PNAS
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**Supplementary Information for**

# SARS-CoV-2 spike engagement of ACE2 primes S2' site cleavage and fusion initiation

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**This PDF file includes:**

Supplementary Methods
Figures S1 to S7

**Supplementary Methods**

Silver staining and mass spectrometry

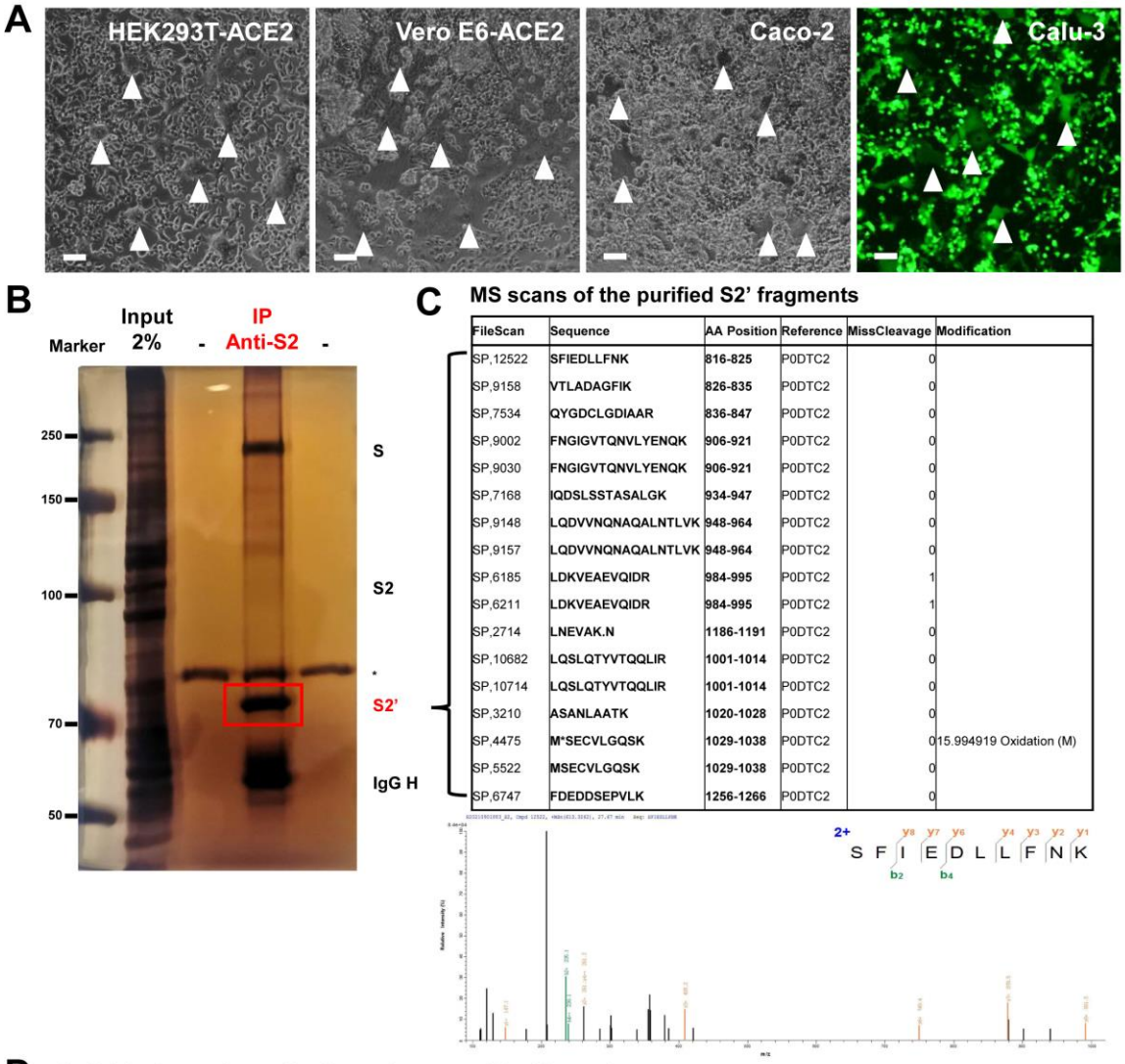
For purification of the S2' protein fragment, $1x10^6$ HEK293T cells transiently expressing S and ACE2 were co-cultured at 1:1 ratio in a T25 flasks. After incubation for 16 hours at $37^oC$, syncytia lysates were washed once in cold PBS before lysed in NP-40 lysis buffer supplemented with 1x EDTA-free protease inhibitor cocktail. Post-nuclear syncytia lysates were incubated with 1.5 µg rabbit anti-S2 antibody for 1 hour at $4^oC$, before immunoprecipitated with Protein A/G magnetic beads. Pull-down proteins were then washed, eluted and boiled in the 2 x Laemmli loading buffer. Total lysates (Input) and immunoprecipitates (IP) were separated by reducing SDS-PAGE on a 7.5% Tris-glycine gel before rinsed in fixation buffer (50% methanol 5% sodium acetate) for 20 min. The gel was sensitized in 0.02% sodium thiosulfate before reacted with 0.1% silver nitrate for 20 min. The gel was eventually developed in 2% sodium carbonate containing 0.045% formalin before rinsed and captured digitally; the S2' band was cut and subjected to in-gel digestion by 12.5 ng/µL trypsin in 25 mM $NH_4HCO_3$.

