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# Proteomic Profiling of SARS-CoV-2 Virus-Host Interactions and Implications for Antiviral Drug Discovery

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*SH generated and validated the cell lines expressing SARS-CoV-2 viral proteins using AP-MS and BioID methods. SH aided sample generation and the validation of protein-protein interactions. SH had important role in manuscript writing and figure preparation.*

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*SH Designed the study, generated the cell lines expressing SARS-CoV-2 viral proteins using and prosessed samples using BioID method, performed of the data-analysis, interpreted the results, wrote the manuscript and prepared final figures.*

# Abbreviations

| | |
|---|---|
| **ACE2** | Angiotensin-Converting Enzyme 2 |
| **ADP** | Adenosine Diphosphate |
| **AP** | Affinity Purification |
| **AP-MS** | Affinity Purification-Mass Spectrometry |
| **ARDS** | Acute Respiratory Distress Syndrome |
| **ATP** | Adenosine Triphosphate |
| **ATPase** | ATP Hydrolyzing Enzyme |
| **BP** | Biological Process (GO category) |
| **BSA** | Bovine Serum Albumin |
| **BioID** | Proximity-Dependent Biotin Identification |
| **BioID-MS** | Proximity-Dependent Biotin Identification coupled with Mass Spectrometry |
| **BirA*** | Mutant *Escherichia coli* biotin ligase used in BioID experiments |
| **CC** | Cellular Component (GO category) |
| **COVID-19** | Coronavirus disease 2019 |
| **DDT** | Dithiothreitol |
| **DIA** | Data-Independent Acquisition |
| **DMV** | Double-Membrane Vesicle |
| **DNA** | Deoxyribonucleic Acid |
| **DRAQ5** | DNA-binding fluorescent dye |
| **E** | Envelope (Protein) |
| **ECL** | Enhanced Chemiluminescence |
| **ERAD** | Endoplasmic Reticulum-Associated Degradation |
| **ERGIC** | Endoplasmic Reticulum-Golgi Intermediate Compartment |
| **FDR** | False Discovery Rate |
| **FRT** | Flippase Recognition Target |
| **Flp** | Flippase (Recombinase) |
| **GFP** | Green Fluorescent Protein |
| **GO** | Gene Ontology |
| **HA** | Hemagglutinin (Tag) |
| **HCI** | High-Content Imaging |
| **HCIP** | High-Confidence Interaction Partners |
| **HEK** | Human Embryonic Kidney (Cells) |
| **IFD** | Induced Fit Docking |
| **IFN** | Interferon |
| **IMAC** | Immobilized Metal Affinity Chromatography |
| **KEGG** | Kyoto Encyclopedia of Genes and Genomes |
| **LC-MS/MS** | Liquid Chromatography coupled with Tandem Mass Spectrometry |
| **M** | Membrane (Protein) |
| **MAC** | Multiple Approaches Combined (Tag) |
| **MF** | Molecular Function (GO category) |

| | |
|---|---|
| **MHC** | Major Histocompatibility Complex |
| **MHC-I** | Major Histocompatibility Complex Class I |
| **MOI** | Multiplicity of infection |
| **MS** | Mass Spectrometry |
| **MS/MS** | Tandem Mass Spectrometry |
| **N** | Nucleocapsid (Protein) |
| **NK** | Natural Killer (Cells) |
| **NSP** | Non-Structural Protein |
| **ORF** | Open Reading Frame |
| **PASEF** | Parallel Accumulation-Serial Fragmentation |
| **PBS** | Phosphate-Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **PKR** | Protein Kinase R |
| **POI** | Protein of Interest |
| **POTS** | Postural Orthostatic Tachycardia Syndrome |
| **PPi** | Inorganic Pyrophosphate |
| **PRR** | Pattern Recognition Receptor |
| **PSM** | Peptide-Spectrum Matches |
| **PTM** | Post-Translational Modification |
| **RBD** | Receptor-Binding Domain |
| **RCT** | Replication-Transcription Complex |
| **RIG-1** | Retinoic acid-Inducible Gene I |
| **RNA** | Ribonucleic Acid |
| **RT** | Room Temperature |
| **RdRp** | RNA-dependent RNA Polymerase |
| **S** | Spike (Protein) |
| **SAINT** | Significance Analysis of INTeractome |
| **SARS-CoV-2** | Severe Acute Respiratory Syndrome Coronavirus 2 |
| **SDS-PAGE** | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| **TBS-T** | Tris Buffered Saline with Tween |
| **TLR** | Toll-Like Receptor |
| **TMPRSS2** | Transmembrane Protease Serine 2 |
| **VOCs** | Variants of Concern |
| **WT** | Wild Type |
| **mRNA** | Messenger Ribonucleic Acid |
| **ssRNA** | Single-Stranded Ribonucleic Acid |

# Tiivistelmä

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) on koronavirus, joka aiheuttaa COVID-19-tautia. Tämä virus levisi nopeasti maailmanlaajuiseksi pandemiaksi, ja viimeisten viiden vuoden aikana COVID-19 on vaatinut yli arviolta kahdeksan miljoonan ihmisen hengen sekä aiheuttanut merkittävää taloudellista haittaa. Vaikka virusta ja sen vaikutuksia on tutkittu intensiivisesti, toistaiseksi markkinoille on saatu vain muutama hyväsytty viruslääke, kuten remdesivir ja Paxlovid, SARS-CoV-2:ta vastaan.

Tässä väitöskirjassa tutkitaan SARS-CoV-2:n ja ihmisen isäntäsolujen välisten proteiinien vuorovaikutuksia, jotka ovat keskeisiä viruksen replikaation ja immuunivasteen väistämisen kannalta. Väitöskirjan tavoitteena on edistää ymmärrystä viruksen ja isäntäsolun välisten vuorovaikutusten molekulaarisista mekanismeista sekä tuoda esiin proteomiikan merkitys viruslääkkeiden kehittämisessä. Tutkimuksessa hyödynnetään muun muassa affiniteettipuhdistusta massaspektrometrialla (AP-MS) ja proteiinien välittömän lähiympäristön biotiinitunnistusta (BioID), joiden avulla pyritään tunnistamaan ne isäntäproteiinit, joita virus käyttää tehokkaasti lisääntyäkseen. Näiden vuorovaikutusten kartoittaminen tarjoaa mahdollisuuden tunnistaa olemassa olevia lääkeaineita, joita voitaisiin käyttää SARS-CoV-2-infektioiden hoitoon lääkkeiden uudelleenkäytön periaatteella.

Lisäksi väitöskirjan osana on kehitetty kattava "hijackome"-tietokanta, joka kokoaa yhteen SARS-CoV-2:n ja eri isäntäproteiinien vuorovaikutuksia useiden virusvarianttien, kuten Alfa-, Beta-, Delta- ja Omikron-varianttien osalta. Proteomi- ja fosfoproteomianalyysien avulla tarkastellaan varianttikohtaisia eroja isäntäsolujen manipuloinnissa, viruksen replikaatiotehokkuudessa ja immuunijärjestelmän väistämisessä. Tämä tarkastelu tarjoaa uutta tietoa siitä, miten virus sopeutuu ja kehittyy säilyttääkseen infektiokyvyn ja välttääkseen immuunipuolustuksen. Tulokset tarjoavat tieteellistä perustaa uusille terapeuttisille strategioille SARS-CoV-2:ta ja mahdollisesti myös muita viruksia vastaan.

# Abstract

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) is a coronavirus that causes COVID-19. The virus rapidly spread into a global pandemic, and over the past five years, COVID-19 has claimed more than eight million lives and caused significant economic disruption. Although several antiviral agents, such as remdesivir and Paxlovid, have been approved for clinical use, only a limited number of effective antiviral drugs targeting SARS-CoV-2 are currently available.

This doctoral thesis investigates the protein–protein interactions between SARS-CoV-2 and human host cells, which are critical for viral replication and immune evasion. The aim of this work is to enhance our understanding of the molecular mechanisms underlying virus–host interactions and to highlight the role of proteomics in antiviral drug discovery. The study employs methods such as affinity purification coupled with mass spectrometry (AP-MS) and proximity-dependent biotinylation (BioID) to identify host proteins that are exploited by the virus to support its replication. Mapping these interactions provides opportunities for drug repurposing by identifying existing compounds that may be effective against SARS-CoV-2.

In addition, this thesis includes the development of a comprehensive "hijackome" database that compiles SARS-CoV-2–host protein interactions across multiple viral variants, including Alpha, Beta, Delta, and Omicron. Proteomic and phosphoproteomic analyses are used to investigate variant-specific differences in host cell manipulation, replication efficiency, and immune evasion strategies. This temporal and variant-level analysis provides novel insights into how emerging variants adapt to sustain infection and evade immune detection. The findings offer a scientific foundation for the development of new therapeutic strategies targeting SARS-CoV-2 and potentially other viral pathogens.

# Index

# I Introduction

The focus of this thesis is to explore the molecular interactions between the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and host cell proteins, which are fundamental to virus replication and evasion on host immune responses. By understanding these interactions, it is possible to identify critical points in the viral life cycle that can be targeted for antiviral drug discovery.

This study employs advanced proteomic techniques, including Affinity Purification-Mass Spectrometry (AP-MS) and Proximity-Dependent Biotin Identification (BioID) to systematically map the interactions between viral and host proteins. These techniques enable the identification of both direct and spatially proximate protein interactions, providing a detailed view of how SARS-CoV-2 hijacks host cellular machinery to support its replication.

The core of this work lies in the comprehensive proteomic analysis of SARS-CoV-2-host interactions, with a special emphasis on how these interactions differ across various viral variants. By characterizing the temporal and spatial protein expression profiles in infected cells, this research uncovers the mechanisms by which different variants of concern, including Alpha, Beta, Delta, and Omicron, enhance viral replication and immune evasion. This detailed proteomic profiling provides essential insights into variant-specific manipulations of host cell processes.

Furthermore, this thesis aims to establish a foundation for antiviral drug repurposing by identifying host proteins that are crucial for viral survival. By targeting these proteins, existing drugs can be rapidly evaluated for their potential to inhibit SARS-CoV-2 replication, offering a promising avenue for the development of broad-spectrum antiviral therapies. The findings from this study furthermore contribute to a growing body of knowledge that can be applied to combat future viral outbreaks by leveraging proteomic approaches in drug discovery.

# 1. COVID-19 disease caused by SARS-CoV-2

The COVID-19 pandemic, caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has posed significant global health challenges since its emergence in late 2019. SARS-CoV-2 is a member of the *Coronaviridae* family, and it shares structural and genetic similarities with other coronaviruses, such as SARS-CoV and MERS-CoV (Rathore and Ghosh 2020).

SARS-CoV-2 primarily targets the Angiotensin-Converting Enzyme 2 (ACE2) receptor, which is abundantly expressed on the surface of epithelial cells in the lungs, heart, kidneys, and other organs. Once bound to the receptor, the virus fuses with the cell membrane and releases its RNA genome into the cytoplasm (Jackson et al. 2022). Inside host cell, the viral RNA is translated into viral proteins using host cell ribosomes. These proteins assemble new viral particles by copying the viral genome and packaging it into new virions (V'kovski et al. 2021). Virus replication often results in direct damage to the infected cells, leading to cell death or by triggering programmed cell death mechanisms such as apoptosis (Yuan et al. 2023).

Virus transmission occurs primarily through respiratory droplets and in contact with contaminated surfaces. Virus can cause a range of symptoms, from mild respiratory illness to severe pneumonia and acute respiratory distress syndrome (ARDS). The severity of the disease is influenced by host factors such as age, comorbidities, and immune response (Harrison, Lin, and Wang 2020). Older adults and those with pre-existing health conditions are at higher risk of severe illness and death. A robust initial immune response can mitigate disease severity, while an overactive immune response can lead to inflammatory damage and multi-organ failure (Nazerian et al. 2022).

When comparing COVID-19 to other circulating diseases with similar transmission type, such as Influenza and common cold (Table 1.), COVID-19 tends to result in more severe complications, especially in vulnerable populations. Co-infections with these viruses can worsen outcomes, increasing the risk of hospitalization and prolonged illness. Complications from COVID-19 extend beyond the acute phase of infection. Many patients experience lingering symptoms for weeks or months after the initial illness, a condition commonly referred to as "long COVID" (Raveendran, Jayadevan, and Sashidharan 2021). These symptoms may include fatigue, shortness of breath, neurological effects, and cardiovascular issues, significantly impacting quality of life.

Efforts to combat the spread of SARS-CoV-2 have included public health measures such as testing, contact tracing, social distancing, mask usage, and vaccination campaigns, which have played crucial roles in reducing transmission. Multiple vaccines, including those based on mRNA and viral vectors, have demonstrated high efficacy in preventing severe disease and death (Fayez *et al.*, 2023). Supportive care, such as oxygen therapy and mechanical ventilation, remains critical for patients with severe respiratory compromise (Murakami et al. 2023; CDC 2024b; WHO 2024; Toussi et al. 2023). The emergence of new variants of SARS-CoV-2 has posed ongoing challenges. Variants with mutations that enhance transmissibility or confer resistance to neutralizing antibodies require continuous monitoring and adaptation of public health strategies (WHO 2023). Despite the availability of vaccines and antiviral treatments, emerging variants of the virus demonstrate enhanced transmissibility, immune evasion, and varying levels of pathogenicity, underscoring the need for ongoing research into virus-host interactions. Understanding the viral genetic composition and molecular protein structures of SARS-CoV-2 is crucial for elucidating its pathogenesis, transmission, and for developing effective therapeutic and preventive measures (Lai et al. 2020).

*Table1. List of Symptoms of SARS-CoV-2 compared to Influenza, and common cold*

| Symptom/Category | SARS-CoV-2 (COVID-19) | Influenza (Flu) | Common Cold |
|---|---|---|---|
| **Fever** | Yes | Yes | Rare |
| **Cough** | Yes | Yes | Mild |
| **Shortness of Breath** | Yes | Rare | No |
| **Fatigue** | Yes | Yes | Sometimes |
| **Loss of Taste or Smell** | Yes | Rare | Rare |
| **Sore Throat** | Yes | Yes | Yes |
| **Muscle or Joint Pain** | Yes | Yes | Rare |
| **Headache** | Yes | Yes | Mild |
| **Runny or Stuffy Nose** | Yes | Yes | Yes |
| **Chills** | Yes | Sometimes | No |
| **Vomiting/Diarrhea** | Rare | Sometimes | No |
| **Pneumonia** | Severe | Possible | No |
| **Acute Respiratory Distress** | Severe | Rare | No |
| **Multiorgan Failure** | Severe | No | No |
| **Blood Clotting Issues** | Possible | No | No |
| **Worsening of Chronic Conditions** | Possible | Yes | No |
| **Death Risk** | 1%–3% | 0.1%–0.2% | Extremely rare |
| **Total Global Cases** | Over 770 million (as of 2023) | 1 billion per year (estimated) | Billions per year (no precise tracking) |
| **Total Global Deaths** | ~7 million confirmed (as of 2023) | 290,000–650,000 per year (estimated) | Extremely rare |

(Flerlage et al. 2021; CDC 2024a; NFID 2024)

## 2. Genome structure and proteins encoded by SARS-CoV-2

SARS-CoV-2 is an enveloped virus with a positive-sense single-stranded RNA genome approximately 29.9 kilobases (kb) in length (Harrison, Lin, and Wang 2020). This genome is one of the largest among RNA viruses and is characterized by several distinct regions encoding structural, non-structural, and accessory proteins (Figure 1). The 5' two-thirds of the SARS-CoV-2 genome encodes two large open reading frames (ORF1a and ORF1b), which are translated into polyproteins pp1a and pp1ab. These polyproteins are subsequently cleaved by viral proteases into 16 non-structural proteins (NSPs 1-16) (Table 2.). The remaining one-third of the genome encodes the four main structural proteins of SARS-CoV-2, which are crucial for the formation of new virions and the virus ability to infect host cells. These structural proteins include the spike (S) protein, the envelope (E) protein, the membrane (M) protein**,** and the nucleocapsid (N) protein**.** The spike protein, in particular, plays a key role in facilitating viral entry by binding to the ACE2 receptor on host cells, making it a primary target for vaccines and antibody-based therapies.

In this section, we will explore the SARS-CoV-2 proteins in greater detail, focusing on the functions of its non-structural and structural proteins and their roles in the viral life cycle during COVID-19 infection. The overall viral cycle and progression of SARS-CoV-2 infection will be further discussed in section 3.

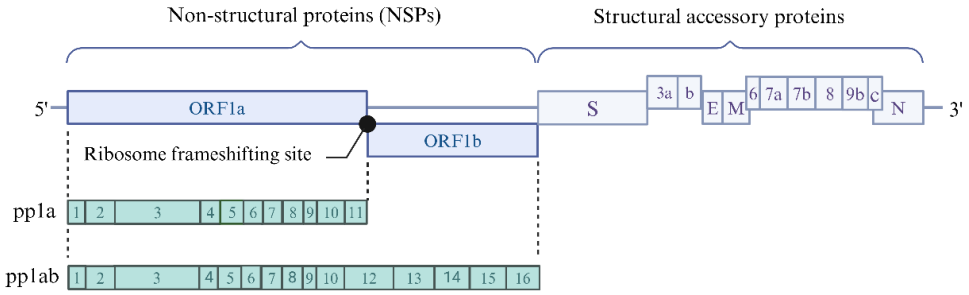


***Figure 1. SARS-CoV-2 genome stucture****. The genome is composed of two large overlapping ORFs, ORF1a and ORF1b, which encode non-structural proteins involved in viral replication and transcription. ORF1a translates directly, while ORF1b is translated following a ribosomal frameshift. The lower panel displays the processing of the polyprotein into individual NSPs. ORF1a is cleaved into NSPs 1 through 11, while ORF1b is cleaved into NSPs 12 through 16. The structural proteins are encoded downstream of ORF1b, including the S, E, M, and N proteins, as well as additional accessory proteins encoded by ORFs 3a, 6, 7a, 7b, 8, 9b, and 9c. Created in BioRender. Huuskonen, S. (2022) BioRender.com/w05e978"*

*Table 2. SARS-CoV-2 proteins and their functions*

| Protein | Function |
|---|---|
| **NSP1** | Suppresses host immune response by inhibiting host mRNA translation and promoting degradation |
| **NSP2** | Interacts with host proteins, possibly affecting cellular processes |
| **NSP3** | Papain-like protease, cleaves polyproteins; involved in viral replication and immune evasion |
| **NSP4** | Involved in double-membrane vesicle formation for replication complexes |
| **NSP5** | Main protease (3CLpro), cleaves viral polyproteins to release functional NSPs |
| **NSP6** | Involved in autophagy and membrane rearrangement |
| **NSP7** | Forms complex with NSP8 to enhance RNA polymerase activity |
| **NSP8** | Cofactor with NSP7, involved in RNA synthesis |
| **NSP9** | Binds to single-stranded RNA, involved in viral RNA replication |
| **NSP10** | Cofactor for NSP14 and NSP16, enhancing their enzymatic activities |
| **NSP11** | Short peptide, potential regulatory role in viral replication, function not well understood |
| **NSP12** | RNA-dependent RNA polymerase (RdRp), crucial for viral RNA replication |
| **NSP13** | Helicase, unwinds RNA duplexes for replication and transcription |
| **NSP14** | Exonuclease, proofreading function; also has N7-methyltransferase activity |
| **NSP15** | Endoribonuclease, processes viral RNA |
| **NSP16** | 2'-O-ribose methyltransferase, involved in RNA capping to evade host immune response |
| **Spike (S)** | Mediates attachment to and entry into host cells via ACE2 receptor |
| **Envelope (E)** | Involved in viral assembly, release, and ion channel activity |
| **Membrane (M)** | Most abundant protein, plays a key role in viral assembly and shape |
| **Nucleocapsid (N)** | Encapsulates viral RNA genome, involved in RNA synthesis and regulation of host cell processes |
| **ORF3a** | Ion channel activity, involved in virus release and apoptosis |
| **ORF6** | Interferes with host immune response, blocks type I interferon signaling |
| **ORF7a** | Inhibits host cell apoptosis, contributes to viral pathogenesis |
| **ORF7b** | Role in viral pathogenesis, not well understood |
| **ORF8** | Modulates immune response, induces endoplasmic reticulum stress |
| **ORF9b** | Antagonizes host immune response by targeting mitochondrial function and suppressing interferon signaling |
| **ORF9c** | Interacts with host proteins, may affect immune signaling and cellular pathways, function not fully understood |

### 2.1 Non-Structural proteins of SARS-CoV-2

Non-stuctural proteins (NSPs), of SARS-CoV-2 are viral proteins produced during the infection process but are not part of the virus structural components (such as the spike, envelope, membrane, and nucleocapsid proteins). These NSPs are crucial for viral replication, transcription, immune evasion, and overall pathogenesis. SARS-CoV-2 produces NSPs from two large polyproteins (pp1a and pp1ab), which are then cleaved by viral proteases into 16 smaller functional proteins (NSP1 to NSP16). In this subsection, we discuss the individual NSPs, highlighting their specific roles in the viral life cycle and interactions with host cellular mechanisms.

