IFITM dependency of SARS-CoV-2 variants of concern

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We have recently shown that a SARS-CoV-2 strain isolated in the Netherlands in February 2020 (NL-02-2020) hijacks interferon-induced transmembrane proteins, especially IFITM2, as entry cofactors for efficient infection. Here, we examined whether SARS-CoV-2 "variants of concern" (VOCs), including the currently dominating delta variant, maintained the dependency on IFITMs for efficient replication. Depletion of IFITM2 reduced viral RNA production from 31- (B.1.1.7) to 755-fold (P.1). In comparison, silencing of IFITM1 had little effect, while knock-down of IFITM3 resulted in an intermediate phenotype. Strikingly, silencing of IFITM2 generally reduced infectious virus production in Calu-3 cells to near background levels. An antibody directed against the N-terminus of IFITM2 inhibited SARS-CoV-2 VOC replication in iPSC-derived alveolar epithelial type II cells. In conclusion, endogenously expressed IFITM proteins (especially IFITM2) are important cofactors for replication of genuine SARS-CoV-2 VOCs, including the Delta variant.

25 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of
26 coronavirus disease 2019 (COVID-19), has caused a devastating pandemic. The reasons for
27 the efficient spread of SARS-CoV-2 are not fully understood but clearly involve the ability to
28 efficiently infect and propagate in human cells. Viral entry depends on binding of the viral
29 Spike (S) protein to the cellular angiotensin-converting enzyme (ACE) 2 receptor and
30 proteolytic processing of the S precursor into the active S1 and S2 subunits[1]. However,
31 additional host factors may affect the efficiency of SARS-CoV-2 entry and play key roles in
32 viral transmission and pathogenesis.

33 We recently demonstrated that interferon-induced transmembrane proteins (IFITMs 1, 2, and
34 3) are required for efficient SARS-CoV-2 infection[2]. This was unexpected, as IFITMs are a
35 family of IFN stimulated genes (ISGs) that are well-known to protect cells against infection by
36 various viral pathogens, such as influenza A, rhabdo-, and human immunodeficiency viruses[3].
37 Inhibitory effects were also reported for highly pathogenic coronaviruses, including SARS-
38 CoV-2[4,5]. However, most evidence was obtained using cells artificially overexpressing IFITM
39 proteins and pseudo-particles containing the S protein of SARS or MERS coronaviruses.

40 The antiviral mechanism of IFITMs is thought to involve alterations in the rigidity and
41 curvature of the cellular membrane, affecting viruses in a broad, unspecific way. In contrast,
42 the SARS-CoV-2 enhancing effect involves specific interactions between the S protein and the
43 N-terminal region of IFITMs, especially IFITM2[2], suggesting that the virus hijacks IFITMs.
44 Consequently, knock-down of endogenous IFITM2 expression in human lung cell lines
45 reduced viral entry and infectious virus production by several orders of magnitude. In addition,
46 IFITM-derived peptides or IFITM targeting antibodies protected gut organoids and
47 cardiomyocytes against infection and cytopathic effects of SARS-CoV-2[2].

48 IFITM2 dependency for efficient infection has been demonstrated using an early European
49 variant of SARS-CoV-2[2] isolated in the Netherlands in February 2020 (NL-02-2020). Since
50 then numerous variants have emerged. Some of them show increased transmission fitness and
51 immune evasion and are thus referred to as "variants of concern" (VOCs). Currently, the WHO
52 has categorized four SARS-CoV-2 variants as VOCs: B.1.1.7, B.1.351, P.1 and B.1.617.2. For
53 simplification, these are also referred to as Alpha, Beta, Gamma and Delta variants,
54 respectively. Compared to the NL-02-2020 isolate, they contain various alterations in their S
55 proteins (Fig. 1A) that might affect ACE2 receptor affinity and proteolytic activation[6].