Mass spectrometry (MS) analysis was performed by Shanghai Applied Protein Technology Co. Ltd. Briefly, a quadrupole Orbitrap mass spectrometer (Q Exactive) mass spectrometer coupled to Easy nLC (ThermoFisher) was operated in the positive ion mode. MS survey scans (300-1800 m/z) were obtained using the higher collision-energy dissociation (HCD) method in a data-dependent acquisition mode. Normalized collision energy was 30 eV and the underfill ratio was set as 0.1%. The instrument was run with peptide recognition mode enabled. MS spectra were searched using MASCOT v2.2 (Matrix Science) against SARS-CoV-2 spike using the Uniprot reference P0DTC2; decoy hits were used to control the false discovery rate.

Immunofluorescence and confocal microscopy

HEK293T cells were seeded overnight onto sterilized poly-D-lysine (100 ug/mL) (Sigma) treated 10 mm coverslips in 24-well plates at a density of $1.5 \times 10^5$ cells per well. After transfection with spike mutants, cells were washed with cold PBS once before fixed with 4% (w/v) paraformaldehyde (PFA) for 20 min. Fixed cells were blocked in 1% (w/v) goat serum in PBS without membrane permeabilization. Anti-SARS-CoV-2 S1 monoclonal antibody in 0.1% (w/v) goat serum in PBS was incubated for 1 hour at room temperature. Coverslips were then washed 3 times with PBS before incubation with goat anti-mouse AlexaFluor555 secondary antibodies (ThermoFisher) for 1 hour at room temperature; secondary-antibody-only controls were performed. Coverslips were washed 3 times before being mounted in neutral mounting medium (Xinyu). Fluorescent images covering various areas on the coverslips were captured at 14-bit depth in monochrome using a 60x oil immersion objective on the Olympus FV-1200 inverted

confocal microscope and subsequently processed using imageJ software (NIH) with scale bars labeled.




**D** **Individual mutations of spike variants used in this study**

| Variants | Pango lineage | Individual point mutations |
|---|---|---|
| Alpha | B.1.1.7 | Δ69-70, Δ144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H |
| Beta | B.1.351 | L18F, D80A, D215G, Δ242-244, R246I, K417N, E484K, N501Y, D614G, A701V |
| Delta | B.1.617.2 | T19R, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N |

**Figure S1, Spike-mediated syncytia formation requires species specific recognition of ACE2.** (**A**) Brightfield images showing syncytia formation 16 hours after HEK293T cells expressing S-WT mixed with HEK293T-ACE2, Vero E6-ACE2 and Caco-2 cells; fluorescent image of HEK293T-S-WT / ZsGreen cells fused with Calu-3 cells. Scale bars are indicative of 50 µm, images are representative of three independent experiments. (**B**) Silver staining image of full-length S, S2 and S2' immunoprecipitated from HEK293T syncytia lysates. Extracted S2' fragment is indicated in red and subjected to mass spectrometry analysis; Image is a representative of two independent experiments. (**C**) Summary list and a representative MS spectrum of S2' peptides detected from MS scans, data is a representative of two independent MS experiments. (**D**) List of

individual point mutations of the SARS-CoV-2 spike Alpha, Beta and Delta variants used in this study.

