Supplemental Information

Expression of SARS-CoV-2 Nonstructural Proteins 3 and 4 can tune the Unfolded Protein Response in cell culture

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Supplemental Figures

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Table S7. Mass spectrometry peptide data of A549 cells (XLSX)

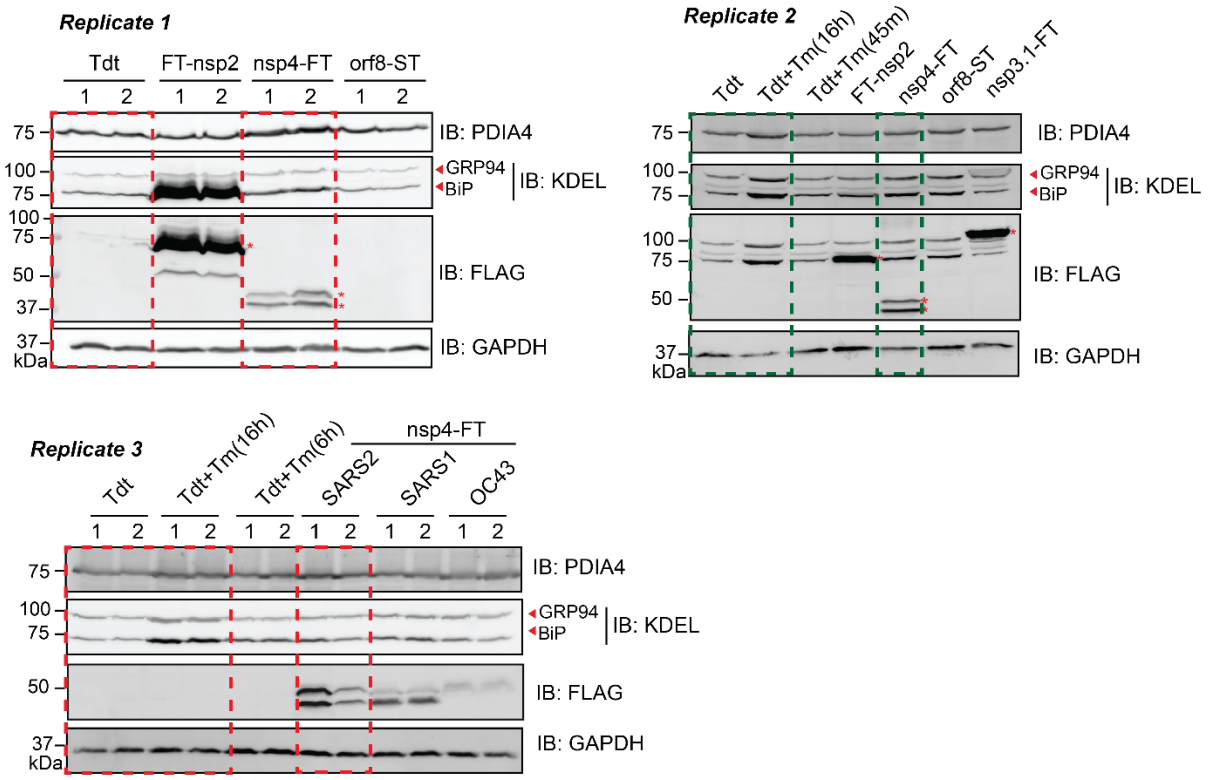
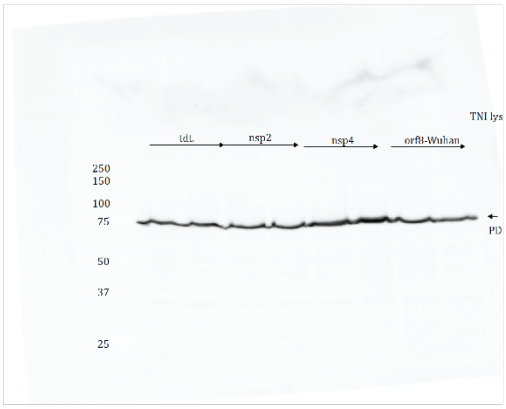


Figure S1. Western blot analysis of HEK293T cells transfected with specified viral proteins or Tdtomato as basal control. Tm treatment was used as a positive control. Green boxed lanes were shown for **Fig. 1c**. Green and red boxed lanes were quantified for **Fig. 1d**. Red asterisks indicate viral proteins. Tdt, tdTomato; Tm, Tunicamycin (1 μg/mL); FT, FLAG-Tag; ST, StrepTag. Corresponding full blots are shown below.

Supplementary Fig.S1 - Replicate 1, Full blots

IB: PDIA4, low contrast



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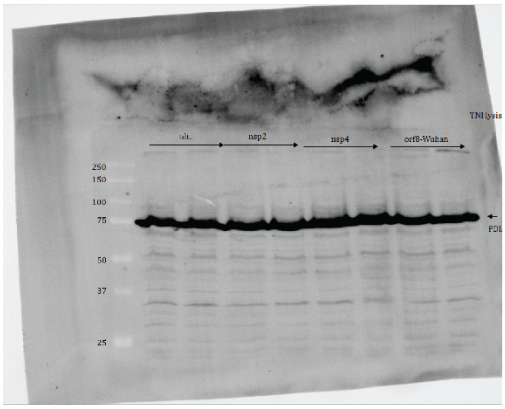
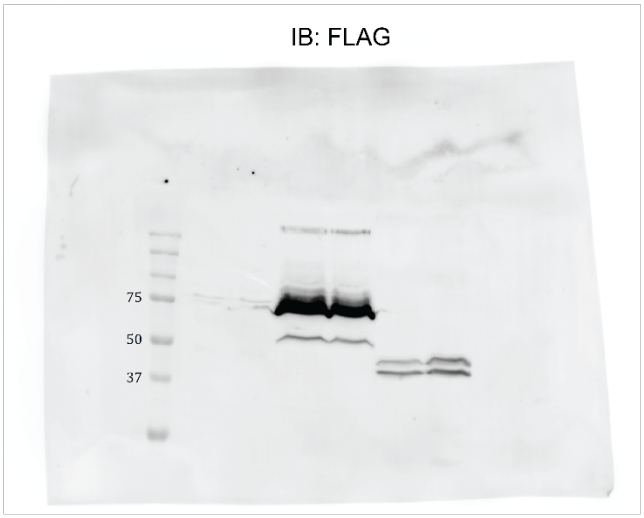


IB: FLAG



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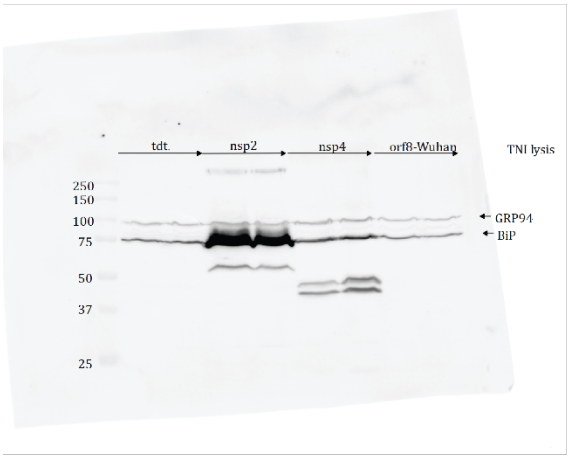

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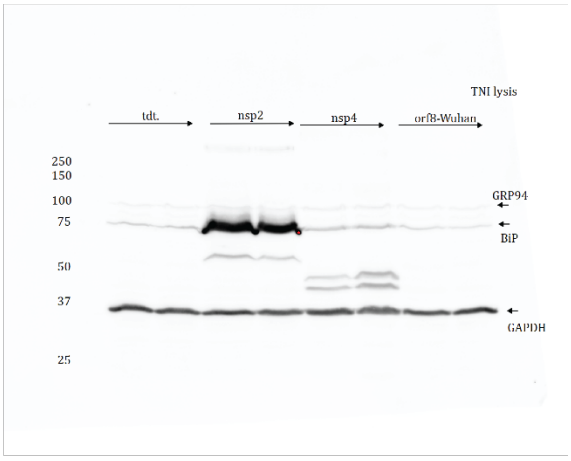

Supplementary Fig.S1 - Replicate 2, Full blots

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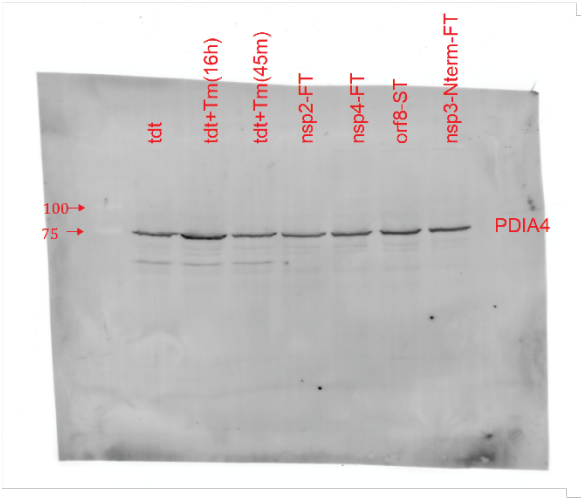

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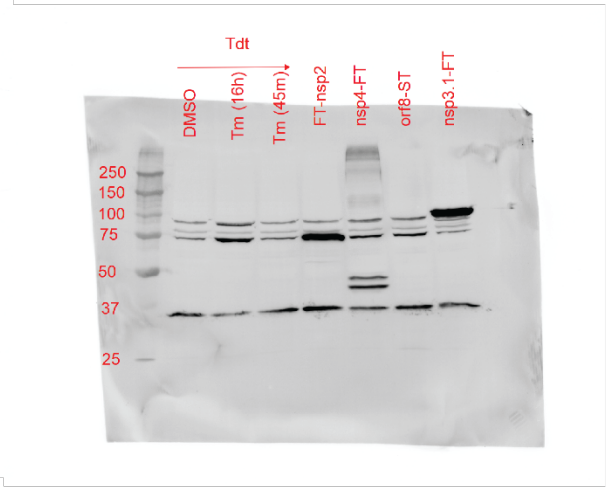

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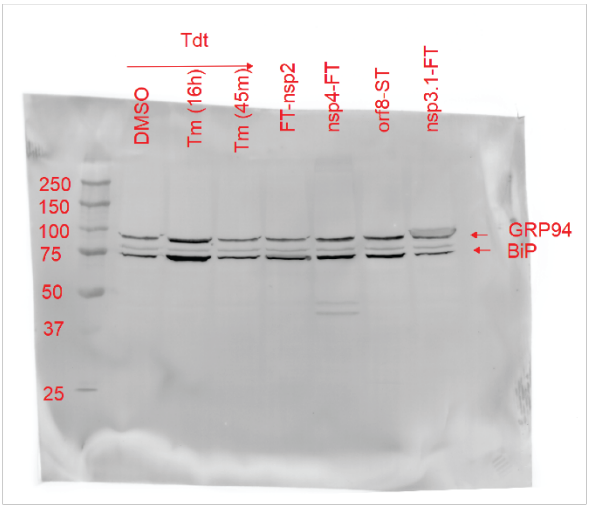
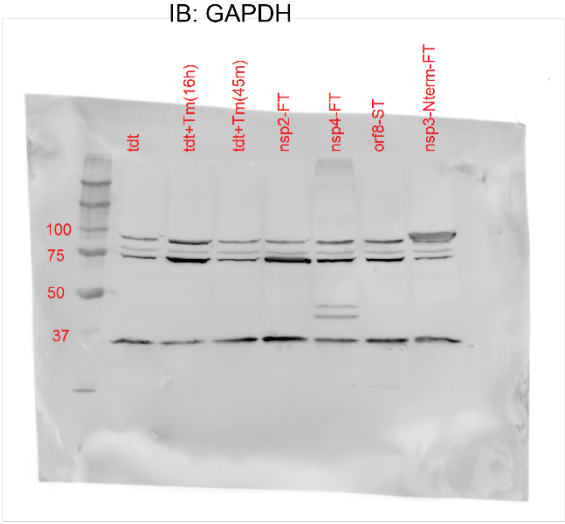


