Pyroptosis of syncytia formed by fusion of SARS-CoV-2 Spike and ACE2 expressing cells

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Abstract

SARS-Cov-2 infected cells fused with the ACE2-positive neighboring cells forming syncytia. However, the effect of syncytia in disease development is largely unknown. We established an *in vitro* cell-cell fusion system and used it to mimic the fusion of SARS-CoV-2 infected cells with ACE2-expressing cells to form syncytia. We found that Caspase-9 was activated after syncytia formation, and Caspase-3/7 was activated downstream of Caspase-9, but it triggered GSDME-dependent pyroptosis rather than apoptosis. What is more, single cell RNA-sequencing data showed that both ACE2 and GSDME were expression in alveolar type 2 cells in human lung. We propose that pyroptosis is the fate of syncytia formed by SARS-CoV-2 infected host cells and ACE2-positive cells, which indicated that lytic death of syncytia may contribute to the excessive inflammatory responses in severe COVID-19 patients.

Results

CoronaVirus Disease 2019 (COVID-19) is an infectious disease associated with systematical multi-organ failure caused by SARS-CoV-2, which mainly infects the lung and upper respiratory system[1,2]. Massive multinucleated syncytia are commonly observed in autopsy of severe COVID-19 patients[3]. It has been reported that the interaction between Spike (S) protein and ACE2 not only mediated the fusion of virus with host cells, but also multinucleated giant cells formation[4,5]. However, the effect of syncytia in disease development is largely unknown.

In order to better observe the formation of syncytia, we established an *in vitro* cell-cell fusion system and used it to mimic the fusion of SARS-CoV-2 infected cells with ACE2-expressing cells to form syncytia. High-content imaging with confocal laser scanning microscope was used to characterize the SARS-CoV-2-S-GFP expressing cells, which showed that SARS-CoV-2-S-GFP was mainly on the cell surface and was co-localized with cell membrane marker PLCδ-PH (Fig. S1a) but not nuclear RFP (Fig. S1e)[6]. In addition, S protein was detected as puncta in cytoplasm with co-localization of Golgi marker GGA1 (Fig. S1b), consisting with reports showing that the S protein was glycosylated in Golgi apparatus[7]. Two endosome-related proteins Rab5a and Rab7a were also co-located with the S protein, indicating the trafficking of S protein was associated with cellular membrane system (Fig. S1c and d). Then, the SARS-CoV-2-S-GFP expressing cell lines, named 2S-GFP-HeLa, were co-cultured with A549 expressing ACE2 cells (PLC-RFP-A549+ACE2) at a 1:1 ratio. Syncytia were observed 4 hours later (Fig. 1a),

and the cell-cell fusion occurred between the cell membranes because of the nuclei were intact (Fig. 1b). Additionally, syncytia were formed in co-culture system regardless of whether they were the same type of cells (2S-GFP-A549 and A549+ACE2, Fig. S2a) or different types of cells (2S-GFP-H1299 and A549+ACE2, Fig. S2b; 2S-GFP-HeLa and A549+ACE2, Fig.1a), or even cells from different species (2S-GFP-L929 and A549+ACE2, Fig. S2c).

It is well known that SARS-CoV-2 and SARS-CoV-1 enter host cells through the same receptor ACE2[8]. To find out whether SARS-CoV-1-S can interact with ACE2 and lead to cell-cell fusion like SARS-CoV-2-S. 293T-ACE2 cells were co-cultured with HeLa cells expressing SARS-CoV-1-S-Flag, or SARS-CoV-2-S-Flag. SARS-CoV-1-S-Flag HeLa cells indeed fused with 293T-ACE2 cells, while the degree of fusion is lower compared with SARS-CoV-2-S-Flag cells (Fig. S2d), even if the expression of S protein of SARS-CoV-1 is higher than SARS-CoV-2 (Fig. S2e). The similar results were obtained when HeLa cells expressing SARS-CoV-1-S-Flag, or SARS-CoV-2-S-Flag were co-cultured with Calu-3, which is a human lung cancer epithelioid cell line expressing endogenous ACE2 and can be infected by SARS-CoV-2 (Fig. S2f and g). Thus, the degree of cell-cell fusion mediated by S protein of SARS-CoV-2 interaction with ACE2 was higher than SARS-CoV-1, which might due to difference in the affinity to ACE2 between the S proteins of SARS-CoV-1 and SARS-CoV-2.

Next, we aimed to investigate the fate of syncytia. To eliminate the cross-talk of different cytosolic contents between different cell types, we co-cultured HeLa cells

expressing SARS-CoV-2-S (HeLa-2S) with HeLa-ACE2, and recorded the process of cell fusion by the real-time observation system (Fig. 1c and Movie-1). We found that syncytia formed upon cell-cell fusion, grew in size steadily, finally, ruptured with LDH release, ATP decrease, and the activity of caspase-3/7 increase. The death was blocked by pan-caspase inhibitor zVAD (Fig. 1d, e and f). We assessed the activation of molecules related to the death. As shown in Fig. 1g, caspase-8/9 and caspase-3/7 were cleaved, suggesting an activation of apoptosis pathway. Interestingly, we also detected cleavage of GSDME. It is known that activation of GSDME is mediated by caspase-3[9,10], and we confirmed the role of caspase in activation of GSDME as zVAD effectively blocked GSDME cleavage. Thus, it can be proposed that syncytia formation led to activation of caspase-8/9 to caspase3/7 cascade. The activated caspase-3 cleaved GSDME and released N-GSDME to permeabilize cell membrane to execute syncytia pyroptosis.