56

57 Here, we examined whether SARS-CoV-2 VOCs, including the currently dominating Delta
58 (B.1.617.2) variant, still depend on IFITMs for efficient infection. We utilized the human
59 epithelial lung cancer cell line Calu-3 because it allows efficient siRNA knockdown (KD) of
60 IFITM expression[2] and iPSC-derived alveolar epithelial type II (iATII) cells as a model for the
61 main target cells of SARS-CoV-2 infection in the distal lung[7]. Both cell types expressed
62 IFITM2 and IFITM3 (Fig. 1B). In agreement with published data[8], ACE2 expression by iATII
63 cells was detectable by FACS (Fig. S1) but not by western blot analysis (Fig. 1B). On average,
64 the Delta VOC produced 3-fold higher levels of viral RNA compared to NL-02-2020 in Calu-
65 3 cells (Fig. 1C). Silencing of endogenous IFITM2 expression in Calu-3 cells reduced viral
66 RNA production from 31- (Alpha) to 754-fold (Gamma). In comparison, KD of IFITM1
67 generally had little effect, while silencing of IFITM3 resulted in 2- to 31-fold reduction of viral
68 RNA yields. Strikingly, the amount of infectious SARS-CoV-2 particles in the cell culture
69 supernatant was generally reduced to background levels upon silencing of IFITM2 (Fig. 1D,
70 1E). Treatment of iATII cells with an antibody targeting the N-terminus of IFITM2 generally
71 reduced viral RNA production in a dose-dependent manner, albeit varying efficiencies (Fig.
72 1F). Most notably, treatment with the α-IFITM2 antibody reduced replication of the Delta
73 VOC in iATII cells by up to 95% (Fig. 1G).

74 In conclusion, our results show that IFITMs (especially IFITM2) are also critical cofactors for
75 efficient replication of current SARS-CoV-2 VOCs including the dominant Delta variant.
76 Notably, the Delta variant replicated to 30-fold higher levels (average vRNA copy numbers of
77 $2.4x10^{11}$) compared to the early NL-02-2020 isolate in iATII cells (Fig. 1F), which agrees with
78 a key role of this cell type *in vivo*[7]. Efficient inhibition of the Delta VOC by an α-IFITM2
79 antibody in this cell type adds to the evidence that IFITM2 may play an important role in
80 SARS-CoV-2 transmission, dissemination and pathogenesis. Our observation that IFITM2
81 dependency is maintained by VOCs also further underlines that this cellular "antiviral" factor
82 represents an interesting target for therapeutic or preventive approaches.
83