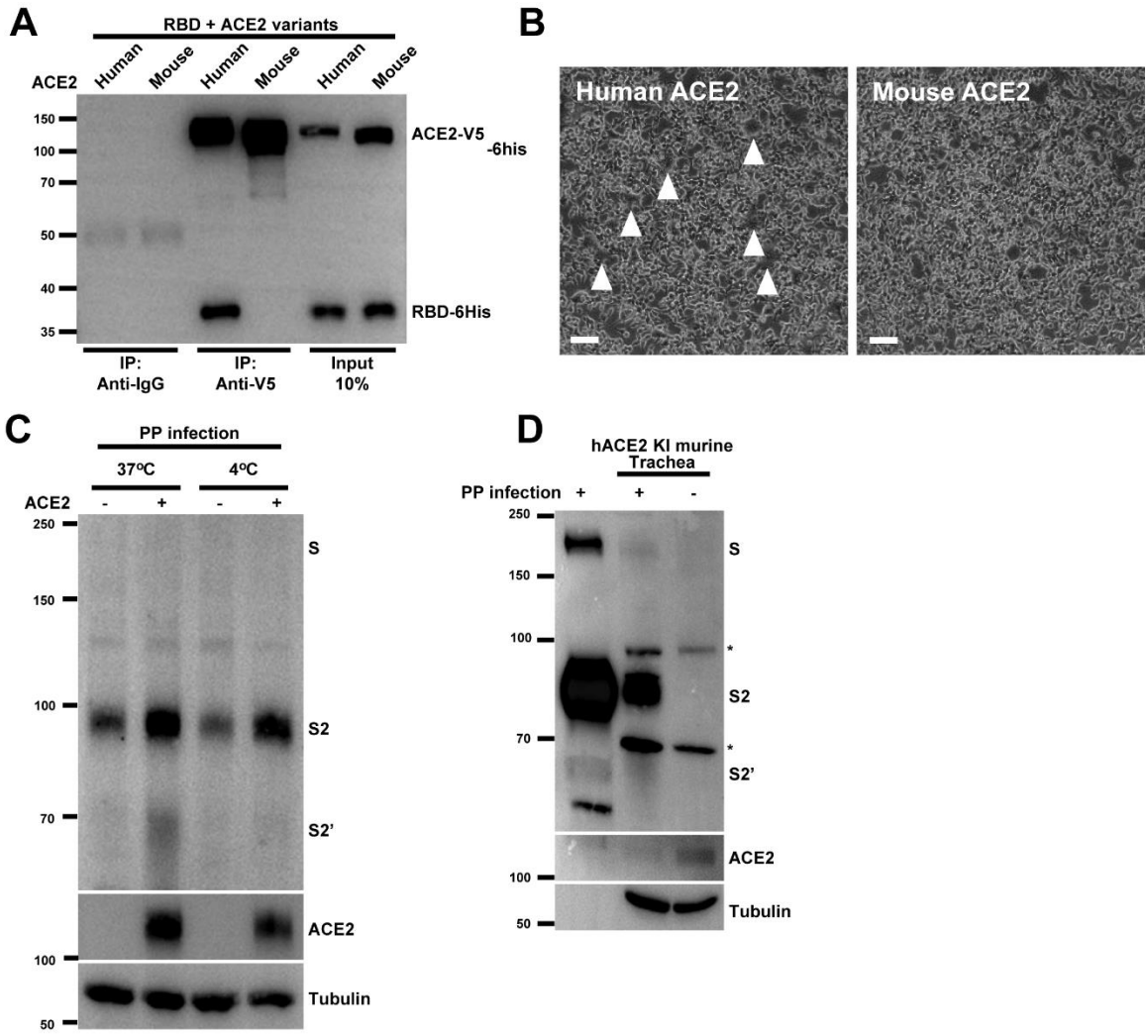



**Figure S2, Spike recognition of functional ACE2 is required for S2' cleavage, syncytia formation and PP infection.** (**A**) Co-immunoprecipitation and input controls of RBD-6his and ACE2 after anti-V5 or anti-IgG pulldown from cell lysates mixed between HEK293T cells expressing human or mouse ACE2-V5-6his and HEK293T cells expressing RBD-6his. RBD and ACE2 variants were detected using anti-his tag antibody. Blots are representative of two independent experiments. (**B**) Bright field images showing syncytia formation 16 hours after HEK293T cells expressing S-WT, co-cultured with HEK293T cells expressing human or mouse ACE2-V5-6his variants; multinuclear syncytia are denoted with white arrow heads, scale bars are indicative of 50 µm. (**C**) Immunoblots showing full-length S, S2, S2', ACE2 and tubulin from HEK293T cells with or without ACE2 expression, these cells were infected with SARS-CoV-2 PPs for 6 hours post-infection (hpi) at 37°C or 4°C as indicated. Blots are representative of two individual repeats. (**D**) Immunoblots showing full-length S, S2, S2', ACE2 and tubulin collected from hACE2 KI murine trachea tissue lysates infected with SARS-CoV-2 PPs for 12 hours. *denotes non-specific bands, blots are representative of three individual repeats.