To define the death pathway underlying syncytia pyroptosis, we knocked out caspase-8 (*Casp8*$^{-/-}$) or caspase-9 (*Casp9*$^{-/-}$) in HeLa cells by CRISPR-Cas9 and expressed ACE2 and SARS-CoV-2-S-Flag in these cells respectively, and then co-cultured these cells in pairs. We observed syncytia formation in *Casp8*$^{-/-}$ and *Casp9*$^{-/-}$ cells without difference from WT cells (Fig. S3a). In contrast, *Casp9* but not *Casp8* deletion blocked syncytia death (Fig. 1i). Further analysis showed that the cleavage of GSDME (N-GE) was significantly decreased in *Casp9*$^{-/-}$ cells (Fig. 1h). Unexpectedly, the 70kD fragment of SARS-CoV-2-S-Flag, which was believed to be processed by protease TMPRSS2 or Cathepsin L during S protein fusion with ACE2,

was disappeared in *Casp9*$^{-/-}$ cells, indicating a linkage between caspase-9 and SARS-CoV-2 S protein cleavage.

To further confirm that GSDME was involved in the death of syncytia, we generated GSDME knock-out (*GE*$^{-/-}$+ACE2, *GE*$^{-/-}$+SARS-CoV-2-S-Flag) and WT HeLa cell lines (*GE*$^{+/+}$+ACE2, *GE*$^{+/+}$+SARS-CoV-2-S-Flag), and co-cultured them. Similarly, GSDME did not affect cell fusion to form syncytia (Fig. S3b and c), but GSDME knock-out significantly inhibited the death of syncytia (Fig. 1j). In addition, GSDME knock-out did not affect the activation of caspases (Fig. 1k), confirming it is downstream of caspases.

Our data indicated that the death of syncytia induced by SARS-CoV-2 infection could be mediated by GSDME-dependent pyroptosis. The existing transcriptomic data[11] showed correlations of the expression between ACE2 and GSDME, especially in the small intestine and testis (Fig. S4). The high-level expression of ACE2 and GSDME in testis could link to the destruction of male reproductive system by SARS-CoV-2 infection[12]. We analyzed single-cell-RNA-sequencing (scRNA-Seq) data from eight normal human lung transplant donors with a total of 42,225 cells[13]. As reported, the expression of ACE2 is concentrated in a special small population of alveolar type 2 (AT2) cells in lung[14] (Fig. S5a), and GSDME is also enrichment in AT2 cells. Thus, GSDME-dependent pyroptosis could occur in SARS-CoV-2 infected AT2 cells (Fig. S5b).

Here, we provide *in vitro* evidence showing that the syncytia formed by fusion of the cells expressing SARS-CoV-2 S protein and ACE2 respectively undergo

pyroptosis (Fig. 1l). The pyroptosis is initiated by components of intrinsic apoptosis pathway and executed by caspase-3/7 mediated activation/cleavage of GSDME. Since scRNA-seq data showed that both ACE2 and GSDME were expression in AT2 cells in human lung, we propose that pyroptosis is the fate of syncytia formed by SARS-CoV-2 infected host cells and ACE2-positive cells. The lytic death of syncytia may contribute to the excessive inflammatory responses in severe COVID-19 patients.

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AUTHOR CONTRIBUTIONS

J.H., Y.Z., H.M. conceived and designed the experiments. H.M., Z.Z., H.L., P.Z., L.L. performed the experiments: generated the cell strains and performed immunofluorescent imaging, and the associated western blot analyses. S.W. and Y.L. analyzed the data from GTEx and scRNA-seq. J.W. helped with discussion and interpretation of results. H.M and J.H wrote the manuscript. All authors provided the final approval of the manuscript.

Competing interests: The authors declare no competing interests.

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Figure legends:

Fig.1 Caspase-9/GSDME trigged pyroptosis of syncytia formed by fusion of SARS-CoV-2 Spike and ACE2 expressing cells.

SARS-CoV-2-S-GFP HeLa cells were co-cultured with PLC-RFP-A549+ACE2 (**a**), or NLS-RFP-A549+ACE2 cells (**b**) at a 1:1 ratio, four hours later, image was scanned by LSM780, the nucleus(blue) was stained by Hoechst; Bar, 20 μm. **c.** The time-phase of cell-cell fusion progress, HeLa-ACE2 co-culture with HeLa-SARS-CoV-2-S cells, images were obtained by Digital Cell Checker for Cell Culture PAULA; Bar, 200 μm; then the LDH release (**d**) , ATP (**e**) , the activity of Caspase-3/7 (**f**) were detected at indicated time , pan-caspase inhibitor, ZVAD(20 uM) was used. LDH release was measured after co-culture for 12 hours as indicated (**i** and **j**). Western blot analysis of the cells collected as indicated, ACE2, Flag, Caspase-8/9/3/7, and GSMDE were probed, β-Actin as loading control (**g**, **h** and **k**). The model of SARS-CoV-2 Spike interaction with ACE2 induced syncytia pyroptosis (**l**). The data were shown as means ± SD , ****P<0.0001, NS, non-significance, and the experiments were replicated more than three times.

Supplementary figure legends:

Fig. S1 The cellular distribution of SARS-CoV-2-S-GFP.

Co-expression of SARS-CoV-2-S-GFP with different markers fused with RFP in HeLa cells: (**a**). PLCδ-PH is the cell membrane marker; (**b**). GGA1 is a Golgi associated protein; (**c**). Rab5A is mainly localized in early endosome membrane or other cytosolic vesicle; (**d**). Rab7A governs early-to-late endosomal maturation; (**e**). NLS-RPF was referred as insert the nuclear localization sequence (NLS) of SV40 at the head of RPF. The images were scanned by LSM780, the nucleus(blue) was stained by Hoechst; Bar, 20 μm.