NSP1 interferes with host cell function primarily by binding to the 40S ribosomal subunit, blocking the translation of host mRNAs. This "host shutoff" activity results in reduced production of proteins essential for innate immunity, including type I interferons and cytokines (Lei et al. 2020). Additionally, NSP1 promotes the degradation of host mRNAs, further contributing to the suppression of immune responses. Importantly, SARS-CoV-2 viral mRNAs are not affected by NSP1 inhibitory effects, allowing viral proteins to be translated efficiently (Fisher et al. 2022; Vazquez et al. 2021; Bujanic et al. 2022). The inhibition of host protein synthesis by NSP1 enables the virus to evade early immune detection, delaying the host antiviral response (Yuan et al. 2021). By preventing the production of interferon-stimulated genes (ISGs), NSP1 diminishes the activation of immune cells responsible for controlling viral spread. This delay in the immune response facilitates higher viral loads and contributes to the severity of COVID-19.

Although less is known about NSP2 compared to other SARS-CoV-2 proteins, studies suggest it influences pathways vital for cellular homeostasis. NSP2 is highly conserved across coronaviruses, underlining its evolutionary importance (Zheng et al. 2021; Naeli et al. 2023). NSP2 disrupts host homeostasis through interactions with proteins involved in intracellular trafficking and organelle maintenance, particularly binding the prohibitin (PHB) complex, which regulates mitochondrial function and cellular stress responses (Zheng et al. 2021). This interaction may alter mitochondrial processes and create conditions conducive to viral replication, though the downstream effects remain unclear. While NSP2 exact role in the viral lifecycle is still being studied, its ability to disrupt signaling and organelle integrity suggests it helps optimize viral propagation. Additionally, NSP2 may indirectly affect the immune response by perturbing cellular homeostasis, leading to dysregulated signaling during infection (Zheng et al. 2021; Naeli et al. 2023).

NSP3 is one of the non-structural proteins encoded by SARS-CoV-2, playing a role in the viral life cycle. Its functions are central to viral replication, immune evasion, and the formation of specialized structures within host cells. One of NSP3 key functions is its papain-like protease (PLpro) activity, which cleaves the viral polyprotein into individual, functional non-structural proteins (Klemm et al. 2020; Yang et al. 2023). Assembling the virus replication-transcription complex (RTC), essential for viral RNA replication. PLpro also deubiquitinates and removes ISG15, small ubiquitin-like protein that plays a crucial role in the immune response, from host proteins, helping SARS-CoV-2 evade the host immune system (Klemm et al. 2020). Another important domain within NSP3 is its macrodomain, which binds to ADP-ribose and reverses ADP-ribosylation, a host defense mechanism (Hoch 2021). This ability to reverse adenosine diphosphate (ADP)-ribosylation enhances the virus ability to counteract cellular defenses, further promoting immune evasion (Hoch 2021; Du et al. 2023). NSP3 additionally plays a structural role in viral replication by interacting with NSP4 and NSP6 to form double-membrane vesicles (DMVs) (Angelini et al. 2013; Yixin Huang et al. 2024). These DMVs protect viral RNA from detection and degradation by the host immune response, providing an environment for viral RNA synthesis (Roingeard et al. 2022).

As previously mentioned, the NSP4, along with NSP3 and NSP6, is involved in the formation of DMVs, which are important for viral replication (Zimmermann et al. 2023; Huang et al. 2024). Given its crucial role in viral replication and membrane rearrangement, NSP4 is considered a potential therapeutic target. Inhibiting its interaction with NSP3 or NSP6, or disrupting its ability to induce membrane rearrangements, could impair the virus ability to replicate (Chakraborty et al. 2022).

NSP5, known as the 3C-like protease (3CLpro) or main protease ($M^{pro}$**)**, is a critical enzyme for SARS-CoV-2 replication (Parmar et al. 2022). It is responsible for cleaving the viral polyprotein into smaller, functional NSPs essential for assembling the virus RTC (Mishchenko and Ivanisenko 2022). NSP5 functions as a protease, specifically cleaving 11 sites within the viral polyprotein. This cleavage releases vital non-structural proteins, including NSP12 (RNA-dependent RNA polymerase) and other components necessary for viral RNA synthesis (Scott et al. 2022; Hillen et al. 2020). Without this cleavage, the viral replication machinery cannot function, making NSP5 a key player in the viral life cycle.

Similar to NSP3 and NSP4, NSP6 is crucial in generating DMVs (Ricciardi et al. 2022). NSP6 interacts with various host cell proteins involved in vesicle trafficking and membrane dynamics, altering intracellular pathways to favor viral replication. These interactions not only support the formation of replication compartments but also may play a role in evading immune detection (Bills, Xie, and Shi 2023; Ricciardi et al. 2022). Studies suggest that NSP6 modulates ER stress and autophagy-related pathways to help the virus control the cellular environment and replicate more efficiently (Jiao et al. 2023). NSP6 is involved in the formation of autophagosomes, which are part of the cell machinery for degrading and recycling cellular components and may help the virus exploit this process for its benefit (Sargazi et al. 2021).

NSP7 and NSP8 are essential components of the SARS-CoV-2 replication machinery, working together as cofactors for NSP12, the RNA-dependent RNA polymerase (RdRp), which is the core enzyme responsible for viral RNA genome replication (Wilamowski, Hammel, et al. 2021). The NSP7-NSP8 complex forms a primase-like structure that enhances the processivity of NSP12, enabling it to synthesize long RNA strands efficiently without frequent dissociation. Additionally, NSP7 and NSP8 stabilize the RdRp complex and help guide the RNA template during replication, ensuring accurate genome copying.

NSP12 itself is a crucial multi-domain enzyme that catalyzes the addition of nucleotides to the growing RNA strand (Peng et al. 2020). It contains a conserved polymerase domain essential for viral RNA synthesis and replication fidelity. This enzyme furthermore interacts with other non-structural proteins, such as NSP13 (a helicase), to further enhance replication efficiency. (Peng et al. 2020; Wilamowski, Hammel, et al. 2021).

NSP9 plays a role in the replication of SARS-CoV-2 by binding viral RNA and facilitating its efficient replication and transcription (Slanina et al. 2021). It is part of the virus RTC. NSP9 is primarily an RNA-binding protein, interacting with ssRNA (Littler et al. 2021). Its ability to bind RNA is essential for viral genome replication, as it helps stabilize the RNA strands during the replication and transcription processes. It works in concert with other viral proteins such as NSP12 and NSP13, helping these proteins carry out the core functions of RNA synthesis (Malone et al. 2022).

NSP10, NSP14**,** and NSP16 work together to facilitate efficient viral replication and help the virus evade immune detection (Wilamowski, Sherrell, et al. 2021; Krafcikova et al. 2020; Yan et al. 2021). NSP10 serves as a regulatory protein that enhances the functions of both NSP14 and NSP16. NSP10 binds to and stabilizes NSP14 and NSP16, ensuring that these proteins can perform their roles effectively in replication and immune evasion.

NSP11 is a short, non-structural protein encoded by the SARS-CoV-2 genome, but its function remains largely unknown (Gadhave et al. 2021; Jin et al. 2022; Tam et al. 2023). While its exact role is unclear, NSP11 is hypothesized to have a regulatory function in viral replication or host-virus interactions. Some studies suggest that it may serve as a byproduct of polyprotein processing rather than an independently functional protein. Further research is needed to determine whether NSP11 plays a significant role in SARS-CoV-2 pathogenesis or replication.

NSP14 has two key functions: it acts as an exoribonuclease (ExoN)**,** which proofreads the viral RNA during replication, and as an N7-methyltransferase, involved in RNA capping (Imprachim, Yosaatmadja, and Newman 2022). The proofreading activity of NSP14 is essential for maintaining the genetic stability of the virus, as it corrects errors that occur during RNA replication (Sarma and Sastry 2022). This makes SARS-CoV-2 less prone to mutations compared to other RNA viruses, allowing it to replicate more efficiently (Krafcikova et al. 2020).

NSP16 is a 2'-O-methyltransferase that works in conjunction with NSP10 to modify the viral RNA cap structure, making it indistinguishable from host RNA (Viswanathan et al. 2020). This modification helps the virus evade recognition by the host immune system, particularly by sensors like MDA5 and retinoic acid-inducible gene I (RIG-I), which detect foreign RNA (Wilamowski, Sherrell, et al. 2021). By masking its RNA, the virus avoids triggering antiviral immune responses. Targeting the interactions between NSP10, NSP14, and NSP16 offers a promising approach for antiviral therapies (Saramago et al. 2021; Kremling et al. 2024).

NSP13 is an RNA helicase that plays a role in the virus replication by unwinding ds-RNA into single strands (Shu et al. 2020; Yue et al. 2022). This activity is essential for viral RNA synthesis, allowing other viral proteins, such as the, RdRp, part of the RTC, to efficiently replicate and transcribe the viral genome. In addition to its RNA helicase activity, NSP13 also functions as an adenosine triphosphatase (ATPase), using the energy from adenosine triphosphate (ATP) hydrolysis to drive the unwinding process (Lawal, Roy, and McCullagh 2024).

NSP15, known as EndoU (endoribonuclease), is a crucial enzyme in SARS-CoV-2 involved in RNA degradation, helping the virus evade immune detection and ensuring efficient replication (Wang and Zhu 2024; Zhang et al. 2023). NSP15 primary function is its uridine-specific endoribonuclease activity, which enables it to cleave RNA at uridine residues (Wang and Zhu 2024). This activity plays a key role in preventing the accumulation of viral double-stranded RNA (dsRNA) during replication. By degrading these RNA intermediates, it prevents the activation of the host immune response, particularly sensors like MDA5 and RIG-I, which detect viral RNA and trigger the production of interferons (Zhang et al. 2023). This mechanism allows SARS-CoV-2 to replicate undetected by the host innate immune system.

## 2.2 Structural and accessory proteins of SARS-CoV-2

The structural and accessory proteins of SARS-CoV-2 are essential for virus structure and function, playing critical roles in virus assembly, infectivity, and interactions with the host immune system. While SARS-CoV-2 has four main structural proteins, it also encodes several accessory proteins. Although these accessory proteins are not required for viral replication, they are crucial for immune evasion, pathogenesis, and host-virus interactions. Each structural and accessory protein serves specific functions that collectively enhance the virus ability to infect and persist within the host. In this subsection we discuss role of structural and accessory proteins of SARS-CoV-2.

The Spike (S) protein is a trimeric transmembrane protein in surface of virion, vital for viral entry into host cells. It mediates attachment to the host cell receptor, ACE2, and subsequent membrane fusion (Nejat, Torshizi, and Najafi 2023). The S protein is composed of two functional subunits: S1 and S2. The S1 subunit contains the receptor-binding domain (RBD) that binds to the ACE2 receptor on host cells, while the S2 subunit facilitates the fusion of the viral and host cell membranes, allowing viral RNA to enter the host cell and initiate infection (Huang et al. 2020).

The N protein encapsulates the viral RNA genome and is involved in RNA synthesis and regulation of host cell processes (Wu et al. 2023). The N protein consists of two distinct domains: the N-terminal domain and the C-terminal domain, both of which can bind RNA (Morse et al. 2022). This protein not only encapsulates the viral RNA genome but also interacts with the M protein to form the viral ribonucleoprotein complex, essential for viral replication and assembly (Morse et al. 2022). In addition to its structural role, the N protein enhances viral RNA synthesis by promoting the function of the RNA polymerase and may regulate host cell processes to aid in immune evasion (Zandi et al. 2022).

The M protein is the most abundant structural protein in SARS-CoV-2 and plays a role in viral assembly (Zhang et al. 2022). As a multi-spanning membrane protein, it has a short N-terminal ectodomain, three transmembrane domains, and a long C-terminal endodomain, which are essential for its structural and functional roles. The M protein interacts with the S, E, and N proteins to facilitate the assembly and budding of new virions, ensuring that the viral components are correctly packaged and released from the host cell (Jackson et al. 2022). This interaction is essential for forming the viral envelope and organizing the assembly of the virus (Dolan et al. 2022).

The E protein is a small, multifunctional membrane structural protein crucial to various stages of the SARS-CoV-2 viral life cycle, including viral assembly, budding, envelope formation, and pathogenesis (Cao et al. 2021a; Zhou et al. 2023). Despite its small size, the E protein plays a role in ensuring the proper formation and release of new virions from the host cell (Zhou et al. 2023). It contains a transmembrane domain that forms ion channels (viroporins), which are important for virus-host interactions and contribute to the virus ability to regulate its environment within host cells, thus enhancing virulence and pathogenicity (Cao et al. 2021a; Zhou et al. 2023). This ion channel activity is also linked to the modification of host cell stress responses, which further promotes viral replication and survival.

ORF3a is a multifunctional accessory protein that plays a significant role in modulating the host immune response and contributing to viral pathogenesis (Zhang et al. 2022). It interferes with host defense mechanisms by impairing lysosomal function, thereby inhibiting the process of autophagy. By inhibiting autophagy, ORF3a prevents the clearance of viral components, which allows for prolonged viral survival within host cells. In addition to disrupting autophagy, ORF3a is also known to induce cell death, including apoptosis and pyroptosis, contributing to tissue damage during infection. The cell death induced by ORF3a, combined with its role in disrupting immune defenses, amplifies the severity of infection and leads to tissue damage in affected organs, particularly in the lungs (Zhang et al. 2022; 2024).

ORF3b, accessory protein, contributes to immune evasion by inhibiting the host interferon type 1 (IFN-I) response, which is important for antiviral defense (Zandi et al. 2022; Konno et al. 2020). ORF3b achieves this by blocking the production of ISGs and disrupting interferon-signaling pathways. This inhibition allows the virus to replicate more effectively within host cells, as the immune system ability to detect and respond to the infection is weakened. Additionally, ORF3b can induce apoptosis in host cells, further contributing to viral pathogenesis. The protein activity not only promotes viral survival but also contributes to the severity of SARS-CoV-2 infection by limiting the

host innate immune response. Its role in immune suppression makes ORF3b a key factor in the virus ability to evade host defenses and cause disease progression.

ORF6, accessory protein, a significant in the virus ability to evade the host immune system. ORF6 primarily interferes with the IFN-I signaling pathway, which is essential for initiating the host antiviral response (Hall et al. 2022). By blocking the nuclear translocation of STAT1 and other interferon-related proteins, ORF6 prevents the activation of ISGs, effectively suppressing the immune response. Additionally, ORF6 has been shown to inhibit the transport of host mRNA from the nucleus to the cytoplasm by interacting with the nuclear pore complex. This further reduces the host cell antiviral response, as it prevents the expression of crucial immune-related genes. Its immune-suppressive functions make ORF6 a key factor in SARS-CoV-2 ability to evade host defenses and contribute to disease severity (Hall et al. 2022; Kato et al. 2021).

ORF7a, accessory protein, contributes to viral pathogenesis by interacting with host immune responses and facilitating viral replication (Arshad et al. 2023; Hou et al. 2023). One of its key functions is interfering with the host IFN-I signaling pathway, which helps the virus evade immune detection and response. ORF7a inhibits the activation of ISGs, weakening the host antiviral defenses. Additionally, ORF7a has been implicated in inducing apoptosis in infected cells, contributing to tissue damage during infection. It promotes the degradation of tetherin (BST-2), a host protein that prevents newly formed viral particles from leaving the host cell (Liu et al. 2022b). By downregulating tetherin, ORF7a facilitates the release of virions, increasing viral spread.

ORF7b, accessory protein, has a relatively less understood function compared to other viral proteins, but it is believed to contribute to the virus ability to evade host immune defenses and assist in viral assembly (García-García et al. 2022). ORF7b is a transmembrane protein, and its localization in the ER and Golgi apparatus suggests that it may play a role in viral budding and the assembly of new visions (Hassan, Choudhury, and Roy 2021). Some studies suggest that ORF7b may contribute to the suppression of the IFN-I, similar to other SARS-CoV-2 accessory proteins, thereby helping the virus evade the host antiviral mechanisms (Xia et al. 2020).

ORF8, accessory protein, plays a role in immune evasion and contributes to the virus pathogenesis. One of its primary functions is downregulating major histocompatibility complex class I molecules (MHC-I) on the surface of infected cells, thereby reducing the host ability to recognize and eliminate infected cells through cytotoxic T cells (Zhang et al. 2021). This allows the virus to evade the adaptive immune response and replicate more efficiently. Additionally, ORF8 promotes the release of

pro-inflammatory cytokines, which can contribute to severe inflammation and cytokine storms observed in critical COVID-19 cases (Lin et al. 2021). ORF8 may also play a structural role in viral replication and assembly, although the precise mechanisms remain to be fully understood.

ORF9b, accessory protein, is involved in immune evasion by interfering with the host innate immune system (Wu et al. 2021; Zandi et al. 2022). ORF9b primarily disrupts the host antiviral response by interacting with the Mitochondrial antiviral-signaling protein (MAVS), a key component of the RIG-I signaling pathway, which is important for triggering the production of IFN-Is. ORF9b also interacts with other host proteins, such as TOM70, to modulate mitochondrial functions, further impairing the immune response and contributing to viral persistence (Jing Wu et al. 2021).

ORF9b function in immune evasion and interaction with host mitochondria highlights its potential as a target for therapeutic interventions aimed at restoring immune function and limiting viral replication.

ORF9c, accessory protein, although its functions are less well-studied compared to other accessory proteins, it is believed having a part in modulating host immune responses (Review: Redondo et al. 2021). ORF9c has been identified as a transmembrane protein that interacts with host cell membranes, potentially contributing to immune evasion and the virus ability to replicate efficiently within host cells. For example, ORF9c may downregulate the activity of proteins involved in the NF-κB signaling pathway, which is critical for the immune response to viral infections (Zandi et al. 2022).

# 3. Life cycle of SARS-CoV-2 in the host cell

SARS-CoV-2 infection begins with virus binding to ACE2 receptor on host cell surface, which facilitates viral entry by membrane fusion and endocytosis (Figure 2.)(V'kovski et al. 2021). Once inside the host cell, viral RNA genome is released into the cytoplasm in the site of replication. The host cell ribosomes then translate viral RNA into a large polyprotein. This polyprotein undergoes autoproteolysis and co-translational cleavage, producing essential NSPs needed for viral replication and transcription. These NSPs form the replication-transcription complex, which RdRp and is responsible for driving subgenomic transcription and replication of the viral RNA genome. Through this RTC, subgenomic RNAs are transcribed and subsequently translated into viral structural and accessory proteins. At this point, the structural proteins, such as nucleocapsid, envelope, membrane, and spike proteins, assemble with the viral RNA genome at the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), leading to the formation of a complete virion.

The newly assembled virions mature and bud into the ERGIC, preparing for release from the host cell. Finally, these mature virions are transported to the cell surface in vesicles and released by exocytosis, allowing them to infect new cells and continue the cycle of infection.

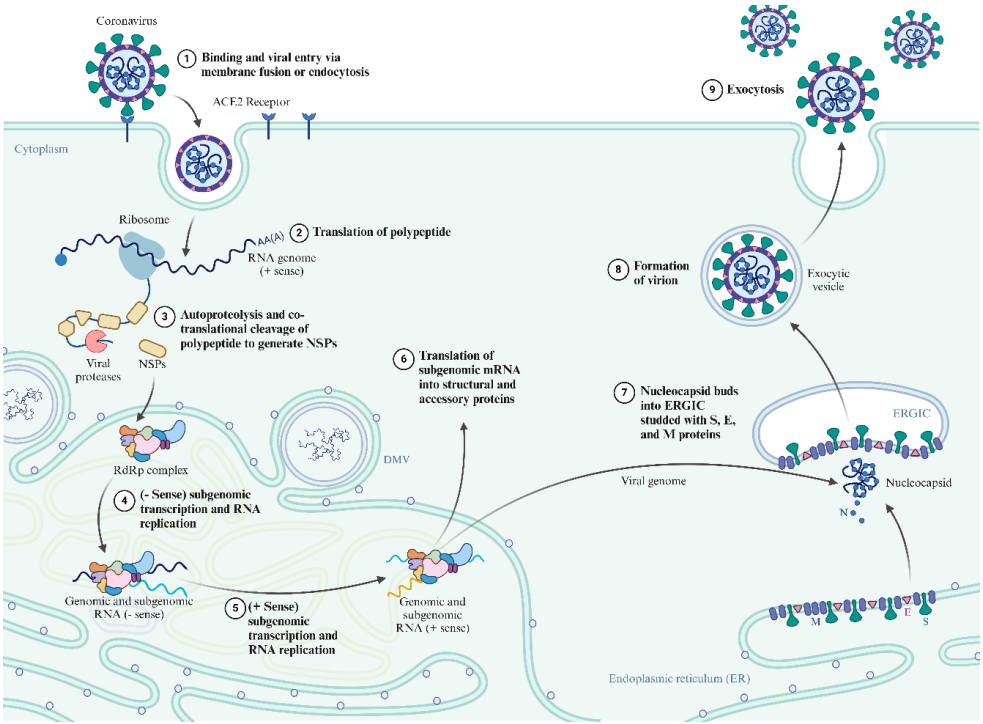


***Figure 2. The viral life cycle of a coronavirus***. *The virus binds to the host cell via the ACE2 receptor, entering through membrane fusion or endocytosis. Once inside, the viral RNA genome, which is of (+) sense, is released into the cytoplasm and translated by the host ribosomes into a polypeptide. This polypeptide is then cleaved by viral proteases to produce NSPs, which form the RTC complex. The RTC synthesizes complementary (-)strand RNA that serves as a template for the production of new genomic RNA and subgenomic RNAs. These subgenomic RNAs are translated into structural and accessory proteins needed for viral assembly. The nucleocapsid, composed of the viral RNA and nucleocapsid protein, buds into the ER-Golgi intermediate compartment, acquiring an envelope with viral proteins. Newly formed virions are then transported to the cell surface in vesicles and released via exocytosis, ready to infect new cells. Template edited from (Hartenian et al. 2020).*

## 3.1 SARS-CoV-2 attachment and entry into host cells

The entry of SARS-CoV-2 into host cells involves a well-orchestrated series of molecular events that primarily hinge on the interaction between the viral S protein and specific host cell factors (Figure 3.) (Peng et al. 2021). Spike protein is composed of two subunits, S1 and S2, each with distinct roles in this process (Huang et al. 2020). The S1 subunit contains the RBD, which is responsible for recognizing and binding to the ACE2 receptor on the surface of host cells. The ACE2 receptor is broadly expressed in various tissues, including the lungs, heart, kidneys, and intestines, which correlates with the ability of the virus to infect multiple organ systems (tropism) (Beyerstedt, Casaro, and Rangel 2021).

