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The exploration of antiviral drug candidates against coronaviruses and the mechanisms

Dissertation

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Table of content

1. List of abbreviations

ACE2	Angiotensin-Converting Enzyme 2
ALV	Alisporivir
APN	AminoPeptidase N
CoIP	Co-ImmunoPrecipitation
CoV	CoronaVirus
COVID-19	COronaVIrus Disease 2019
CsA	Cyclosporine A
Cyp	Cyclophilin
CypA	Cyclophilin A
CypB	Cyclophilin B
CypC	Cyclophilin C
CypD	Cyclophilin D
CypI	Cyclophilin Inhibitor
DMSO	DiMethyl SulfOxide
DMV	Double-Membrane Vesicle
DNA	DeoxyriboNucleic Acid
DPP4	DiPeptidyl Peptidase IV
dsRNA	double-stranded RNA
E	Envelope protein
eCypA	extracellular CypA
eCypB	extracellular CypB
ER	Endoplasmic Reticulum
FCoV	Feline CoV
FDA	The United States Food and Drug Administration
HCoV	Human CoronaVirus
HCoV-229E	Human CoronaVirus 229E
HCoV-NL63	Human CoronaVirus NL63
HCoV-OC43	Human CoronaVirus OC43
HE	Hemagglutinin-Esterase protein
IBV	Infectious Bronchitis Virus
iCypA	intracellular CypA

ITZ	ITraconaZole
LTPs	Lipid Transfer Proteins
M	Membrane protein
MERS-CoV	Middle East Respiratory Syndrome CoronaVirus
MHV	Mouse Hepatitis Virus
N	Nucleocapsid protein
NFAT	Nuclear Factor of Activated T cells
Nsp	Nonstructural protein
ORPs	OSBP-related proteins
OSBP	OxySterol Binding Protein
PDCoV	Porcine DeltaCoronaVirus
PEDV	Porcine Epidemic Diarrhea Virus
PI4P	PhosphatidylInositol 4-Phosphate
PPIs	Protein-Protein Interactions
PPIase	Peptidyl-Prolyl cis-trans Isomerase
RLR	RIG-I-like Receptor
RNA	RiboNucleic Acid
RO	Replication Organelle
S	Spike protein
SADS-CoV	Swine Acute Diarrhea Syndrome-Coronavirus
SARS	Severe Acute Respiratory Syndrome
Y2H	Yeast Two Hybrid-Screening

2. List of publications

Scientific publications summarized in this thesis:

Publication I:

Yang, F., Liu, C., **Li, P. (co-first author),** Wu, A., Ma-Lauer, Y., Zhang, H., Su, Z., Lu, W., von Brunn, A., & Zhu, D. (2023). Targeting Cyclophilin A and CD147 to Inhibit Replication of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and SARS-CoV-2-Induced Inflammation. Mol Pharmacol, 104(6), 239-254. https://doi.org/10.1124/molpharm.122.000587

Publication II:

Ma-Lauer, Y., **Li, P. (co-first author),** Niemeyer, D., Richter, A., Pusl, K., von Brunn, B., Ru, Y., Xiang, C., Schwinghammer, S., Liu, J., Baral, P., Berthold, E. J., Qiu, H., Roy, A., Kremmer, E., Flaswinkel, H., Drosten, C., Jin, Z., & von Brunn, A. (2024). Oxysterole-binding protein targeted by SARS-CoV-2 viral proteins regulates coronavirus replication. Frontiers in Cellular and Infection Microbiology, 14. https://doi.org/10.3389/fcimb.2024.1383917

3. My contribution to the publications

3.1 Contribution to Publication I

For Publication I with the title “Targeting Cyclophilin A and CD147 to Inhibit Replication of SARS-CoV-2 and SARS-CoV-2-Induced Inflammation”, published in Molecular Pharmacology on November 15, 2023, I conducted co-immuno-precipitation and split YFP assay to examine the protein-protein interactions between SARS-CoV-2 spike protein and host protein CD147 (S-CD147), CD147 and CypA (CD147-CypA), CypA and SARS-CoV-2 nucleocapsid protein (CypA-N). I contributed in writing the paper and revising it as well. I was one of the co-first authors of this publication.

3.2 Contribution to Publication II

For Publication II with the title “Oxysterole-binding protein targeted by SARS-CoV-2 viral proteins regulate coronavirus replication”, published in Frontiers in Cellular and Infection Microbiology on July 25, 2024, I contributed to carrying out experiments, writing part of the manuscript, analyzing data, and preparing several figures.

To be specific, I wrote the method part of Yeast Two Hybrid (Y2H) screening (2.10) and Statistical analysis (2.11), summarized the primer list (Table S1) and constructed some plasmids used for several experiments in the paper. I analyzed the raw data to generate figures in Figure1G, 1I and 1J. Additionally, I conducted the experiments in Figure 2A and Figure S2 and also wrote the corresponding figure legends. I contributed to Figure 3E, 3F and 3G by conducting experiments, and then analyzing and describing them. Moreover, I also provided the graphic summary in Figure S7. Furthermore, I contributed to the submission, revision and proofreading.

4. Introduction

4.1 Coronaviruses

Coronaviruses, or the *Coronaviridae* Family, contains four genera, the Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. As a common feature, coronaviruses own the largest enveloped, single-stranded positive RNA genome (about 30Kb) carrying a large gene termed as ORF1a/b (which is subsequently translated into two polyproteins pp1a and pp1ab), genes of four general structural proteins, and a variable number of accessory genes.

The four structural proteins common for all coronaviruses are spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N) [1]. Some coronaviruses from the Betacoronavirus genus, e.g. human coronavirus (HCoV) OC43 and HKU-1, have an extra structural protein, the hemagglutinin-esterase protein (HE),[2, 3]. The N protein, a crucial structural protein for all coronaviruses, is capable of forming a capsid to package the genome, protect it, and is involved in viral replication and viral pathogenesis [4].

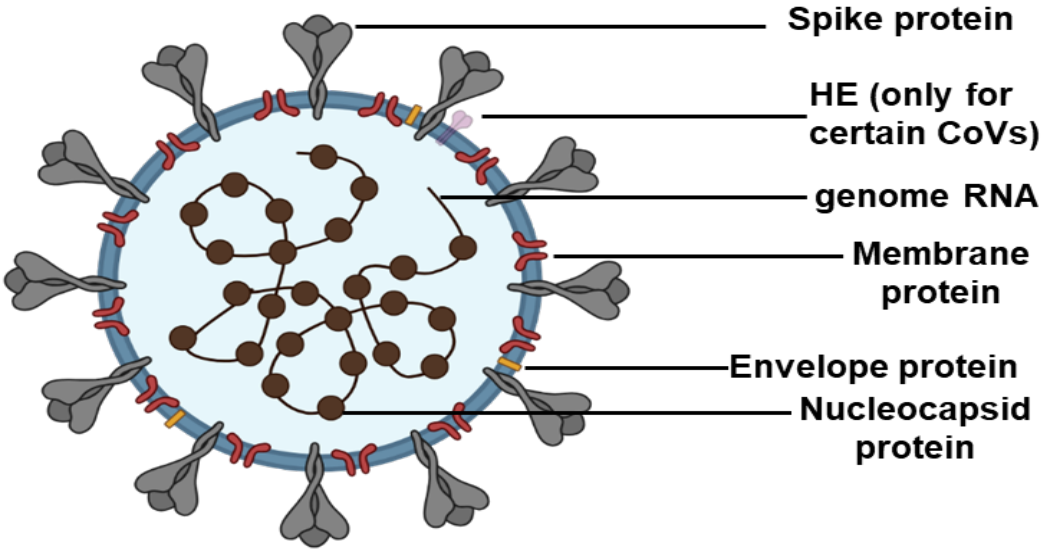

Figure 1 The general structure of coronavirus

The Nonstructural proteins (Nsps) in coronaviruses are important and indispensable for their enzymatic activities and other fundamental functions. For instance, Nsp3 and Nsp5 are two proteinases responsible for the mature and

release of almost all nonstructural proteins [5, 6]. Nsp12 is the RNA-dependent RNA polymerase in charge of replication and methylation [7]. Among all nonstructural proteins, Nsp3 is a multi-domain protein and the largest nonstructural viral protein (1945 amino acids) coded by coronaviruses with several important functions such as proteinase activity. Besides the aforementioned proteinase activity, it is also involved in post-translation modifications by deubiquitination or deISGylation [8]. Nsp3 has a high frequency of mutations, making it a primary target for evolutionary pressure in coronaviruses [9]. Nsp3 is the main component to form the replication organelle (RO) together with Nsp4 [10]. The replication of coronaviruses depends on the formation of RO. Viruses utilize the endoplasmic reticulum (ER) membrane to form Double-Membrane Vesicles (DMVs) accommodating the replication process. Another nonstructural protein, Nsp6 also contributes to the viral replication. It connects RO to ER membranes and mediates lipid transfer between RO and ER [11]. The accessory proteins of coronaviruses are a group of unique proteins interspersed within or among structural-protein encoding genes. Although they are not pivotal to several coronaviruses' replication [12], some accessory proteins are involved in the immune response escape and viral pathogenesis [13, 14]. Given the important and complicated functions of those viral proteins, researchers are interested in studying the virus-host interactions and looking for therapeutic targets that antagonize viral replication.

The replication cycle of coronaviruses starts from the host cell attachment mediated by S protein. Coronaviruses use peptidases as their host receptors. Whereas most Alphacoronaviruses except NL63 utilize Aminopeptidase N (APN) as their cellular receptor [15], NL63 and Betacoronaviruses such as SARS-CoV and SARS-CoV-2 share the same receptor ACE-2 [16-19]. The most representative coronaviruses and their corresponding receptors are listed in table 1.

Table 1 coronaviruses and their host receptors

Virus	Receptor	References
Alphacoronaviruses		
HCoV-229E (human)	APN	[20]
HCoV-NL63 (human)	ACE2	[16]
TGEV (pig)	APN	[21]

PEDV (pig)	APN	[22]
Betacoronaviruses		
MHV (mouse)	mCEACAM	[23]
SARS-CoV (human)	ACE2	[18]
MERS-CoV (human)	DPP4	[24]
HCoV-OC43 (human)	9-O-Acetylated sialic acid	[25]
HCoV-HKU1 (human)	9-O-Acetylated sialic acid	[26]
SARS-CoV-2 (human)	ACE2	[27]

4.1.1 Coronaviruses in animals

Coronaviruses are well-studied in the field of veterinary medicine because coronaviruses from all four genera (Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus) can infect both wild and domestic animals among a wide range of species [28]. The Alphacoronavirus and Betacoronavirus mostly infect mammals, whereas Gammacoronavirus and Deltacoronavirusare are typically found in avian hosts. The representative of avian coronaviruses is the Infectious Bronchitis Virus (IBV) which propagates among chickens and other domestic birds [29]. Pig is another susceptible host for several coronaviruses. To date, six coronaviruses have been identified to infect pigs so far. Among them, Porcine Epidemic Diarrhoea Virus (PEDV), Porcine Deltacoronavirus (PDCoV), and Swine Acute Diarrhea Syndrome-CoronaVirus (SADS-CoV) cause acute gastroenteritis in piglets, resulting in death of piglets and huge economic loss [30, 31]. Bats play a critical role in the propagation, evolution, and cross-species transmission (also called “spillover”) of coronaviruses as the reservoir hosts for many coronaviruses. It is reported that more than 4,800 coronavirus sequences have been identified in bats, accounting for 30% of all bat viruses that have been sequenced [32]. Bats as the reservoir hosts generally show tolerance to infection with coronaviruses[33] even though some bats in experimental environments exhibit slight tissue damage [34]. Most researchers believe the coronaviruses circulating in mammals originated from bat-borne coronaviruses [35]. For example, five out of

seven human coronaviruses can be traced back to bat coronaviruses (SARS-CoV, MERS-CoV, SARS-CoV-2, HCoV-229E and HCoV-NL63) [27, 36].

4.1.2 Mild human coronaviruses

Up to now, there are seven coronaviruses that can be transmitted from person to person, namely HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2. Among them, the first four viruses can be classified as mild human coronaviruses, given they typically cause mild upper respiratory tract illnesses, accounting for about fifteen to thirty percent of adult colds [37]. The first two human coronaviruses, HCoV-229E and HCoV-OC43 were identified in the mid-1960s and were regarded as the only two human coronaviruses for decades till 2002. The virus HCoV-229E was isolated by Tyrrell and Bynoe [38] in a sample obtained from an adult patient with common cold (originally called B814). Hamre and Procknow [39] successfully cultivated the virus and named it 229E. The discovery of HCoV-OC43 happened in approximately the same period. McIntosh et al [40] isolated multiple similar strains following the same technique adopted by Tyrrell and Bynoe. The second human coronavirus HCoV-OC43 was then identified via organ culture [41]. HCoV-229E and HCoV-OC43 taxonomically belong to Alphacorona- and Betacoronaviruses, respectively.

Between 2002-2003, a worldwide pandemic caused by the first known highly pathogenic coronavirus SARS-CoV drew the attention of scientists and researchers. The increase of studies on coronavirus since the outbreak of SARS led to the discovery of two novel human coronaviruses, HCoV-NL63 and HCoV-HKU1 [42].

The third mild human coronavirus is HCoV-NL63, which was initially isolated from a seven-month-old infant in the Netherlands [43]. The last type of mild human coronavirus, HCoV-HKU1, was documented in 2004 from a 71-year-old male patient [44]. Different from HCoV-NL63 (verified as an Alphacoronavirus), HCoV-HKU1 has been ascertained to be a Betacoronavirus.

Normally, the mild human coronaviruses only cause self-limiting infection in the upper respiratory tract with mild symptoms. However, in young children, old people, and individuals with underlying health problems or diseases, even mild

coronaviruses can lead to severe symptoms, especially induced by severe infection and inflammation in the lower respiratory tract [15]. A retrospective study in France [42] found around 10% respiratory specimens from children (80% under 5 years old) to be positive for HCoV-NL63 and more than one-third of the patients to have severe lower respiratory tract infection. Another epidemiological study conducted in Hong Kong showed that HCoV-HKU1 tended to infect children with underlying disease and cause severe symptoms such as pneumonia, acute bronchiolitis, asthmatic exacerbation and febrile seizures [45].

The epidemic of those mild human coronaviruses features seasonal patterns: onset during October-November and offset between April-June [46].

4.1.3 Highly pathogenic human coronaviruses (SARS-CoV and MERS-CoV)

There are three highly pathogenic human coronaviruses that have emerged in the 21st century so far: SARS-CoV in 2002-2003, MERS-CoV in April 2012 and SARS-CoV-2 in December 2019.

The Severe Acute Respiratory Syndrome (SARS) is the first lethal disease with high mortality caused by a coronavirus. The virus that caused the worldwide-pandemic during 2002-2003 was then named as SARS-CoV. The SARS disease first occurred in Southern China in November 2002 and its range then expanded to at least 27 countries. It caused more than 8000 cases and more than 900 deaths in total [47, 48]. The SARS pandemic was brought under control within a period of eight months through a coordinated global effort initiated by the WHO. By July 2003, the SARS-CoV transmission from person to person had ceased and the global outbreak was declared over [49].

The virus enters cells through the binding of spike protein to the host receptor angiotensin-converting enzyme 2 (ACE2). Thereafter, the viral genome is released into the cytoplasm of the host cell. The expression of the SARS-CoV genome starts with the (cap-dependent) RNA translation, which produces polyprotein 1a (pp1a) and polyprotein pp1ab (by ribosomal frameshifting), which is longer than pp1a. Then the viral replication is initiated by the viral proteins pp1a and pp1ab together with cellular factors [50].

The primary symptoms in the patients infected with SARS are fever, chills, cough, and other flu-like discomfort [51]. Approximately 70% of these patients present with symptoms such as respiratory distress and a continuous elevation in body temperature. [52]. Acute respiratory distress syndrome is typical among severe cases and the major complication is respiratory failure [53].

In 2012, a second novel strain of highly pathogenic human coronavirus emerged, namely Middle East Respiratory Syndrome Coronavirus (MERS-CoV), which caused a major outbreak in the Middle East. As of May 2012, there had been more than 2500 cases globally and around 900 deaths according to WHO public data reports [54]. In total, 27 countries reported cases of MERS, whereby 80% cases were reported by Saudi Arabia [55, 56]. MERS-CoV is a zoonotic pathogen. Camels are the intermediate hosts directly mediating the virus transmission to humans, even though evidence shows that bats are the original host species [57-59]. The host binding receptor for MERS-CoV is DiPeptidyl Peptidase IV (DPP4) [60].

MERS-CoV is more pathogenic than SARS-CoV, given the high mortality rate among confirmed cases (about 35%). The clinical symptoms of MERS are similar to those of SARS, i.e. upper and/or lower respiratory infections [61]

4.1.4 Human coronavirus SARS-CoV-2 and COVID-19

The third life-threatening, lethal human coronavirus emerged in late December 2019. First described in Wuhan, China, the WHO formally designated the novel coronavirus as "Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)" on February 11, 2020. The disease caused by SARS-CoV-2 is designated "COronaVIrus Disease 2019," or "COVID-19." [62]. So far, 227 countries or regions are involved in this pandemic, with over 700 million cases and 7 million deaths [63, 64]. Besides the heavy burden to public human health, the pandemic has also resulted in deep recession and huge economic loss globally. In May 2023, the WHO announced the end of the emergency phase of COVID-19, however, SARS-CoV-2 is still circulating among populations all over the world.