IB: GAPDH



Supplementary Fig.S1 - Replicate 3, Full blots

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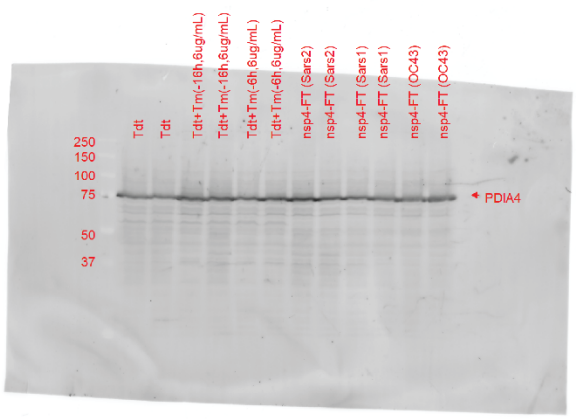

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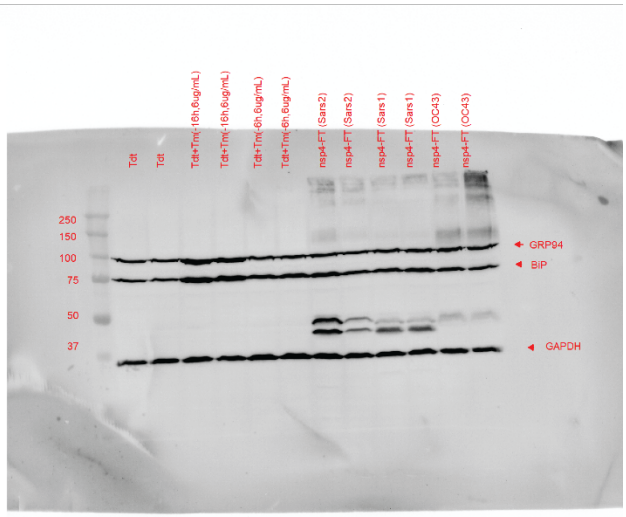

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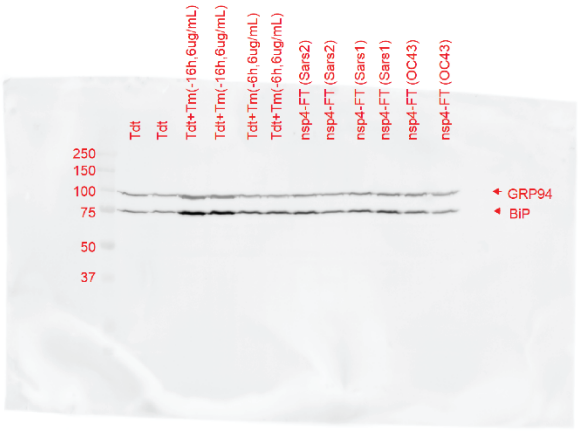

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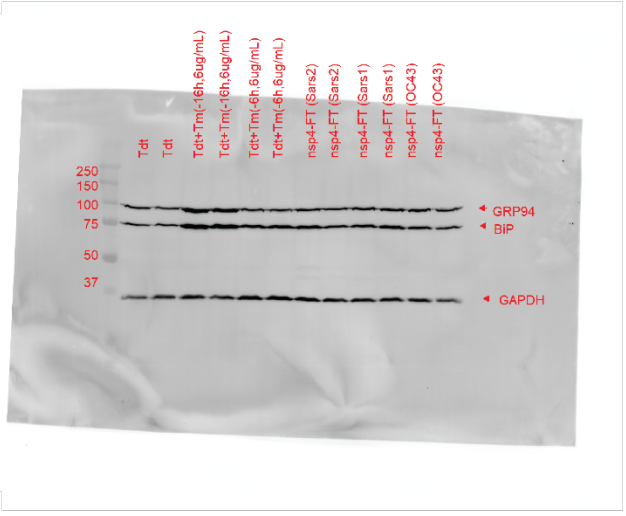


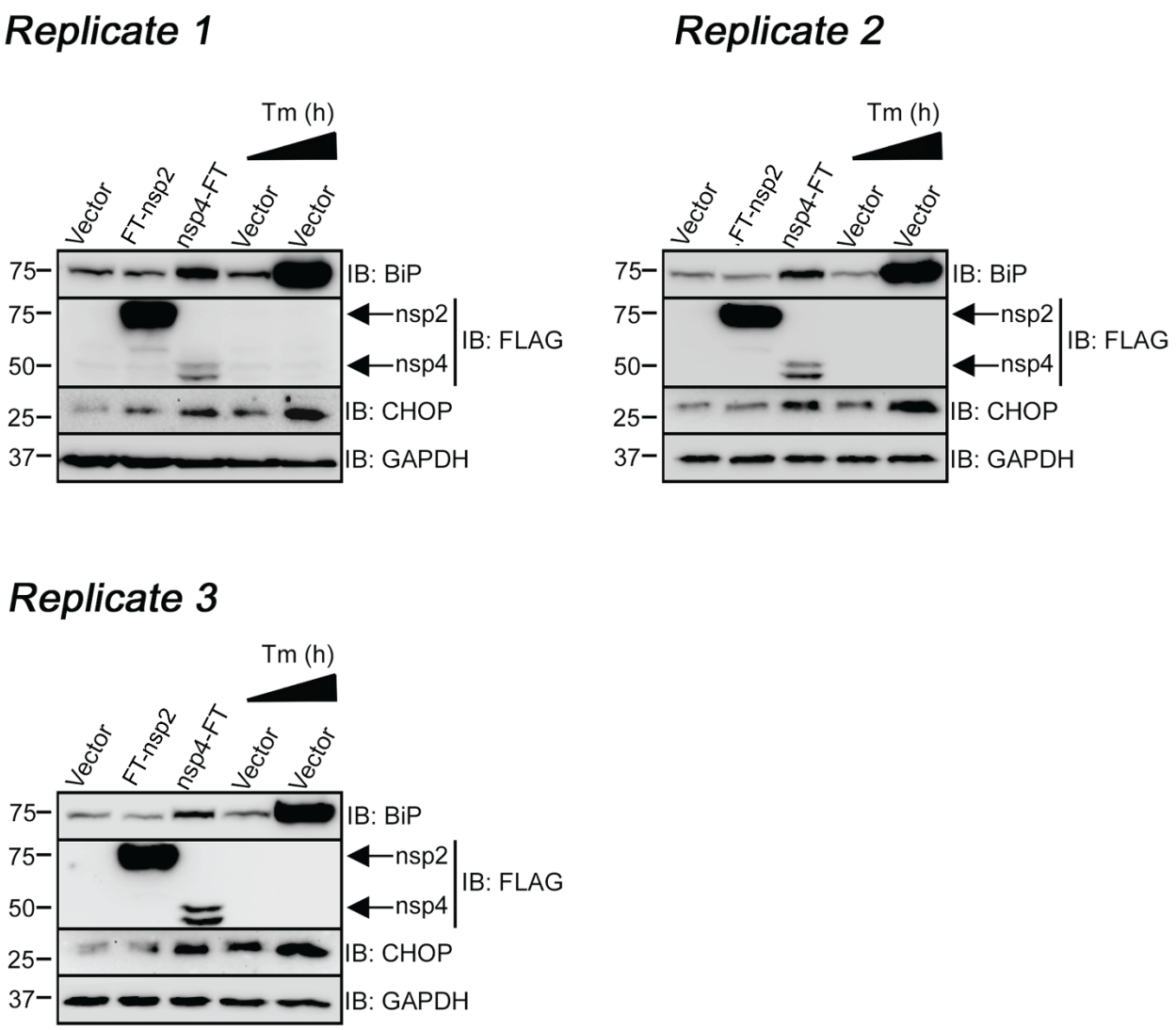
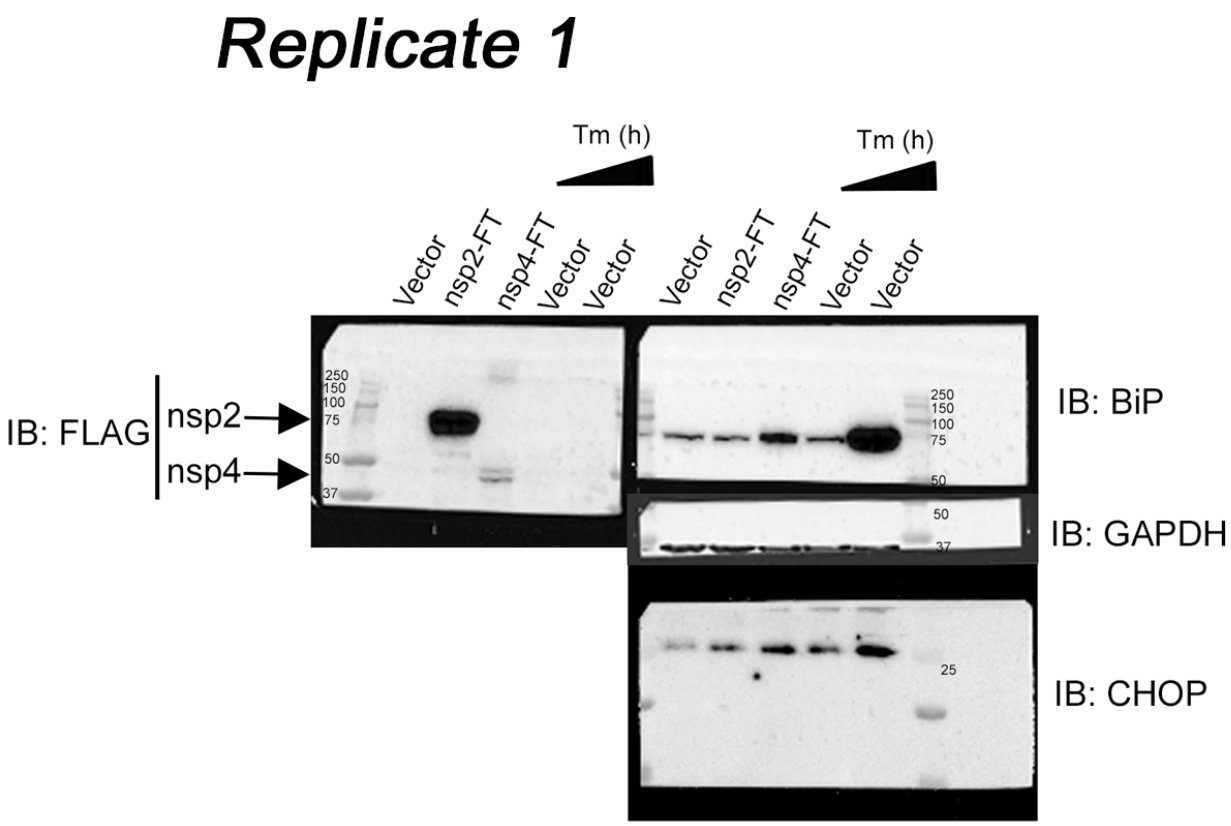
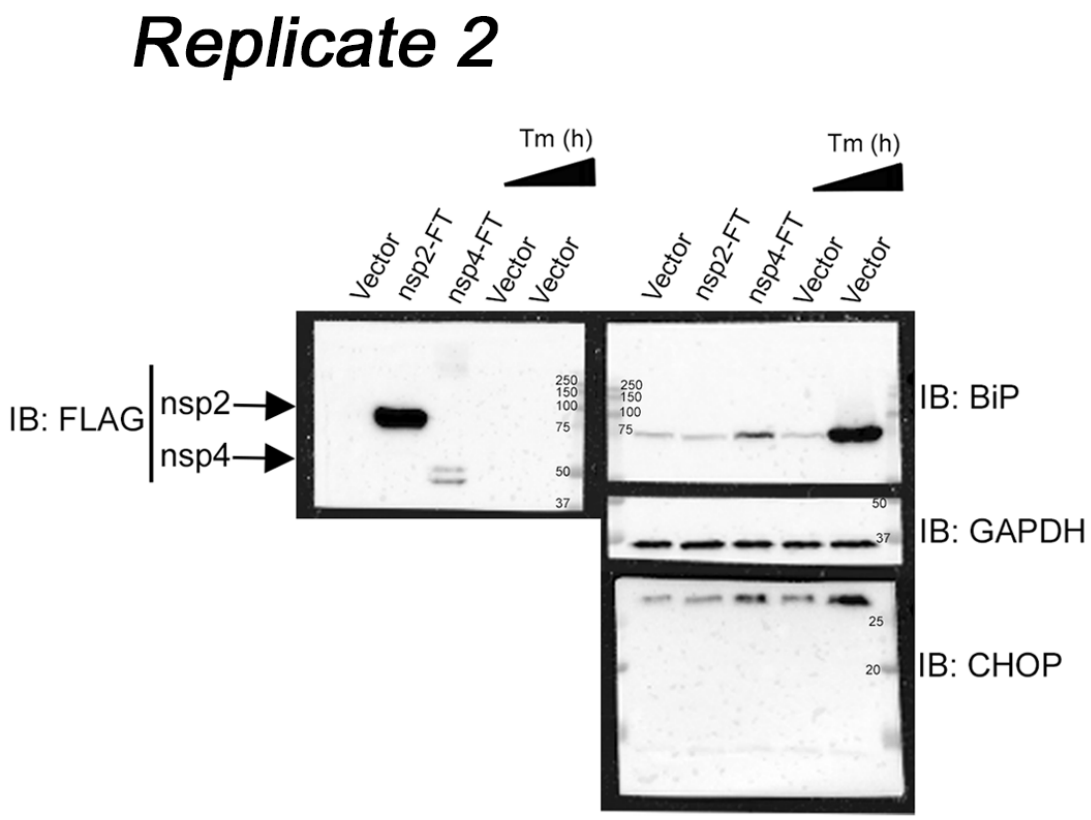


Figure S2. Blots quantified for **Fig. 1f**. Replicate 2 blot was shown in **Fig. 1e**. WT-HEK293T cells were transfected with empty vector or SARS-CoV-2 FT-nsp2 or nsp4-FT, with control vector samples treated for 4 or 20 h Tunicamycin (5 µg/mL). Western blotting for M2-FLAG, BiP, and CHOP shows marked upregulation of BiP and CHOP in nsp4-FT expressing cells. Corresponding full blots are shown below.

