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SUPPLEMENTAL MATERIAL

S1 Fig. Gating strategy for spike expression and characterization of cell lines used

(A) Gating strategy to measure spike expression at the surface of transfected HEK 293Tn cells. Spike was detected in singlet viable cells after surface labeling with the human mAb 129. The top row shows cells transfected with the empty control vector, while the bottom row shows cells transfected with the WT wuhan spike vector. The percentage of spike+ cells is reported in red.

(B) Expression of the receptors ACE2 and TMPRSS2 in the cell lines used in the study. Cells were surface-labeled for ACE2, permeabilized, and then labelled intracellularly for TMPRSS2. The top row shows labeling with the isotype-AF647 control, while the bottom row shows labeling with the ACE2-AF647 antibody.

S2 Fig. Total levels of spike surface expression measured by S2 subunit labeling

(A-D) Spike-transfected cells were labeled with S2-specific monoclonal antibodies, GTX-S2 and mAb10, to evaluate total spike expression at the cell surface. The percentage of S2+ cells (A, B) and mean fluorescence intensity (MFI) of S2 labeling in live cells (C, D) normalized to WT is reported. Means +/- SD are shown for n=3 independent experiments.

(E-G) The ratio of spike surface expression measured in the G614 backbone to that in the D614 backbone is reported, using either the percentage (E, F) or the MFI of spike+ cells (G, H) for measurement. Statistics evaluating whether each ratio is different from 1 were measured by one sample t-tests. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

S3 Fig. The D614 mutation promotes S1 subunit retention in producer cells

The different forms of spike present into HEK 293Tn cells transfected for GFP-pseudotyped viruses (PV) production were analyzed by Western blotting on cell lyzates.

(A) Representative Western blot showing the S1 (green) and S2 (red) spike subunits, and the actin protein (red) used for normalization at the bottom of the gel. The expected size of molecular weight markers in indicated in kD on the left. The uncleaved spike precursor S0 is visible in yellow due to the superposition of green and red fluorescence.

(B) Quantitation of total spike expressed (S0 + S2), reported to actin content, and normalized to WT. The fluorescence intensity of each band was quantified with the Image Studio Lite software.

(C) Quantitation of total cleaved spike, measured by the S2/S0 ratio, and normalized to WT.

(D) Quantitation of S1 subunit retention in producer cells, measured by the S1/S2 ratio, and normalized to WT.

(B-D) Statistics were measured by one-way ANOVA, with the Holm-Sidak's correction for multiple comparisons. Mutants in the D614 backbone were compared to the WT D614 spike, while mutants in the G614 backbone were compared to the WT G614 spike.

(E-G) The ratio of total spike expressed (E), total cleaved spike (F), and S1 retention (G) measured for spikes in the G614 backbone to that in the D614 backbone is reported. Statistics evaluating whether each ratio is different from 1 were measured by one sample t-tests. (B-G) Means +/- SD are shown for n=3 independent experiments. * $p<0.05$; ** $p<0.01$.

S4 Fig. Gating strategy for the analysis of GFP-pseudovirus infectivity

(A) Gating strategy to measure the infectivity pseudotyped virus (PV) expressing the GFP reporter gene in HEK-ACE2 cells. GFP expression was evaluated at day 2 post-infection, by measuring the percentage of GFP+ cells in the singlet viable cells. A negative control corresponding to infection with 0.25 µg p24 equivalent of Empty PV is reported.

(B) Infectivity of PV pseudotyped with the different spikes studied. A representative infection experiment in HEK-ACE2-TMPRSS2 cells inoculated with 0.25 µg of p24 equivalent is shown. PV infectivity is measured by the percentage of GFP+ cells reported in the top right corner of each plot.

S5 Fig. Dose response analysis of pseudovirus infectivity and target cell viability

(A) The infectivity of pseudotyped viruses (PV) carrying the different spikes was analyzed in HEK-ACE2 cells induced or not for TMPRSS2 expression. Infections were carried out with serial 2x dilutions of PV ranging from 0.5 to 0.625 µg of p24 equivalent. The percentage of GFP+ cells obtained in HEK-ACE2 cells (left) and HEK-ACE2-TMPRSS2 cells (right) is reported.

(B) The percentage of viable cells, measured by exclusion of the Aqua viability dye in the singlet cell gate, is reported for each infection condition.

(A,B) PV carrying a spike with the D614G mutation are labeled in red. Means +/- SD are shown for n≥3 independent experiments, except for the T716I mutant at the highest dose where n=2.

