

Identification of Transmembrane and Extracellular Host Proteases that Promote Human CoV Entry and Syncytium Formation

Rory Mulloy

This thesis is submitted to the Faculty of Medicine in partial fulfillment of the requirements for the degree of Master of Science with specialization in Microbiology & Immunology

Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine, University of Ottawa

© Rory Mulloy, Ottawa, Canada, 2021

Abstract

Coronaviruses (CoVs) comprise a family of enveloped viruses that cause respiratory disease in humans, including CoV disease 2019 (COVID-19), caused by severe-acute respiratory syndrome CoV-2 (SARS-CoV-2). For CoV infection to occur, the CoV spike (S) protein must mediate fusion between the viral and host membranes. This entry process can also be repurposed during infection to promote cell-to-cell fusion, further contributing to viral spread. To trigger fusion, S must bind its cognate receptor and be cleaved by host proteases. Identifying cellular proteases capable of triggering CoV fusion is critical to understand CoV entry, tropism, and cell-cell spread, however the range of proteases capable of promoting CoV fusion has not been fully explored. Here, using fusion and entry assays, I provide evidence implicating matrix metalloproteinase-9 (MMP-9) as a fusion trigger for SARS-CoV-2 and HCoV-229E. Additionally, I show MMP-9 expression is upregulated during CoV infection, highlighting its potential relevance as a CoV triggering factor.

Acknowledgements

Firstly, I would like to thank the members of my amazing lab(s). In Ottawa, I want to thank Alex, Kathy, and Gen, who spent the early months of the pandemic in the lab with me and were my only form of social contact. I want to formally apologize to the three of you for my taste in music; I am amazed you tolerated it for so long. Corina and Shirley, thank you for providing leadership and sharing your grad school survival guides on ‘what-to-do’ and ‘what-not-to-do’. Yuxia and David, thank you for the support and cheerful spirits. And of course, Marceline, thank you for giving me the opportunity join this global initiative to better characterize this novel virus during a pandemic. Truly (and hopefully), a once in a lifetime experience. Thank you for trusting me to work on this project and supporting the collaboration with the Corcoran lab and my move to the University of Calgary.

In Calgary, I want to thank the whole Corcoran lab, but in particular Mariel and Carolyn for making me feel welcome, mostly through excessive sarcasm and incredibly nerdy trivia. You both are clearly geeks and somehow have convinced me to follow in your footsteps. And to Jenn, thank you for taking me into your lab when I was an inexperienced undergrad at Dalhousie and then taking me back into your lab as a visiting MSc student in Calgary. Clearly, I have fooled you twice.

I would also like to acknowledge caffeine. All my successes were because of you, and all my failures were due to a lack of you.

Lastly, I want to thank Lane and Jon and my family for always picking up the phone and chatting with me while I was simultaneously doing experiments. Thanks for reminding to not take it all too seriously.

Table of Contents

<i>List of abbreviations</i>	<i>VI</i>
<i>List of figures</i>	<i>VIII</i>
<i>List of tables</i>	<i>IX</i>
CHAPTER 1 - INTRODUCTION	1
1.1 Coronaviruses	1
1.1.1 The Coronaviridae family	2
1.1.2 Endemic human coronaviruses.....	2
1.1.3 Epidemic human coronaviruses	3
1.1.4 Coronavirus virion structure and genome organization	5
1.1.5 Tissue tropism	8
1.1.6 CoV infectious cycle	9
1.1.6.2 CoV translation, transcription, and genome replication	14
1.1.6.3 CoV assembly & egress.....	17
1.1.6.4 CoV syncytiogenesis	18
1.2 Membrane fusion	19
1.2.1 Cellular membrane fusogens	20
1.2.2 Viral membrane fusogens.....	25
1.2.2.1 Fusogen priming.....	25
1.2.2.2 Receptor binding	29
1.2.2.3 Fusogen triggering.....	30
1.3 Matrix metalloproteinases	33
1.3.1 MMP family and structure	33
1.3.2 MMP regulation	34
1.3.2.1 MMP post-transcriptional regulation in processing bodies.....	36
1.3.2.2 MMP pro-enzyme activation and biological inhibition.....	37
1.3.3 MMPs and CoVs	37
1.4 Rationale and objectives	38
CHAPTER 2 - MATERIALS & METHODS	40
2.1 Cell culture	40
2.2 Solutions and reagents	41
2.3 Protease prediction sites using PROSPER	41
2.4 Plasmids	42
2.5 Transfections	42
2.6 Immunoblots	43
2.7 Inhibitor treatment	44
2.8 Cell-cell fusion assays	44
2.8.1 Two-population cell-cell fusion assay.....	44
2.8.2 One-population cell-cell fusion assay	45
2.9 Recombinant lenti pseudoviruses	45
2.9.1 Lenti-pseudovirus production	45
2.9.2 Pseudovirus concentration.....	45
2.9.3 LacZ pseudovirus titration	46

2.10 Replication-competent HCoV-229E.....	47
2.10.1 HCoV-229E propagation.....	47
2.10.2 Tissue-culture infectious dose 50% (TCID ₅₀) titration	47
2.10.3 HCoV-229E infection	49
2.10.4 Recombinant soluble MMP-9 treatment	49
2.11 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)	49
2.12 Fluorescence and immunofluorescence microscopy.....	51
2.12.1 Live-cell fluorescence microscopy.....	51
2.12.2 Immunofluorescence staining	51
2.12.3 Immunofluorescence microscopy	52
2.12.4 229E entry quantification using CellProfiler	52
2.12.5 Syncytial nuclei indexing	53
2.12.6 Processing body quantification	53
2.13 Statistical analysis	53
CHAPTER 3 - RESULTS.....	54
3.1 SARS-CoV-2 S mediates TMPRSS2 independent cell-cell fusion that is abrogated by MMP inhibition.....	54
3.2 TMPRSS2 and TMPRSS13 rescue Tapi-2 inhibition of SARS-CoV-2 syncytia formation	57
3.3 SARS-CoV-2 S TMPRSS2-independent fusion is abrogated by chemical inhibition of MMP-9	57
3.4 SARS-CoV-2 pseudovirus entry pathway is cell-type dependent and is facilitated by promiscuous protease usage.....	60
3.5 229E syncytia formation, but not entry is abrogated by Tapi-2 and GI-X in HUVECs.....	65
3.6 229E infection of HUVECs elicits processing body disassembly with and increased MMP-9 gene expression.....	69
3.7 Soluble MMP-9 facilitates cathepsin-independent 229E infection.....	69
CHAPTER 4 - DISCUSSION	74
4.1 Overview	74
4.2 Identification of MMP-9 and TMPRSS13 as activators of SARS-CoV-2 spike-mediated cell-cell fusion	75
4.3 CoV pseudovirus entry is cell-type specific and is sensitive to various protease inhibitions, including MMP-9 inhibition	81
4.4 Chemical inhibition of MMP-9 in HUVECs does not impact 229E viral entry but abrogates virus-mediated syncytium formation	84
4.4 Concluding remarks	92
References	94
Contribution of collaborators	108
Curriculum Vitae	109

List of abbreviations

ACE2: Angiotensin converting enzyme 2
APN1: Aminopeptidase N1
ARE: Adenylate/uridylate-rich elements
CCoV: Canine coronavirus
CCR5: C-C chemokine receptor type 5
CD4: Cluster differentiation 4
CDC: Center for disease control and prevention
CoV: Coronavirus
COVID-19: Coronavirus disease of 2019
CXCR4: C-X-C chemokine receptor type 4
DC-SIGN: Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
DMEM: Dulbecco's Modified Essential Medium
DMSO: Dimethyl sulfoxide
DMV: Double membrane vesicle
dsRNA: Double-stranded RNA
E: Envelope protein (coronavirus)
EboV: Ebolavirus
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
EGM-2: Endothelial cell growth medium
EMEM: Eagle's Minimal Essential Medium
ER: Endoplasmic reticulum
ERGIC: Endoplasmic reticulum-Golgi intermediate complex
FBS: Fetal bovine serum
FDA: Food and drug administration
FFU: Focus forming units
FIPV: Feline infectious peritonitis virus
FP: Fusion peptide
GFP: Green fluorescent protein
GP: Glycoprotein (ebolavirus)
GP41: Glycoprotein (retrovirus)
GP120: Glycoprotein (retrovirus)
HA: Hemagglutinin (influenza A virus)
HCQ: Hydroxychloroquine
HE: Hemagglutinin esterase (coronavirus)
HEK293t: Human embryonic kidney 293 cells large T antigen
HeV: Hendravirus
HIV: Human immunodeficiency virus
Hpi: Hours post-infection
HR1: Heptad repeat 1
HR2: Heptad repeat 2
HUVEC: Human umbilical vein endothelial cell
IAV: Influenza A virus
IBV: Infectious bronchitis virus

IL: Interleukin
 L-SIGN: Lymph node-specific intracellular adhesion molecule-3-grabbing non-integrin
 M: Membrane protein (coronavirus)
 MAPK: Mitogen-activated protein kinase
 MERS: Middle East respiratory syndrome
 MERS-CoV: Middle East respiratory syndrome
 MeV: Measles virus
 MHV: Mouse hepatitis virus
 MMP: Matrix metalloproteinase
 MOI: Multiplicity of infection
 M7G: methyl-7 guanosine cap
 N: Nucleocapsid protein (coronavirus)
 NSP: Non-structural protein
 ORF: Open reading frame
 PAMP: Pathogen-associated molecular pattern
 PB: Processing body/P-body
 PBS: Phosphate buffered serum
 PRR: Pattern recognition receptor
 PSQ: Penicillin-streptomycin glutamine
 PTM: Post-translational modification
 TGF- β : Tumor-growth factor-beta
 TIMP: Tissue inhibitor of metalloproteases
 TLR: Toll-like receptor
 TM: Transmembrane domain
 TMPRSS: Type 2 transmembrane serine protease
 TNF α : Tumor-necrosis alpha
 TRS: Transcription regulation sequence
 t-SNARE: Target-soluble N-ethylmaleimide-sensitive factor attachment protein receptor
 RBD: Receptor binding domain
 RBP: RNA-binding protein
 RNA: ribonucleic acid
 RNP: Ribonucleoprotein
 S: spike protein (coronavirus)
 SARS: Server-acute respiratory syndrome
 SARS-CoV-1: Severe-acute respiratory syndrome coronavirus-1
 SARS-CoV-2: Severe-acute respiratory syndrome coronavirus-2
 sgRNA: Subgenomic RNA
 STAT-3: Signal transducer and activator of transcription 3
 ssRNA: Single-stranded RNA
 UTR: Untranslated region
 VEGF: Vascular endothelial growth factor
 VLP: Virus-like particles
 VSV: Vesicular stomatitis virus
 v-SNARE: Vesicle-soluble N-ethylmaleimide-sensitive factor attachment protein receptor
 Zn²⁺: Zinc

List of figures

Introduction

Figure 1.1 Human coronavirus cladogram and virion and genome structure.....	6
Figure 1.2 Schematic representation of coronavirus infectious cycle.	10
Figure 1.3 Coronavirus transcription, subgenomic RNA synthesis, and genome replication.	16
Figure 1.4 Membrane fusion upon fusion with cellular and viral fusogens according to the hemifusion stalk model.	23
Figure 1.5 Schematic representation class I viral membrane fusion according to the hemifusion stock model.	26
Figure 1.6 Schematic depiction of MMP-9 regulatory mechanisms.	35

Materials & Methods

Figure 2.1 Tissue-culture infectious dose 50% (TCID ₅₀) of HCoV-229E in MRC5 cells.	48
---	----

Results

Figure 3.1 SARS-CoV-2 S mediates TMPRSS2-independent cell-cell fusion.	56
Figure 3.2 TMPRSS2 and TMPRSS13 can overcome Tapi-2-mediated syncytia inhibition to promote SARS-CoV-2 S cell-cell fusion.	58
Figure 3.3 Single population cell-cell fusion assay recapitulates SARS-CoV-2 TMPRSS2-independent fusion, which can be abrogated by MMP-9 inhibitor GI-X.	61
Figure 3.4 SARS-CoV-2 pseudovirus entry is cell-type dependent and can be blocked by Tapi-2 and GI-X. Cells including.....	64
Figure 3.5 229E infection of HUVECs is diminished by E64d and unchanged by Camostat, Tapi-2, and GI-X treatment.	67
Figure 3.6 229E syncytium formation is abrogated by Tapi-2 and GI-X treatment.	68
Figure 3.7 229E infection of HUVECs reduces PB numbers which correlates with an increase of MMP-9 mRNA abundance.	70
Figure 3.8 Soluble MMP-9 rescues E64d-mediated 229E entry inhibition in HUVECs.	72

Discussion

Figure 4.1 Differential furin and MMP-9 cooperativity likely impacts S fusogenic activity.....	80
Figure 4.2 Proposed model for 229E infection and syncytium formation in HUVECs.	88
Figure 4.3 MMP-9 proteolysis could promote or prevent CoV viral fusion competency depending on timing of proteolysis.	91

List of tables

Table 2.1 Source of plasmids used	42
Table 2.2 Source and final concentration ranges of protease inhibitors used.....	44
Table 2.3 qPCR primer sequences	50
Table 2.4 Primary and secondary antibodies used for immunofluorescence.....	52

CHAPTER 1 - INTRODUCTION

1.1 Coronaviruses

Coronaviruses (CoVs) comprise a large family of enveloped RNA viruses that are infamous for having a diverse host-range and a remarkable propensity for interspecies zoonosis, resulting in sporadic outbreaks in the human population. In the last two decades, there have been three such zoonotic events, with the emergence of severe-acute respiratory syndrome coronavirus-1 (SARS-CoV-1), Middle East respiratory coronavirus (MERS-CoV) and most recently with the emergence of severe-acute respiratory syndrome coronavirus-2 (SARS-CoV-2), leading to an unprecedented global health crisis (Woo et al., 2009; Zhou et al., 2020). Since the discovery of the first CoV, infectious bronchitis virus (IBV), isolated from chicken embryos in 1937 (Beaudette & Hudson, 1937), CoVs have been discovered in a diversity of animals, including humans. In addition to these three epidemic CoVs, four CoVs have been found to circulate seasonally in the human population, typically causing a mild respiratory disease (Hamre & Procknow, 1966; Tyrrell & Bynoe, 1966; Van Der Hoek et al., 2004; Woo et al., 2005). As an enveloped virus, to gain access to host machinery and replicate, CoVs must mediate membrane fusion between the viral and host lipid membrane. Membrane fusion is one of the first and most critical barriers that must be overcome for CoV infection to occur. The remarkable ability for CoVs to cross species barriers could, in part, be attributed to the plasticity of CoV fusion requirements during infection of a target cell. However, despite the pervasiveness and importance of CoVs as human and veterinary pathogens, the molecular mechanisms responsible for CoV fusion are incompletely understood.

1.1.1 The Coronaviridae family

All CoV members belong to the *Coronaviridae* family in the *Nidovirales* order (Fung & Liu, 2019; Snijder et al., 2013). Based on genomic similarity, the *Coronaviridae* family contains four genera: *alpha* (α), *beta* (β), *gamma* (γ), and *delta* (δ) CoVs (International Committee on Taxonomy of Viruses, 2012). Seven CoVs have been shown to infect and cause disease in humans: α -CoVs NL63 and 229E; and β -CoVs OC43, HKU1, SARS-CoV-1 SARS-CoV-2, and MERS-CoV (Figure 1.1A).

1.1.2 Endemic human coronaviruses

Of the CoVs known to infect humans, they can be categorized into endemic CoVs, which continuously circulate within the human population, and epidemic CoVs, which are a result of recent zoonosis from animal reservoirs. The endemic CoVs include 229E, NL63, OC43, and HKU1. These viruses seasonally circulate and are one of the major causative agents of the common cold (Corman et al., 2019; Dominguez et al., 2009; W. Zhou et al., 2013). Although, infection with these endemic CoVs typically causes a mild upper respiratory tract infection, children and immunocompromised patients can progress to more severe disease. In particular, there have been reports of OC43 and 229E infection triggering neurological pathologies, yet it is unclear if this is a result of viral neurotropism or a result of an immunopathogenic response to viral infection (Arbour et al., 2000; Burks et al., 1980; Stewart et al., 1992).

Phylogenetic analysis indicates that the predecessors of these endemic CoVs entered the human population via historic zoonotic events. 229E and NL63 are thought to have originated in bats whereas OC43 and HKU1 likely originated in rodents (Corman et al., 2018; Corman et al., 2015). OC43 is thought to have emerged around 1890, a time coinciding with worldwide reports

of outbreaks of a respiratory disease (Vijgen et al., 2005). This could suggest that initially these endemic CoVs triggered more severe disease, however decades of human transmission has led to more mild disease outcomes.

1.1.3 Epidemic human coronaviruses

In the last two decades, three novel CoVs have crossed into the human population causing severe respiratory diseases. In November 2002, SARS-CoV-1 (called SARS-CoV at the time) emerged from the Guangdong province in Southeast China (Drosten et al., 2003). SARS-CoV-1 is believed to have originated from bats and was introduced to the human population through various intermediated hosts, including palm civets and raccoon dogs (Drosten et al., 2003; Guan et al., 2003; Wendong Li et al., 2005). In humans, SARS-CoV-1 causes severe-acute respiratory syndrome (SARS), which typically includes flu-like symptoms followed by an atypical pneumonia, respiratory failure, and often death. There were over 8096 recorded SARS-CoV-1 infections between 2002 and 2004, resulting in approximately 774 deaths, with a case fatality of 9.6% (Center for disease control and prevention [CDC], 2017). However, implementation of targeted health measure prevented SARS-CoV-1 human-to-human spread, ending the SARS-CoV-1 epidemic (Petersen et al., 2020). Many of the suspected intermediate hosts were culled to prevent re-emergence; there have been no documented cases of SARS-CoV-1 infection since 2004.

In 2012, another novel CoV, MERS-CoV, emerged in Jeddah, Saudi Arabia (Zaki et al., 2012). MERS-CoV is also thought to have arisen in bats, however it was introduced to the human population through dromedary camels (De Wit et al., 2016; Reusken et al., 2013). Infection with MERS-CoV causes Middle East respiratory syndrome (MERS), which has a

similar presentation to SARS, but a higher case-fatality rate of 35% (CDC, 2019). MERS-CoV transmits poorly between humans, however it continues to re-emerge into the human population from camels causing localized outbreaks (Corman et al., 2015; Cui et al., 2019).

In December 2019 there were clusters of an acute respiratory illness in the Hubei province in central China. The infectious cause of this disease was rapidly identified as a novel β -CoV with 79% genomic identity to SARS-CoV-1, thus the virus was named SARS-CoV-2 (Lu et al., 2020). SARS-CoV-2 is also thought to have emerged from bats, yet currently it is unclear how it entered the human population. The disease manifestations of SARS-CoV-2, called coronavirus disease of 2019 (COVID-19), can result in severe respiratory disease similar to other epidemic CoVs, however these severe manifestations seem to occur much less frequently upon infection with SARS-CoV-2 (Petersen et al., 2020). Instead, a large proportion of SARS-CoV-2 infections result in mild disease or asymptomatic infections, lowering the case-fatality rate of SARS-CoV-2 to about 2% (Johns Hopkins CoV resource centre, accessed June 6, 2021). Although these features contribute to a less virulent virus, they also support a much more transmissible one. For example, because SARS-CoV-1 caused severe disease in humans and could only be transmitted after the onset of symptoms, most of the transmission occurred within hospitals, allowing targeted public health measures to be extremely effective (Petersen et al., 2020; Wu et al., 2020). Asymptomatic infections and mild disease outcomes shifted the spread of SARS-CoV-2 from hospitals to community-associated spread, making containment extremely challenging (Petersen et al., 2020).

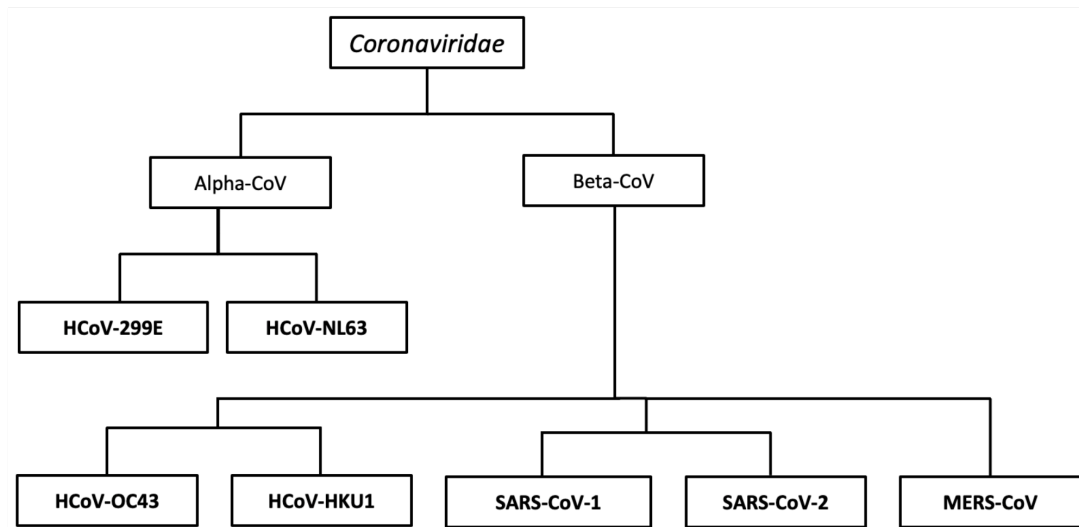
In addition to these epidemiological factors, there are also molecular mechanisms that contribute to the enhanced transmission of SARS-CoV-2 compared to SARS-CoV-1. Notably, SARS-CoV-2 has acquired traits that allows the virus to more easily enter host cells, increasing

the likelihood of transmission (Johnson et al., 2021; Petersen et al., 2020; Wölfel et al., 2020). Ultimately, epidemiological and virological factors have resulted in the sustained global spread of SARS-CoV-2 resulting in over 3.7 million deaths as of June 2021 (Johns Hopkins CoV resource centre, accessed June 6, 2021).

