**Supplementary materials**

**Mass Spectrometry Analysis of SARS-CoV-2 Nucleocapsid Protein Reveals Camouflaging Glycans and Unique Post-Translational Modifications**

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## Supplementary Materials and Methods

**Expression and purification of SARS-CoV NCP**

Full-length severe acute respiratory syndrome coronavirus (SARS-CoV) nucleocapsid phosphoprotein (NCP) gene (NC\_004718.3) was cloned into pcDNA3.1 vector with a C-terminal 2 X Strep tag. HEK293T cells or Vero cells were transfected with the plasmid using Lipofectamine2000 Transfection Reagent (Invitrogen, 11668019). After 40-60 hours, the cells were lysed in 1 mL cold immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) supplemented with 0.5% Nonidet P 40 Substitute (NP-40, Solarbio), complete EDTA-free protease inhibitor cocktail (Roche, 11873580001) for 30 minutes at 4 °C. The lysates were then centrifuged for 30 min at 15000xg. The remaining lysate was incubated with 30 μL of Strep-Tactin Sepharose beads (IBA Lifesciences) that had been washed in IP buffer and suspended in 0.6 mL IP Buffer. Bead incubation proceeded for 2h with constant rotation at 4 °C. The beads were washed three times with 1 mL of IP buffer containing 0.05% NP-40 followed by one final wash in detergent-free IP Buffer in a fresh detergent-free tube. Proteins were eluted by agitating beads in 40 μL IP buffer supplemented with 2.5 mM D-Desthiobiotin (IBA Lifesciences) on a vortex mixer at room temperature for 30 minutes.

**LC-MSMS experiments:**

Peptides (~0.5 μg) were reconstituted in 2% acetonitrile (ACN) and 0.1% formic acid (FA) and then enriched on an Acclaim PepMap 100 reversed-phase pre-column (20 mm X 75 μm, 5 μm, Thermo Scientific#164535). Peptide separation was carried out by an Acclaim PepMap 100 reversed-phase column (250 mm X 75 μm, 2 μm, Thermo Scientific#164941) on an U3000 nanoUPLC system (Thermo Scientific) at a flow rate of 400 nL/min. The mobile phase A contains 2% ACN, 0.1% FA, and the mobile phase B contains 98% ACN, 0.1% FA. The total analysis time per injection was 120 min. The gradient was designed as follows: 0-6 min, 3% B; 6-7 min, 3%-5% B; 7-70 min, 5%-18% B; 70-90 min, 18%-32% B; 90-100 min, 32%-80% B; 100-110 min, 80% B; 110-120 min, 80%-3% B.

The nanoLC was coupled to an Orbitrap Q-Exactive HFX mass spectrometer (Thermo). The source was operated at 2.3 kV. For post-translational modification (PTM) characterizations (including phosphorylation and O18 deamidation profiling), the data-dependent analysis (DDA) scheme included a full MS survey scan from 350 to 1,800 Th at the resolution of 120,000 FWHM (at m/z 200 Th) with automatic gain control (AGC) set to 1e6 and MS1 maximum injection time (MIT) set to 60ms, followed by MS2 scans of 20 most intense peaks selected for fragmentation by higher-energy collision dissociation (HCD), with normalized collision energy (NCE) set to 27%. The MS2 spectra were acquired at 15,000 FWHM resolution with AGC set to 1e5 and MS2 MIT set to 60 ms. For intact glycopeptide analysis, the above DDA method was modified as follows: full MS survey scan was set from 800 to 2,300 Th with AGC set to 3e6 and MS1 MIT set to 120ms, followed by 20 MS2 spectra acquired with AGC set to 5e5 and MS2 MIT set to 250 ms, while HCD with step NCE was set at 21%, 27%, 38%.

**Supplementary Figures**

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**Figure S1** Mass spectra showing the identification of peptides containing phosphorylation in S176 (**A**) and T379 (**B**) in SARS-CoV-2 NCP. NCP, nucleocapsid phosphoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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**Figure S2** Mass spectra showing the identification of deglycopeptides with N77 (**A**) and N269 (**B**) labeled with O18 deamidation, mass spectra of intact glycopeptides containing N77 (**C**) and N269 (**D**) glycan in SARS-CoV-2 NCP. NCP, nucleocapsid phosphoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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**Figure S3 A**. Alignment analysis to coronaviruses sequences related to K169, K374 and K388 ubiquitination sites in SARS-CoV-2 NCP. Homologous sites were marked red. Uniprot Accession ID and Entry were list on the left. CVHN5, human coronavirus HKU1; CVH22, human coronavirus 229E; CVHNL, human coronavirus NL63; CVHOC, human coronavirus OC43; BCHK3, bat coronavirus HKU3; CVM1, murine coronavirus 1; IBVB, avian infectious bronchitis virus; MERS, Middle East respiratory syndrome-related coronavirus. **B**. Immunoprecipitated SARS-CoV NCP were immunoblotting analyses for ubiquitination and ISGylation. NCP, nucleocapsid phosphoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Fig3S.tif**Figure S4** Alignment analysis to coronaviruses sequences related to N77 and N269 glycosylation sites in SARS-CoV-2 NCP. Homologous glycosylation sites were marked red and *N*-glycosylation sequons (N-X-S/T, X≠P) were enclosed by box. Uniprot Accession ID and Entry were list on the left. CVHN5, human coronavirus HKU1; CVH22, human coronavirus 229E; CVHNL, human coronavirus NL63; CVHOC, human coronavirus OC43; BCHK3, bat coronavirus HKU3; CVM1, murine coronavirus 1; IBVB, avian infectious bronchitis virus; MERS, Middle East respiratory syndrome-related coronavirus; NCP, nucleocapsid phosphoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

**Supplementary Tables**

**Table S1: Summary of all PTM identification on SARS-CoV-2 NCP (Excel table)**

**Table S2: Summary of phosphorylated sites identified on SARS-CoV-2 NCP by this and previous studies (Excel table)**

**Table S3: Summary of glycan and O18-labeled deamidation identification on SARS-CoV-2 NCP (Excel table)**