S6 Fig. Effect of protease inhibitors on pseudovirus infectivity

The TMPRSS2 inhibitor Camostat and the cathepsin inhibitor E64D evaluated for their capacity to block pseudovtyped virus (PV) infectivity, alone or in combination. Infectivity was measured in relative luciferase

units (RLU) for n=3 independent experiments, with 2 technical replicates per experiment. Conditions included untreated samples (grey) and samples treated with Camostat (blue), E64D (green), or the combination of the two inhibitors (red). Infectivity was analyzed in the following cell lines: HEK-ACE2 (A), HEK-ACE2-TMPRSS2 (B), Calu-3 (C), U2OS-ACE2 (D), and U2OS-ACE2-TMPRSS2 (E). The names of PV carrying the D614G mutation are reported in red.

S7 Fig. Dose response to protease inhibitors

(A, B) The TMPRSS2 inhibitor Camostat and the cathepsin inhibitor E64D were tested at 3 different doses (50 µM, 100 µM, and 200 µM) for their effects on PV infectivity in U2OS-ACE2 cells (A) and U2OS-ACE2-TMPRSS2 cells (B). Infectivity was measured in relative luciferase units (RLU) for n=1 experiment with 2 technical replicates for each point. Conditions included untreated samples (grey bars) and samples treated with E64D (green bars) or Camostat (blue bars).

(C-E) Viability in cells treated or not with protease inhibitors was evaluated by the proportion of cells that did not stain for the Aqua Live/Dead dye at day 1 (D1) and day 2 (D2) post-treatment. (C) Gating strategy to measure the percentage of viable cells in U2OS-ACE2 cells at D1. (D, E) Viability change in U2OS-ACE2 (D) and U2OS-ACE2-TMPRSS2 (E) cells treated with E64D (green lines, top graph) and Camostat (blue lines, bottom graph) between D1 and D2.