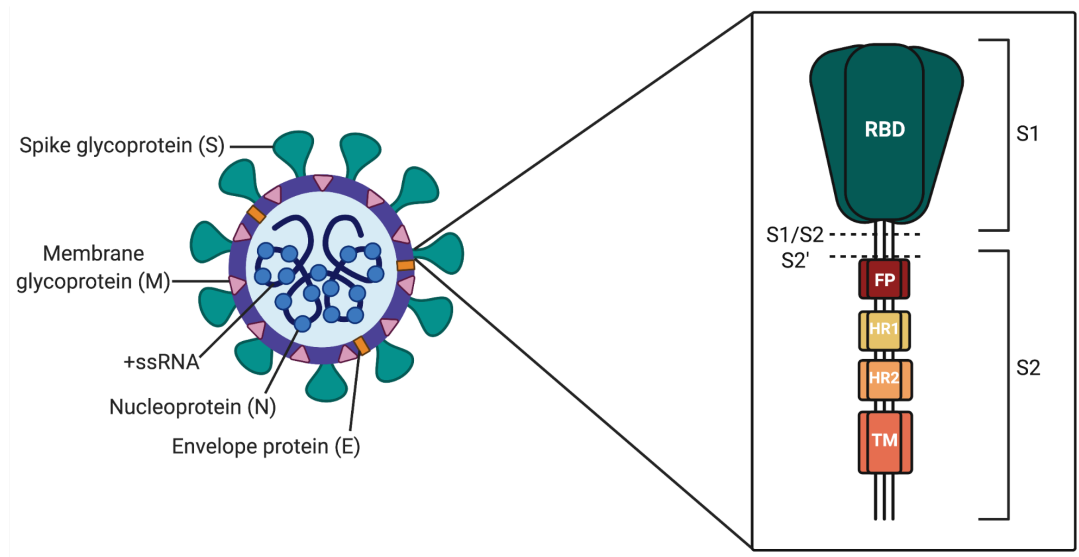
1.1.4 Coronavirus virion structure and genome organization

CoVs are pleomorphic enveloped viruses approximately 80-120 nm in diameter (Belouzard et al., 2012). All CoVs are studded with large trimeric spike (S) glycoproteins giving them a crown-like appearance or a *corona* in Latin (Figure 1.1B). For all CoVs, S is solely responsible for receptor binding and mediating membrane fusion. In addition to S, β -CoVs OC43 and HKU1 contain membrane-bound hemagglutinin-esterase (HE), which is believed to be important for viral egress (Bakkers et al., 2017). Other structural proteins in the virion include the membrane (M) protein and envelope (E) protein, both of which are also transmembrane glycoproteins. M is the most abundantly expressed protein in the viral particle and is critical for viral envelope formation and integrity (Siu et al., 2008). E is a minor constituent within the viral particle and is thought to facilitate viral budding during egress (Ruch & Machamer, 2012). Lastly, the nucleocapsid protein (N) is a phosphoprotein that binds the viral RNA genome forming a helical nucleocapsid, thereby physically linking the viral genome to the envelope (Li, 2016). CoVs have the largest genomes amongst RNA viruses, ranging from 26-35 kilobases of non-segmented single stranded RNA, containing a 5' m⁷G cap and a 3' poly(A) tail (Enjuanes et al., 2006; Kim et al., 2020).

A)



B)



C)

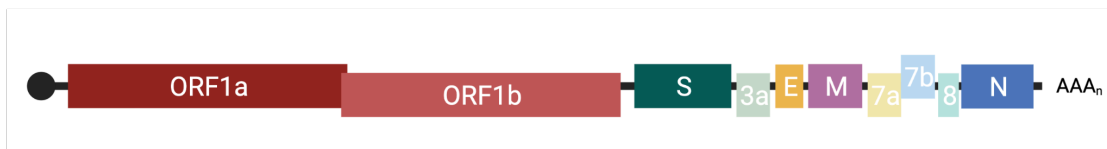


Figure 1.1 Human coronavirus cladogram and virion and genome structure. Figure caption on following page.

Figure 1.1 Human coronavirus cladogram and virion and genome structure. A) A simplified cladogram of all CoVs known to infect and transmit in humans. **B)** Schematic representation of CoV particle. CoVs have a host-derived lipid envelope embedded with at least three structural proteins: envelope protein (E), membrane glycoprotein (M), the spike glycoprotein (S); HKU1 and OC43 have an additional viral transmembrane protein, hemagglutinin esterase (HE). Within the viral particle, CoVs contain a positive-sense single-stranded RNA genome bound by nucleoprotein (N). The zoom panel depicts a diagram of a CoV S trimer. S contains two major subunits, S1 and S2. S1 is N-terminal and is most distal to the viral envelope. S1 contains the receptor-binding domain (RBD; teal). S2 makes up the C-terminus of S and is membrane-spanning. S2 contains the fusion machinery, including the fusion peptide (FP; red), located at the N-terminal portion of S2, two heptad repeat regions (HR1; yellow and HR2; light orange), and a transmembrane domain (TM; orange). S has two cleavage sites between the S1 and S2 subunits; the S1/S2 cleavage site, and the S2' cleavage site, located immediately upstream of the FP. **C)** Simplified schematic of the SARS-CoV-2 genome including two large open-reading frames (ORF1a and ORF1b) followed by structural and accessory proteins, spike (S), ORF3a, envelope (E), membrane (M), ORF7a, ORF7b, ORF8, and nucleoprotein (N). Figure was made using biorender.com

1.1.5 Tissue tropism

The tropism of a virus refers to the ability of a virus to successfully infect a given cell type (cellular tropism), tissue type (tissue tropism), or an organism (host tropism/range). In addition to having a broad host tropism, CoVs can also infect various tissues and cell types. In their hosts, CoVs primarily infect the respiratory and gastrointestinal tracts, however in humans, CoV infection in the upper and lower respiratory tract is most common (Laporte et al., 2021; Wölfel et al., 2020). SARS-CoV-1 and MERS-CoV have a high tropism for the lower respiratory tract, which is thought to contribute to the progression to pneumonia and disease severity (Laporte et al., 2021; Raj et al., 2013). Differently, the endemic CoVs have a high tropism for the upper respiratory tract, which has been suspected to promote more efficient transmissibility (Wölfel et al., 2020; Zou et al., 2020; Zumla et al., 2016). SARS-CoV-2 appears to readily infect both the upper and lower respiratory tracts, which is thought to contribute to its highly transmissible and pathological nature (Wölfel et al., 2020; Zou et al., 2020).

Animal studies have shown that various cell-types in the respiratory tract can be infected during SARS-CoV-1 and SARS-CoV-2 infection, but ciliated respiratory epithelial cells and type II pneumocytes appear to be the primary targets (Rockx et al., 2020; To et al., 2004; Zhu et al., 2020). In addition to the respiratory tract, SARS-CoV-2 antigens and RNA have been detected in the intestine, liver, and kidneys of patients with severe COVID-19. In these tissues, viral antigens can be found in both epithelial and vascular endothelial cells (Gupta et al., 2020; Liu et al., 2021; Puelles et al., 2020; Rosen et al., 2021). These findings suggest that SARS-CoV-2 possess extrapulmonary tissue tropism.

1.1.6 CoV infectious cycle

1.1.6.1 Viral attachment, penetration, and uncoating

As obligate intracellular parasites, viruses require host cellular machinery to facilitate all stages of their infectious cycle. The first step of a viral infectious cycle is viral entry, which can be divided into three phases: viral attachment, penetration, and uncoating. To mediate entry into a host cell, all enveloped viruses, including CoVs, face the challenge of getting a hydrophilic virus particle across a hydrophobic membrane. To overcome this obstacle, enveloped viruses mediate attachment to the target cell, followed by fusion between the viral envelope and cellular membrane, resulting in the delivery of the viral genome to the target cell (Figure 1.2). Here, I define viral entry as all events occurring between viral release from a producer cell to membrane fusion with a target cell. For CoVs, this entry process is facilitated by the S protein.

CoV entry begins with non-specific electrostatic interactions between the viral glycoprotein, S, and the surface of the target cell. These non-specific interactions increase the likelihood of receptor binding with S and promote efficient docking (Thépaut et al., 2020). CoVs are able to exploit various cell surface molecules to facilitate attachment. Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and lymph node-SIGN (L-SIGN) have been shown to enhance entry for various CoVs (Jeffers et al., 2004; Marzi et al., 2004; Regan & Whittaker, 2008; Thépaut et al., 2020). Although attachment factors increase the likelihood of viral entry, they are not sufficient for completing entry.

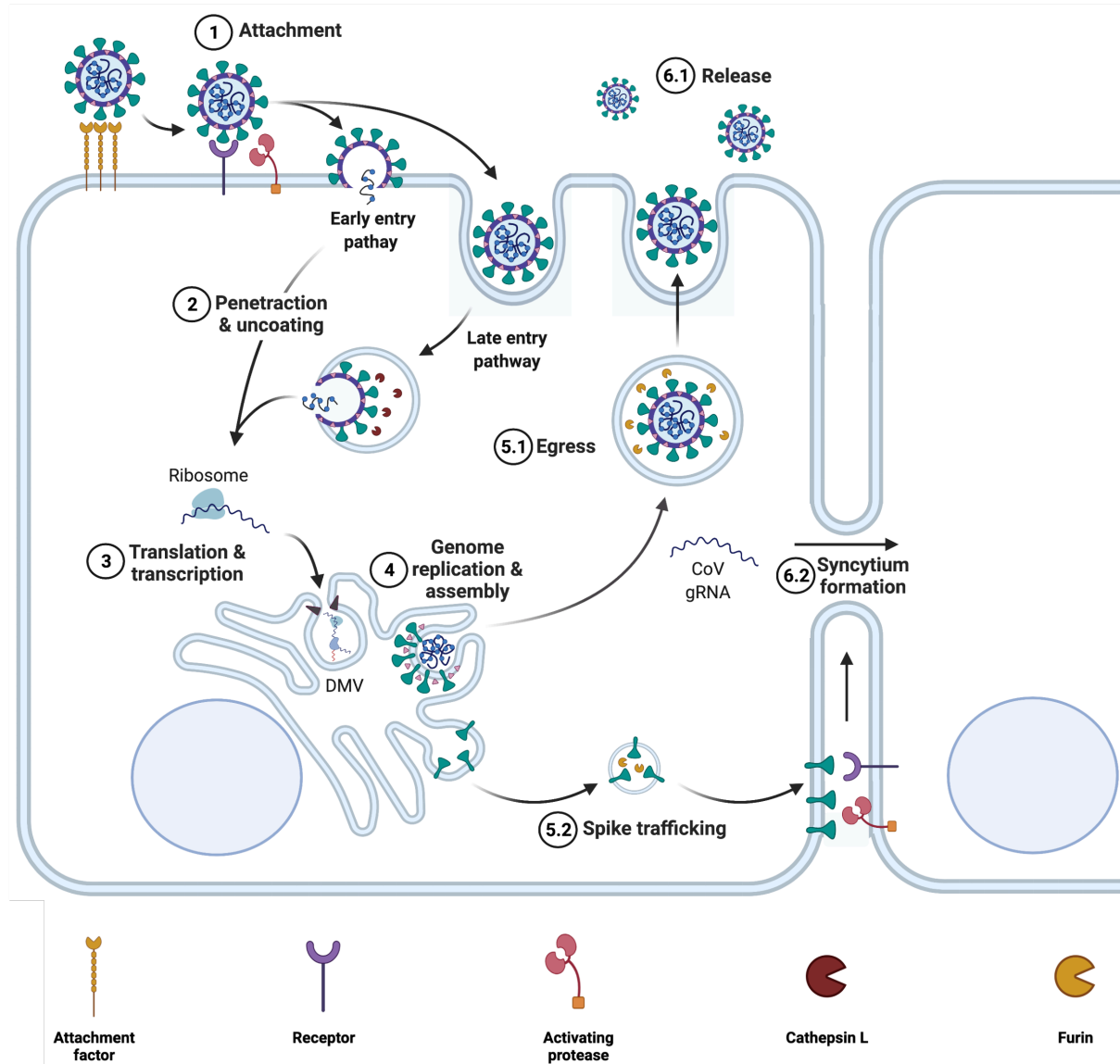


Figure 1.2 Schematic representation of coronavirus infectious cycle. Figure caption on following page.

Figure 1.2 Schematic representation of the coronavirus infectious cycle. (1) CoV particles can mediate non-specific attachment to target cells by interacting with surface-associated attachment factors. Following attachment, CoV S mediates interaction with cognate cellular receptor on the plasma membrane. (2) If there is access to a cellular protease that can sufficiently process the CoV S (cleavage at the S2' site or both the S1/S1 and the S2' sites), then the virus envelope will fuse with the plasma membrane, termed 'early entry pathway'. If S is not sufficiently proteolytically processed at the surface of a target cell, the CoV particle will undergo receptor-mediated endocytosis. Upon delivery to the acidic late endosome, S proteolysis can be facilitated by cathepsin L, triggering virus fusion with the endosomal membrane, termed 'late entry pathway'. Both entry pathways ultimately result in the delivery of the CoV genome to the cytoplasm. (3) CoV genomic RNA can be directly translated by host ribosomes. Viral gene products produce invaginations in the endoplasmic reticulum termed double membrane vesicles (DVMs), in which viral transcription and genome replication occur. (4) Following genomic replication, a viral genome is packaged into the assembling viral particle in the ERGIC. (5.1) During viral egress through the exocytic pathway, some CoV S proteins can be proteolytically processed at the S1/S2 junction by furin. (6.1) The virus is then trafficked to the cell surface by exocytosis and released into the extracellular space. (5.2) Alternatively, instead of assembling with other CoV structural proteins, a subset of S can be localized to the plasma membrane of the infected cell. (6.2) Provided that neighbouring cells express the viral receptor and necessary S proteolysis occurs, S can mediate fusion between the cellular membrane of the infected cell and the membrane of neighbouring cells, generating multinuclear syncytia. Upon syncytium formation, unpackaged CoV genomic RNA (gRNA) pass through the fusion pore and readily access host machinery in the neighbouring cell, thus contributing to viral propagation.

To complete entry, fusion between the viral and cellular membranes must occur. To initiate fusion, the viral fusion protein must undergo extensive conformational changes to assume a fusion-competent configuration (White et al., 2008). These conformational changes are, in part, elicited by receptor binding. In this way, S-receptor binding could be analogous to a lock and key, whereas attachment factors are more like a welcome mat. Many of the CoV receptors have been determined and interestingly, several distantly related CoVs share the same receptors. Feline infectious peritonitis CoV (FIPV), canine CoV (CCoV), and human CoV 229E all use aminopeptidase N (APN1) and SARS-CoV-1, SARS-CoV-2, and NL63 all use angiotensin-converting enzyme-2 (ACE2) (Hoffmann, Kleine-Weber, Schroeder, et al., 2020; Wendong Li et al., 2005); however, the significance of receptor conservation has yet to be determined.

Following receptor binding, the last requirement for fusion is a triggering signal. To continue with the lock and key analogy, if receptor binding is a key to a lock, the triggering signal is turning the key to open the door. Viruses employ various triggers to elicit fusion, including low pH, co-receptor binding, and in the case of CoVs, proteolytic processing of the S protein (Belouzard et al., 2009; White et al., 2008). The cellular location where S triggering occurs dictates where fusion occurs. Depending on protease availability and S protein specificity, S triggering can either occur at the cell surface or within the late endosome/lysosome of a susceptible cell (Belouzard et al., 2012; Huang et al., 2006).

S triggering at the cell surface will result in virus-cell fusion with the plasma membrane, directly facilitating viral uncoating. This entry strategy is termed ‘early entry’ (Figure 1.2). Many viruses, including human immunodeficiency virus (HIV), herpesviruses, measles virus (MeV) and CoVs can mediate virus-cell fusion at the plasma membrane (White et al., 2008). For CoVs, early entry is contingent on access to S-activating host proteases on the cell surface. One of the

best characterized plasma membrane proteases capable of activating CoVs is transmembrane serine protease 2 (TMPRSS2). TMPRSS2 has been shown to promote early entry for numerous CoVs, including all seven human CoV (Bertram et al., 2013; Gierer et al., 2013; Glowacka et al., 2011; Hoffmann, Kleine-Weber, Schroeder, et al., 2020; Phillips et al., 2017; Shirato et al., 2011, 2018). TMPRSS2 is highly expressed throughout the human respiratory tract and therefore TMPRSS2-mediated entry is thought to be the dominant entry strategy upon CoV infection (Shirato et al., 2018).

Without access to a triggering protease at the surface of a target cell, the surface-bound virion can be subjected to receptor-mediated endocytosis, where it relies on endosomal proteases to facilitate fusion triggering. CoVs have been found to use clathrin-dependent endocytosis, as well as micropinocytosis to enter target cells (Inoue et al., 2007; Millet & Whittaker, 2018; H. Wang et al., 2008). The virus will then transit through the endocytic pathway until endosomal acidification occurs, where the low pH-activated endosomal protease cathepsin L can cleave S to initiate virus-endosome fusion (Kawase et al., 2009; Millet & Whittaker, 2015; Shirato et al., 2011; Simmons et al., 2005; T. Tang et al., 2020). In this way, although CoV entry is not directly dependent on low pH, low pH is required for cathepsin-mediated entry. In addition to serving as a trigger for CoV fusion, cathepsin L has been shown to mediate fusion for various other viruses, such as ebolaviruses (EboVs) and hendravirus (HeV) (Chandran et al., 2005; Pager & Dutch, 2005). Although CoV early and the late entry pathways are reminiscent of other viral entry strategies, the ability to access both pathways highlights the plasticity of CoV entry.

Both entry pathways ultimately result in the delivery of the viral genome to a target cell, however each entry strategy has unique consequences on the viral infectious cycle. During the late entry pathway, the CoV particle is passively trafficked through the endocytic network until

endosomal maturation and acidification enables cathepsin L-mediated fusion. In this way, endosomal recycling prior to acidification will prevent successful viral entry (Vale-Costa & Amorim, 2016). Furthermore, during transit through the endocytic network, CoVs can encounter various endosomal-localized pattern recognition receptors (PRRs), such as toll-like receptors-3 and -7 (TLR3 and TLR7). These PRRs can recognize the stowaway virion and stimulate an antiviral immune response to restrict viral propagation (Lester & Li, 2014). During early entry, direct viral fusion with the plasma membrane circumvents both of these obstacles to efficiently complete viral entry. In fact, for SARS-CoV-1, early entry was found to be 100-1000 times more efficient than the late entry pathway (Matsuyama et al., 2005). Thus, the surface proteases that promote early entry have a significant impact on cellular susceptibility and tissue tropism.

1.1.6.2 CoV translation, transcription, and genome replication

CoV entry will result in the delivery of the viral genome into the host cytoplasm. The CoV genome consists of a single stranded positive sense RNA molecule about 30 kilobases in length. The first two-thirds of the CoV genome consists of two large open-reading frames (ORFs) ORF1a and ORF1b. The later third of the genome encodes the aforementioned structural proteins (S, E, M, N) interspersed with viral accessory proteins (Figure 1.1C). Because the CoV genome contains a m⁷G cap and a poly(A) tail, it is recognized by eukaryotic translation factors and can be directly translated by host ribosomes. In this way, following entry, ORF1ab is translated to produce one of two large polyproteins, the choice of which depends on utilization of a ribosomal frameshifting site. Polyprotein1a/1ab is subsequently autoproteolytically processed to produce 11-16 non-structural proteins (NSPs). These NSPs encode all the requisite function for viral genome copying and replication, one of which is the viral RNA-dependent RNA polymerase.

The production of viral structural proteins and various accessory proteins are stoichiometrically fine-tuned by the synthesis of sub-genomic (sgRNAs) (Sola et al., 2011). These genes are directly preceded by slippery transcription-regulating sequences (TRSs) with base-pairing affinity to a common leader TRS in the 5'UTR of the CoV genome (Figure 1.3). During viral negative strand synthesis, the viral polymerase will bind to the 3' UTR of the genomic RNA and begin copying the genome producing a complementary strand. Continuous negative strand synthesis will provide a template for positive genomic RNA synthesis, however CoVs can also undergo discontinuous negative strand synthesis. During negative strand replication, the polymerase can slip on a TRS and reattach at the leader TRS, generating a truncated or sgRNA. Polymerase slippage at various TRSs followed by joining to a common leader sequence creates a nested set of capped and polyadenylated sgRNAs (Sola et al., 2011). Furthermore, TRSs have defined slippage likelihoods, which is thought to enable fixed molar ratios of these viral proteins (Nedialkova et al., 2010).

As a consequence of sgRNA production, CoVs produce an abundance of double-stranded (ds)RNA intermediates. Mammalian cells recognize dsRNA as a pathogen-associated molecular pattern (PAMP) and have evolved mechanisms to sense dsRNA resulting in the activation of an antiviral response. One mechanism CoVs employ to limit detection by host sensors is by sequestering viral PAMPs within double membrane vesicles (DMVs) (Klein et al., 2020; Knoops et al., 2008) (Figure 1.2). CoV proteins nsp3, 4, and 6 form DMVs by altering the membrane curvature of the endoplasmic reticulum, forming invaginations where viral transcription and translation can occur without triggering cellular immune detection (V'kovski et al., 2021).