84 **Fig. 1. Effects of IFITM proteins on replication of SARS-CoV-2 variants.** (**A**) Mutations
85 in the Spike proteins of SARS-CoV-2 VOCs (Beta, blue; Alpha, green; Gamma, yellow; Delta,
86 red) compared to the SARS-CoV-2 (NL-02-2020) strain. (**B**) Immunoblot of Calu-3 and iATII
87 cells left uninfected (c) or infected with the indicated SARS-CoV-2 variants. Whole-cell lysates
88 were stained with the indicated antibodies. An unspecific signal was observed in the Calu-3
89 control lane stained with the CoV-2 N antibody. (**C**) Viral N RNA levels in the supernatant of
90 Calu-3 cells, collected 48 h post-infection with SARS-CoV-2 (MOI 0.05). Cells were
91 transfected with control (CTRL) or IFITM targeting siRNAs as indicated. Bars represent the
92 mean of 3 to 4 independent experiments (±SEM). (**D**) Impact of IFITM2 siRNA knock-down
93 on infectious SARS-CoV-2 yields. Supernatants derived from Calu-3 cells treated with control
94 (CTRL) of IFITM2 siRNA two days after infection with the SARS-CoV-2 NL-02-2020 or
95 VOCs were serially diluted and added to Caco-2 cells seeded in 96-well plates. Five days later
96 cells were examined for CPE, fixed and stained with crystal violet. Productively infected wells
97 appear transparent since cells are eliminated and detached by the virus. (**E**) Infectious SARS-
98 CoV-2 particles in the supernatant of Calu-3 cells treated with control or IFITM2 targeting
99 siRNAs. Bars represent the mean of one experiment performed with eight technical replicates
100 (±SD) shown in panel D. (**F**) Quantification of viral N RNA levels in the supernatant of iATII
101 cells treated with α-IFITM2 antibody (20, 40, or 80 µg/ml) or Remdesivir (10 µM) 1 h before
102 infection (SARS-CoV-2, MOI 0.5), collected 48 h post-infection. Bars represent the mean of
103 three independent experiments. (**G**) Average percentage of reduction of vRNA levels in the
104 supernatants of (E) compared to the untreated control.
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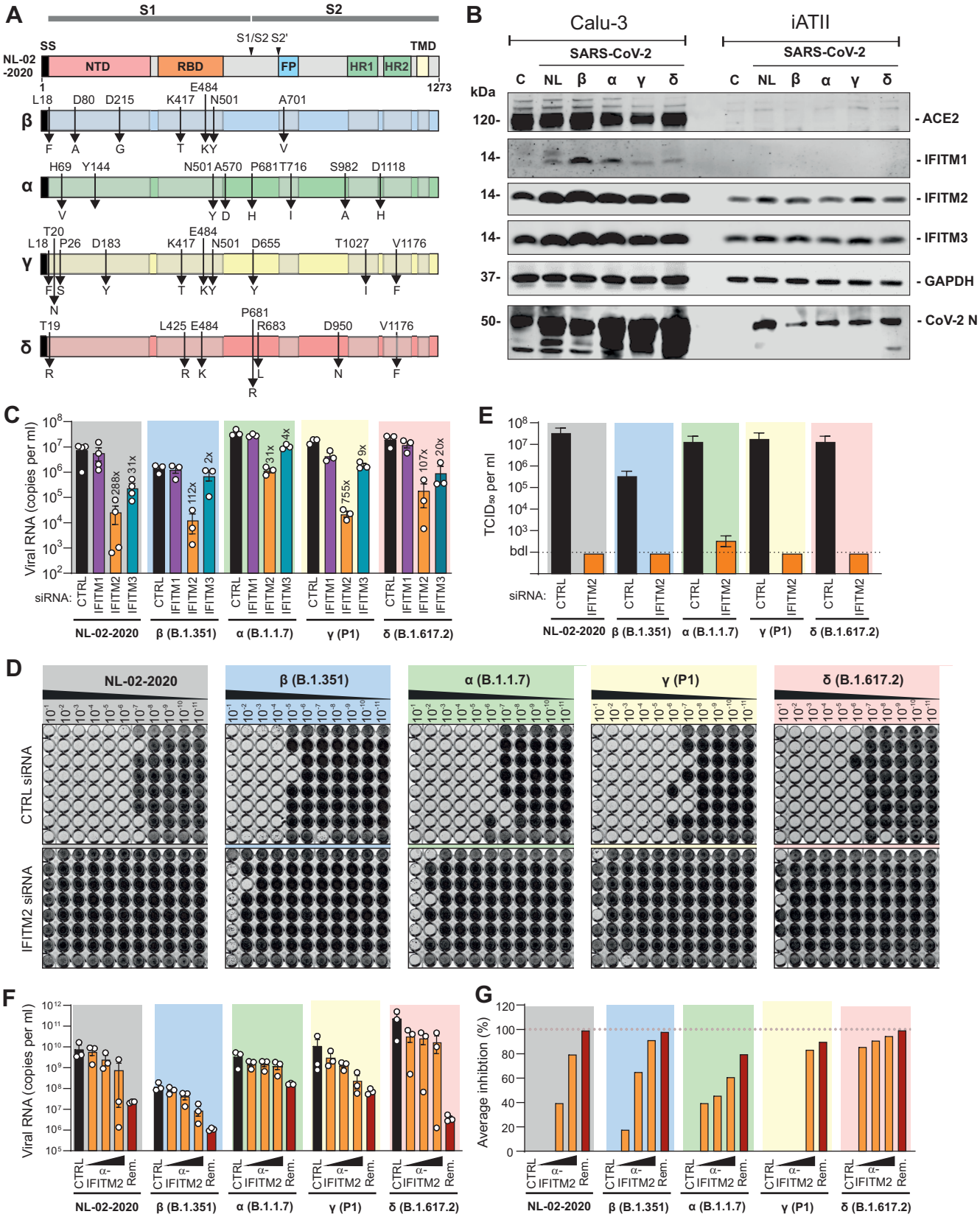
COMPETING INTERESTS

