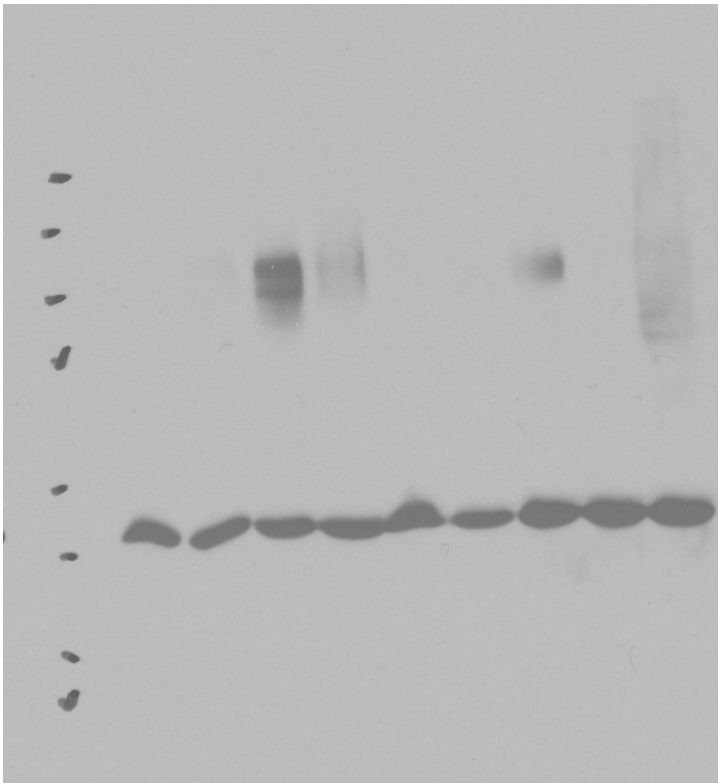
TMPRSS2



ACE2

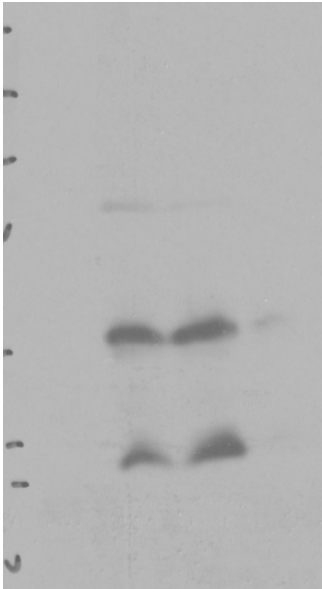


b-actin

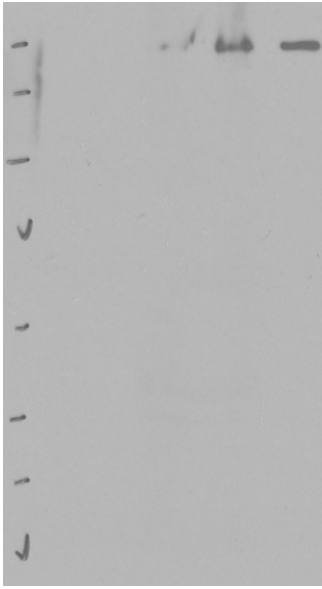


Supplementary Figure 3b

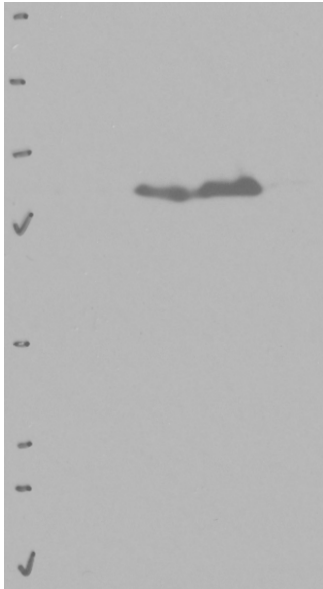
TMPRSS2



ZO-1

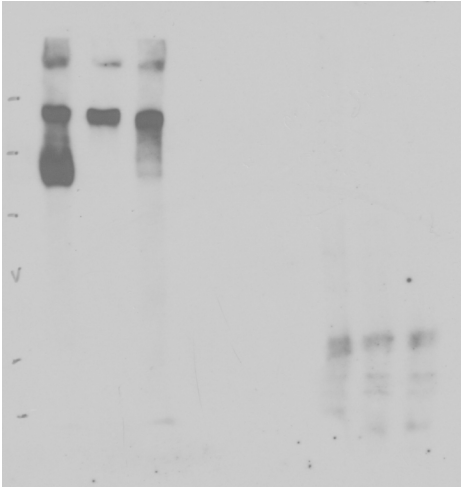


HSP90

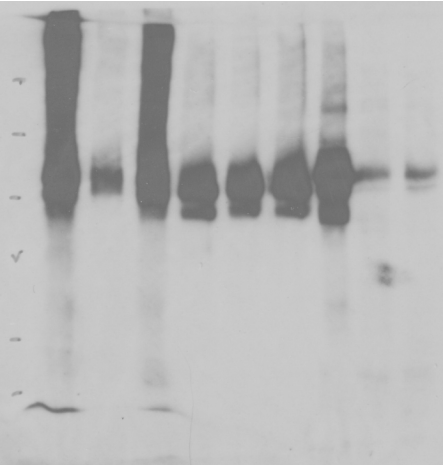


Supplementary Figure 6a.

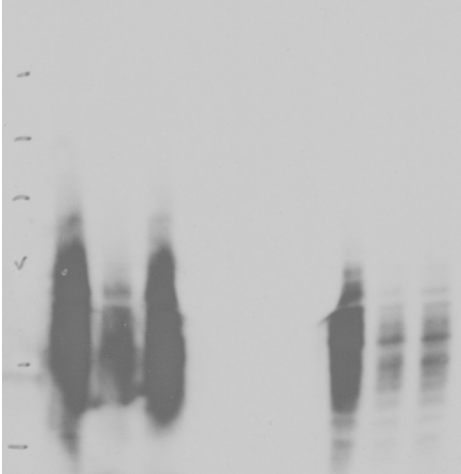
TMPRSS2



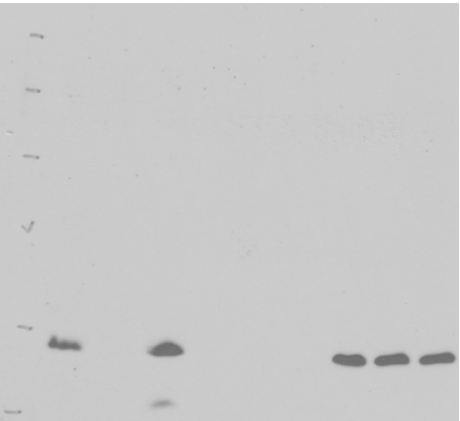
ACE2



CD63



TSG101



Supplementary figure 11e.

TMPRSS2

