

Supplementary Information for:
The sequence and structural integrity of the SARS-CoV-2 Spike protein
transmembrane domain is crucial for viral entry

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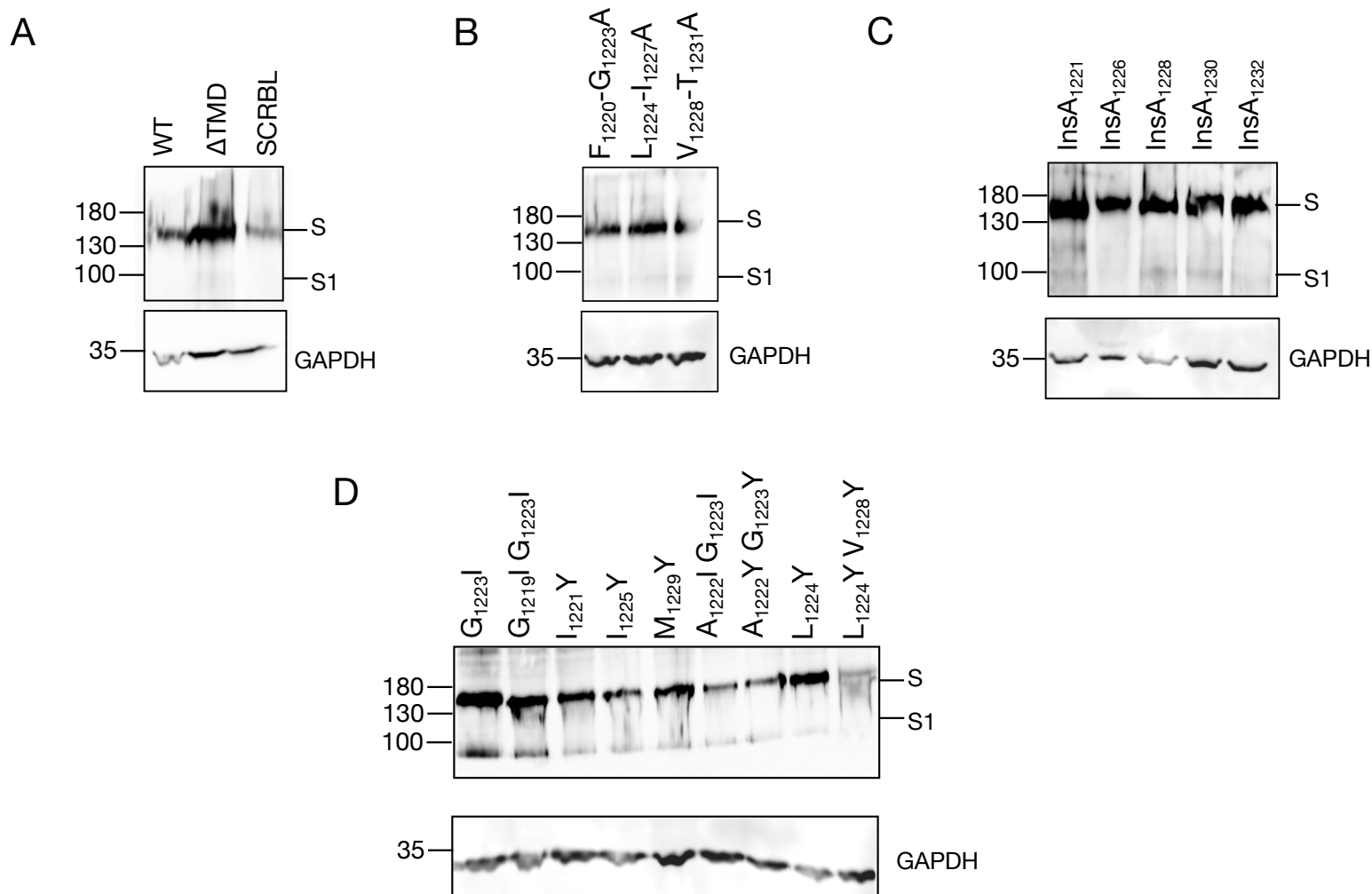
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