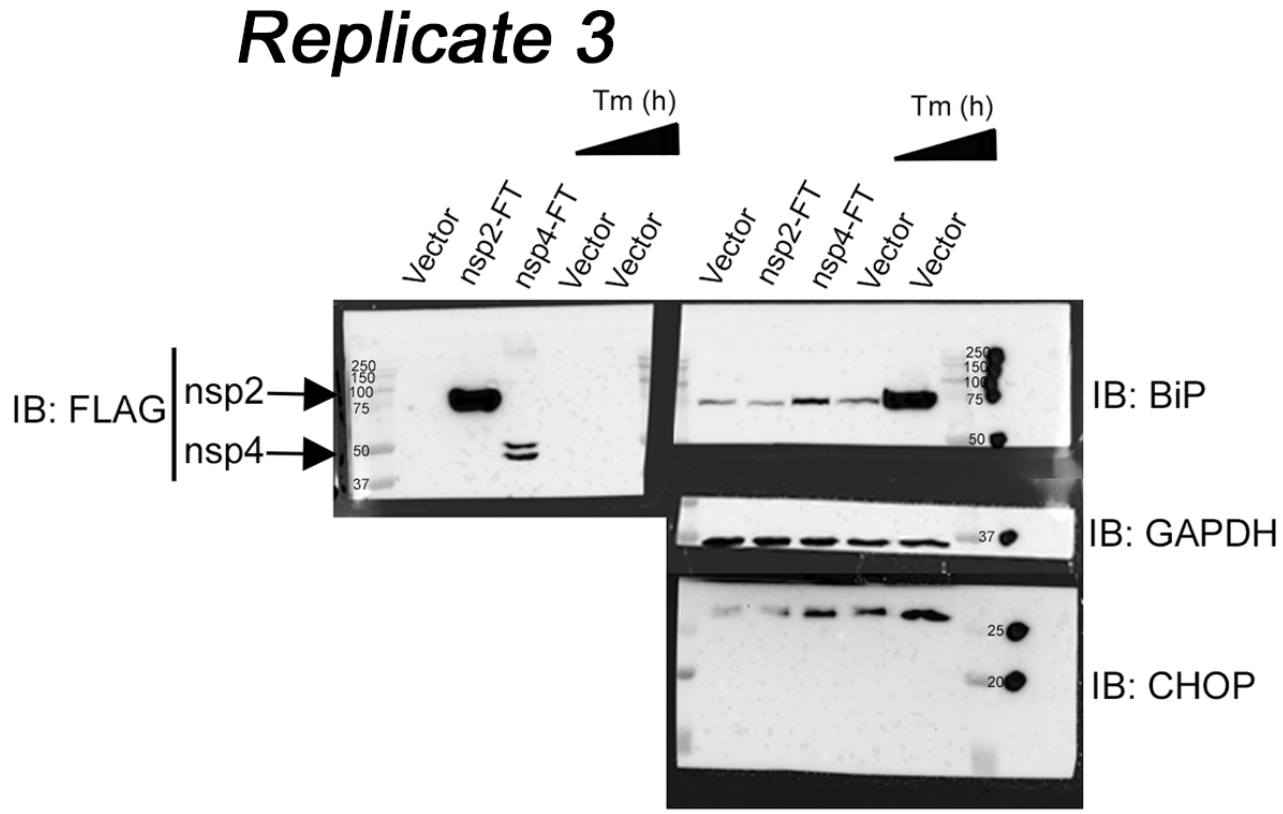
Supplemental Fig. S2, Replicate 1 Full blots



Supplemental Fig. S2, Replicate 2 Full blots



Supplemental Fig. S2, Replicate 3 Full blots



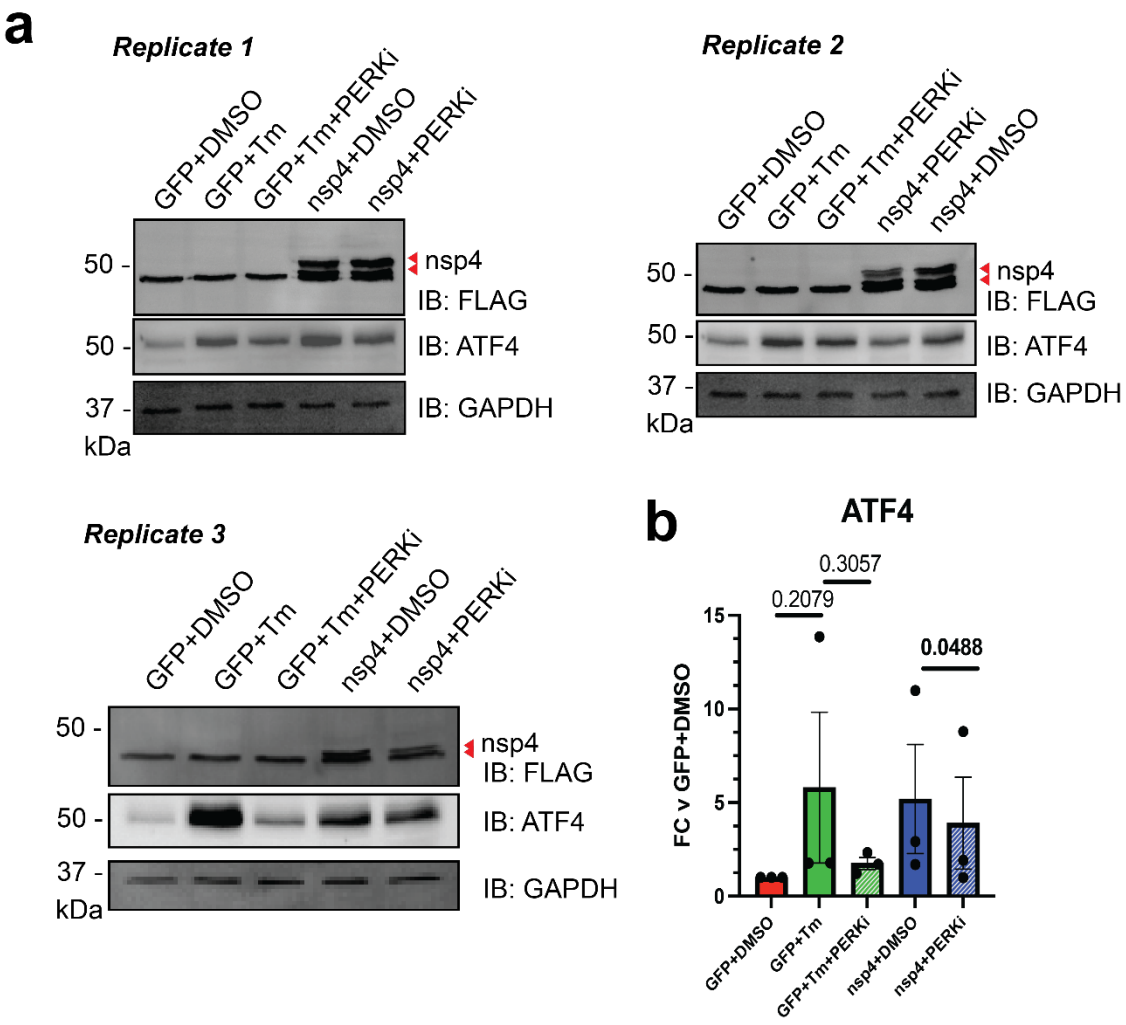
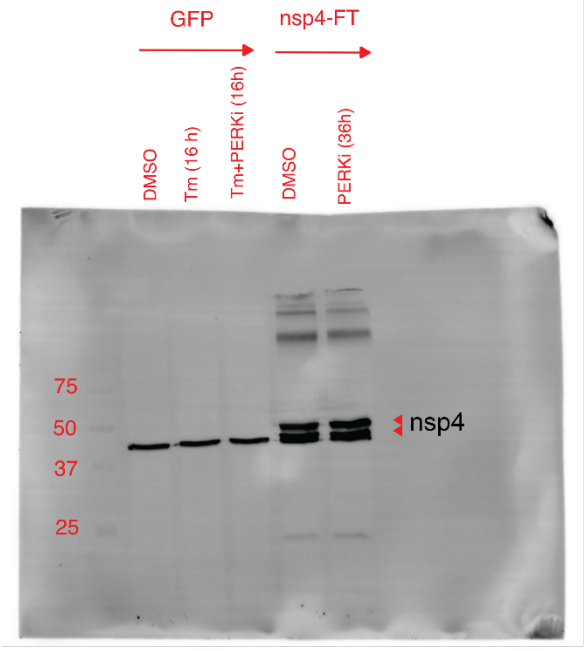


Figure S3. Effect of PERK inhibition on ATF4 upregulation mediated by nsp4.
HEK293T cells were transfected with GFP or SARS-CoV-2 nsp4-FT, with GFP samples treated with either Tunicamycin (Tm, 1 µg/mL) or Tunicamycin and PERK inhibitor II GSK2656157 (PERKi, 1 µM) for 16h and nsp4-FT samples treated with PERK inhibitor II GSK2656157 (PERKi, 1 µM) for 36h.

- **a)** Western blots were probed for FLAG, ATF4, and GAPDH. Corresponding full blots are shown below.
- **b)** Quantification of Western blots in **(a)**, normalized to GAPDH band intensities and relative to basal control (GFP+DMSO). n = 3, mean ±SEM. Paired T-test was used to test significance, $p<0.05$ considered statistically significant.