S1 Table. Primers used for spike mutagenesis and sequencing

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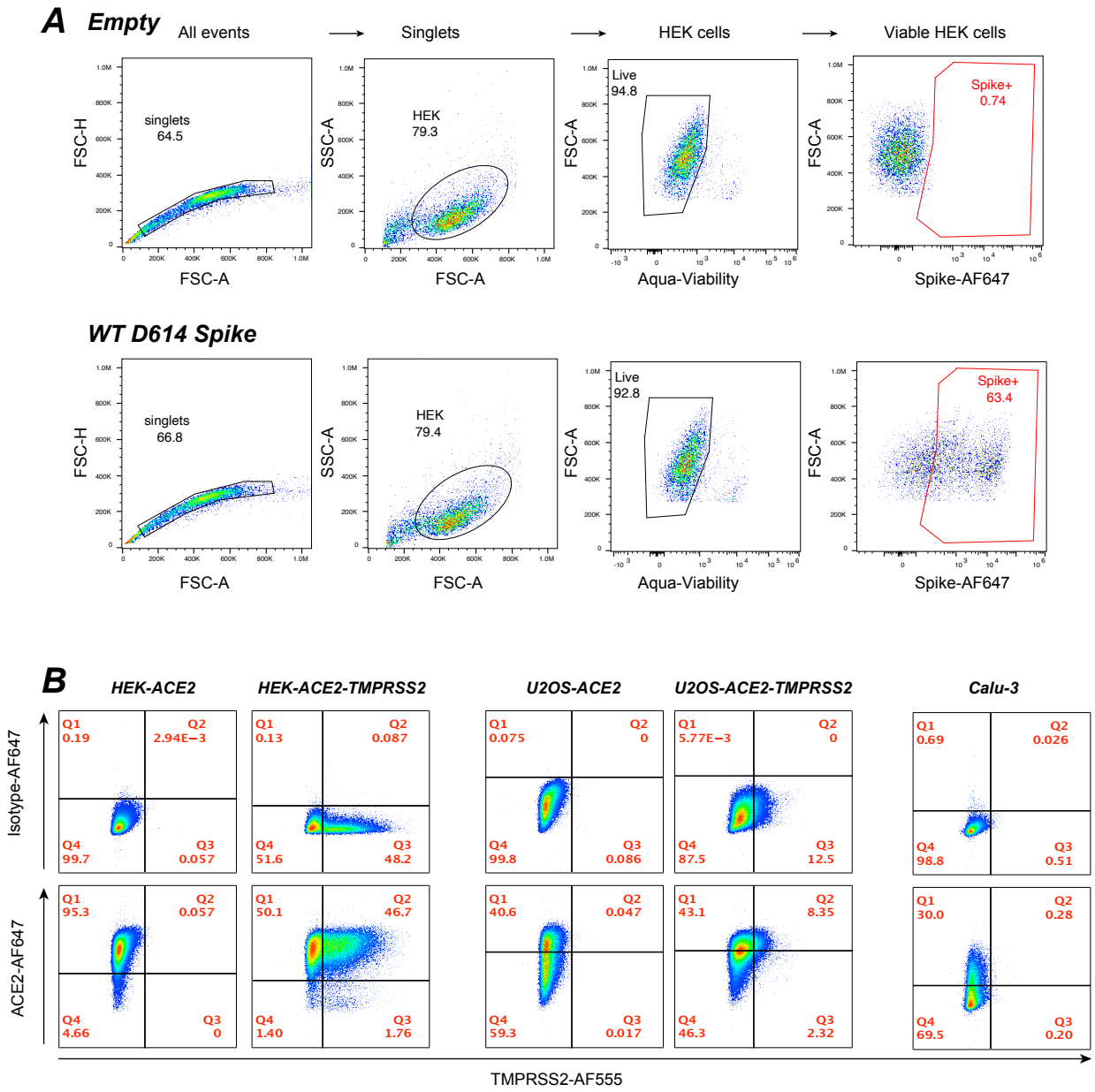
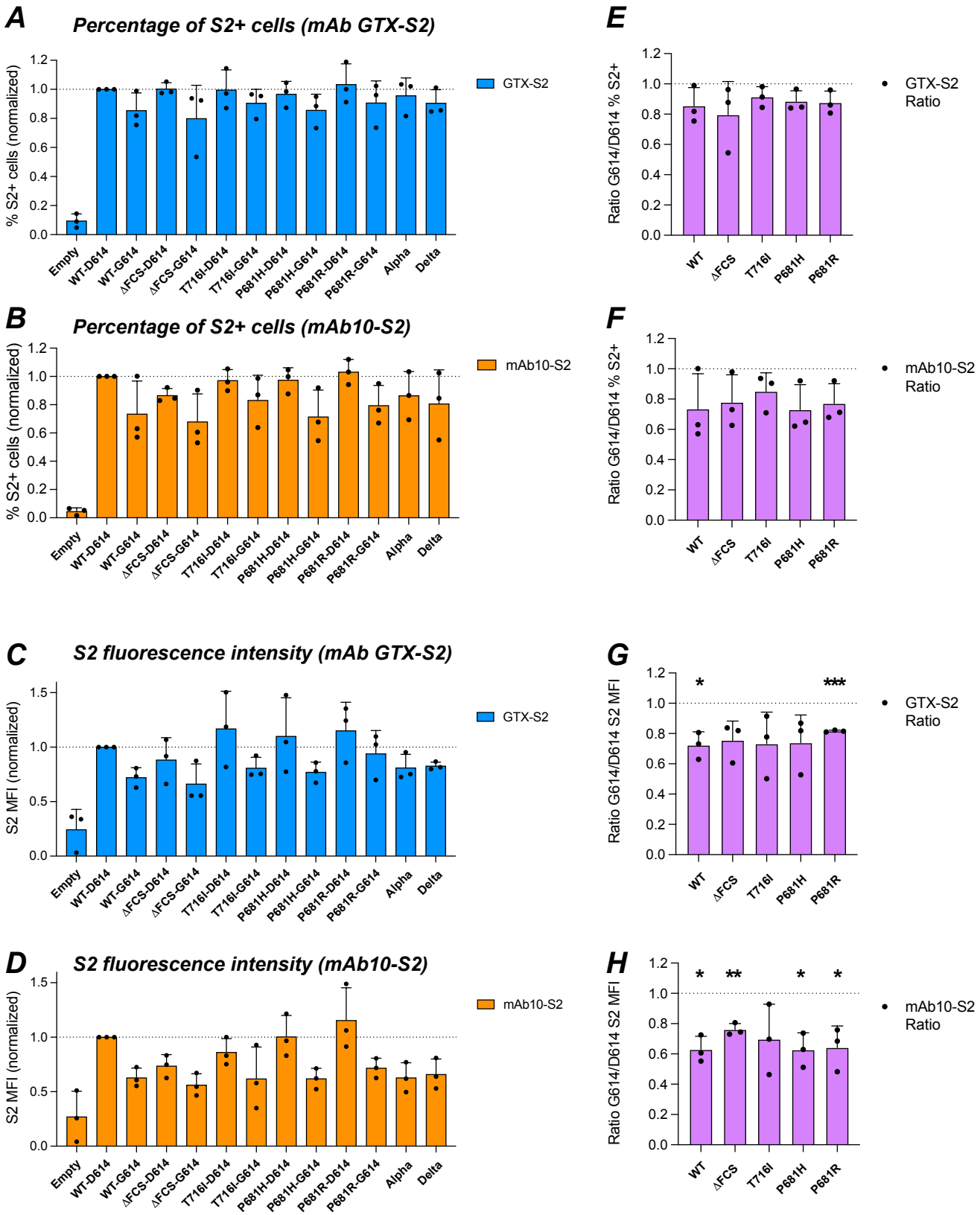
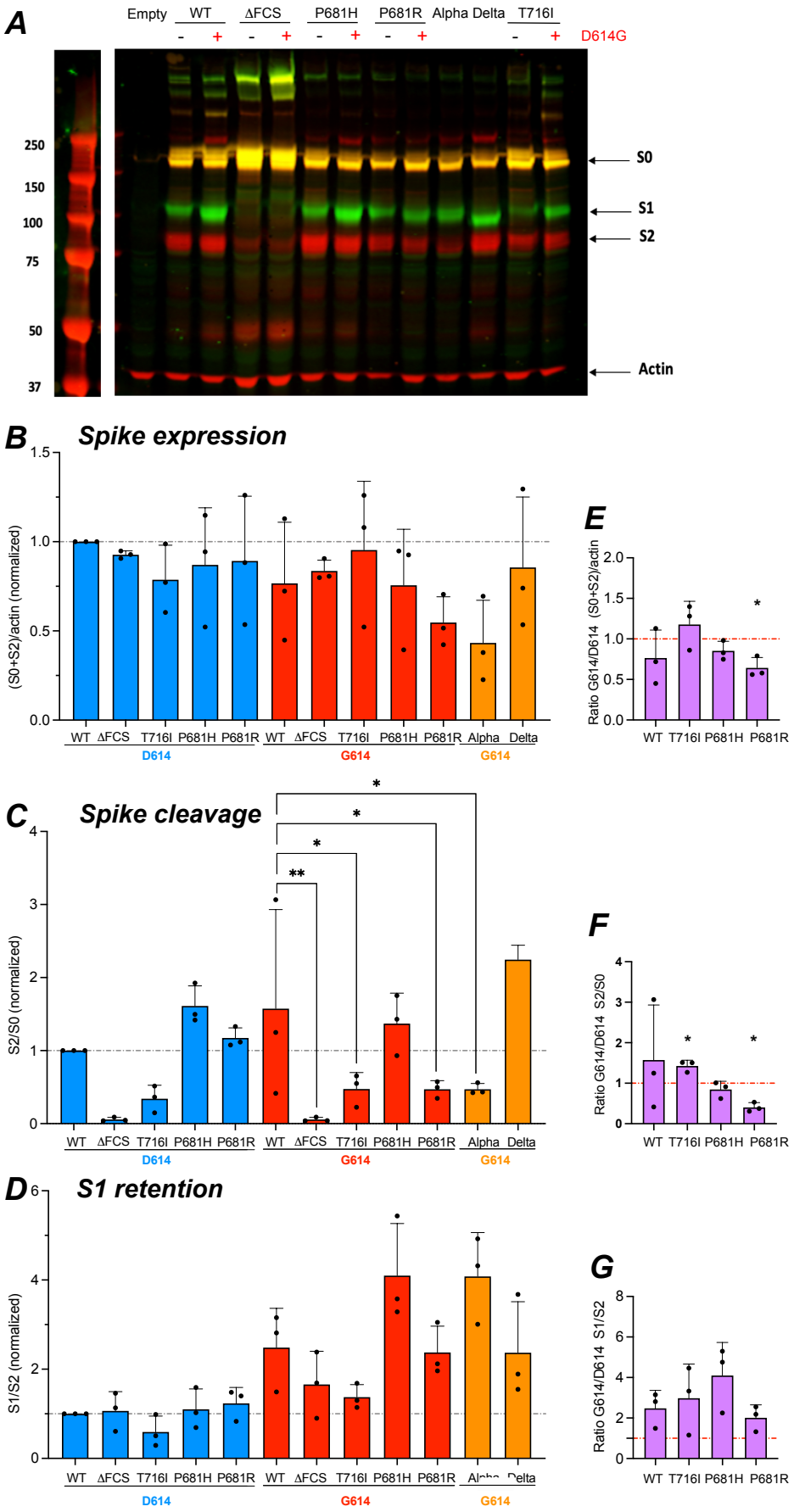