Fig. S2:

PLC-RFP-A549+ACE2 cells were co-cultured with SARS-CoV-2-S-GFP-A549 cells (**a**), SARS-CoV-2-S-GFP-H1299 cells (**b**), SARS-CoV-2-S-GFP-L929 cells (**c**) at a 1:1 ratio, four hours later, image was scanned by LSM780, the nucleus(blue) was stained by Hoechst; Bar, 20 μm. HeLa-SARS-CoV-1-S, or HeLa-SARS-CoV-2-S cells were co-cultured with 293T-ACE2 cells (**d**), or Calu-3 cells (**f**), four hours later, images were obtained by Axio Observer 5; Bar, 100 μm. Western bolt analyzed the samples collected as indicated (**e** and **g**), anti-Flag and β-Actin were probed.

Fig. S3：

a. Generated Caspase-8(*Casp8*$^{-/-}$), Caspase-9(*Casp9*$^{-/-}$) knock-out HeLa cells by CRISPR-Cas9; then transfected these cells with ACE2, or SARS-CoV-2-S-Flag to obtained wild-type HeLa (WT+ACE2; WT+2S-Flag); Caspase-8 knock-out (*Casp8*$^{-/-}$+ACE2; *Casp8*$^{-/-}$+2S-Flag); Caspase-9

knock-out (*Casp9*$^{-/-}$+ACE2; *Casp9*$^{-/-}$+2S-Flag); then co-culture these cells, images were obtained by Axio Observer at 5 hours indicated for syncytia formation, and 12 hours for cell death; Bar, 100 μm.

b. Images were obtained by Axio Observer at 5 hours indicated for syncytia formation as indicated; Bar, 100 μm.

c. Western blot analyzed the GSDME-KO cell lines.

Fig. S4 The expression profile of GSDME and ACE2.

Data from the Genotype-Tissue Expression (GTEx) Project for tissues.

Fig. S5 scRNA-seq profile of GSDME and ACE2 in human lung

a. Cellular populations identified. Cells were clustered using a graph-based shared nearest neighbor clustering approach and visualized using a t-distributed Stochastic Neighbor Embedding (tSNE) plot. AT1 = alveolar type □; AT2 = alveolar type □.

b. Violin plots representing expression probability distributions across clusters.