Once the S1 subunit has successfully engaged the ACE2 receptor, the viral spike protein undergoes a series of proteolytic cleavages that are necessary for subsequent steps in viral entry (Peng et al. 2021; Huang et al. 2020). These cleavages occur at two specific sites within the spike protein: the S1/S2 junction and the S2' site. Proteolysis at the S1/S2 boundary is typically facilitated by host proteases such as furin or cathepsins, while cleavage at the S2' site is executed by the transmembrane protease, serine 2 (TMPRSS2). The cleavage of the spike protein at the S1/S2 site dissociates the S1 subunit from S2, leaving the S2 subunit poised for activation.

The S2 subunit, once activated, undergoes a dramatic conformational change that brings the viral and host cell membranes into close proximity. This membrane fusion process is driven by the heptad repeat regions (HR1 and HR2) within the S2 subunit, which form a six-helix bundle structure that pulls the viral and host membranes together, facilitating the fusion of the viral lipid envelope with the host cell membrane (Tang et al. 2020). The fusion event allows the viral RNA genome to be released directly into the host cell cytoplasm, where it can initiate the replication and translation processes necessary for viral propagation.

There are two main pathways through which SARS-CoV-2 can enter host cells: receptor-mediated endocytosis and direct membrane fusion. In the endocytic pathway, the virus-ACE2 complex is internalized into endocytic vesicles (Jackson et al. 2022). The acidic environment within these vesicles triggers further conformational changes in the spike protein, promoting fusion between the viral envelope and the vesicle membrane, releasing the viral genome into the cytoplasm. In contrast, membrane fusion occurs at the host cell surface, bypassing endocytosis, and is facilitated by TMPRSS2 cleavage, which primes the spike protein for immediate fusion with the plasma membrane (Xinyu Li et al. 2023). The virus ability to use both entry pathways enhances its infectivity and capacity to infect a wide range of cell types.

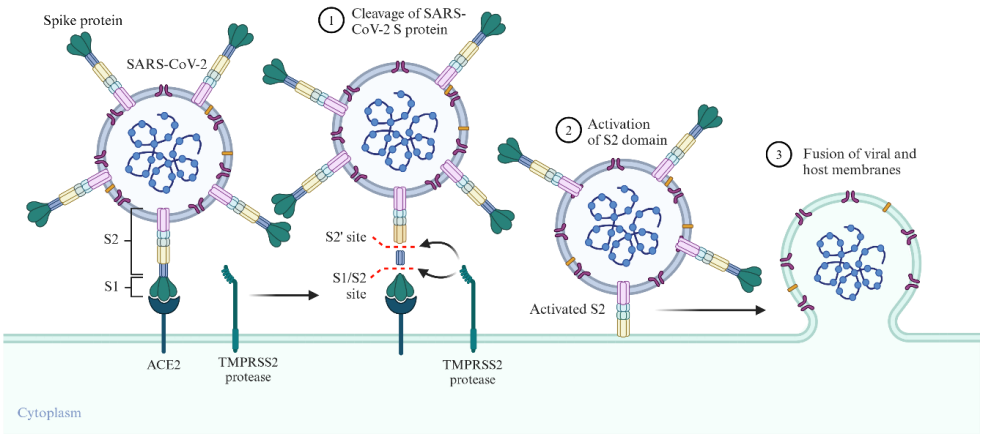


***Figure 3. SARS-CoV-2 entry to host cell.*** *Initially, the viral spike protein, which consists of two subunits, S1 and S2, binds to the ACE2 receptor on the surface of the host cell through its S1 subunit. This attachment allows the virus to latch onto the host cell. Following this, the TMPRSS2 protease cleaves the spike protein at two critical sites: the S1/S2 cleavage site and the S2' site. This cleavage activates the S2 domain of the spike protein, which undergoes a structural change. This activation is crucial for the fusion of the viral and host cell membranes. The fusion allows the viral RNA to enter the host cell cytoplasm, initiating the viral replication process. Together, the ACE2 receptor, TMPRSS2 protease, and the conformational changes in the spike protein facilitate the virus successful entry into the host cell. Created in BioRender. Huuskonen, S. (2024) BioRender.com/d27q581*

## 3.2 Viral genome release and replication in SARS-CoV-2 infection

Once inside the host cell, the SARS-CoV-2 genome is released into the cytoplasm, where replication and gene expression begin. SARS-CoV-2, as an RNA virus, completes its entire replication cycle within the cytoplasm (Malone et al. 2022; V'kovski et al. 2021). Upon entry, the positive-sense, single-stranded viral RNA genome is immediately recognized by host ribosomes, which begin translating it into viral proteins essential for replication, assembly, and immune evasion. This RNA genome functions as mRNA, directly encoding structural proteins such as the S, N, M, and E protein, all of which are crucial for the formation of new viral particles.

Viral replication occurs within specialized structures called RTCs, which are formed on modified host membranes derived from the endoplasmic reticulum (Mishchenko and Ivanisenko 2022). Within these complexes, the viral RdRp, synthesizes new copies of the viral genome and subgenomic RNAs (Malone et al. 2022). The subgenomic RNAs encode structural proteins and accessory proteins needed for assembling new virions (Zhang et al. 2022). Several NSPs play essential roles in facilitating these processes: NSP3 assists in RTC formation and cleaving viral polyproteins into functional units; NSP5 (Mpro) acts as the main viral protease; and NSP4 and NSP6 modify host membranes to support RTCs (Zimmermann et al. 2023; Bills, Xie, and Shi 2023; Scott et al. 2022; Altincekic et al. 2024). Additionally, NSP7 and NSP8 act as cofactors to enhance RdRp activity, while NSP14 ensures replication fidelity by providing proofreading activity during RNA synthesis(Peng et al. 2020; Wilamowski, Hammel, et al. 2021; Sarma and Sastry 2022).

SARS-CoV-2 tightly regulates viral gene expression to ensure efficient production of viral proteins necessary for replication and assembly (Jackson et al. 2022). These viral proteins, including both structural and non-structural proteins, manipulate host cell machinery to create a favorable environment for viral replication while suppressing the host immune response (Kakavandi et al. 2023).

## 3.3 Assembly, maturation, and egress of SARS-CoV-2 particles

As viral proteins and RNA accumulate within the host cell, new SARS-CoV-2 particles begin to assemble and mature (Jackson et al. 2022). This assembly process typically takes place at the ERGIC, a key cellular location where the viral components are brought together. Within the ERGIC, the viral RNA genome is encapsulated by N proteins, forming the core of the virus. Simultaneously, the structural proteins—S, M, and E—are integrated into the lipid bilayer, which the virus acquires as it buds off from the host cell membrane (de Haan and Rottier 2005; Wu et al. 2023; Wang, Carreras-Sureda, and Demaurex 2023).

During assembly, the S proteins, which buds from the surface of the viral envelope, undergo critical post-translational modifications, such as glycosylation (Zhang et al. 2024). These modifications are crucial for the spike proteins' ability to mediate the virus attachment to host cell receptors, particularly the ACE2 receptor, and for evading the host immune system (Wong and Saier 2021; de Haan and Rottier 2005).

The final step in SARS-CoV-2 life cycle is the release of new viral generation from the host cell, a process known as egress (Prydz and Saraste 2022). Once the viral particles have fully formed and acquired their envelope, they are released into the extracellular space. From there, the newly formed virions can go on to infect neighboring cells within the same host or be transmitted to new hosts through respiratory droplets, contributing to the spread of the infection.

# 4. Host immune response to viral COVID19 infection

Host immune responses to SARS-CoV-2, is a complex and multifaceted process that plays a critical role in determining the outcome of the infection in multicellular environment (Sapir et al. 2022). The immune response can range from effective viral clearance, leading to recovery, to an overactive response that can result in severe disease and complications. In this section, we discuss the key components of the host immune response to SARS-CoV-2, including innate and adaptive immunity, the role of cytokines, and the mechanisms underlying immune dysregulation that contribute to disease severity.

## 4.1 Innate immune response

Innate immunity froms first line of defense of the host against SARS-CoV-2 infection, and is this crucial for preventing viral spread and replication (Schiuma et al. 2022). When the virus enters the respiratory tract, epithelial cells, along with immune cells like macrophages and dendritic cells, detect the virus using pattern recognition receptors (PRRs). Key PRRs involved in SARS-CoV-2 detection include toll-like receptors (TLRs) 3, 7, and 8, which recognize viral single-stranded RNA (ssRNA) (Liu et al. 2022a; Bortolotti et al. 2021; Manan et al. 2022). These TLRs activate transcription factors like NF-κB, IRF3, and IRF7, driving the production of type I interferons (IFN-α and IFN-β) and pro-inflammatory cytokines such as IL-6 and TNF-α (Bortolotti et al. 2021).

Type I interferons establish an antiviral state by upregulating ISGs, which inhibit viral processes like replication and protein synthesis (Lukhele, Boukhaled, and Brooks 2019). Proteins like MxA block replication, while PKR inhibits viral protein synthesis. The type I interferon response also activates natural killer (NK) cells, which recognize and destroy infected cells by releasing perforin and granzymes (Ramírez-Labrada et al. 2022). Additionally, pro-inflammatory cytokines recruit immune cells like monocytes and neutrophils to amplify the antiviral response and present antigens to adaptive immune cells (Guo et al. 2024b; Lukhele, Boukhaled, and Brooks 2019). In SARS-CoV-2 infection, dysregulation, particularly overproduction of cytokines, can lead to a "cytokine storm," causing tissue damage and severe conditions like ARDS (Montazersaheb et al. 2022).

## 4.2 Adaptive immune response

In addition to the innate immune response, the adaptive immune response is activated after the initial phase, providing a more targeted and sustained defense against the virus. Adaptive immunity involves the activation of both T cells and B cells, each playing a critical role in controlling and eliminating the infection.

Cytotoxic T cells (CD8+ T cells) play a central role in the adaptive immune response by identifying and destroying infected cells that present viral peptides on major histocompatibility complex (MHC) class I molecules. Upon activation, CD8+ T cells release perforin and granzymes, inducing apoptosis in infected cells and halting viral replication. Meanwhile, helper T cells (CD4+ T cells) enhance the activity of cytotoxic T cells and B cells by releasing cytokines that support immune activation. This helps recruit more immune cells and amplifies the overall antiviral response. In cases of severe COVID-19, dysregulation of the T cell response can occur (Kalfaoglu et al. 2021). T cell exhaustion, a state of reduced functionality due to prolonged antigen exposure, is marked by the upregulation of inhibitory receptors like programmed cell death protein 1 (PD-1) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), leading to diminished cytokine production and weakened cytotoxic activity.

Finally, B cells are crucial in eliminating the virus by producing neutralizing antibodies that specifically target viral antigens, particularly the RBD in the S protein (Röltgen and Boyd 2021). By binding to the RBD, these antibodies block SARS-CoV-2 from engaging with the ACE2 receptor on host cells, preventing the virus from entering and infecting new cells (Wang et al. 2022). Some B cells differentiate into memory B cells, enabling a rapid response upon re-exposure to the virus.

## 4.3 Long-term immune response, memory, and long COVID

Following recovery from COVID-19, the immune system typically develops immunological memory against SARS-CoV-2 (Diani et al. 2022; Cox et al. 2023; Hajissa et al. 2022). Memory T cells and B cells are critical for long-term protection, enabling a rapid and effective response upon re-exposure to the virus. The presence of neutralizing antibodies contributes to short-term protection by blocking viral entry into host cells; however, antibody levels in circulation usually decline within a few months following infection or vaccination. Long-term immunity depends more on memory B cells, which can swiftly produce neutralizing antibodies upon re-infection, and memory T cells, which assist in eliminating infected cells and coordinating the immune response.

Both memory B and T cells can persist for extended periods—ranging from several months to years—though their longevity and functional capacity vary among individuals. Factors such as age, disease severity, and the nature of the infecting viral variant influence this variability. Importantly, emerging SARS-CoV-2 variants with spike protein mutations may partially escape antibody-mediated recognition, potentially compromising the durability and effectiveness of B cell–mediated protection.

While most infected individuals fully recover from SARS-CoV-2 infection in few weeks, some suffer from lingering symptoms that affect multiple organ systems and can substantially impact their quality of life. This condition, known as long COVID or post-acute sequelae of SARS-CoV-2 infection (PASC), refers to a spectrum of symptoms that continue for weeks, months, or even longer after the acute phase has resolved resolved (Saniasiaya, Islam, and Abdullah 2021; Carfì et al. 2020; T. Jiao et al. 2024). The prevalence of long COVID varies across studies but is estimated to affect approximately 10–30% of those infected with SARS-CoV-2 (Sapna et al. 2023). It can impact individuals of all ages, including those who had mild or asymptomatic infections, although it is more commonly reported in people who experienced severe disease. The pathophysiology of long COVID remains incompletely understood, but proposed mechanisms include the persistence of viral components leading to chronic immune activation, a dysregulated or maladaptive immune response, autonomic nervous system dysfunction (e.g., postural orthostatic tachycardia syndrome, POTS), and endothelial dysfunction contributing to symptoms such as fatigue, dyspnea, and chest pain.

# 5. Immune evasion by SARS-CoV-2

SARS-CoV-2 has several strategies to evade the host immune response. These include; delaying interferon response, modulating host cell death, and antigenic variation (Minkoff and tenOever 2023). In this section, we delve into the molecular mechanisms underlying these evasion strategies and explore how they contribute to viral persistence, immune dysregulation, and disease severity.

## 5.1 Delaying interferon response

One of the viral strategies is to evade the host immune system is by delaying the production of interferons (Rubio-Casillas, Redwan, and Uversky 2022). Interferons, particularly IFN-α and IFN-β, play a key role in the early defense against viral infections by establishing an antiviral state, inhibiting viral replication, and activating immune cells such as NK and cytotoxic T cells (Mertowska et al. 2023). SARS-CoV-2 interferes with interferon production and signaling at multiple levels.

The virus can inhibit pattern recognition receptors like RIG-I and MDA5, which are responsible for detecting viral RNA and initiating interferon production (Rehwinkel and Gack 2020; Minkoff and tenOever 2023). Additionally, SARS-CoV-2 proteins, such as NSP1, NSP6, and ORF6, block downstream signaling pathways, including the activation of MAVS, TBK1, and IRF3, which are essential for the transcription of interferon genes (Li et al. 2020; Vazquez et al. 2021). The virus is able to suppress the activation of the JAK-STAT pathway, which is necessary for interferon signaling and the expression of ISGs (Znaidia et al. 2022). By delaying or suppressing the interferon responses, SARS-CoV-2 can replicate unrestricted in the early stages of infection.

## 5.2 Modulating host cell death

SARS-CoV-2 has evolved mechanisms to modulate host cell death, particularly through the inhibition of apoptosis, a crucial form of programmed cell death that acts as an innate defense mechanism against viral infections (Yuan et al. 2023; Morais da Silva et al. 2022). Apoptosis typically entails the systematic dismantling of the cell, which includes DNA fragmentation, cell shrinkage, and ultimately, phagocytosis by immune cells without triggering inflammation, before virus can complete the viral life cycle. However, SARS-CoV-2 disrupts this apoptotic pathway, thereby extending the lifespan of infected cells (Yuan et al. 2023; Li et al. 2022). The virus achieves this by interfering with key regulators of apoptosis, such as the Bcl-2 family proteins, caspases, and signaling pathways like the intrinsic pathway and the extrinsic pathway. For instance, viral proteins such as the NSP1, NSP13 and ORF3a have been implicated in modulating apoptosis, with some promoting cell survival by inhibiting pro-apoptotic factors, and others selectively triggering apoptosis in ways that benefit viral replication (Zhang et al. 2022; Kim, Kim, and Song 2021).

## 5.3 Antigenic variation

SARS-CoV-2 exhibits antigenic variation, a process by which mutations, particularly in the S protein, lead to alterations in viral epitopes—the specific regions of the virus proteins recognized by the host immune system (Cosar et al. 2022; Mittal, Khattri, and Verma 2022). The S protein is the primary antigenic target for neutralizing antibodies generated either through natural infection or vaccination (Fayez et al. 2023). Point mutations in the S protein, especially within the RBD, can change the conformation of epitopes, making them less recognizable to pre-existing neutralizing antibodies (Figure 4.). For example, mutations like E484K, found in several variants of concern (VOCs), alter a key residue in the RBD that is targeted by many potent neutralizing antibodies (Harvey et al. 2021; Weisblum et al. 2020).

Similarly, the N501Y mutation, present in variants such as Alpha, Beta, and Gamma, increases the binding affinity of the spike protein to the ACE2 receptor while also contributing to immune evasion by subtly altering the epitope landscape, making it harder for antibodies to bind effectively (Liu, Liu, Plante, et al. 2022). Mutations in VOCs not only contribute to immune evasion but may also enhance the virus transmissibility and pathogenicity. For example, the D614G mutation, which became dominant early in the pandemic, increases the stability of the spike protein, facilitating more efficient viral entry into host cells and resulting in higher viral loads (Weissman et al. 2021; Korber et al. 2020). When combined with mutations that allow immune evasion, these changes can lead to variants that spread more easily and potentially cause more severe disease, placing additional strain on public health systems.

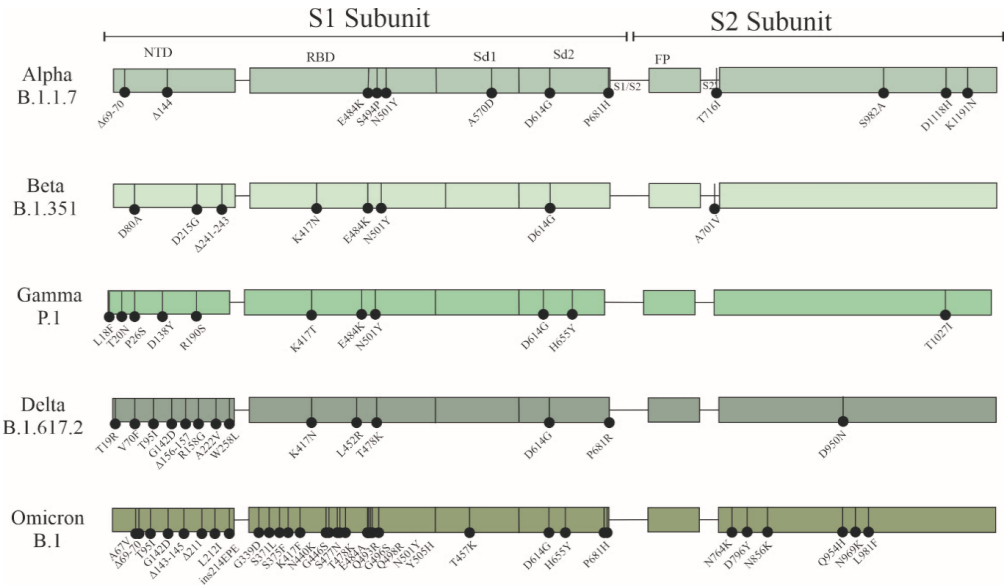


***Figure 4. Iillustration of the mutations in the spike (S) protein of several SARS-CoV-2 variants****, focusing on the differences between the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1) variants. The spike protein is divided into two main subunits: S1, which contains regions like the N-terminal domain, receptor-binding domain (RBD), and subdomains 1 and 2 (Sd1, Sd2); and S2, which includes the fusion peptide (FP), and regions involved in viral entry into host cells. These mutations, particularly in the RBD and other functional regions, are important because they can alter the virus ability to infect cells, evade immune responses, and affect transmissibility.*

# 6. SARS-CoV-2 variants

VOCs of SARS-CoV-2 represent specific strains of the virus that have acquired mutations with significant public health implications (Table 3.) (WHO 2023). These mutations can increase the virus transmissibility, alter disease severity, or reduce the effectiveness of vaccines, treatments, and public health measures. Several VOCs have been identified, each presenting unique challenges in the ongoing effort to control the COVID-19 pandemic.