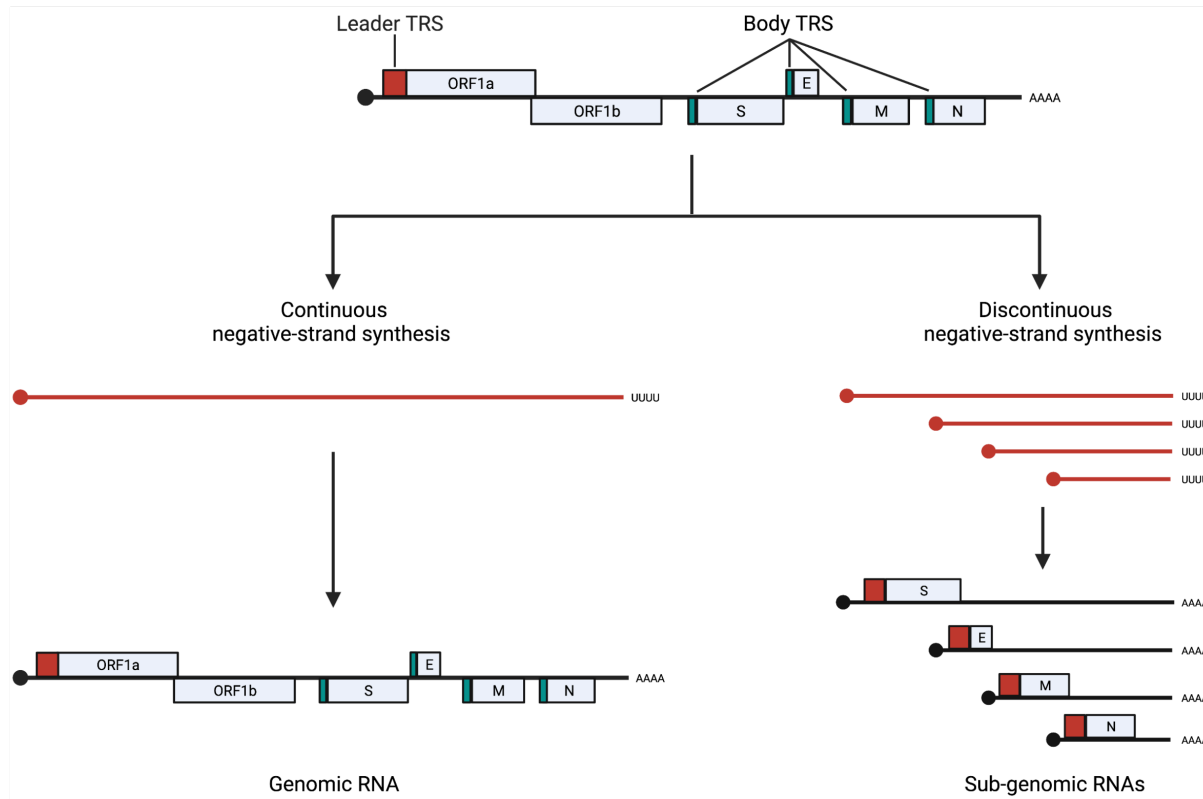


Figure 1.3 Coronavirus transcription, subgenomic RNA synthesis, and genome replication. CoVs contain a single-stranded positive sense RNA genome, which has a 5' m7G cap and 3' poly(A) tail. Viral genes encoding structural and accessory proteins are preceded by body transcription regulating sequences (TRS). The 5' untranslated region (UTR) of the viral genome contains a common leader TRS that has complementarity to the body TRS sequences. During infection, synthesis of the viral RNA-dependent-RNA polymerase enables viral transcription and genome replication. Genome replication requires the synthesis of a full-length complementary negative-sense copy the viral genome, which can subsequently be amplified to produce many positive-sense viral genomes that can incorporate into nascent virions. However, instead of continuous negative-strand synthesis, the viral polymerase can slip on the body TRS sequence, and reattach at the leader TRS, generating a 5' nested set of truncated negative-sense RNAs. The viral polymerase can then transcribe these truncated negative-sense RNAs into positive-sense sub-genomic RNAs that can be readily translated by host ribosomes to produce the CoV structural and accessory proteins. Figure was made using biorender.com

1.1.6.3 CoV assembly & egress

The assembly of nascent CoV virions occurs within the ER-Golgi intermediate complex (ERGIC) and requires the colocalization of structural key proteins and the N-coated viral genome. The membrane protein M is integral for CoV assembly and particle formation. M is targeted to the ERGIC, where it will associate with other viral structural proteins and the genomic packaging sequence (Hsieh et al., 2005). Although M-M and M-E interactions are sufficient to generate viral-like particles (VLPs), during infection M has been shown to interact with other structural proteins N and S (Corse & Machamer, 2003; Siu et al., 2008). Additionally, SARS-CoV-1 N is thought to contribute to viral envelope formation, as N co-expression with M and E significantly increases VLP production; however, the role of N during egress remains obscure (Siu et al., 2008).

E is a small integral membrane protein and is highly expressed within the infected cell despite being a minor constituent of the viral particle (Schoeman & Fielding, 2019; Siu et al., 2008). The precise role of E is still debated, however accumulation of SARS-CoV-1 E within the ERGIC is thought to destabilize cellular ionic gradients, alter membrane curvature, and perhaps trigger membrane scission during viral budding (Ruch & Machamer, 2012; Schoeman & Fielding, 2019). CoV budding occurs when the forming viral envelope pinches off from the Golgi membrane. The nascent virus will be enclosed in a cellular vesicle and transit to the plasma membrane to be released into the extracellular space.

During viral assembly, viral structural proteins will undergo extensive post-translational modifications (PTMs) including glycosylation and proteolysis. The N-terminus of the S protein is heavily glycosylated, which is thought to shield various immunogenic epitopes from immune detection (Grant et al., 2020). In addition to glycosylation, some CoV S proteins can be

proteolytically processed by furin and furin-like proteases (Millet & Whittaker, 2015). S proteolysis will be extensively reviewed in the following section, but it is believed that furin processing lowers the threshold for S fusogenic activation upon infection of neighbouring cells.

1.1.6.4 CoV syncytiogenesis

During viral assembly, structural proteins congregate to form the nascent virion. However, CoVs share a perplexing trait; instead of colocalizing with other structural proteins, a subset of S is localized to the plasma membrane and decorates the surface of an infected cell (Figure 1.2). Consequently, if neighbouring cells bear the cognate viral receptor and a protease capable of cleaving S, such as TMPRSS2, the plasma membrane of the infected cell can fuse with neighbouring cells, forming a multi-nucleated cell or syncytium (Bussani et al., 2020; Hoffmann et al., 2020; Li et al., 2003). CoVs are not the only viruses known to cause syncytia; many enveloped viruses, such as MeV and HIV trigger syncytium formation (Cathomen et al., 1998; Sodroski et al., 1986). However, unlike CoVs, these viruses assemble and bud from the plasma membrane, thus it could be argued that syncytium formation is an incidental biproduct of the viral egress process. CoV syncytium formation is peculiar because it requires plasma membrane localization of the viral fusogen, where it cannot become part of a forming viral particle. Given that this is a conserved feature amongst various CoVs, it is likely that CoV syncytium formation provides some functional importance to the viral infectious cycle. One hypothesis is that viral syncytium formation serves as an alternative strategy to mediate viral genome spread from cell to cell, whilst limiting extracellular immune detection. In addition to mediating viral dissemination, syncytium formation tends to be extremely cytotoxic, likely contributing to worsened tissue damage and disease outcomes (Higuchi et al., 2000; Le Boeuf et

al., 2017; Leroy et al., 2020). This is evident in cell culture, as expression of a syncytium-inducing viral protein will cause monolayer fusion followed by simultaneous cell death (Sodroski et al., 1986). The mechanism by which syncytia induce cytotoxicity is unknown, but many have speculated it could be attributed to various biological processes, such as cell stress due to unfavourable membrane curvature, ionic homeostatic imbalance due to fusion events, or programmed cell death triggered by polyploidy (Ciechonska et al., 2014; Perfettini et al., 2005; Zhivotovsky & Kroemer, 2004). As syncytia can be found in the lungs of COVID-19 patients, it is likely that these fusion events contribute to worsened disease outcomes (Bussani et al., 2020). From membrane fusion during viral entry, the formation of DMVs, budding, and syncytium formation, CoVs have evolved intricate mechanisms of manipulating host membranes to facilitate the viral life cycle.

1.2 Membrane fusion

Biological membranes separate the cell interior from the extracellular environment and enables intracellular compartmentalization. Membrane fusion is vital to various fundamental biological processes, such as organelle trafficking, fertilization, and tissue development (Jahn et al., 2003). Enveloped viruses also rely on membrane fusion to facilitate entry and syncytium formation.

Membrane fusion is the merger of two distinct lipid membranes into one continuous bilayer. For fusion to occur, the outer leaflets must be brought into close membrane apposition, which is strongly opposed by electrostatic repulsion of the negatively charged phospholipid headgroups. The energy required to overcome these barriers is facilitated by proteinaceous fusogens (Chernomordik & Kozlov, 2008; Jahn et al., 2003). Although the precise mechanism

for membrane fusion has not been resolved, the prevailing theory involves the formation of a transient hemi-fusion stalk (Chernomordik & Kozlov, 2008). This model proposes that upon close membrane apposition, the protein fusogen destabilizes the hydrophilic-hydrophobic boundary allowing for distortion of the lamellar arrangement of the outer leaflets.

Destabilization allows the outer leaflets of both membranes to fuse, while the inner leaflets remain distinct, forming a highly unstable hourglass structure called a hemi-fusion stalk (Chernomordik & Kozlov, 2008). As the hemi-fusion stalk widens, the inner leaflets fuse, forming a fusion pore (Figure 1.4A). At this stage, the two cells are connected by one continuous lipid membrane and can readily exchange luminal contents. To resolve unfavourable membrane curvature, the fusion pore expands, completing the fusion process.

1.2.1 Cellular membrane fusogens

Membrane fusion is a universal process that facilitates various programs in eukaryotic organisms but can be broadly characterized into extracellular and intracellular fusion.

Extracellular fusion, also known as cell-to-cell fusion or syncytiogenesis, is critical for the development of both basic and complex organisms (Jahn et al., 2003). Examples of syncytium formation include the formation of myotubes, helping synchronize skeletal muscle fibre contractions, the development of the syncytiotrophoblast, which forms the protective layer for the fetus in mammalian development, and fertilization during sexual reproduction (Jahn et al., 2003; Shi et al., 2009).

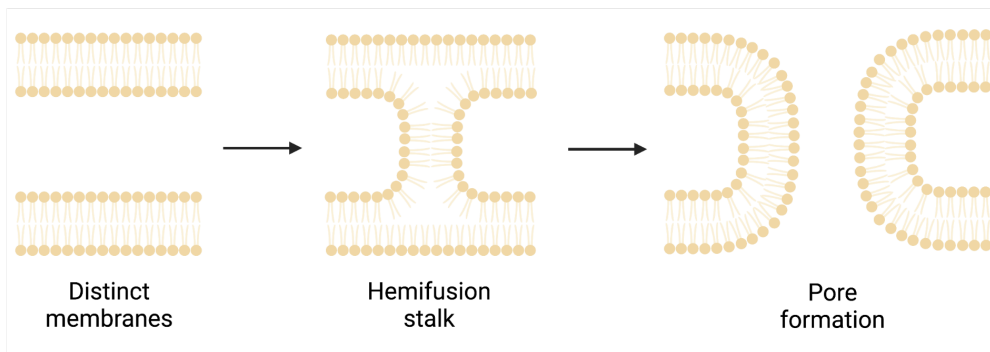
Myotube formation is the best characterized extracellular fusion model. Myotube formation is an essential step in mammalian muscle tissue development where multiple mononucleated myoblasts fuse to form elongated, multinucleated myofibers (Petrany & Millay,

2019). Myoblast fusion requires the expression of two surface proteins, the aptly named myomaker and myomerger (Figure 1.4B). Myomaker is a seven-pass membrane protein expressed on skeletal muscle cells. The ectopic expression of myomaker in fibroblasts is sufficient to enable fusion with a myotube but does not trigger fusion with other myomaker-expressing fibroblasts (Petrany & Millay, 2019). Only when it is co-expressed with myomerger can fibroblast homotypic fusion occur. Myomerger is a small single-pass membrane protein that has a long extracellular domain. Interestingly, myomaker and myomerger mediate different aspects of the fusion process, acting independently of one another. Myomaker drives hemi-fusion stalk formation, yet is unable to trigger pore formation, whereas myomerger cannot generate a hemi-fusion stalk but is sufficient for pore-formation. Although both proteins are capable of performing their functions in the absence of the other, in tandem, they achieve myoblast fusion to create a mature myotube.

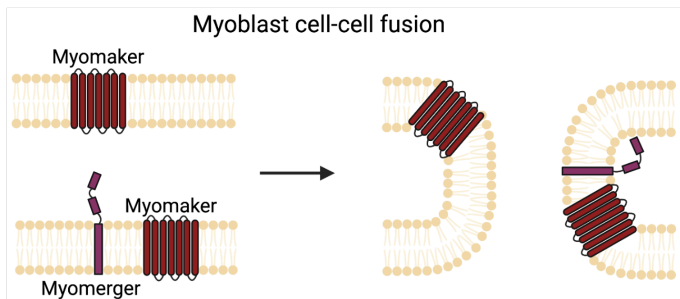
Unlike extracellular membrane fusion, which tends to be reserved to specific cell types, intracellular membrane fusion occurs in all eukaryotic cells. Vesicles are in a perpetual state of flux, merging and budding from intracellular membranes, forming an intricate network that supports all aspects of cellular homeostasis and development. The prototypical intracellular fusogens are the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family. SNAREs are localized on both the vesicle membrane (v-SNARE) and on the target membrane (t-SNARE) (Figure 1.4C) (Han et al., 2017; Yoon & Munson, 2018). SNAREs are transmembrane proteins that have a short luminal tail and a large cytoplasmic terminus, which contains conserved sequences called SNARE motifs. When in close proximity, the SNARE motifs between t- and v-SNARES bind and coil together, generating a four-helix bundle. Formation of this helical bundle brings the opposing membranes together, overcoming

the electrostatic repulsion, allowing membrane fusion to occur (McNew et al., 1999). Membrane fusion appears to require a conserved sequence of events, yet protein fusogens have evolved unique strategies to facilitate this process. Although extracellular and intracellular fusion occurs as a result of several multiprotein complexes, requiring fusion partners on both opposing membranes, they do so in remarkably different ways.

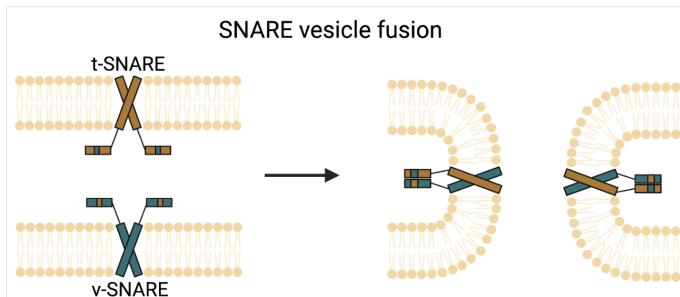
A)



B)



C)



D)

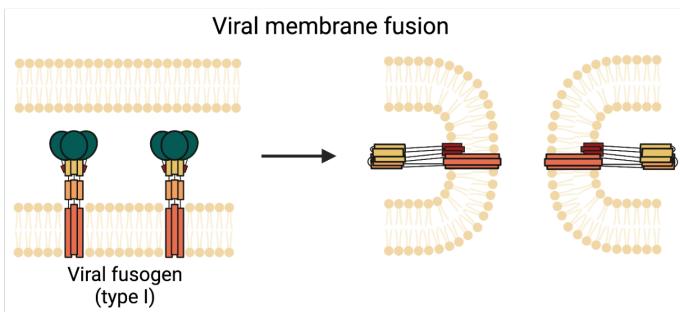


Figure 1.4 Membrane fusion upon fusion with cellular and viral fusogens according to the hemifusion stalk model. Figure caption on following page.

Figure 1.4 Membrane fusion upon fusion with cellular and viral fusogens according to the hemifusion stalk model. **A)** Two distinct membranes transition to a hemifusion stalk conformation where outer leaflets merge, but inner leaflets remain distinct. Hemifusion is resolved by merging of the inner leaflets, resulting in the formation of a fusion pore. This fusion pore formation process is facilitated via protein fusogens; examples are shown in **B-D**. **B)** Myomaker and myomerger function cooperatively to mediate myoblast cell-cell fusion to make myofibrils during muscle cell development. **C)** SNARE proteins are intracellular fusogens required for vesicle fusion. In this simplified schematic, a vesicle-localized SNARE protein (v-SNARE) and target membrane-localized SNARE (t-SNARE) interact via complementary domains forming a coil-coil structure that brings the opposing membranes into close apposition, helping to overcome the barrier of pore formation. **D)** Viral membrane fusions can also mediate pore formation. Shown is a prototypical class I viral fusogen, such as CoV S. Schematic adapted from (Petrany & Millay, 2019). Figure was made using biorender.com

1.2.2 Viral membrane fusogens

1.2.2.1 Fusogen priming

All enveloped viruses and some non-enveloped viruses encode membrane fusogens to mediate viral entry and/or syncytium formation (White et al., 2008). In contrast to cellular membrane fusogens, which require protein contributors on both membranes, viral fusogens mediate heterotypic fusion (Figure 1.4C). Heterotypic viral fusion is possible with the use fusion peptides (FPs). FPs are typically composed of 15-25 hydrophobic amino acids that will anchor into the host membrane (Epand, 2003). Viral fusion proteins can share very few structural characteristics, yet mechanistically, viral fusion typically follows a canonical sequence of events: a priming step to transition the viral fusogen to a metastable fusion-competent configuration, a triggering step to initiate the fusion process, formation of an extended intermediate enabling intercalation of the FP into the host membrane, and retraction of the extended intermediate bringing the membranes in close proximity overcoming the kinetic barrier to mediate membrane merger (White et al., 2008) (Figure 1.5). Based on their structural characteristics, viral fusogens can be grouped into three classes. Class I viral fusogens protrude from the viral particle and are predominantly made of alpha helices; these are some of the best studied and include human pathogens such as influenza A virus (IAV), HIV, EboV, and CoVs (Bosch et al., 2003; White et al., 2008). Class II fusogens consist largely of beta sheet structures and lay flat on the viral particle until initiation of the fusion process. Examples of class II fusion proteins include hepatitis C virus, dengue virus, and chikungunya virus (White et al., 2008). Class III viral fusogens are a hybrid of alpha helical and beta sheet structures and include rhabdoviruses, including vesicular stomatitis virus (VSV). Here, I will discuss class I fusion proteins, with an emphasis on CoV S.

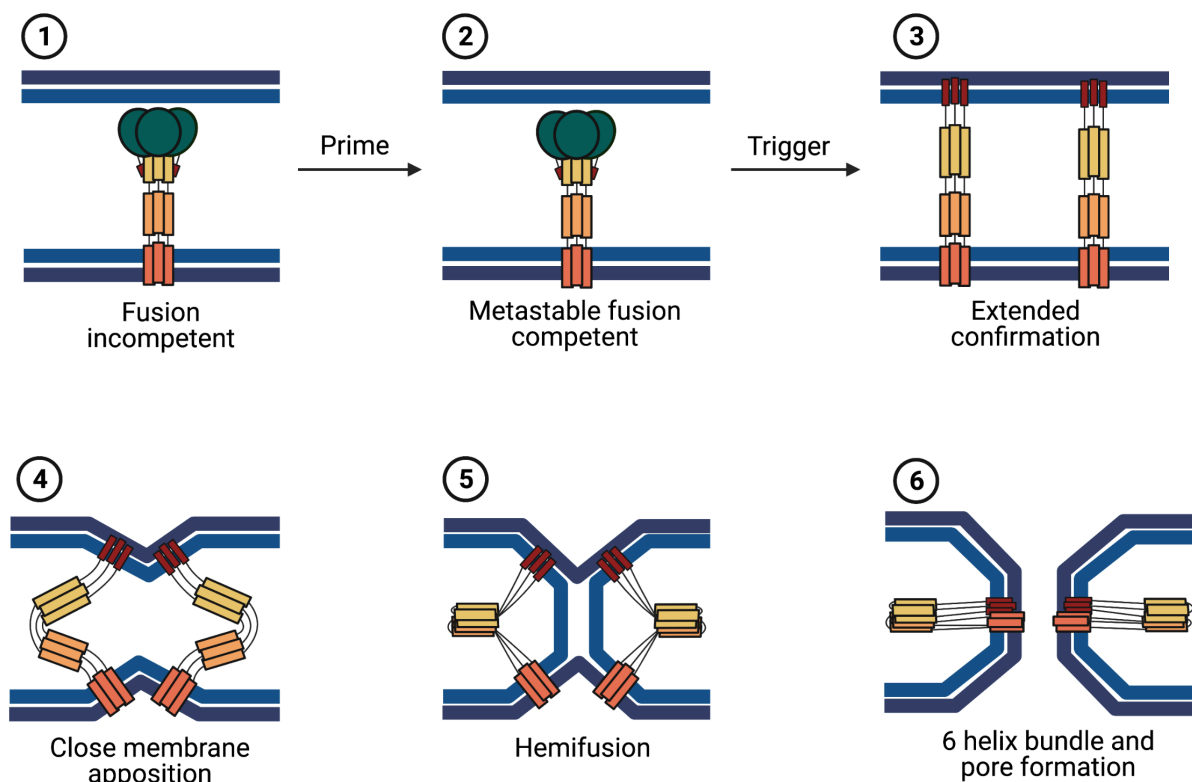


Figure 1.5 Schematic representation class I viral membrane fusion according to the hemifusion stock model. This schematic depicts a class I viral fusogen trimer that does not require other viral glycoproteins to facilitate fusion (eg. CoV S, IAV HA, HIV env). These viral fusogens contain the receptor binding subunit (teal) and the fusion machinery (fusion peptide; red, heptad repeat 1; yellow, heptad repeat 2; light orange, and a transmembrane domain; dark orange). Upon synthesis, viral fusogens are in a fusion-incompetent form (1) and undergo a priming step. For many class I viral fusogens, priming is facilitated by proteolytic processing, often separating the receptor-binding subunit and the fusogenic subunit (For CoVs priming is proteolytic cleavage at the S1/S2 interface). Priming shifts the fusogen into a fusion-competent metastable configuration (2). A triggering signal will liberate the fusion peptide and allow the viral fusogen to assume an extended intermediate conformation where the fusion peptide (red) can intercalate into the cellular membrane (3). The triggering signal is extremely variable between viral species; for CoVs it is a secondary proteolytic cleavage event proximal to the fusion peptide at a site called S2'. Receptor-binding (not shown) is thought to occur between steps 1-3. Following intercalation into the opposing membrane, the fusogen retracts, overcoming membrane electrostatic repulsion to achieve close membrane apposition (4). As the complementary heptad repeats interact allowing the two distal leaflets to coalesce forming an hour-glass shaped hemifusion stalk (5). The unfavourable hemifusion stalk structure is quickly resolved by merging the proximal leaflets, generating a fusion pore (6). At this point the viral fusogen has assumed a low-energy conformation. Schematic adapted from (White et al., 2008). Figure was made using biorender.com

Class I viral fusogens are formed as trimers of a single chain precursor protein. Many class I viral fusogens, such as IAV hemagglutinin (HA) and CoV S, contain a receptor binding subunit, located most distal to the viral envelope, and a fusogenic subunit, located proximal to the viral envelope (Figure 1.1B). The fusogenic subunits share many structural similarities: a transmembrane domain (TM), which anchors the fusogen to the viral envelope, a large extracellular portion with two heptad-repeat sequences (HR1 and HR2) separated by a flexible linker, and either an internal or external FP (Figure 1.1B – FP in red).