Supplemental Fig. S3 - Replicate 1, Full Blots



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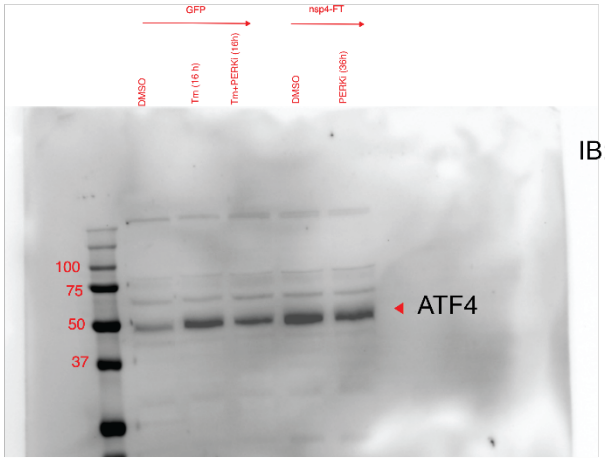
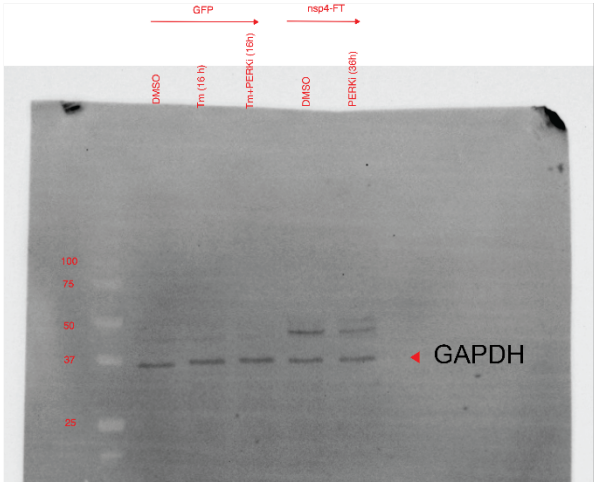
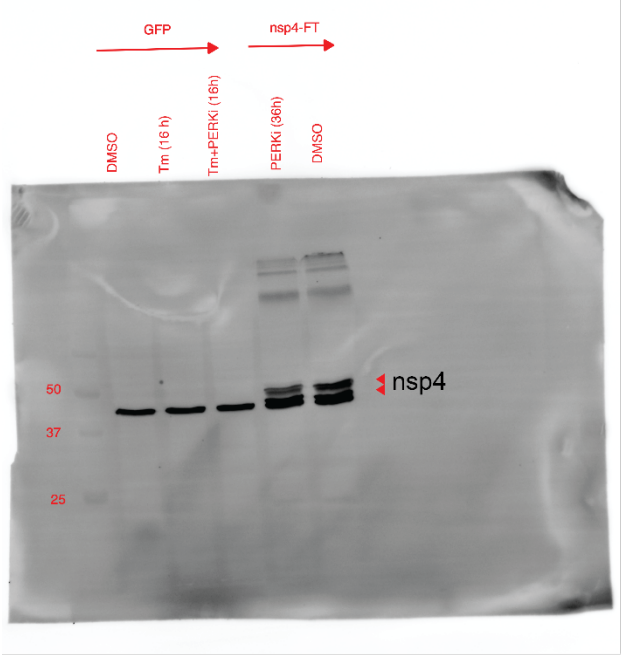


IB: ATF4

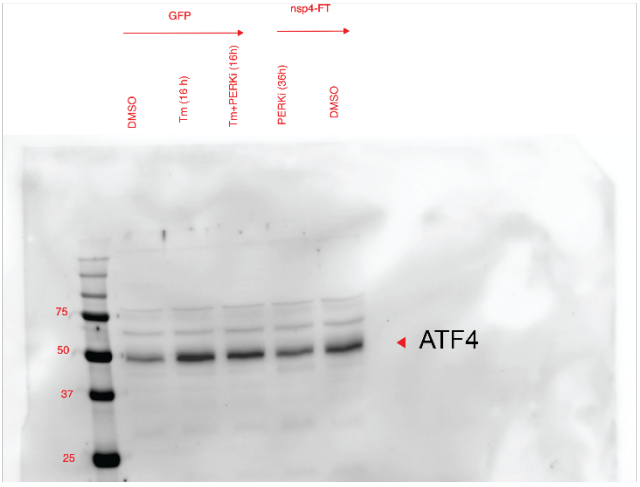


IB: GAPDH

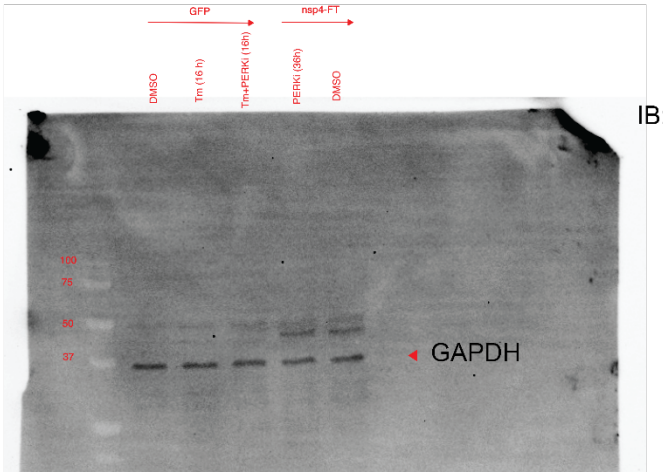
Supplemental Fig. S3 - Replicate 2, Full Blots



IB: FLAG

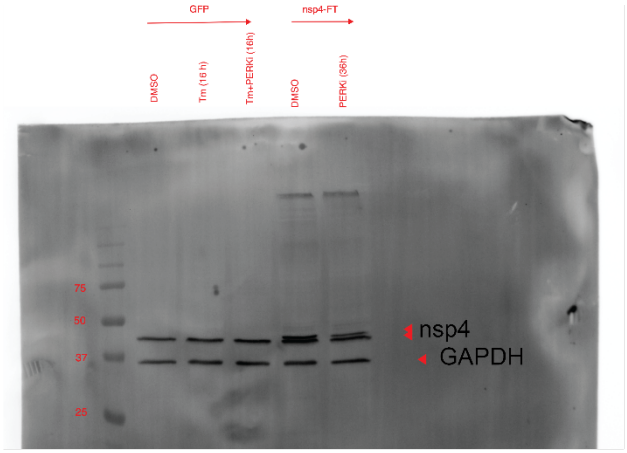


IB: ATF4

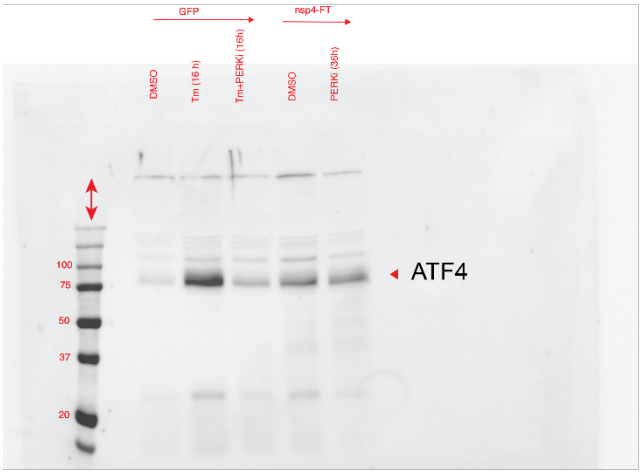


IB: GAPDH

Supplemental Fig. S3 - Replicate 3, Full Blots



IB: FLAG
IB: GAPDH



IB: ATF4

Note: Ladder was imaged separately and shifted down erroneously as indicated by the red arrow.

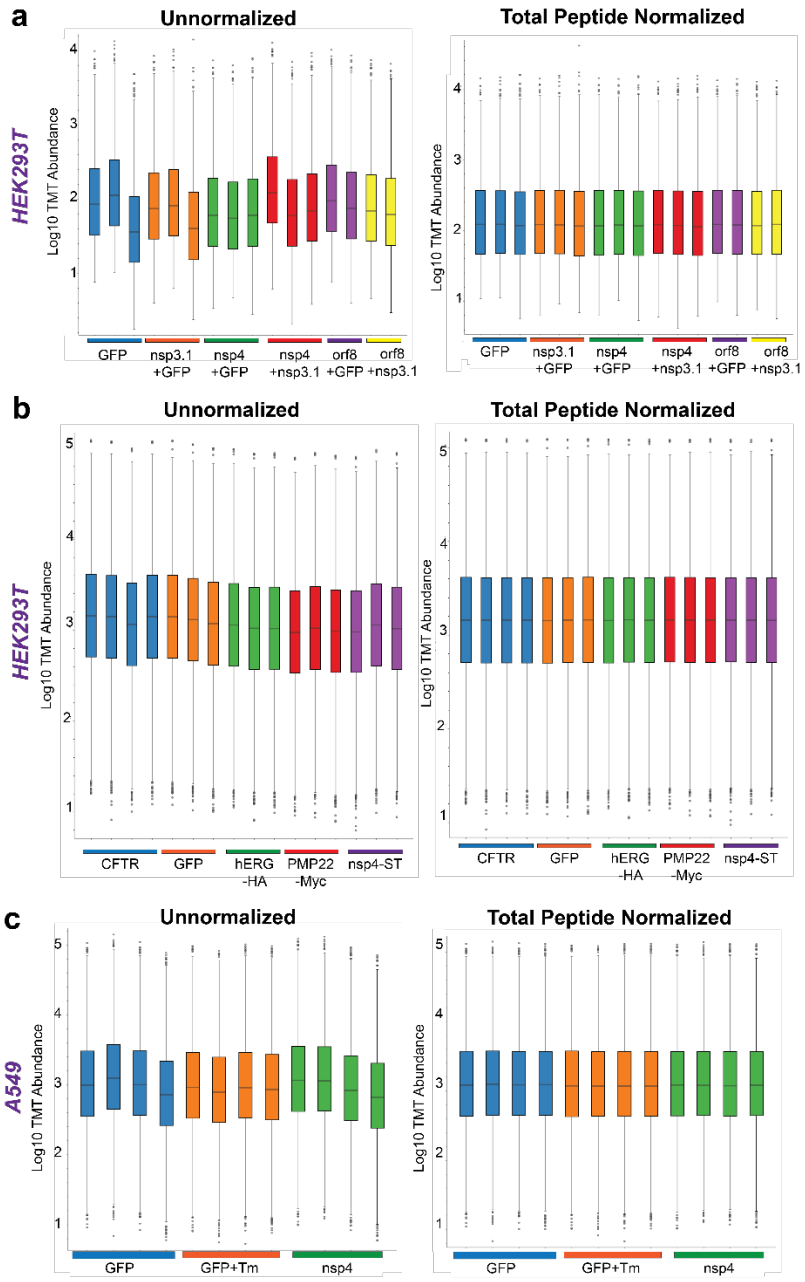


Figure S4. HEK293T **(a,b)** or A549 **(c)** cells were transfected with GFP (basal control) or corresponding proteins, lysates harvested and lysed at 40 h post-transfection, labeled with TMTpro isobaric labels, and analyzed by tandem mass spectrometry (LC/MS-MS). Identified proteins were normalized by total peptide amount.

a) Unnormalized and normalized log10 TMT abundances for MS experiment analyzing HEK293T UPR, corresponding to **Fig. 2,3**. See **Supplemental Tables S2, S3**.

b) Unnormalized and normalized log10 TMT abundances for HEK293T cells expressing transmembrane protein panel, corresponding to **Supplemental Fig. S8**. See **Supplemental Tables S4, S5**.

c) Unnormalized and normalized log10 TMT abundances for MS experiment analyzing A549 UPR, corresponding to **Fig. 2**. See **Supplemental Tables S6, S7**.

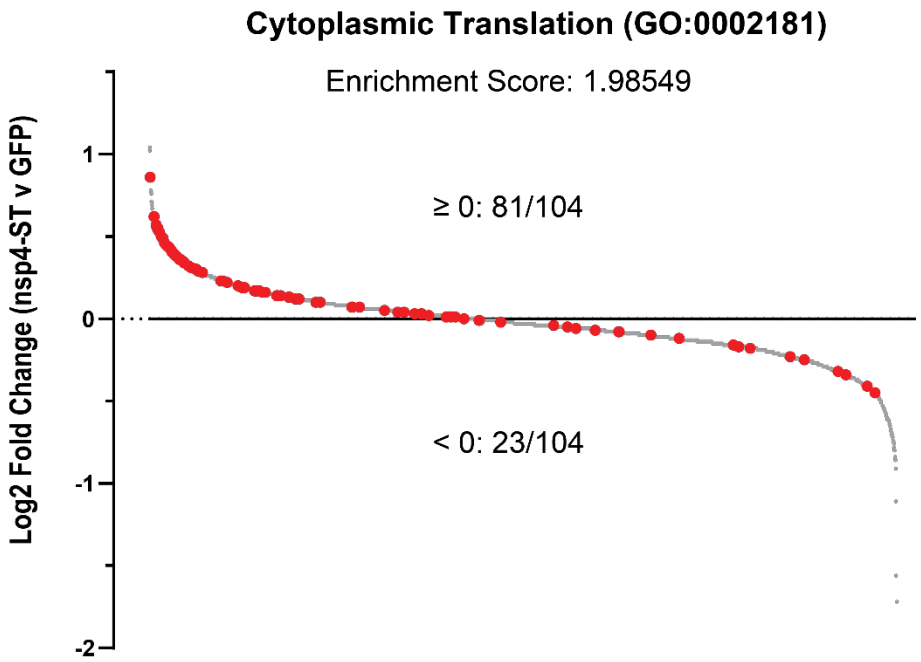


Figure S5. Waterfall plot of proteome in HEK293T cells expressing SARS-CoV-2 nsp4-ST vs GFP control (corresponding to Fig. 2).
A ranked list enrichment analysis was applied to identified proteins based on Log2 Fold Change (nsp4-ST vs GFP) (using the STRING database[1]). Only one Gene Ontology (GO) Biological Processes term was significantly enriched (Cytoplasmic Translation, GO:0002181), with 81/104 genes having a positive Log2 Fold Change and 23/104 genes having a negative Log2 Fold Change. Proteins within the Cytoplasmic Translation GO Term are highlighted in red.