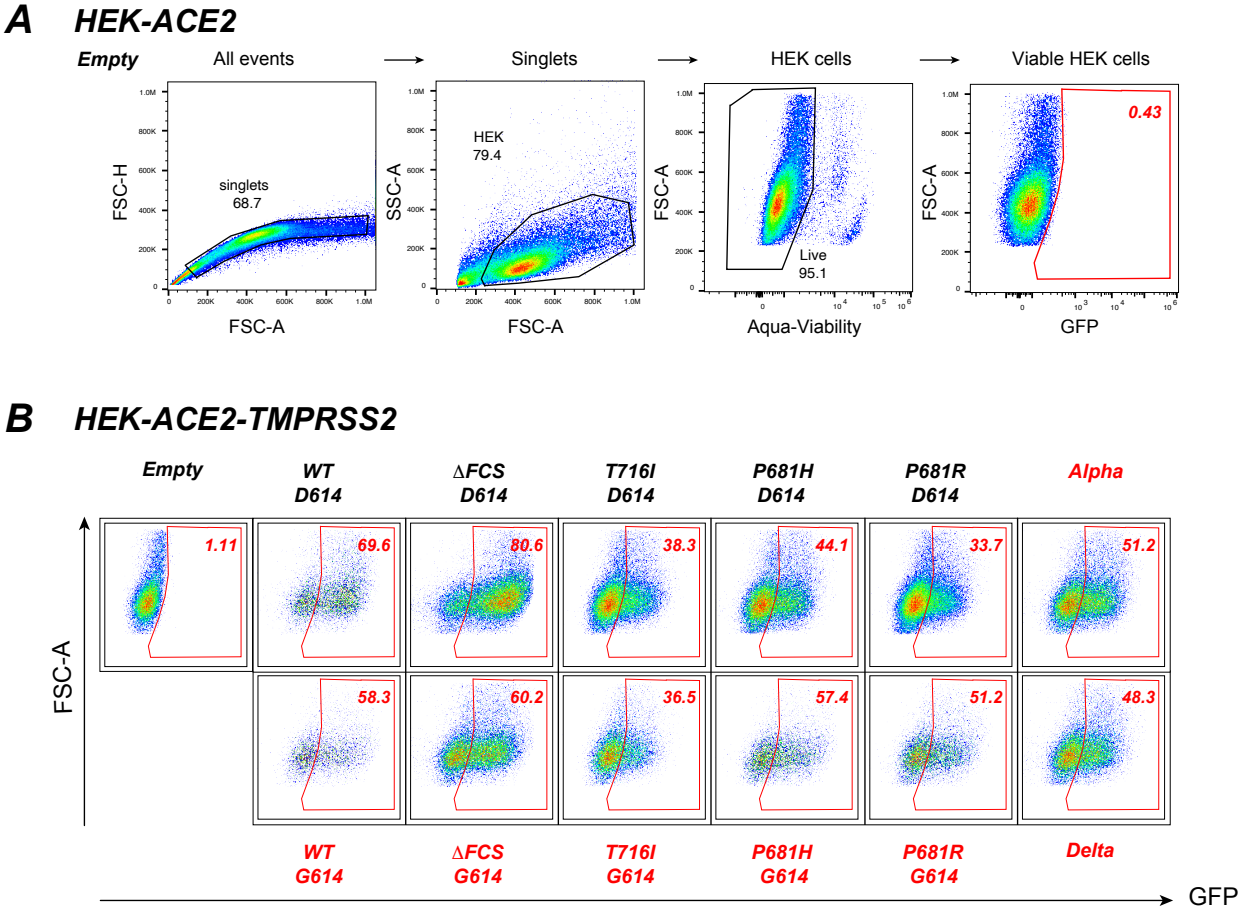
Supplementary Figure 1 Gellenoncourt et al.