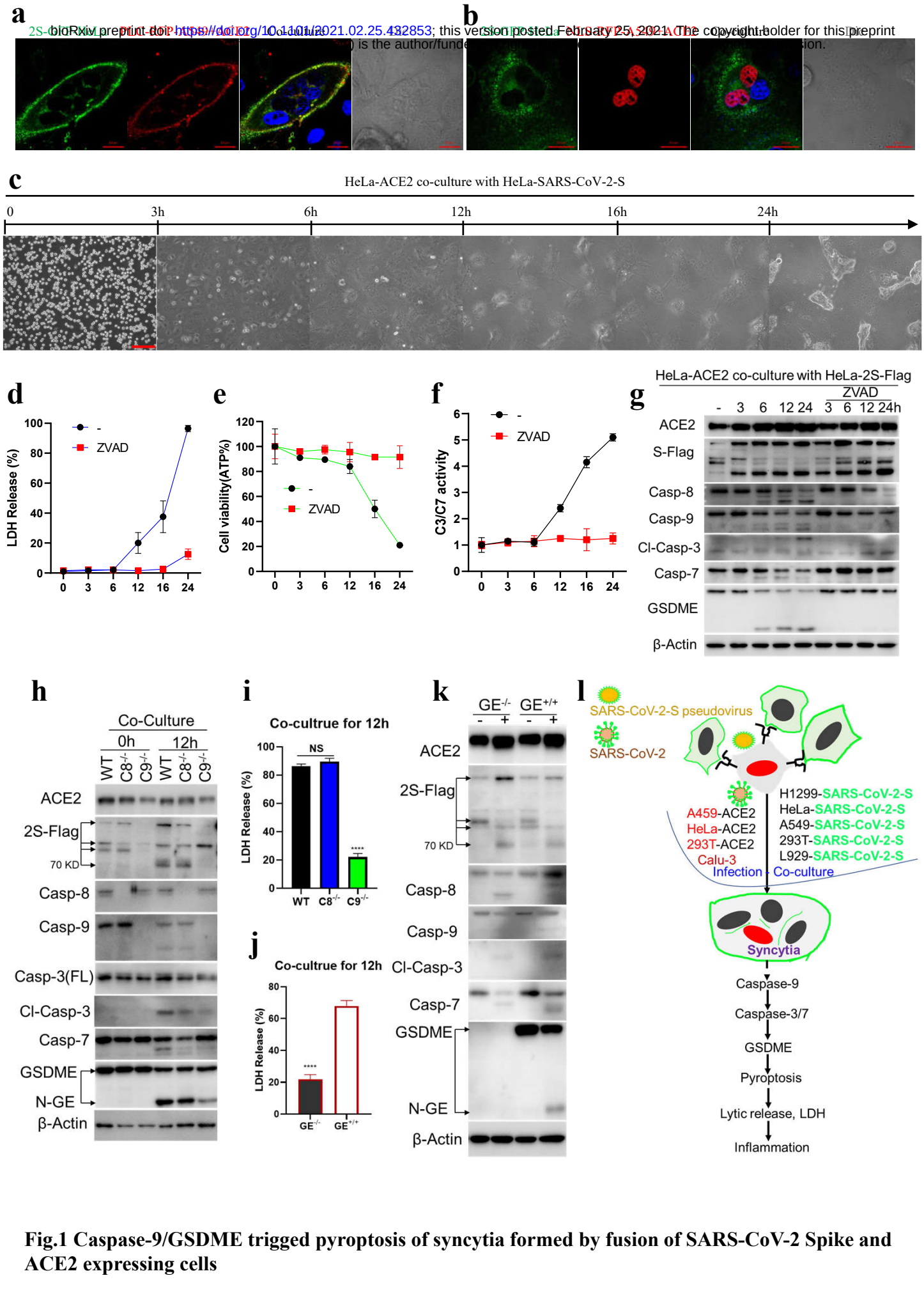


Fig.1 Caspase-9/GSDME trigged pyroptosis of syncytia formed by fusion of SARS-CoV-2 Spike and ACE2 expressing cells