SARS-CoV-2 is very closely related to SARS-CoV, especially since they share over 80% similarity in their genomes [65]. Given the high similarity, SARS-CoV-

2 has a similar structure of viral proteins as SARS-CoV, encoded by the 30kb genome. Like other RNA viruses, SARS-CoV-2 evolves rather quickly. The variants of SARS-CoV-2 which substantially influence the disease severity, and enhance the transmission capacity or decrease the effectiveness of vaccines are called Variants Of Concern (VOCs). Even though SARS-CoV-2 showed limited evolution in the first 8 months since the outbreak, three VOCs (later named as Alpha, Beta, and Gamma) emerged almost simultaneously in different parts of the world. After the identification of these variants, scientists observed a shift of dominant strain Delta, and subsequently Omicron [66, 67].

Fever, dry cough, and fatigue are some common clinical features of COVID-19 patients. Severe cases may also have dyspnea. Smell and taste disorders are another hallmark symptom among COVID-19 patients [68, 69]. To slow down the COVID-19 pandemic, more than a hundred vaccines were developed and more than 18 of them were approved to human populations [70]. Based on the research done by Thibault Fiolet, et al., most COVID-19 vaccines are effective in protecting people from the original strain and VOCs [71]. The current circulating variants of interest from WHO reports are BA.2.86 and JN.1 from the Pango lineage, suggesting that SARS-CoV-2 is still an actively evolving virus.

4.1.5 Therapeutic targets for coronavirus disease

The ongoing circulation of SARS-CoV-2 and the substantial burden caused by the COVID-19 pandemic to both global public health and economics require a concerted effort to solve this problem. Even though vaccines and approved drugs likeNirmatrelvir/ritonavir are available, it is still vital to explore more potential therapeutic targets, especially in the host, in order to be prepared for further evolving variants and novel coronaviruses.

A hint for the therapeutic target selection can be derived from either academic research or clinical experience. Most biological reactions and functions rely on Protein-Protein Interactions (PPIs), emphasizing the importance of identifying antiviral targets by screening virus-host interactions. The screening can be conducted in either bioinformatic or experimental approaches [72]. Our group utilizes Yeast Two-Hybrid (Y2H) assay to search protein pairs between a variety of host genes and SARS-CoV-2 orfeome. The Y2H technique, which was published in late 1980s, is still used as one of the most capable, fast and

inexpensive platforms to identify interacting protein pairs [73]. The yeast two-hybrid assay exploits the modular nature of eukaryotic transcription factors by bringing the DNA-binding domain and the activation domain into close proximity through protein interactions to activate reporter genes [74]. The potential targets discovered from PPIs can be viral proteins and/or host proteins. Considering the rapid genomic mutation rate of coronaviruses, we concentrate on identifying host cell targets that could serve as potential therapeutic intervention points for the treatment of a broad spectrum of coronavirus-related diseases. In earlier studies our group identified cyclophilins as a binding partner for Nsp1 of SARS-CoV. Additionally, inhibition of cyclophilins by the inhibitor Cyclosporine A (CsA) impaired the replication of all coronaviruses tested [75]. Thus, the cyclophilin family is a group of target proteins of significant interest in our research for anti-coronavirus drugs, and this protein family is considered to have the potential to serve as a therapeutic target.

My doctoral research starts from the virus-host protein-protein interaction screening to look for promising host targets and effective inhibitors, and further elucidate the antiviral mechanisms. This study focuses mainly on two promising host proteins or protein families, namely cyclophilins and the OxySterol Binding Protein (OSBP). In the next chapters, I will introduce cyclophilins and OSBP as effective therapeutic targets for inhibiting coronavirus replication.

4.2 Cyclophilin

Cyclophilins (Enzyme Commission number 5.1.2.8) are a subset of a larger protein group called immunophilins, which contain Peptidyl-Prolyl cis-trans Isomerase (PPIase) activity. The first cyclophilin was discovered in 1984 as an active protein in pig kidney with PPIase activity [76]. Cyclophilins are highly conserved and highly abundant across eukaryotes and prokaryotes [77, 78]. So far, there are at least 17 diverse Cyclophilins in human cells [79], and seven major representatives including Cyclophilin A (CypA, 18 kDa), Cyclophilin B (CypB, 22 kDa), Cyclophilin C (CypC) and Cyclophilin D (CypD). Generally, cyclophilins have high affinity for the immunosuppressive drug Cyclosporin A (CsA). Although Cyclophilins are mostly distributed intracellularly (in the cytosol, ER, mitochondria, or the nucleus), some members can be secreted as extracellular factors [80].

Cyclophilins have been an area of intense interest for researchers for many years, and their functions are extensively studied across plants, humans, and microorganisms. Their broad spectrum of functions in various hosts includes cell signaling [81], regulation at the transcriptional level [82], regulation on the cell cycle [83], etc. For example, CypA regulates the activation of RIG-I-like Receptor (RLR) Signaling Pathway by ubiquitination [84]. Cyclophilins are involved in a broad field of human diseases, like cancer [85], neurodegenerative disorder [86], metabolic-related diseases [87] and infectious disease [88, 89]. In this study, I mainly focus on CypA and CypB, two main representatives of human Cyclophilin family with the most abundant expression. From the virus-host interaction screening between CypA or CypB and the orfeome of SARS-CoV-2, several possible protein-protein interactions were observed. For example, CypA binds to Nsp3, and CypB is identified as a general partner for Nsp3, Nsp4, Nsp6 and several accessory proteins, as well as structural protein E and M.

4.2.1 Cyclophilin A

Cyclophilin A is the most ubiquitous Cyclophilin in mammalian cells, accounting for approximately 0.1-0.6% of the total cytosolic proteins [90]. CypA was first purified from bovine thymocytes and identified as the host target and receptor for the immunosuppressive drug CsA [91]. CypA is an 18 kDa, archetypal protein distributed in all mammalian tissues [92]. It was initially regarded as an intracellular protein because it lacked an export sequence [93]. However, in 1992, a surprising and initially underappreciated finding was reported that Cyclophilin A, is secreted by macrophages in response to inflammatory stimuli [94].

The intracellular CypA (iCypA) regulates several pathways including protein folding, trafficking and signaling [90]. On the other hand, the extracellular CypA (eCypA) also participates in the pathogeneses of many diseases [80]. First, CypA plays a critical role in cardiovascular diseases as it regulates vascular remodeling [95] and contributes to atherosclerosis. As CypA is a proinflammatory cytokine [96], it is closely linked to inflammatory diseases. Second, CypA is also involved in the pathogenesis of neurodegenerative diseases, e.g. Alzheimer's disease [97] and Amyotrophic Lateral Sclerosis [98].

Moreover, CypA influences viral infection and replication. CypA is also found to restrict the replication of influenza A virus [99]. However, it appears to act more as a proviral factor to other viruses. CypA interacts with HIV capsid protein and accessory proteins, facilitating the viral life cycle [100-102]. There is also evidence that CypA enhances the HCV replication by binding to HCV non-structural proteins [103, 104]. Recently, a paper showed that CypA promotes the replication of poxvirus by antagonizing TRIM5α [105].

4.2.2 Cyclophilin B

CypB is another abundant cyclophilin in host cells which is of great interest to researchers. It was first discovered in 1991 [106]: it is about 22 kDa with a structure containing the conserved PPIase active site resembling CypA [77]. The main difference between CypA and CypB is that CypB mainly locates at the endoplasmic reticulum (ER) [106] and processes a signal export sequence targeting CypB to the ER protein secretory pathway [77]. CypB is the first human cyclophilin protein found to be secreted extracellularly as it was discovered in human milk [107]. Interestingly, the CsA binding site of CypB overlaps with the ER retention signal, which results in the secretion of CypB under CsA treatment [108, 109]. Like eCypA, extracellular CypB (eCypB) is also believed to be a pro-inflammatory factor. The receptor of eCypA and eCypB is the same, which is CD147 [110, 111].

CypB is also closely associated with many human diseases. For example, CypB is found to increase prolactin-induced proliferation, probably contributing to the malignant progression of breast cancer [112, 113]. Besides CypA, CypB is also found to play an essential role during the HCV replication [114]. Especially, CypB binds to the RNA-dependent RNA polymerase of HCV, which can be abolished by CsA treatment [115].

4.3 Cyclophilin inhibitor

Cyclophilins play important roles in cell signaling, protein folding and trafficking, cell cycle and T cell activation [90]. As mentioned above, Cyclophilins, especially CypA and CypB, are closely associated with many human diseases,

including cancers, metabolic-related diseases, inflammatory diseases, and infectious diseases. Given these reasons, Cyclophilins have emerged as a group of host therapeutic targets, drawing extensive interest among scientists. As a consequence, Cyclophilin inhibitor has become an attractive topic, and a number of novel unimmunosuppressive drug have emerged since the first Cyclophilin inhibitor CsA came out.

4.3.1 Cyclosporin A and non-immunosuppressive derivatives

Cyclosporin A (CsA) was the first Cyclophilin-targeting inhibitor to be identified. It was primarily isolated in 1970s from the fungus Tolypocladium inflatum [116]. The discovery of CsA stemmed from a screening program to look for immunosuppressive drug candidates or compounds in the 1960s. After Dr J. Borel gave an important presentation about CsA and the immunosuppression *in vitro* and *in vivo* at the British Society of Immunology conference in 1976, the agent named CsA stimulated interests of clinicians from the field of organ transplantation. Since then, doctors tested CsA in organ transplant recipients and in 1983, CsA was officially approved by The United States Food and Drug Administration (FDA), becoming one of the first-line immunosuppressive drugs [117, 118].

There are two significant binding sites in the molecular structure of CsA: one is the Cyclophilin binding site (encompassing amino acid residues 1, 2, 9, 10, and 11 in Fig.2) and the other targets calcineurin (including the amino acid residues 4,5,6 and 7, see Fig.2) [119]. Calcineurin is a calcium/calmodulin-dependent serine/threonine protein phosphatase, which dephosphorylates transcription factors including Nuclear Factors of Activated T-cells (NFATs). The dephosphorylated NFATs will be subsequently transferred from the cytoplasm to the nucleus. CsA suppresses the activation of calcineurin by binding to Cyclophilins and forming a ternary complex (between CsA, Cyclophilin A and calcineurin), preventing the dephosphorylation and the translocation of NFATs and finally suppressing the T cell-mediated immune responses [120, 121].

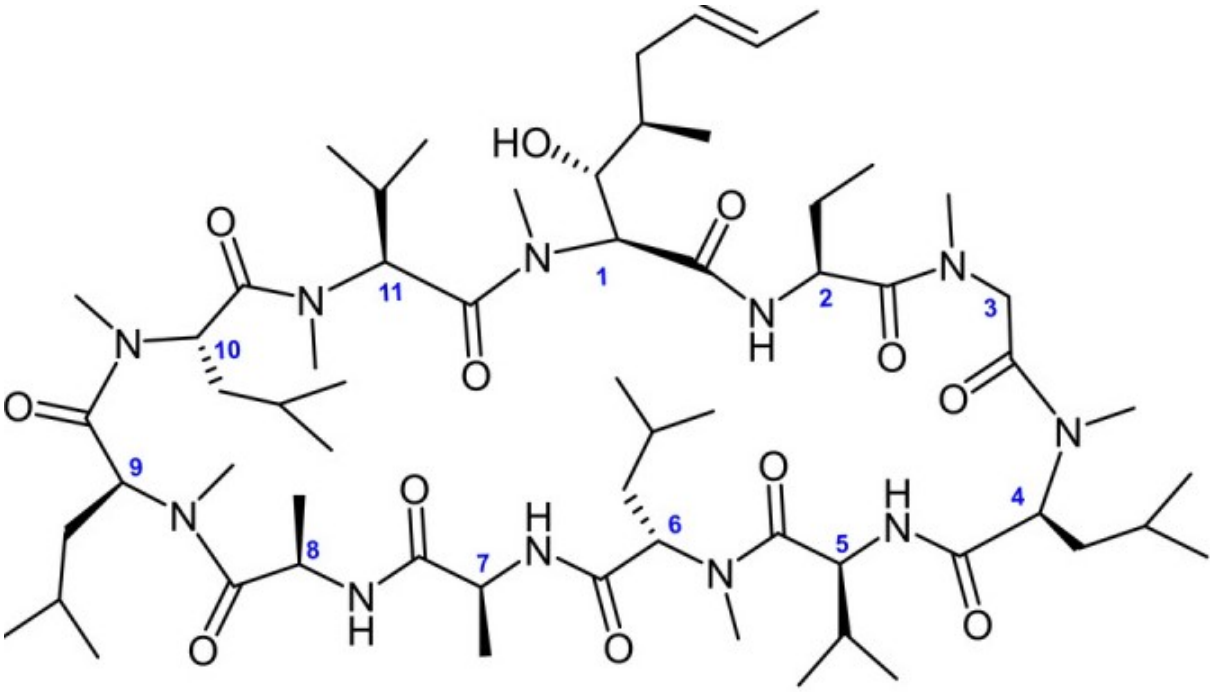

Figure 2 The structural formula of CsA (source: Non-Immunosuppressive Cyclophilin Inhibitors [119]).

However, the immunosuppressive activity of CsA is a significant drawback. This is particularly problematic for treating viral infections, where maintaining immune function is crucial. The pathophysiological functions of Cyclophilins and the demand for reducing the side effects on the immune system encouraged efforts for chemical modification on CsA devoid of immunosuppressive activity. To achieve this goal, the technical route that can be used to develop non-immunosuppressive derivatives of CsA is clear: enhance or maintain the binding affinity to Cyclophilin while abolishing the binding to calcineurin [119].

The first non-immunosuppressive derivative was developed in the 1980s when scientists tried to figure out the mechanism of immunosuppression and the exact function of Cyclophilin [122]. A highly effective modification on CsA to reduce immunosuppressive activity is at position 4, the [MeLeu]4 residue, which is the main binding site for calcineurin. This conclusion led to the successful development of NIM-811, replacing N-methylleucine by N-methylisoleucine (Fig.3) [123]. Another well-known CsA derivative is Alisporivir (ALV). It is constructed based on a combination of modifications on both position 3 and position 4 (Fig.4). Both NIM-811 and ALV exhibit high efficiency to inhibit PPIase activity and meanwhile, substantially reducing the immunosuppression [124, 125]. There are other CsA derivatives with modifications at other positions,

for example, [MeAla]6-CsA and [D-Lys]8-CsA analogues. They also exhibit decreased immunosuppressive capacity but binding is affinity similar to CsA [126, 127].