***Table 3. SARS-CoV-2 VOCs.*** *Each variant is listed with its name, detection date, and location. The key mutations section highlights significant genetic changes, particularly in the spike protein, which affect the variant behavior. Transmissibility compares how easily each variant spreads, while severity shows its impact on health, including risks of hospitalization and mortality. Lastly, immune evasion indicates the variant ability to bypass immunity from previous infections or vaccines, leading to potential reinfections.*

| Variant | First Detected | Key Mutations | Transmissibility | Severity | Immune Evasion |
|---|---|---|---|---|---|
| Alpha (B.1.1.7) | Sep 2020 (UK) | N501Y, D614G, P681H | ~1/2 times more transmissible than original strain | Increased hospitalization risk | Limited immune evasion |
| Beta (B.1.351) | May 2020 (South Africa) | K417N, E484K, N501Y | Similar to Alpha | Higher severity than original strain | Higher immune evasion compared to Alpha |
| Gamma (P.1) | Nov 2020 (Brazil) | K417T, E484K, N501Y | ~2 times more transmissible than original strain | Increased hospitalization risk | High immune evasion |
| Delta (B.1.617.2) | Oct 2020 (India) | L452R, T478K, P681R | ~1/2 times more transmissible than Alpha | Increased severity, associated with higher mortality risk | Moderate immune evasion |
| Omicron (BA.1) | Nov 2021 (South Africa) | S371L, S373P, S477N, G496S, Q498R | Highly transmissible, more than Delta | Lower severity than Delta | High immune evasion, reinfections reported |
| Omicron (BA.5) | Early 2022 (South Africa) | F486V, R493Q | Even more transmissible than BA.1 | Generally mild, but can cause severe illness in vulnerable populations | Strong immune evasion, evades prior Omicron immunity |

The Alpha variant (B.1.1.7), first identified in the United Kingdom in September 2020, is characterized by the N501Y mutation in the spike protein (WHO 2023; Rambaut et al. 2020). This mutation enhances the binding affinity of the spike protein to the ACE2 receptor, making the virus more transmissible—an estimated 50-70% more than the original strain (Liu, Liu, Plante, et al. 2022). While vaccines remain effective against Alpha, some studies suggest a slight reduction in efficacy, and this variant has been associated with an increased risk of hospitalization.

The Beta variant (B.1.351), first detected in South Africa in May 2020, carries several notable spike protein mutations—N501Y, E484K, and K417N—that enhance immune evasion by reducing the binding efficiency of neutralizing antibodies produced by prior infection or vaccination (Liu, Liu, Plante, et al. 2022; Pondé 2022). This had raised concerns about vaccine efficacy, as some vaccines show reduced effectiveness against Beta in preventing mild to moderate disease, though they still offer strong protection against severe outcomes.

The Gamma variant (P.1), first identified in Brazil in November 2020, shares key mutations with Beta, including N501Y and E484K, contributing to increased transmissibility and immune evasion (Banho et al. 2022). Like Beta, Gamma is associated with a higher risk of reinfection and a reduction in vaccine-induced immunity, though vaccines continue to protect against severe disease caused by this variant.

The Delta variant (B.1.617.2), which emerged in India in October 2020, includes mutations such as L452R and P681R that increase both transmissibility and immune evasion(Liu, Liu, Johnson, et al. 2022; Motozono et al. 2021). Delta has been particularly concerning due to its significantly higher transmissibility—up to 60% more than Alpha—and its association with more severe disease, leading to increased hospitalizations and deaths. Although vaccines are slightly less effective at preventing infection with Delta, they remain highly effective at preventing severe disease and death.

The Omicron variant has multiple sublineages, with BA.1 and BA.5 being among the most significant (WHO 2023). First identified in South Africa in November 2021, Omicron BA.1 is marked by over 30 mutations in the spike protein, contributing to significant immune evasion (Sharma et al. 2022). While BA.1 is highly transmissible, it appears to cause milder disease compared to Delta. However, it has led to a surge in breakthrough infections due to its ability to evade immunity from both previous infections and vaccinations. The BA.5 subvariant, which shares many mutations with BA.1 but also has additional changes like L452R and F486V, further enhances the virus ability to evade immune defenses (Tallei et al. 2022; Cao et al. 2022). As of March 2025, Omicron-derived sublineages continue to dominate globally, though newer variants such as JN.1

and other XBB-descendant lineages have emerged and surpassed BA.5 in prevalence. These newer subvariants maintain many of the immune escape features of earlier Omicron strains but also possess additional spike protein mutations that further reduce the efficacy of neutralizing antibodies induced by previous infection or vaccination. Updated bivalent and monovalent vaccines targeting Omicron-related antigens have been deployed to enhance protection, but continuous viral evolution underscores the need for ongoing surveillance and vaccine adaptation.

# 7. Ananlyses of virus-host interactions using proteomic approaches

Understanding virus–host interactions is fundamental to deciphering the mechanisms of viral infection, pathogenesis, and immune evasion. Proteomic approaches—such as global proteome profiling and protein–protein interaction (PPI) mapping—provide powerful tools for investigating these interactions at a molecular level. Proteome studies allow researchers to quantify changes in host and viral protein expression during infection, revealing how viruses reprogram host cellular processes. In parallel, protein–protein interaction analyses identify physical contacts between viral and host proteins, shedding light on how viruses hijack key cellular pathways to promote replication and evade immune responses. Together, these complementary strategies offer deep insights into the molecular interplay between viruses and their hosts and can guide the development of targeted antiviral therapies and immune-based countermeasures.

## 7.1 Mapping virus-host protein interactions

Proteomic techniques like Affinity Purification-Mass Spectrometry (AP-MS) and proximity labelling are key tools for mapping protein-protein interactions and understanding the spatial organization of viral and host proteins during infection (Liu et al. 2018) (Figure 5.). AP-MS identifies direct interactions by tagging viral proteins and isolating them with their host protein partners, followed by mass spectrometry analysis to reveal interaction networks critical for viral survival and replication. In the case of SARS-CoV-2, this method has been used to isolate viral proteins and their host interactions, providing insights into processes like replication and immune evasion (Liu et al. 2021).

Proximity labeling techniques, such as BioID, enable the identification of proteins in the immediate vicinity of a viral protein within living cells (Figure 5.). BioID fuses a viral protein with a biotin ligase (BirA*) that biotinylates nearby proteins. These labeled proteins are then isolated and identified using mass spectrometry. BioID is particularly effective for detecting transient or weak interactions that might be missed by traditional AP-MS methods, making it highly valuable for viral studies where interactions are often dynamic and temporally regulated (Liu et al. 2021). BioID also helps map the spatial organization of viral proteins within host cells, especially within specialized replication compartments, offering critical insights into the virus manipulation of host processes (Liu et al. 2020).

Data-Dependent Acquisition (DDA) is a widely used mass spectrometry (MS) technique for identifying and quantifying proteins in complex samples. In DDA, an initial survey scan (MS1) detects all peptides in the sample, after which the most abundant peptides are selected for fragmentation in a secondary scan (MS2). The fragment ion spectra are then analyzed to identify the proteins through database matching. This approach is particularly useful for AP-MS and BioID samples, as it enables detailed characterization of interaction networks and spatially relevant proteins, providing insight into dynamic protein interactions and cellular processes.

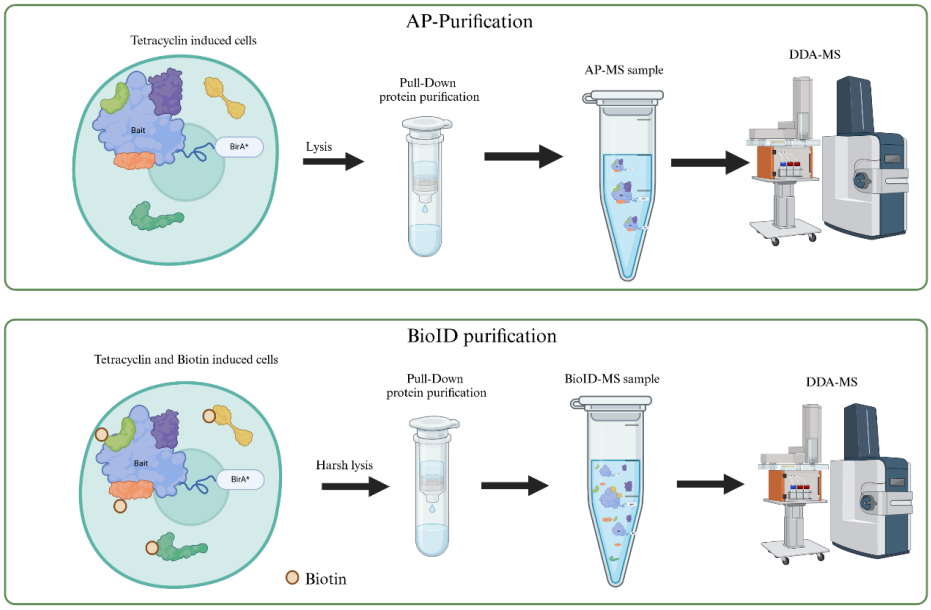


***Figure 5. Affinity Purification (AP) and BioID Purification.*** *In AP-purification, a bait protein is used to pull down direct interacting partners, isolating protein complexes that are physically bound to the bait. The result is a purified sample containing these direct interactors. In contrast, BioID*

*purification uses a bait protein fused to a biotin ligase, which labels nearby proteins with biotin in vitro. This combination of methods allows for the identification of both direct interactors and proteins that are in close proximity to the bait. Created in BioRender. Huuskonen, S. (2024) BioRender.com/v28b985*

## 7.2 Applications of Protein-tagging in viral research

The MAC-tag (Multiple Approaches Combined Tag) system is a versatile and powerful tool in both cellular and viral research, particularly beneficial for studying viral-host interactions (Liu et al. 2018; 2020; 2021; 2023b). The MAC-tag combines both methods into a single construct, allowing simultaneous use of AP-MS and BioID, thereby improving the detection and mapping of both physical and functional protein interactions with high sensitivity and reproducibility. The tag includes elements such as the StrepIII-tag for AP-MS and the BirA* biotin ligase for BioID, enabling researchers to analyze protein complexes and localization in a streamlined and efficient manner.

By tagging viral or host proteins involved in viral replication with the MAC-tag, researchers can map the surrounding protein environment in infected cells, offering valuable insights into viral mechanisms and potential therapeutic targets. The MAC-tag system adaptability is further enhanced by versions such as the MAC2-tag (BioID2) and MAC3-tag (UltraID), which improve labeling efficiency and expand its range of applications.

A typical MAC-tag workflow involves generating cell lines that express MAC-tagged bait proteins, purifying these proteins along with their interactors, and using mass spectrometry (MS) analysis to map detailed interaction networks. This integrated approach has proven highly effective for studying viral mechanisms and identifying protein-protein interactions, greatly enhancing our understanding of the cellular and molecular contexts involved in infection and other biological processes.

## 7.3 Global proteomics and phosphoproteomics of SARS-CoV-2

Global proteomics enables a comprehensive analysis of all proteins expressed within host cells, offering insights into how viral infections, such as SARS-CoV-2, alter cellular processes (Figure 6.). By comparing the proteomes of infected and uninfected cells, researchers can detect proteins that are up- or downregulated in response to infection, which aids in understanding how viruses reprogram host cell pathways to enhance replication. This approach also reveals the host stress responses and immune signaling pathways activated during infection.

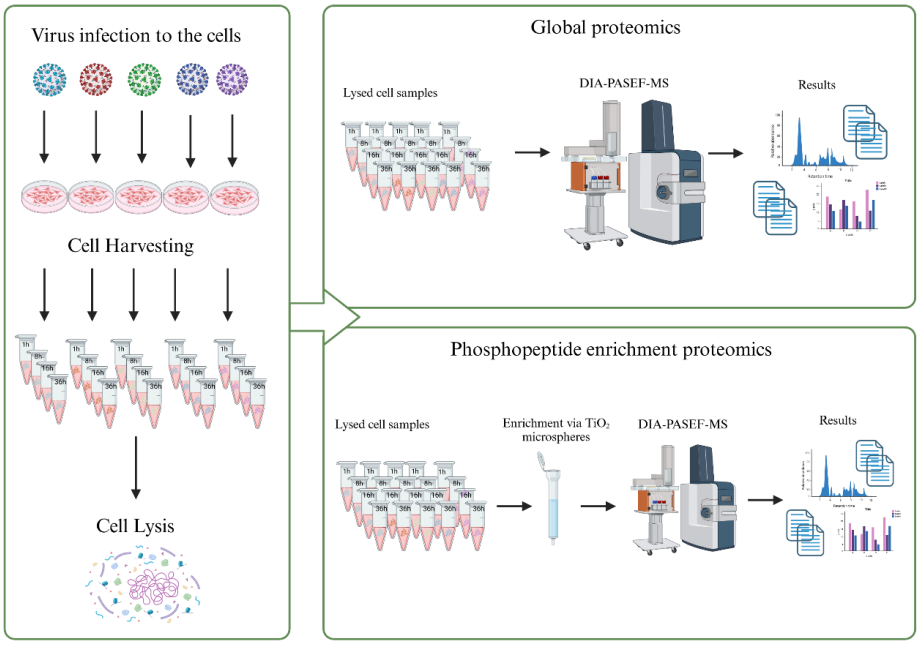


***Figure 6. Workflow for analyzing virus-infected cells using global proteomics and phosphopeptide enrichment.*** *The process begins with virus infection of host cells, followed by cell harvesting and lysis to obtain protein samples. For global proteomics, lysed cell samples are analyzed using Data-Independent Acquisition-Parallel Accumulation–Serial Fragmentation Mass Spectrometry (DIA-PASEF-MS), yielding a comprehensive profile of protein expression changes in response to infection. In parallel, phosphopeptide enrichment is performed on the lysed cell samples using CAE-Ti-IMAC microspheres to selectively capture phosphorylated peptides, focusing on phosphorylation-mediated signaling events. The enriched phosphopeptides are then analyzed by DIA-PASEF-MS. Data is processed with DIA-NN software, facilitating the quantification and analysis of both global proteome changes and phosphorylation events. Created in BioRender. Huuskonen, S. (2024) BioRender.com/h25n416*

While global proteomics provides a broad overview of protein expression, it often overlooks post-translational modifications (PTMs) like phosphorylation, which play a pivotal role in regulating cellular functions. To address this limitation, phosphopeptide enrichment is employed to selectively isolate phosphorylated peptides from the complex peptide mixture. This step is essential because phosphorylated peptides are typically present in low abundance compared to non-phosphorylated peptides and can be easily overshadowed during analysis. Phosphopeptide enrichment combined with liquid chromatography–mass spectrometry (LC-MS) is a powerful method to analyze phosphorylated peptides and proteins. Phosphoproteomics specifically focuses on the systematic analysis of phosphorylated proteins. Phosphorylation, the addition of a phosphate group to serine, threonine, or tyrosine residues, is crucial for regulating protein function, signaling pathways, and various cellular processes. Phosphorylation events, generally mediated by protein kinases, play a critical role in viral infections. Viruses like SARS-CoV-2 have evolved mechanisms to exploit the host phosphorylation machinery, often by mimicking cellular proteins or presenting motifs that resemble phosphorylation sites. This allows them to interact with host kinases, ultimately hijacking cellular signaling pathways and modifying host functions to facilitate viral replication.

Sample preparation begins with virus deactivation, a critical step to ensure biosafety during the analysis of infectious samples. Deactivation is achieved using UV irradiation, which disrupts the viral genome while preserving protein integrity, followed by lysis buffer, which denatures proteins and inactivates the remaining viral particles. This dual approach ensures both safety and sample integrity for downstream analysis. The subsequent steps include protein concentration measurement, reduction, alkylation, digestion, and peptide desalting, yielding samples for both global proteome and phosphoproteome analysis. For phosphopeptide enrichment, CAE-Ti-IMAC (Carboxyamidomethyl-Modified Titanium Dioxide Immobilized Metal Affinity Chromatography) microspheres are employed to capture phosphorylation events (Zhou et al. 2013). These microspheres are prepared through conjugation with titanium sulfate ($TiSO_4$), which provides robust binding specificity to phosphate groups.

In Data-Independent Acquisition (DIA), the mass spectrometer fragments all ions within a predefined mass range simultaneously, creating a comprehensive set of fragment ions (Meier et al. 2020). In Parallel Accumulation–Serial Fragmentation (PASEF), ions are accumulated in a trap, released in packets, and fragmented as they pass through a collision cell. This approach enables parallel ion fragmentation while preserving ion mobility separation, allowing for broad detection of phosphorylation patterns across numerous proteins.

Mass spectrometry data is processed using DIA-NN, a software specifically designed to analyze DIA data. DIA-NN identifies and quantifies peptides and proteins in complex samples with high precision and minimal missing values (Demichev et al. 2022). This information is then refined through analytical methods, facilitating detailed examination of phosphorylation networks modulated by viral infection. This enables the identification of modified amino acid residues on proteins and reveals signaling pathways critical to viral replication and host responses.

By combining global proteomics and phosphoproteomics, researchers capture a comprehensive view of molecular changes during viral infection. Sampling at various time points post-infection allows the construction of a timeline that tracks host manipulation by different viral variants, from early entry to immune response modulation. For instance, SARS-CoV-2 variants induce distinct phosphorylation profiles at various infection stages, providing insights into the dynamics of the viral infection process.

Mapping phosphorylation networks and identifying key viral proteins or host kinases involved in these pathways highlight potential drug targets to disrupt viral replication or enhance immune responses. The identification of specific kinases involved in SARS-CoV-2 infection, for example, offers targets for intervention, potentially hindering viral exploitation of host survival pathways. Furthermore, this method allows for cross-viral comparisons, facilitating the discovery of shared phosphorylation-dependent mechanisms among viruses, which could contribute to the development of broad-spectrum antiviral therapies.

# 8. Role of proteomics in antiviral drug discovery

Proteomics plays a crucial role in drug discovery by enabling researchers to identify and characterize both viral and host proteins essential for viral infection, replication, and immune evasion. These insights are critical for developing antiviral drugs, particularly against rapidly evolving viruses like SARS-CoV-2. In antiviral drug development, two main strategies are pursued: virus-targeting and host-targeting drugs (Clercq 2004; Everts et al. 2017; von Delft et al. 2023). Virus-targeting drugs aim to inhibit viral proteins essential in replication, assembly, or entry into host cells. In contrast, host-targeting drugs focus on disrupting viral infections by targeting host proteins that viruses exploit, reducing the likelihood of development of drug resistance as they affect host mechanisms rather than the virus directly, and are thus less likely to be circumvented by evolutionary mechanisms, and are thus less likely to be circumvented by evolutionary mechanisms.

Proteomics studies have identified several SARS-CoV-2 proteins as key drug targets by enabling detailed insights into viral protein structure, function, and interaction within host cells. Using high-resolution techniques like mass spectrometry, it is possible to map the viral proteome, highlighting proteins essential for viral replication, immune evasion, and host interaction. For example. NSP12, the RNA-dependent RNA polymerase (RdRp), works with NSP7 and NSP8 to stabilize its function, (Wu et al. 2022). Drugs like remdesivir inhibit NSP12, causing premature termination of viral RNA synthesis. For NSP5, the main protease (Mpro), processes viral polyproteins, and protease inhibitors like nirmatrelvir (in Paxlovid) block viral replication by binding to NSP5 active site (Iketani et al. 2023; Dawood 2023; Altincekic et al. 2024). For NSP13, an RNA helicase, is vital for unwinding viral RNA, and inhibiting its activity can halt viral replication (Romeo et al. 2022; Pitsillou et al. 2022).

Other potential targets include NSP9, which is involved in RNA replication. Disrupting its RNA-binding or dimerization could hinder viral replication (Farias et al. 2021). Targeting interactions between NSP10, NSP14, and NSP16—proteins involved in RNA proofreading and capping—also offers promising antiviral strategies (Saramago et al. 2021; Kremling et al. 2024). NSP15, which plays a role in immune evasion, could be a target for restoring immune detection of viral RNA (Saramago et al. 2022).

Proteomics has also been instrumental in understanding the S protein, which mediates viral entry into host cells and is the primary target for vaccines like Pfizer-BioNTech and Moderna (Dai and Gao 2021). The N protein, essential for viral RNA packaging and replication, is another therapeutic target (Royster et al. 2023). Similarly, E protein, despite its small size, is involved in viral assembly and release, making it a potential target for inhibiting viral particle release (Zhou et al. 2023). Accessory proteins like ORF8, which helps the virus evade immune detection, and ORF9b, which interferes with host mitochondria, also represent promising drug targets for restoring immune function and reducing viral replication (Li et al. 2020; Gil et al. 2020; Wu et al. 2021).