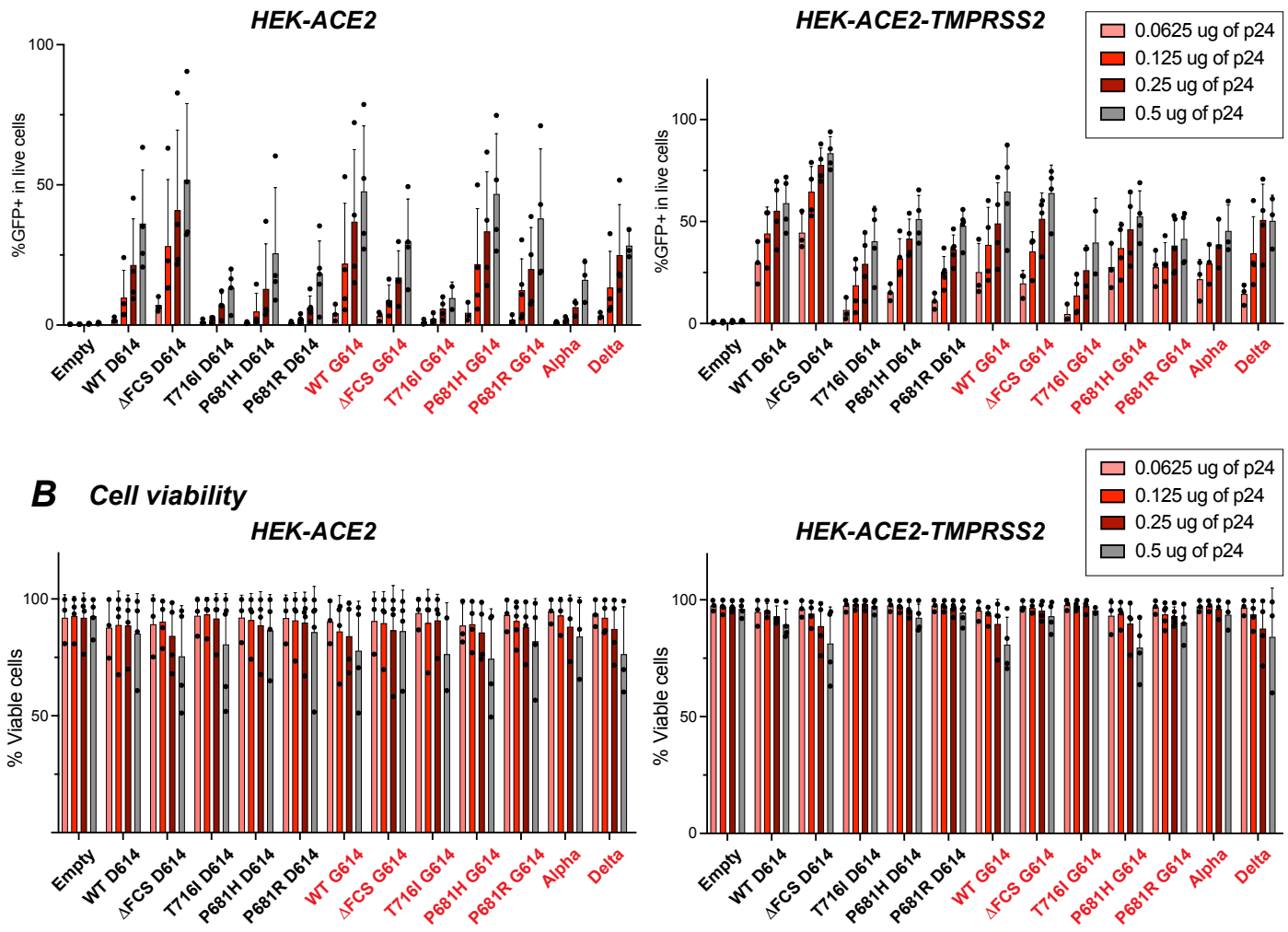
Supplementary Figure 2 Gellenoncourt et al.