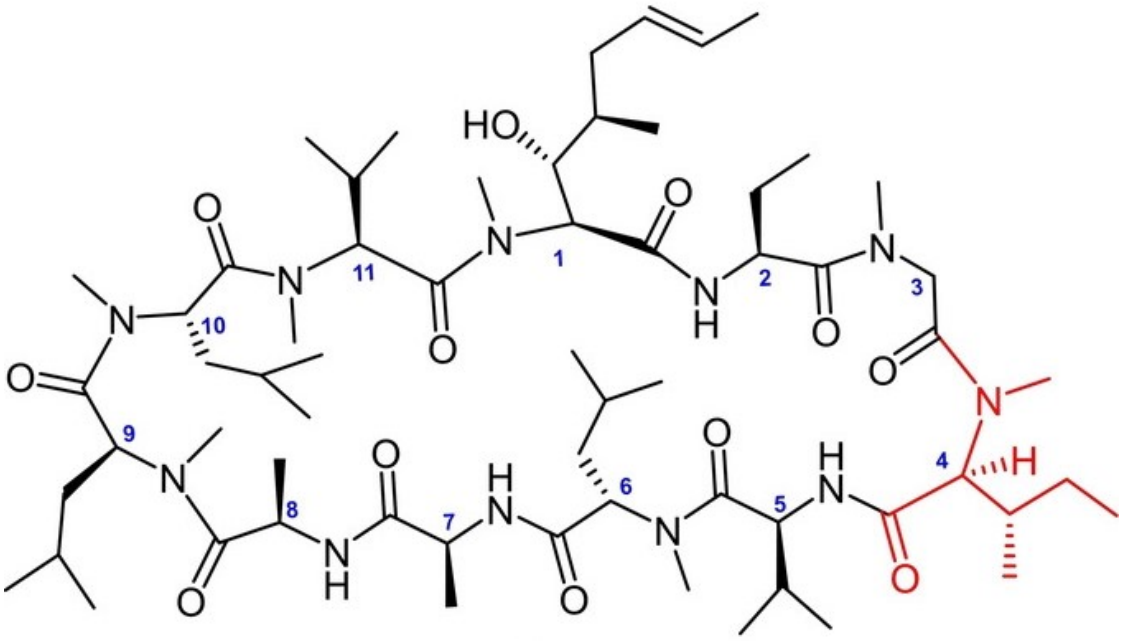

Figure 3 The structural formula of NIM-811 (source: Non-Immunosuppressive Cyclophilin Inhibitors [119])

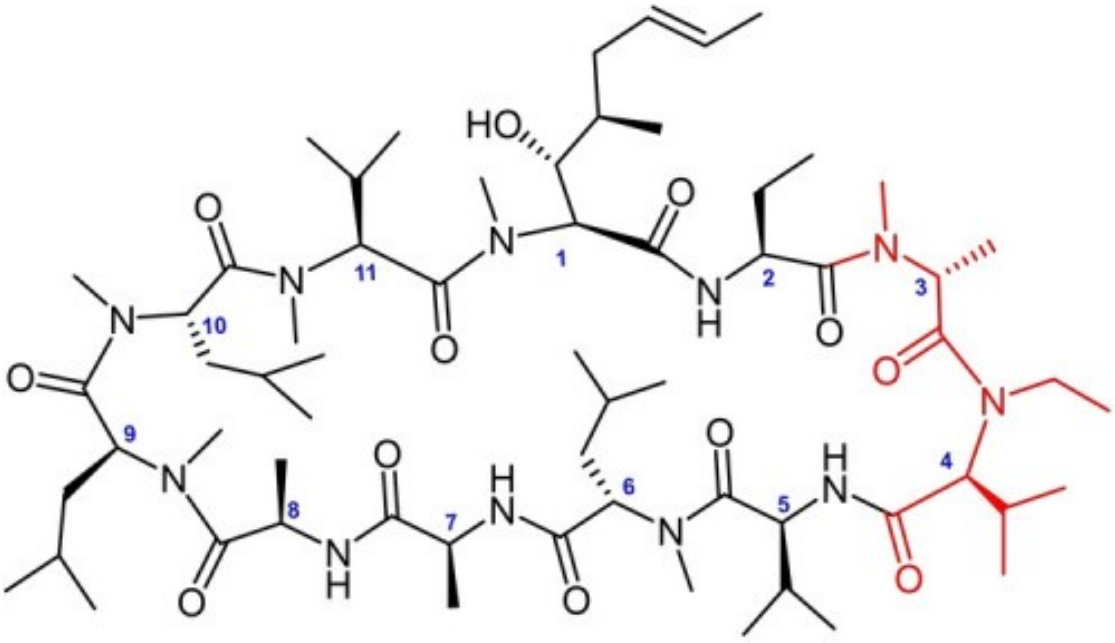

Figure 4 The structural formula of ALV (source: Non-Immunosuppressive Cyclophilin Inhibitors [119])

4.3.2 Cyclophilin inhibitors and viral replication

Cyclophilins, especially CypA, play a significant role in viral replication and infection. A number of studies showed that CypA and CypB are involved in the proliferation process of the influenza A virus, flaviviruses, retrovirus, and coronaviruses [128-131]. Not surprisingly, CsA and its analogs as Cyclophilin inhibitors exhibit strong inhibitory effects on the Cyp-regulated viral infection. They are reported as potent antiviral drugs against HCV and HIV infection [123, 125, 132, 133]. Some clinical trials also showed benefits of CsA combination therapy in patients with chronic HCV or HIV infection. [134-137].

The mechanism of CsA and other CypI against HCV replication is well studied. By silencing the expression of several human Cyclophilins, researchers found CypA and CypB to be involved in HCV replication [115, 138, 139]. On the other hand, HCV viral proteins NS5A and NS5B were identified as factors influencing susceptibility of HCV replication to CsA [140]. One of the reasonable approaches adopted by CypI is that the drugs inhibit the formation of viral replication organelle by targeting the CypA-NS5A interaction [141].

CypIs are also a group of promising therapeutics for coronavirus infection. A number of in vitro studies showed that CsA and non-immunosuppressive compounds could inhibit Feline CoV (FCoV) in a dose-dependent manner [142]. De Wilde et al. discovered that a broad range of CoVs are inhibited by CsA, including SARS-CoV, HCoV-229E and mouse hepatitis virus (MHV) [143]. Our group has published a series of papers since 2011, demonstrating that CypIs are promising pan-coronavirus inhibitors. These inhibitors effectively block the replication of coronaviruses across all genera, including SARS-CoV, HCoV-229E, HCoV-NL63, feline coronavirus, and avian infectious bronchitis virus [75, 144, 145]. At least two independent studies showed that both CsA and ALV reduced the replication of SARS-CoV-2 in vitro [146, 147]. A phase-2 clinical trial on the efficacy of ALV to treat COVID-19 patients was initiated during the pandemic (EudraCT 2021-004020-15) [148].

It is well established that CsA and its analogues inhibit viral replication including a variety of coronaviruses. However, it is still unclear if the inhibitory effect of CypIs is related to CypA or CypB. In my project, I attempted to clarify the roles

of CypA and CypB facing different coronaviruses and futher to explore the mechanisms of the antiviral activity of CypIs.

4.4 OSBP and its inhibitors

As a key component of biological membranes, lipids are mainly generated in the ER and play an essential role in cellular metabolism and bioactivities. The organelle-specific lipid composition requires complex and sophisticated control of lipid transport. The lipid translocation occurs in three manners: via vesicles, diffusion and lipid transfer proteins (LTPs). Human OSBP is an important and conserved member in the LTP protein family [149].

OSBP is a protein mediating lipid transfer by exchanging PhosphatidylInositol 4-Phosphate (PI4P) between ER and trans-Golgi network (TGN) [150, 151]. OSBP was first discovered by in 1985 by Taylor and Kandutsch [152], featuring binding affinity with oxysterols in the cytosol. In 1989, scientists reported successful purification of OSBP from hamster liver for the first time, which laid a solid foundation to support the research in cholesterol metabolism [153]. The OSBP gene located at human chromosome 11 encodes an 800 amino acid protein with 3 functional domains: a PI4P binding domain, a short ER-targeting signal, and a highly conserved oxysterol-binding protein-related domain [154].

OSBP has been identified to be involved in a number of human diseases. The first group of diseases is closely related to lipid and/or cholesterol metabolism, for example fatty liver disease and diabetes [155-157]. A large body of evidence suggests that OSBP or OSBP-Related Proteins (ORPs) also contribute to the occurrence and progression of different cancers. The rapid proliferation of malignant tumor cells requires a high supply of “nutrition and resource”, and lipid as a necessary component of membranes, is extensively demanded by cancerous cells. For example, ORP4 expression has been repeatedly identified as a key factor in the development of several types of cancer, including lung cancer, breast cancer, and leukemia [158]. Additionally, more and more studies reveal that OSBP plays an essential role in viral infections. Knockdown of OSBP was found to inhibit Dengue virus replication [159]. OSBP is recruited to the viral replication organelle, providing cholesterol to support viral replication [160]. Many other RNA viruses besides HCV, like poliovirus and enterovirus,

similarly hijack OSBP to utilize the lipid transfer system to facilitate viral replication [161, 162]. Several small molecules including OSW-1, TTP-8307, and itraconazole (ITZ), can effectively restrict viral replication by targeting OSBP, indicating that OSBP is a potential therapeutic target for virus-related infectious diseases [163]. In my research, the OSBP-specific inhibitor ZJ-1 reduced the replication of a number of coronaviruses, including HCoV-NL63, 229E, OC43, SARS-CoV-2, and murine hepatitis virus (MHV). Moreover, OSBP is found to be involved in the replication organelle as well and enhance the stability of viral proteins of orf3a, orf7a and orf7b. All those results support that OSBP also plays an important role in the replication process of coronaviruses and is a promising therapeutic target for coronavirus-related disease.

5. Summary

Coronaviruses are a group of emerging pathogens, attracting more and more attention in recent years. So far, scientists have identified seven coronaviruses which have the capacity to infect and circulate in human beings, namely HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2. Since 2002, there have been three life-threatening outbreaks caused by highly pathogenic human coronaviruses within two decades. The ongoing pandemic of COVID-19 has led to more than 770 million cases and more than 7 million deaths. It has also led to a huge economic loss worldwide and to some extent, to a global recession. In order to explore therapeutic targets for coronavirus-related diseases, it is necessary to map virus-host protein-protein interactions. In this study, cyclophilins are identified as a group of general host targets interacting with viral proteins from pan-coronaviruses. The family of cyclophilins comprises a conserved class of proteins that possess peptidyl-prolyl cis-trans isomerase activity. Cyclophilin A (CypA) and Cyclophilin B (CypB) are the most abundant human Cyclophilins among 17 members of this large protein family. They participate in protein folding, trafficking, signaling, cell cycle and T-cell activation. Given their important functions, CypA and CypB are involved in many human diseases, including cancers, neurodegenerative disorders, metabolic-related diseases, inflammatory diseases, and infectious diseases. Cyclophilin inhibitors, CsA and its analogues are found to have the capacity to inhibit viral replication in HIV and HCV studies. Researchers, including our group, have reported that CsA and its analogues could inhibit viral replication of HCoV-NL63, 229E, SARS-CoV-2, and several animal coronaviruses of veterinary relevance, indicating that they are promising pan-coronaviruses inhibitors. However, the exact mechanism behind the inhibitory phenotype is still unclear.

In my thesis research, we aim to elucidate the functions of CypA and CypB during human coronavirus replication by measuring the viral replication in Cyclophilin knockout and overexpression Huh7 cells. The screening and verification of virus-host protein-protein interactions is another focus of my project. We are trying to clarify the inhibitory mechanisms by analyzing the protein-protein interactions and the involved pathways and to confirm whether CypA and CypB could be potential drug targets to counter coronavirus infection.

My first publication shows that Cyclophilin inhibitors suppress SARS-CoV-2 replication by interfering with the binding of intracellular CypA to the nucleocapsid protein of SARS-CoV-2. The extracellular CypA binds to the host receptor CD147. CD147 is also considered by some scientists to be the second receptor mediating SARS-CoV-2 entry. We therefore speculate that CD147 may be another therapeutic target in addition to CypA.

Other experiments during my study (displayed in the appendix) focuses on the mechanisms by which CsA and its other analogues inhibit coronavirus replication. However, the impact of cyclophilin on coronaviruses and the manner in which it acts vary among different coronaviruses. We confirm that CypA is the main target of CsA against HCoV-229E replication, as it can counteract the CsA and restore viral growth. CypB may play a more important role in SARS-CoV and SARS-CoV-2 infection. CypA and CypB are involved in forming the replication organelle of SARS-CoV-2 by binding to Nsp3, Nsp4, and Nsp6. The nucleocapsid is another important viral protein that provides clues to understanding replication mechanisms. Our findings suggest that the nucleocapsid protein of SARS-CoV-2 hijacks Cyclophilins by using CypA and CypB from the RLR pathway to evade the immune response.

My second publication investigates the role of oxysterol-binding protein (OSBP) in coronavirus replication. Our findings indicate that OSBP also acts as a helper factor supporting viral replication among diverse coronaviruses. The OSBP inhibitor ZJ-1 reduces OSBP expression and viral proliferation of HCoV-NL63, 229E, OC43, SARS-CoV-2, and MHV. Protein-protein interaction studies confirm that OSBP is targeted by several SARS-CoV-2 proteins, including Nsp3, Nsp4, Nsp6, and several accessory proteins. As Nsp3, Nsp4 and Nsp6 form the replication organelle, we suppose that OSBP plays a role in this process in order to promote replication, via similar approaches as cyclophilins. Furthermore, OSBP also stabilizes viral proteins such as orf3a, orf7a, and orf7b. Disturbing the interaction of OSBP and several coronavirus proteins could be the potential inhibitory mechanisms adopted by the OSBP-Inhibitor ZJ-1.

6. Zusammenfassung

Coronaviren sind eine Gruppe neu auftretender Krankheitserreger, die in den letzten Jahren immer mehr Aufmerksamkeit auf sich ziehen. Bis jetzt haben Wissenschaftler sieben Coronaviren identifiziert, die Menschen infizieren und im menschlichen Körper zirkulieren, nämlich HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, und SARS-CoV-2. Seit 2002 kam es innerhalb von zwei Jahrzehnten drei lebensbedrohlichen Ausbrüchen durch hochpathogene menschliche Coronaviren. Die laufende COVID-19 Pandemie hat zu mehr als 770 Millionen Infizierten und über 7 Millionen Todesfällen geführt. Die Pandemie hat außerdem weltweit zu enormen wirtschaftlichen Verlusten und in gewissem Maße zu einer globalen Rezession geführt. Um therapeutische Ziele für Krankheiten im Zusammenhang mit Coronaviren zu erforschen, ist es notwendig, die Wechselwirkungen zwischen Virus und Wirtsprotein zu darstellen. In dieser Studie werden Cyclophiline als eine Gruppe allgemeiner Wirtsproteine identifiziert, die mit viralen Proteinen von Pan-Coronaviren interagieren. Die Familie der Cyclophiline umfasst eine erhaltende Klasse von Proteinen, die über Peptidyl-Prolyl-Cis-Trans-Isomerase-Aktivität verfügen. Unter den 17 Mitgliedern dieser großen Proteinfamilie sind Cyclophilin A (CypA) und Cyclophilin B (CypB) die am häufigsten vorkommenden menschlichen Cyclophiline. Sie sind an der Proteinfaltung, dem Proteintransport, der Signalübertragung, dem Zellzyklus, und der T-Zell-Aktivierung beteiligt. Aufgrund ihrer wichtigen Funktionen spielen CypA und CypB bei vielen Erkrankungen des Menschen eine Rolle, darunter Krebs, neurodegenerative Erkrankungen, metabolische Erkrankungen, Entzündungserkrankungen und Infektionskrankheiten. In HIV- und HCV-Studien wurde festgestellt, dass Cyclophilin-Inhibitoren, u.a. Cyclosporin A (CsA) und ihre Analoga, die Virusreplikation hemmen können. Forscher, darunter auch unsere Gruppe, haben berichtet, dass CsA und die Analoga die virale Replikation von HCoV-NL63, 229E, SARS-CoV-2 und mehreren tierischen Coronaviren von veterinärmedizinischer Relevanz hemmen könnten, was darauf hindeutet, dass sie vielversprechende Pan-Coronavirus-Inhibitoren sind. Der genaue Mechanismus hinter bei Hemmphänotyp ist jedoch noch unklar.

In meiner Promotionsarbeit wollen wir die Funktionen von CypA und CypB während der Replikation des menschlichen Coronavirus durch Messung der Virusreplikation in Cyclophilin-Knockout- und -Überexpressions-Huh7-Zellen aufklären. Die Virus-Wirt-Protein-Protein-Interaktionen sind ein weiterer Schwerpunkt meines Projekts. Wir versuchen, die Hemmmechanismen durch Analyse vieler Wechselwirkungen und Signalwege aufzuklären und zu bestätigen, ob CypA und CypB die Hauptziele für Medikamente zur Reduzierung der Coronavirus-Infektion sind. Meine erste Publikation zeigt, dass Cyclophilin-Inhibitoren die Replikation von SARS-CoV-2 unterdrücken, indem sie die Bindung von intrazellulärem CypA und dem Nukleokapsidprotein von SARS-CoV-2 stören. Das Wirtsprotein CD147 ist der Rezeptor für extrazelluläres CypA. CD147 wird von einigen Forschern auch als der zweite Rezeptor angesehen, der den Eintritt von SARS-CoV-2 in die menschliche Zelle vermittelt. Wir spekulieren daher, dass CD147 neben CypA ein weiteres therapeutisches Ziel sein könnte.

Weitere Experimente während meiner Studie (siehe Anhang) konzentriert sich auf die Mechanismen, durch die CsA und andere Analoga die Replikation von Coronaviren hemmen. Unsere Ergebnisse zeigen, dass CypA und CypB eine wichtige Rolle bei der Replikation von Coronaviren spielen. Knockout oder Überexpression von CypA und CypB beeinflusst die virale Replikation mit Spezies- oder Stammspezifität. Wir bestätigen, dass CypA das Hauptziel von CsA gegen die HCoV-229E-Replikation ist, da es der Hemmfunktion des Arzneimittels entgegenwirken kann. CypB könnte bei SARS-CoV und SARS-CoV-2 eine wichtigere Rolle spielen. CypA und CypB sind an der Bildung des Replikationsorganells von SARS-CoV-2 beteiligt, indem sie an Nsp3, Nsp4 und Nsp6 binden. Das Nukleokapsid Protein ist ein weiteres wichtiges virales Protein, welches Hinweise zu Replikationsmechanismen gibt. Unsere Ergebnisse legen nahe, dass das Nukleokapsidprotein von SARS-CoV-2 Cyclophiline entführt, indem es CypA und CypB aus dem RLR-Signalweg nutzt, um der Immunantwort zu entgehen.