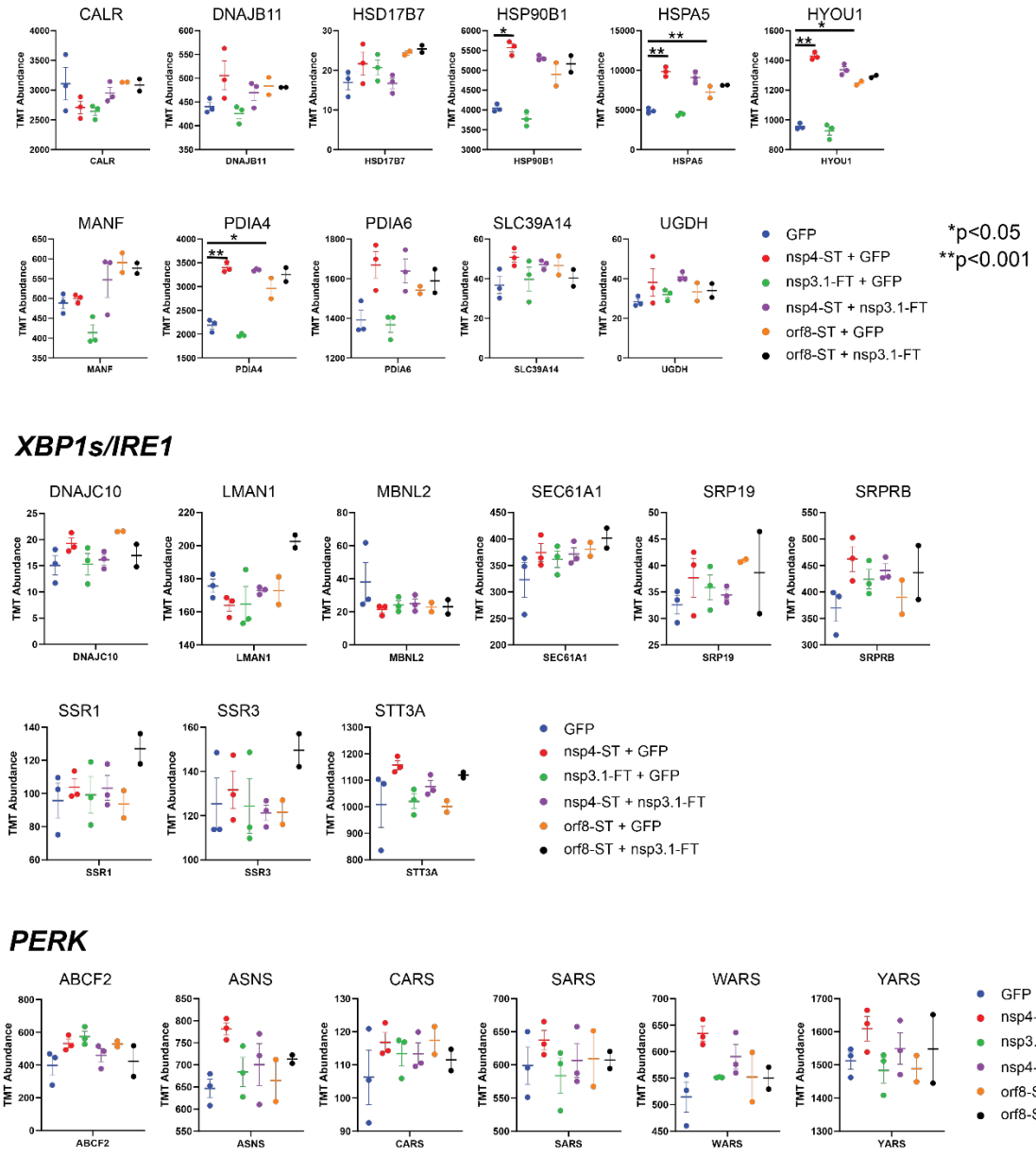


Figure S6. Normalized TMT abundance levels of individual Unfolded Protein Response (UPR) pathway protein markers in HEK293T cells expressing various viral proteins (corresponding to Fig. 2, 3). Adjusted p-value calculated using a T-test, controlling for FDR by the Benjamini-Hochberg method. *p<0.05, **p<0.001

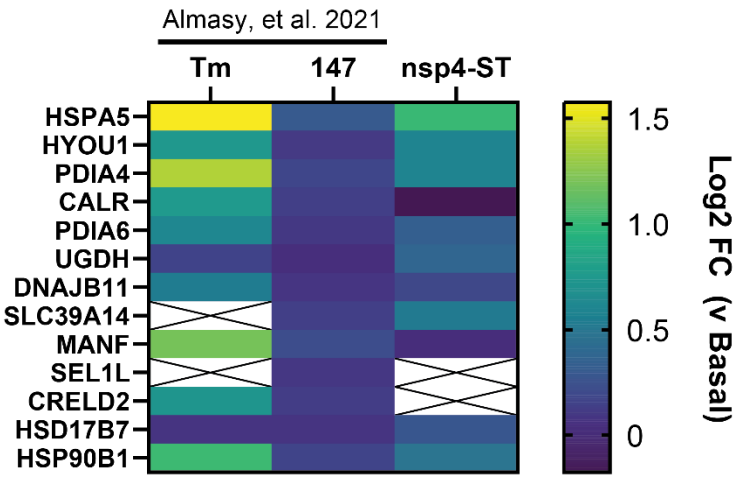


Figure S7. ATF6 marker log2 fold change enrichment over basal conditions in HEK293T cells with treatment of Tm (1 µg/mL, 16 h), **147** (10 µM, 16 h), or nsp4-ST expression. Samples were quantified by TMTpro-based LC/MS-MS. Basal conditions are Tdtomato transfection with DMSO for drug treatment or GFP transfection for nsp4-ST treatment. Previously published data from Almasy, Davies, Plate *MCP* 2021 is annotated[2].

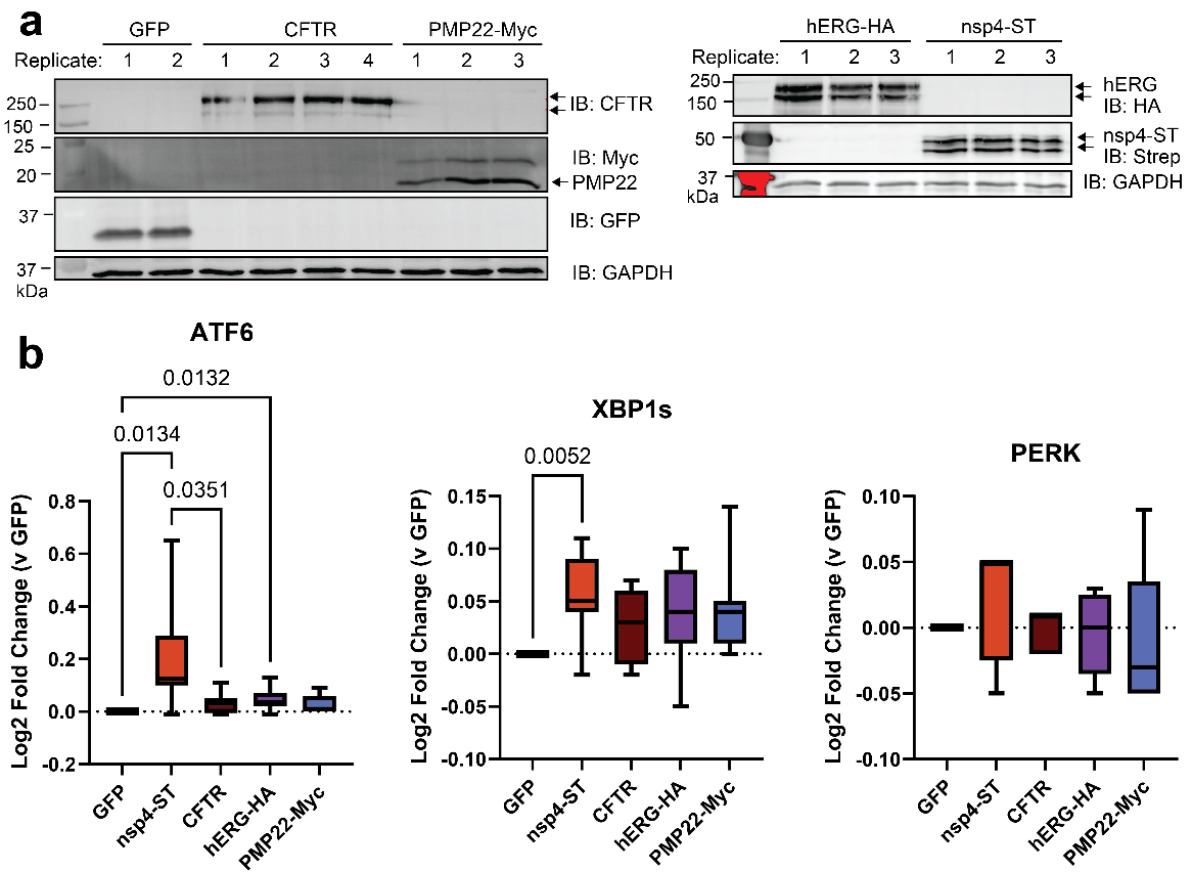


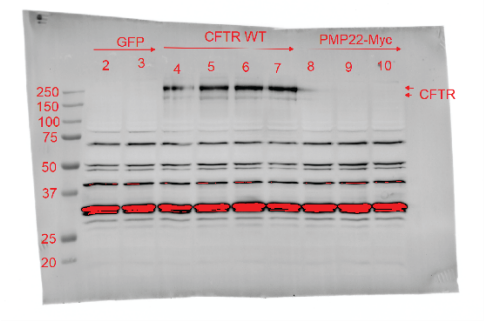
Figure S8. Effect of various multi-pass transmembrane proteins on the UPR as compared to nsp4. Nsp4-ST, WT Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), HA-tagged human Ether-a-go-go Related Gene (hERG-HA), or myc-tagged peripheral myelin protein 22 (PMP22-Myc) were expressed in HEK293T cells and harvested 40 h post-transfection.

a) Western blot confirmation of protein expression in HEK293T lysates.

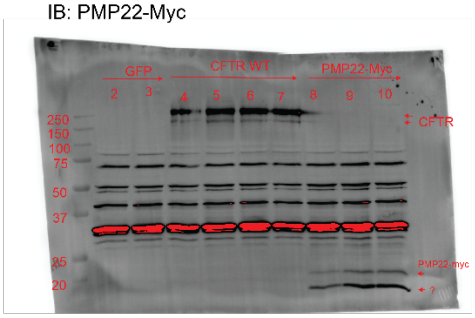
b) UPR branch reporter protein levels were measured by global proteomics using tandem mass spectrometry (LC/MS-MS) and compared to GFP negative control. One-way ANOVA with Geisser–Greenhouse correction and post-hoc Tukey's multiple comparison test was used to determine significance, $p<0.05$ considered significant, significant p values shown; 1 MS run, n = 3-4 biological replicates. See **Supplemental Tables S4, S5** for mass spectrometry data set.

