Supplemental Data Fig. 1: ΔQTQTN SARS-CoV-2 replication.

a, Virus stock titer of WT and ΔQTQTN SARS-CoV-2 from Vero E6. b, Plaque morphology of WT and ΔQTQTN in Vero E6. c-d, Competition assay between WT and ΔQTQTN SARS-CoV-2 at a ratio of 1:0 (c) and 0:1 (d) WT:ΔQTQTN, showing RNA percentage from next generation sequencing.
Supplemental Data Fig. 2: Hamster infection with ΔQTQTN SARS-CoV-2.

a, Survival curve (based on euthanasia criteria of >20% weight loss) following infection of WT or ΔQTQTN SARS-CoV-2 (n=10). b, Principal component analysis (PCA) plot of hamster lung samples. c, DESeq2 analysis of mapped genes between WT (purple) and ΔQTQTN (orange) at 2 dpi (left) with upregulated genes indicated in volcano plot (right). d, DESeq2 analysis of mapped genes between WT (purple) and ΔQTQTN (orange) at 4 dpi (left) with upregulated genes indicated in volcano plot (right).
Supplemental Data Fig. 3: ΔQTQTN SARS-CoV-2 replication in TMPRSS2-expressing Vero E6.
Viral titer from TMPRSS2-expressing Vero E6 infected with WT or ΔQTQTN SARS-CoV-2 at an MOI of 0.01 (n=3). Data are mean ± s.d. Statistical analysis measured by two-tailed Student’s t-test. *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001.
Supplemental Data Fig. 4: QTQVN and QVQVN SARS-CoV-2 replication and spike processing.

a, Virus stock titer of WT, QTQVN, and QVQVN SARS-CoV-2 in Vero E6. b, Plaque morphology of QTQVN and QVQVN in Vero E6. c, Quantification by densitometry of full-length (FL), S1/S2 cleavage product, and S2' cleavage product from western blot experiments of glycosylation mutants in Calu-3 2B4. d-e, Purified WT (black), ΔQTQTN (red), QTQVN (blue), and QVQVN (green) SARS-CoV-2 virions from Vero E6 (d) and TMRPSS2-expressing Vero E6 (e) probed with anti-S or anti-N antibody (upper). Full-length (FL), S1/S2 cleavage product, and S2' cleavage product are indicated. Quantification of densitometry of FL, S1/S2, and S2' normalized to N shown (lower). Results are representative of two experiments.
| Virus                 | Coverage [%] | # Peptides | # Unique Peptides | Annotated Sequence | Modifications                                                                 | Abundance          | EThcD | HCD |
|----------------------|--------------|------------|-------------------|--------------------|-----------------------------------------------------------------------------|--------------------|-------|-----|
| SARS-CoV-2 WT        | 91           | 250        | 56                | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | Carbamidomethyl [C3; C16; C25] Phospho [Y14] HexNAc(2)Hex(4)Fuc(1) [N11] | 4.31E+05           | 1.76E+06 |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | Deamidated [N12] Phospho [Y14] HexNAc(2)Hex(5)Fuc(1) [N11]               | Not Detected       | Below Quantitation Limit |
| SARS-CoV-2 QTQVN     | 93           | 284        | 92                | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xPhospho [Y14] 1xHexNAc(2)Hex(3)Fuc(1) [N11] | Not Detected       | 5.61E+05 |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xDeamidated [N12] 1xPhospho [Y14] 1xHexNAc(2)Hex(3)Fuc(1) [N11] | Not Detected       | 5.61E+05 |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xPhospho [Y14] 1xHexNAc(2)Hex(2)Fuc(1) [N11] 1xHexNAc(1)Hex(1)Fuc(1) [S13] | 3.51E+06           | 6.46E+06 |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xPhospho [Y] 1xHexNAc(2)Hex(1)Fuc(2) [S] 1xHexNAc(3)Hex(3)Fuc(1) [S/T] 1xHexNAc(1)Hex(1)Fuc(1) [S13] 1xHexNAc(4)Hex(3)Fuc(1) [S27] | Not Detected       | Below Quantitation Limit |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xPhospho [S13] 1xHexNAc(4)Hex(3)Fuc(1) [N11] 1xHexNAc(2)Hex(1)Fuc(1) [N11] 1xHexNAc(2)Hex(1)Fuc(2) [S27] 1xHexNAc(4)Hex(3)Fuc(2) [S27] | Below Quantitation Limit | Not Detected |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xHexNAc(4)Hex(7)NeuAc(1) [N11] | Not Detected       | Below Quantitation Limit |
| SARS-CoV-2 QVQVN     | 93           | 272        | 78                | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xPhospho [Y14] 1xHexNAc(2)Hex(4)Fuc(1) [N11] | 5.49E+05           | 7.28E+05 |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xDeamidated [N12] 1xHexNAc(3)Hex(5) [N11] | 1.21E+05           | 2.32E+05 |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xPhospho [Y] 1xHexNAc(2)Hex(5)Fuc(1) [N11] | 3.28E+05           | 1.59E+06 |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xDeamidated [N12] 1xPhospho [Y14] 1xHexNAc(2)Hex(5)Fuc(1) [N11] | Not Detected       | Below Quantitation Limit |
|                      |              |            |                   | [R].AGCLIGAEHVNSYECPIGACASYQTQVNSPR.[R]                             | 3xCarbamidomethyl [C3; C16; C25] 1xHexNAc(4)Hex(7)NeuAc(1) [N11] | Not Detected       | Below Quantitation Limit |
Supplemental Data Table S1. Quantification of Nanoflow-LC-MS/MS analysis of QTQTN glycosylation mutants.

Modifications and quantification of peptide spanning Spike 647-682 for WT, QTQVN and QVQVN