Meine zweite Publikation untersucht die Rolle des Oxysterol-bindenden Proteins (OSBP) bei der Coronavirus-Replikation. Unsere Ergebnisse deuten darauf hin, dass OSBP als Hilfsfaktor fungiert, der die Virusreplikation bei verschiedenen Coronaviren unterstützt. Der OSBP-Inhibitor ZJ-1 reduziert die

OSBP-Expression und die Virusproliferation von HCoV-NL63, 229E, OC43, SARS-CoV-2 und dem Maushepatitisvirus (MHV). Protein-Protein-Interaktionsstudien bestätigen, dass OSBP von mehreren SARS-CoV-2-Proteinen targeted wird, darunter Nsp3, Nsp4, Nsp6 und mehreren akzessorischen Proteinen. Da Nsp3, Nsp4 und Nsp6 das Replikationsorganell bilden, nehmen wir an, dass OSBP bei diesem Vorgang eine Rolle spielt, um die Replikation zu fördern. OSBP stabilisiert auch virale Proteine wie orf3a, orf7a und orf7b. Die Manipulation der Interaktion von OSBP mit mehreren Coronavirus-Proteinen könnte der potenzielle Hemmmechanismus sein, der durch den OSBP-Inhibitor ZJ-1 angepasst wird.

7. Publication I

Targeting Cyclophilin A and CD147 to Inhibit Replication of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and SARS-CoV-2-Induced Inflammation

Authors: Fan Yang*, Chenglong Liu*, **Pengyuan Li***, Aihua Wu*, Yue Ma-Lauer, Hao Zhang,

Zhuang Su, Wei Lu, Albrecht von Brunn, and Di Zhu

*: Fan Yang, Chenglong Liu, Pengyuan Li and Aihua Wu contributed equally to this study

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DOI: 10.1124/molpharm.122.000587

8. Publication II

Oxysterole-binding protein targeted by SARS-CoV-2 viral proteins regulates coronavirus replication

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Zhendong Jin and Albrecht von Brunn

*: Yue Ma-Lauer and Pengyuan Li contributed equally to this study

Frontiers in Cellular and Infection Microbiology, 2024, Vol. 14

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9. Appendix

In this section, I have included some unpublished data, results, and analyses that are essential for understanding the entire research project and assessing my work.

General description and highlights:

The immunosuppressive drug Cyclosporin A (CsA) and its non-immunosuppressive derivatives, which target the host cyclophilin family, have shown effective inhibition of several coronaviruses, including SARS-CoV-2, *in vitro*. However, the precise antiviral mechanisms remain unclear. Our study demonstrates the critical role of cyclophilin A (CypA) and cyclophilin B (CypB) in coronavirus replication. I observed that the replication of HCoV-229E is reduced by half in CypA knockout Huh7 cells, but not in CypB knockout Huh7 cells. Transient overexpression of CypA can reverse the inhibitory effects of CsA, facilitating HCoV-229E replication. For attenuated SARS-CoV-2 sCPD9, CypB plays a more important role influencing the viral replication. SARS-CoV-2 exploits CypA in Huh7 cells by upregulating CypA expression and interacting with non-structural protein 3 (Nsp3) and nucleocapsid protein (N). On the other hand, CypB binds Nsp3, Nsp4, Nsp6, and N, and these interactions are disrupted by CsA. Besides those nonstructural proteins and N, CypB is found to interact with diverse accessory and structural proteins of SARS-CoV-2 as well. Additionally, CypA and CypB play crucial roles in the host innate immune response by interacting with RIG-I and MAVS, yet SARS-CoV-2's N protein competes with RIG-I and MAVS for CypA or CypB binding. Thus, coronaviruses hijack CypA and CypB to suppress antiviral responses and enhance viral replication. My findings advance the understanding of CsA's antiviral mechanisms against coronaviruses and support the potential repurposing of CsA and its analogues for treating coronavirus variants or novel coronaviruses.

- All of the results indicated that Cyclophilins were important to the replication of coronaviruses, but the involved mechanisms differed depending

on the species of coronaviruses. The replication of HCoV-229E partially relied on CypA, but the proliferation of sCPD9 was highly influenced by CypB.

- For HCoV-229E, CypA counteracted the inhibition of CsA to the viral replication.
- For SARS-CoV-2 and sCPD9, endogenous CypA was enhanced by the viral infection, which suggested SARS-CoV-2 exploited CypA to facilitate viral infection, eg. positively regulating Nsp3.
- CypIs decreased the cellular protein level of CypB and viral proteins (Nsp3, Nsp4 and Nsp6 of SARS-CoV-2), and CypB bound to these three viral proteins involved in RO formation, which demonstrated a possible mechanism that CypIs impaired the replication through CypB. Intracellular CypB was secreted after CypI treatment, resulting in reduced protein levels of Nsp3, Nsp4 and Nsp6, finally leading to impaired formation of ROs and reduced replication.
- SARS-CoV-2 Nsp3 and N proteins were reported to evade innate immune response by suppressing the IFN pathways. Both Nsp3 and N bound to CypA and CypB, but only N could compete with RIG-I and MAVS to interact with Cyps, implying N might suppress IFNs via binding to Cyps. The interactions between N and Cyps were abolished by CsA, suggesting another plausible mechanism adopted by CypI, preventing N from evading immune responses, thereby restoring the antiviral functions of cyclophilins.

1. Materials and methods

1.1 Plasmids

Most plasmids were constructed by Gateway cloning using pDONR 207/223 for BP clonase and tag-carrying expression vectors for LR clonase reactions [164, 165]. pDEST-RFP and pDEST-GFP or pDEST-ct-RFP and pDEST-ct-GFP destination vectors carry RFP and GFP genes at the N- or C-terminus of a human gene or viral open reading frame (ORF) to be expressed, respectively. Split YFP expression plasmids pDEST-c-myc-YFP^N/pDEST-ct-c-myc-YFP^N and pDEST-HA-YFP^C/pDEST-ct-HA-YFP^C carry the N-terminal (YFP^N, aa 1–155) and C-terminal (YFP^C, aa 156–239) fragments of YFP, respectively. In pDEST-`ct´ vectors the YFP^N or YFP^C fragments are located to the C-terminus of the respective gene of interest. All primers used in this study are listed in Supplementary Table S1.

Human gene sequences correspond to the indicated sources: full-length CypA (PPIA, CCDS5494.1), short CypA (sPPIA, CCDS 75592.1) and CypB (PPIB, CCDS 10191.1), RNA sensor RIG-I (RIG-I, CCDS 6526.1), and mitochondrial antiviral signaling protein (MAVS, CCDS 33437.1). SARS-CoV-2 pCG1-Nsp3 (aa 1-1363) expression plasmid is described in [166]. As full-length CoV Nsp3 ORFs tend to be unstable during cloning *E. coli,* I obtained several codon-optimized Nsp3 ORFs from SARS-CoV, SARS-CoV-2 and HCoV-NL36 [167]. These sequences were confirmed by whole plasmid sequencing (Mikrosynth AG, Switzerland and Eurofins Genomics Germany GmbH).

Nsp3 of the Wuhan isolate was further subdivided into several fragments: N-terminal (aa 1-412), SUD (aa 413-745), PLp (aa 746-1064), NAB-ßSM (aa 1065-1414) and C-terminal (aa 1415-1945) [168]. For the construction of CypA or CypB knockout cells oligo-dimers of gRNA primers (Supplementary Table 1) were cloned into plasmid lentiCRISPR V2 (Addgene plasmid #52961) after digestion with the restriction enzyme BsmBI-V2 (New England Biolabs, R0739S). Lentiviral plasmids for CypA or CypB overexpression were constructed by transferring PPIA or PPIB genes from pDONR207 into the Gateway destination vector pLenti CMV/TO Puro DEST (Addgene plasmid # 17293). Yeast-2-Hybrid (Y2H) assays were performed following LR cloning of SARS-CoV-2 and host genes from pDONR207 into Gateway destination

vectors pGBKT7g, pGBKT7Cg, pGADT7g or pGADCg by LR cloning as described [169]. For the NanoLuc two-hybrid (N2H) assay, four gateway-compatible vectors were designated as N1, N2, C1 and C2 as described [170]. The intact protein NanoLuc was divided into two fragments F1 and F2. The four vectors used in the N2H assay represented F1 or F2 located at either N- or C-terminus of the Gateway cloning site or the inserted target gene. Plasmids for the detection of type I interferon pathway activation including pIFN-beta (fused with Firefly luciferase activity), pISRE (fused with Firefly luciferase activity) and pCR3-Flag-RIG-I CARD 1-284 (CARD) were described [166, 171].

1.2 Cells and transfection

Human Embryonic Kidney HEK293, HEK293T cells, human hepatocellular carcinoma cells Huh 7 were maintained in Dulbecco's modified Eagle´s medium (Gibco, 11965092) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Non-cancerous human bronchial epithelium BEAS-2B ACE2 (stably expressing angiotensin-converting enzyme 2 (ACE2) [172] were cultured at standard conditions in Dulbecco´s modified Eagle´s medium/nutrient Mixture F-12 (DMEM/F12, Gibco, 11320033) containing 10% (v/v) fetal bovine serum, 1% (v/v) HEPES (Gibco, 15630056), 100 U/ml penicillin, and 100 µg/ml streptomycin. All cell lines were grown at the condition with 5% CO2 at 37°C degree and checked for mycoplasma contamination regularly. HEK293 or 293T cells were transfected with plasmids by using Lipofectamine 3000 Reagent (Invitrogen, L3000015) or 25 kDa polyethyleneimine (PEI) at 50% or 80% confluency, respectively.

1.3 Virus stock preparation and replication

HCoV-NL63, -229E, -OC43 and HCoV-229E virus expressing Renilla luciferase (HCoV-229E-Rluc) were prepared as described [75, 173]. Attenuated sCPD9 was prepared in Vero E6 cells following the protocol of the provider [174]. Titers of the viral stocks were determined by either qPCR or plaque titration assay. Generally, the infection assay was done following this procedure: cells seeded in either 24- or 48-well plates at 80% confluency were inoculated with virus-containing serum-free DMEM for 3-10 days at 33°C with 5% CO2 after removing the supernatant. To compare the proliferation of 229E-Rluc on various

Huh7 wildtype cells and cyclophilin knockout (KO) or overexpression (OV) polyclones, cells prepared in 96-well plates were infected with 229E-Rluc at MOI=1 and cultured at 33°C with 5% CO2 for 2 more days. To check the replication of SARS-CoV, a SARS-CoV replicon [175] carrying the Renilla luciferase gene (SARS-CoV-Rluc) was transfected into various HEK293T polyclones using Lipofectamine 3000 Reagent and recorded the luminescent activities after 48 h. Replication of HCoV-NL63 and -OC43 in different cell polyclones was determined by probe-based qPCR assay. Replication of 229E-Rluc was examined by measuring Renilla luciferase activity. Replication of sCPD9 was measured mainly through qPCR and Western blot (by checking the protein level of N protein).

1.4 RNA isolation and qPCR

RNA was isolated from either supernatants or cell lysates using the ISOLATE II RNA Mini Kit (Bioline, BIO-52073). The presence of viral RNA was analyzed by probe-based qPCR (Luna® Probe One-Step RT-qPCR 4X Mix with UDG, New England Biolabs, M3019 or SensiFAST™ Probe Hi-ROX One-Step Kit, Bioline). Procedures were performed according to the manufacturer's instructions. Primer and probe sequences are listed in Supplementary Table S1.

1.5 Luciferase assay, cell viability and fluorescence measurement

After discarding the growth medium, cells were lysed with 20ul/well (96-well plate) in 1x Lysis Buffer (provided by Promega kits) for at least 15 minutes. The lysate was then transferred to a non-transparent, white polystyrene 96-well microplate (Greiner Bio-One). Renilla or firefly luciferase activities were measured in a CLARIOstar microplate reader (BMG LABTECH) using the Renilla luciferase assay system (Promega, E2820) or the luciferase assay system for firefly luciferase activity (Promega, E1501). Cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7570). The detailed protocols followed the instructions provided by the manufacturer.

1.6 Immunofluorescence staining

Mock-infected and virus-infected cells seeded on 8-well chamber slides were washed with 1x PBS (Gibco) and fixed for at least 1 hour in 4% PFA (Carl Roth GmbH) in PBS. Cells were then permeabilized for 15 minutes in 0.1% Triton X-100 (Carl Roth GmbH) in PBS and blocked for 1 hour in PBS buffer containing 5% BSA (Carl Roth GmbH) and 0.2% Tween-20 (Sigma-Aldrich Chemie). Rat anti-N 21H2-1-1 (of SARS-CoV-2, 1:200 [173]), mouse anti-dsRNA J2 (SCICONS) at 1:1000 and rabbit anti-CypA (Abcam, Ab3563, 1:500) or anti-CypB (Invitrogen, PA1-027A, 1:1000) primary antibodies were diluted in blocking medium and then applied to cells overnight at 4°C in a humid atmosphere. After three washes with PBS, the cells were incubated with secondary antibodies AlexaFluor®647 anti-rat (1:500, Invitrogen, A48265), AlexaFluor®555 anti-mouse (1:500, Invitrogen, A21424) or FITC anti-rabbit (1:1000, Sigma Aldrich, F0382) at indicated dilutions in PBS supplemented with 5% goat serum (Bio&SELL GmbH, ZIE.SE.0100) and 0.2% Tween-20. Cells were incubated for 1 hour at room temperature in the dark. They were then stained with DAPI (Sigma Aldrich, D9542) diluted 1:1000 in PBS for 10 minutes in the dark and washed three times with PBS. Finally, images were taken using a Nikon TiE confocal microscope with a Nikon 100×/1.45 NA oil immersion objective.

1.7 Yeast two-hybrid screening

Yeast two-hybrid screening between CypA (PPIA), short CypA (sPPIA), CypB (PPIB) and the full SARS-CoV-2 orfeome was performed using competent yeast cells (PJ69-7A) grown on amino acid starvation medium according to our published protocol [173]. All SARS2-CoV-2 genes were cloned into pGBKT7g bait vector encoding leucine and host genes (PPIA, sPPIA and PPIB) were cloned into pGADCg prey vector expressing tryptophan. Positive interaction results were verified by the growth of yeast colonies on both double dropout (without Leu and Trp) and triple dropout (without Leu, Trp and His) selection plates. Combinations found positive in yeast two-hybrid assays were later evaluated by N2H assay and/or co-immunoprecipitation (CoIP).

1.8 N2H assay

HEK293T cells were seeded in 96-well plates and transfected with N1 or C1, N2 or C2 plasmids fused to target genes. Cells were then lysed with 20 µl NP-40 lysis buffer (1%NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) and treated with 20 µl substrate containing 0.5% Nano-Glo® Luciferase Assay Substrate (Promega, #N113A) in 1x Nano-Glo® Blotting Buffer (Promega, #N242A) for measurement. NanoLuc activities were measured using a CLARIOstar microplate reader.

1.9 CoIP and Western blot

GFP- or RFP-fused proteins and their interaction partners were co-precipitated with GFP (or RFP) trap agarose beads (ChromoTek & Proteintech Germany, gta-20 or rta-20) according to the manufacturer's standard protocol. Briefly, plasmids were transfected into HEK293 cells in a 6-well plate for 24-48 hours using lipofectamine. Whole cell lysate was then collected from cells lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA and 0.5% NP-40. The eluate samples containing the GFP or RFP complexes were bound to the corresponding agarose beads after incubation for 2 h at 4 ^{0}C. Protein samples in 1x SDS-PAGE sample buffer were analyzed by Western blot assay as described in detail [145]. The antibodies used in the Western blot are listed in Supplementary Table S2.

1.10 Lentiviral production, transduction and generation of polyclonal KO and OV cell pools

Originally, I attempted to construct single cell-selected CypA and CypB KO and OV cell clones. However, expression phenotypes were too inhomogeneous to yield consistent results. Consequently, stable CypA or CypB KO and OV polyclonal cell lines were constructed for this study. Corresponding gRNA suggested by the Synthego website (https://design.synthego.com/#/) or from published data were cloned into plasmid lentiCRISPR V2 (Addgene #52961). The replication-defective pseudo-typed lentiviruses encoding gRNA sequences and Cas9 protein were produced in HEK293T cells co-transfected with lentiviral plasmid, psPAX2 (Addgene #12260) and vesicular stomatitis virus G protein (VSV-G) (Addgene plasmid #8454) under selection for puromycin (2-4 µg/ml) for 2-3 days. The supernatant was then collected and added to Huh7 cells to

transduce the target gene knockout for a further 3-4 days. Lentiviruses designed to overexpress CypA or CypB were generated in a similar manner using plasmid pLenti CMV/TO Puro DEST (Addgene #17293). Successful knockout or overexpression was assessed by standard Western assays using the appropriate antibodies. As the cell polyclones were pool-selected, they were only used for up to 10 passages in addition to frequent WB checks to ensure quality.

1.11 Multiple protein alignment and comparison

The HCoV viral sequences were obtained from the NCBI database. The Nsp3 and N protein sequences were obtained from the complete sequences of SARS-CoV-2 (NCBI reference sequence: NC_045512.2), SARS-CoV (NCBI reference sequence: NC_004718. 3), MERS (NCBI reference sequence: NC_019843.3), NL63 (GenBank: JQ765575.1), 229E (GenBank: KF514433.1) and OC43 (GenBank: KF530099.1). The similarity among those sequences representing by the percentage of identical amino acids were compared to SARS-CoV-2 calculated by ClustalW (https://www.genome.jp/tools-bin/clustalw).