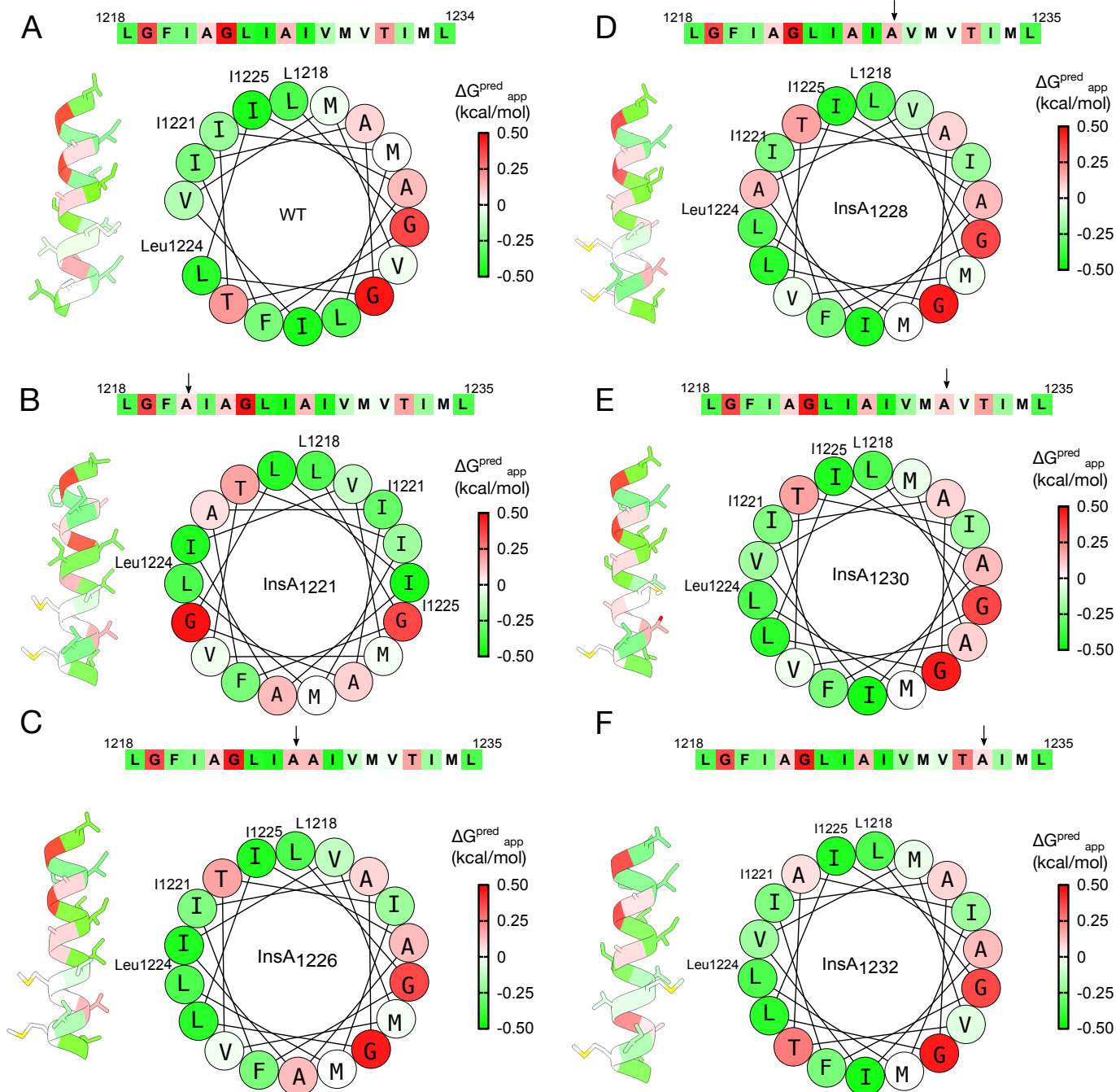
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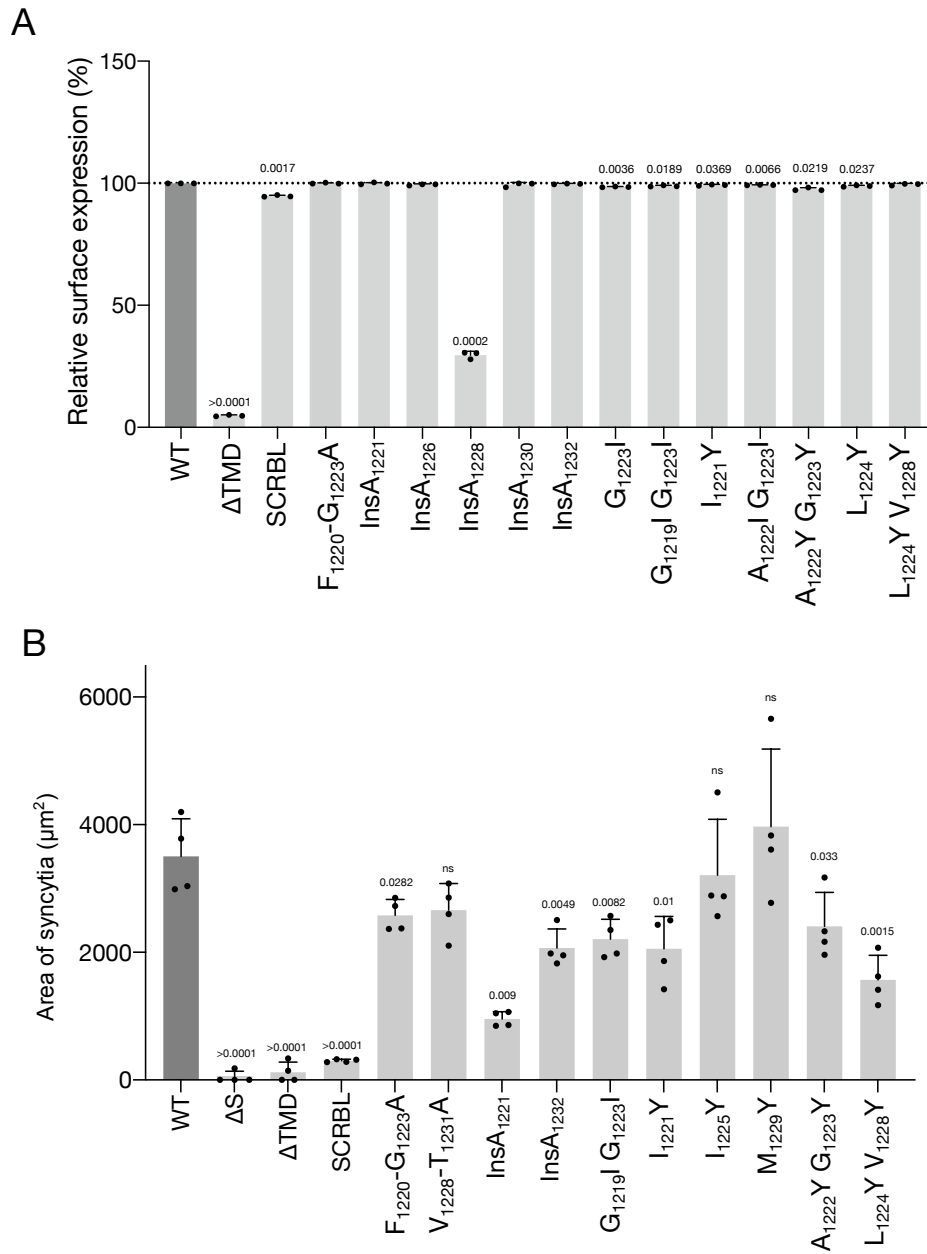
Supplementary Figures 1-7.