Supplemental Fig. S8 – Full Western blots

IB: CFTR



IB: PMP22-Myc



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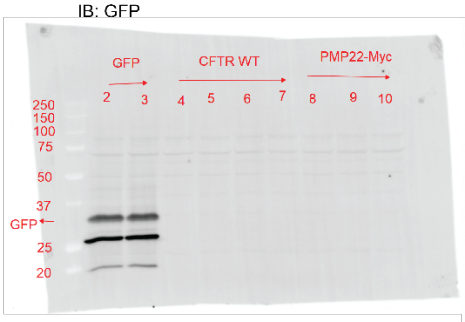
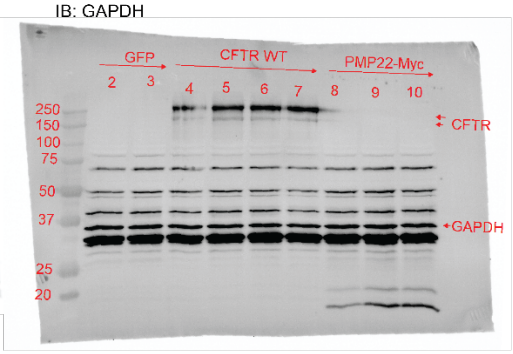
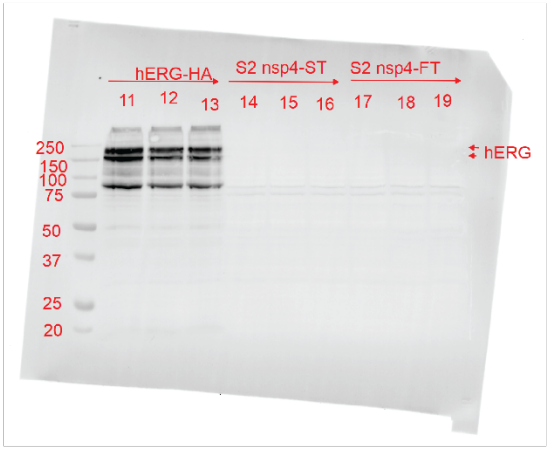


IB: GAPDH



IB: HA



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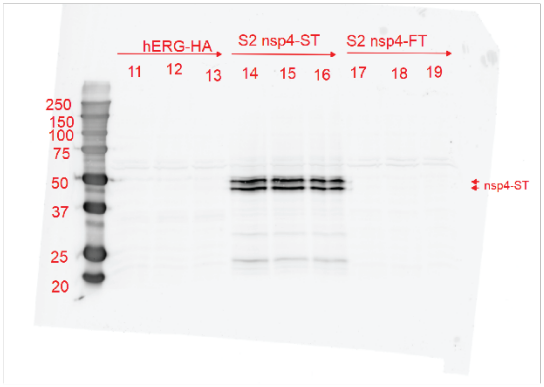

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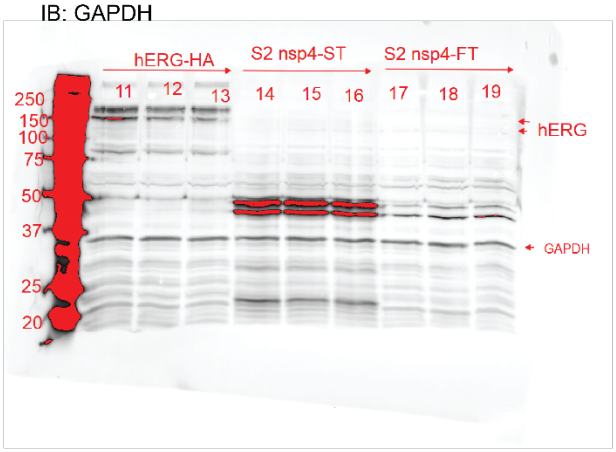


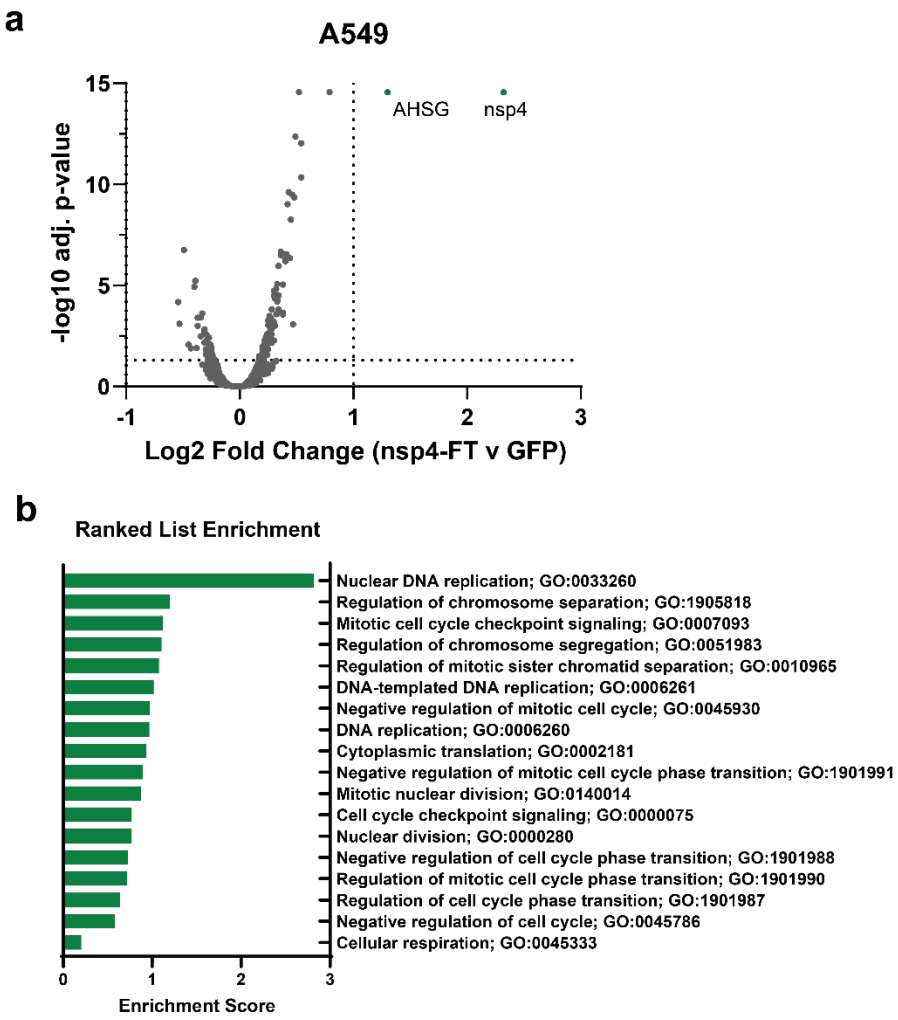


Figure S9. Global proteome analysis of A549 cells expressing SARS-CoV-2 nsp4-FT vs GFP control (corresponding to Fig. 2).

a) Volcano plot of global proteome, with cut-offs indicating Log2 FC (vs GFP) < -1 or > 1, adjusted p-value < 0.05. Common contaminant proteins were filtered out. Proteins passing cut-offs are annotated. Adjusted p-value calculated using a T-test, controlling for FDR by the Benjamini-Hochberg method. N = 4, 1 MS run.

b) A ranked list enrichment analysis was applied to identified proteins based on Log2 Fold Change (nsp4-FT vs GFP) (using the STRING database[1]).

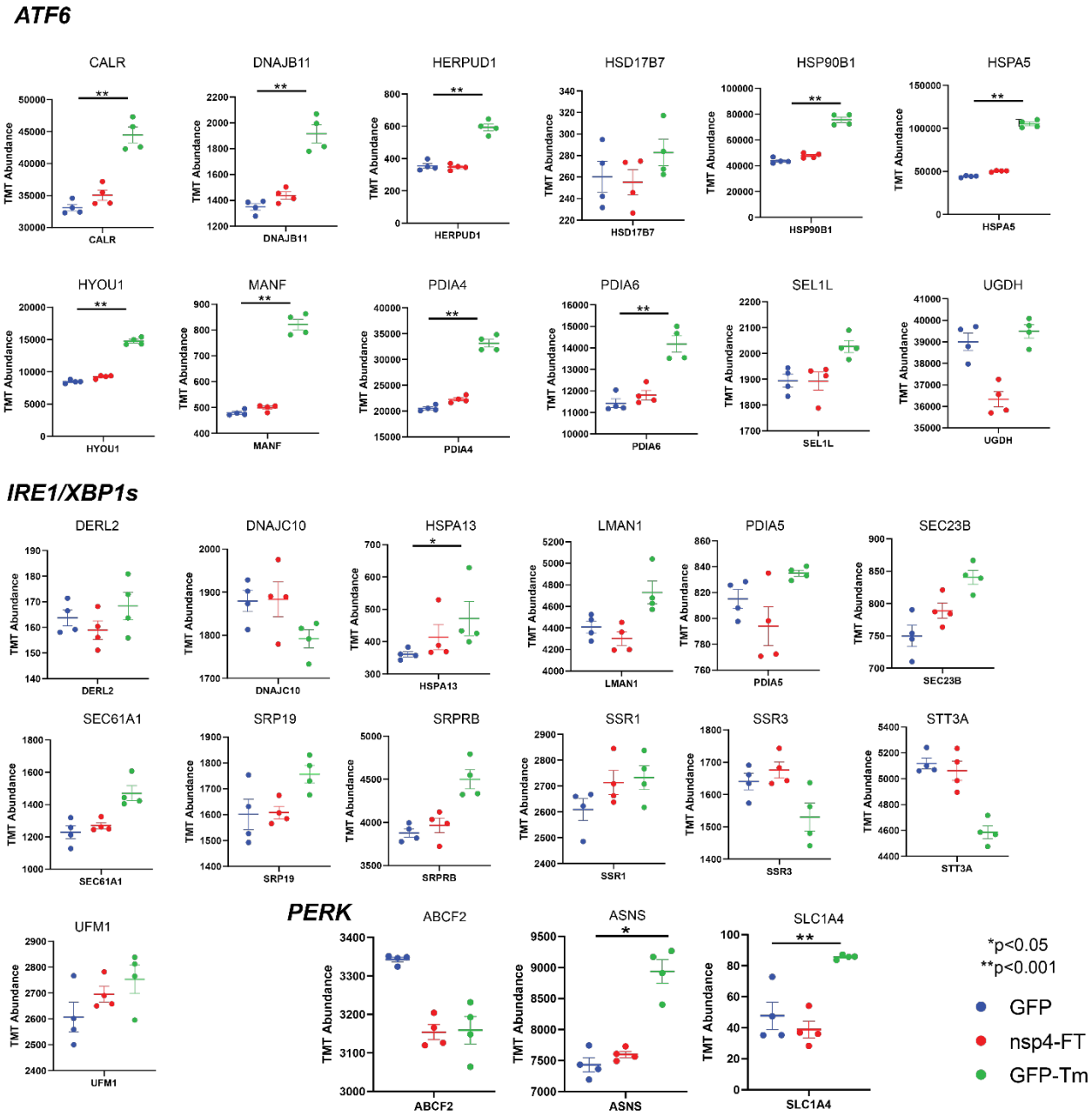


Figure S10. Normalized TMT abundance levels of individual Unfolded Protein Response (UPR) pathway protein markers in A549 cells expressing nsp4-FT or treated with Tm (corresponding to Fig. 2). Adjusted p-value calculated using a T-test, controlling for FDR by the Benjamini-Hochberg method. *p<0.05, **p<0.001