1.12 Statistical analysis

The qPCR and luciferase measurement data were presented as the mean ± standard deviation of triplicate or quadruplicate values. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test for multiple-group analysis or Student's t-test for two-group comparison. All statistical analyses were performed using GraphPad Prism 10 software with a significance level of $\alpha=0.05$. All graphs were also generated using GraphPad Prism 10, including both the raw data and the statistical analysis performed within this software. The symbols in the graphs indicated the following levels of significance of the p-values: "ns" (not significant): $p>=0.05$, an asterisk (*): $0.01<p<0.05$, two asterisks (**): $0.001<p<0.01$, three asterisks (***): $p<0.001$, four asterisks (****): $p<0.0001$.

2. Results

2.1 CsA and its analogue ALV inhibit various human coronaviruses

Huh7 cells, commonly used in HCV research, have been shown to be susceptible to coronaviruses, particularly SARS-CoV-2 [176, 177]. Our previous studies [144, 145] demonstrated that CsA and its analogs Alisporivir (ALV) and NIM811 effectively inhibited the replication of HCoV-229E-Rluc in Huh7.5 and of NL63 in Caco-2 cells at low micromolar concentrations.

Here, I assessed the inhibition of CsA on the replication of three different human coronaviruses (Figure S1). The replication of HCoV-229E-RLuc, which was represented and measured by Renilla luciferase activity, was markedly reduced by CsA at both 24 and 48 hours post-infection (h.p.i) in Huh7 cells (Figure S1A). CsA treatment also reduced the replication of NL63 and OC43 in Huh7 cells, as quantified by probe-based qPCR targeting cellular nucleocapsid genes (Figure S1B and C). Furthermore, 20 μM CsA or ALV treatment also reduced the replication of the attenuated SARS-CoV-2 sCPD9 in qPCR assay (Figure 1A). In our earlier studies we had shown that the inhibitors do not substantially influence the cell viability at 20 μM.

Inhibition of the various CoVs by CsA and ALV was further confirmed by a series of immunofluorescence assays in Huh7 cells (Figure S2). Replication of each HCoV was demonstrated by dsRNA staining indicating active replication (red). No dsRNA was detected in the presence of both, CsA and ALV, indicating their effectiveness against the three seasonal HCoVs as well as sCPD9.

The intracellular CypB was reduced under CsA or ALV treatment. It is noteworthy that CypB in HCoV-229E-infected Huh7 cells only formed special bleb-like structures, which I previously identified as ER components [144]. NL63, OC43, sCPD9 viruses did not show this phenotype. Those results suggested that CsA was a promising effective inhibitor to general coronaviruses.

2.2 CypA but not CypB is induced in the presence of SARS-CoV-2

CsA targets both CypA and CypB [178]. These cyclophilins are abundant in human cells and play crucial roles in viral infection and immune responses [179]. However, their specific functions during the coronavirus replication cycle remain unclear. To elucidate these roles, I examined the protein levels and cellular distribution of endogenous CypA and CypB in the presence of different HCoVs.

Western blot analysis (Figure 1B) revealed an induction of CypA, but not CypB, in Huh7 cells infected with SARS-CoV-2 (MOI=1, 48h) or sCPD9 (MOI=0.01,

48h) compared to mock-treated cells. Immunofluorescence staining was used to visualize the distribution of CypA, CypB, and dsRNA, a marker of viral replication for positive-stranded RNA viruses [180]. The results showed increased expression of CypA in SARS-CoV-2-infected cells, which co-localized with dsRNA (Figure 1C, left). These effects were not observed for CypB (Figure 1C, right).

Similar experiments with sCPD9, including additional anti-N antibody staining (Figure S3), showed only slight induction of CypA in infected cells (orange circle) compared to neighboring uninfected cells (yellow circle) and no co-localization with dsRNA. CypB levels remained unchanged regardless of viral replication (orange circle versus yellow circle). The dsRNA signals co-localized with N, confirming the anti-N antibody as an effective marker of HCoV replication. I also evaluated CypA and CypB protein levels in Huh7 and BEAS-2B ACE2 cells, comparing mock-infected cells to those infected with NL63, 229E, and OC43 for 2 days. Both CypA and CypB levels were unaffected by these seasonal coronaviruses (Figure 1D).

I demonstrated through immunofluorescent staining that intracellular CypB levels, but not CypA levels, were reduced under CsA or ALV treatment in both mock-treated and HCoV-229E-infected Huh7 cells (Figure S2). Additionally, HCoV-229E infection induced bleb-like structures associated with the endoplasmic reticulum (ER) [144]. This finding is confirmed in the present study(Figure S2). Extending our investigation to infections with HCoV-NL63, HCoV-OC43, and sCPD9 (Figure S2), I observed a similar reduction in CypB levels, but not CypA levels, in the presence of CsA and ALV. However, I did not observe re-localization of CypB to bleb-like structures with these viruses, regardless of the presence of CypIs. Thus, CypB re-localization appears to be specific to HCoV-229E infection.

2.3 CypA plays an important role in HCoV replication

To further explore the functions of CypA and CypB in coronavirus replication, I constructed polyclonal KO cell pools selected by puromycin selection.

Although many coronaviruses including SARS-CoV-2 [181] proliferate better in Huh7.5 cells, I primarily used Huh7 in this study because of a RIG-I mutation present in Huh7.5 cells. As cyclophilins are extensively involved in cellular

innate immune responses, the mutation in the host viral RNA sensor RIG-I could interfere with the normal functions of cyclophilins, leading to biased and unreliable conclusions.

By knocking out CypA or CypB in Huh7 cells using lentivirus and puromycin selection, I generated polyclonal Huh7-CypA-KO (A1), Huh7-CypB-KO (BIII), and the control polyclonal cells Huh7-V2 (with the same lentiviral backbone) (Figure 2A). As a result, I observed a 50% reduction in the replication of HCoV-229E-Rluc in Huh7-CypA-KO (A1) cells at 48 h.p.i. compared to control Huh7-V2 and Huh7-CypB-KO (BIII) (Figure 2B). In contrast, the replication of sCPD9 was reduced only in Huh7-CypB-KO (BIII) cells, as demonstrated by qPCR assay (Figure 2C).

To determine the effect on SARS-CoV replication, a SARS-CoV replicon expressing Renilla luciferase [175] was transfected into 293T-Cyp-KO cells (Figure 2D), as Huh7 cells were poorly transfectable for replicons with large sizes. The graph in Figure 2E showed suppression of the SARS-CoV replicon in both CypA and CypB KO cells. All of the results indicated that Cyclophilins were important to the replication of coronaviruses, but the involved mechanisms differed depending on the species of coronaviruses. The replication of HCoV-229E partially relyed on CypA, but the proliferation of sCPD9 was highly influenced by CypB.

2.4 CsA inhibits HCoV-229E-Rluc mainly through CypA

CsA and other CypIs are known to block viral replication in viruses such as HIV, HCV, and coronaviruses. Until now it was not clear if CsA inhibits viruses by directly antagonizing CypA or CypB.

To confirm whether CsA inhibits viral replication via CypA, I designed an experiment approach to evaluate if transient transfection of external HA-tagged CypA (HA-PPIA) could counteract CsA during HCoV-229E-Rluc replication in CypA and CypB KO cells. Due to the low transfection efficiency of Huh7 cells and the inability of the Huh7 cells to withstand the combined stresses of transfection and infection, the 293T-CypA-KO (A1) and 293T-CypB-KO (BIII) cells were used in this assay by transfecting them with Aminopeptidase N (APN, host receptor for HCoV-229E) and varying amounts of HA-tagged CypA (HA-PPIA) or HA-tagged CypB (HA-PPIB) plasmids. The cells were infected with

HCoV-229E-Rluc the next day and treated with 2 μM CsA (approximately the IC_{50} value from previous study [144]) 2 hours later.

At 24 h.p.i., the CsA-reduced luciferase activity gradually increased with higher levels of plasmid-encoded CypA in 293T-CypA-KO (A1) cells (Figure 3A). Conversely, external CypB did not reverse the inhibitory effect of CsA in 293T-CypB-KO (BIII) cells (Figure 3B). These results support our hypothesis that CypA is the primary target for CsA in inhibiting HCoV-229E replication.

2.5 Overexpression of cyclophilins enhances coronavirus replication

Our earlier findings indicated that CypA is upregulated and co-localizes with dsRNA in SARS-CoV-2-infected cells (Figure 1C), suggesting a potential pro-viral role for CypA in coronaviruses. To investigate this further, I constructed stable 293T and Huh7 cells overexpressing either CypA or CypB. These cells were transfected with essential viral entry receptors: APN for HCoV-229E and ACE2 for NL63, SARS-CoV, and SARS-CoV-2, which are not naturally expressed in 293T cells.

Overexpression of CypA significantly increased the replication of HCoV-229E-Rluc in 293T cells transfected with the APN vector (Figures 4A and 4B). This effect was also observed with the SARS-CoV replicon in 293T cells (Figure 4C) and with HCoV-229E-Rluc in Huh7 cells overexpressing CypA (Huh7-CypA-OV) (Figure 4E).

Additionally, I assessed the replication of sCPD9 in Huh7 cells overexpressing CypA and CypB (Huh7-CypA-OV and Huh7-CypB-OV) (Figure 4D). I assumed that a second CypB band above the main band results from the recognition of CypB isomers by the antibody (also shown on the product page of the antibody selling company). Interestingly, CypB overexpression substantially enhanced sCPD9 infection, as demonstrated by qPCR tested on both cellular and supernatant RNA (Figure 4F) and a strongly increased Nucleocapsid band in Western blot analysis (Figure 4G). These results suggested that CypB might have a pro-viral effect on sCPD9, as indicated by the enhanced N protein levels shown in the Western blot (Figure 4G).

2.6 Both CypA and CypB bind to Nsp3 in SARS-CoV and SARS-CoV-2

Our lab identified key cellular proteins that interact with SARS-CoV viral proteins [75]. Previous protein-protein interaction (PPI) screenings of the SARS-CoV orfeome revealed interactions such as the SARS-CoV Unique Domain (SUD) and the PLpro protease with the cellular E3 ubiquitin ligase RCHY1, contributing to p53 degradation [164]. In this study, I conducted similar screenings for SARS-CoV-2-host PPI, identifying that Nsp3, when divided into five functional fragments and cloned into Y2H expression vectors (Figure 5A), bound both CypA and CypB, while Nsp4 and Nsp6 bound to CypB in a Y2H assay (Figure 5B).

To further examine these interactions, I used CoIP (Figure 5C) and N2H (Figure 5D) assays in HEK293 cells, cloning the five Nsp3 fragments into a GFP vector for CoIP pull-down. Results indicated that CypA mainly bound to the N-terminal fragment of Nsp3 (Figure 5E), while CypB bound to the NAB-ßSM domains and the N-terminal fragment of Nsp3 (Figure 5F). Notably, CypA and CypB also bound to Nsp3 of SARS-CoV, but not to Nsp3 of NL63, as shown in the N2H assay (Figure S4A). The strain specificity was attributed to the varying similarity of Nsp3 sequences among coronaviruses, with high similarity between SARS-CoV and SARS-CoV-2 (Supplementary Table S3).

The interactions between Nsp3 and CypA or CypB could be abrogated by the treatment of CsA (Figure 5G) or ALV (Figure S4B) in HEK293 cells. Those figures also demonstrated that overexpression CypA enhanced the protein level of Nsp3, and CsA or ALV treatment antagonized this induction. This phenotype was more noticeable in Figure S4C with the noticeable induction of Nsp3 protein level in cells overexpressing GFP-tagged CypA. Furthermore, western blot analysis confirmed that both CsA or ALV could reduce the protein level of Nsp3 but not the GFP control (GFP cloned into the same vector to replace Nsp3) (Figure 5H) in HEK293 cells.

To determine the necessity of CypA or CypB for Nsp3 expression, I transfected the plasmid encoding full-length Nsp3 of SARS-CoV-2 into 293T-Cyp-KO cells and control cells (293T-V2). Western blot images (Figure S4D) revealed that 20 µM CsA suppressed Nsp3 protein levels in both 293T-V2 and 293T-CypA-KO (A1) cells, but not in 293T-CypB-KO (BIII) cells. Notably, Nsp3 protein levels in BIII cells were markedly reduced compared to the other samples. This result demonstrated the correlation between the CypB protein level and the

expression of Nsp3. The expression of Nsp3 highly likely depended on the expression of CypB.

CypB was captured by RFP-Nsp4 or Nsp6 using RFP-trap, with their interactions largely reduced by 24-hour ALV treatment (Figure S4E). However, CypA was not pulled down under the same conditions. The protein levels of RFP-tagged Nsp4 or Nsp6 were visible only in RFP-trap purified samples, and their levels were largely decreased in the presence of ALV, similar to the change of CypB levels in the total lysate (input) samples. I assumed that Nsp4 and Nsp6 might be influenced by CypB in the similar manner of regulating Nsp3 of SARS-CoV-2 by CypB.

Besides Nsp3, Nsp4 and Nsp6, a number of accessory proteins and structure proteins of SARS-CoV-2 were found as potential interacting partners of CypB by Y2H screening. Further examination by CoIP assay in HEK293 cells demonstrated that ORFs 3a, 7a, 7b, and 9c, and structural proteins E and M interacted with CypB (Figure S4F), which explained the importance of CypB in regulation of SARS-CoV-2 replication again.

2.7 N protein of SARS-CoV-2 is another target for both CypA and CypB

The nucleocapsid (N) protein of coronaviruses represents another important target for our research. Our lab previously demonstrated that CypA bound to the N protein of HCoV-229E [144] and SARS-CoV-2 [182]. The CoIP results (Figure 6A) showed that both CypA and CypB bound to the HA-tagged N protein in HEK293 cells, with this interaction being inhibited by 20 μM CsA treatment. Unlike Nsp3, the expression levels of N-HA were not affected by CsA or ALV in 293 cells (Figure 6B).

I also performed a sequence similarity analysis of the nucleocapsid proteins from various human coronaviruses (HCoVs) including NL63, 229E, OC43, SARS-CoV, MERS, and SARS-CoV-2 using ClustalW. The analysis revealed that the N protein was more conserved compared to Nsp3 among HCoVs. Specifically, SARS-CoV and SARS-CoV-2 shared the highest sequence identity, at 89.74% (Supplementary Table S3).

2.8 Nsp3 and N of SARS-CoV-2 impair host innate immune responses

Similar to numerous viruses, SARS-CoV-2 has the capability to evade the innate immune system through various strategies, such as viral antagonism, evasion of detection, and modulation of inflammatory responses [183]. Recent findings have shown that Nsp3 and Nsp5, which encode proteases of SARS-CoV-2, can cleave proteins involved in the host's innate immune response [184]. We examined the effect of Nsp3 on the production of type-I interferon (IFN) by performing IFN-beta or interferon stimulated response element (ISRE) promoter-driven luciferase assays in HEK293 cells. The results demonstrated that Nsp3 dampened either IFN-beta or ISRE responsive luciferase activity induced by Sendai virus (SEV) (Figure S4G). The SEV worked as a stimulus to boost cellular innate immune response thus the luciferase activity could be detectable.

The N protein has been widely characterized for its function in suppressing the innate immune response. N proteins from both SARS-CoV and SARS-CoV-2 inhibited TRIM25-mediated RIG-I activation and IFN-beta production [185, 186].

The Cyclophilin family affects viral infection, immunity, and inflammatory responses [179]. Wei Liu et al. [84] discovered that CypA enhanced RIG-I-mediated antiviral immune responses by regulating the ubiquitination of RIG-I and MAVS. In our study, CoIP assays showed that both CypA and CypB physically bound to RIG-I and MAVS (Figure 6C and 6D). The interactions between RIG-I or MAVS and Cyps were further confirmed using the N2H assay (Figure 6E). Notably, the CypA-enhanced production of type-I interferon was not impaired by Cyp inhibitor ALV (Figure 6F), which suggested boosting immune responses did not rely on the enzyme activity of CypA.

To explore if proteins of SARS-CoV-2 participate in Cyp-mediating induction of type-I interferons, I conducted the competitive N2H assays using the same combinations in Figure 6E, but with an additional 10% plasmids of YFP^c-tagged Nsp3, N or the ‘empty’ backbone. The results indicated that N protein likely antagonized the interactions between RIG and CypA or CypB (Figure S4H) and significantly reduced the binding signals of MAVS and Cyps (Figure 6G).

3. Summary

In summary, CypA and CypB are potentially important targets for pan-coronavirus strategies. Coronaviruses hijack and exploit host cyclophilins to facilitate their replication, likely contributing to the formation of DMVs. Our research suggests two mechanisms by which CsA and ALV inhibit SARS-CoV-2 replication: (a) by disrupting the interactions of Nsp3 and N with CypA and CypB, and by reducing Nsp3 expression; and (b) by preventing N from evading immune responses, thereby restoring the antiviral functions of cyclophilins. This study enhances our understanding of how CsA and its analogues inhibit viral replication and provides a foundation for developing therapeutics against evolving coronaviruses.