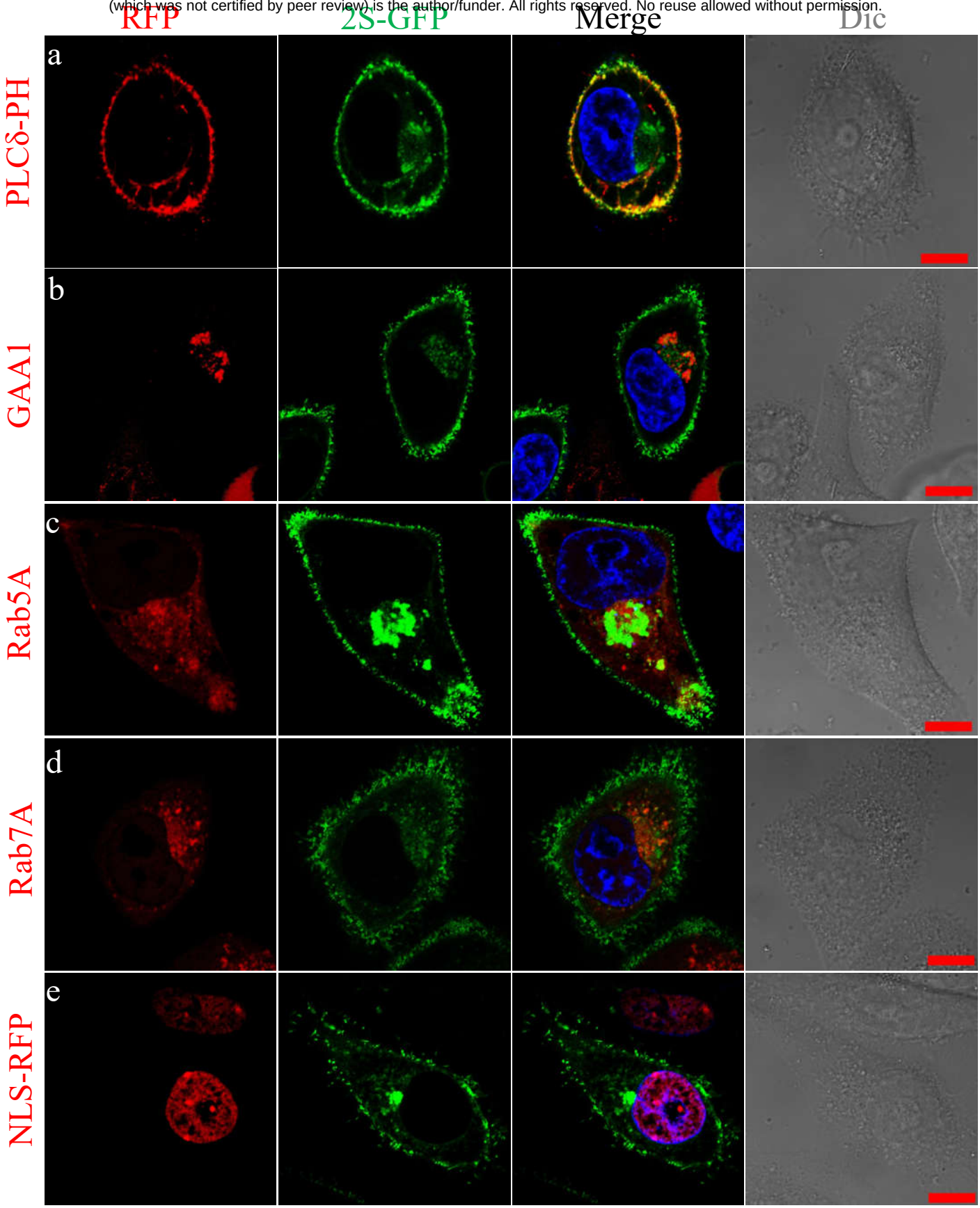
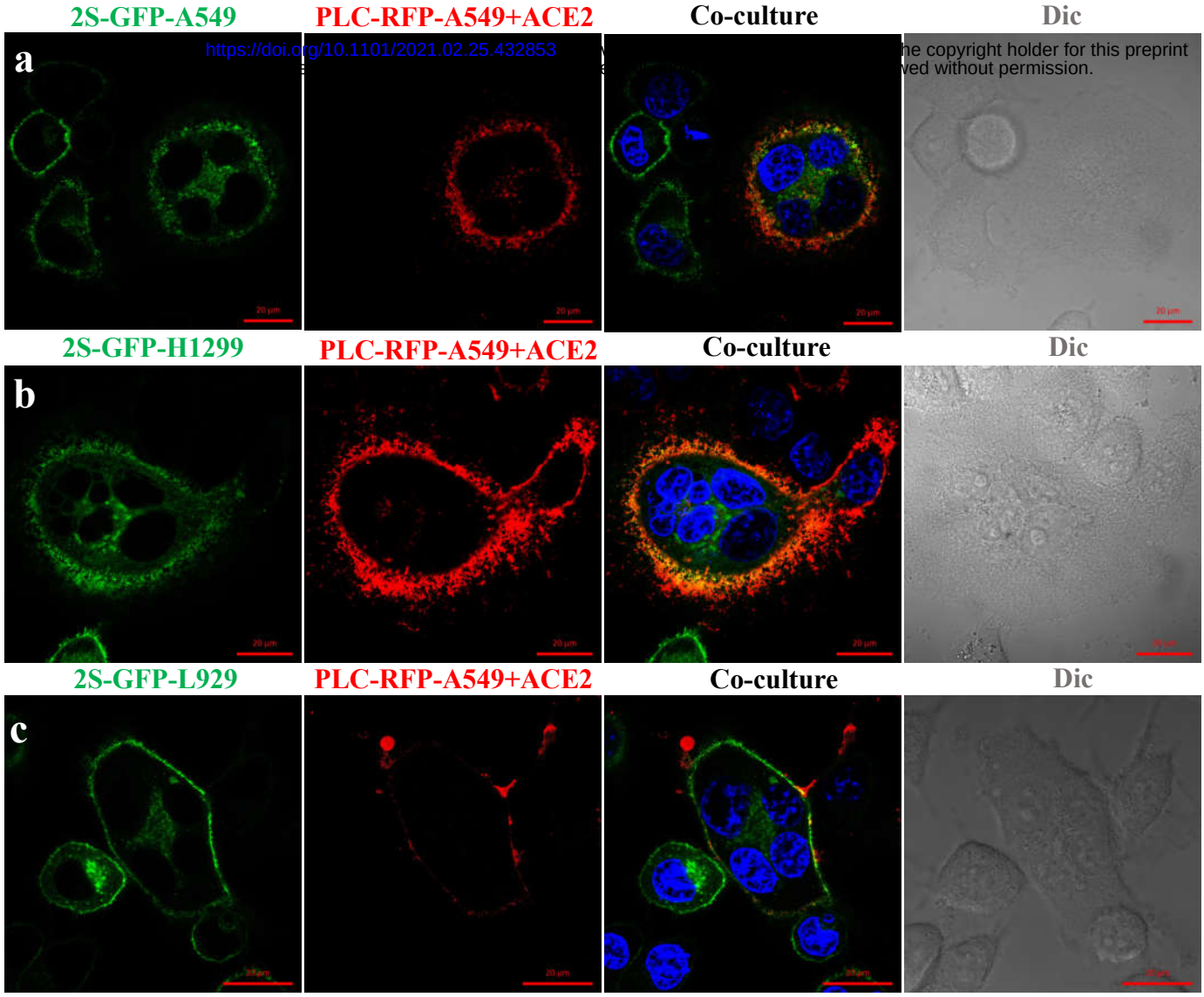