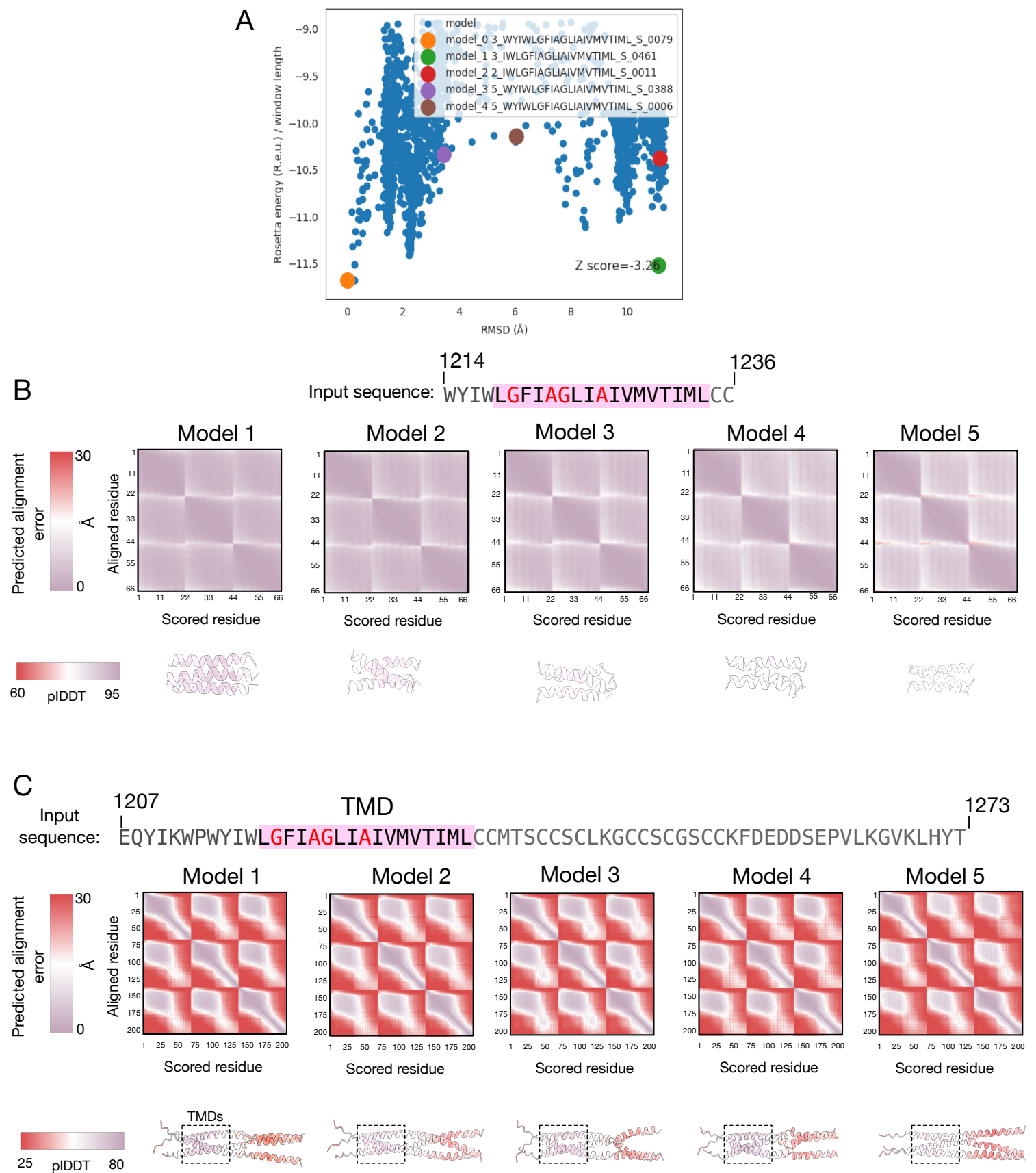
Supplementary Figure 1. Expression levels of SARS-CoV-2 S protein mutants. Analysis by western blotting of SARS-CoV-2 S protein mutants. A. Expression levels of WT, ΔTMD, and SCRB L mutants. B. Expression levels of SARS-CoV-2 S protein mutants including alanine substitutions Phe1220-Gly1223, Leu1224-Ile1227, and Val1228-Thr1231. C. Expression levels of SARS-CoV-2 S protein mutants including alanine insertions in positions 1221, 1226, 1228, 1230, and 1232 (InsA1221, InsA1226, InsA1228, InsA1230, and InsA1232 respectively). D. Expression levels of SARS-CoV-2 S protein mutants including point mutations, G1223I, G1219I G1223I, I1221Y, I1225Y, M1229Y, A1222I G1223I, A1222Y G1223Y, L1224Y, L1224Y V1228Y. Endogenous GAPDH was used as a loading control for all experiments.