The priming step for these fusogens is mediated by a proteolytic cleavage event between the receptor binding unit and the fusogenic subunit. For many viral fusogens, including HA, this cleavage step occurs directly adjacent to the FP, such that the fusogenic domain has a N-terminal or external FP. For other viral fusogens, like CoV S, this proteolytic priming event does not occur adjacent to the FP, resulting in an internally situated FP (Belouzard et al., 2009) (Figure 1.1B – S1/S2 cleavage). In addition to exposing the FP, priming allows the fusogen to assume a fusion-competent state with a metastable conformation. Metastability refers to a protein that has not yet attained the minimum free-energy conformation (White et al., 2008). However, to achieve minimum free energy, the protein must undergo extensive irreversible changes. Metastability is fundamental for viral fusion, because the transition to a stable conformation provides a source of stored energy necessary to overcome the high costs of membrane fusion.

In the case of CoV S, this priming event is the cleavage of the precursor form of S, S₀, into S1 and S2 subunits by furin or trypsin-like proteases (Millet & Whittaker, 2015). Furin proteases are ubiquitously expressed serine proprotein convertases that reside in the ERGIC and trans Golgi network (Millet & Whittaker, 2015). Furin performs proteolytic modifications for a variety of cellular proteins at highly conserved polybasic amino acid sequences R-X-X-R, where

X is any amino acid. A diversity of viruses, including EboV glycoprotein (GP), HIV envelope glycoprotein gp120, and certain CoV S proteins also use furin proteolysis to mediate this fusogen priming step during viral egress (Millet & Whittaker, 2015). SARS-CoV-2, MERS-CoV, OC43, and HKU1 S proteins all contain a furin cleavage site at the S1/S2 junction, while SARS-CoV-1 S does not contain a furin cleavage site, despite sharing approximately 77% amino acid identity with SARS-CoV-2 S (Hoffmann, Kleine-Weber, Schroeder, et al., 2020; Lokman et al., 2020). The higher transmission of SARS-CoV-2 compared to SARS-CoV-1 has been attributed to the presence of this furin cleavage site (Johnson et al., 2021).

In addition to furin, most CoVs can also be primed by trypsin-like proteases, namely TMPRSS2 (Bertram et al., 2013; Laporte et al., 2021; Shirato et al., 2011; Shulla et al., 2011). TMPRSS2 is a membrane-bound serine protease, part of the TMPRSS super family (Szabo & Bugge, 2008). Unlike furin proteolysis, TMPRSS2 cleavage occurs on the cell surface of the target cell. In addition to TMPRSS2, other trypsin-like serine proteases have been shown to trigger CoV fusion, including thermolysin, elastase, and TMPRSS11d (Belouzard et al., 2010; Bertram et al., 2013; Matsuyama et al., 2005).

TMPRSS proteases are a diverse family of membrane-bound serine proteases. Many of the TMPRSS members have been identified exclusively due to homology to other TMPRSS proteases and for this reason are largely uncharacterized (Barré et al., 2014; Kyrieleis et al., 2007). These proteases can have multiple isoforms, adding an additional layer of complexity. However, TMPRSS proteases do share common structural features, such as a N-terminal cytoplasmic tail, a single transmembrane domain, and a C-terminal extracellular trypsin-like protease domain (Kyrieleis et al., 2007). All TMPRSS proteases are synthesized as pro-enzymes and are catalytically activated on the cell surface by other proteases, including other TMPRSS

proteases (Barré et al., 2014). TMPRSS proteases are thought to have diverse biological roles, as dysregulated expression can result in a variety of disorders, including cancer, skin defects, and anemia (Finberg et al., 2008; Madsen et al., 2014; Scott et al., 2001; Vaarala et al., 2001).

The RNA expression profile of TMPRSS members shows distinct and diverse tissue tropism, as RNA can be found in many tissues including the respiratory tract, kidneys, pancreas, spinal cord, ovaries, testes, and bladder. RNA from multiple different TMPRSS members was found throughout the respiratory tract. For example, TMPRSS7 and 11e are expressed in the salivary glands, TMPRSS2, 5, and 11a are expressed within the trachea, and TMPRSS3 and 13 are expressed in the lungs (Kido & Okumura, 2008; Scott et al., 2001; Szabo & Bugge, 2008; Vaarala et al., 2001). While the substrate specificity for TMPRSS proteases is incompletely understood, many TMPRSS proteases, including TMPRSS2 can cleave at mono- or poly- basic residues, with a preference for arginine (R) (Baron et al., 2013; Barré et al., 2014; Szabo & Bugge, 2008). Importantly, it is unclear which other TMPRSS proteases can act on CoV S to promote viral entry.

1.2.2.2 Receptor binding

CoVs exploit a wide variety of cellular receptors to mediate entry. Although for CoVs, receptor binding is not a classical fusion triggering factor, it is believed that receptor binding promotes triggering (Benton et al., 2020). Structural analysis for MERS-CoV S revealed that prior to receptor binding, the secondary cleavage site necessary for fusion triggering (S2' site) is buried, and thus is inefficiently accessed by host proteases (Pallesen et al., 2017). Only upon receptor binding could the S2' site be exposed to allow triggering to occur (Pallesen et al., 2017). Furthermore, Benton et al (2020) found that SARS-CoV-2 S binding to ACE2 promoted

dissociation of the S1 subunit, exposing the fusogenic S2 subunit for triggering and fusion to occur (Benton et al., 2020).

1.2.2.3 Fusogen triggering

Transition from the pre-fusion metastable arrangement to the post-fusion low-energy conformation is required for fusion to occur. Because this transition is typically an irreversible process, it must be tightly regulated. Attachment of the virus to the host cell through receptor binding is a requirement for infection, however receptor binding is often not sufficient to trigger fusion. Instead, the fusion-competent protein must be activated by an environmental stimulus or “trigger”. This triggering event facilitates a conformation change resulting in the extension of the fusion protein and exposure of the amphipathic FP to the target membrane (White et al., 2008) (Figure 1.5). Despite having a similar outcome, viruses employ a range of triggers to facilitate this conformational change. The most common fusion triggers include exposure to low pH, co-receptor binding, or a secondary proteolytic cleavage event.

Low pH is a common triggering requirement for a diversity of viruses, including orthomyxo-, rhabdo-, bunya-, alpha-, flavi-, and some retroviruses (Côté et al., 2009; White & Whittaker, 2016). These viruses use the low pH environment of endosomes to elicit conformational changes necessary for fusion, namely structural changes to promote the release and insertion of the FP into the target membrane. IAV HA is one of the best characterized low pH-dependent fusogens. Like CoVs, proteolytic priming separates the precursor HA₀ into the receptor binding (HA₁) and fusogenic (HA₂) subunits (White et al., 2008). The HA FP is externally situated, meaning it is located directly adjacent to the cleavage site on the N terminus of the HA₂ subunit. However, in the pre-fusion metastable structure, the FP is folded back into

the base of HA₂ and fixed in place due to a salt bridge (White et al., 2008). Upon HA₁ binding to the cellular receptor, sialic acid, the virion is endocytosed by the target cell, where proton pumps on the endosomal membrane trigger endosomal acidification. Protonation of HA₂ destabilizes the salt bridge, resulting in the extension of HA₂ and insertion of the FP into the endosomal membrane (White et al., 2008).

Another triggering factor viruses use to mediate fusion is co-receptor binding. The envelope protein (Env) of HIV is proteolytically processed into two subunits: the receptor binding gp120; and the fusogenic gp41. Like IAV HA₂, gp41 contains an N-terminal externally situated FP, which is retracted in its pre-fusion conformation (B. Chen, 2019; Weissenhorn et al., 1997). Interaction between gp120 and the cellular receptor CD4 on the cell surface enables viral docking, but like HA, receptor binding is not sufficient for fusion. Instead of relying on acidification to release the FP, HIV fusion is triggered by binding to a co-receptor, either CXCR4 or CCR5 (White et al., 2008).

For CoVs, fusion is triggered by a secondary proteolytic cleavage event on the S protein. Differently than IAV HA₂ and HIV gp41, which have externally situated FP in their post-priming conformation, CoV S priming occurs by cleavage at the S1/S2 junction resulting in an internally situated FP (Figure 1.1B). For CoV fusion to occur, the internal FP must be released via a secondary proteolytic cleavage event directly adjacent to the FP at the S2' site (Belouzard et al., 2009) (Figure 1.1B). S2' cleavage is mandatory for S fusogenic activity, however the protease that performs this second cleavage event and the location at which this triggering proteolytic step occurs influence the viral entry route and the tropism of different CoVs (Millet & Whittaker, 2015).

Cathepsin L is one of the best-known proteases that cleaves the S2' site for all human CoVs except NL63 (Huang et al., 2006). Cathepsin L is a ubiquitously expressed cysteine endopeptidase that functions in nutrient degradation and protein turnover (Lankelma et al., 2010). Cathepsin L is typically localized to endosomes and lysosomes and is enzymatically active at low pH (Lankelma et al., 2010). In this way, although CoV fusion does not require low pH, cathepsin-L-mediated CoV fusion is indirectly pH-dependent. CoV proteolysis by cathepsin L will trigger fusion with the endosomal membrane, termed the endosomal or late entry pathway.

For various CoVs, including SARS-CoV-1 and SARS-CoV-2, the late entry pathway is favoured during infection in most cells in culture. This can be circumvented by treating the virus with trypsin during infection (Matsuyama et al., 2005). In fact, it has been shown that various trypsin-like proteases, including TMPRSS2, can cleave both the S1/S2 and the S2' sites for most CoVs (Matsuyama et al., 2005; Shirato et al., 2018). In this way, TMPRSS2 can facilitate both the priming and triggering step. Because of this, TMPRSS-mediated S2' cleavage results in viral fusion with the plasma membrane, avoiding the endocytic pathway. Since TMPRSS proteases, including TMPRSS2, are highly expressed in the respiratory tract, TMPRSS2-dependent entry is thought to be the dominant entry strategy during human CoV infection in vivo. Furthermore, S is capable of mediating membrane fusion, regardless of whether the membranes belong to a virus or a cell. For this reason, if TMPRSS2, or any other protease capable of cleaving S2', is present on a neighbouring susceptible cell, S will mediate syncytium formation. Although various trypsin-like proteases have been implicated CoV early entry and syncytiogenesis, the relationship between CoV fusion and many of TMPRSS protease has not been determined.

Regardless of the stimuli, fusogen triggering will enable the formation of an extended intermediate allowing the FP to intercalate and latch into the host membrane (Figure 1.5).

Finally, the extended fusogen will collapse into a low-energy post-fusion state due to HR1-HR2 coiling, forming a 6-helix bundle. This collapse to a low-energy conformation provides the energy to bring the membranes into close proximity and initiates the membrane merger, resulting in CoV infection or syncytium formation (Figure 1.5).

1.3 Matrix metalloproteinases

1.3.1 MMP family and structure

Two major groups of proteases that populate the plasma membrane include transmembrane serine proteases, like TMPRSS2, and matrix metalloproteinases (MMPs). MMPs comprise a large family of transmembrane or secreted proteases. MMPs are zinc (Zn^{2+})-dependent endopeptidases that trigger extracellular matrix (ECM) remodeling and degradation during various physiological processes, such as wound healing, embryonic development, and immune cell infiltration (Collier et al., 1988; Mondal et al., 2020). In addition to their physiological roles, aberrant MMP activity can have diverse pathological roles, including rheumatoid arthritis and tumour invasion and metastasis (Burrage et al., 2006; Liotta et al., 1980).

There are 26 members of the MMP family that are subclassified based on substrate specificity. For example, collagenases (MMP-1, -8, -13, and -18), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), and gelatinases (MMP-2 and -9). MMP-2 and MMP-9, also known as gelatinase A and B, respectively, can degrade gelatin, laminin, fibronectin, and type IV and V collagens (Mondal et al., 2020). In addition to its ECM substrates, MMP-9 also cleaves and activates various cell surface and pericellular molecules, such as tumor-necrosis

alpha (TNF α), tumor-growth factor-beta (TGF- β), and interleukin-1 beta (IL-1 β) (Manicone & McGuire, 2008).

All MMPs share at least three domains: An N-terminal signal peptide domain, responsible for MMP secretion from the cell; a pro-domain, which inhibits enzymatic activity by sequestering Zn²⁺; and a catalytic domain that is responsible for proteolytic activity and substrate specificity (Eckhard et al., 2016). In addition to these canonical domains, MMP-9 also has a fibronectin domain and hemopexin-like domain, both of which are thought to promote gelatinase activity.

1.3.2 MMP regulation

MMP expression is largely restricted to a subset of cells, including endothelial cells, fibroblasts, and immune cells, such as macrophages and neutrophils (Kessenbrock et al., 2010; Taraboletti et al., 2002). In these cell types, most MMPs are maintained at low levels, but can be rapidly upregulated in response to a physiological stimulus. Various MMPs, including MMP-9, can be induced in response to inflammatory mediators, such as IL-6 (Ding et al., 2019; Kothari et al., 2014). IL-6 is a pleiotropic inflammatory cytokine that can be produced in response to viral infections, including during infection with SARS-CoV-2 (Y. Tang et al., 2020). Kothari et al (2014) found that in primary mouse macrophages, IL-6-mediated MMP-9 synthesis is MAPK^{ERK1/2} (mitogen-activated protein kinase)-dependent and STAT-3 (signal transducer and activator of transcription 3)-independent. In addition to transcriptional activation, the production of enzymatically active MMPs requires passage through various regulatory steps, including post-transcriptional regulation, pro-enzyme activation, and interactions with biological MMP inhibitors (Gaffney et al., 2015) (Figure 1.6).

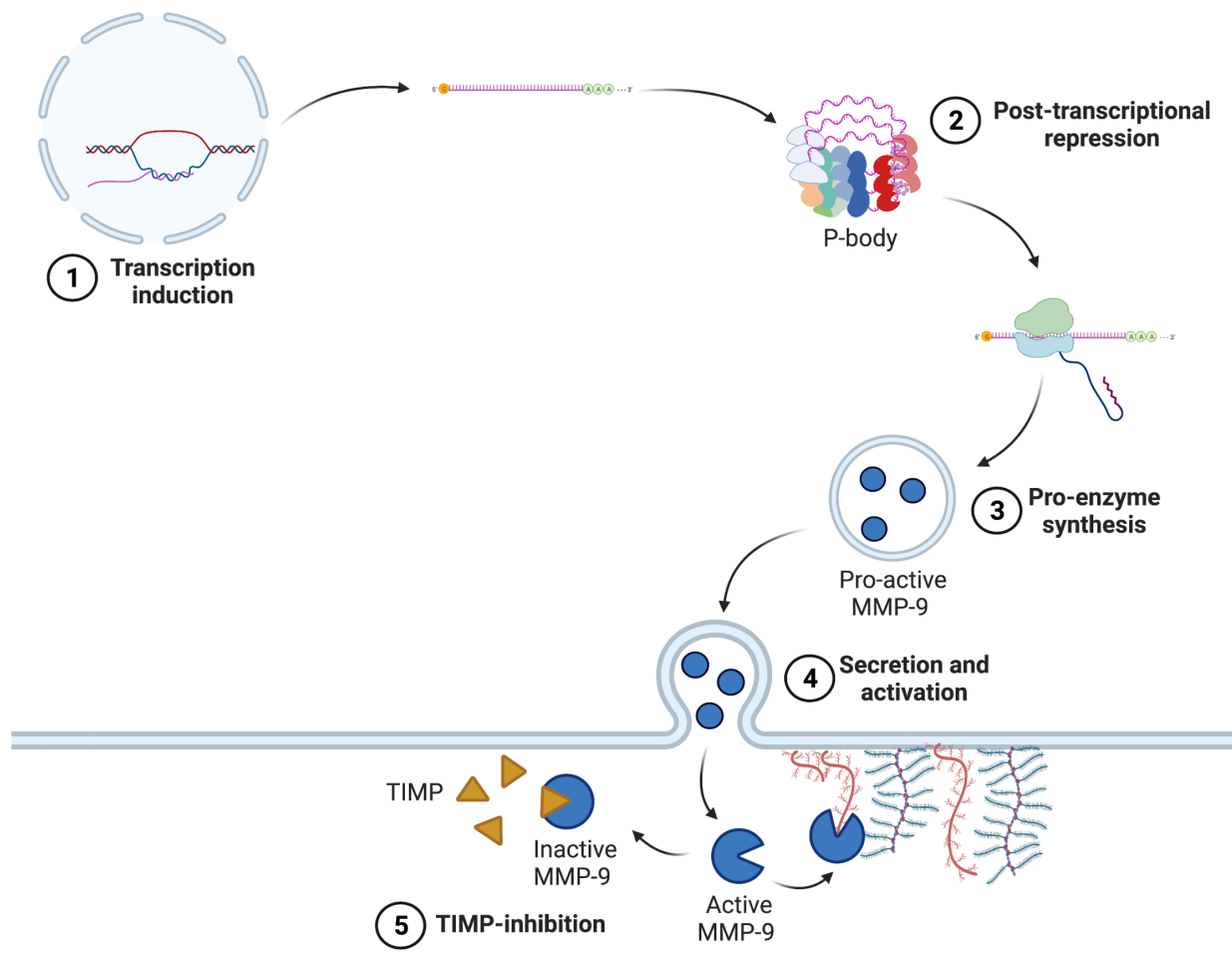


Figure 1.6 Schematic depiction of MMP-9 regulatory mechanisms. (1) MMP-9 transcription can be stimulated by various inflammatory mediators, such as IL-6. MMP-9 mRNA contains a destabilizing AU-rich element (ARE) in its 3'UTR. As an ARE-mRNA, MMP-9 likely can be post-transcriptionally repressed via sequestration and/or decay within processing bodies (P-bodies) (2). MMP-9 mRNA that is not repressed (i.e., following P-body disassembly) can be translated into an inactive pro-enzyme form (3). Pro-MMP-9 is secreted from the cell into the ECM and can be activated by other MMPs or autoactivated (4). Active MMP-9 can digest the ECM. (5) However, active MMP-9 can be inhibited by TIMPs in the ECM. This multi-level regulatory cascade serves to fine-tune MMP-9 proteolytic activity. Figure was made using biorender.com

1.3.2.1 MMP post-transcriptional regulation in processing bodies

Many MMP mRNAs, including MMP-1 and -9, contain adenylate-uridylylate rich elements (AREs) in their 3' untranslated region (3'-UTR) (Akool et al., 2003; Scott et al., 2001). 3'-UTR-AREs are commonly found in immunomodulatory and cancer-associated mRNAs and serve as a cis-acting regulatory component. Various RNA-binding proteins (RBPs) will shuttle these ARE-containing mRNAs to cytoplasmic granules called processing bodies or P-bodies (Qi et al., 2012). P-bodies are constitutive, cytoplasmic ribonucleoprotein granules that are the sites of post-transcriptional regulation via mRNA sequestration and decay. Since P-bodies are constitutively present, they contribute to the low levels of these immunomodulatory mRNAs (cytokines, growth factors, MMPs) by suppressing their translation and/or mediating their decay (Bakheet et al., 2006). Although the mechanism by which ARE-mRNAs are repressed/degraded has not been fully elucidated, our group and others have shown that with the reduction of visible P-bodies, ARE-mRNAs are no longer repressed and therefore are free to be translated (Blanco et al., 2014; Corcoran et al., 2012; Franks & Lykke-Andersen, 2007). In this way, the disassembly of P-bodies enables the cell to rapidly alter its proteome.

The precise role of P-bodies during viral infection is unclear, although viral infection typically results in the disassembly of P-bodies (Reineke & Lloyd, 2013). One hypothesis is that in addition to regulating cellular mRNAs, P-bodies can also degrade viral RNAs. Alternatively, viral disassembly of P-bodies could lead to conditions optimal for viral replication. We have found that the human CoV, OC43 infection of human umbilical vein endothelial cells (HUVECs) resulted in the disassembly of P-bodies, however it is unclear if this impacted MMP-9 mRNA abundance (Robinson et al., 2020).

1.3.2.2 MMP pro-enzyme activation and biological inhibition

MMPs are initially produced as inactive pro-enzymes or zymogens. Pro-MMPs remain latent due to steric inhibition of the Zn^{2+} ion in the catalytic site by a cysteine residue called a 'cysteine switch' (Nagase et al., 2006). Removal of the cysteine switch via proteolysis will activate MMP enzymatic activity. MMP activation typically occurs after secretion in the ECM due to serine protease activity or proteolysis from other MMPs.