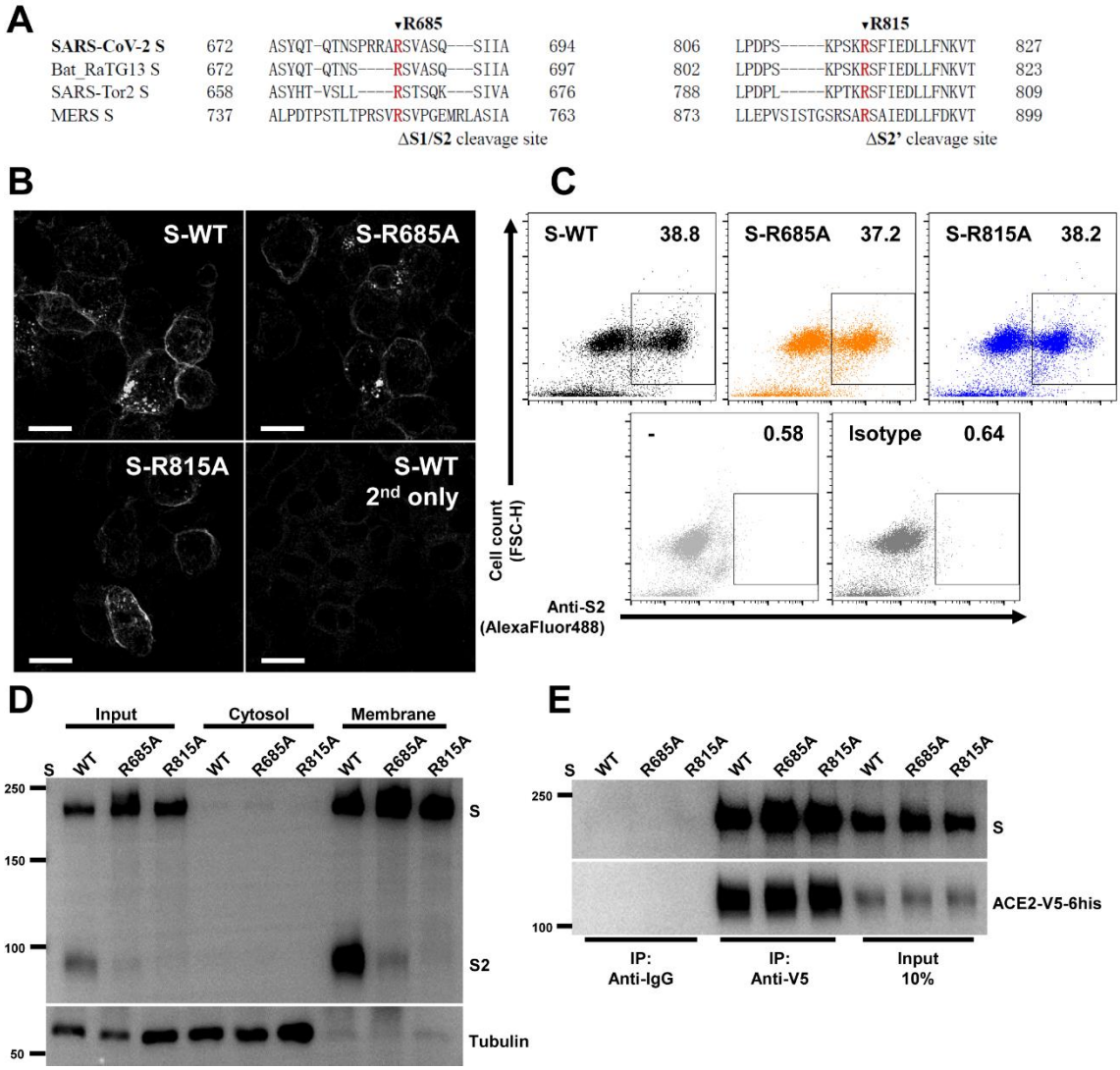



**Figure S3, Spike S2 and S2' mutants are localized on the plasma membrane after expression.** (**A**) Amino acid sequence alignment of coronaviruses S proteins at the SARS-CoV-2 R685 and R815 loci. Conserved S1/S2 cleavage site arginine residue is highlighted in bold and red; putative cleavage sites are indicated by dark arrow heads. (**B**) Representatives of confocal projections from cell surface immunostaining of S1 in HEK293T cells expressing S-WT, S-R685A and S-R815A mutants. Scale bars are indicative of 20 μm and images are representative of two independent experiments. (**C**) Representative FACS plots showing gating strategy and percentage of positive fluorescent signals (AlexaFluor488) detected from membrane staining of S2 on HEK293T cells (FSC-H) transiently expressing S-WT, S-R685A and S-R815A. **-** denotes control HEK293T cells without S expression. Data are representative of two individual experiments. (**D**) Immunoblots showing the membrane association of S and S2 in HEK293T cells expressing S-WT, S-R685A and S-R815A. Blots are representative of two independent experiments. (**E**) Co-immunoprecipitation and input controls of full-length S after anti-V5 or anti-

IgG pulldown from cell lysates mixed between HEK293T cells expressing human or mouse ACE2-V5-6his and HEK293T cells expressing S-WT, S-R685A or S-R815A mutants. S was detected using anti-S2 (upper panel) antibody, or ACE2 protein detected with anti-ACE2 (lower panel) antibody, blots are representative of two independent experiments.