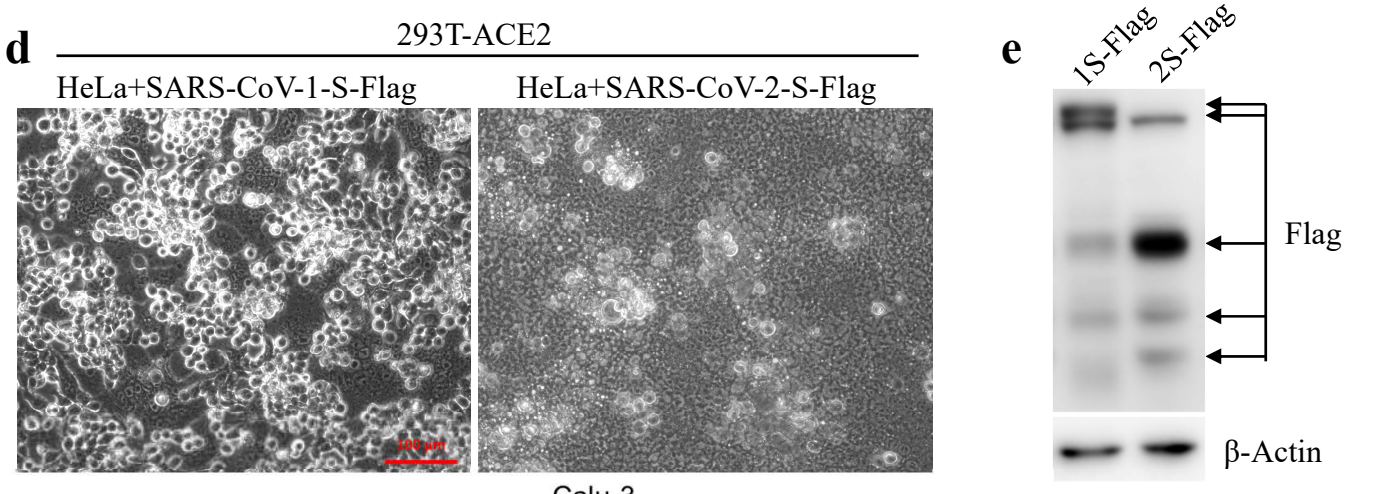


Fig. S1 The cellular distribution of SARS-CoV-2-S-GFP.