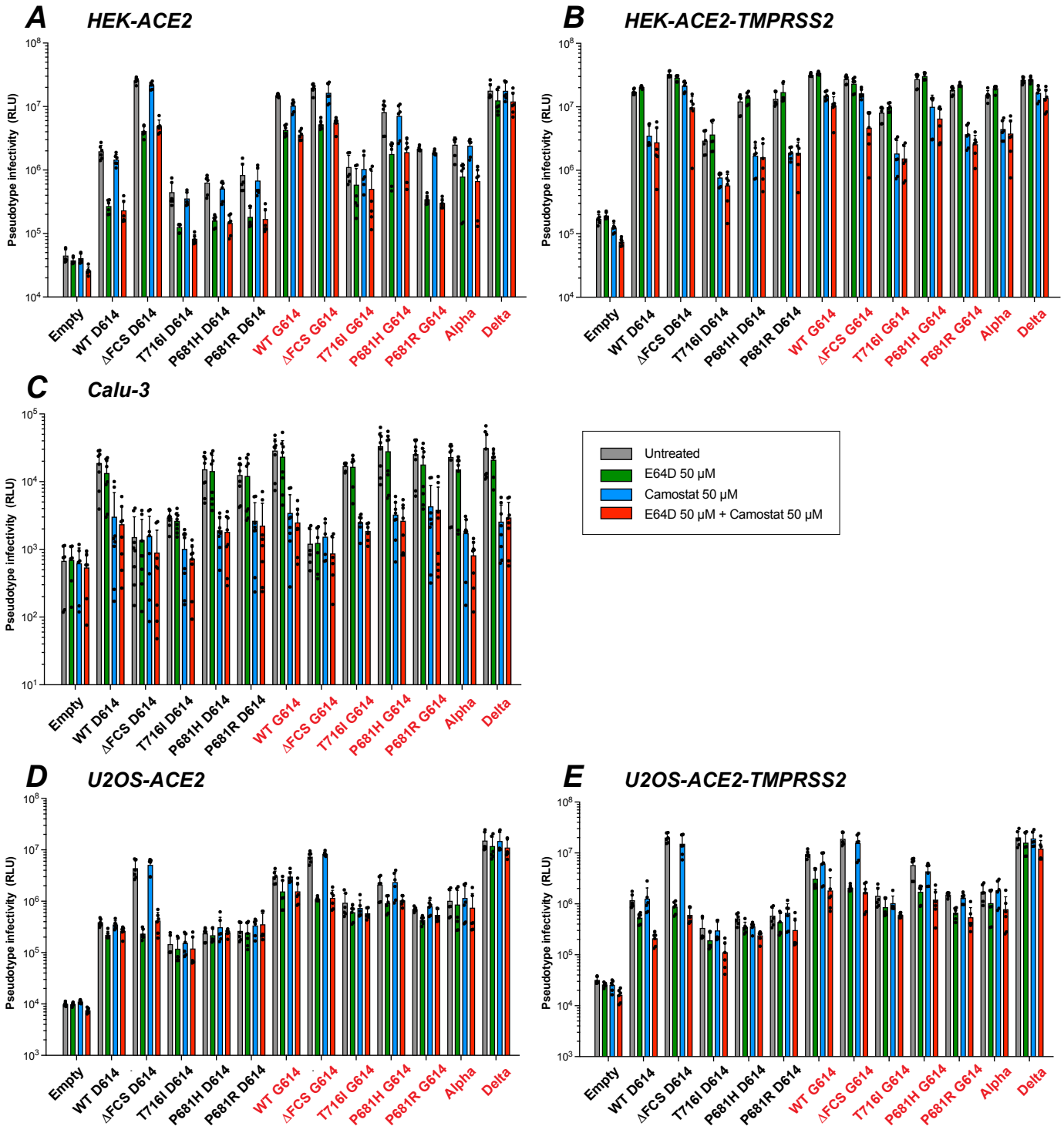
Supplementary Figure 3 *Gellenoncourt et al.*