Figure legend

Figure 1: Effects of CoV infection on CypA and CypB in Huh7, Huh7.5 and BEAS-2B-ACE2 cells.

A) qPCR analysis reveals the reduction of sCPD9 replication in Huh7 cells after CypI treatment. Cells were infected with attenuated SARS-CoV-2 (sCPD9) at MOI=0.01 or mock infection for 96 hours. RNA samples isolated from 100µl supernatant were analyzed to measure the replication of sCPD9. ****: p<0.0001.

B) Western blot analysis reveals induction of CypA but not of CypB in SARS-CoV-2-infected (left) or sCPD9-infected (right) Huh7 Cells. Cells were infected by SARS-CoV-2 (MOI=1) or sCPD9 (MOI=0.01) for 48 hours. Whole protein lysates were analyzed for the levels of SARS-CoV-2 N protein, endogenous CypA, CypB and the loading control beta-actin using specific antibodies.

C) Immunofluorescence analysis of CypA (not CypB) induction and co-localization with dsRNA in SARS-CoV-2-infected Huh7 cells. Huh7 cells prepared in 8-well chamber slides were infected with SARS-CoV-2 (MOI=0.1, 24 hours) and treated with 0.1% DMSO before fixation. Cells were then incubated with anti-CypA (left) or anti-CypB (right), anti-dsRNA antibodies, followed by DAPI staining before imaging. Images were taken by confocal microscopy with 100X objective. Scale bar:10µm

D) Western blot analysis reveals unchanged endogenous CypA and CypB expression levels upon infection with HCoV-NL63, -229E, and -OC43. Huh7 (left) or BEAS-2B ACE2 (right) cells plated in 6-well plates were infected with HCoV-NL63 (MOI=0.01), -229E (MOI=1) or -OC43 (MOI=0.1) for 48 hours. Protein samples were analyzed for the expression of various HCoV N proteins, endogenous CypA, CypB and the loading control vinculin. Note, the mouse mab 1H11 cross-reacted with HCoV-NL63 and 229E N proteins.

Figure 2: Replication of coronaviruses in Huh7 and HEK293T-CypA and -CypB knockout (KO) cells.

A) Verification of CypA and CypB KO in Huh7 cells by Western blot assay. Huh7-CypA-KO (A1), Huh7-CypB-KO (BIII), and the control cell (Huh7-V2) were

plated in a 12-well plate with equal cell numbers. Cells were lysed using NP40 lysis buffer after overnight culture for protein collection. Western blot analysis was performed with anti-CypA, anti-CypB, and anti-beta-actin antibody.

B) CypA KO suppression of 229E-Rluc growth in Huh7 cells at 48 h.p.i. Renilla luciferase assays were conducted on Huh7-CypA-/CypB-KO cells and control cells infected with HCoV-229E-Rluc at MOI=1 for 48 hours. Viral replication was assessed by measuring Renilla luciferase activity using a microplate reader. ***: $p<0.001$.

C) Inhibition of sCPD9 replication in Huh7-CypB-KO (BIII) cells in qPCR assay. Huh7-Cyp-KO cells seeded in a 48-well plate were infected with sCPD9 (MOI=0.0002). After 2 hours, the inoculum was removed, and cells were washed by DPBS twice. RNA samples from cell lysates were isolated 48 h.p.i. (n=3). ns: not significant, **: $p<0.01$.

D) Verification of CypA- or CypB-KO in HEK293T cells by Western blot assay. Cell clones seeded in a 12-well plate were lysed and processed for Western blot analysis using antibodies targeting CypA, CypB, and vinculin (as a loading control).

E) Impairment of SARS-CoV-Rluc replication in HEK293T-CypA-KO (A1) and HEK293T-CypB-KO (BIII) cells. Cell clones from panel C were seeded in a 96-well plate and transfected with the SARS-CoV-Rluc replicon for 48 hours. Cells were harvested to measure Renilla luciferase activity. *: $p<0.05$, **: $p<0.01$.

Figure 3: CsA inhibits HCoV-229E-Rluc replication mainly through CypA.

A) External CypA transfection counteracts CsA inhibition at low dose.

293T-CypA-KO (A1) cells were plated in a 48-well plate and transfected with an APN receptor-expressing plasmid and either an "empty" HA vector or HA-PPIA fusion constructs at a 1:3 ratio using Lipofectamine. Increasing doses of HA-tagged CypA (HA-PPIA) replaced the same amount of HA vector starting from the third group on the left, as indicated on the x-axis. Details of the transfection were listed in the table below the Western blot panels. HCoV-229E-Rluc was added at MOI=1, and 2 μM CsA or DMSO solvent (at the same dilution) was applied 4 h.p.i. The luciferase assay was conducted at 24 h.p.i (n=3). Concurrently, similar cells prepared in a 24-well plate were treated in the same manner but at double the dose due to the increased cell quantity. Proteins

extracted from these cells were used for Western blot to confirm the expression of APN and HA-tagged CypA. Note: The HA tag of the HA cloning vector alone was too small to be detected.

B) External CypB transfection does not counteract CsA inhibition at low dose.

293T-CypB-KO (BIII) cells prepared in a 48-well plate were transfected with the receptor APN and HA vector at a 1:3 ratio using Lipofectamine. Similar to Figure 3A, cells were infected with HCoV-229E-Rluc (MOI=1) and treated with 2 µM CsA or DMSO solution after transfection (details shown in the table). The luciferase assay was conducted at 24 h.p.i (n=3). A parallel Western blot was performed to visualize the transfection. 293T-CypB-KO (BIII) cells prepared in a 24-well plate were treated in the same manner but at double the dose due to the increased cell quantity. Proteins extracted from these cells were used for Western blot to confirm the expression of APN and HA-tagged CypB. Note: The HA tag alone was too small to be visualized.

Figure 4: Replication of coronaviruses in HEK293T and Huh7 cells upon stable cyclophilin overexpression.

A) Verification of CypA or CypB overexpression in HEK293T cells by Western blot assay. Cells plated in 12-well plates were transfected with APN receptor-expressing plasmid and lysed at 24 h.p.t. Subsequently, protein samples were processed for Western blot using antibodies targeting CypA, CypB, APN and beta-actin (as a loading control).

B) Promotion of 229E-Rluc replication in 293T APN-transfected cells upon CypA overexpression. 293T-CypA (A9) OV, CypB (B7) OV and the control cell clone 293T-control were seeded in 48-well plates 24 hours before the experiment. Cells were then transfected with plasmid expressing APN overnight and infected with HCoV-229E-Rluc (MOI=1) for 48 hours. ns: not significant ($p \geq 0.05$), **: $p < 0.01$.

C) CypA overexpression promotes the propagation of the SARS-CoV-Rluc replicon. 293T cells used in Figure 4B were prepared in 96-well plates and transfected with SARS-CoV-Rluc for 48 hours. Cells were harvested to measure Renilla luciferase activity. *: $p < 0.05$.

D) Verification of CypA or CypB overexpression in Huh7 cells by Western blot assay. Cell clones seeded in 12-well plates were lysed and processed for Western blot using antibodies targeting CypA, CypB, and vinculin (as a loading control).

E) Promotion of 229E-Rluc replication in Huh7-CypA-OV cell. Renilla luciferase assays were conducted on Huh7-CypA/B-OV cells and control cell clone Huh7-control infected with HCoV-229E-Rluc at MOI=1 for 72 hours. Viral replication was assessed by measuring Renilla luciferase activity using a microplate reader. ns: not significant, ***: $p<0.001$.

F) Enhancement of sCPD9 replication in Huh7 CypB overexpression cells. Huh7-Cyp-OV cells seeded in a 48-well plate were infected with sCPD9 (MOI=0.0002). After 2 hours, the inoculum was removed, and cells were washed with DPBS twice. RNA samples from cell lysates and supernatants were isolated at 72 h.p.i (n=3). ns: not significant, **: $p<0.01$, ***: $p<0.001$.

G) Western blot analysis of sCPD9 replication in Huh7-Cyp-OV cells. Huh7 cells were seeded in a 12-well plate and infected with sCPD9 (MOI=0.0002) for 48 hours, except for the mock well. Protein samples isolated from the corresponding wells were examined for sCPD9 replication using the anti-N 21H2-1-1 antibody.

Figure 5: Interactions of CypA and CypB with SARS-CoV-2 Nsp3

A) Annotations of SARS-CoV-2 Nsp3 fragments. Nsp3 was divided into five fragments, each representing different domains. These fragments were cloned as follows: N terminal (aa 1-412), SUD (aa 413-745), PLp (aa 746-1064), NAB-βSM (aa 1065-1414) and C terminal (aa 1415-1945).

B) Y2H interaction between sPPIA (short isoform 105 aa, [CCDS 75592.1]) or CypB (PPIB [CCDS 10191.1]) and viral genes. The yeast cells transformed with both prey and bait plasmids could only grow on triple-dropout (without Leu, Trp and His) plates if the bait expressing SARS-CoV-2 protein interacted with the host protein encoded by the prey plasmid. The results showed that the protein expressed by the sPPIA gene interacted with N terminal fragment of Nsp3, and CypB interacted with Nsp3 C-terminus, Nsp4 and Nsp6. All host proteins and viral proteins encoded by plasmids did not interact with the products from empty Y2H vectors.

C) CypA and CypB bind to Nsp3 aa (1-1363) of SARS-CoV-2 in a CoIP assay. HEK293 cells in a 6-well plate were transfected with plasmids as indicated in the figure. SARS-CoV-2 Nsp3 was pulled down by CypA or CypB with GFP trap.

D) CypA and CypB bind to SARS-CoV-2-Nsp3 (codon-optimized full length, FL) in the N2H assay. HEK293 cells transfected overnight with indicated plasmids were assessed for Nano luciferase activity by plate reader to confirm the interactions. The mean of background signal from untreated cells was taken as 1. As displayed next to the chart, Nanoluc gene was divided into two fragments: F1 and F2. In this assay, Nsp3 fused with F1 at N-terminal (N1-Nsp3) and cyclophilins fused with F2 at N-terminal (N2-PPIA and N2-PPIB). N1 and N2 were the empty vectors expressing F1 and F2.

E) and F) Interactions between CypA or CypB and Nsp3 fragments from SARS-CoV-2. HEK293 cells were co-transfected with HA-tagged PPIA and GFP-fused Nsp3 fragments, or GFP vector control for 24 hours. Cells were then lysed and purified by GFP trapping for CoIP assay.

G) Reversal of CypA or CypB binding to SARS-CoV-2-Nsp3 (FL) by CsA treatment. HEK293 cells co-transfected with plasmids shown in the figure were treated with 20μM CsA or DMSO 4 h.p.t. After 24 hours, cells were lysed following standard protocol for CoIP assay.

H) Impairment of SARS-CoV-2-Nsp3 (FL) expression by Cyp inhibitors. HEK293 cells transfected with either Nsp3 or GFP cloned into HA-YFPc vector were treated with 20μM CsA, 20μM ALV or DMSO with the same dilution 4 h.p.t. Protein samples were collected for western blot analysis after 24 hours.

Figure 6: SARS-CoV-2 N protein binding to cyclophilins and SARS-CoV-2 Nsp3 or N antagonizing immune response

A) Blockage of binding CypA or CypB to SARS-CoV-2 N protein by CsA treatment. HEK293 cells co-transfected with plasmids shown in the figure were treated with 20μM CsA or DMSO 4 h.p.t. After 48 hours, cells were lysed following standard protocol for CoIP assay.

B) Influence of Cyp inhibitors on the expression of SARS-CoV-2 N protein. HEK293 cells transfected with either N or GFP cloned into HA-YFP^{C} vector

were treated with 20μM CsA, 20μM ALV or DMSO with the same dilution 4 h.p.t. Cells were lysed at 24 h.p.t for western blot.

C) and D) Interaction of CypA and CypB with RIG-I and MAVS in CoIP assay. HEK293 cells prepared in 6-well plates were transfected with GFP vector, GFP-tagged PPIA or PPIB, plus RIG-I (C) or MAVS (D) with HA tag. Proteins collected from cells after overnight transfection were purified by GFP-trap and analyzed by CoIP.

E) Interaction of CypA and CypB with RIG-I and MAVS in N2H assay. HEK293 cells transfected with indicated plasmids overnight were measured by plate reader for the Nano luciferase activity. The mean of background signal from untransfected cells was regarded as 1. The interactions were verified by excluding the possibility of unspecific signals from empty vectors (data of the first 3 groups).

F) CARD-induced type-I interferon (IFN-beta) was not changed by ALV. HEK293 cells were transfected with CARD (acted as stimuli), HA-PPIA or HA vector and pIFN-beta. The induction of IFN-beta was assessed by a firefly luciferase reporter under the control of the promotor. Cells were subsequently treated with 20μM ALV 4 hours after transfection for 24 hours.

G) SARS-CoV-2 N protein competition with MAVS for binding to CypA and CypB. HEK293 cells in 96-well plates were transfected with MAVS in N1-vector and PPIA or PPIB in N2 as shown in Figure 4E, plus 10% more plasmid of Nsp3, N, or just the vector as described in the figure. The Nanoluc signals generated from Cyp-MAVS binding were significantly reduced by the small amount of N plasmid compared to the control group. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Figure S1: CsA suppression of HCoV replication.

A) Inhibition of 229E-Rluc in Huh7 cells, quantified by Rluc expression. Huh7 cells were infected with 229E-Rluc at MOI=1 for 24 or 48 hours in a 96-well plate. CsA (20 μM) was applied to the cells 4 h.p.i. Renilla luciferase activities, representing viral replication, were recorded (n=3 for each point).

B) and C) Relative inhibition of NL63 and OC43 replication in Huh7 cells by 20μM CsA, quantified by probe-based qPCR targeting cellular nucleocapsid genes. Huh7 cells were infected with NL63 (MOI=0.01) or OC43 (MOI=0.01) for 24 or 48 hours before cellular RNA samples were isolated for

qPCR (n=3, pooled samples with double technical replicates). Data were compared with internal control beta-actin.

Figure S2: Infection Inhibition and Downregulation of CypB by CsA and ALV in Huh7 Cells

Huh7 cells prepared in 8-well chamber slides were infected with HCoV-NL63, 229E, OC43, sCPD9 or mock at indicated MOI. After 2-hour inoculation, cells were treated with 20μM CsA, ALV or DMSO solvent for 48 hours. Cells were then incubated with anti-CypA or anti-CypB, anti-dsRNA antibodies, followed by DAPI staining before imaging. Images were taken by confocal with 100X objective. Scale bar: 10μm.

Figure S3: Co-localization of dsRNA and N in Huh7 cells infected with sCPD9

Huh7 cells prepared in 8-well chamber slides were infected with SARS-CoV-2 sCPD9 at MOI=0.003 for 48 hours. Cells were then incubated with anti-CypA or anti-CypB, anti-dsRNA, and anti-N antibodies, followed by DAPI staining before imaging. Images were taken by confocal with 100X objective. The infected cell examples were circled by the orange dotted line and the uninfected cell examples were labeled in red. Merge 1: triple channels with CypA or CypB, dsRNA and DAPI. Merge 2: triple channels with CypA or CypB, N and DAPI. Merge 3: quadruple channels with CypA or CypB, dsRNA, N and DAPI. Scale bar: 10μm.

Figure S4: Effects of cyclophilin inhibitors on the binding of CoV proteins to cyclophilins

A) CypA and CypB bind to Nsp3 of SARS-CoV but not NL63. HEK293 cells were transfected with Nsp3 (of SARS-CoV or NL63) in N1 vector and CypA or CypB in N2 vector by Lipofectamine for 24 hours. Nanoluc assay was carried out on the cells lysed by NP40 to explore their bindings (n=3). ***: $p<0.001$, ****: $p<0.0001$.

B) Reversal of CypA or CypB binding to SARS2-Nsp3 aa (1-1363) by ALV treatment. HEK293 cells transfected with corresponding plasmids were treated with 20μM ALV and lysed for CoIP after 24 hours. The Western blot result

showed that Nsp3 aa (1-1363) was pulled down by GFP-fused CypA or CypB in the presence of DMSO vehicle, but not after ALV treatment.

C) Induction of Nsp3 aa (1-1363) protein level by transient overexpression of CypA. HEK293 cells transfected with corresponding plasmids were treated with 20μM ALV and lysed after 48-hour drug treatment. The samples were then used in the western blot assay to clarify the influence of CypA or CypB transfection on the protein level of Nsp3 with DMSO or ALV treatment.

D) The expression of SARS2 Nsp3 (FL) in 293T polyclonal cells with drug treatment. The 293T CypA KO (A1), CypB KO (BIII) and the control cell clone (V2) in 12-well plate were transfected with HA-YFP^{C}-Nsp3 of SARS-CoV-2 and after that treated with 20μM CsA for 24 hours. The protein levels of Nsp3 were checked in different cell clones with inhibitor or only the solvent by western blot.