Additionally, MMPs have biological inhibitors called tissue inhibitors of metalloproteinases, or TIMPs. TIMPs comprise a family of secreted proteins that can inhibit a range of metalloproteases (Brew & Nagase, 2010; Jackson et al., 2017). Although the mechanism of inhibition is unclear, it is believed to result from reversible interactions with the catalytic site, rendering the MMP inactive. In this way, the balance between MMP and TIMP expression is critical in determining the MMP proteolytic activity. From transcription induction, post-transcriptional regulation in P-bodies, zymogen activation, and TIMP inhibition, MMP activation is an intricately regulated process.

1.3.3 MMPs and CoVs

As MMPs can be upregulated in response to an inflammatory insult, there have been various reports of increased MMP expression concurrent with viral infections, including during HCoV-229E infection (J. Chen et al., 2017; Chung et al., 2004; Desforges et al., 2007; Hui et al., 2020; P. Wang et al., 2008; Yeo et al., 2002). Most recently, it has been reported that patients with severe COVID-19 have increased levels of secreted MMP-9 in their lungs (Ueland et al., 2020). Phillips et al (2017) found that a mouse CoV, mouse hepatitis virus type JHM strain SD (MHV-JHM.SD) early entry and syncytium formation could be abrogated by treatment with a

broad spectrum MMP inhibitors. However, entry of a related mouse CoV, MHV-2, was not impacted by MMP inhibition (Phillips et al., 2017). This finding suggests that MMPs could play a role in fusion for some CoVs and not others, however further analysis is warranted.

1.4 Rationale and objectives

CoVs have adopted versatile strategies to drive virus-cell and cell-cell fusion, which likely contributes to their success as human and veterinary pathogens. This fusion versatility could be explained as a result of promiscuous protease usage, enabling multiple viral entry strategies, however the diversity of protease usage has not been fully appreciated. With the onset of the SARS-CoV-2 pandemic, various small molecule inhibitors have been proposed as potential antiviral therapeutics to target proteases necessary for SARS-CoV-2 fusion. One of these compounds is hydroxychloroquine (HCQ), a lysosomotropic agent that prevents endosomal/lysosomal acidification, thereby inhibits cathepsin L activity. However, as the cathepsin L or late pathway is not the preferred pathway during infection of respiratory cells, HCQ had very limited antiviral activity, with substantial adverse effects that worsened disease outcomes (T. Ou et al., 2021). Due to its lack of efficacy, the FDA has since revoked HCQ usage in COVID-19 treatment. Instead, a TMPRSS inhibitor, camostat, is currently in clinical trials for the use against TMPRSS2-dependent SARS-CoV-2 entry (National Library of Medicine - NCT04608266, 2020). Further characterization of the viral requirements for fusion would allow for the identification of other small molecule antivirals for treatment of SARS-CoV-2.

The current understanding of CoV protease usage centers around furin, cathepsin L, and TMPRSS2. I hypothesize that there are likely more cellular proteases that can promote CoV

entry and syncytium formation. Here, the goal was to identify other proteases that are able to promote CoV entry, with an emphasis on SARS-CoV-2. To address this aim, my objectives were two-fold: first, to use a cell-cell fusion assay to determine if other proteases are able to drive CoV fusion; and second, to test if these proteases can improve infection with SARS-CoV-2 S-pseudotyped virus and a human CoV replicative virus. I found that in addition to TMPRSS2, SARS-CoV-2 S-mediated syncytium formation can be mediated by TMPRSS13. Furthermore, SARS-CoV-2 and 229E can undergo serine protease-independent cell-cell fusion that is abrogated by chemical inhibition of MMP-9. Chemical inhibition of MMP-9 also negatively impacted SARS-CoV-2 entry with S-pseudotyped virus but did not impact SARS-CoV-1 S-pseudotype entry. This work provides initial evidence that human CoVs, including SARS-CoV-2, use diverse proteases to trigger S protein-mediated membrane fusion during entry or syncytium formation. This observation has important implications for our understanding of human CoV infection and tropism as well as cell-to-cell spread and pathogenesis, which could be relevant to various human and veterinary CoVs.

CHAPTER 2 - MATERIALS & METHODS

2.1 Cell culture

All cells were maintained at 37°C in a humidified chamber with 5% CO₂ and 20% O₂ unless otherwise stated. Human embryonic kidney 293t cells (HEK293t) (ATCC), HEK293t-ACE2 (A kind gift from Dr. Michael Farzan), human hepatoma-derived cells (Huh7.5) (a kind gift from Dr. Morgan Fullerton), and fibrosarcoma cells (HT1080) (ATCC), were cultured in Dulbecco's Modified Essential Medium (DMEM) (Gibco) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (1% PSQ) (Wisent) and 10% heat-inactivated fetal bovine serum (HI-FBS) (Sigma). Human lung adenocarcinoma cells (Calu-3) and immortalized human lung fibroblast cells (MRC-5) were cultured in Eagle's Minimal Essential Medium (EMEM) (ATCC) supplemented with 1% PSQ and 10% HI-FBS. Calu3 were sub-cultured at 90% confluency with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher). Other cells were sub-cultured at 90% confluency using 0.05% trypsin-EDTA (Thermo Fisher) and the media was refreshed every 48 hours or as needed.

Primary human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in endothelial cell growth medium (EGM-2) (Lonza). EGM-2 consists of endothelial base medium (EBM-2) (Lonza) supplemented with 2% FBS, 0.04% hydrocortisone, 0.4% human fibroblast growth factor-B, 0.1% vascular endothelial growth factor (VEGF), 0.1% long arginine-3 insulin-like growth factor, 0.1% ascorbic acid, 0.1% human epidermal growth factor, gentamycin sulfate (30 µg/mL), amphotericin (15 ng/mL), and 0.1% heparin. HUVECs were seeded onto tissue culture plates pre-coated with 0.1% (w/v) porcine gelatin (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) (Thermo Fisher). EGM-2 was refreshed every 48 hours or as needed. HUVECs were sub-cultured using 0.05% trypsin-EDTA at ratios of 1:3 onto 6-well tissue culture

plates and 1:6 onto 12-well tissue culture plates. For immunofluorescence, HUVECs were seeded onto 18mm round #1.5 glass coverslips (Electron Microscopy Sciences) pre-coated with porcine gelatin as above. All experiments were completed using HUVECs between passage 4 and 8.

2.2 Solutions and reagents

X-gal staining solution was prepared as follows: 0.1 M phosphate buffer (pH 7.3) supplemented with 2 mM MgCl₂, 5 mM potassium ferrocyanide (K₄Fe(CN)₆·3H₂O; Sigma-Aldrich), 5 mM potassium ferricyanide (K₃Fe(CN)₆; Sigma-Aldrich). X-gal staining solution was stored at 4°C away from light.

Poly-D-lysine was prepared by diluting poly-D-lysine powder (Sigma-Aldrich) with sterile water to 50 µg/mL working stock. Working stock was stored at 4°C.

Crystal violet staining solution (0.5%) was prepared by diluting 0.5% w/v crystal violet powder (Sigma-Aldrich) in 8 parts ddH₂O and 2 parts 100% methanol.

2.3 Protease prediction sites using PROSPER

Coronavirus spike amino acid sequences were acquired from NCBI and aligned using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo). SARS-CoV-2 Hu-1 (GenBank QOS45029.1); SARS-CoV-1 (GenBank QJE50589.1); MERS-CoV (GenBank QBM11748.1); HCoV-OC43 (GenBank AIX10763.1); HCoV-HKU1 (GenBank AYN64561.1); HCoV-229E (GenBank APT69890.1); HCoV-NL63 (GenBank APF29063.1); MHV-JHM-SD (GenBank P22432.1); and MHV-2 (GenBank AAF19386.1). PROSPER protease prediction server was used to determine putative cleavage sites within S1/S2 and S2' sites. PROSPER

(prosperous.erc.monash.edu) is an *in silico*, open-access server that identifies protease cleavage sites including aspartic, cysteine, metallo, and serine proteases based on primary-tertiary protein structure and support-vector machine learning.

2.4 Plasmids

Table 2.1 Source of plasmids used

Plasmid	Source
TMPRSS(x)-pLX-TRC317-puromycin	Millipore Sigma
pLJM1-EV	Gift from C. McCormick (Dalhousie University)
SARS-CoV-1-Spike-pcDNA3.1	Gift from M. Farzan (Scripps Research Institute)
SARS-CoV-2-Spike-pcDNA3.1	BioBasic (Canada)
pMD2.G (VSV-G)	Gift from Didier Trono (Addgene #12259)
psPAX2	Gift from Didier Trono (Addgene #12260)
ACE2-pcDNA3.1	Gift from H. Choe (The Scripps Research Institute)
LTR-GFP	Gift from J. Cunningham (Brigham and Women's Hospital)
LV-LacZ	Gift from J. Cunningham (Brigham and Women's Hospital)

2.5 Transfections

HEK293t or HT1080 cells were seeded into 10 cm or 15 cm tissue culture dishes to achieve 80% confluency the following day. Cells were washed with PBS and refreshed with DMEM containing 10% FBS and 1% PSQ. A reaction mixture consisting of a plasmid DNA, jetPRIME reagent (PolyPlus), and jetPRIME buffer (PolyPlus) was made as per manufacturer's instructions. The mixture was mixed thoroughly and added dropwise to cells. The media was changed 24-48 hours post-transfection (hpt) depending on experimental design.

2.6 Immunoblots

HEK293t cells were seeded in 12-well plates to achieve 80% confluency after 24 h. Once 80% confluent, cells were transfected with the indicated TMPRSS protease-encoding plasmid or an empty vector control as described above. All TMPRSS proteases were encoded from a pLX-TRC317-puromycin backbone and a carboxy-terminal V5 tag (GKPIPNNPLLGLDST). 24 hpt, cells were washed once with cold PBS and lysed in cold lysis buffer (1% Triton X-100, 0.1% IGEPAL CA-630, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5). Cell lysates were thawed on ice. 5% 2-mercaptoethanol (Sigma) and 0.1% bromophenol blue (Sigma) was added to lysates. Samples were then heated at 95°C for 5 mins. Protein samples were resolved using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to ImmunoBlot polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h at RT with blocking buffer (5% skim milk powder dissolved in 25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20 [Tris-buffered saline with Tween-20;TBST]). TMPRSS proteases were detected using anti-V5 tag antibody raised in a mouse (abcam) diluted 1:2000 in blocking buffer. PVDF membranes were incubated with anti-V5 antibody overnight at 4°C. Excess unbound antibody was removed from the membrane by washing with TBST. Membrane was then incubated with anti-mouse IgG conjugated to horseradish peroxidase (HRP) diluted 1:10,000 in blocking buffer rocking for 1 h at RT. Membranes were washed with TBST and exposed to Clarity western ECL blotting substrate (BioRad) as per manufacturer's instructions. Chemiluminescence was visualized on a ChemiDoc Imaging System (BioRad).

2.7 Inhibitor treatment

All inhibitors were diluted in dimethyl sulfoxide (DMSO) to a 10 mM stock solution and aliquoted to avoid repeated freeze-thaws. Additional dilutions were conducted in the appropriate cell media (Table 2.2).

Table 2.2 Source and final concentration ranges of protease inhibitors used.

Inhibitors	Company	Catalogue	Concentration
E64d	Sigma	E864	5 -10 μ M
Camostat	Sigma	SML0057	20 - 25 μ M
GI-254023X	Sigma	SML0789	5 - 40 μ M
Tapi-2	Sigma	SML0420	20 - 40 μ M

2.8 Cell-cell fusion assays

2.8.1 Two-population cell-cell fusion assay

HEK293t cells were seeded into a 12 well plate to reach 80% confluency the following day. One population of HEK293t cells were co-transfected with GFP, ACE2, and the indicated TMPRSS protease of empty vector. A second population of HEK293t cells were co-transfected with SARS-CoV-1 or SARS-CoV-2 S and mCherry. 24 h post-transfection both populations were resuspended using versene (Gibco). The cells were pelleted and resuspended in complete DMEM, and the two populations were co-cultured at a 1:1 ratio in 12 well plates. Protease inhibitors were added simultaneous with co-culture as indicated. 4-6 hours post co-culture cells were imaged using ZOE fluorescent imager (BioRad) using excitation and emission spectra of 480/17 nm and 517/23, respectively. At least 3 fields were captured per treatment.

2.8.2 One-population cell-cell fusion assay

HEK293t-ACE2 cells were co-transfected with GFP and SARS-CoV-1 or SARS-CoV-2 S and a TMPRSS protease. Protease inhibitors were added directly following transfection as indicated. 16 h post-transfection cells were imaged using the ZOE fluorescent imager as above.

2.9 Recombinant lenti pseudoviruses

2.9.1 Lenti-pseudovirus production

A second-generation recombinant lentivirus system was used in this study, which is comprised of the following: a viral envelope gene, the CoV spike (CoV-S), or vesicular stomatitis virus G (VSV-G); a packaging plasmid for HIV structural and transactivator genes Gag, Pol, Rev, and Tat (psPAX2); and a transfer plasmid which contains viral long-terminal repeats and psi packaging signal. In this study the transfer plasmid contained a LacZ reporter gene. Plasmids were co-transfected into an 80% confluent 15-cm dish of HEK293ts using 10 ng of Env (CoV-S or VSV-G), 5 ng psPAX2, and 5 ng LV-LacZ. 48 hpt virus-containing supernatant was collected and filtered through a 0.45 µm PES sterile membrane filter (VWR) and stored at 4°C. Fresh complete DMEM was added to cells. Pseudovirus-containing supernatant was harvested again at 72 hpt and 96 hpt.

2.9.2 Pseudovirus concentration

Virus-containing supernatants were combined and concentrated by ultra-centrifugation through a sucrose cushion, as follows: 30 mL polycarbonate ultracentrifuge tubes (Beckman Coulter) were filled with a combination collected supernatants. 5 mL of 20% (w/v) sucrose diluted in PBS and filter sterilized with a 0.22 µm PES membrane, was added to the bottom of the ultracentrifuge tube. The virus was pelleted into the sucrose cushion by ultracentrifugation at

at 20 000 rpm for 1.5 h, 4°C (Beckman Coulter Optima XPN-100 ultracentrifuge, SW32Ti rotor). Following ultracentrifugation, the pellet was decanted and resuspended in 300 µL PBS overnight at 4°C. Concentrated virus was aliquoted and stored at -80°C for single use to avoid freeze-thawing.

2.9.3 *LacZ* pseudovirus titration

96-well tissue culture plates were pre-coated with poly-D-lysine for 30 min, then left to dry at room temperature for 1 h. HEK293t-ACE2 cells were seeded onto the pre-coated 96-well tissue culture plate to achieve 80% confluency the following day. Serial dilutions of concentrated pseudovirus were made by diluting pseudovirus in complete DMEM supplemented with 5 µg/mL hexadimethrine bromide (polybrene) (Sigma). Cells were infected with diluted pseudovirus in triplicate. 24 h post-infection (hpi), the media was refreshed with complete DMEM. 48 hours post-infection (hpi) the cells were washed with PBS and fixed with 10% formaldehyde for 15 minutes at RT. During this time, X-gal staining solution was pre-warmed at 42°C. Formaldehyde was removed and cells were washed twice with PBS. Once warm, X-gal staining solution was supplemented with 1 mg/ml X-gal (Thermo Fisher) and mixed vigorously, before adding 100 µL to each well. The plate was sealed with parafilm and left overnight at 37°C. The following day the staining solution was removed, and the plate was visualized using a compound light microscope. Pseudovirus infected cells were stained blue. Infected cells were manually counted for the entire well. Because lentiviruses will be passed on to daughter cells upon cell division, two to three yellow cells in contact were scored as a single positive cell. The pseudovirus dilution that yielded ~200 focus-forming units (FFU) per well were used for further infections. Pseudovirus titre was calculated as follows: Titre = FFU per well / vol of virus used (mL).

Cells were seeded in a 96-well format to achieve 95% confluency 48 hpi. HEK293t-ACE2 cells were seeded onto poly-D-lysine coated plates as above. Cells were treated with the indicated inhibitor at 37°C for 1 h before infection. The media was then removed and replaced with pseudovirus-containing media with polybrene. 24 hpi the media was refreshed and 48 hpi the cells were fixed and stained as above.

2.10 Replication-competent HCoV-229E

2.10.1 HCoV-229E propagation

HCoV-229E (ATCC, VR-740 isolate) was propagated in MRC-5 cells as follows. MRC-5 cells were seeded into a T75 tissue-culture flask to achieve 80% confluency the following day. 229E viral stock was diluted in serum-free, antibiotic-free, EMEM to achieve 0.01 multiplicity of infection (MOI). The cells were rinsed in PBS, then 1 mL of diluted viral inoculum was added to the flask. The flask was incubated at 33°C for 1 h, gently rocking the flask every 10 min to maximize viral adsorption. After the 1 h incubation, viral inoculum was removed, and cells were covered in fresh EMEM containing 2% FBS and 1% PSQ. Cells were incubated for 4 days at 33°C. After 4 days, the virus-containing supernatant was harvested, centrifuged at 2000 rpm for 5 min, then aliquoted and stored at -80°C for single use.

2.10.2 Tissue-culture infectious dose 50% (TCID₅₀) titration

MRC-5 cells were seeded into a 96-well tissue-culture plate to achieve 80% confluency the following day. 10-fold serial dilutions were made from virus stock by diluting in serum-free, antibiotic-free media. Cells were washed with PBS then overlaid with 50 µL of viral inoculum per well, with each dilution done in triplicate. The plate was incubated at 33°C for 1 h, gently rocking the plate every 10 mins. The viral inoculum was removed and replaced with EMEM

containing 2% FBS and 1% PSQ. Cells were incubated at 33°C for 5 days. After 5 days, cells were fixed with 10% formaldehyde for 1 h at room temperature (RT) then stained with crystal violet for 2 min at RT. Wells were washed with water and left to dry. Wells were scored based on viral-mediated clearing (Figure 2.1). Infectious titre was determined using the Reed and Muench method (Reed & Muench, 1938) as in Figure 2.1B & C.

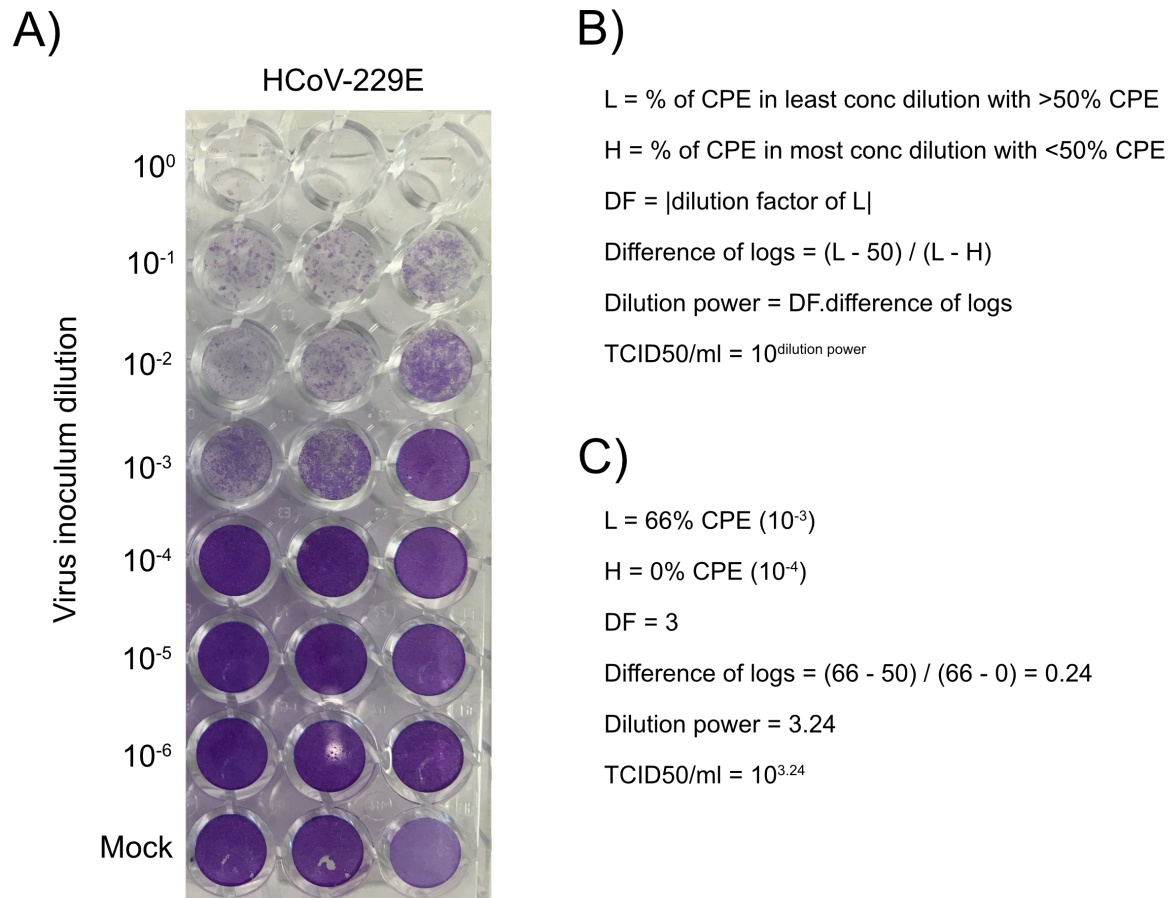


Figure 2.1 Tissue-culture infectious dose 50% (TCID₅₀) of HCoV-229E in MRC5 cells. A) Virus-induced CPE scoring after MRC-5 infection HCoV-229E. 5dpi cells were fixed and stained with crystal violet and scored binarily based on CPE. **B)** Reed and Muench TCID₅₀ calculation. **C)** Sample calculation based on A.