The treatment landscape for SARS-CoV-2 has evolved significantly, with several antiviral drugs, monoclonal antibodies, and other therapeutics approved or in use under emergency authorization (Table 4.). Treatments such as previously mentioned remdesivir**,** and Paxlovid have been widely used to reduce the severity of COVID-19 in infected patients (Lui and Guaraldi 2023; CDC 2024b). Additionally, monoclonal antibodies targeting the spike protein have been deployed to prevent viral entry, although their effectiveness has diminished with the emergence of highly immune-evasive variants like Omicron (Liu et al. 2023a; Guo et al. 2024a).

Current treatments aim to reduce viral load, mitigate severe disease, and prevent hospitalization, but challenges remain due to the rapid evolution of the virus and its ability to develop resistance. Ongoing clinical trials are investigating both virus-targeting and host-targeting therapies, along with combination treatments, to improve effectiveness and reduce resistance. Proteomic approached aids drug research identifying novel drug targets against the SARS-CoV-2 and potential future epidemics and pandemics.

***Table 4. Ongoing and Completed Clinical Trials for COVID-19 Treatments.*** *overview of antiviral and immunomodulatory treatments in various stages of clinical evaluation or already in use.*

| Drug/Treatment | Mechanism of Action | Phase of Trial | Status | Outcome/Remarks |
|---|---|---|---|---|
| **Remdesivir** | RdRp inhibitor, halts viral RNA synthesis | Phase III | Approved (FDA) | Effective in reducing recovery time for hospitalized patients |
| **Paxlovid (nirmatrelvir/ ritonavir)** | Protease inhibitor (NSP5) | Phase III | Approved (FDA EUA) | Reduces hospitalization and death in high-risk patients |
| **Molnupiravir** | Induces viral RNA mutagenesis | Phase III | Approved (FDA EUA) | Limited efficacy compared to other antivirals; primarily for mild-to-moderate cases |
| **Baricitinib** | JAK inhibitor, reduces inflammation | Phase III | Approved (FDA EUA) | Effective for reducing severe symptoms and mortality in hospitalized patients |
| **Tocilizumab** | IL-6 receptor antagonist, reduces cytokine storm | Phase III | Approved (FDA EUA) | Used in conjunction with corticosteroids to reduce severe inflammation |
| **Casirivimab/ Imdevimab** | Monoclonal antibodies targeting the spike protein | Phase III | Emergency Use Authorization (EUA) | Reduced effectiveness against Omicron and other variants |
| **Sotrovimab** | Monoclonal antibody, neutralizes the virus | Phase III | Emergency Use Authorization (EUA) | Retained some activity against Omicron variants, but limited availability |
| **Favipiravir** | RdRp inhibitor, halts viral replication | Phase II/III | Ongoing | Mixed results; approved for emergency use in some countries |
| **Fluvoxamine** | SSRI, modulates immune response | Phase II/III | Ongoing | Shows promise in reducing severity in early-stage COVID-19 |
| **Dexamethasone** | Corticosteroid, reduces inflammation | Phase III | Approved | Effective in reducing mortality in severe cases, especially in ventilated patients |

# II Study aims

**Research Questions and Hypotheses**

This study aims to investigate the key protein-protein interactions between viruses and host cells during infection. Specifically, it seeks to understand how these interactions influence viral replication, immune evasion, and pathogenesis. Additionally, the study explores whether proteomics can be effectively used to identify novel antiviral drug targets and contribute to therapeutic intervention development.

The specific aims of studies I, II, and III were as follows:

I. To comprehensively map the virus–host protein–protein interaction network of the Wuhan-Hu-1 SARS-CoV-2 strain using affinity purification mass spectrometry (AP-MS) and BioID proximity labeling, and to identify virus–host interactions potentially involved in viral replication and pathogenesis, with the aim of evaluating their potential as antiviral drug targets.

II. To optimize a phosphopeptide enrichment protocol suitable for infection models, enabling reliable and reproducible detection of phosphorylation events in host cells during SARS-CoV-2 infection.

III. to map SARS-CoV-2 variants of concern (VOCs), alter virus–host interactions and modulate host cell signaling pathways. This included mapping interaction profiles of viral proteins across variants and conducting phosphoproteomic analyses to identify infection-induced changes in host signaling and phosphorylation dynamics, with the goal of uncovering mechanisms of immune evasion and potential therapeutic targets.

# III Materials and methods

## 1. Cloning (I & III)

We acquired entry plasmid vectors containing our target genes from the libaries, which includes the ORFeome and MGC Libraries. These libraries are maintained by the Genome Biology Unit with support from HiLIFE, the Faculty of Medicine at the University of Helsinki, and Biocenter Finland. The ORFeome collection is a comprehensive repository of open reading frames (ORFs) that facilitates high-throughput functional studies of genes.

To generate stable cell lines, we employed Gateway cloning techniques. Gateway cloning is a recombination-based cloning method that allows for the efficient transfer of DNA fragments between different vectors without the need for restriction enzymes (Liu et al. 2018; 2020). Using this technique, we fused the gene of interest to the MAC-tag-C destination vector (Addgene, Plasmid #108077). The MAC-tag is a versatile tag used for multiple applications, including protein purification and detection. For validating protein interactions, we similarly used Gateway cloning to attach the gene of interest to a modified pDEST40 vector, which features a 3×V5 C-terminal tag. The 3×V5 tag is a sequence of three V5 epitopes that enhance the detection and purification of the protein of interest. This cloning strategy and its applications were described by Liu et al. in 2018 and 2020.

## 2. Mutagenesis (III)

To generate specific point mutations of SARS-CoV-2 variants, we employed site-directed mutagenesis on plasmids containing single ORF using the Q5 High-Fidelity DNA Polymerase (NEB, M0491L). The primers necessary for these mutations were ordered from Eurofins Genomics. The design of the mutations was informed by data from the GISAID database (GISAID.ORG) and the WHO classification of variants of concern or interest. This included key variants such as Alpha (B.1.1.7 and Q lineages), Beta (B.1.351 and its descendent lineages), Gamma (P.1 and its descendent lineages), Delta (B.1.617.2 and AY lineages), and Omicron (B.1.1.529, BA.1, and BA.5 lineages.). Primer design can be found in Article III material.

# 3. Cell culture (I & III)

The Flp-In™ 293 T-REx (Invitrogen, R78007), HEK293 (ATCC® CRL-1573™), Vero E6 (ATCC® CRL-1586™), Vero E6-TMPRSS2-H10, Calu-1 (ATCC® HTB-54™), and U2-OS (ATCC® HTB-96™) cell lines were cultured under manufacturer-recommended conditions. These cell lines originate from human (*Homo sapiens*) tissues, with Flp-In™ 293 T-REx and HEK293 cells derived from human embryonic kidney (HEK) cells, U2-OS cells from a human osteosarcoma (bone cancer) tumor, and Calu-1 cells from human lung epidermoid carcinoma. Vero E6 and Vero E6-TMPRSS2-H10 cells were derived from the kidney of an African green monkey, with Vero E6-TMPRSS2-H10 cells stably expressing transmembrane serine protease 2 (TMPRSS2), enhancing viral entry and replication efficiency. Each cell line was maintained in its respective growth medium: Flp-In™ 293 T-REx cells in Dulbecco Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S); U2-OS cells in McCoy's 5A Medium (ATCC) supplemented with 10% FBS and 1% P/S; Calu-1 cells in RPMI 1640 Medium (Gibco) supplemented with 10% FBS and 1% P/S; Vero E6 and Vero E6-TMPRSS2-H10 cells in DMEM supplemented with 10% FBS, 1% P/S, and 1% L-glutamine, with Vero E6-TMPRSS2-H10 cells additionally maintained under selective pressure with 5 µg/mL blasticidin (InvivoGen) to preserve TMPRSS2 expression.

Transfection and generation of stable cell lines were performed as described by (Liu et al. 2020), with isogenic cell lines generated to ensure consistent transgene expression at a specific genomic location and enhance reproducibility. Flp-In™ 293 T-REx cells were co-transfected with MAC-tagged bait or green fluorescent protein (GFP) constructs along with the pOG44 vector (Invitrogen, V600520) using FuGENE 6 (Promega), allowing site-specific integration via Flippase recombinase (Flp) at an Flippase recognition target (FRT) site. After 48 hours, stable integrants were selected using hygromycin B (100 µg/mL; Invitrogen) for three weeks. Positive clones were expanded to 80% confluence in ten 150-mm plates (CELLSTAR, Greiner), with cells from five plates used per biological replicate. To induce protein expression, cells were treated with 1 µg/mL tetracycline (Sigma-Aldrich, T3383-25G) and 50 µM biotin (Thermo Fisher, 29129) for 24 hours before harvesting, ensuring controlled gene expression and functional biotinylation.

## 4. Western blot analysis (I)

Cell lysate samples were collected prior to protein purification on beads. Protein concentrations were measured using the Bradford reagent (Bio-Rad, 500-0006), and the concentrations were standardized using Laemmli Sample Buffer (Bio-Rad, 1610737). The samples were then loaded and separated using a 4–20% gradient SDS-PAGE gel (Bio-Rad, 4561096). After electrophoresis, the proteins were transferred to a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad). The membrane was blocked with 5% milk in TBS-T (0.1% Tween-20) for 1 hour at room temperature.

Primary antibodies were diluted in fresh blocking buffer and incubated with the membrane overnight at 4°C. The following primary antibodies were used: Hemagglutinin (HA) mouse monoclonal antibody (BioLegend, PRB-101C, 1:2,000 dilution) and anti-α-tubulin monoclonal antibody (Abcam, ab184613, 1:1,000 dilution). The membrane was then incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:2,000 dilution), enabling detection via Enhanced Chemiluminescence (ECL) reagent (GE Healthcare, RPN2209).

## 5. Infection experiments (III)

SARS-CoV-2 variants were isolated from nasopharyngeal samples and propagated in Vero E6 or VeroE6-TMPRSS2-H10 cells (Rusanen et al. 2021). The specific variants used were: FIN34-21 (Alpha variant, GenBank ON532062.1), FIN32-21 (Beta variant, OK448476.1, EPI_ISL_3471851), FIN37-21 (Delta variant, OK626882.1, EPI_ISL_2557176), and Omicron variants FIN56-21 (BA.1.1, EPI_ISL_8586102) and FIN61-22 (BA.5, OP435368, EPI_ISL_13118918). Viral stocks were grown in VeroE6-TMPRSS2-H10 cells in DMEM supplemented with 2% fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin, and titrated using a median Tissue Culture Infectious Dose (TCID50) assay.

For infection experiments, VeroE6-TMPRSS2-H10 cells were seeded the day before infection at densities of $3 \times 10^6$ cells per 10 cm² dish, $0.4 \times 10^6$ cells per well in a 6-well plate, and $0.075 \times 10^6$ cells per well in a 24-well plate. Cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin to ensure optimal growth and viability.

At the time of infection, the medium was replaced with DMEM supplemented with 2% fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin, and this medium was used for the duration of the experiment. To enhance the efficiency of infection, SARS-CoV-2 variants were added in half of the final designated medium volume to achieve the desired multiplicity of infection (MOI), which reflects the ratio of infectious virus particles to target cells. Following 1 hour of incubation at 37°C with 5% $CO_2$, the remaining half volume of the medium was added. Notably, the inoculated virus was not removed to allow continuous viral replication.

The final titers of the inoculated virus ranged from 20,000 to 70,000 TCID50/mL, depending on the experiment and variant used (Belik et al. 2023; Ardito et al. 2017). For proteome and RNA analyses, final titers were 70,000 TCID50/mL (Fin-34), 58,000 TCID50/mL (Fin-37), and 25,000 TCID50/mL (Fin-32, Fin-56, and Fin-61). For immunofluorescent analyses, final titers were 56,000, 46,400, and 20,000 TCID50/mL, respectively. Samples were collected at 1 hpi, 8 hpi, 16 hpi, and 36 hpi, with uninfected cells used as controls.

# 6. Infection rate estimation (III)

For infection rate estimation, VeroE6-TMPRSS2-H10 cells were infected with 0.1–0.28 × $10^5$ TCID50 of the respective SARS-CoV-2 variant per well in a 24-well plate. Following infection, cells were fixed with 4% formaldehyde for 1 hour, washed with phosphate-buffered saline (PBS), and stored in PBS at +4°C until immunofluoresence analysis.

To prepare samples for immunofluorescence, cells were blocked and permeabilized with 0.5% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) in PBS (Gibco) for 30 minutes at room temperature. Primary antibodies against SARS-CoV-2 N (gift from Sari Maljanen and Ilkka Julkunen) and S1 proteins were diluted in 3% BSA in PBS and incubated with the samples for 1 hour at room temperature. After three washes (10 minutes each in PBS), secondary antibodies (Goat α-rabbit IgG (H+L) Alexa Fluor Plus 488, Thermo Fisher Scientific) and DAPI (Life Technologies, 1:5,000 dilution) were applied for 1 hour at room temperature in the dark. The cells were then washed three times for 10 minutes in 3% BSA in PBS before imaging.

Fluorescently labeled cells were imaged using the EVOS FL Auto imaging system (Life Technologies). Image processing was conducted in Fiji (a distribution of ImageJ). StarDist2D plugin was used for nuclei segmentation, while a binary classifier was trained using the Trainable Weka Segmentation plugin to differentiate between infected and uninfected cells. Approximately 20% of the

images from the first repetition were used for training, while the remaining 80% were used for validation. The trained algorithm was then applied to subsequent image sets for infection quantification. (Arganda-Carreras et al. 2017).

## 7. RNA sample preparation (III)

For protein and RNA analyses, cells were seeded at $3 \times 10^6$ cells per 10 cm² dish. On the day of infection, cells were inoculated with $3–8 \times 10^5$ $TCID_{50}$ of each SARS-CoV-2 variant per 10 cm² dish (MOI of 0.1–0.2).

At the time of protein sample collection, plates were placed on ice, and the culture medium was removed. Cells were washed once with ice-cold Tris-buffered saline (TBS), followed by the addition of 0.5 mL of freshly prepared 8 M urea solution (in 50 mM ammonium bicarbonate, $NH_4HCO_3$) supplemented with Phosphatase Inhibitor Cocktail (Roche) per dish. Cells were then scraped off, and the lysates were kept on ice before storage at −80°C until further analysis.

For RNA sample collection, cells were infected with $0.5–1.4 \times 10^5$ $TCID_{50}$ of the respective SARS-CoV-2 variant per well in a 6-well plate. At collection, plates were placed on a cold plate, and the medium was removed. Cells were washed once with ice-cold PBS to eliminate residual medium and contaminants. Next, 0.3 mL of RA1 buffer (Macherey-Nagel) supplemented with 20 mM dithiothreitol (DTT) was added per well. RA1 buffer effectively lyses cells while preserving RNA integrity, and DTT acts as a reducing agent to prevent oxidation of RNA molecules. The lysate was carefully mixed by pipetting, kept on ice, and stored at −80°C until analysis.

## 8. NanoString (I and III)

The NanoString platform is a highly sensitive and specific method for measuring gene expression. Flp-In™ 293 T-REx cells and generated viral ORF stable cells were harvested to obtain the necessary amount of RNA (~100 ng) using a RNeasy® Mini Kit (Qiagen, Hilden, Germany, 74004). The extracted RNA was then added to the NanoString nCounter gene expression platform (NanoString Technologies, Seattle, USA) and hybridized using the NanoString nCounter® Human v1.1 Pan-Cancer Immune Profiling Panel, which targets 770 transcripts.

Gene expression data were analyzed using nSolver™ 4.0 analysis software (NanoString Technologies). Positive controls were included to adjust for possible variations between samples. Data normalization was performed using housekeeping genes present in the panel, which serve as internal controls to account for any variations in RNA input or processing efficiency. Additionally, negative

control subtraction was applied to correct for background noise. To facilitate comparison between different samples, log2 transformation was applied to the normalized gene counts, allowing for the calculation of fold changes in gene expression between the samples and untransfected control cells.

# 9. Global and phosphoproteomics sample preparation (II and III)

For protein sample preparation, VeroE6-TMPRSS2-H10 cells were infected with 3–8 × $10^5$ $TCID_{50}$ of the respective SARS-CoV-2 variant per 10 $cm^2$ dish. At the time of sample collection, plates were placed on a cold plate to maintain cellular integrity, and the culture medium was removed. Cells were washed once with ice-cold Tris-buffered saline (TBS) to eliminate residual medium and debris.

To lyse cells, 0.5 mL of freshly prepared ice-cold 8 M urea solution (in 50 mM ammonium bicarbonate, $NH_4HCO_3$) supplemented with Phosphatase Inhibitor Cocktail (Roche) was added per 10 $cm^2$ dish. Urea, a chaotropic agent, denatures and solubilizes proteins, while $NH_4HCO_3$ maintains pH stability, and phosphatase inhibitors prevent protein dephosphorylation. Cells were scraped off using a cell scraper, and lysates were kept on ice before storage at −80°C.

For quantitative proteomics and phosphoproteomics analysis, lysates were further processed by centrifugation at 16,000 × g for 10 min at 4°C to remove cellular debris. Protein concentration was determined using a BCA protein assay kit (Thermo Fisher), ensuring equal protein input (250 µg per sample). To facilitate protein reduction and alkylation, samples were treated with Tris(2-carboxyethyl) phosphine (TCEP) and iodoacetamide, respectively. The urea concentration was then reduced to <2 M by dilution with 50 mM ammonium bicarbonate (AMBIC) before digestion with Sequencing Grade Modified Trypsin (Promega) at a 1:50 to 1:20 (w/w) ratio at 37°C for 16 hours.

Following tryptic digestion, peptides were desalted using C18 Macrospin columns (Nest Group) to remove contaminants and salts. A portion (~50 µg) of the desalted peptides was reserved for whole proteome analysis, while the remaining (~250 µg) was subjected to phosphopeptide enrichment.

For phosphopeptide enrichment, the peptide mixture was loaded onto stage-tips packed with $TiO_4$-IMAC microspheres, prewashed with loading buffer (80% acetonitrile/6% trifluoroacetic acid, TFA). $TiO_4$-IMAC microspheres selectively captured phosphopeptides, while nonspecific peptides were removed with sequential washes of 50% acetonitrile/6% TFA/200 mM NaCl and 50% acetonitrile/0.1% TFA. Phosphopeptides were eluted using 10% $NH_4OH$, followed by centrifugation to collect the eluate. The eluate was lyophilized and stored for subsequent LC-MS/MS analysis.

## 10. Affinity -and proximity labeling purification (I and III)

In the AP-MS method, cell samples were lysed in ice-cold lysis buffer containing 0.5% IGEPAL, 50 mM HEPES (pH 8.0), 150 mM NaCl, 50 mM NaF, 1.5 mM $NaVO_3$, 5 mM EDTA, and protease inhibitors (Sigma-Aldrich), a composition optimized to maintain protein stability and preserve protein-protein interactions (Liu et al. 2018). In the BioID-MS approach, cell pellets were lysed using a buffer containing 0.1% sodium dodecyl sulfate (SDS) in addition to the same components. SDS is a strong anionic detergent, that disrupts protein-protein interactions. After lysis, lysates were sonicated and treated with Benzonase® Nuclease (Santa Cruz Biotechnology) to degrade nucleic acids and reduce viscosity. Cleared lysates were obtained by centrifugation, followed by a one-step purification using Strep-Tactin® Sepharose® resin (IBA).

For LC-MS preparation, purified protein lysates were treated with 5 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) at 37°C for 20 minutes to disrupt disulfide bonds. Samples were then alkylated with 10 mM iodoacetamide at room temperature for 20 minutes. Proteins were digested with 1.5 µg of Sequencing Grade Modified Trypsin (Promega) at 37°C for 16 hours. The peptides were acidified with 10% trifluoroacetic acid (TFA) and desalted using BioPureSPN MINI C18 columns (The Nest Group, Inc.). The dried peptides were reconstituted in 30 µL of Buffer A (0.1% TFA and 1% acetonitrile in HPLC water), diluted in a 1:19 ratio with HPLC water containing 0.1% formic acid, and loaded into Evotips (Evosep) for LC-MS analysis.

## 11. Fluorescence microscopy of bait proteins (I and III)

U2-OS cells were transfected with the specified viral bait ORF and fixed in 4% paraformaldehyde in PBS. Paraformaldehyde is a fixative that preserves cell structure and protein integrity by cross-linking proteins. Bait proteins were detected with an anti-V5 antibody (Invitrogen, 37-7500, 1:1,000 dilution), followed by an Alexa Fluor 488-conjugated secondary antibody. Actin, a major component of the cytoskeleton, was stained with Alexa Fluor 594-conjugated phalloidin (Thermo Fisher), and nuclei were stained with DAPI. After staining, the cells were washed twice to remove excess antibodies and imaged in PBS. Imaging was performed using an Intelligent Imaging Innovations (3i) Marianas Inverted Spinning Disk (Yokogawa CSU-X1 M1 5,000 rpm) microscope, equipped with an Andor Neo sCMOS camera and solid-state lasers (405 nm/100 mW, 488 nm/150 mW, 561 nm/50 mW, 640 nm/100 mW).