The authors declare no competing interests.

Fig. 1

Nchioua et al.,



SUPPLEMENTAL MATERIAL AND METHODS

Cell culture. Calu-3 (human epithelial lung adenocarcinoma) cells were cultured in Minimum Essential Medium Eagle (MEM, Sigma, Cat#M4655) supplemented with 10% (upon and after viral infection) or 20% (during all other times) heat-inactivated fetal bovine serum (FBS, Gibco, Cat#10270106), 100 units/ml penicillin, 100 µg/ml streptomycin (ThermoFisher, Cat#15140122), 1 mM sodium pyruvate (Pan Biotech, Cat#P04-8010), and 1x non-essential amino acids (Sigma, Cat#M7145). Vero E6 cells (Cercopithecus aethiops derived epithelial kidney, ATCC) and TMPRSS2-expressing Vero E6 cells (kindly provided by the National Institute for Biological Standards and Control (NIBSC), No. 100978) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Cat#41965039) supplemented with 2.5% (upon and after viral infection) or 10% (during all other times) heat-inactivated FBS (Gibco, Cat#10270106), 100 units/ml penicillin, 100 µg/ml streptomycin (ThermoFisher, Cat#15140122), 2 mM L-glutamine (Gibco, Cat#25030081), 1 mM sodium pyruvate (Pan Biotech, Cat# P04-8010), 1x non-essential amino acids (Sigma, Cat#M7145) and 1 mg/mL Geneticin (Gibco, Cat#10131-019) (for TMPRSS2-expressing Vero E6 cells). Caco-2 cells (human epithelial colorectal adenocarcinoma, kindly provided by Prof. Holger Barth (Ulm University)) were grown in the same media as Vero E6 cells but with supplementation of 10% heat-inactivated FBS.

Human induced Alveolar Type 2 cells (iATII) were differentiated from BU3 NKX2-1^{GFP};SFTPC^{tdTomato} induced pluripotent stem cells[1] (iPCSs, kindly provided by Darrell Kotton, Boston University and Boston Medical Center) and maintained as alveolospheres embedded in 3D Matrigel in CK+DCI media, as previously described[2]. For infection studies, iATII cells were cultured as 2D cultures on Matrigel-coated plates in CK+DCI medium + 10 µM Y-27632 (Tocris, Cat#1254) for 48h before switching to CK+DCI medium on day 3.

SARS-CoV-2 stocks. The SARS-CoV-2 variant B.1.351 (Beta), 2102-cov-IM-r1-164 was provided by Prof. Michael Schindler (University of Tübingen) and the B.1.617.2 (Delta) variant by Prof. Florian Schmidt (University of Bonn). The BetaCoV/Netherlands/01/NL/2020 (NL-02-2020) and B.1.1.7. (Alpha) variants were obtained from the European Virus Archive. The hCoV-19/Japan/TY7-503/2021 (Brazil P.1) (Gamma) (#NR-54982) isolate was obtained from the BEI resources. SARS-CoV-2 strains were propagated on Vero E6 (NL-02-2020, Delta), VeroE6 overexpressing TMPRSS2 (Alpha), CaCo-2 (Beta) or Calu-3 (Gamma) cells. To this end, 70-90% confluent cells in 75 cm² cell culture flasks were inoculated with the SARS-CoV-2 isolate (multiplicity of infection (MOI) of 0.03-0.1) in 3.5 ml serum-free medium. The cells were incubated for 2h at 37°C, before adding 20 ml medium containing 15 mM HEPES (Carl Roth, Cat#6763.1). Virus stocks were harvested as soon as strong cytopathic effect (CPE) became apparent. The virus stocks were centrifuged for 5 min at 1,000 g to remove cellular debris, aliquoted, and stored at -80°C until further use.