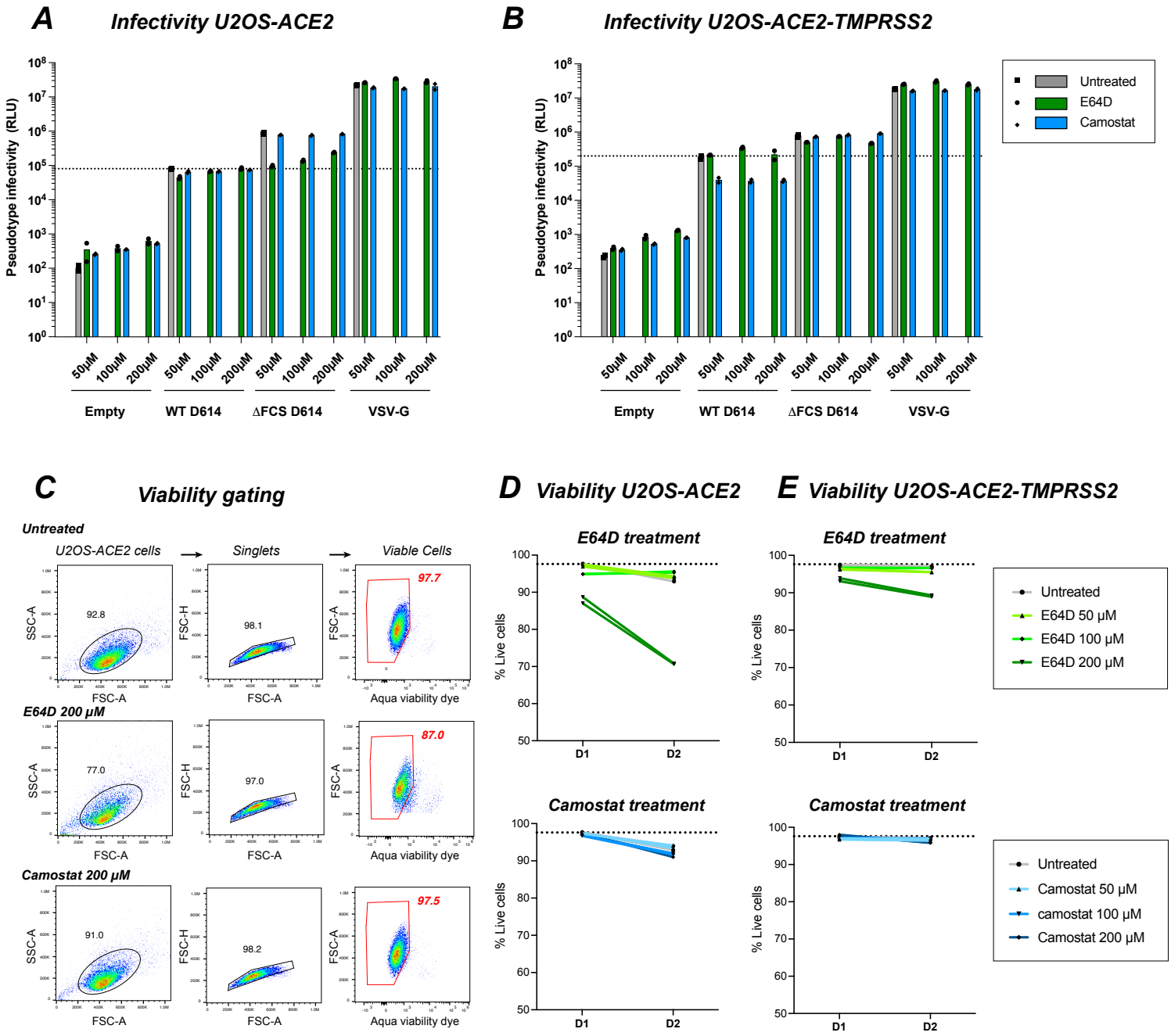
Supplementary Figure 4 Gellenoncourt et al.