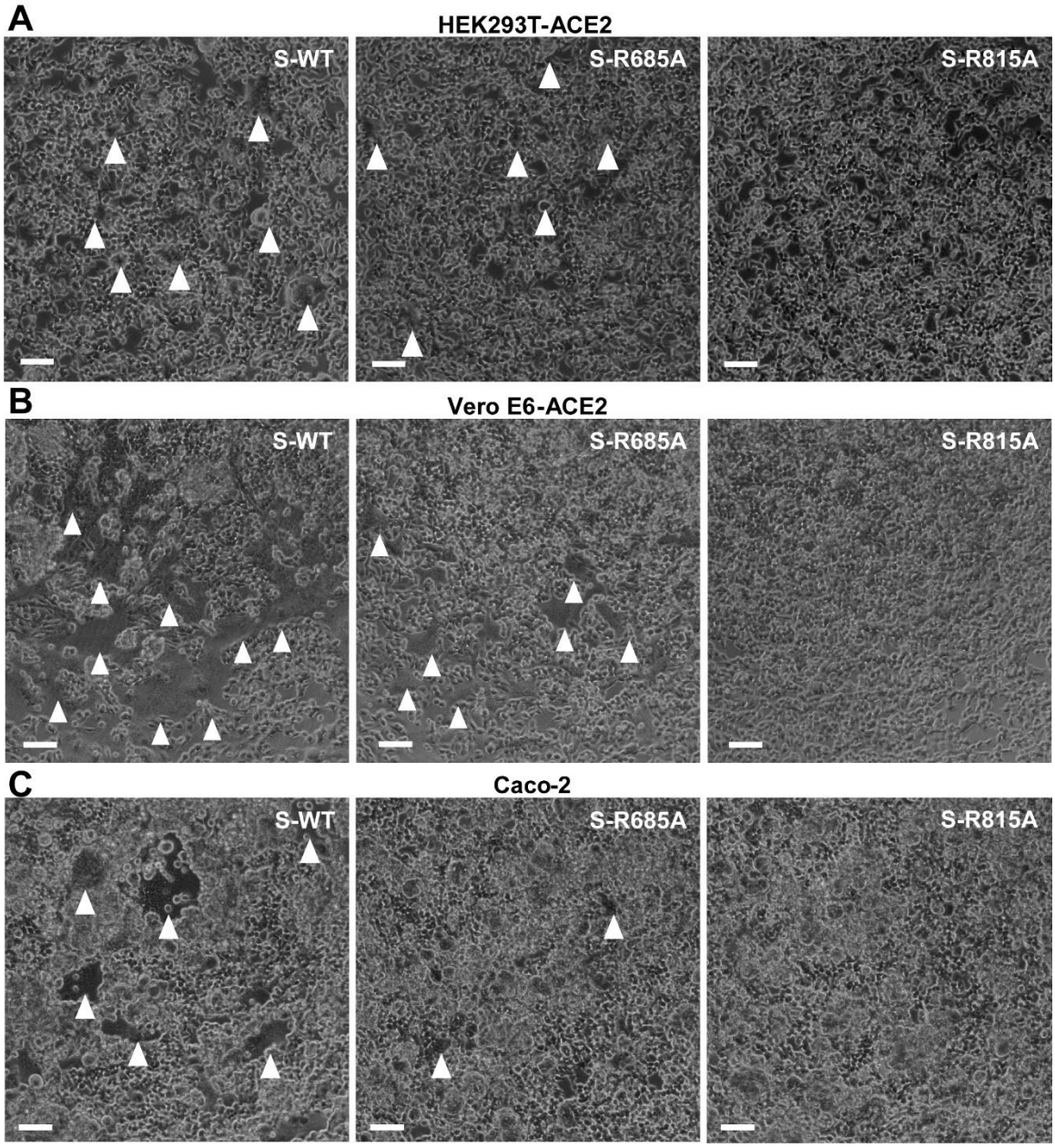



**Figure S4, Spike arginine 815 is essential for syncytia formation in various cell lines expressing human ACE2.** Representative bright field images showing syncytia formation 16 hours after HEK293T cells transiently transfected with S-WT, S-R685A and S-R815A mixed with HEK293T-ACE2 (**A**), Vero E6-ACE2 (**B**), and Caco-2 (**C**) cells. Multinuclear syncytia are denoted with white arrow heads, scale bars are indicative of 50 µm, images are representative of at least two independent experiments.