Cells were imaged using a 20×/0.8 Plan-Apochromat Ph2 WD = 0.55 M27 objective lens (Zeiss). The spinning disk confocal system allows for high-speed and high-resolution imaging of fluorescently labeled cells. NSP3-expressing cells were further imaged with a Leica Stellaris 8 Falcon microscope, using HyD X and HyD S detectors, diode 405 and WLL SuperK Extreme lasers, and a Leica HC PL APO CS2 63×/1.20 W objective lens. The voxel size was 0.068 × 0.068 × 0.356 µm; Pinhole 1 AU.

## 12. Mass spectrometry analysis (I, II and III)

The desalted samples were analyzed using the Evosep One liquid chromatography system, coupled with a Bruker timsTOF Pro mass spectrometer, via a CaptiveSpray nano-electrospray ion source. Peptides were separated using an 8 cm × 150 µm column packed with 1.5 µm C18 beads, following a 60-sample-per-day method with a 21-minute gradient. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B).

For BioID and affinity purification (AP) samples, mass spectrometry (MS) analysis was performed in positive-ion mode using data-dependent acquisition (DDA) in Parallel Accumulation Serial Fragmentation (PASEF) mode with the DDA-PASEF-short_gradient_0.5s-cycletime method. Data-independent acquisition (DIA) MS analysis was also conducted in positive-ion mode using the DIA-PASEF method, optimized based on DDA-PASEF runs for each sample (Meier et al. 2020). The "tims Control" software was used to adjust parameters for phosphorylation enrichment and total proteome analysis to optimize PASEF-windows based on the detected ion cloud.

Raw data from total proteome and phosphoproteomics experiments were processed using DIA-NN software (version 1.8.1) (Demichev et al. 2020). Parameters included settings for missed cleavages, variable modifications (N-terminal methionine excision, cysteine carbamidomethylation, methionine oxidation, and N-terminal acetylation), peptide length, precursor charge, and m/z range for both precursors and fragment ions. For phosphoproteomics, methionine oxidation was excluded.

AP and BioID raw data were processed with FragPipe v17.1 using MSFragger against human UniProtKB entries and SARS-CoV-2 proteins from GISAID (Global Initiative on Sharing All Influenza Data) (Yu et al. 2020). Modifications included cysteine carbamidomethylation, N-terminal acetylation, methionine oxidation, and biotinylation on lysine and N-termini. Trypsin was used as the enzyme, allowing two missed cleavages, with label-free quantification and default instrument parameters. Results included Spectral Counts (SC) from peptides with FDR < 0.01 from Philosopher.

## 13. Identification of high condifience interactions (I, II and III)

Significance Analysis of INTeractome (SAINT) is a statistical tool utilized in the analysis of protein-protein interaction (PPI) data obtained from affinity purification-mass spectrometry (AP-MS) experiments. SAINT aims to distinguish true protein interactions from background noise and nonspecific interactions, thus enabling the identification of high-confidence interactions. The web tool (http://proteomics.fi/) integrated with SAINT-express version 3.6.3 (Teo et al. 2014) served as the statistical approach for identifying specific high-confidence interactions from MS data. High-confidence interactions (HCIs) were characterized by an estimated protein-level Bayesian False Discovery Rate (BFDR) of ≤ 0.05. Moreover, our analysis incorporated an in-house contaminant GFP library for Proximity Dependent Biotinylation with a cutoff frequency of ≥ 50%, coupled with an average spectral count fold change ≥ 3 to eliminate non-specific interactors. Common mass-spectrometry contaminants were also removed from the results. The outcomes were bait normalized to ensure accuracy and reliability.

## 14. MS-microscopy analyses (I and III)

In the MS-microscopy analyses, we calculated the averaged peptide-spectrum match (PSM) values for each prey protein associated with any bait of interest. These values were then uploaded to the web tool (http://proteomics.fi/) to assess and determine their subcellular distribution. This approach allows us to gain insights into the localization patterns of the identified proteins within the cell, providing valuable information about their functional roles and interactions in specific cellular compartments.

## 15. Data pre-processing (I and III)

For AP/BioID data, we first performed bait normalization to account for any differences in expression levels between bait proteins. Next, missing values in the dataset were imputed using the QRILC (Quantile Regression-based Imputation using Locally Constant functions) method. This imputation technique was implemented using the imputeLCMD R package in R (version 4.3.1). Additionally, we conducted median normalization on the data as another normalization step before further analysis. This normalization process helps to adjust for any systematic variations in the data, ensuring that subsequent analyses are robust and accurate. For global proteome samples and phosphopeptide enriched samples, follows similar patter, naturally without bait normalization.

## 16. Correlation analysis (I, II, and III)

In correlation analysis, we conducted a differential abundance analysis to compare protein levels across different sample groups. Log2 fold change and p-values were calculated using Python (version 3.9.7) and the ttest_ind function from the scipy.stats package. Subsequently, p-values were corrected using the Benjamini-Hochberg method from the multitest module of the statsmodels.stats package. Visualization of the results was performed using the Plotly Python library.

## 17. Gene ontology enrichment analysis (I, II and III)

In our Gene Ontology (GO) enrichment analysis, we utilized the Database for Annotation, Visualization, and Integrated Discovery (DAVID) to explore the functional characteristics of the proteome dataset. Our primary objective was to identify enriched GO terms and pathways that provide insights into the molecular functions of the identified proteins. Our investigation covered three main aspects of gene ontology: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF).

DAVID enabled us to identify GO terms that demonstrated significant enrichment within our protein dataset. Additionally, we delved into the complex network of cellular pathways by employing KEGG and Reactome pathway analyses (Huang, Sherman, and Lempicki 2009b; 2009a). This comprehensive approach allowed us to gain a deeper understanding of the biological processes and molecular functions associated with the proteins under study.

## 18. Preparation for docking and druggability assessment (I)

The protein data bank (PDB) identifiers for potential target proteins identified during interaction analysis were obtained using structural data from UniProt (https://www.uniprot.org/). Preference was given to crystal structures that included bound small molecules or cofactors. Each selected protein underwent preparation through a customized workflow designed for structural processing (Schrödinger Release 2020-3: Schrödinger KNIME Extensions, Schrödinger, LLC, New York, NY, 2020).

This workflow involved four main steps: (a) retrieving and converting protein structures into Maestro format, (b) preparing the protein structure, (c) aligning structures, and (d) exporting the processed structures into a Maestro project for further examination. During protein preparation (step b), hydrogen atoms were added, bond orders were assigned, and water molecules located more than 5 Å away from heteroatoms were removed. Additionally, the hydrogen bonding network was refined, and energy minimization was performed using the OPLS3e force field until the root-mean-square deviation for heavy atoms reached a convergence threshold of 0.3 Å. The initial crystal structure for each target protein served as the template for structural alignment (step c).

To identify the most appropriate docking receptors, the structures were visually inspected and validated. A second protein preparation step was conducted in Schrödinger Maestro (Schrödinger Release 2020-3: Maestro, Schrödinger, LLC, New York, NY, 2020), where missing side chains were reconstructed using Prime, and solvent molecules, ions, and crystallization additives were removed before optimizing the hydrogen bond network. Following this, ligands were extracted from the structures, and SiteMap (Halgren 2009; Halgren 2007) was used to evaluate druggability.

# 19. Database preparation for molecular docking (I)

Drug molecules were sourced from multiple databases, including DrugBank (Knox et al. 2011), ChEMBL (Bento et al. 2014), the NPC browser (R. Huang et al. 2011), and SciFinder (scifinder.cas.org). The collected compounds underwent manual pre-processing to retain primarily drug-like molecules. This involved filtering out inorganic compounds, commonly encountered substrates and cofactors such as ATP and NADPH, and applying a molecular weight threshold of 700 g/mol.

Following this refinement, 5,518 unique compounds remained. These were subsequently used to generate a Phase database, prepared using the LigPrep module from Schrödinger Suite 2020-3 (Schrödinger Release 2020-3: Phase, Schrödinger, LLC, New York, NY, 2020). Default settings were applied during processing, while preserving specified chiralities where applicable.

## 20. Docking (I)

For each docking receptor, a corresponding grid (docking parameter file) was generated using the Glide module in Schrödinger. The center of the binding site was defined by calculating the centroid of the crystallographic ligand. For kinase proteins, specific hydrogen bond constraints were applied to the hinge-region amide to enhance the accuracy of virtual screening.

The prepared grids were then employed in a multi-step virtual screening workflow using Glide. Initially, a high-throughput virtual screening (HTVS) docking was performed to filter compounds efficiently. The top 20% of hits from this stage were subjected to a more refined docking process using standard precision mode (Schrödinger Release 2020-3: Glide, Schrödinger, LLC, New York, NY, 2020). The highest-scoring 200 ligands from the standard precision results were further analyzed through extra precision docking to achieve more accurate binding predictions.

At each stage of docking, the van der Waals potentials of ligands were adjusted using a scaling factor of 0.6 for atoms with partial charges up to 0.15 e, ensuring optimized interactions. Final hit selection was based on a detailed visual assessment of the top-ranked ligands from both standard- and extra precision docking, prioritizing favorable ligand conformations and interactions within the protein-binding site.

## 21. Image-based drug screen with SARS-CoV-2 (I)

Drugs were dissolved in either DMSO or water according to the manufacturer guidelines and dispensed into the wells of CellCarrier-96 Ultra Microplates (PerkinElmer, 6055302) at six different concentrations using the Echo 550 acoustic liquid handler (Labcyte) at the FIMM High Throughput Biomedicine Unit, HiLIFE, University of Helsinki, Finland. Calu-1 cells were then seeded into the pre-dosed plates at a density of 10,000 cells per well in standard cell culture medium.

Following cell seeding, the plates were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 10. As controls, mock-infected cells (neither virus-infected nor drug-treated) exposed to DMSO were included to assess the effects of DMSO on virus inhibition and cell viability. Additionally, virus-infected but untreated cells served as an infection control.

After 48 hours of incubation at 37°C, cells were fixed using 4% paraformaldehyde (PFA). Immunofluorescence staining was then performed on the fixed cells, and imaging was conducted using the Opera Phenix HT microscope.

## 22. Drug response quantification (I)

Cells in the acquired images were segmented by expanding the nuclear region, identified through nuclear staining, and classified into N protein-positive and N protein-negative populations. Classification was based on intensity and morphological features using a training set of 150 cells derived from both mock-infected and virus-infected control samples as previously mentioned in section 5.

To determine drug efficacy, a four-parameter (4PL) logistic regression model was applied to fit dose–response data, allowing the calculation of half-maximal inhibitory concentrations (IC50) and the area under the curve (AUC) for each drug. The virus-infected control (without drug treatment) served as the baseline reference (Bailer 1988; Jaki, Wolfsegger, and Ploner 2009).

Curve fitting and AUC analysis were used to quantify both viral inhibition (measured by the count of N protein-positive cells) and drug-induced cytotoxicity (assessed by total nuclei count). These analyses were performed using Prism 4.2 (GraphPad Software, San Diego, California, USA). A higher AUC value indicated a greater drug effect. Drugs were classified as effective antiviral candidates if they exhibited an AUC value (N protein-positive cell count) exceeding 100 with a positive curvature.

## 23. In silico protein structure preparation (III)

For each protein of interest, structural models were obtained from the AlphaFold Protein Structure Database (Jumper et al. 2021, AlphaFold, accessed August 14, 2024). Additionally, all available X-ray diffraction and electron microscopy (EM) structures were retrieved from the RCSB Protein Data Bank (Berman et al. 2000, RCSB PDB, accessed August 14, 2024).

To ensure consistency, experimental structures underwent preprocessing using a custom workflow developed in Python 3.12 with BioPython 1.83. Structures containing fewer than 80 amino acids

were excluded. In multi-protein complexes, only the relevant chains were extracted, and for homo-multimeric proteins, the biologically relevant assembly was prioritized, with monomeric structures retained as a fallback. Non-protein components such as water, ions, and solvents were removed, except for essential cofactors, including heme groups, iron-sulfur clusters, NADH, NADPH, FAD, and FMN.

Protein structures were further refined using the Schrödinger Protein Preparation Wizard (Sarma and Sastry 2022; chrödinger Release Notes - Release 2022-4' 2024) with default parameters. This process included adding hydrogen atoms, assigning bond orders and cofactor charge states where necessary, and determining amino acid protonation states using PROPKA at pH 7. Following this, the hydrogen bonding network was optimized, and restrained energy minimization was performed using the OPLS4 force field under standard conditions.

## 24. Pocket druggability calculations (III)

Druggability assessment and site prediction were performed using Schrödinger SiteMap (T. A. Halgren, 2007, 2009; Schrödinger Release Notes, 2022-4). Target proteins were analyzed for the presence of well-defined druggable pockets, with a druggability score (DScore) of 0.8 or higher considered indicative of a druggable site (Halgren 2009).

For shallower, more hydrophobic sites commonly associated with protein-protein interactions (PPIs), a modified SiteMap protocol was applied based on the method described by Loving et al. (2014) (Loving, Lin, and Cheng 2014). This approach utilized specific parameters, including grid = 0.35, maxdist = 10, and dtresh = 5.0, to refine pocket identification. To enhance druggability evaluation, a modified descriptor, DScore+, was computed by adding 0.3 * hydrophobicity to the base DScore. Pockets with volumes ranging from 160 to 800 Å³ were classified as druggable PPI sites, whereas larger cavities were categorized as 'large PPI sites.' Pockets smaller than 160 Å³ were designated as cryptic sites.

Cryptic pockets with a DScore+ of 1.3 or higher were subjected to further analysis using a two-step induced fit docking (IFD) pipeline, incorporating Schrödinger Glide and Prime for structural refinement (Sherman et al. 2006; Schrödinger Release Notes, 2022-4). Given that SiteMap validation was primarily conducted using X-ray crystallographic structures, separate evaluations were performed for electron microscopy (EM) structures and AlphaFold models to account for potential limitations in resolution and structural uncertainty.

## 25. Assessment of known ligand profiles (III)

Ligands in the experimental structures were collected using the RCSB GraphQL API, and their structures were standardized and analyzed with RDKit v2023.09.4. Parameters such as molecular weight (MW), heavy atom count, hydrogen-bond donors and acceptors, and logP were calculated, and ligands were categorized as follows:

- **Extended drug-like**: MW 200-650 Da, at least 10 heavy atoms.
- **Rule-of-Five (RoF) compliant**: MW < 500 Da, hydrogen-bond donors ≤ 5, acceptors ≤ 10, logP < 5 (Lipinski 2004)
- **iPPI-like violations**: Up to two RoF violations, including MW ≥ 500 Da or logP > 5, were accepted for PPIs.

## 26. Classification by druggability confidence (III)

To assess the druggability confidence of each protein, proteins were assigned scores ranging from "most likely druggable" (4) to "not druggable" (0). For structures analyzed by EM or AlphaFold models, druggability was classified as "likely druggable" (3). Discrepancies between datasets led to classifications of "potentially druggable" (2) or "possibly not druggable" (1). Proteins with known iPPI ligands were considered "potentially druggable" (2), as RoF violations may impact oral availability but not necessarily drug-likeness. For literature and TTD analysis, targets with at least 100 PubMed results were flagged as "research targets," and clinical, patented, or established targets were classified as "druggable." These scores were combined to generate an overall druggability confidence score, with higher scores indicating greater druggability likelihood.

## 27. Co-immunoprecipitation (I and III)

Co-Immunoprecipitation (Co-IP) is a widely used biochemical technique employed to investigate protein-protein interactions within a biological sample. It allows researchers to identify and validate interactions between proteins of interest, shedding light on complex molecular networks and signaling pathways.

For Co-IP validation, HEK293 cells (5 × 10^5 per well) were seeded in a 6-well plate and co-transfected with Strep-HA-tagged prey (500 ng) and V5-tagged bait (500 ng) constructs using Fugene 6 transfection reagent. Following 24 hours of transfection, cells were washed with ice-cold PBS and lysed with 1 ml of HENN lysis buffer per well on ice. The HENN lysis buffer composition included 50 mM HEPES (pH 8.0), 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.5% IGEPAL, 1 mM DTT, 1 mM PMSF, 1.5 mM Na3VO4, and 1x Protease inhibitor cocktail.

The cell lysate was collected and centrifuged at 16,000 g for 20 minutes at 4°C to obtain a clear supernatant. In parallel, 30 µl of Strep-Tactin Sepharose resin (50% suspension) was washed twice with 200 µl of HENN lysis buffer using a microcentrifuge tube. The clear lysate was then added to the pre-washed Strep-Tactin beads and incubated for 1 hour on a rotation wheel at 4°C. After the incubation period, the beads were collected by centrifugation and washed three times with 1 ml of HENN lysis buffer. Each wash step involved centrifugation at 4,000 g for 30 seconds at 4°C. Following the final wash, 60 µl of 2x Laemmli sample buffer was added directly to the beads, and the samples were boiled at 95°C for 5 minutes. The resulting samples were then utilized for immunodetection via dot-blot analysis, enabling the validation of protein-protein interactions.

For the cell-based immunoprecipitation, compounds were added to the culture medium of HEK293 cells for 24 h before harvesting. Strep-HA-tagged GFP with V5-tagged LacZ construct was used as a negative control. The compounds BMS-863233 (XL413, CAS: 1169562-71-3) and methotrexate (CL-14377, CAS: 59-05-2) were purchased from Selleck Chemicals. Cells were, each for 24 h, untreated or treated with 5 µM and 50 µM BMS-863233 (dissolved in water) or 1 µM and 10 µM methotrexate (dissolved in DMSO). Each condition was repeated three times to generate the average trend.

## 28. Dot-blot (I and III)

The Dot-Blot analysis employed the Bio-Dot Microfiltration apparatus. A nitrocellulose membrane was pre-washed with TBS, and 10 µL of the CO-IP sample was applied at the center of the well, drained under vacuum. The membrane was blocked with 5% fat-free milk in TBS-T (0.05% Tween-20 in TBS) for 60 minutes at room temperature with gentle shaking. After blocking, it was incubated with the primary antibody (mouse anti-V5 at a 1:5,000 dilution) in TBS-T overnight at 4°C.

Following overnight incubation, the membrane was washed three times with TBS-T for 10 minutes each to remove unbound primary antibody. The secondary antibody (goat anti-mouse IgG conjugated with HRP at a 1:2,000 dilution) was then applied for 60 minutes at room temperature with gentle shaking. After incubation, the membrane was washed again and treated with Amersham ECL Prime solution for 5 minutes before imaging using the iBright Imaging Systems.

The membrane was stripped using Restore Plus Stripping buffer for 15 minutes, re-blocked with 5% fat-free milk in TBS-T for 60 minutes, and then incubated with a different primary antibody (Rabbit anti-HA at a 1:2,000 dilution) overnight at 4°C. Similar washing and secondary antibody steps followed (goat anti-rabbit IgG conjugated with HRP at a 1:2,000 dilution). After another round of washing and imaging, the ECL Prime solution was reapplied, and the blot was imaged again to detect HA-tagged proteins. This allowed the detection of both V5-tagged bait and HA-tagged prey proteins, confirming their interaction through dot-blot analysis.

# IV Results and discussion

## 1. Proteomic and phosphoproteomic Analysis

Proteomics has become an essential tool in the study of viral infections, particularly in the investigation of SARS-CoV-2. In this research, advanced proteomic methodologies—including phosphopeptide enrichment, affinity purification mass spectrometry (AP-MS), and BioID proximity labeling—were employed to generate a comprehensive map of interactions between SARS-CoV-2 and host cellular machinery. These complementary techniques enable the identification of post-translational modifications, direct protein–protein interactions, and spatially restricted protein environments, providing a multidimensional view of virus–host dynamics. In this Results and Discussion section, findings from all three studies (I, II, and III) are synthesized and discussed collectively to highlight key patterns and biological insights. The results are divided into two main parts. In the first part, proteomic findings—including protein–protein interactions and phosphoproteomic data—are presented and analyzed, with emphasis on the cellular pathways enriched by these interactions. In the second part, we explore how these high-confidence interactions (HCIs) can inform drug target identification, therapeutic intervention strategies, and opportunities for drug repurposing in the context of SARS-CoV-2 infection.

To understand SARS-CoV-2 interactions and its protein profile, the virus–host interactions of the original Wuhan-Hu-1 strain (I) was first analysed. protein–protein interactions were successfully identified using both affinity purification mass spectrometry (AP-MS) and BioID proximity labeling for all 29 viral proteins encoded by the Wuhan-Hu-1 SARS-CoV-2 genome (Figure 7). These interactions covered all viral open reading frames (ORFs) and included binding with 18 host bait proteins, each potentially playing a critical role in viral replication and host–pathogen dynamics.

Analysis revealed a list of 693 hub proteins that interact with both viral and host proteins, suggesting their central role in SARS-CoV-2 infection biology. Additionally, a detailed host receptor/cofactor interaction network was constructed, identifying a total of 5351 high-confidence interactions (HCIs)—of which 2224 were detected via AP-MS and 3127 via BioID-MS—across 18 human bait proteins localized to various cellular compartments. As anticipated, BioID-MS identified a greater number of high-confidence interactions compared to AP-MS. Specifically, we detected 3454 viral bait–prey interactions and 3,127 host bait–prey interactions with BioID-MS, versus 2216 viral bait–prey and 2224 host bait–prey interactions with AP-MS. These results underscore the strength of

BioID-MS in capturing transient and spatially proximal interactions that may be missed by conventional AP-MS approaches.