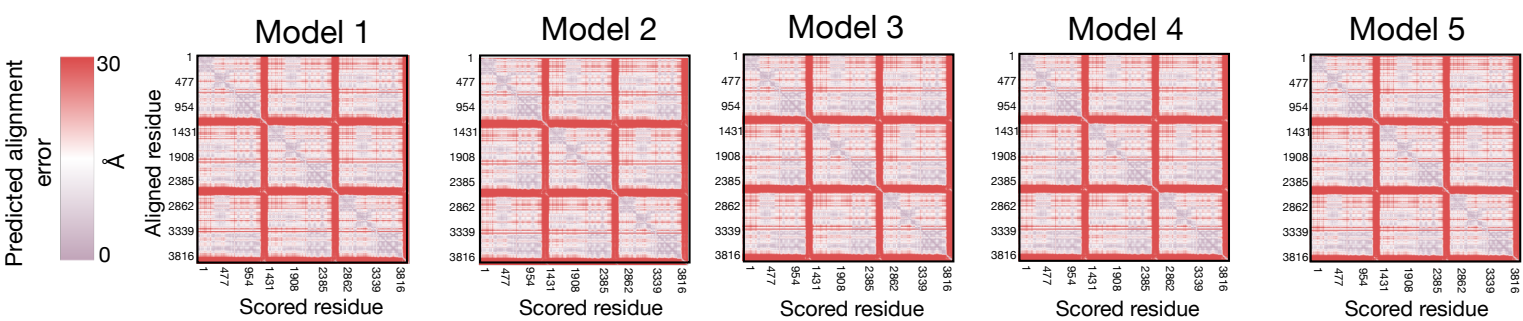
Supplementary Figure 2. Helical wheel diagram for SARS-CoV-2 S protein TMD hydrophobic core. A-F. The SARS-CoV-2 S protein TMD hydrophobic core sequence (top, residues 1218-1234), AlphaFold3 models (bottom left), and helical wheel representations (bottom right). Residues are represented with a color code based on their predicted ΔG of insertion relative to their position within the membrane (<https://dgpred.cbr.su.se/index.php?p=home>).



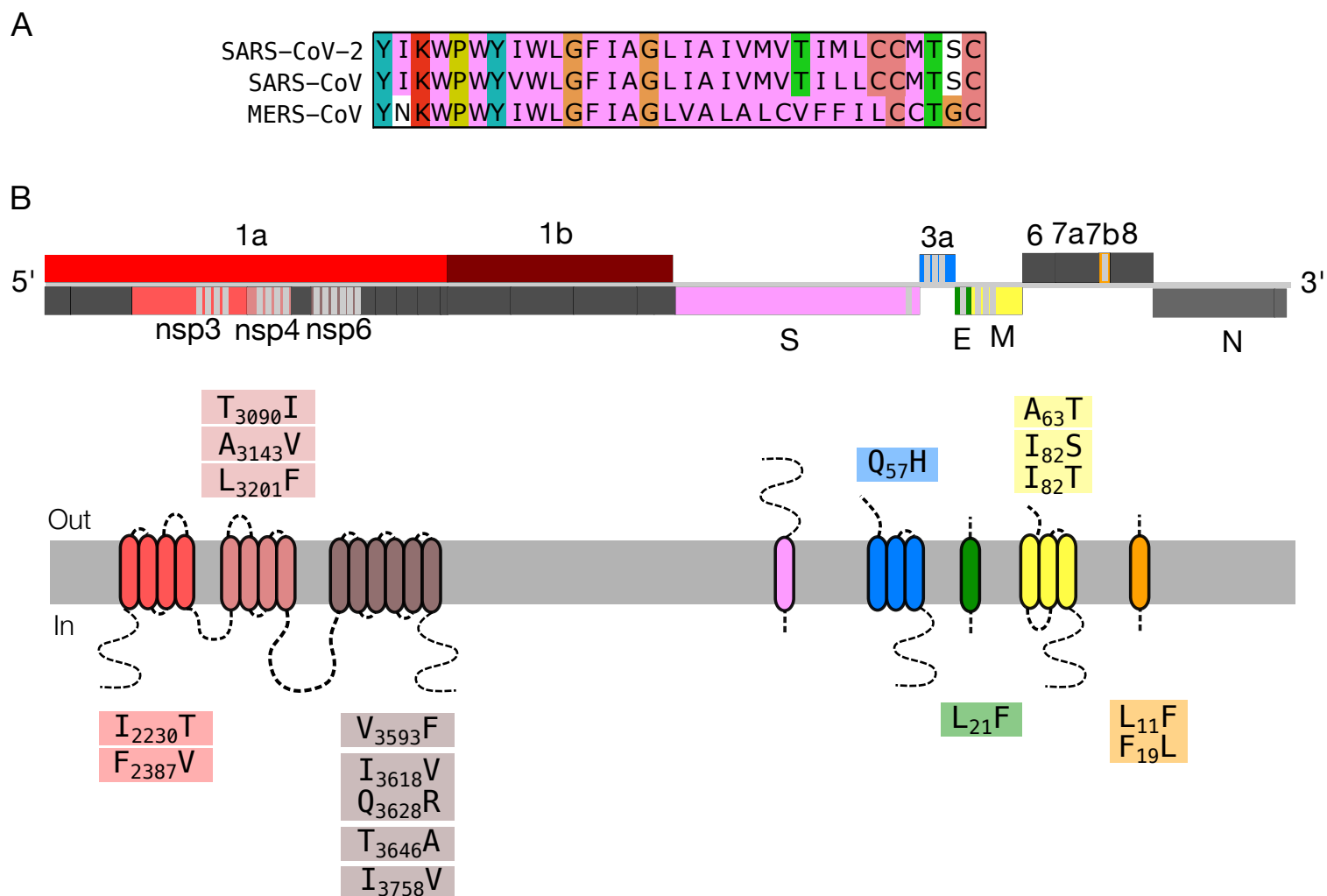
Supplementary Figure 3. Analysis of S surface expression by flow cytometry and fluorescence microscopy . A. Relative surface expression of all Spike mutants tested in this assay. The figure shows the percentage of SARS-CoV-2 S protein signal on the cell surface. Samples were normalized to the SARS-CoV-2 S protein WT. The significant p-values (<0.05) for individual one-sample t-tests vs. WT are indicated above each bar. **B.