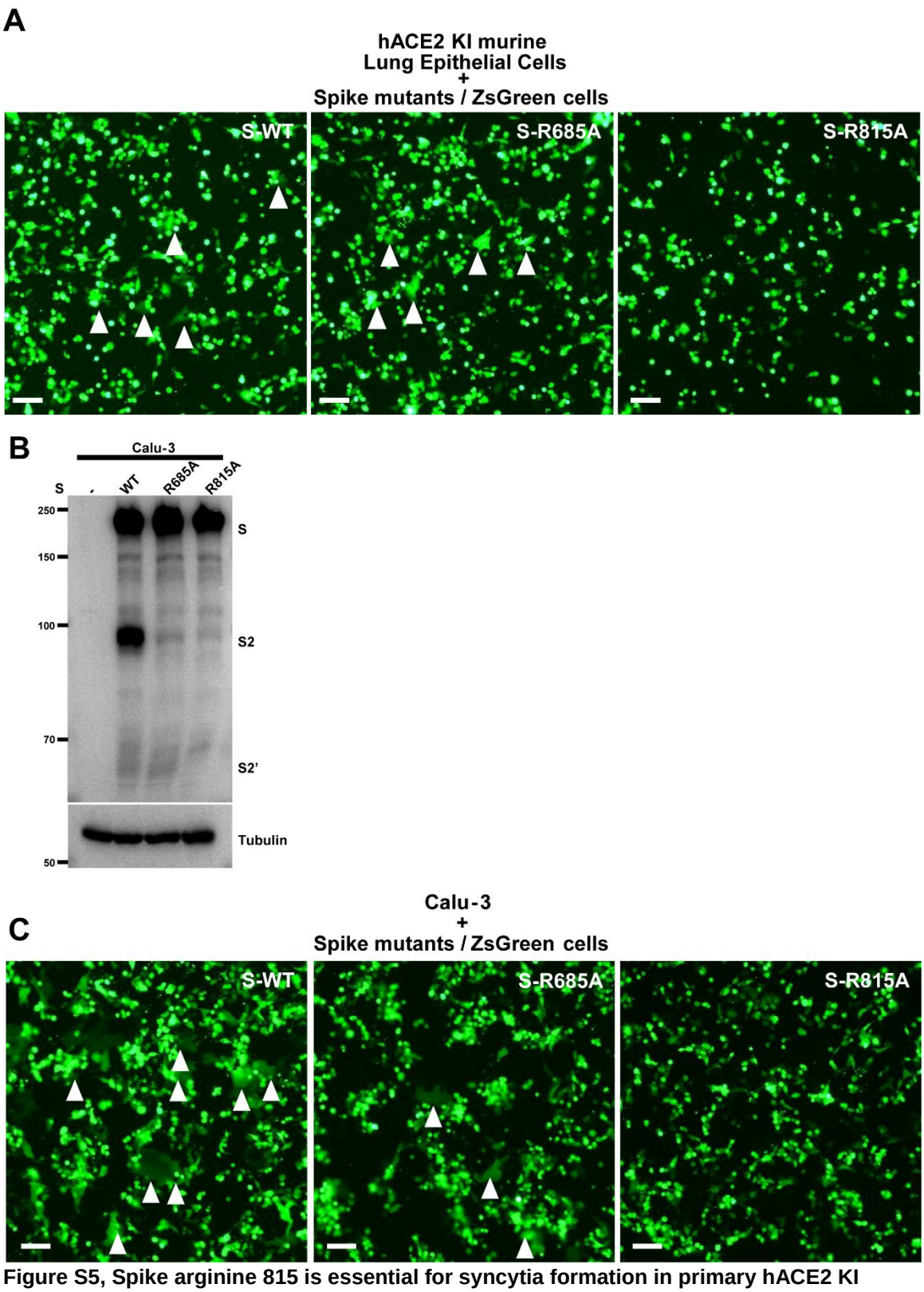



**Figure S5, Spike arginine 815 is essential for syncytia formation in primary hACE2 KI murine cells and human Calu-3 cells.** (**A**) Fluorescent images of ZsGreen showing syncytia formation in hACE2 KI murine lung epithelial cells co-cultured with HEK293T cells expressing S-

WT, S-R685A or S-R815A co-transfected with ZsGreen plasmid. (**B**) Immunoblots showing the full-length S, S2, S2' from HEK293T cells expressing S-WT, S-R685A or S-R815A co-cultured with human lung Calu-3 cells. Blots are representative of three individual repeats. (**C**) Fluorescent images of ZsGreen for the detection of Calu-3 syncytia formation with HEK293T cells expressing S-WT, S-R685A or S-R815A co-transfected with ZsGreen plasmid. Multinuclear syncytia are denoted with white arrow heads, scale bars are representative of 50 μm, images are representative of at least two independent experiments.