2.10.3 HCoV-229E infection

HUVECs were seeded at a 1:6 ratio into a gelatin-coated 12-well tissue culture plate or onto coverslips, as described above. The following day, HUVECs were washed with PBS, then infected with 100 μ L of $1 \times 10^{3.5}$ TCID₅₀/mL 229E, diluted in serum-free DMEM. Cells were incubated in viral inoculum for 1 h at 37°C, gently rocking every 10 mins, after which the viral inoculum was replaced with 1 mL fresh EGM-2 and cells were incubated at 37°C for the indicated time period. To study 229E entry, HUVECs were pre-treated with the indicated inhibitor diluted in fresh EGM-2 and incubated at 37°C for 30 mins. Following inhibitor treatment, HUVECs were washed with PBS then inoculated with 229E, as detailed above. To study syncytium formation, protease inhibitors were added directly after viral adsorption and remained in the media until the end of the experiment.

2.10.4 Recombinant soluble MMP-9 treatment

Recombinant human MMP-9 (BioVision) was resuspended in filter-sterilized 30% glycerol to a concentration of 10 units/ μ L and stored at -20°C. Recombinant MMP-9 was directly added to HCoV-229E inoculum 30 mins before viral adsorption at concentration of 3 units/mL (3 U/mL) and incubated at 37°C. After 1 h of viral adsorption, virus inoculum containing MMP-9 was removed and cells were washed with PBS and replaced with fresh media without MMP-9.

2.11 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Following treatment, cells were lysed for total RNA with RLT buffer (Qiagen) containing 1% 2-mercaptoethanol and stored at -80°C. RNA lysates were thawed on ice and total RNA was extracted using RNeasy Mini Kit according to the instructions provided by the manufacturer (Qiagen). The concentration of extracted RNA was determined using Thermo Fisher NanoDrop

One^C spectrophotometer. 250–500 ng of RNA was reverse transcribed using qScript cDNA SuperMix (QuantaBio). Complementary DNA (cDNA) was diluted 1:20 with nuclease-free water for all qPCR reactions. qPCR was performed using SsoFast EvaGreen qPCR master mix (BioRad) and the following reaction conditions: 4 µL diluted cDNA, 5 µL SsoFast EvaGreen master mix, and 1 µL of a 2 µM mixture of forward and reverse primers (Table 2.3).

qPCR was conducted in a 96-well plate using the BioRad CFX Connect Real-Time system thermocycler. Samples were subjected to the following cycling conditions: 2 min at 98°C, then cycling 39X between 98°C for 2 s and 56°C for 5 s, incubation at 65°C for 10 s, and 95°C for 2 s. To ensure a single product was produced from each reaction, following every run a melt curve was determined by increasing the temperature from 75°C by increments of 0.2°C/10 s to 95°C to ensure single product amplification. All samples were run in duplicate and the average values were normalized to the housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT). The cycle threshold was calculated by the CFX connect (BioRad). The fold-change of mRNA expression levels was determined using the $\Delta\Delta C_t$ quantification method as follows:

$$\Delta C_t = (\text{average } C_t \text{ of target gene}) - (\text{average } C_t \text{ of house-keeping gene})$$

$$\Delta\Delta C_t = \Delta C_t^{\text{experimental condition}} - \Delta C_t^{\text{control condition}}$$

$$\text{Relative fold-change} = 2^{-\Delta\Delta C_t}$$

Table 2.3 qPCR primer sequences

Primer	Primer Sequence
HPRT-1 - Forward	5' CTTTCCTTGGTCAGGCAGTATAA 3'
HPRT-1 - Reverse	5' AGTCTGGCTTATATCCAACACTTC 3'
MMP-9 - Forward	5' GTGGTGTCTCACGAAGGGAT 3'
MMP-9 - Reverse	5' GCAGGTTCGACGTGAAGGC 3'

2.12 Fluorescence and immunofluorescence microscopy

2.12.1 Live-cell fluorescence microscopy

One- and two-population fusion assays were imaged using ZOE fluorescent imager (BioRad) using excitation and emission spectra of 480/17 nm and 517/23, respectively. At least 3 fields were captured per treatment.

2.12.2 Immunofluorescence staining

Cells were seeded onto gelatin-coated coverslips (#1.5, Electron Microscopy Sciences) and after experimental manipulation, fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) diluted in PBS for 10 min at RT. Following fixation, cells were washed three times with PBS, then permeabilized with 0.1% v/v Triton X-100 (Sigma-Aldrich) diluted in PBS for 10 minutes at RT. Cells were washed with PBS three times before being blocked with 1% human AB serum (Sigma) for 1 h at RT. Coverslips were then incubated with primary antibodies, diluted in 1% human AB serum as denoted in Table 2.4. Coverslips were incubated with primary antibodies overnight at 4°C in a humidified chamber. Following primary antibody incubation, cells were washed three times with PBS then incubated with one or more fluorescently-tagged secondary antibodies and Hoechst (diluted in 1% AB serum, Table 2.4) for 1 h at RT. Cells were washed three times with PBS before being mounted onto a microscope slide (FisherBrand) with Prolong Gold Antifade mounting media (Thermo Fisher) and allowed to cure overnight at RT.

Table 2.4 Primary and secondary antibodies used for immunofluorescence

Antibody	Species	Company	Catalogue	Dilution	Incubation Temp
Vimentin	Rabbit	CST	D21H3	1:500	4°C
J2 (dsRNA)	Mouse	Sigma	MABE1134	1:100	4°C
DDX6	Rabbit	Bethyl	A300-461A	1:1000	4°C
Hoechst	N/A	Thermo Fisher	H3570	1:10 000	RT
AlexaFluor 555 conjugated to anti-rabbit IgG	Donkey	Thermo Fisher	A31572	1:1000	RT
AlexaFluor 488 conjugated to anti-mouse IgG	Chicken	Thermo Fisher	A21200	1:1000	RT

2.12.3 Immunofluorescence microscopy

Coverslips were visualized with the Zeiss AxioObserver Z1 fluorescent wide-field microscope (Charbonneau Microscopy Facility, University of Calgary), with filtered LED illumination at the 20X or 40X oil immersion objectives. For each biological replicate, channel exposures and intensities were unchanged throughout imaging. Images were processed using the Zen Blue Software.

2.12.4 229E entry quantification using CellProfiler

Cells were imaged at 20X magnification and at least 3 fields of view were obtained, imaging 80+ cells per condition. Cells were scored as infected based on J2 (dsRNA) staining using an unbiased image analysis pipeline generated in the open-access image analysis software CellProfiler (cellprofiler.org) (Kamentsky et al., 2011). The pipeline used here was designed by M Kleer. Briefly, nuclei were detected as primary objects using manual thresholding of the Hoechst-stained image. Cell boundaries were defined and masked using the propagate function

as a discrete distance from the nucleus (60 pixels and 80 pixels for 20X and 40X, respectively). The cytoplasm masks containing J2 staining were defined as ‘infected’; all other cells were defined as ‘uninfected’. Due to fusogenic nature of 229E infection in HUVECs, all nuclei within a J2-positive syncytium were scored as infected. The relative infection was calculated by the number of ‘infected cells’ divided by total nuclei.

2.12.5 Syncytial nuclei indexing

Cells were imaged at 20X magnification and at least 3 fields of view were obtained, imaging 80+ cells per condition. The syncytial index was calculated as syncytial nuclei (nuclei contained within a single cell, syncytium), counted manually, divided by total nuclei. A syncytium was defined as a cell with three or more nuclei to account for dividing cells.

2.12.6 Processing body quantification

Processing bodies were quantified using CellProfiler as described above. To quantify P-body puncta, the cytoplasm mask was applied to the DDX6 channel. Background staining was removed using “Enhance Speckles” function. Using “global thresholding with robust background adjustments”, puncta with a defined size and intensity range were quantified. Size and intensity thresholds were unchanged between biological replicates. Processing body numbers for ‘infected’ and ‘uninfected’ cells were quantified and exported to GraphPad Prism 8.0 for analysis.

2.13 Statistical analysis

Experimental data was analysed using GraphPad Prism 8.0 software. A one-way ANOVA with a Dunnett’s post hoc analysis was used for all experiments comparing multiple means to the control. Statistical significance was determined as $p < 0.0332$ (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****)

CHAPTER 3 - RESULTS

3.1 SARS-CoV-2 S mediates TMPRSS2 independent cell-cell fusion that is abrogated by MMP inhibition

For CoV entry and syncytium formation to occur, S must facilitate membrane fusion. One of the requirements for S-mediated fusion is proteolytic processing of S by host proteases (Millet & Whittaker, 2015). Therefore, characterizing host proteases that can trigger S fusion is critical to understanding CoV entry and syncytium formation. To investigate if other cellular proteases are capable of cleaving SARS-CoV-2 S to trigger fusion at the cell surface, I sought to screen various proteases using cell-cell fusion assays. Given that CoV syncytium formation and the early entry pathway have the same requirements for fusion (receptor binding, proteolytic priming and triggering prior to endocytosis), cell-cell fusion assays serve as a proxy to study proteases that promote the early entry pathway and syncytium formation.

To mimic viral-cell fusion, I used a two-population cell-cell fusion assay. In short, a population of HEK293t cells, expressing GFP, ACE2, and the protease of interest (termed target cells), were co-cultured with a population of HEK293t cells, expressing mCherry and the S of either SARS-CoV-1 or SARS-CoV-2 (termed effector cells). In this way, after co-culture, the production of syncytia between effector and target cells indicated successful protease processing of the S (Figure 3.1A). I observed that SARS-CoV-1 S-mediated syncytium formation was dependent on ectopic expression of both TMPRSS2 and ACE2, as cell-cell fusion only occurred with TMPRSS2⁺/ACE2⁺ target cells and not TMPRSS2⁻/ACE2⁺ cells. Additionally, to specifically attribute SARS-CoV-1 S syncytium formation to TMPRSS2 over-expression, I treated the cells with a TMPRSS inhibitor camostat. I found that camostat treatment abrogated SARS-CoV-1 S syncytium formation (Figure 3.1B).

When I applied this system to SARS-CoV-2 S, I found that unlike SARS-CoV-1, SARS-CoV-2 S triggered syncytium formation with TMPRSS2-/ACE2+ target cells. Although I observed that TMPRSS2 co-expression enhanced SARS-CoV-2 syncytium formation, fusion clearly occurred in the absence of TMPRSS2. Furthermore, camostat treatment did not eliminate SARS-CoV-2 syncytium formation upon co-culture with TMPRSS2+/ACE2+ target cells; instead camostat reduced syncytium formation to a level comparable to fusion with TMPRSS2-/ACE2+ target cells (Figure 3.1B). Given that S proteolysis is mandatory for CoV fusion, together these findings suggest that a surface protease endogenously expressed by HEK293t cells is capable of activating SARS-CoV-2 S, but not SARS-CoV-1 S fusogenicity.

As a first step, I tried to categorize this unknown protease by family type. To do this, I used a two-population cell-cell fusion assay with TMPRSS2-/ACE2+ target cells. Without ectopic protease expression, cell-fusion could only occur upon S activation by endogenous proteases. During co-culture, I incorporated a broad-spectrum TMPRSS inhibitor (camostat) or a broad-spectrum MMP inhibitor (Tapi-2). Consistent with my previous findings, I found that syncytium formation occurred in the presence of camostat, suggesting that the endogenous activator is likely not due to endogenous expression of TMPRSS2 or another TMPRSS protease. However, no cell fusion events were observed upon treatment with MMP inhibitor Tapi-2, suggesting that the unknown endogenous protease could be an MMP (Figure 3.1C).

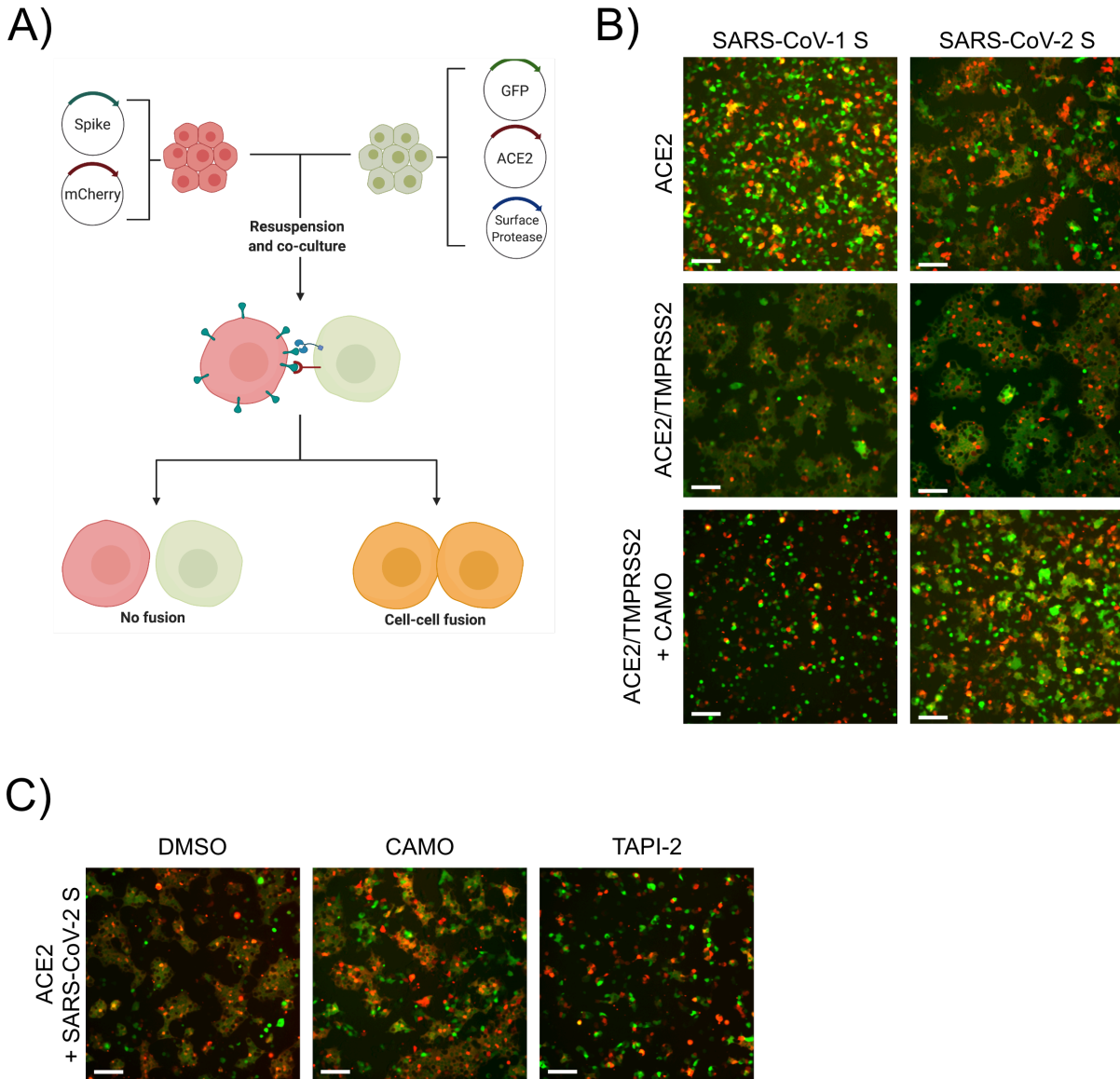


Figure 3.1 SARS-CoV-2 S mediates TMPRSS2-independent cell-cell fusion. **A)** The two-population cell-cell fusion assay was developed by co-transfecting one population of HEK293t cells with mCherry and S from either SARS-CoV-1 or SARS-CoV-2, and co-transfected the second population with GFP, ACE2, and the surface protease of interest. 24 h post-transfection the two populations were resuspended and co-cultured. Figure was made using biorender.com **B)** SARS-CoV-1 S or SARS-CoV-2 S-expressing cells (green) were co-cultured with cells expressing ACE2 or ACE2/TMPRSS2 (red). 25 μ M camostat (TMPRSS inhibitor) was added simultaneous with co-culture. **C)** SARS-CoV-2 S-expressing cells (green) were co-cultured with ACE2-expressing cells (red). Camostat (25 μ M), Tapi-2 (40 μ M; MMP-inhibitor), or DMSO (vehicle control) was added during co-culture. Cells were imaged 2-3 h post co-culture. These data represent n=3. Scale bar = 100 μ m.

3.2 TMPRSS2 and TMPRSS13 rescue Tapi-2 inhibition of SARS-CoV-2 syncytia formation

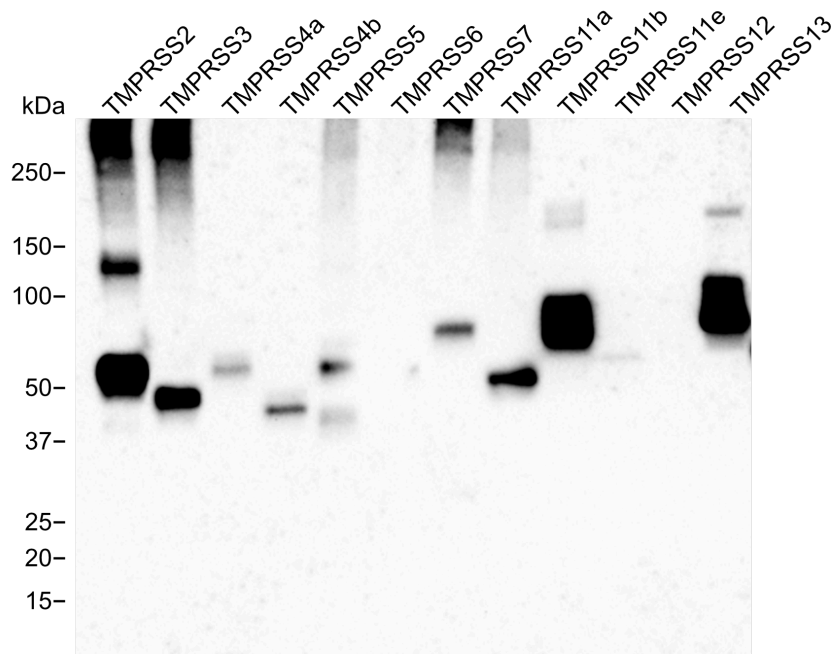
To assess if other members of the TMPRSS family are also able to promote SARS-CoV-2 S fusion, I acquired expression plasmids for various other TMPRSS proteases, including TMPRSS 3, 4a, 4b, 5, 6, 7, 11a, 11b, 11e, 12, and 13. I was not able to detect expression for TMPRSS6 or TMPRSS12, therefore they were omitted from further experimentation (Figure 3.2A).

Because SARS-CoV-2 S can mediate fusion in the absence of protease ectopic expression, to determine which TMPRSS proteases can facilitate SARS-CoV-2 S fusion, I eliminated the endogenous protease-mediated fusion by treating cells with Tapi-2 and attempted to rescue cell-cell fusion by expressing select TMPRSS proteases. Consistent with my previous findings, I found that Tapi-2 treatment blocked syncytium formation in the empty vector (EV) transfected cells (Figure 3.2B). Whereas both TMPRSS2 and TMPRSS13 expression induced robust syncytium formation (Figure 3.2B). TMPRSS3, 4a, 4b, 5, 7, 11a, 11b, and 11e did not rescue syncytium formation.

3.3 SARS-CoV-2 S TMPRSS2-independent fusion is abrogated by chemical inhibition of MMP-9

To validate my findings from the two-population cell-cell fusion assay with a simpler assay, I used a one population syncytium assay wherein HEK293t-ACE2 cells were transiently transfected with GFP, TMPRSS2, and the S of SARS-CoV-1 or SARS-CoV-2, circumventing the need for co-culture (Figure 3.3A). Consistent with the two-population cell-cell fusion assay, using a one-population fusion assay, I found that SARS-CoV-1 S fusion was dependent on TMPRSS2 ectopic expression, whereas SARS-CoV-2 S triggered fusion without TMPRSS2

A)



B)

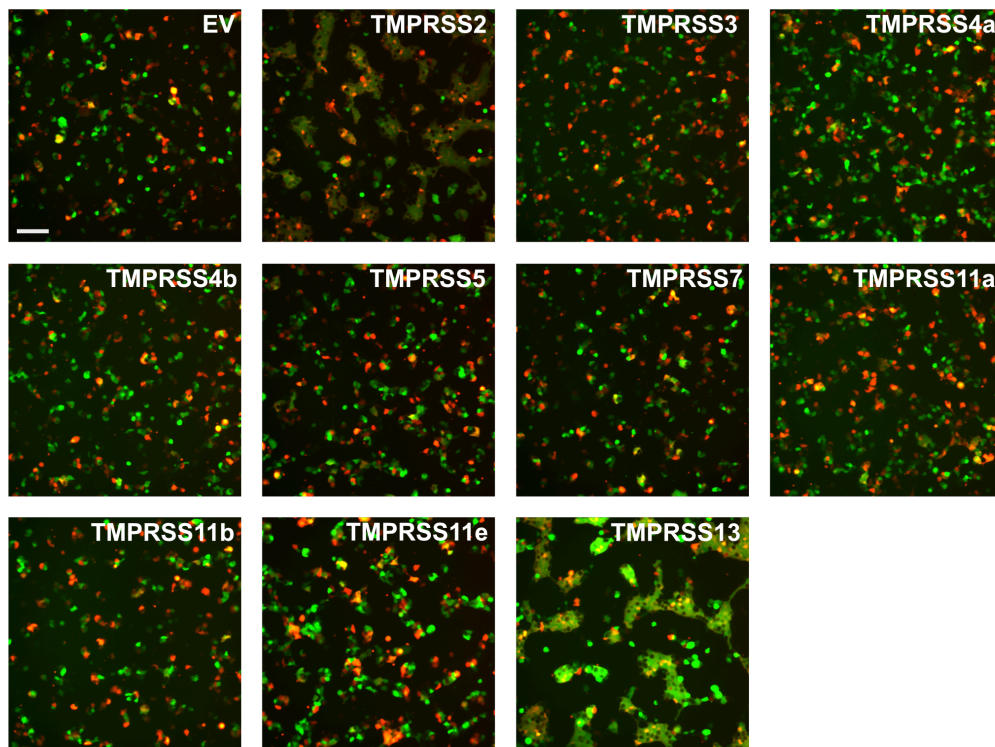


Figure 3.2 TMPRSS2 and TMPRSS13 can overcome Tapi-2-mediated syncytia inhibition to promote SARS-CoV-2 S cell-cell fusion. Figure caption can be found on the following page.