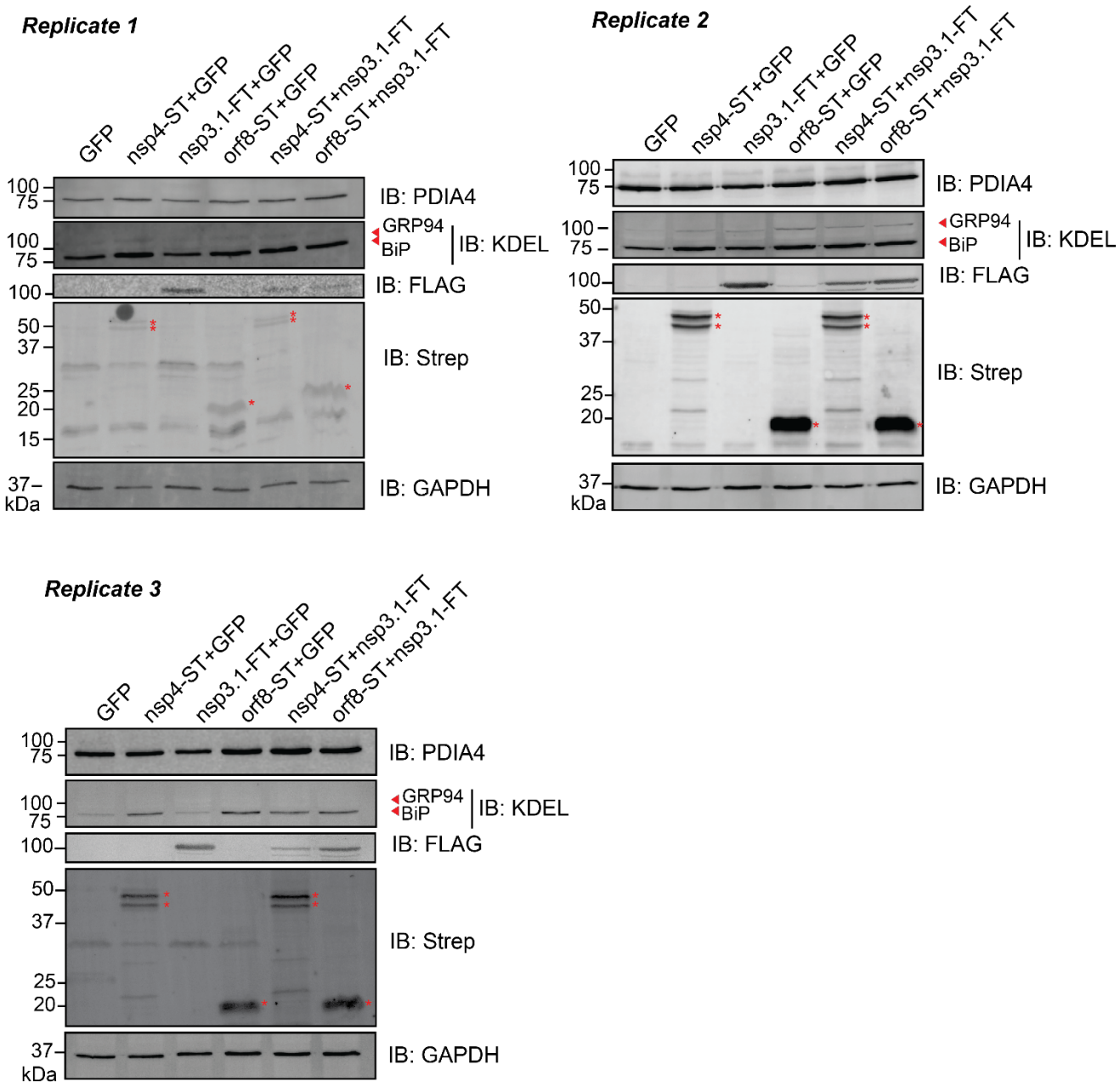
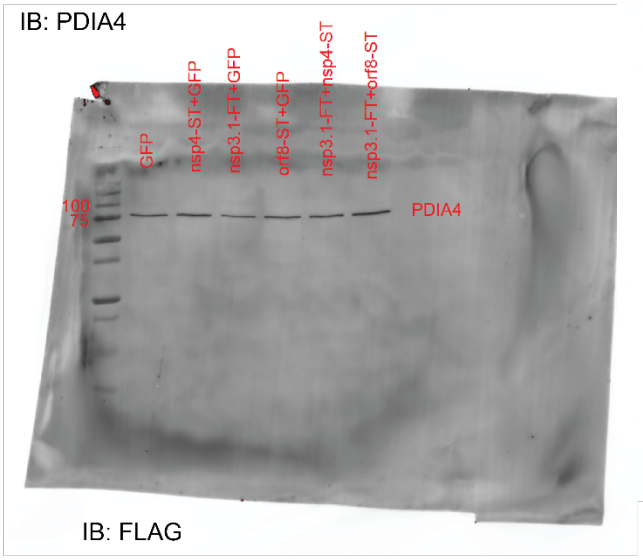
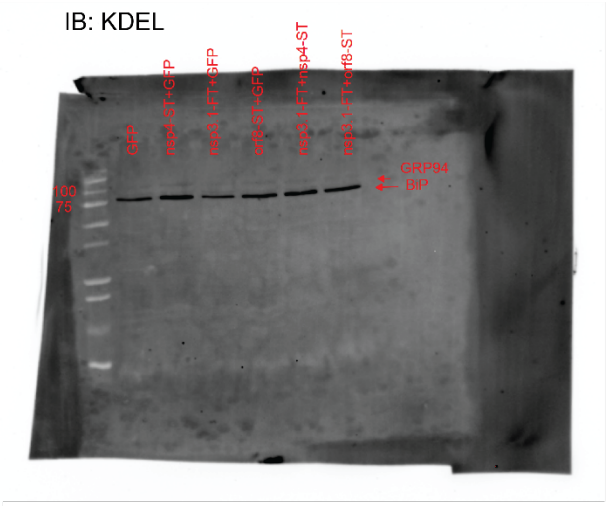


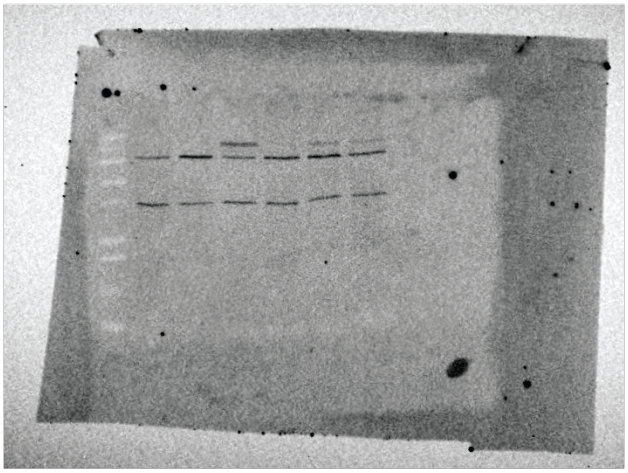
Figure S11. Blots quantified for **Fig. 3c**, replicate 2 was shown in **Fig. 3b**. Red asterisks indicate viral proteins. Corresponding full blots are shown below.

Supplemental Fig. S11 – Replicate 1 Full Western Blots

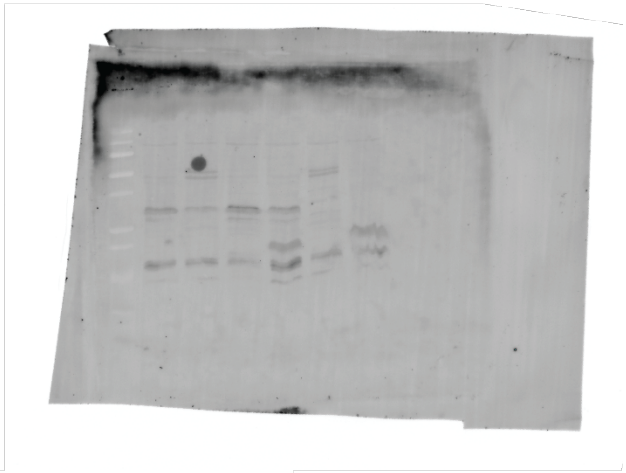




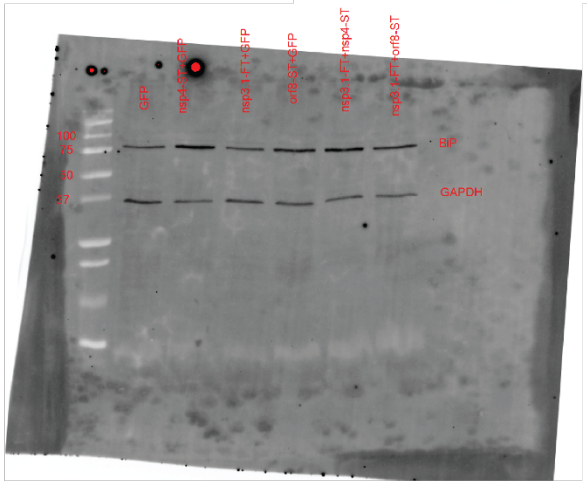
IB: FLAG



IB: Strep

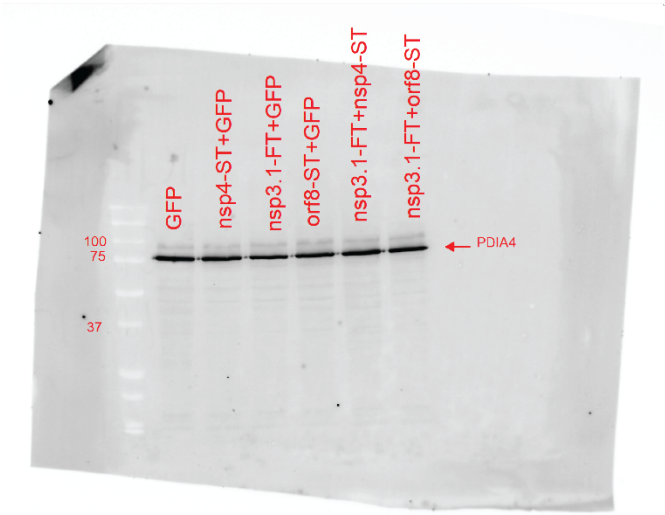


IB: GAPDH

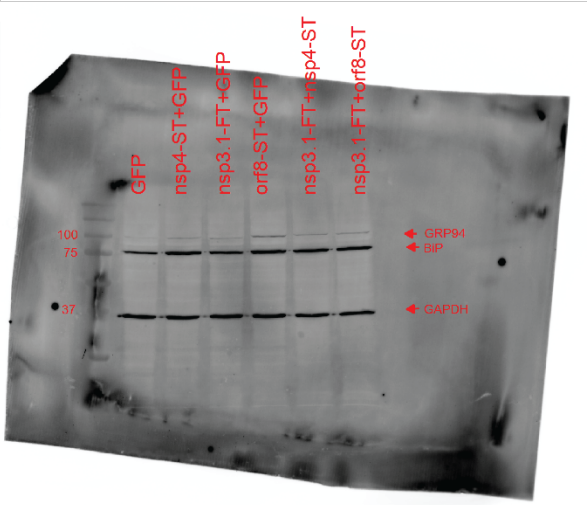


Supplemental Fig. S11 – Replicate 2 Full Western Blots

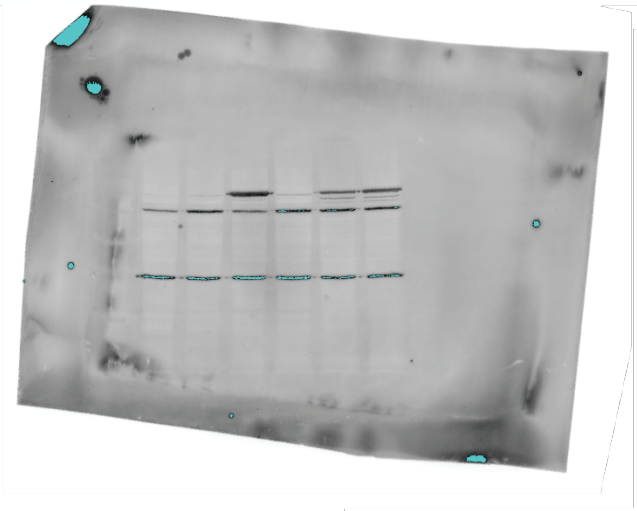
IB: PDIA4



IB: KDEL



IB: FLAG



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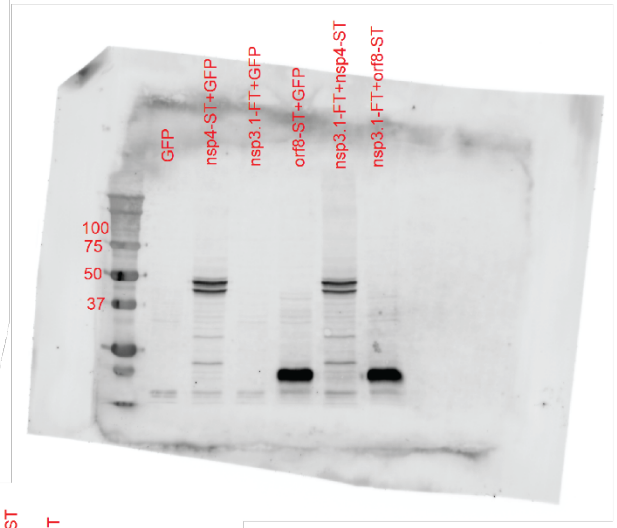
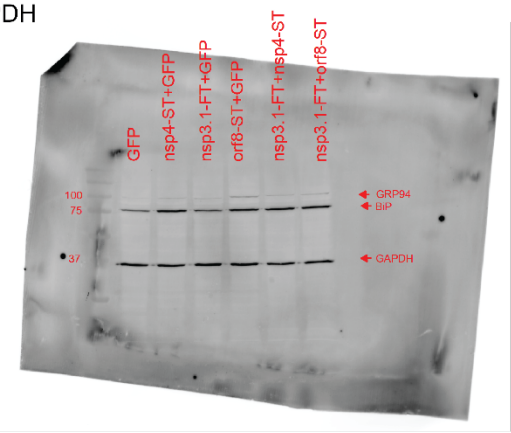
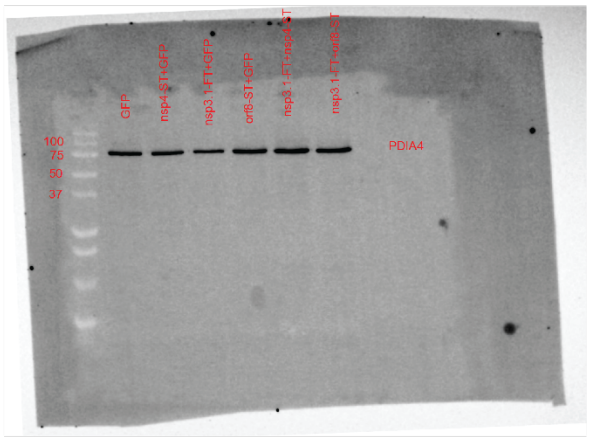