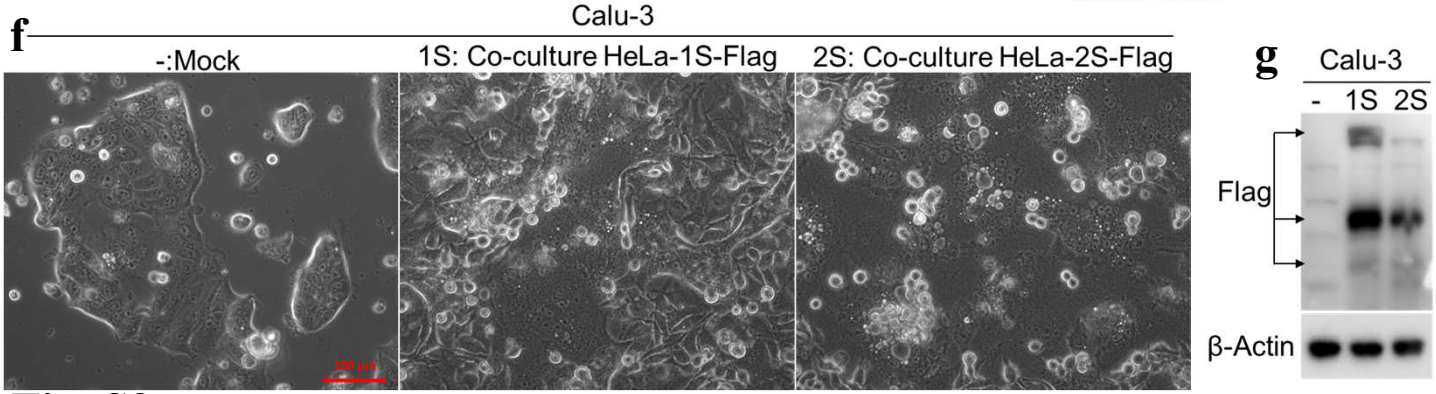
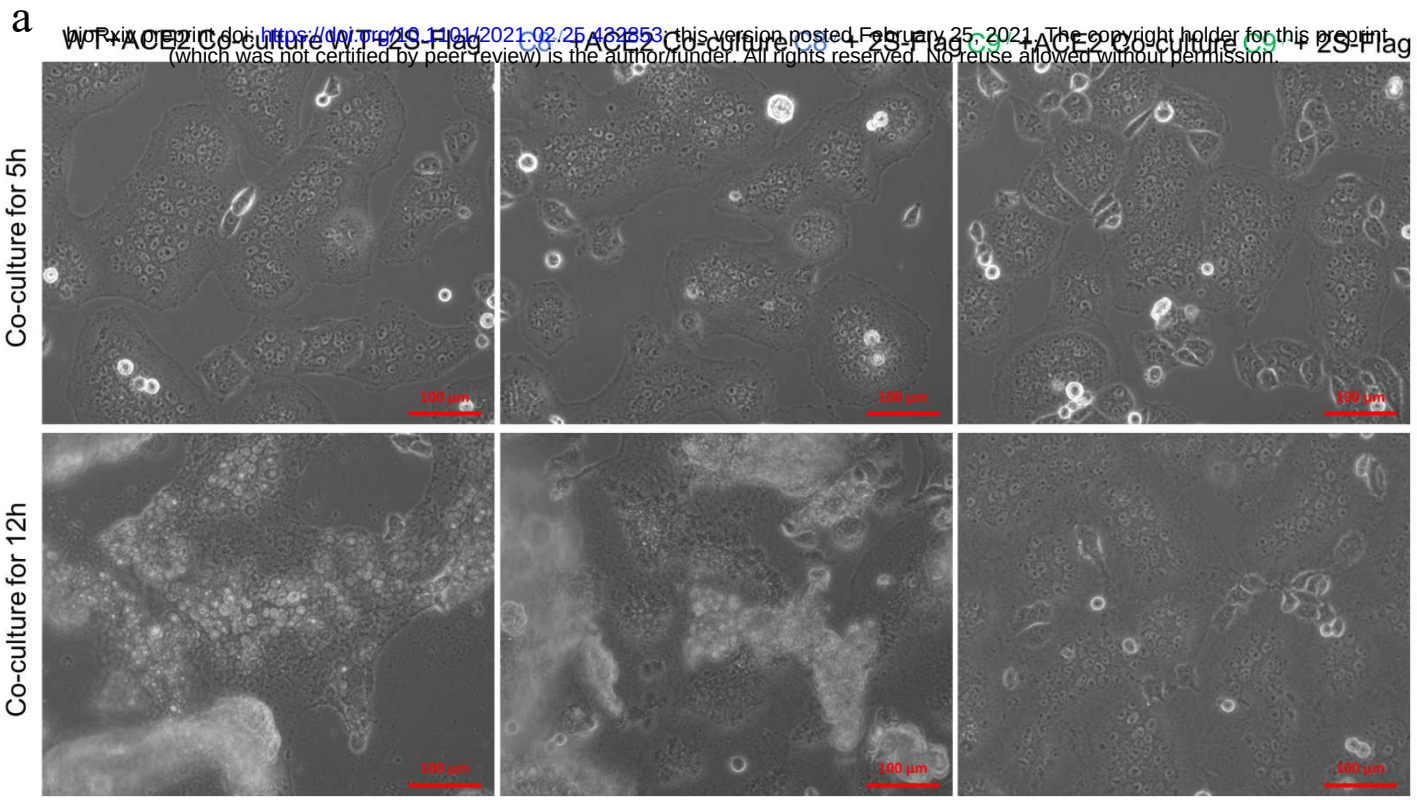
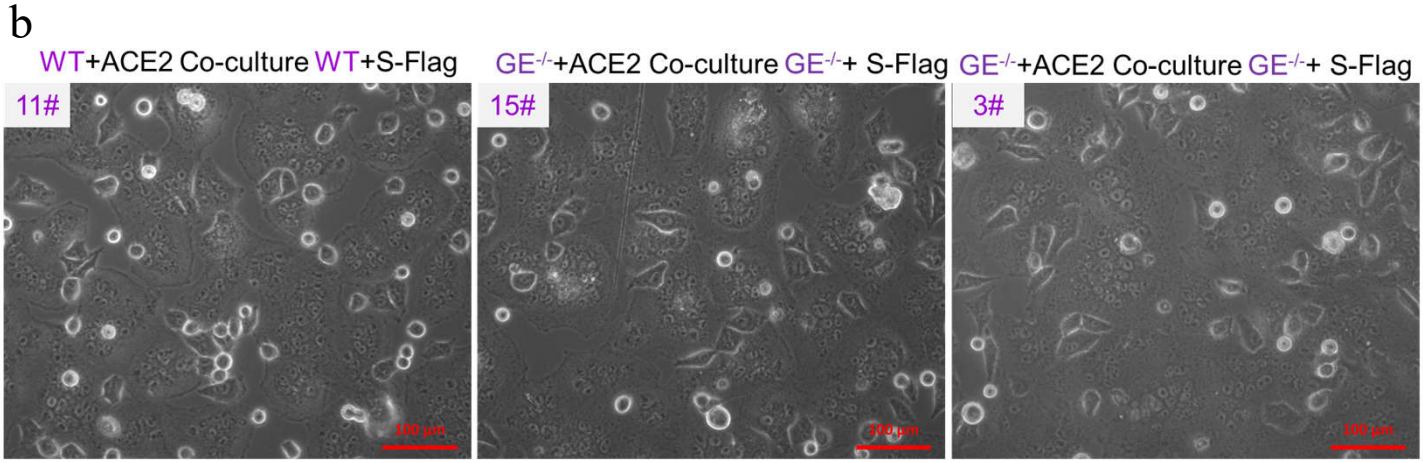