E) Interference of SARS-CoV-2 Nsp4/Nsp6 and CypB binding by ALV. The CoIP/RFP trap pulldown of RFP-Nsp4/Nsp6 and HA-PPIB was performed in HEK293 cells in the presence of 45μM ALV. The bindings were largely impaired with ALV treatment for 24 hours.

F) Binding of CypB to several accessory proteins and structural proteins of SARS-CoV-2. HEK293 cells seeded in 6 well plates were transfected with corresponding combinations of plasmids (1 well) for 24 hours. Cellular extracts were applied to RFP-trap columns and analyzed by Western blot. Viral ORFs tested positive for interaction with CypB by Y2H were chosen for the analysis. Interaction was observed for orf3a, 7a, 7b, 9c and E, and weakly for orf9b and M.

G) Downregulation of IFN-beta and ISRE promoters by Nsp3. HEK293 cells in 96-well plate transfected with pIFN-beta or pISRE promotor plasmids fused with firefly luciferase reporter and Nsp3 or the vector were infected by Sendai viruses the next day imitating RLR activation by SARS-CoV-2 for another 24 hours. The modulation of innate immune pathway was measured by the firefly luciferase activity (n=4). ****: $p<0.0001$.

H) The interactions of CypA/CypB and RIG-I were possibly influenced by N of SARS-CoV-2. HEK293 cells in 96-well plate were transfected with RIG-I in Nanoluc C2-vector and PPIA or PPIB in N1 as Fig. 4E, plus 10% more plasmid of Nsp3, N or just the vector as described in this figure. The Nanoluc signals

generated from Cyp-RIG-I binding were potentially reduced by the small amount of N plasmid compared to the control group. ns: not significant.

Figure 1

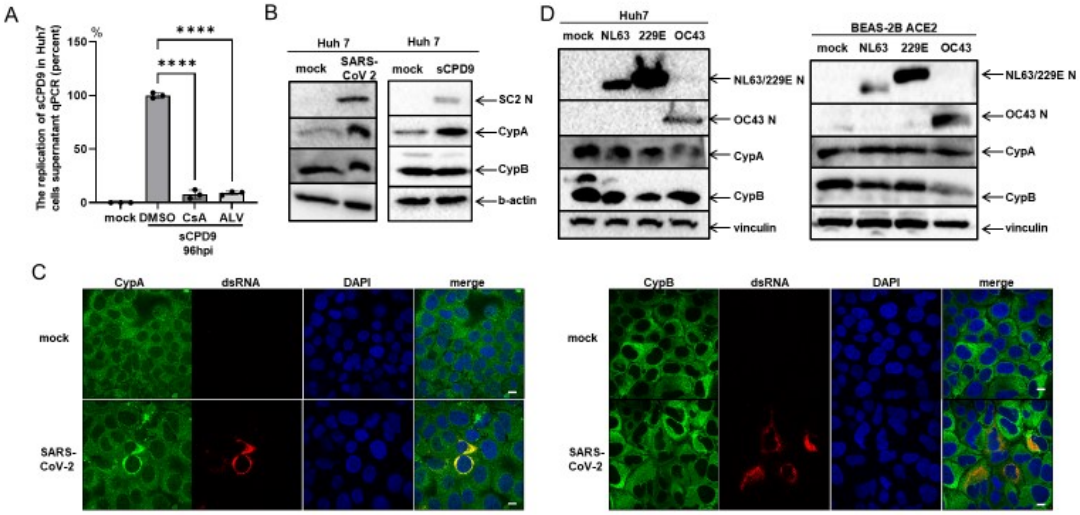


Figure 2

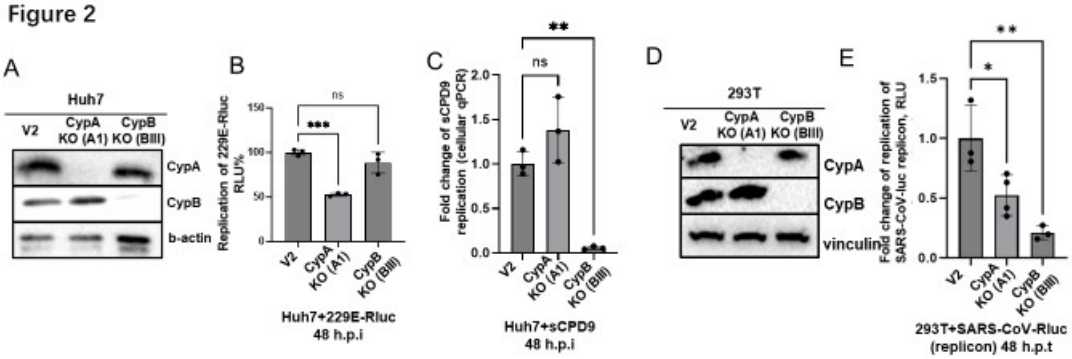
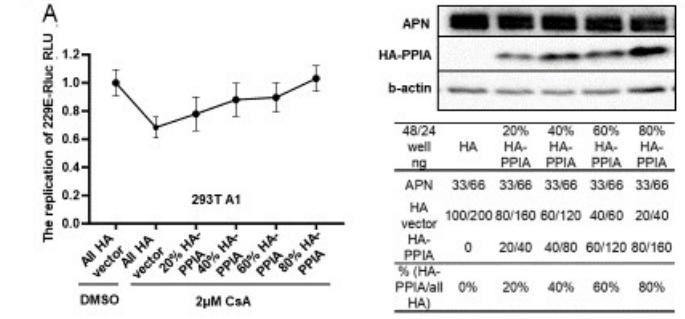
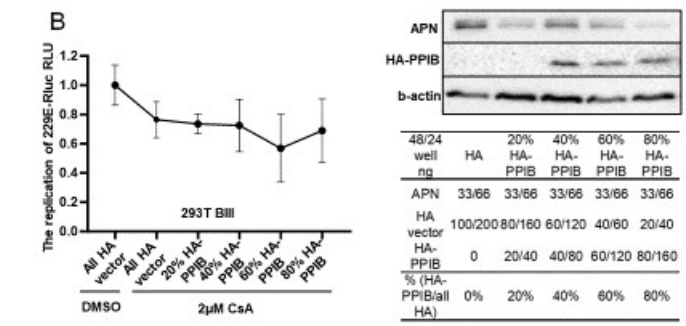


Figure 3



48/24 well ng	HA	20% HA-PPIA	40% HA-PPIA	60% HA-PPIA	80% HA-PPIA
APN	33/66	33/66	33/66	33/66	33/66
HA vector	100/200	80/160	60/120	40/60	20/40
HA-PPIA	0	20/40	40/80	60/120	80/160
% (HA-PPIA/all HA)	0%	20%	40%	60%	80%



48/24 well ng	HA	20% HA-PPIB	40% HA-PPIB	60% HA-PPIB	80% HA-PPIB
APN	33/66	33/66	33/66	33/66	33/66
HA vector	100/200	80/160	60/120	40/60	20/40
HA-PPIB	0	20/40	40/80	60/120	80/160
% (HA-PPIB/all HA)	0%	20%	40%	60%	80%