Sequencing of SARS-CoV-2 VOCs. Virus stocks were inactivated and lysed by adding 0.3 ml TRIzol Reagent (ambion, Cat#132903) to 0.1 ml virus stock. Viral RNA was isolated using the Direct-zol RNA MiniPrep kit (ZymoResearch, Cat#R2050) according to manufacturer's instructions, eluting the RNA in 50 µl DNase/RNase free water. The protocol to prepare the viral RNA for sequencing was modified from the nCoV-2019 sequencing protocol V.1. For reverse transcription, the SuperScript IV First-Strand Synthesis System (Invitrogen, Cat#18091050) was used with modified manufacturer's instructions. First, 1 µl random hexamers (50 ng/µl), 1 µl dNTPs mix (10 mM each), and 11 µl template RNA (diluted 1:10 in DNase/RNase free water) were mixed, incubated at 65°C for 5 min and placed on ice for 1 min. Next, 4 µl SSIV Buffer, 1 µl DTT (100 mM), 1 µl RNaseOUT RNase Inhibitor, and 1 µl SSIV Reverse Transcriptase were added to the mix, followed by incubation at 24°C for 5 min, 42°C for 50 min, and 70°C for 10 min. To generate 400 nt fragments in PCR, the ARTIC nCoV-2019 V3 Primer set (IDT) and the Q5 Hot Start High-Fidelity 2X Master Mix (NEB,

Cat#M0494S) were used with modified manufacturer's instructions. The primers pools 1 and 2 were diluted to a final concentration of 10 µM and a reaction with each primer pool was set up as follows, 4 µl respective primer pool, 12.5 µl Q5 Hot Start High-Fidelity 2X Master Mix, 6 µl water, and 2.5 µl cDNA. The PCR was performed as follows, 98°C for 30 s, 30 cycles of 98°C for 15 s and 65°C for 5 min, and hold at 4°C. The PCR products were run on a 1% agarose gel to check for the presence of fragments at the correct size. The products from primer pool 1 and primer pool 2 for each variant were pooled, diluted and quantified by Qubit DNA HS kit (Thermo Fisher, Cat#Q32851). The sequencing amplicon pools were diluted to 0.2 ng/µl and tagmented with Nextera XT library prep kit (Illumina, Cat#FC-131-1024). Nextera libraries were dual-barcoded and sequenced on an Illumina NextSeq1000 instrument. The obtained sequenced reads were demultiplexed and mapped against the SARS-CoV-2 reference genome (NC_045512.2) with *BWA-MEM*[3]. Pileup files were generated from the mapped reads using *Samtools*[4]. The mapped reads and the pileup file were used to construct the consensus sequence with the *iVar* package[5] using default settings.

IFITM knock-down. At 24 h and 96 h post-seeding, 150,000 Calu-3 cells, seeded in 24-well plates, were transfected with 20 µM of non-targeting siRNA or IFITM1, IFITM2 or IFITM3 specific siRNAs using Lipofectamine RNAiMAX (Thermo Fisher, Cat#13778100) according to the manufacturer's instructions. 6 h after the second transfection, Calu-3 cells were infected with the various SARS-CoV-2 variants at an MOI of 0.05. 6 h post-infection, the inoculum was removed, the cells washed once with PBS and supplemented with fresh media. 48 h post infection, supernatants were harvested for qRT-PCR analysis.