Supplementary Figure 5 Gellenoncourt et al.



Supplementary Figure 6 Gellenoncourt et al.



Supplementary Figure 7 Gellenoncourt et al.

Supplementary Table 1 : Primers used for spike mutagenesis and sequencing

Primer name	Primer sequence (5' – 3')	Application
Del19_F	TGATAACGAGCGCGCCTC	Mutagenesis (Del19)
Del19_R	GCAGCAGCTTCCGCAACT	
ΔFCS_F	TCGAAGTGTGGCCAGCCAG	Mutagenesis (ΔFCS mutant)
ΔFCS_R	CTATTGGTCTGGGTCTGATAGG	
P681H_F	ACCAATAGTCATCGACGAGCCC	Mutagenesis (P681H mutant)
P681H_R	CTGGGTCTGATAGGAGGC	
T716I_F	GCTATCCCCATCAACTTCACC	Mutagenesis (T716I mutant)
T716I_R	AATGCTGTTGTTGGAGTAG	
H681R_F	ACCAATAGTCGTCGACGAGCC	Mutagenesis (P681R mutant)
H681R_R	CTGGGTCTGATAGGAGGC	
D614G_F	CTGTACCAGGGCGTGAATTGCACAGAGGTG	Mutagenesis (D614G mutant)
D614G_R	AACGGCCACCTGGTTGCT	
phCMV_F	CTCTTTCCTACAGCTCCTGG	Spike Sequencing
phCMV_R	TAGCCAGAAGTCAGATGCTC	
Spike_1F	ATCTACAGCAAGCACACCCC	
Spike_1R	TGATGTTGATGCCGATGG	
Spike_2F	TTACCTGTACCGGCTGTTCC	
Spike_2R	CCGTTACAAGGGGTGCT	
Spike_3F	AGAACAGCGTGGCCTACTCC	
Spike_3R	ATGGACACAGGCAGGATCTC	
Spike_4F	AGGACGTGGTCAACCAGAAT	
Spike_4R	GCTCAGGATATCGTTCAGCA	