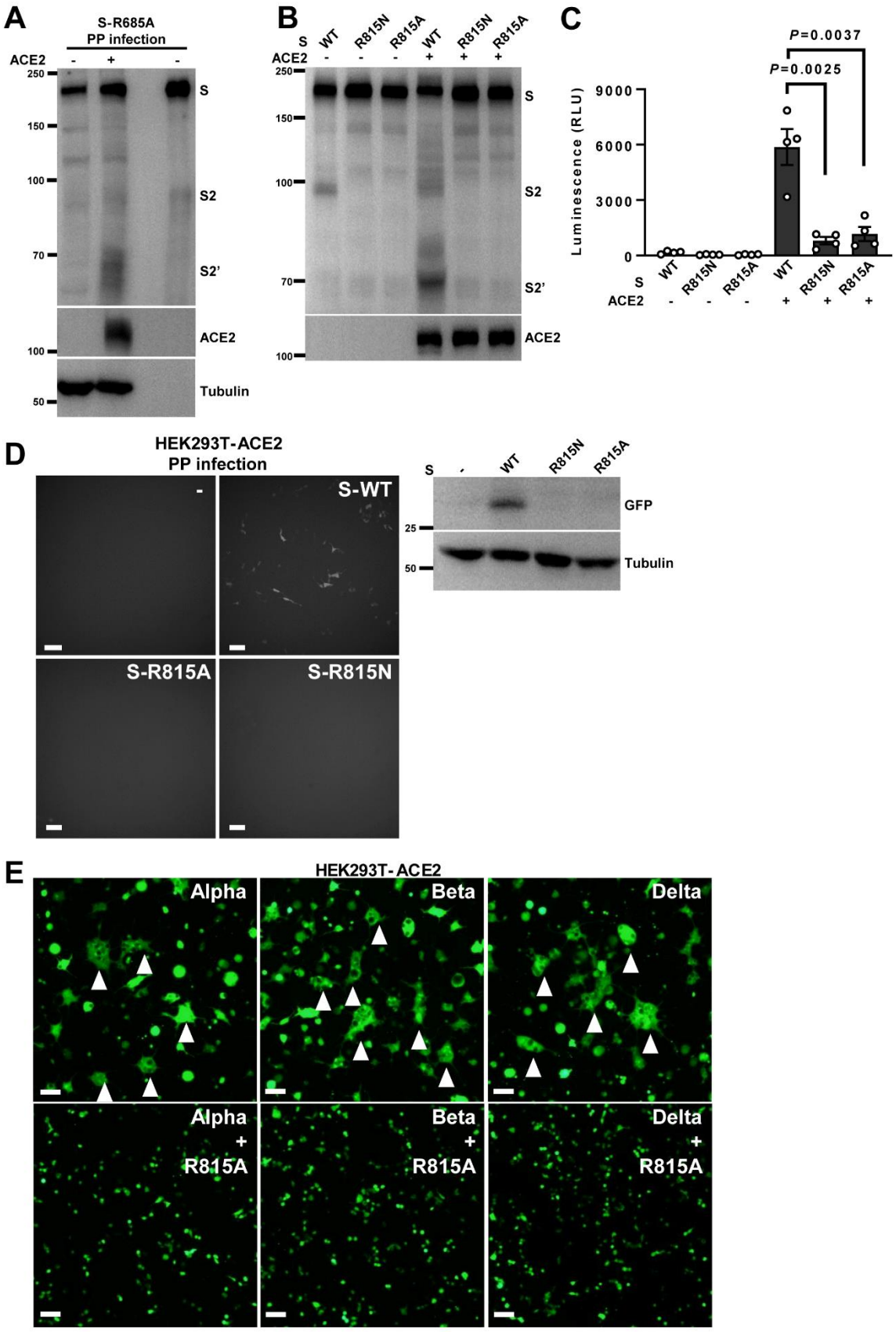

A
S-R685A
PP infection
ACE2
S
S2
S2'
ACE2
Tubulin
B
S
ACE2
WT
R815N
R815A
S
S2
S2'
ACE2
C
P=0.0037
P=0.0025
Luminescence (RLU)
D
HEK293T-ACE2
PP infection
S-WT
S-R815A
S-R815N
GFP
Tubulin
E
HEK293T-ACE2
Alpha
Beta
Delta
Alpha + R815A
Beta + R815A
Delta + R815A

**Figure S6, Spike arginine 815 is essential for syncytia formation and viral infection. (A)** Immunoblots showing the full-length S, S2, S2', ACE2 and tubulin detected from HEK293T cells infected for 6 hours by PP prepared using the spike R685A mutant. Blots are representative of three individual repeats. (**B**) Immunoblots showing the full-length S, S2, S2' and ACE2 from HEK293T cells transiently expressing S-WT, S-R815N or S-R815A co-cultured with HEK293T-ACE2 cells for 16 hours. Blots are representative of four independent experiments. (**C**) Luciferase activity (RLU) measured from HEK293T cells co-expressing S-WT, S-R815N or S-R815A and Cre, co-cultured with *Stop-Luc*-expressing cells transfected with ACE2; cells expressing *Stop-Luc* without ACE2 transfection were used as the negative control. Data shown are mean ± SEM from four independent experiments. *P* values were obtained by one-way ANOVA with Sidak's *post hoc* test and are indicated on figures. (**D**) Fluorescent images showing eGFP-positive HEK293T-ACE2 cells infected with PPs prepared using S-WT, S-R815N, or S-R815A mutants, scale bars are representative of 50 μm (left panel). PPs infected HEK293T-ACE2 cell lysates were collected for immunoblots for eGFP expression (right panel). Images and blots are representative of three independent experiments. (**E**) Fluorescent images showing syncytia formation from HEK293T cells co-expressing a ZsGreen reporter and spike Alpha, Beta and Delta variants with or without the R815A mutation, co-cultured with ACE2-expressing cells for 16 hours. Multinuclear syncytia are denoted with white arrow heads, scale bars are indicative of 50 μm, images are representative of at least three independent experiments.