IB: GAPDH

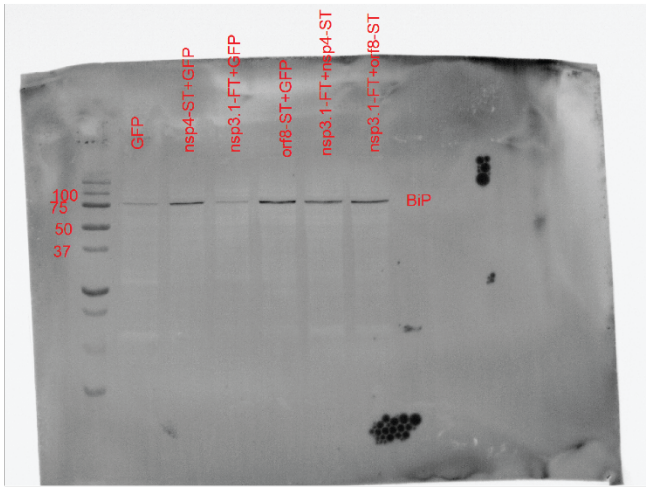


Supplemental Fig. S11 – Replicate 3 Full Western Blots

IB: PDIA4



IB: KDEL



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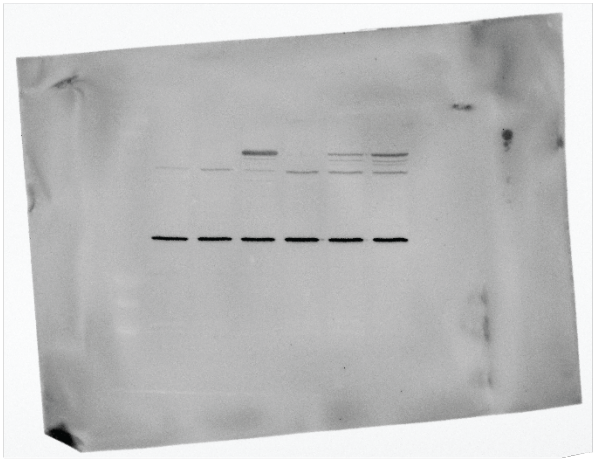
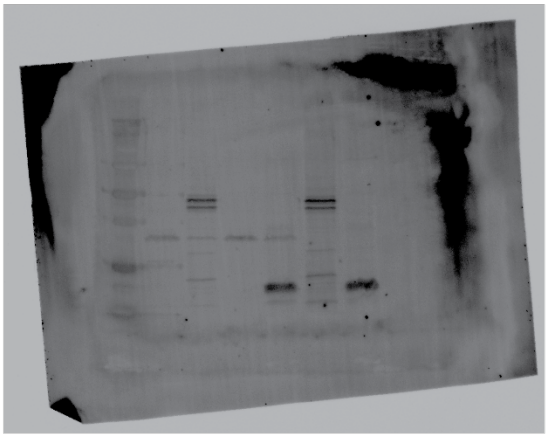
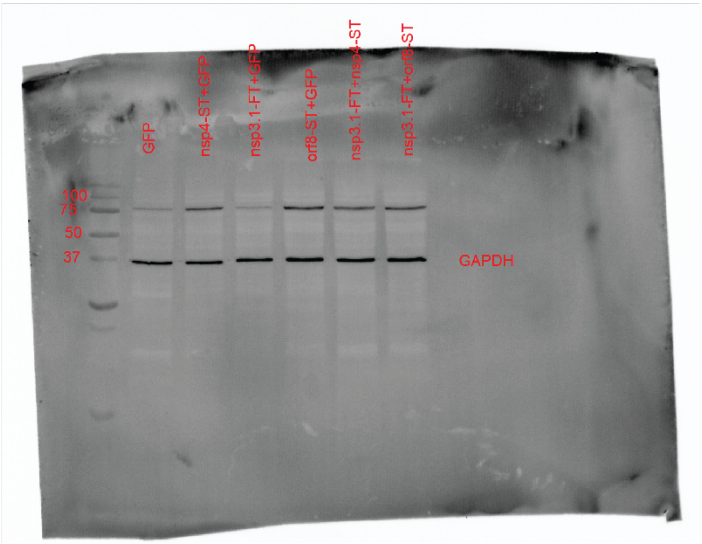


IB: Strep



IB: GAPDH



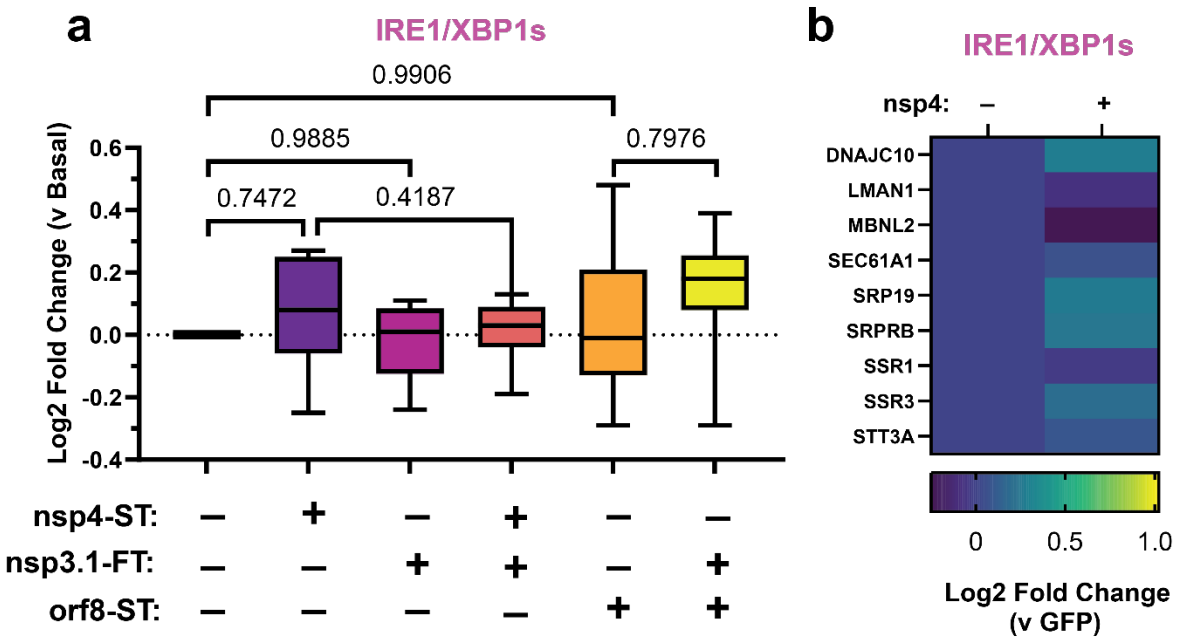


Figure S12. Upregulation of IRE/XBP1s proteomics protein markers.

a) Global proteomics analysis of IRE1/XBP1s protein marker levels in the presence of SARS-CoV-2 nsp4-ST, nsp3.1-FT, orf8-ST, or specified combinations. Box-and-whisker plot shows median, 25th and 75th quartiles, and minimum and maximum values. One-way ANOVA with Geisser–Greenhouse correction and post-hoc Tukey's multiple comparison test was used to determine significance, p<0.05 considered significant; 1 MS run, n = 2-3 biological replicates. See **Supplemental Tables S2, S3** for mass spectrometry data set.

b) Individual IRE1/XBP1s pathway protein markers in the absence or presence of nsp4-ST, from **(a)**.

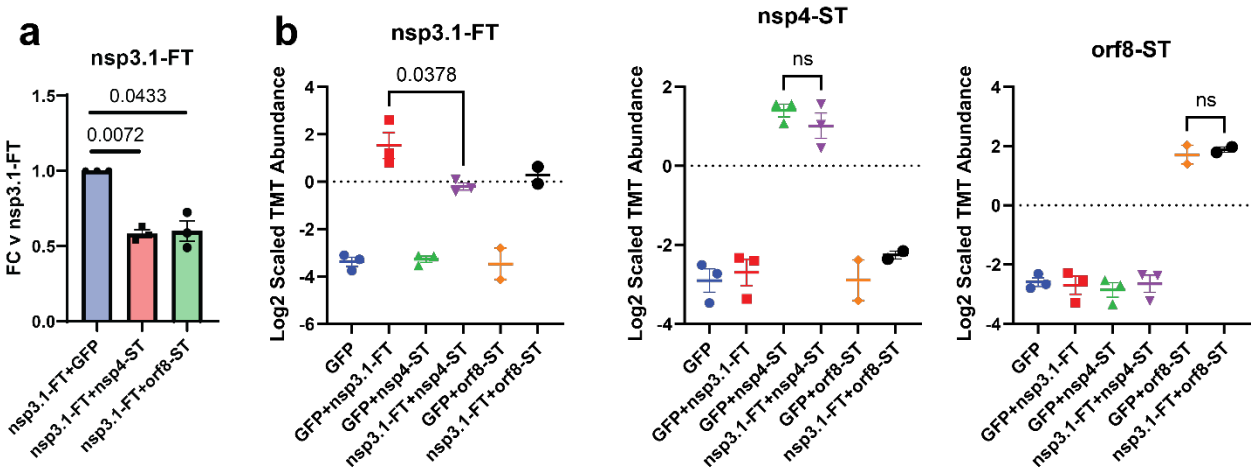


Figure S13. Viral protein abundance levels.

a) Quantification of the nsp3.1-FT bands shown in **Fig. 3, Supplemental Fig. S11**. n = 3. T-test for significance between samples, *p*-value annotated.

b) SARS-CoV-2 nsp3.1-FT, nsp4-ST, and orf8-ST protein abundances (log2 scaled TMT abundances) in experiment corresponding to **Fig. 3.** n = 2-3 biological replicates, 1 MS run, T-test for significance between indicated samples, *p*-value annotated.

References

(1) Szklarczyk, D.; Kirsch, R.; Koutrouli, M.; Nastou, K.; Mehryary, F.; Hachilif, R.; Gable, A. L.; Fang, T.; Doncheva, N. T.; Pyysalo, S.; Bork, P.; Jensen, L. J.; von Mering, C. The STRING Database in 2023: Protein–Protein Association Networks and Functional Enrichment Analyses for Any Sequenced Genome of Interest. *Nucleic Acids Res.* **2023**, *51* (D1), D638–D646. https://doi.org/10.1093/nar/gkac1000.

(2) Almasy, K. M.; Davies, J. P.; Plate, L. Comparative Host Interactomes of the SARS-CoV-2 Nonstructural Protein 3 and Human Coronavirus Homologs. *Mol. Cell. Proteomics* **2021**, *20*, 100120. https://doi.org/10.1016/j.mcpro.2021.100120.