** The area of the syncytia was calculated from fluorescent images. Each dot represents a single measurement. Bars show the average and st. deviation of four images. The p-value was assessed by an unpaired t-test against WT condition. Values higher than 0.05 were considered non-significant (ns).



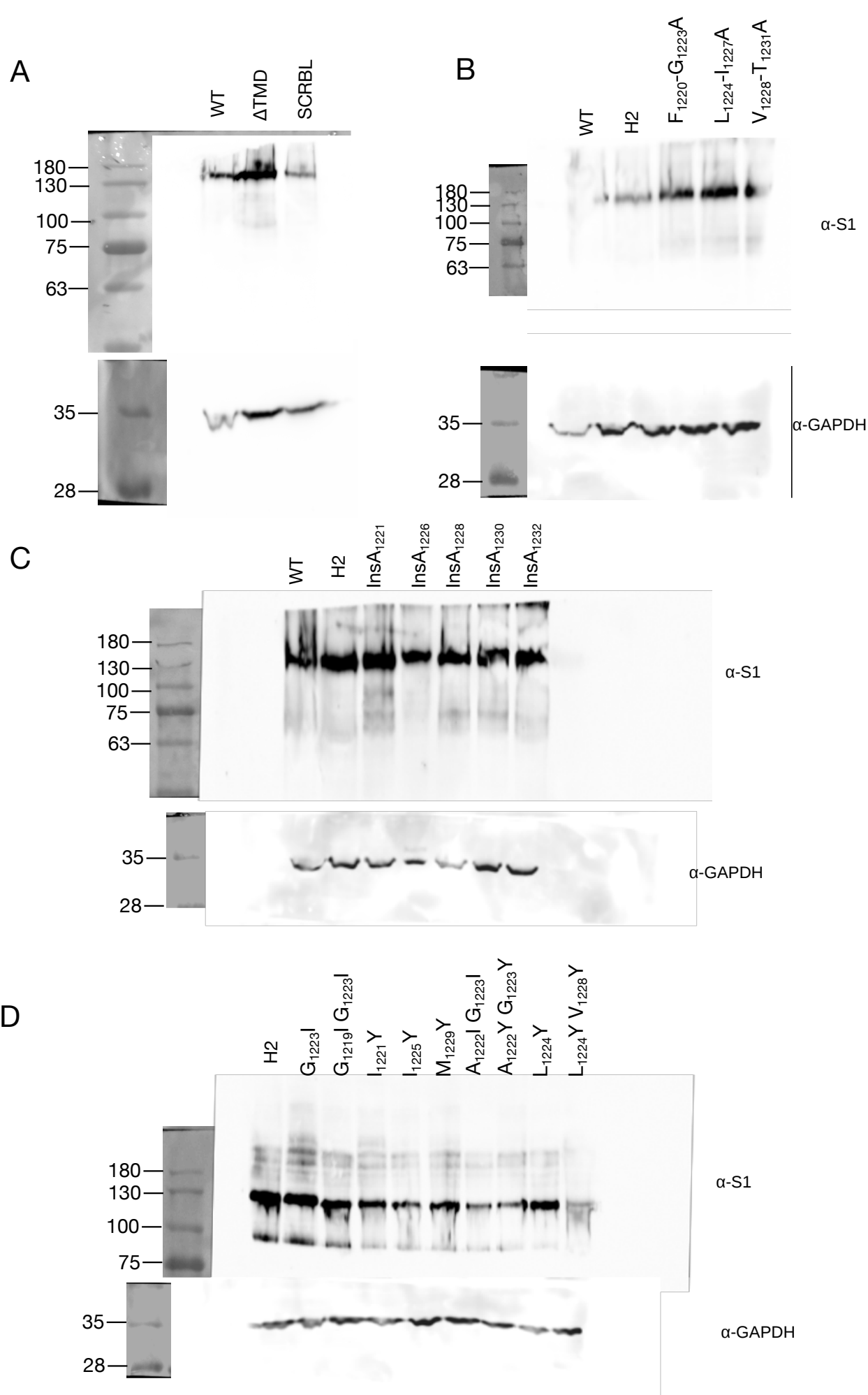
Supplementary Figure 4. Statistical output of SARS-CoV-2 S protein TMD trimer models. A. Scatterplot distribution for the S TMD homodimer modeling by TMHOPP. Dots represent each of the models for the S TMD's homotrimers. rmsd is calculated from the lowest-energy model. The selected models are highlighted in five different colors. B-C. Predicted alignment error (top) for different AlphaFold3 models. The b-factor of each residue is colored by pIDDT values on the predicted structural models (bottom). Two inputs were applied: one covering just the TMD (B, residues 1214-1236) or including the N-terminal TMD flanking region up to the C-terminal end of the protein (C, residues 1207-1273).



Supplementary Figure 5. Statistical output of full-length SARS-CoV-2 S protein trimer models. Predicted alignment error (top) for different AlphaFold3 models. The b-factor of each residue is colored by pLDDT values on the predicted structural models.



Supplementary Figure 6. Mutational landscape of SARS-CoV-2 transmembrane proteome. A. Sequence alignment of Spike protein TMDs from SARS-CoV-2, SARS-CoV-1 and MERS-CoV. Hydrophobic residues are colored in pink, while the rest are colored by the ClustalX color scheme. B. (Top) Schematic representation of SARS-CoV-2 genome. ORFs that codify for membrane proteins are colored, and regions that codify for TMDs are highlighted in gray. (Bottom) Schematic representation of SARS-CoV-2 non-structural, structural and accessory membrane proteins. Accumulated mutations in different TMDs are highlighted in colors.



Supplementary Figure 7. Uncropped western blots shown in this study. A-D. Uncropped western blots from Supplementary Figures 1A-D, respectively. A digital image of the membrane containing the molecular marker is included on the left side of each blot.