Figure 4

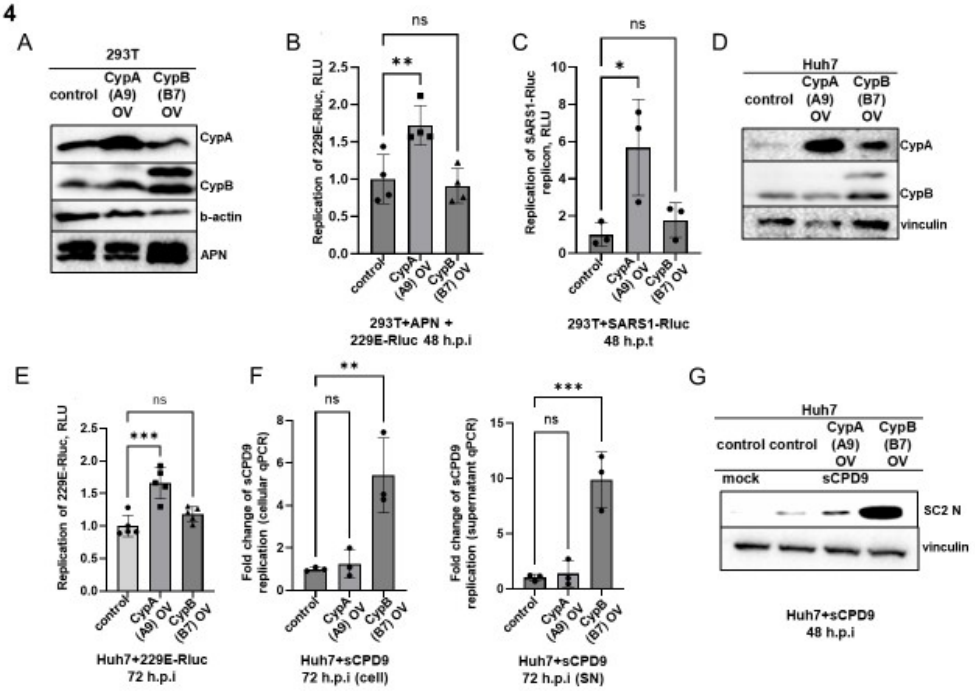


Figure 5

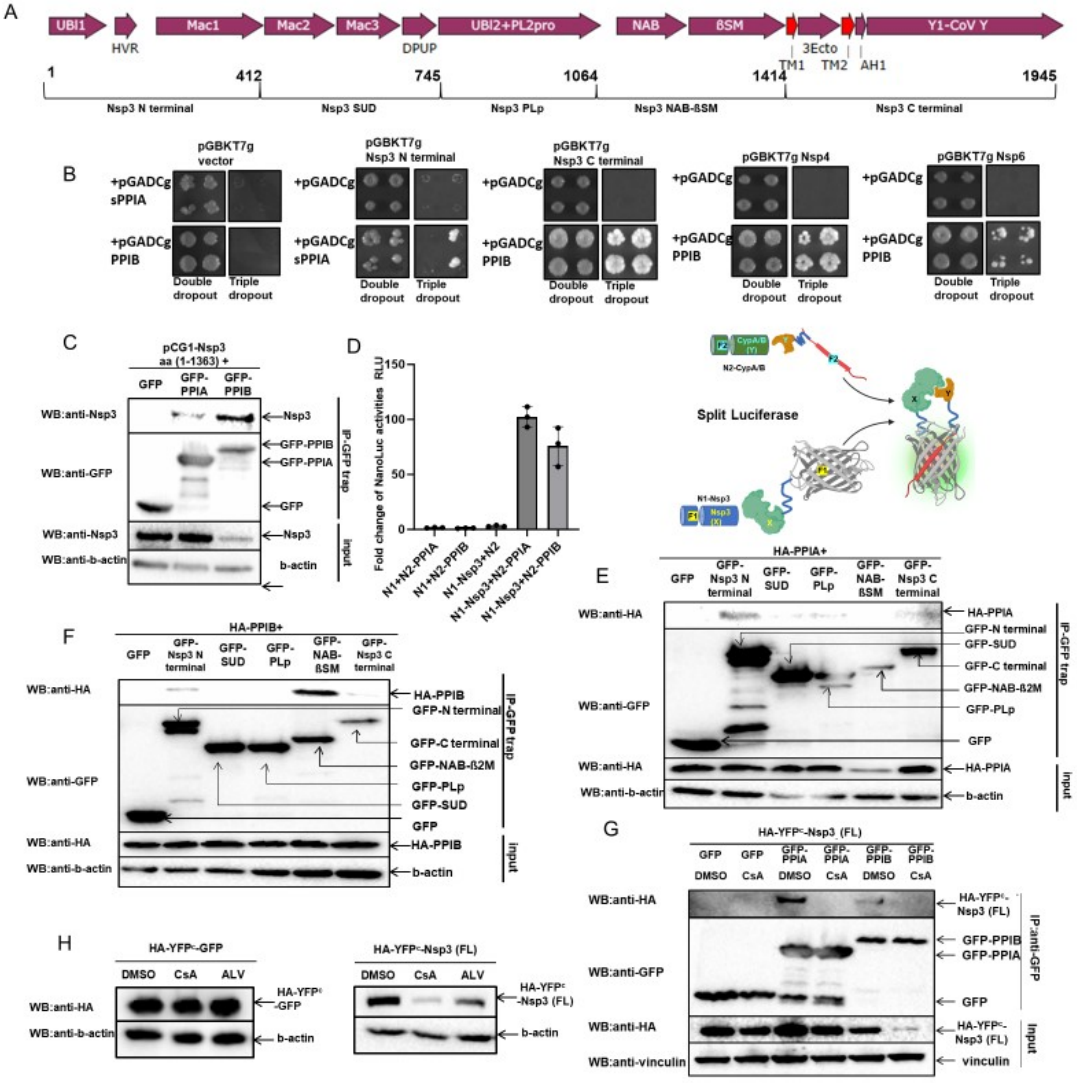


Figure 6

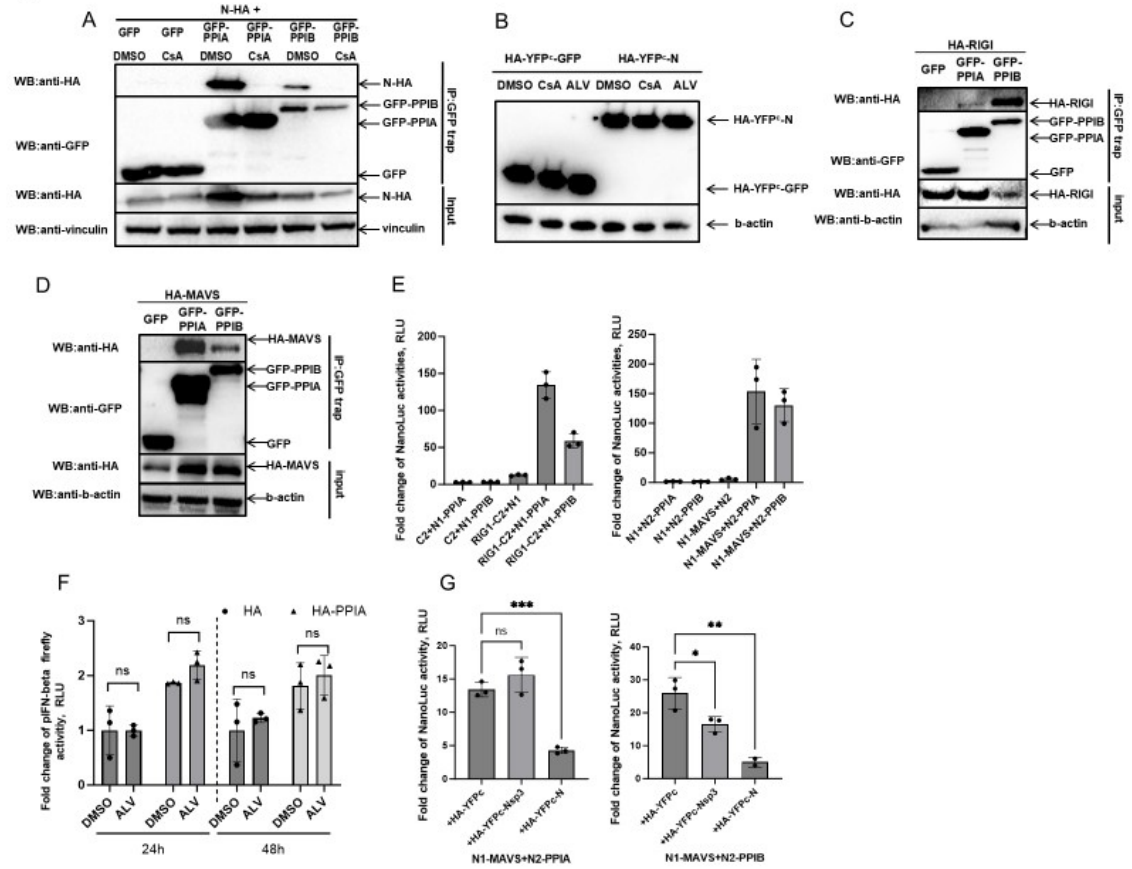


Figure S1

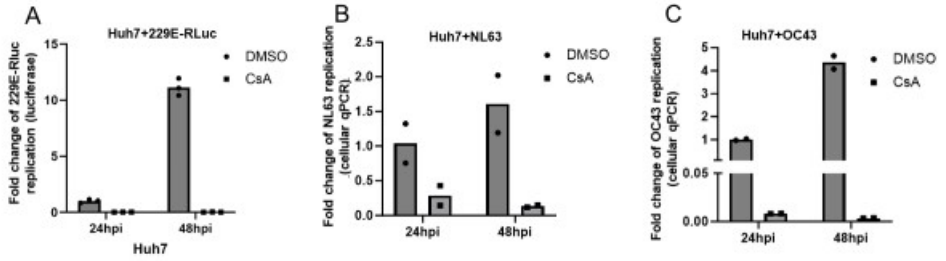


Figure S2

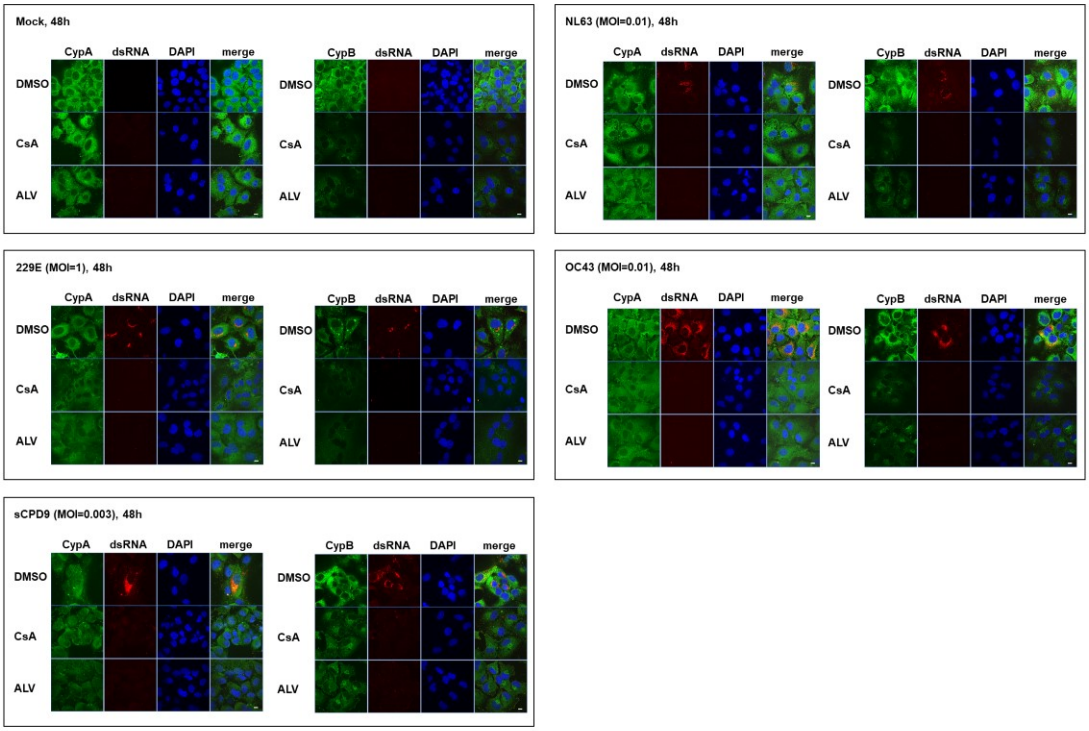


Figure S3

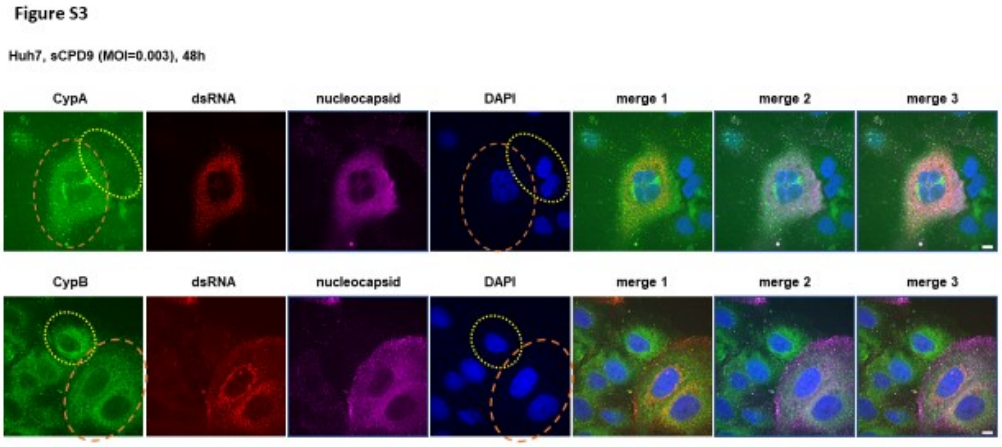


Figure S4

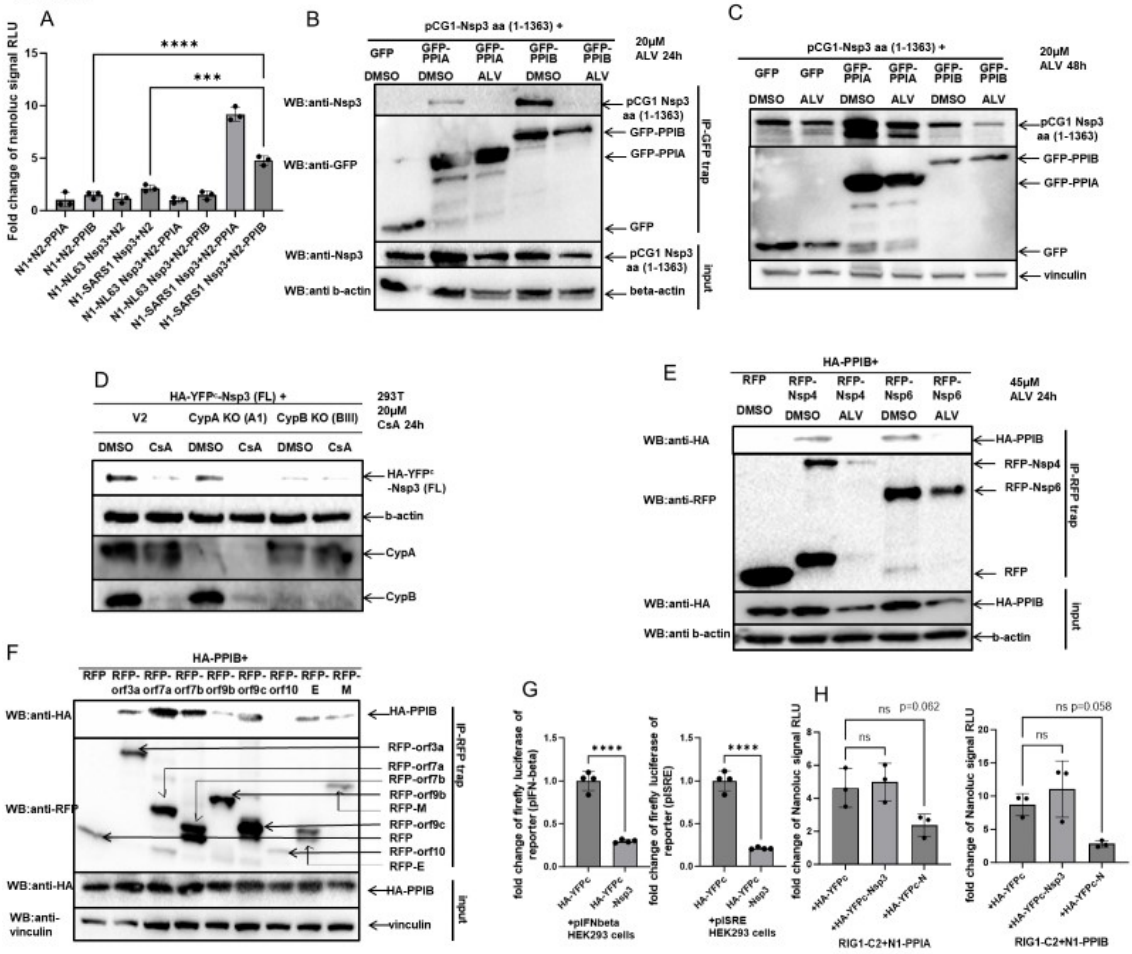

A
Fold change of nanoluc signal RLU
****
***
N1+N2-PPIA
N1+N2-PPIB
N1-NL63 Nsp3+N2
N1-SARS1 Nsp3+N2
N1-NL63 Nsp3+N2-PPIA
N1-NL63 Nsp3+N2-PPIB
N1-SARS1 Nsp3+N2-PPIA
N1-SARS1 Nsp3+N2-PPIB
B
pCG1-Nsp3 aa (1-1363) +
GFP
GFP-PPIA
GFP-PPIB
DMSO
ALV
20μM ALV 24h
WB:anti-Nsp3
WB:anti-GFP
WB:anti-Nsp3
WB:anti b-actin
pCG1 Nsp3 aa (1-1363)
GFP-PPIB
GFP-PPIA
GFP
pCG1 Nsp3 aa (1-1363)
beta-actin
IP-GFP trap
input
C
pCG1-Nsp3 aa (1-1363) +
20μM ALV 48h
pCG1 Nsp3 aa (1-1363)
GFP-PPIB
GFP-PPIA
GFP
vinculin
D
HA-YFPc-Nsp3 (FL) +
293T 20μM CsA 24h
V2
CypA KO (A1)
CypB KO (BIII)
CsA
HA-YFPc -Nsp3 (FL)
b-actin
CypA
CypB
E
HA-PPIB+
RFP
RFP-Nsp4
RFP-Nsp6
45μM ALV 24h
WB:anti-HA
WB:anti-RFP
HA-PPIB
RFP-Nsp4
RFP-Nsp6
RFP
IP-RFP trap
input
b-actin
F
HA-PPIB+
RFP
RFP-orf3a
RFP-orf7a
RFP-orf7b
RFP-orf9b
RFP-orf9c
RFP-orf10
RFP-E
RFP-M
WB:anti-vinculin
vinculin
G
fold change of firefly luciferase of reporter (pIFN-beta)
fold change of firefly luciferase of reporter (pISRE)
HA-YFPc
HA-YFPc -Nsp3
+pIFNbeta HEK293 cells
+pISRE HEK293 cells
H
fold change of Nanoluc signal RLU
ns
p=0.062
p=0.058
+HA-YFPc
+HA-YFPc-Nsp3
+HA-YFPc-N
RIG1-C2+N1-PPIA
RIG1-C2+N1-PPIB

Table S1. Primer List

primer name	primer sequence
PPIA-att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GGT CAA CCC CAC CGT GTT CTT CGA C-3'
PPIA-att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA TTC GAG TTG TCC ACA GTC AGC AAT GG-3'
sPPIA aa61 att for	5'GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GTG TCA GGG TGG TGA CTT CAC ACG-3'
PPIB-att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GCT GCG CCT CTC CGA ACG CAA CAT G-3'
PPIB-att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA CTC CTT GGC GAT GGC AAA GGG CTT C-3'
PPIA gRNA1 for	5'-CAC CGG TAC CCT TAC CAC TCA GTC T-3'
PPIA gRNA1 rev	5'-AAA CAG ACT GAG TGG TAA GGG TAC C-3'
PPIB gRNAIII for	5'-CAC CGC CAG GGT GGA GAC TTC ACC A-3'
PPIB gRNAIII rev	5'-AAA CTG GTG AAG TCT CCA CCC TGG C-3'
Beta-actin qPCR for	5'-TCC TGA GCG CAA GTA CTC CG-3'
Beta-actin qPCR rev	5'-CTG ATC CAC ATC TGC TGG AAG G-3'
Beta-actin qPCR probe	5´6-FAM-ATCGGCGGCTCCATCCTG-BHQ1-3´
HCoV-OC43 qPCR for	5'-CGC CGC CTT ATT AAA GAT GTT G-3'
HCoV-OC43 qPCR rev	5'-GGC ATA GCA CGA TCA CAC TTA GG-3'
HCoV-OC43 qPCR probe	5'-FAM-AAT CCT GTA CTT ATG GGT TGG GAT T-BHQ1-3'
HCoV-NL63 qPCR for	5′-CTT CTG GTG ACG CTA GTA CAG CTT AT-3′
HCoV-NL63 qPCR rev	5′-AGA CGT CGT TGT AGA TCC CTA ACA T-3′
HCoV-NL63 qPCR probe	5′-FAM-CAG GTT GCT TAG TGT CCC ATC AGA TTC AT-TAMRA-3′
SARS-CoV-2 qPCR for	5′-GAC CCC AAA ATC AGC GAA AT-3′
SARS-CoV-2 qPCR rev	5′-TCT GGT TAC TGC CAG TTG AAT CTG-3′
SARS-CoV-2 qPCR probe	5′-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
SARS-CoV-2 nsp3 (aa1-412) att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GGC ACC AAC AAA GGT TAC TTT TGG TGA TG-3'
SARS-CoV-2 nsp3 (aa1-412) att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM CTT ATC ATC TTG TTT TCT CTG TTC AAC TG-3'
SARS-CoV-2 nsp3-PLp att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GGA AGT GAG GAC TAT TAA GGT GTT TAC AAC-3'
SARS-CoV-2 nsp3-PLp att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM ATA AGT AAC TGG TTT TAT GGT TGT TGT GTA ACT G-3'

SARS-CoV-2 nsp3-SUD att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GAA AAT CAA AGC TTG TGT TGA AGA AGT TAC-3'
SARS-CoV-2 nsp3-SUD att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM AGA AAG AAG TGT CTT AAG ATT GTC AAA GG-3'
SARS-CoV-2 nsp3 (aa1065-1414) att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GAA ATT GGA TGG TGT TGT TTG TAC AGA AAT TG-3'
SARS-CoV-2 nsp3 (aa1065-1414) att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM CCA AAT TAT AAT ATT TAT CAG TTT AGA AAA ATT AGG-3'
SARS-CoV-2 nsp3 (aa1547-1945) att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GTG GTT AAT AAT TAA TCT TGT ACA AAT G-3'
SARS-CoV-2 nsp3 (aa1547-1945) att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM ACC ACC CTT AAG TGC TAT CTT TGT TGT TAC-3'
SARS-CoV-2 nsp4 att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GAA AAT TGT TAA TAA TTG GTT GAA GCA G-3'
SARS-CoV-2 nsp4 att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTCT CMC TGC AAA ACA GCT GAG GTG ATA G-3'
SARS-CoV-2 nsp6 att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GAG TGC AGT GAA AAG AAC AAT CAA GG-3'
SARS-CoV-2 nsp6 att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM CTG TAC AGT GGC TAC TTT GAT AC-3'
SARS-CoV-2 orfN att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT GTC TGA TAA TGG ACC CC-3'
SARS-CoV-2 orfN att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM GGC CTG AGT TGA GTC AGC AC-3'
RIG-1 att for	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CTT CAT G AC CAC CGA GCA GCG ACG CAG CCT GCA AGC-3‘
RIG-1 att rev	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCM TTT GGA CAT TTC TGC TGG ATC AAA TGG TAT C-3'
MAVS att for	5´-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGC CAT G CC GTT TGC TGA AGA CAA GAC CTA TAA GTA TAT C-3
MAVS att rev	5´-TTG TAC CAC TTT GTA CAA GAA AGC TGG GTC TCM GTG CAG ACG CCG CCG GTA CAG CAC CAC CAG G-3

Table S2: Antibody List

target	Strain, company	dilution	source
anti-CypA	Ab3563, Abcam	1:500	rabbit
anti-CypB	PA1-027A, Invitrogen	1:800	rabbit
anti-SARS1/2 N	4H2-1-1, hybridoma supernatant, lab-made	1:10	mouse
anti-SARS-CoV-2 N (purified)	21H2-1-1, lab-made	1:200	rat
anti-SUD(Nsp3)	7g9-1-1, hybridoma supernatant, lab-made	1:5	mouse
anti-HCoV N (NL63/229E)	1H11, Ingenasa	1:400	mouse
anti-vincullin	V9264, sigma	1:1000	mouse
anti-beta-actin	A3854, sigma	1:100 000	HRP conjugated
anti-RFP	MA5-15257, Invitrogen	1:1000	mouse
anti-GFP	A6455, Invitrogen	1:1000	rabbit
anti-HA	Clone 3F10, Roche	1:1000	rat
anti-rabbit	P0217, DAKO	1:1000	
anti-mouse	A9917, sigma	1:10 000	
anti-rat	A9037, sigma	1:5000	
anti-MERS N	GTX134868, GeneTeX	1:1000	rabbit
anti-CD13(APN)	Ab108382, Abcam	1:1000	rabbit
Anti-OC43 N	MAB9012, Merckmillipore	1:1000	mouse
Anti-dsRNA J2	J2, SCICONS	1:1000	mouse

Anti-rabbit FITC	F0382, sigma	1:1000	
Anti-mouse Alexa555	A21424, Invitrogen	1:500	
Anti-rat Alexa647	A48265, Invitrogen	1:500	

Table S3 the similarity of the protein sequences of Nsp3 and N in coronaviruses

Strains	Nsp3	Nucleocapsid
SARS-CoV-2	100%	100%
SARS-CoV	75.96%	89.74%
MERS-CoV	20.51%	38.74%
HCoV-OC43	19.33%	26.25%
HCoV-229E	14.20%	15.94%
HCoV-NL63	12.99%	22.28%

Table legend:

The protein sequences of the indicated coronaviruses were downloaded from NCBI database. The similarity of these protein sequences was provided by ClustalW after calculating the percentage identity shared by those sequences compared to SARS-CoV-2.

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12. Curriculum vitae

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