Figure 3.2 TMPRSS2 and TMPRSS13 can overcome Tapi-2-mediated syncytia inhibition to promote SARS-CoV-2 S cell-cell fusion. **A)** V5-tagged TMPRSS protease expression plasmids were transfected into HEK293t cells and total protein was harvested 24 h post-transfection. Cell lysates were subjected to immunoblotting using the V5 antibody. These data represent n=1. **B)** HEK293t cells expressing SARS-CoV-2 S (red) were co-cultured with HEK293t cells expression ACE2 and the indicated V5-tagged TMPRSS proteases (green), which were detectable from A, or empty vector (EV). Concurrent with co-culture, cells were treated with 40 μ M Tapi-2. Cells were imaged 2-3 h post co-culture. These data represent n=3. Scale bar = 100 μ m.

expression, supporting the notion that another endogenous protease can process and activate SARS-CoV-2 S, but not SARS-CoV-1 S (Figure 3.3B). Recapitulating my findings from the two-population assay, I found that SARS-CoV-2 TMPRSS-independent fusion was not impacted by TMPRSS inhibitor camostat, but was abrogated by MMP inhibitor Tapi-2 (Figure 3.3C). Cathepsin inhibitor E64d also did not inhibit syncytium formation.

Furthermore, *in situ* protease substrate specificity analysis using PROSPER (prosper.erc.monash.edu.au) identified putative MMP-9 cleavage sites at the S1/S2 junction and the S2' site for various CoVs (Figure 3.3D; red underlined motifs). This included predicted MMP-9 cleavage at the S1/S2 region for MERS-CoV, 229E, and HKU1, and in S2' region of HKU1, 229E, MHV-JHM-SD and notably, SARS-CoV-1 and both SARS-CoV-2-Hu1 and the B.1.1.7 variant of concern (VOC). Informed by these *in situ* analysis, I incorporated MMP-9 inhibitor GI-254023X (GI-X) into the cell-cell fusion assay. I observed that GI-X treatment blocked syncytium formation to a similar extent as Tapi-2 treatment, suggesting that MMP-9 could be responsible for TMPRSS2-independent SARS-CoV-2 syncytium formation.

3.4 SARS-CoV-2 pseudovirus entry pathway is cell-type dependent and is facilitated by promiscuous protease usage

Using the cell-cell fusion assay, I found that GI-X or Tapi-2 blocked SARS-CoV-2 S-mediated cell-cell fusion, but did not impact SARS-CoV-1 S cell-cell fusion. To address if this fusion inhibition occurred during viral entry, I generated lentiviral pseudoviruses that bear the S of either SARS-CoV-1 or SARS-CoV-2. Thus, the pseudoviruses would undergo proteolytic processing and entry like their authentic CoV counterparts; however, rather than a viral genome, the pseudoviruses encode a reporter gene, *LacZ*, as a transgene. To characterize the protease

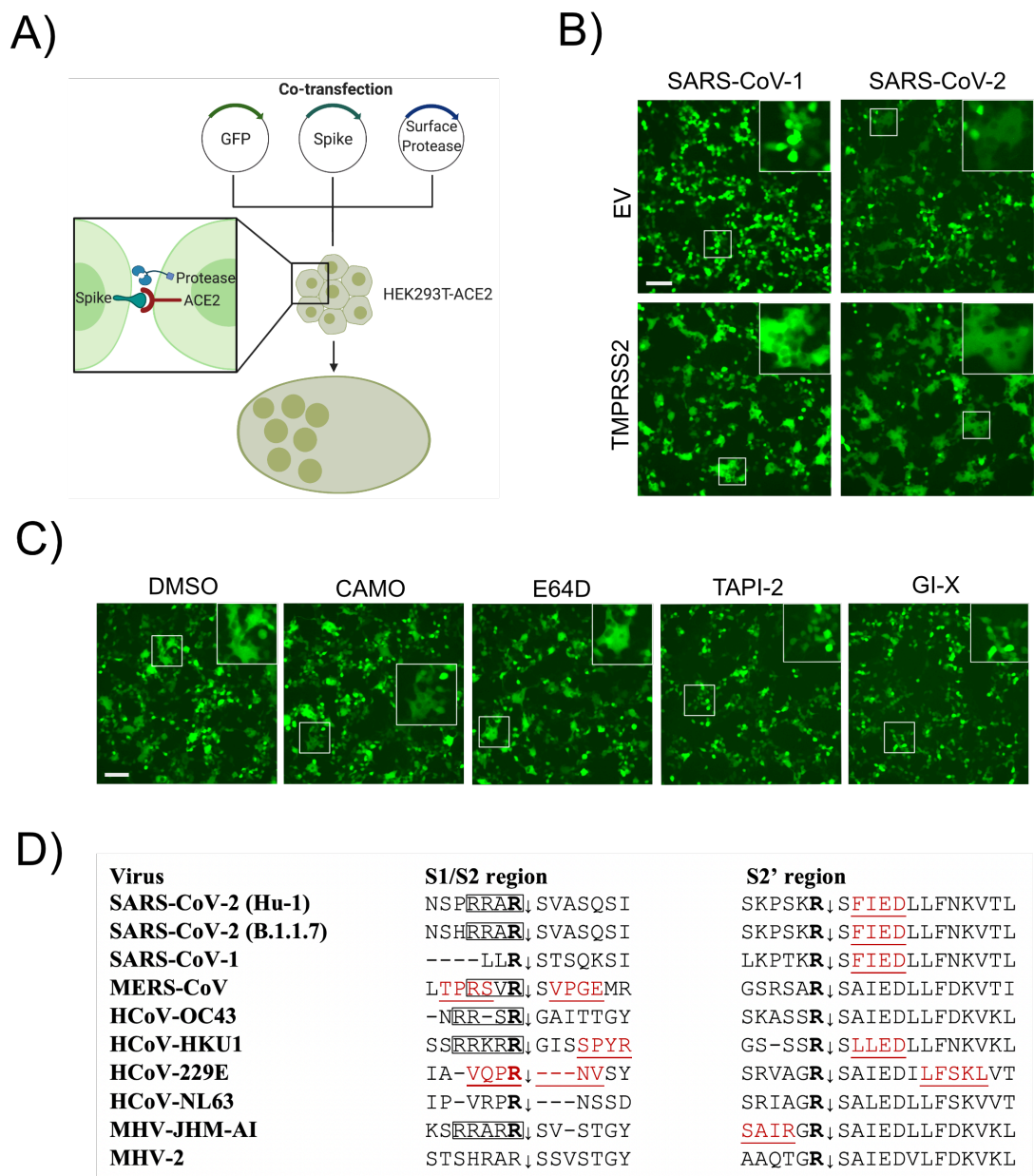


Figure 3.3 Single population cell-cell fusion assay recapitulates SARS-CoV-2 TMPRSS2-independent fusion, which can be abrogated by MMP-9 inhibitor GI-X. Figure caption can be found on following page.

Figure 3.3 Single population cell-cell fusion assay recapitulates SARS-CoV-2 TMPRSS2-independent fusion, which can be abrogated by MMP-9 inhibitor GI-X. **A)** Single-population cell-cell fusion assay was developed by co-transfecting HEK293t-ACE2 cells with GFP, SARS-CoV-1 or -2 S, and TMPRSS2 or empty vector (EV). Figure was made using biorender.com **B)** SARS-CoV-1 S or SARS-CoV-2 S was co-expressed with TMPRSS2 or EV. **C)** Protease inhibitors camostat (25 μ M; TMPRSS inhibitor), E64d (10 μ M; cathepsin inhibitor), Tapi-2 (40 μ M; MMP inhibitor), GI-X (10 μ M; MMP-9 inhibitor), or DMSO (vehicle control) was added directly after transfection. Cells were imaged 16 h post-transfection. These data represent n=3. These data were collected with equal contribution from K. Fu and A. Phan. **D)** S1/S2 and S2' regions were aligned using www.ebi.ac.uk/Tools/msa/clustalo. Arrows indicate the canonical cleavage my trypsin-like proteases. *In situ* protease cleavage predictor PROSPER (prosper.erc.monash.edu.au) identified putative MMP-9 cleavage sites on various human CoV S S1/S2 and S2' regions (red underlined motifs). Polybasic furin cleavage sites are indicated as rectangles. Human CoV S were acquired from NCBI. SARS-CoV-2 Hu-1 (GenBank QOS45029.1); SARS-CoV-1 (GenBank QJE50589.1); MERS-CoV (GenBank QBM11748.1); HCoV-OC43 (GenBank AIX10763.1); HCoV-HKU1 (GenBank AYN64561.1); HCoV-229E (GenBank APT69890.1); HCoV-NL63 (GenBank APF29063.1); MHV-JHM-SD (GenBank P22432.1); and MHV-2 (GenBank AAF19386.1). Scale bar = 100 μ m.

requirements for SARS-CoV-1 and SARS-CoV-2 entry in various cell types, I used a panel of protease inhibitors. To ensure protease inhibitors did not impact transgene integration, I also generated lentivirus pseudotyped with the G protein from vesicular stomatitis virus (VSV), which does not require proteolytic processing (Gaudin, 2000; Kima et al., 2017).

We screened four different cell-lines, including HEK293t-ACE2, Calu-3, Huh7.5, and HT1080-ACE2 cells. During pseudovirus entry in HEK293t-ACE2 cells, I found that E64d treatment reduced SARS-CoV-1 and -2 pseudoviruses entry by 90% and 77%, respectively (Figure 3.4A). Although camostat did not impact entry of either CoV pseudoviruses, Tapi-2 treatment modestly reduced entry for both SARS-CoV-1 and SARS-CoV-2 pseudoviruses by 18% and 35%, respectively (Figure 3.4A).

CoV pseudotype entry in Calu-3 cells revealed that both SARS-CoV-1 and SARS-CoV-2 pseudoviruses predominantly enter in a TMPRSS-dependent pathway, as entry was severely reduced upon camostat treatment (94% and 88%, respectively) (Figure 3.4B). Whereas, both broad-spectrum MMP inhibitor Tapi-2 and MMP-9 inhibitor GI-X did not significantly reduce entry for either SARS-CoV-1 or SARS-CoV-2 pseudoviruses. Furthermore, both viruses were modestly impacted by E64d treatment, suggesting that the late entry pathway has a limited role for SARS-CoV-1 and SARS-CoV-2 entry in Calu-3 cells.

Upon infection with SARS-CoV-1 and SARS-CoV-2 pseudoviruses in Huh7.5, I found that SARS-CoV-1 entry was predominantly sensitive to late entry pathway inhibition, as E64d reduced entry by 90% (Figure 3.4C). SARS-CoV-2 pseudovirus entry also appeared to be sensitive to late pathway inhibition, but to a lesser extent, as E64d reduced entry by ~40%. In fact, none of the inhibitors tested could reduce SARS-CoV-2 pseudovirus entry more than 50%.

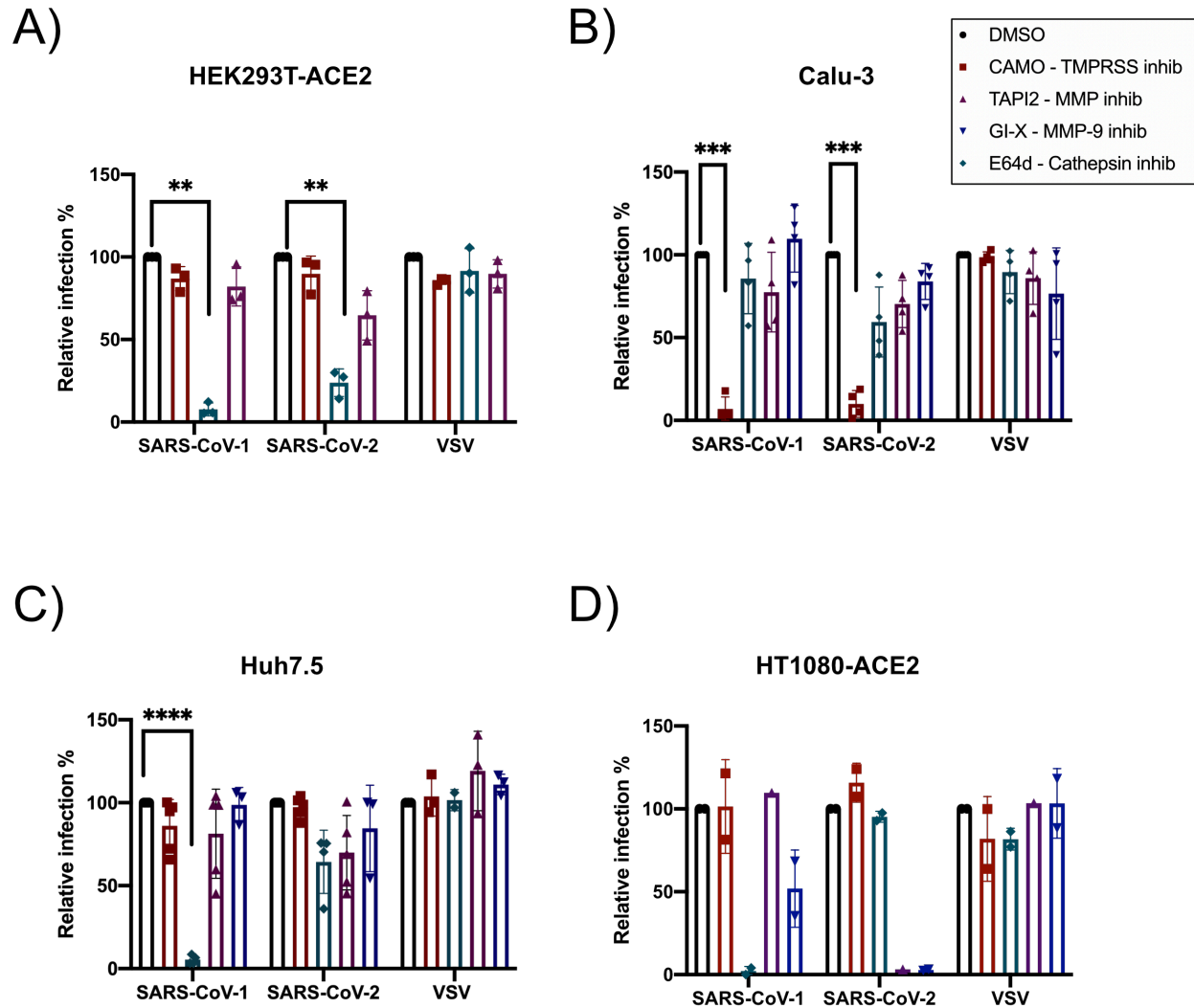


Figure 3.4 SARS-CoV-2 pseudovirus entry is cell-type dependent and can be blocked by Tapi-2 and GI-X. Cells including. A) HEK293T-ACE2, n=3, B) Calu-3, n=4, C) Huh7.5, n≥2, D) HT1080-ACE2, n≥1, were pre-treated for 1 h with the 25 μ M Camostat, 10 μ M E64d, 10 μ M, 40 μ M Tapi-2, 10 μ M GI-X or DMSO. HT1080 cells were treated with 40 μ M GI-X. Cells were then infected with lentiviral pseudoviruses bearing the SARS-CoV-2 S, SARS-CoV-1 S, or VSV-G. After 48 h cells were fixed and supplemented with X-gal and β -galactosidase overnight at 37°C. Infected cell were quantified manually for the entire well of each condition as follows. Relative infection was calculated as infected cells in the indicated inhibitor treatment relative to infection in DMSO treatment. The impact of inhibitors on infection compared to DMSO was analyzed using a one-way ANOVA and Dunnett's post-hoc analysis. $p < 0.0332$ (*), 0.0021 (), 0.0002 (***), < 0.0001 (****); error bars represent SD. K. F contributed equally to HEK293t-ACE2, Calu-3, and Huh7.5 pseudovirus entry assays. HT1080-ACE2 pseudovirus experiments were conducted by K.F & C.S. n=2, except Tapi-2, n=1.**

Lastly, fellow graduate students in the Côté lab screened SARS-CoV-1 and SARS-CoV-2 pseudovirus entry in HT1080-ACE2. They found that both CoV pseudovirus entry was unchanged in response to camostat (Figure 3.4D) (K. Fu & C. Stewart, unpublished data). Furthermore, SARS-CoV-1 pseudovirus entry occurred predominantly via the late entry pathway, as E64d completely inhibited viral entry. However, SARS-CoV-2 pseudovirus entry was unchanged in response to E64d treatment. Instead, SARS-CoV-2 pseudovirus entry was completely inhibited by Tapi-2 and GI-X treatment, suggesting that SARS-CoV-2 pseudovirus entry into HT1080-ACE2 cells is dependent on MMP activity, whereas SARS-CoV-1 does not. We also found that SARS-CoV-1 pseudovirus entry was modestly reduced in response to GI-X, but not Tapi-2 treatment. Taken together, these data show that for both SARS-CoV-1 and SARS-CoV-2, viral entry is cell-type specific. In HEK293t-ACE2, Calu-3, and Huh7.5 cells both pseudoviruses appear to have similar protease requirements, however upon infection of HT1080-ACE2 cells, SARS-CoV-1 pseudovirus entry was sensitive to cathepsin inhibition, whereas SARS-CoV-2 pseudovirus entry was MMP-dependent.

3.5 229E syncytia formation, but not entry is abrogated by Tapi-2 and GI-X in HUVECs

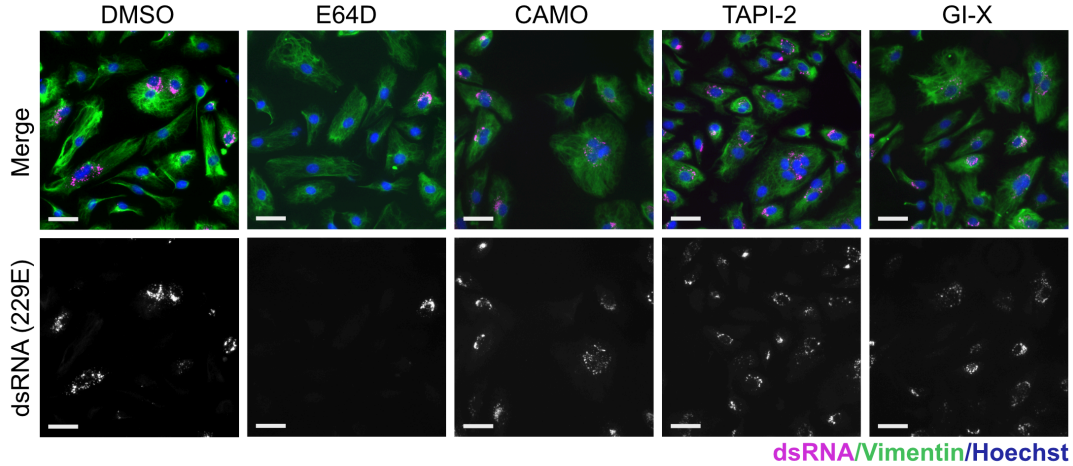
Given that chemical inhibition of MMP-9 altered SARS-CoV-2 pseudotype entry in a cell type-dependent manner, I sought to determine if this remains true in the context of authentic coronavirus infection. To test this in a containment-level 2 facility, I developed an infectious model for a related human CoV, 229E in primary human umbilical vein endothelial cells (HUVECs). Cells were fixed 12 h post-infection (hpi) and dsRNA was stained to distinguish 229E-infected cells, and vimentin was stained to visualize cell morphology. I found that HUVECs were susceptible to 229E infection, and notably, infection with 229E triggered robust

formation of multinucleated syncytia (Figure 3.5A & 3.6A). Furthermore, *in situ* protease prediction analysis with PROSPER identified putative MMP-9 cleavage sites at both the S1/S2 and the S2' of 229E S (Figure 3.3D). If true, this means that MMP-9 may be the sole protease necessary for 229E S priming and triggering.

To determine the protease requirements for 229E entry in HUVEC, cells were treated with camostat, E64d, Tapi-2, GI-X, or vehicle control 30 min prior to infection with 229E. Using dsRNA staining as a proxy for successful entry, I found that camostat, Tapi-2, and GI-X had no impact on 229E entry. However, E64d pre-treatment resulted in a 4-fold reduction in dsRNA+ cells, suggesting that entry is cathepsin-mediated and neither TMPRSS proteases nor MMPs are playing a role during 229E entry in HUVECs (Figure 3.5).

Given that I show that 229E infection of HUVECs induced syncytium formation, to address the impact of these proteases exclusively on syncytium formation, HUVECs were first infected with 229E, then treated with the panel of protease inhibitors 1 hpi. By measuring the ratio of syncytial nuclei (≥ 3 nuclei in a cell) to total nuclei, I found that whereas camostat treatment had no impact on syncytium formation, Tapi-2 and GI-X completely abrogated syncytium formation, implying that 229E syncytium formation could rely on MMP-9 (Figure 3.6A&B). Taken together, these data suggest that 229E entry and syncytium formation are facilitated by different proteases.