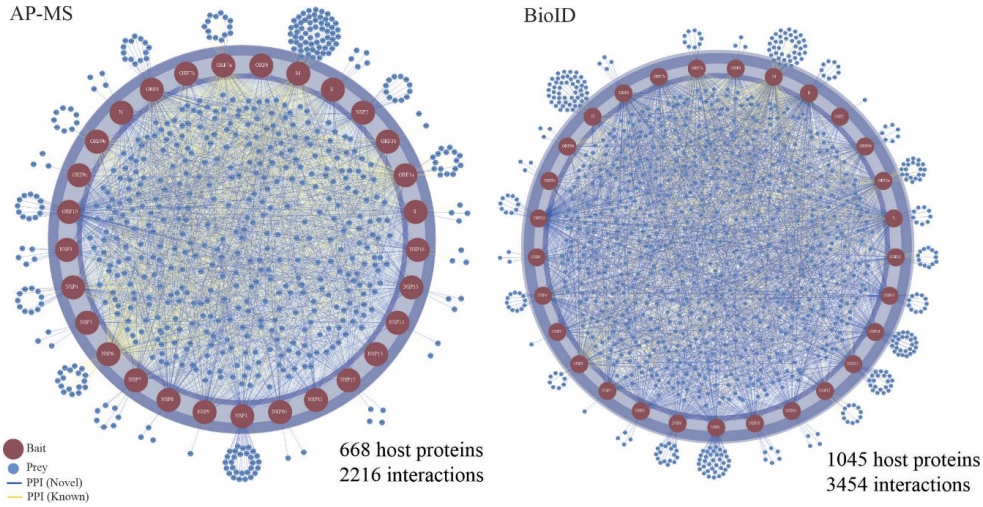


***Figure 7. AP-MS and BioID interaction network from Article I, Wuhan-Hu-1 strand produced in HEK Flp-in T-REX 293 cells****. Both networks represent the interactions between viral proteins (red circles, labeled as baits) and host proteins (blue circles). The left panel shows the AP-MS network, capturing 668 host proteins with 2,216 interactions. The right panel shows the BioID network, identifying 1045 host proteins and 3454 interactions. In both networks, prey proteins (blue) are connected to bait proteins by lines representing PPI. Novel interactions are highlighted, and known interactions are marked with distinct colors.*

Building upon this foundational research, we extended our investigation to focus on the variants of concern (VOCs) of SARS-CoV-2, specifically Alpha, Beta, Gamma, Delta, Omicron BA.1, and Omicron BA.5 (III). By employing both BioID and phosphopeptide enrichment techniques (II), we systematically mapped host-viral interactions for each VOC, allowing us to pinpoint key phosphorylation events and variant-specific interactions that play critical roles in viral replication and immune evasion. From our global proteomic analysis, we detected and quantified a comparable number of host proteins across all samples, with the number of identified proteins ranging from 7529 in mock-infected cells to 7851–7900 in SARS-CoV-2 VOC-infected cells. Of these proteins, 312 were found to be unique to SARS-CoV-2-infected cells (Figure 8.).

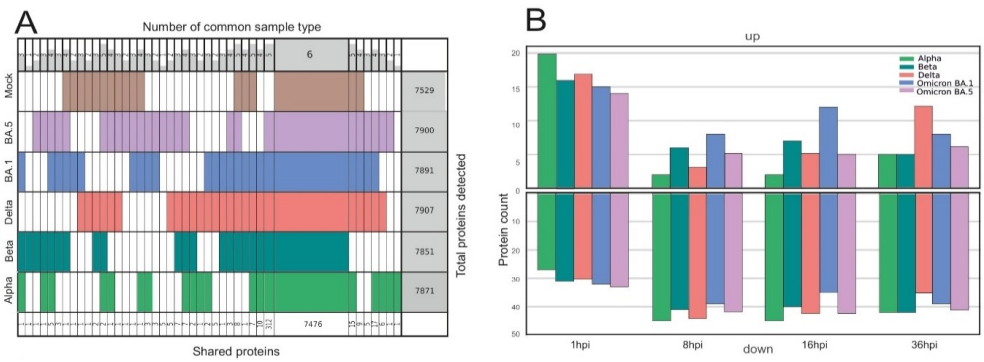


***Figure 8. Comparative proteome analysis across SARS-CoV-2 variants and control samples produced in VeroE6-TMPRSS2-H10 cells from Aricle III. A)*** *Super Venn diagram representing the total number of proteins quantified in each sample group at all time points. Each row corresponds to a distinct sample, with the variants color-coded: Alpha (light green), Beta (dark green), Delta (coral), Omicron BA.1 (blue), Omicron BA.5 (lilac), and Control (brown). The numbers at the bottom of each column indicate the total number of proteins shared within that group, while the numbers at the top reflect the number of sample groups contributing to the shared set. The rightmost column presents the total number of proteins detected in each sample group.* ***B)*** *Bar chart displaying the total number of proteins exhibiting either upregulation (above the line) or downregulation (below the line) compared to the previous sample time point. Each column set corresponds to a different time point: 1 hp), 8 hpi, 16 hpi, and 36 hpi. The bars are color-coded by variant as in panel A.*

We further analyzed the phosphoproteomes of SARS-CoV-2 VOC-infected cell proteins across multiple time points, identifying a total of 2500 unique phosphosites from 1395 distinct proteins. This comprehensive analysis enabled us to investigate the mutation-induced changes in virus-host protein interactions across different VOCs.

Finally, we performed sequence analysis of five SARS-CoV-2 variants, identifying 50 distinct mutations of interest (as defined by the WHO) across 12 viral proteins (NSP2, NSP3, NSP5, NSP6, NSP12, NSP13, S, ORF3a, E, ORF7a, ORF8a, and N) (Figure 9.). Isogenic stable cell lines for BioID analysis. This approach resulted in a robust dataset consisting of 5654 HCIs and 1151 high-confidence interacting proteins (HCIPs) for both the Wuhan-Hu-1 viral ORFs and the 50 mutated SARS-CoV-2 ORFs, Providing further insight into the virus-host interaction landscape.

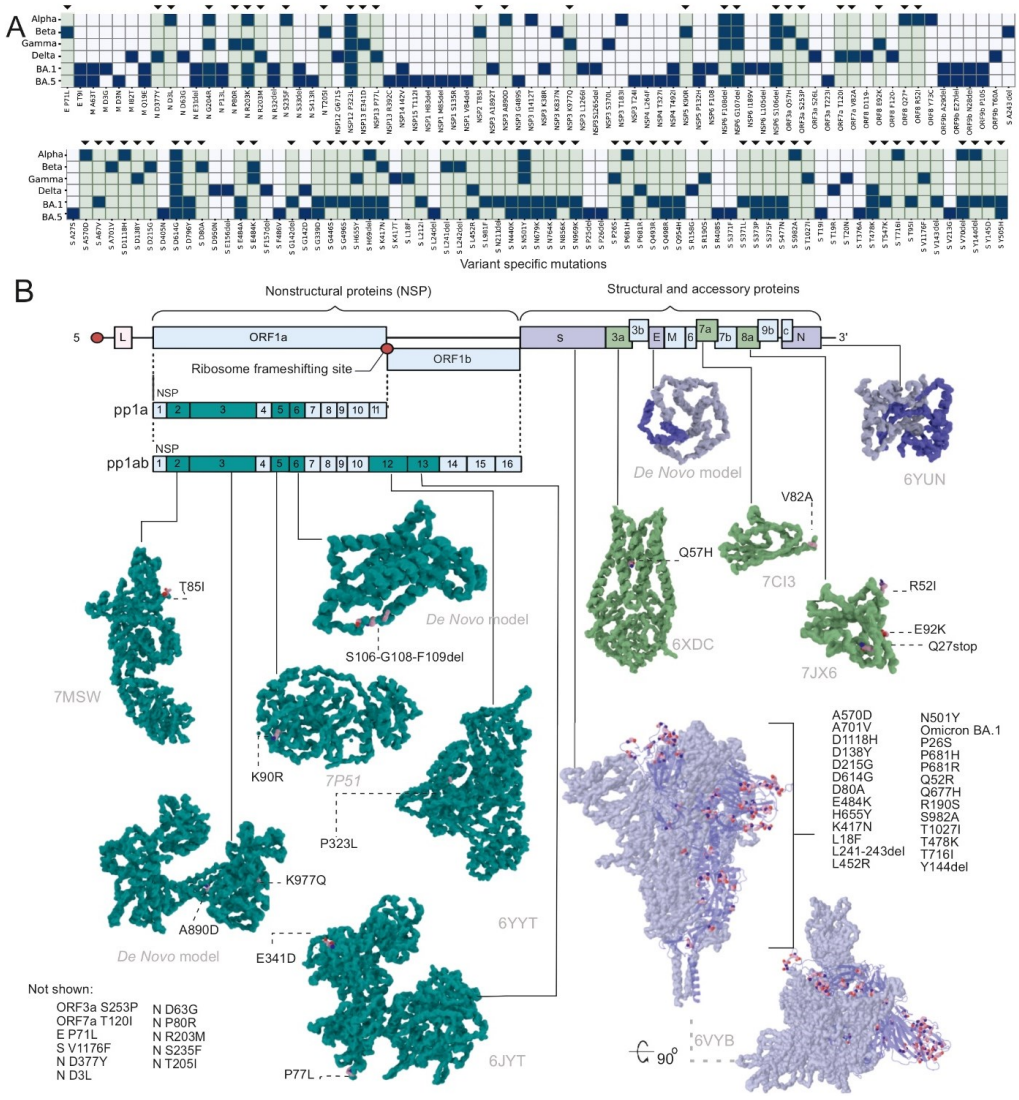


***Figure 9. Variant-specific mutations and structural models of SARS-CoV-2 proteins from Article III. A****) An overview of all mutations detected in SARS-CoV-2 variant proteins (dark blue). Mutations selected for this study are highlighted in green and small arrows at the top of the columns.* ***B****) Genomic architecture of SARS-CoV-2. The genome comprises 14 ORFs, encoding structural proteins S, E, M, and N, non-structural proteins (pp1a and pp1ab), and nine accessory proteins. The protein structures of 12 selected viral proteins with their corresponding PDB IDs (RCSB PDB database) are shown with variant-specific mutations (light pink). Structural proteins are shown in slate blue, other ORFs are in limon. NSPs cleaved from pp1A and pp1AB are depicted in teal.*

These observed HCIs and HCIPs from all three studies were further characterized using Gene Ontology (GO) analysis, focusing on biological processes (BP), cellular components (CC), and molecular functions (MF). Additionally, we applied Reactome and KEGG pathway enrichment analyses to further elucidate the intricate mechanisms of viral infection and the host-pathogen interactions present in our pool of samples (figure 10.) (Huang, Sherman, and Lempicki 2009b). Through GO analysis, we identified several biological processes significantly affected by SARS-CoV-2 infection. These included pathways related to Rho GTPase signaling, exploitation of the endoplasmic reticulum-associated degradation (ERAD) pathway, and mRNA editing and immune evasion mechanisms. These enriched pathways are discussed in detail in the following subsections.

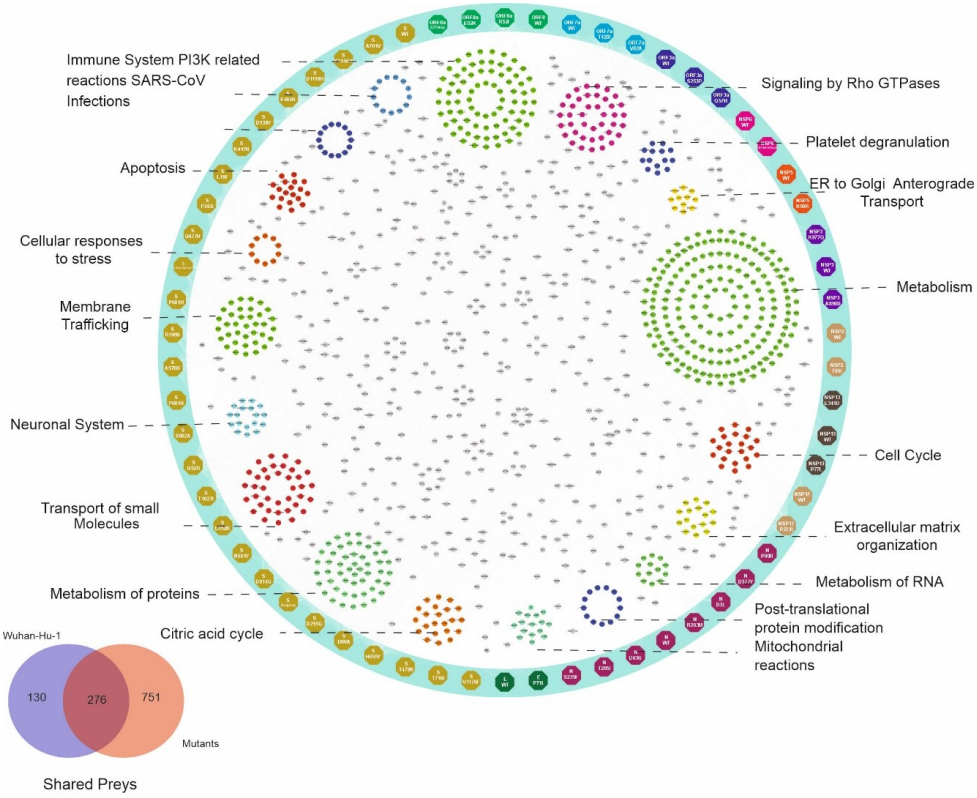


***Figure 10. Reactome enriched results from BioID data from Article III.*** *SARS-CoV-2 high-confidence interactome map with 50 mutated SARS-CoV-2 proteins expressed in Flip-in T-Rex HEK 293 cells. The enriched Reactome term clusters display interactions with preys with the corresponding terms. The Venn diagram showing the prey overlap detected by wild type and mutated ORFs.*

## 1.1 Rho GTPase signaling and cytoskeletal manipulation

Subsequent results from the HCI enrichment analysis revealed a consistent upregulation of Rho GTPase signaling components across all SARS-CoV-2 variants examined (I and III). Rho GTPases are critical regulators of the cytoskeleton, which plays an essential role in maintaining cell shape, facilitating movement, and enabling intracellular transport (Chi et al. 2013). The manipulation of Rho GTPase signaling by SARS-CoV-2 suggests a conserved viral strategy to exploit host cell structural dynamics for its own advantage. By modulating Rho GTPase pathways, SARS-CoV-2 likely enhances its ability to enter host cells, navigate intracellular environments, and promote the assembly and release of new viral particles. The upregulation of these pathways across different variants indicates that this mechanism is a key aspect of SARS-CoV-2 pathogenicity, providing the virus with a reliable means to control host cell architecture and function.

In addition to the consistent upregulation of Rho GTPase signaling, our analysis revealed variant-specific phosphorylation patterns in kinases and phosphoproteins/phosphosites, suggesting that different VOCs may employ distinct regulatory mechanisms to hijack host cell signaling (Figure 11). This underscores the evolutionary adaptation of the virus and highlights the complexity of its interaction with host cellular pathways.

Reactome enrichment analysis of BioID data emphasized the significance of viral protein interactions with Rho GTPase signaling molecules. These interactions were particularly evident in S-protein mutants (D138Y, D215G, D80A, E484K, L241-243del, L452R, P681H, R190S, Y144del), NSP12 (P323L), ORF3a (Q57H), ORF7a (T120I, V82A), and NSP6 (S106-108del), when compared to the Wuhan-Hu-1 strain. The Rho GTPase pathway, which governs various cellular processes (Nayak et al., 2013), was notably activated or altered in variants such as Alpha, Beta, Gamma, Delta, and Omicron BA.1/BA.5. In particular, S, NSP6, and ORF7a mutants demonstrated distinct signaling cascades and cellular responses.

Drugs targeting Rho GTPase signaling could interfere with the virus ability to manipulate the host cytoskeleton, thereby disrupting viral entry and replication. Given the consistent upregulation of Rho GTPase signaling across multiple variants, such a therapeutic approach could be broadly effective against various strains of SARS-CoV-2.

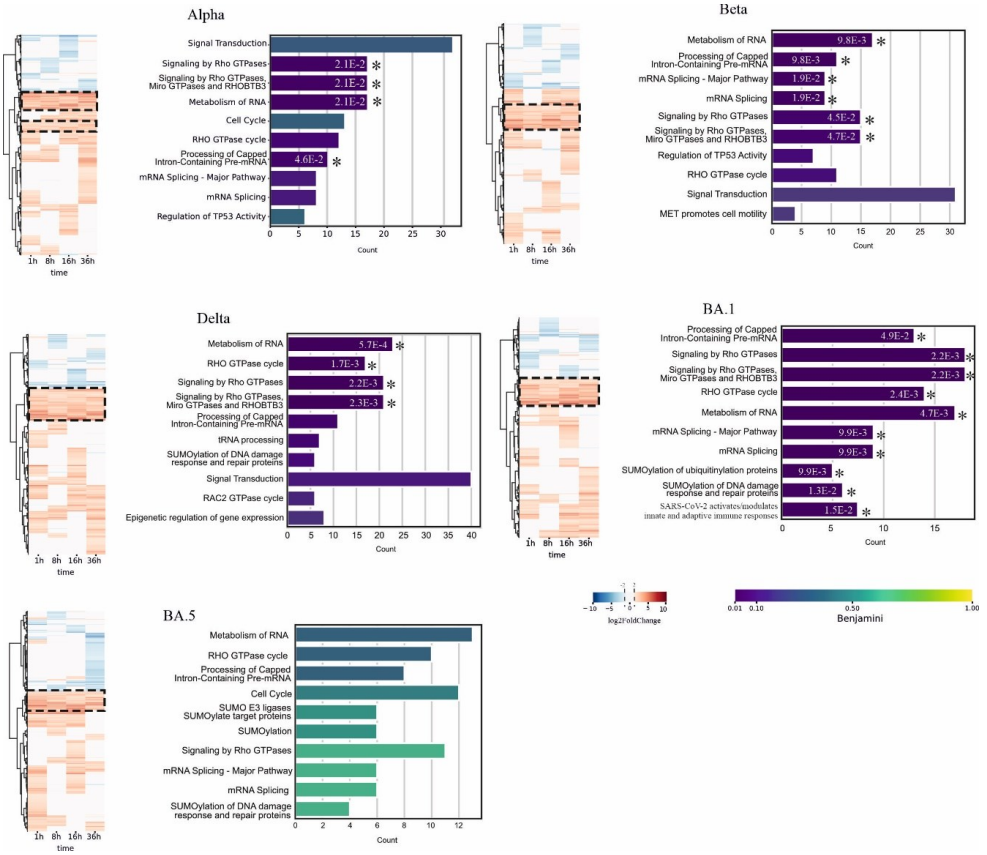


***Figure 11. Phosphoproteome analysis of SARS-CoV-2 variant infection from Article III.*** *Cluster plot delineating the temporal dynamics of protein phosphorylation across time points during SARS-CoV-2 variant infections. Comprehensive comparison analysis was conducted between variants and Control cells to identify the variant specific up-(red) or down-(blue) regulation. Numbers ontop of arrows representsnumber of uniqueproteins detected in boxed upregulated cluster Selected clusters of consistently upregulated phosphorylation events were subjected to Reactome pathway enrichment analysis using the DAVID functional annotation tool. Top 10 pathway and processes terms by count are shown with the corresponding enrichment adjusted p-value (Benjamini). An asterisk adjacent to the bar denotes significant threshold of enrichment terms ($p<0.05$), with the corresponding adjusted p-value.*

## 1.2 Exploitation of the endoplasmic reticulum-associated degradation pathway

SARS-CoV-2 is thought to enter host cells primarily via clathrin-mediated endocytosis, allowing the virus to exploit vesicular trafficking pathways while avoiding early detection by the immune system. Following ACE2 receptor binding, the virus is internalized into clathrin/AP2-coated vesicles, transported into RAB5-positive early endosomes, and subsequently uncoated in late endosomes or lysosomes, where the viral genome is released and replication begins (Bayati et al. 2021; Cesar-Silva et al. 2022). This entry mechanism has been considered advantageous for efficient viral dissemination and immune evasion.

Interactions between SARS-CoV-2 and components of the endocytosis and endoplasmic reticulum–associated degradation (ERAD) pathways were investigated (I and III). A total of 47 host proteins involved in endocytosis were found to interact with viral proteins. The greatest number of interactions was detected for viral proteins M (27 interactions), ORF3a (26), ORF7a (19), NSP6 (18), and S (15). These results suggest that endocytic pathways are widely targeted by multiple viral components to facilitate entry and intracellular trafficking. It is proposed that these findings may offer potential therapeutic angles by identifying host components—such as CDC42 and clathrin/AP2 complex members—as possible targets to block viral entry.