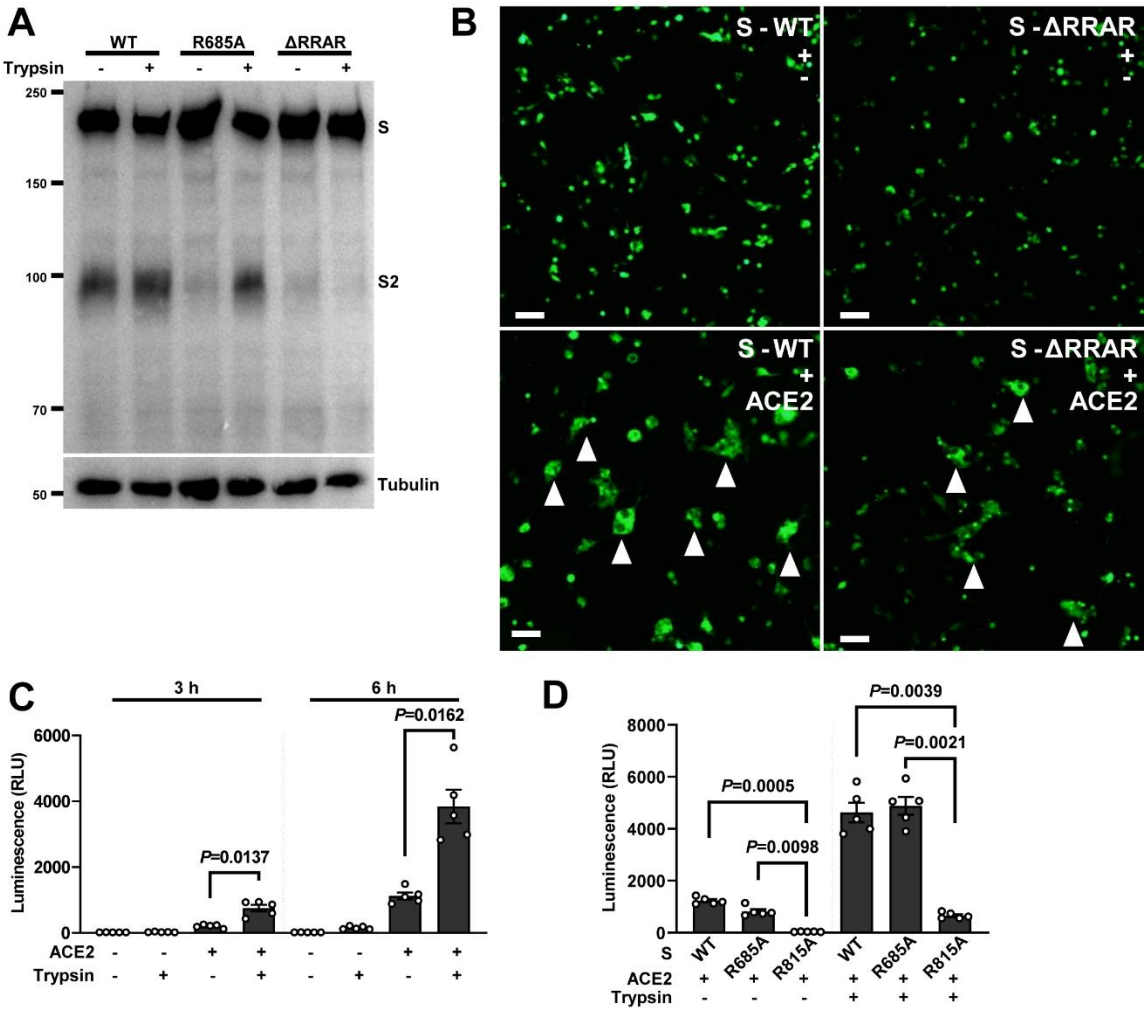



**Figure S7, Trypsin enhances spike-driven cell-cell fusion in the presence of ACE2.** (**A**) Immunoblots showing trypsin-cleaved the full-length S and S2 collected from HEK293T cells transiently expressing S-WT, S-R685A and S-ΔRRAR mutants, treated without or with 5 μg/mL trypsin for 1 hour. Blots are representative of two individual experiments. (**B**) Fluorescent images showing syncytia formation from HEK293T cells co-expressing a ZsGreen reporter and spike with or without ΔRRAR mutation, incubated with ACE2-expressing cells for 16 hours. Multinuclear syncytia are denoted with white arrow heads, scale bars are indicative of 50 μm, images are representative of at least three independent experiments. (**C**) Luciferase activity (RLU) detected from cell-cell fusion lysates, treated without or with 10 μg/mL trypsin for 3 and 6 hours using the system described in Figure 1G. Data are shown as individual points with mean ± SEM from five independent experiments. (**D**) Luciferase activity (RLU) detected from S-WT, S-R685A or S-R815A spike variants induced cell-cell fusion lysates in the absence or presence of 10 μg/mL trypsin for 6 hours, employing the system described in Figure 1G. Data are shown as individual points with mean ± SEM from five independent experiments. *P* values were obtained by one-way ANOVA with Sidak's *post hoc* test and are indicated on figures.