qRT-PCR. *N* (nucleoprotein) transcript levels were determined in supernatants collected from SARS-CoV-2 infected Calu-3 cells 48 h post-infection as previously described[6]. Total RNA was isolated using the Viral RNA Mini Kit (Qiagen, Cat#52906) according to the manufacturer's instructions. RT-qPCR was performed as previously described[7] using TaqMan

Fast Virus 1-Step Master Mix (Thermo Fisher, Cat#4444436) and a OneStepPlus Real-Time PCR System (96-well format, fast mode). Primers were purchased from Biomers (Ulm, Germany) and dissolved in RNAse free water. Synthetic SARS-CoV-2-RNA (Twist Bioscience, Cat#102024) or RNA isolated from BetaCoV/France/IDF0372/2020 viral stocks quantified via this synthetic RNA (for low Ct samples) were used as a quantitative standard to obtain viral copy numbers. All reactions were run in duplicates. Forward primer (HKU-NF): 5'-TAA TCA GAC AAG GAA CTG ATT A-3'; Reverse primer (HKU-NR): 5'-CGA AGG TGT GAC TTC CAT G-3'; Probe (HKU-NP): 5'-FAM-GCA AAT TGT GCA ATT TGC GG-TAMRA-3'.

Inhibition by IFITM2 antibody and Remdesivir. 30,000 iATII cells were seeded as single cells in 96-well plates coated for 1 h at 37 °C with 0.16 mg/ml Matrigel (Corning, Cat#356238) diluted in DMEM/F12 (Thermo Fisher, Cat#11330032), 24 h later cells were treated with increasing concentrations (20, 40, 80 µg/ml) of α-IFITM2 (Cell Signaling, Cat#13530 S) or Remdesivir (Selleck Chemicals Cat#S8932) (10 µM). One h post-treatment, cells were infected with SARS-CoV-2 VOCs with an MOI of 0.5. 6 h post-infection, cells were washed once with PBS and supplemented with fresh medium. Thereafter, day 0 wash CTRL was harvested. 48 h post-infection supernatants were harvested for qRT-PCR analysis.

Western blot. To determine the expression of cellular and viral proteins, infected Calu-3 (MOI 0.2, 48 h post infection) or iATII (MOI 0.5, 48h post infection) cells or uninfected controls were washed in PBS and subsequently lysed in Western blot lysis buffer (150 mM NaCl, 50 mM HEPES, 5 mM EDTA, 0.1% NP40, 500 µM Na3VO4, 500 µM NaF, pH 7.5) supplemented with protease inhibitor cocktail (Roche, Cat#11697498001). After 5 min of incubation on ice, samples were centrifuged (4 °C, 20 min, 20,817 g) to remove cell debris. The supernatant was transferred to a fresh tube, the protein concentration was measured by Nanodrop and adjusted using Western blot lysis buffer. Western blotting was performed as

previously reported. Proteins were stained using primary antibodies against IFITM1 (α-IFITM1, Cell Signaling Cat#13126S, 1:1,000), IFITM2 (α-IFITM2 Cell Signaling Cat#13530 S, 1:1,000), IFITM3 (α-IFITM3 Cell Signaling Cat#59212S, 1:1,000), ACE2 (Rabbit polyclonal anti-ACE2 Abcam, Cat#ab166755, 1:1,000); rat anti-GAPDH (Biolegend Cat#607902, 1:1,000) and SARS CoV-2 N (anti-SARS-CoV-2 N Sino Biologicals Cat#40588-V08B, 1:1,000) and Infrared Dye labeled secondary antibodies (LI-COR IRDye). Membranes were scanned using an Odyssey infrared imager and band intensities were quantified in Image Studio Lite Version 5.0.

$TCID_{50}$ Endpoint titration. 10,000 Caco-2 cells were seeded in 96-well F-bottom plates. One day later, infectious supernatants were serially diluted and added to the cells. Cells were then incubated for 5 days and monitored for CPE. $TCID_{50}$/mL was calculated according to Reed and Muench.