Concurrently, significant exploitation of ER processing and ERAD pathways was also observed. A total of 41 ER-associated proteins were identified as interaction partners of viral ORFs, including M, ORF7a, ORF3a, ORF10, S, ORF8, NSP6, E, and ORF6. The disruption of ER homeostasis and induction of ER stress appear to play a critical role in viral replication and immune modulation (Fung and Liu 2014a). Post-translational modifications of viral proteins—particularly S, E, and M—were likely processed within the ER, triggering the expression of ER stress–related chaperones such as CANX, HSPA5, PDIA3, and PDIA4 (Ma et al. 2008; Fukushi et al. 2012) (Figure 12.).

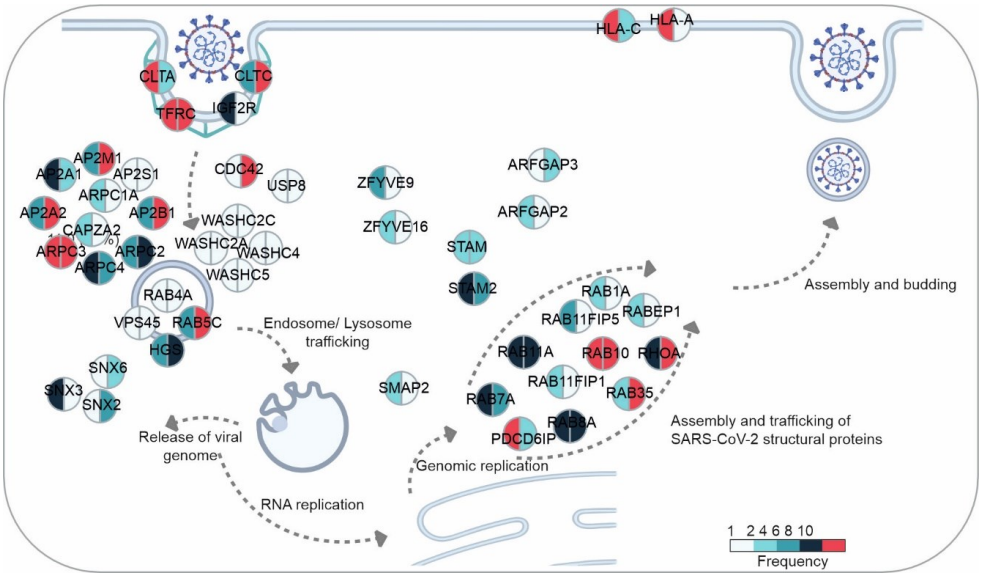


***Figure 12. ERAD pathway related proteis detected in Article I.*** *KEGG Enriched proteins were illustrated by the schematic diagram relevant to endocytosis (P-value: 5.84 × $10^{-15}$) and protein processing in ER (P-value: 1.80 × $10^{-22}$). The color of each slice in a node corresponds to the frequency of occurrence of the prey that was detected in virus–host interactome (left half slice) and host–receptor interactome (right half slice).*

Prolonged ER stress has been shown to lead to apoptosis, but in the case of SARS-CoV-2, it may also facilitate the formation of double-membrane vesicles (DMVs), which serve as replication compartments. This process is believed to be supported by COPII-coated vesicles and proteins such as SEC23/24 and LMAN1 (Wolff, Limpens, et al. 2020; Wolff, Melia, et al. 2020). Overexpression of viral proteins has also been associated with the accumulation of misfolded proteins, which in turn activates the ERAD pathway to maintain ER function (Byun et al. 2014).

The involvement of the ERAD pathway in viral infections has been previously reported for several viruses. For instance, HIV-1 has been shown to hijack ERAD to degrade MHC-I molecules, thereby impairing antigen presentation and facilitating immune evasion (Byun et al. 2014; Zou et al. 2022). Likewise, flaviviruses such as dengue and Zika virus utilize ERAD components to modulate host protein folding and reduce ER stress, helping the virus avoid detection by innate immune sensors (Verhaegen and Vermeire 2024). In the context of coronaviruses, viral proteins have been found to trigger ER stress and interact with ERAD components, thereby balancing viral replication needs and innate immune activation (Fung, Huang, and Liu 2014b). These studies suggest that ERAD serves not only as a quality control system but also as a regulatory node that viruses exploit to evade immune responses while maintaining ER function during infection.

GO biological process enrichment (BP) of BioID samples revealed associations between specific SARS-CoV-2 variant mutations and ERAD-related processes (III). Notably, mutations in NSP12 (P323L) and ORF8a (E92K), as well as multiple Spike mutations (e.g., P681H, T478K), were linked to increased engagement with ER stress pathways. Quantitative proteomics data further confirmed that upregulation of ERAD components was more pronounced in certain variants. For example, in cells infected with the Beta variant, DERL1—a key regulator of retrotranslocation in the ERAD pathway—was significantly upregulated during later stages of infection (Sugiyama et al. 2021). This suggests that heavier reliance on the ERAD pathway may confer a replication or immune evasion advantage. Enhanced interactions with ERAD components were especially noted for mutated forms of NSP12 and ORF8.

Furthermore, RETREG1, an autophagy-related regulator of ERAD, was found to be consistently downregulated in the Alpha and Omicron BA.5 variants, while in other variants its expression re-emerged during the middle or late stages of infection (Mookherjee et al. 2021). This suggests that variant-specific modulation of ER stress and autophagy signaling may contribute to differences in replication efficiency or host adaptation strategies.

Taken together, these findings indicate that SARS-CoV-2 systematically exploits both endocytic and ER-associated degradation pathways across multiple variants, and that proteomic profiling can reveal conserved host–virus interfaces that may be of high value for therapeutic intervention and drug repurposing.

## 1.3 mRNA editing and immune evasion in SARS-CoV-2

In addition to altering host signaling and protein trafficking pathways, SARS-CoV-2 appears to manipulate host mRNA processing mechanisms to promote viral gene expression and evade immune responses. Although not directly measured in this study, our enrichment analyses revealed host processes related to RNA metabolism, mRNA splicing, and translation regulation, suggesting that viral proteins may interfere with these pathways during infection.

This is supported by previous studies showing that SARS-CoV-2 suppresses host mRNA stability and alternative splicing, thereby prioritizing viral mRNA translation and reducing host antiviral gene expression (Banerjee et al. 2020; Gu et al. 2022). This interference is particularly important for dampening interferon (IFN) responses, a key component of early antiviral immunity (I).

Several viral mutations have been associated with altered immune evasion strategies (III). For example, NSP13 mutations have been shown to reduce activation of innate immune sensors, highlighting the protein's role in evading host defenses and suggesting its potential as a drug target (Vazquez et al. 2021; Spratt et al. 2021). Similarly, mutations in NSP12 (P323L) and ORF7a (V82A, T120I)—common in the Delta variant—have been linked to suppression of interferon signaling (Cao et al. 2021; Xia et al. 2020). The nucleocapsid (N) protein also contributes to immune evasion by interfering with host RNA sensors such as RIG-I, which detect viral RNA in the cytosol. Mutations in the N protein, such as D63G and P80R, found in Gamma and Delta variants, have been associated with enhanced viral replication (Oh and Shin 2021; Chen et al. 2021).

Furthermore, alternative splicing is believed to be exploited by the virus to downregulate host antiviral proteins and modulate cytokine signaling. This mechanism may allow the virus to fine-tune the host immune response in a variant-specific manner. For instance, Beta and Omicron variants have been associated with altered activity of host splicing factors, potentially contributing to enhanced immune evasion while maintaining high transmissibility (Oh and Shin 2021).

Taken together, these findings from the literature complement our observed enrichment of RNA-related processes in host–virus interaction networks and highlight how mutations in SARS-CoV-2 proteins may reinforce the virus's ability to manipulate post-transcriptional gene regulation for immune evasion and replication efficiency.

# 2. Therapeutic implications and aplications

The repurposing of existing drugs to target the Wuhan-Hu-1 strain of SARS-CoV-2 was investigated by focusing on viral proteins with available crystal structures and druggable binding pockets (I). A virtual screening approach was employed to evaluate 5518 drugs, resulting in the identification of 59 potential repurposing candidates targeting 15 viral proteins. Many of these compounds had previously been reported to possess antiviral, anti-inflammatory, antibacterial, or anticancer properties, supporting their potential utility against SARS-CoV-2 (Figure 13.).

Among the identified drugs, three antidiabetic agents (licogliflozin, pioglitazone, and MK-0767) may have a potential effect on SARS-CoV-2 infection based on the screening results. Pioglitazone, for instance, has shown in clinical trials that treatment may have certain biochemical effects, such as lowering interleukin (IL)-3 levels, however, it does not independently provide clinical benefits to COVID-19 patients with type 2 diabetes in hospital settings (Baagar et al. 2024). While pioglitazone has been explored for its potential benefits in COVID-19 treatment, licogliflozin and MK-0767 have not been investigated in clinical trials for this purpose.

The in vitro antiviral activity of several candidate drugs was assessed using an image-based drug screen that monitored viral N protein expression. Ten drugs available in house were evaluated in this study. Six drugs—baicalein, methotrexate (MTX), guadecitabine, PX-478, mizoribine, and BMS-863233—demonstrated promising antiviral effects, with MTX and guadecitabine standing out for their potential to inhibit SARS-CoV-2 replication. MTX, in particular, showed potential by inhibiting the DEAD-box RNA helicase DDX39B (Rao and Mahmoudi 2022), which is known to interact with viral ORFs and host receptors. Since DDX39B is essential for viral replication, its inhibition by MTX suggests a possible mechanism for reducing viral replication and impeding viral entry into host cells.

However, rest of the compounds did not display effects at the protein-protein interaction (PPI) level. For example, BMS-863233 did not alter PPIs, likely due to the broad spectrum of target molecules it affects or potential toxicity at higher concentrations. Moreover, the study identified core viral processing subnetworks with biologically relevant targets, suggesting that repurposing drugs to target these proteins could mitigate disease severity. Shared targets among these subnetworks, such as guadecitabine dual interaction with mitochondrial Rho GTPase 2 (RHOT2) and SAR1a, provide an opportunity for multi-target drug therapies.

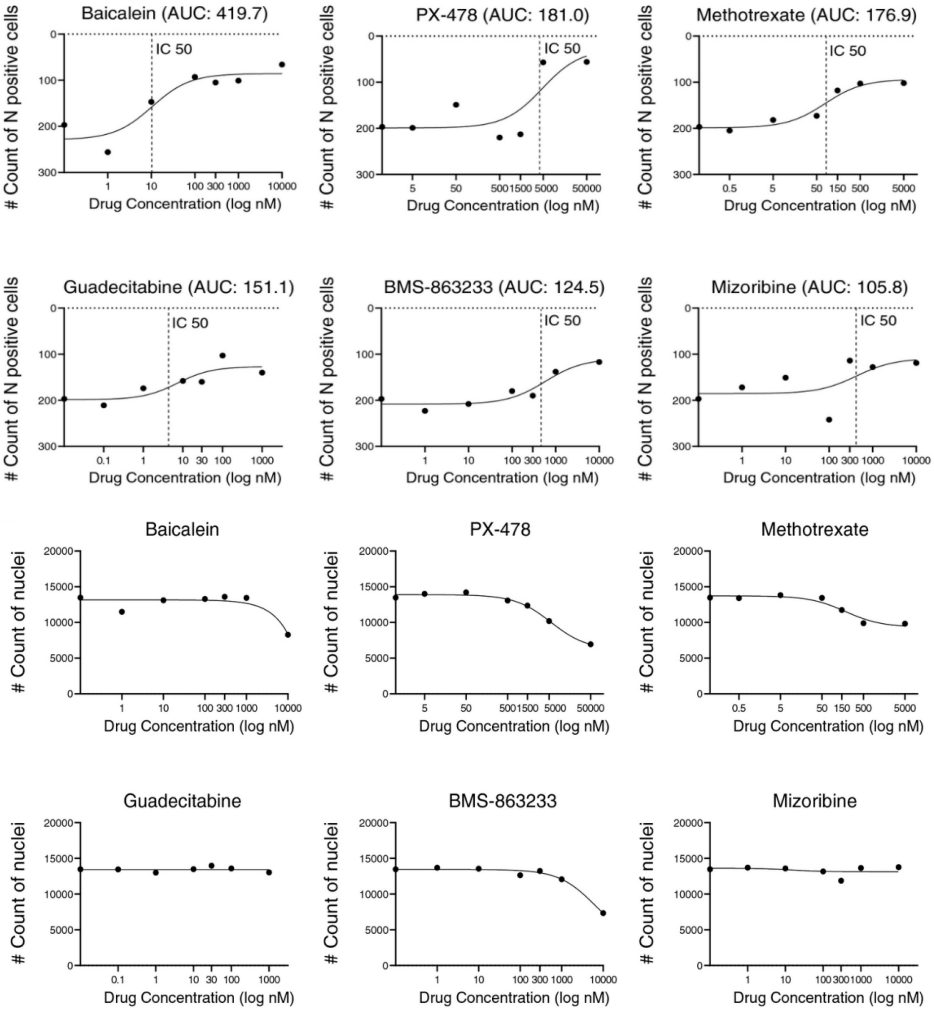


***Figure 13. The dose–response curves for six drugs with potential antiviral effects in Calu-1 cells from Article I.*** *The drug-induced inhibition of virus infection (count of viral-N protein-positive cells) was quantified using the area under the curve (AUC) values (top two rows). Alternatively, the effect of drugs on cell viability in the presence of the virus, e.g., cytotoxicity, was evaluated by the count of nuclei (last two rows).*

Expanding the scope from the Wuhan-Hu-1 strain, by exploring druggable targets across multiple SARS-CoV-2 VOCs (III). By analyzing a dataset of 1 474 HCI proteins, the study evaluated druggability based on structural analysis, including X-ray crystallography, electron microscopy, and AlphaFold models (Figure 14). Of these proteins, 1126 were found to have druggable pockets suitable for small molecule binding, while 680 were predicted to have druggable PPI sites. In addition, 61 proteins were identified with cryptic binding sites that could be activated by ligand binding. This approach highlights the potential of targeting dynamic protein conformations, expanding the range of druggable targets. (Loving, Lin, and Cheng 2014)

A key finding from this analysis is the identification of 273 proteins that bind ligands with properties that satisfy Lipinski Rule of Five, meaning these ligands likely have favorable characteristics for oral bioavailability. According to Lipinski criteria, such ligands typically have a molecular weight under 500 Daltons, a partition coefficient (LogP) of less than 5, no more than 5 hydrogen bond donors, and no more than 10 hydrogen bond acceptors (Lipinski 2004). In contrast, proteins with binding profiles similar to inhibitors of PPIs (iPPIs) were highlighted. Furthermore, the study identified 69 proteins as already established drug targets, with 91 others currently under clinical investigation. An additional 129 proteins have been researched to some extent, suggesting a wealth of potential for further drug development. Among these proteins, 60 protein had major changes in expression, phosphorylation, and PPIs, 46 were predicted to possess druggable deep binding sites, and 14 had druggable PPI sites. These proteins represent promising candidates for the development of novel anti-coronaviral therapies.

One of the most curious aspects of this research is the identification of phosphorylation-dependent processes as key drivers of SARS-CoV-2 pathogenicity. Targeting these phosphorylation events—particularly those conserved across multiple variants—could offer a new avenue for antiviral therapy. Kinase inhibitors, widely used in cancer treatment, may be repurposed to disrupt the virus manipulation of host cell signaling pathways, reducing viral replication and spread. The variant-specific findings also point to the potential for personalized medicine in COVID-19 treatment. For example, therapies targeting phosphorylation events unique to the Delta variant may be particularly effective in treating severe cases, while different strategies may be needed for Omicron or other variants. In addition to therapeutic interventions, these findings have significant implications for vaccine development. Vaccines that target conserved phosphorylation-dependent mechanisms across SARS-CoV-2 variants could provide broader and more durable protection.

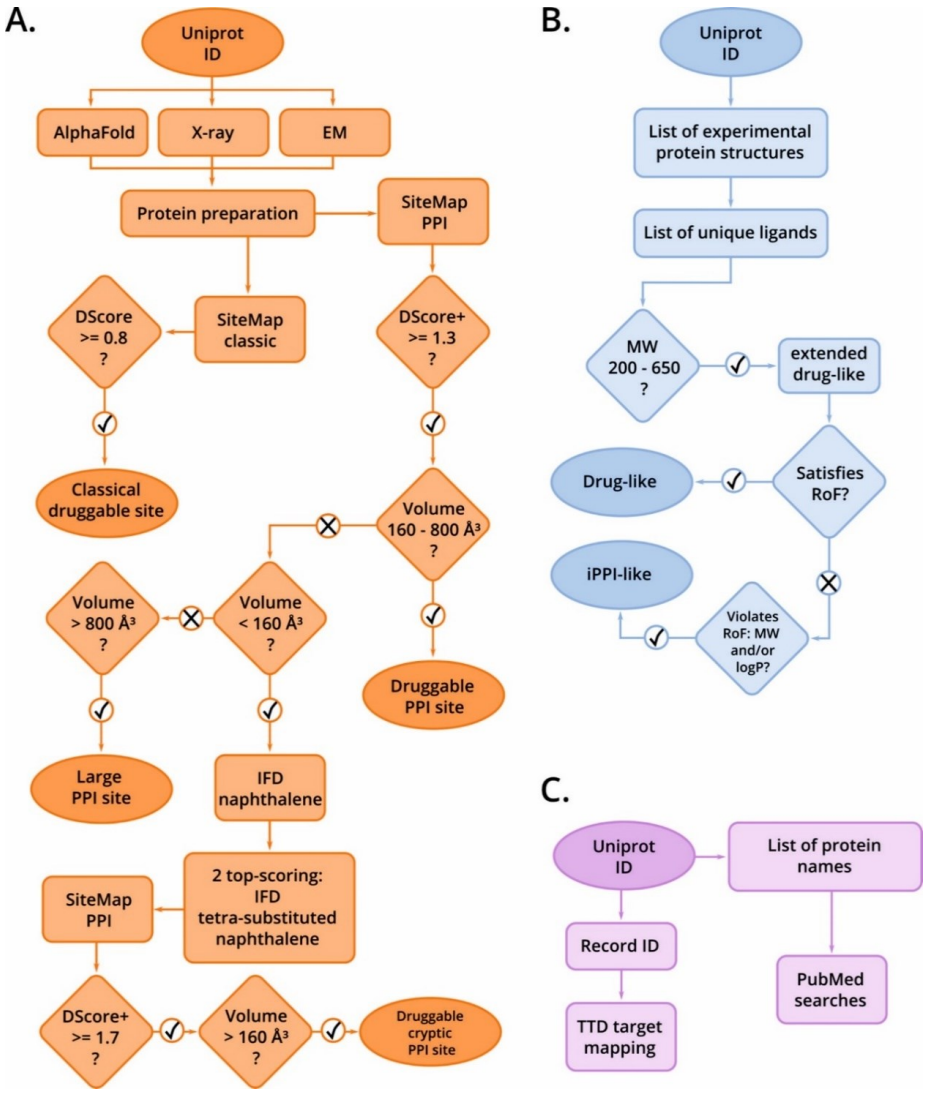


***Figure 14. Schematic illustrations of workflows for target druggability assessmen from article III***
**A**.*) Structure-based pocket prediction and druggability analysis workflow with Schrödinger SiteMap and induced fit docking (IFD) steps to open cryptic pockets with limited protein flexibility modeling. PPI = protein: protein interaction.* ***B)****. Evaluation of properties of ligands in experimental target structures. RoF = Rule-of-Five; MW = molecular weight; iPPI = PPI inhibitor.* ***C)****. Workflow to assess literature coverage by PubMed searches and cross-analysis with the Therapeutic Target Database (TTD)*

# V Conclusion and future perspectives

The research presented in this thesis contributes to the broader understanding of SARS-CoV-2 biology by providing detailed insights into the molecular interactions between the virus and host cells. The use of advanced proteomic and phosphoproteomic techniques has uncovered events that are central to the virus ability to replicate and evade immune detection.

These findings have significant implications for the development of antiviral therapies and vaccines, as they highlight the importance of targeting phosphorylation-dependent processes in the fight against COVID-19. The identification of conserved signaling pathways, such as, ERAD, mRNA processing, and Rho GTPase signaling, suggests that some therapeutic strategies may be effective against multiple variants, while the variant-specific findings underscore the need for personalized approaches.

Future research should continue to explore the molecular mechanisms of SARS-CoV-2 pathogenicity, particularly as new variants emerge. The ongoing evolution of the virus, driven by selective pressures such as immune responses and antiviral treatments, will likely result in the emergence of new strategies for evading host defenses. Understanding these strategies at the molecular level will be crucial for staying ahead of the virus and developing effective countermeasures.

Expanding research to encompass a broader range of viruses through comparative proteomics enhances our understanding of viral biology, host-pathogen interactions, and viral pathogenesis mechanisms. Comparative analyses of viral proteomes across different viral families allow identifying conserved structural motifs, functional domains, and protein-protein interaction networks shared among diverse viruses.

# VI Acknowledgements

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