Fig. S2





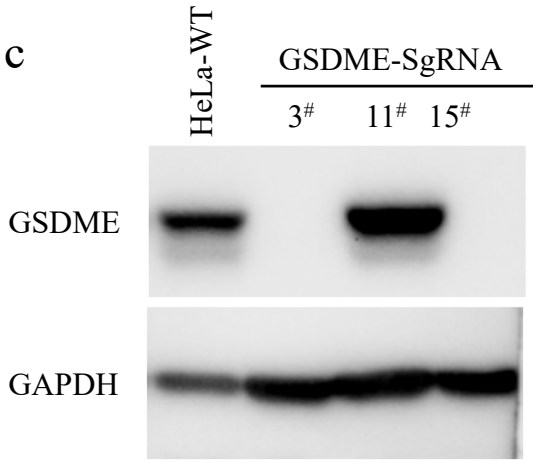


Fig. S3

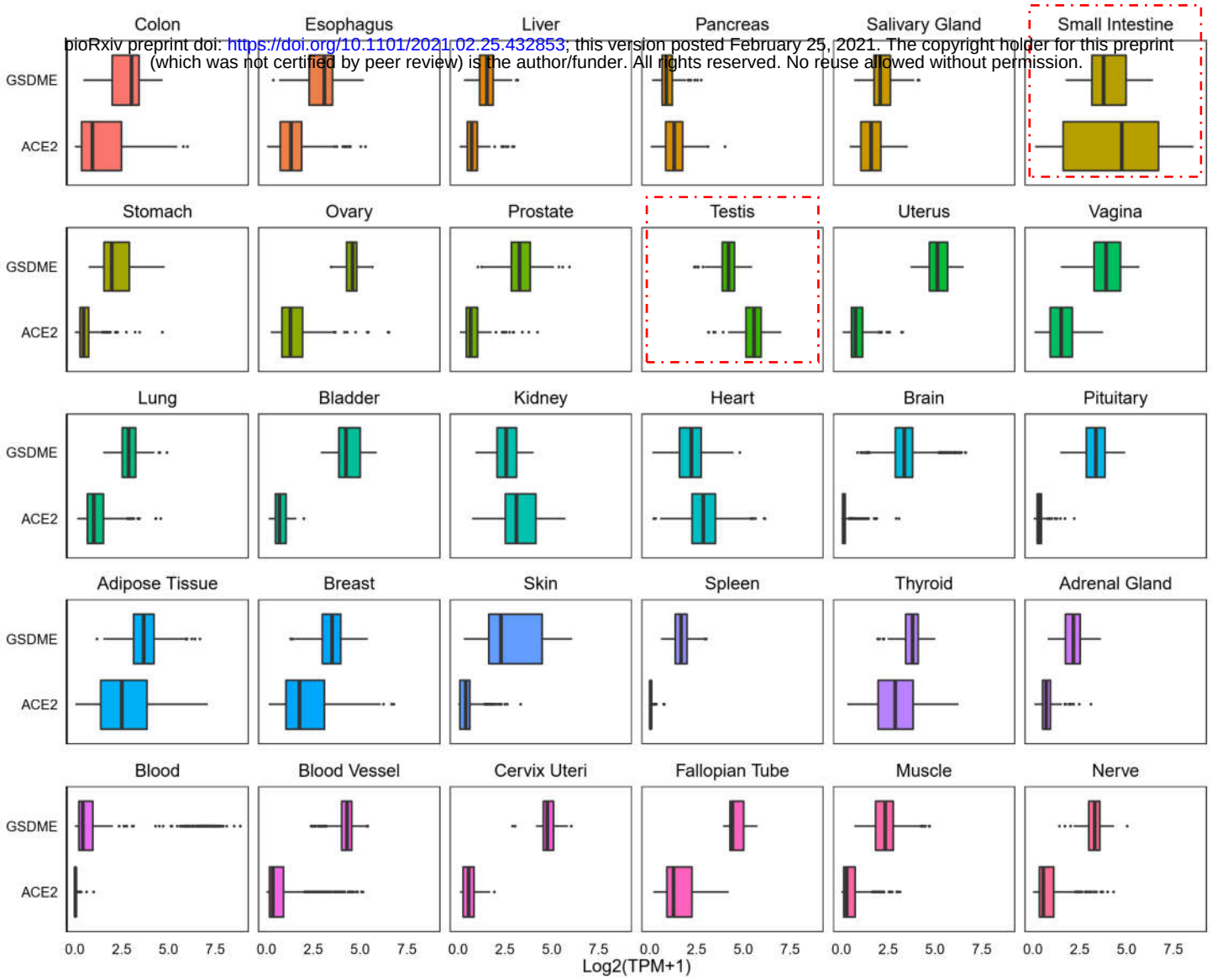


Fig. S4 The expression profile of GSDME and ACE2.

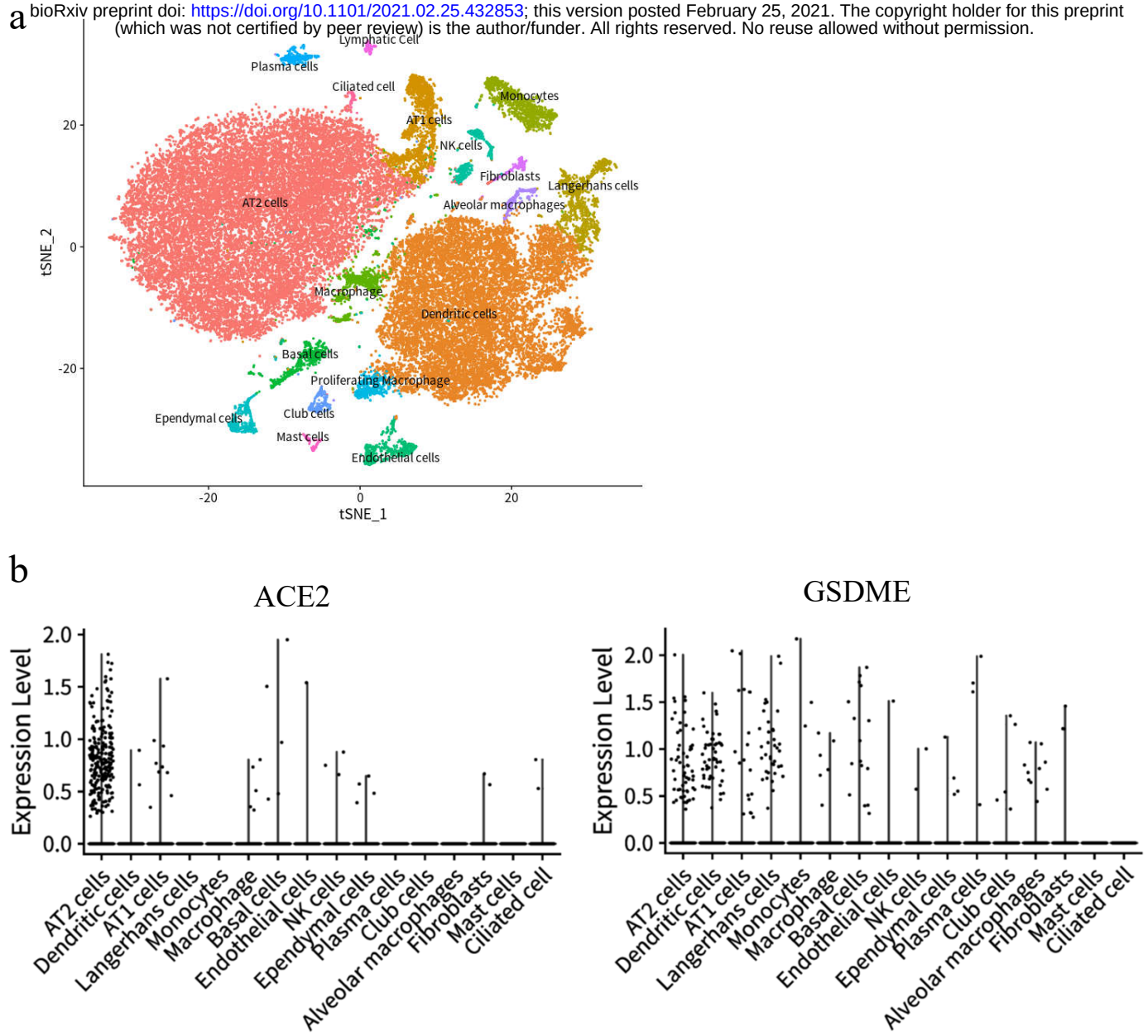


Fig. S5 scRNA-seq profile of GSDME and ACE2 in human lung