Flow cytometric analysis. 60,000 iATII cells or Calu-3 cells were incubated for 1 h at 4°C with equal protein concentrations of control rabbit IgG (Diagenode, Cat#C15410206) or 1/200 dilution of rabbit anti-ACE2 (Abcam, Cat#ab166755) diluted in FACS buffer (PBS, 1% FBS), washed three times in PBS, stained for 30min with 1/400 dilution of goat anti-rabbit AF647 (Invitrogen, Cat#A27040), fixed in 1% PFA and analyzed using a BD FACS Canto II flow cytometer.

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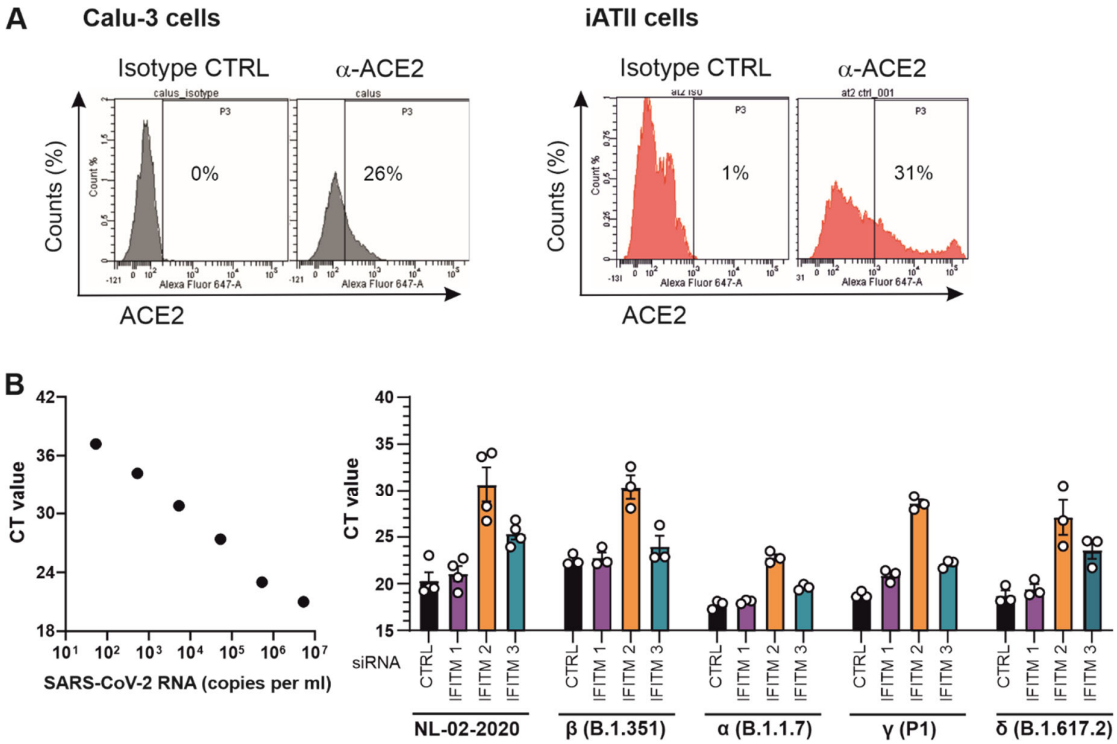

Supplementary Figure 1. ACE2 expression and impact of IFITM siRNA knock-down on SARS-CoV-2 replication in Calu-3 cells. (**A**) Flow cytometric analysis of surface ACE2 expression in Calu-3 and iATII cells. (**B**) Standard curve (left) and raw qRT-PCR CT values (right) obtained using supernatants of Calu-3 cells collected 2 days post-infection. Bars represent mean values (+/- SEM) of four to three independent experiments each measured in technical duplicates.