A)



B)

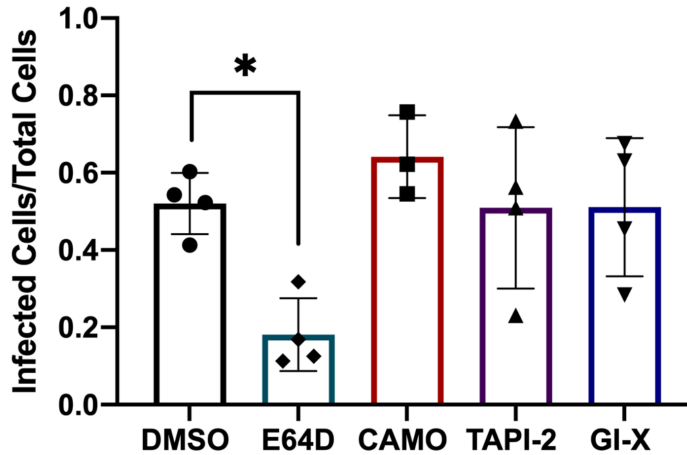


Figure 3.5 229E infection of HUVECs is diminished by E64d and unchanged by Camostat, Tapi-2, and GI-X treatment. HUVECs were pre-treated with 5 μ M E64d, 20 μ M Camostat, 20 μ M Tapi-2, 5 μ M GI-X, or vehicle control (DMSO) for 1 h before infection with $1 \times 10^{3.5}$ TCID₅₀/mL 229E. 12 hpi cells were fixed. **A)** Cells were stained for dsRNA (pink), vimentin (green; for cellular structure), and nuclei (blue). Images were captured at 20X magnification with at least 3 fields per condition and <80 cells per each biological replicate. **B)** Relative infection was quantified using CellProfiler based on dsRNA+ cells. These data represent n=3 or 4. A one-way ANOVA and Dunnett's post-hoc analysis was performed. $p < 0.0332$ (*); error bars represent SD. Scale bar = 50 μ m.

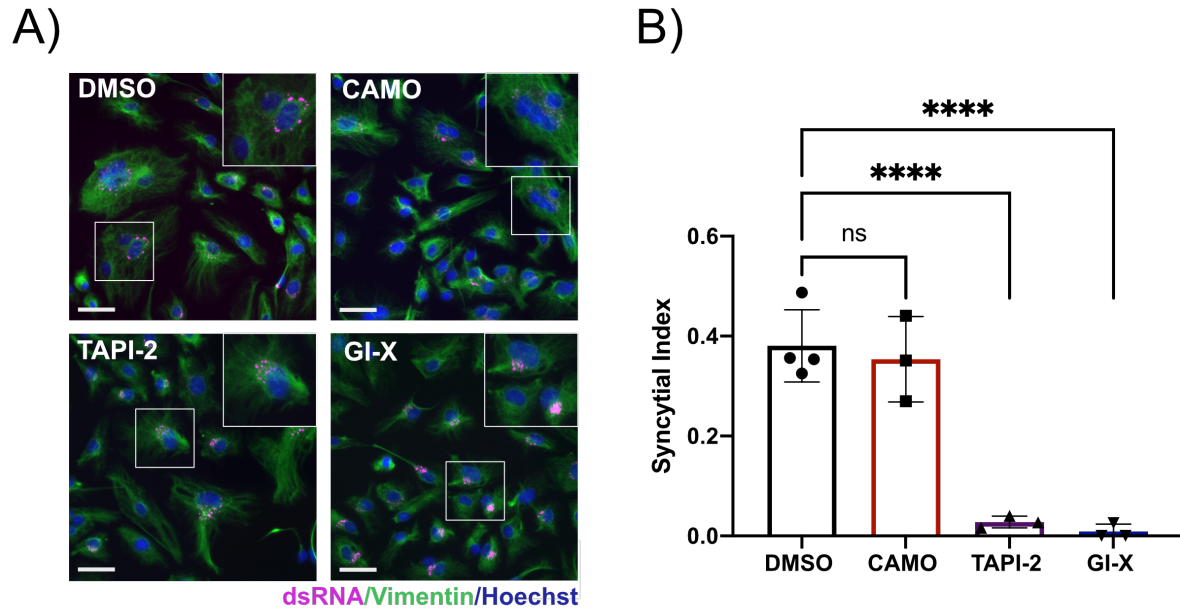


Figure 3.6 229E syncytium formation is abrogated by Tapi-2 and GI-X treatment. HUVECs were treated with 20 μ M Camostat, 20 μ M Tapi-2, 5 μ M GI-X, or vehicle control (DMSO) 1 h post-infection with $1 \times 10^{3.5}$ TCID₅₀/mL 229E. 12 hpi cells were fixed. **A)** Cells were stained for dsRNA (pink), vimentin (green; for cellular structure), and nuclei (blue). Images were captured at 20X magnification with at least 3 fields per condition and <80 cells per each biological replicate. **B)** Total cells were quantified using CellProfiler based on nuclei and syncytial nuclei were quantified manually and defined as >3 nuclei within a cell. The syncytial index represents syncytial nuclei relative to total nuclei per treatment. These data represent n=3. A one-way ANOVA with a Dunnett's post-hoc analysis was performed. $p < 0.0332$ (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****); error bars represent SD. Scale bar = 50 μ m.

3.6 229E infection of HUVECs elicits processing body disassembly with and increased MMP-9 gene expression

Our lab has previously shown that relative abundance of ARE-containing mRNAs are inversely correlated with P-body levels, and that infection with human CoV OC43 resulted in P-body loss and subsequent increased levels of various ARE-mRNAs (Robinson et al., 2020). Given that MMP-9 mRNA contains an ARE in the 3'UTR (Akool et al., 2003), I wondered if 229E infection would decrease P-bodies and cause a corresponding increase in MMP-9 mRNA abundance. To determine the effect of 229E infection on P-bodies, 229E-infected HUVECs were fixed at various times post-infection and stained for a viral marker (dsRNA) to denote infected cells, and the P-body-resident RNA helicase, DDX6 (previously shown to label PBs as in Shimada et al., 2019). I observed that P-bodies were lost during the course of 229E infection, but not lost over time in mock treated cells (Figure 3.7A). These results show that P-body counts were modestly reduced 6 hpi and diminished greater than 7-fold by 12 hpi (Figure 3.7B). To determine if reduced PB numbers coincide with changes in MMP-9 mRNA abundance, I harvested total RNA from 229E or mock infected cells 6 hpi and performed RT-qPCR for MMP-9. Consistent with the observed reduction of PBs, the steady-state levels of MMP-9 mRNA increased 3-fold compared to uninfected cells (Figure 3.7C).

3.7 Soluble MMP-9 facilitates cathepsin-independent 229E infection

To directly test if MMP-9 can facilitate 229E entry, soluble human MMP-9 that lacks the pro-domain was added to the viral inoculum for 30 min prior to HUVEC infection. Cells were then fixed 12 hpi and stained for dsRNA and vimentin (Figure 3.8A). I observed a very slight (~10%) increase in relative infection upon MMP-9 pre-treatment compared to MMP-9-

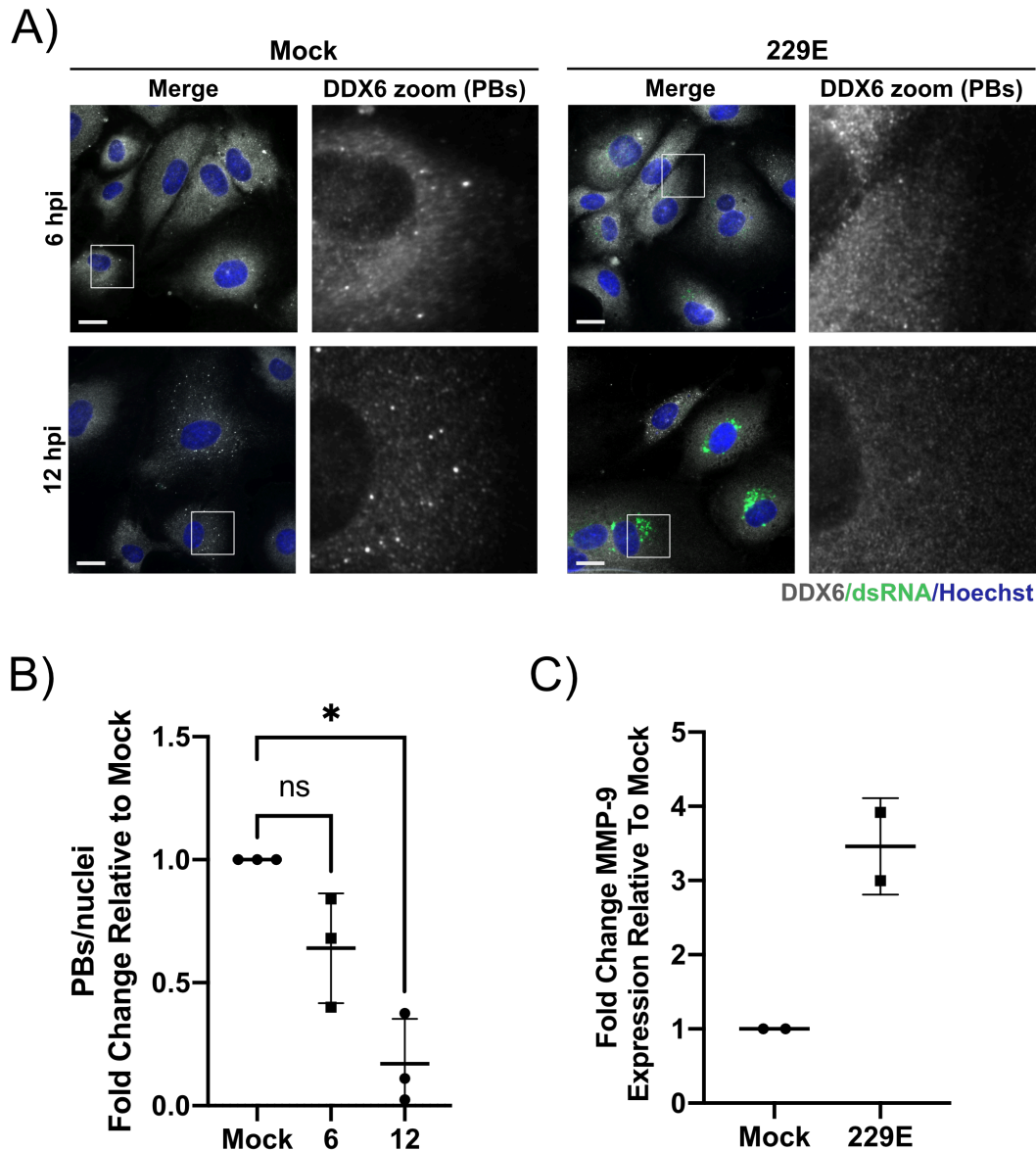


Figure 3.7 229E infection of HUVECs reduces PB numbers which correlates with an increase of MMP-9 mRNA abundance. **A)** HUVECs were infected with 229E ($1 \times 10^{3.5}$ TCID₅₀/mL) or mock infected. Samples were fixed at indicated times post-infection and stained for dsRNA (green; virus), DDX6 (white; PBs), and nuclei (blue). Images were captured at 40X magnification with oil-emersion with at least 5 fields per condition and <80 cells per each biological replicate. **B)** Using images from A, DDX6 puncta in mock or 229E-infected cells were quantified using CellProfiler. Quantification represents fold-change relative to mock infected cells. A one-way ANOVA with a Dunnett's post-hoc analysis was performed. $p < 0.0332$ (*); error bars represent SD. These data represent $n=3$. **C)** RT-qPCR was performed for MMP-9 mRNA on mock-infected or 229E-infected HUVECs at 6 hpi. $n=2$; error bars represent SD. Scale bar = 20 μ m.

negative mock treatment. Given that I previously showed that 229E entry in HUVECs is sensitive to E64d treatment, to improve the dynamic range of this assay, I pre-treated HUVECs with E64d prior to infection with MMP-9-treated 229E. Consistent with my previous findings, I found that E64d treatment reduced 229E infection compared to without E64d. Furthermore, I found that compared to the MMP-9-negative treatment, MMP-9 pre-treatment increased relative infection by ~2-fold (Figure 3.8C-D).

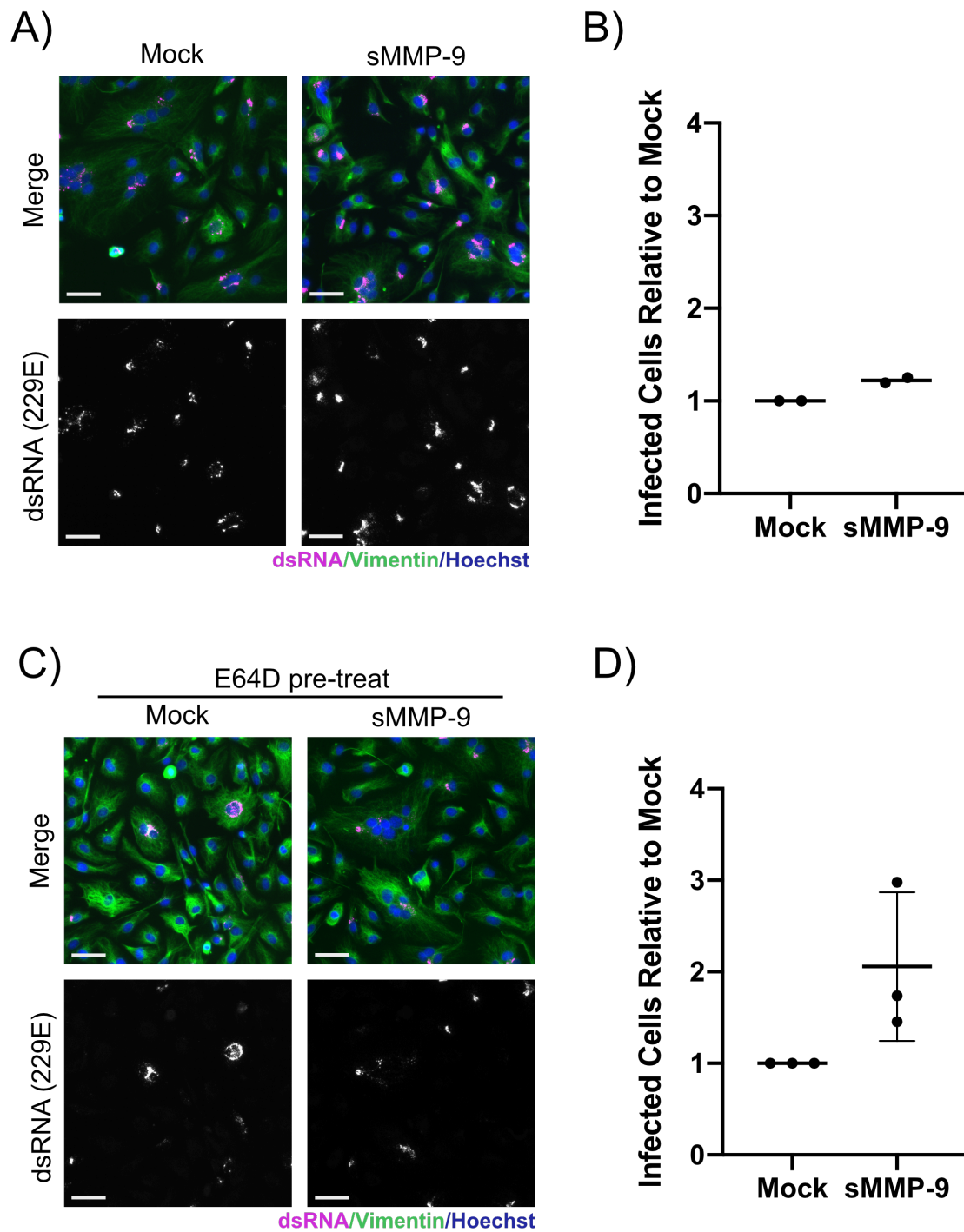


Figure 3.8 Soluble MMP-9 rescues E64d-mediated 229E entry inhibition in HUVECs.
Figure caption can be found on following page.

A) 3 U/mL of soluble human MMP-9 lacking the inhibitory pro-domain was added during 229E viral adsorption ($1 \times 10^{3.5}$ TCID₅₀/mL) of HUVECs. 12 hpi samples were fixed and stained for dsRNA (pink; virus), vimentin (green; cellular structure), and nuclei (blue). Images were captured at 20X magnification with at least 5 fields per condition and <100 cells per each biological replicate. **B)** Using images from A, 229E-infected cells were quantified using CellProfiler. Quantification represents fold-change relative to mock, where no sMMP-9 was added. This data represents n=2; error bars represent SD. **C)** HUVECs were treated with 5 μ M E64d for 1 h before being infected with 229E in the presence of 3 U/mL sMMP-9 as in A. **D)** Using images from C, infected cells were quantified as in B. This data represents n=3; error bars represent SD. Scale bar = 50 μ m.

CHAPTER 4 - DISCUSSION

4.1 Overview

The emergence of SARS-CoV-2 has led to over 173 million infections and 3.7 million deaths as of June 2021, resulting in an unprecedented public health crisis with far-reaching economic and societal implications (Johns Hopkins CoV resource centre, accessed June 6, 2021). SARS-CoV-2 is the seventh member of the CoV family to enter into the human population and given that these viruses seem have a remarkable ability for crossing inter-species boundaries, it likely will not be the last. The success of this family of viruses as human pathogens and their ability to cross species boundaries is, in part, facilitated by their extremely adaptable fusion requirements. Membrane fusion is one of the first and most critical barriers that must be overcome for CoV infection to occur. CoV fusion requires receptor binding and activation of the viral fusion protein (Belouzard et al., 2012; White et al., 2008). This activation is mediated by proteolytic processing of S by host proteases at two distinct sites: the S1/S2 junction and the S2' site (Belouzard et al., 2009, 2012). Access to S-activating proteases is required for CoV fusion, however different CoVs use distinct proteases to complete these two cleavage events (Millet & Whittaker, 2015; J. E. Park et al., 2016; Phillips et al., 2017). Characterizing CoV protease specificity is critical to understand CoV entry, tropism, and syncytium formation, however the full spectrum of host proteases used to facilitate CoV fusion remains incomplete.

The goal of this thesis work was to identify other cellular protease capable of triggering CoV fusion, with an emphasis on SARS-CoV-2 and 229E. Using cell-cell fusion assays in tandem with pseudovirus and replication-competent virus infection assays, I identified TMPRSS13 as a novel activator of SARS-CoV-2 fusion. I also provide evidence implicating MMP-9 as a fusion activator for both SARS-CoV-2 and 229E cell-cell fusion. Additionally, I

show preliminary evidence proposing a new mechanism used by CoVs to regulate MMP-9 levels by the disassembly of processing bodies. The identification of proteases that facilitate CoV fusion is necessary to understand the fundamental biology of this fascinating group of viruses, but also can help explain the diverse CoV tropism and inform the rational design of antiviral therapeutics.

4.2 Identification of MMP-9 and TMPRSS13 as activators of SARS-CoV-2 spike-mediated cell-cell fusion

Because *in vivo* CoVs favour the early entry pathway via virion fusion at the cell surface (Hoffmann, Kleine-Weber, & Pöhlmann, 2020; Matsuyama et al., 2010), I decided to direct my focus to identifying SARS-CoV-2 S-activating proteases localized to the plasma membrane or extracellular matrix. To do this, I used cell-cell fusion assays, as they would provide a method to specifically study S proteolysis and fusion at the plasma membrane, thus serving as a proxy to CoV early entry and syncytiogenesis.

Prior to the emergence of SARS-CoV-2, all six human CoVs had been shown to use TMPRSS2 to facilitate early entry (Bertram et al., 2013; Gierer et al., 2013; Glowacka et al., 2011; Shirato et al., 2011, 2018). Consistent with the pervasive use of TMPRSS2, we and others found that TMPRSS2 also facilitates SARS-CoV-2 S fusion (Buchrieser et al., 2020; Hoffmann, Kleine-Weber, Schroeder, et al., 2020). However, I also found that SARS-CoV-2 S-mediated syncytium formation occurred in the absence of TMPRSS2 ectopic expression, suggesting that TMPRSS2 was not the only protease in this system that promoted SARS-CoV-2 S fusion (Figure 3.1B & 3.3B). This finding had two important implications: firstly, this indicated that a protease endogenously expressed by HEK293t-ACE2 cells was capable of facilitating SARS-CoV-2

fusion; and secondly, the TMPRSS-independent fusion would make screening proteases by ectopic expression more challenging than anticipated. Although TMPRSS2 co-expression increased SARS-CoV-2 fusion, due to the TMPRSS-independent fusion, this assay would not have the sensitivity to detect proteases with less potent triggering potential.

The observation that SARS-CoV-2 S can spontaneously trigger cell-cell fusion in the absence of ectopic protease expression implied that an endogenous protease was capable of processing SARS-CoV-2 S. In this way, instead of screening for potential S-triggering proteases by ectopic expression, I attempted to characterize this endogenous protease using small molecule protease inhibitors. I found that TMPRSS-independent fusion could be eliminated upon treatment with the broad-spectrum MMP inhibitor Tapi-2, implying that the endogenous protease could be an MMP. This finding not only provided preliminary evidence indicating the identity of this endogenous protease, but by removing SARS-CoV-2 S spontaneous fusion, it offered a method to screen the panel of TMPRSS proteases.

By pre-treating with Tapi-2 to remove TMPRSS-independent fusion, I was able to rescue syncytium formation by ectopically expressing TMPRSS2 as well as TMPRSS13. TMPRSS13 is expressed in several epithelial tissues, including the nasal and oral cavities, esophagus, lungs, and stomach (Kido & Okumura, 2008; Madsen et al., 2014; Zmora et al., 2014). Thus, TMPRSS13 expression in these tissues could promote SARS-CoV-2 entry and syncytium formation, contributing to its broad tissue tropism. In agreement with this data, another group has since reported that ectopic expression of TMPRSS2 and TMPRSS13 enhanced SARS-CoV-2 pseudovirus entry (Laporte et al., 2021). Laporte et al (2021) also found that TMPRSS13 enhanced SARS-CoV-1 and MERS-CoV pseudovirus entry, but not 229E pseudovirus entry.

In addition to TMPRSS13, using a similar cell-cell fusion assay in HEK293t-ACE2 cells, Ou et al (2020) found that TMPRSS4, 11A, and 11E also promoted SARS-CoV-2 S fusion. However, problematically, the authors did not include a negative control for fusion, and all the proteases tested appeared to have triggered fusion (Ou et al., 2020). Thus, from these data it is impossible to differentiate SARS-CoV-2 S TMPRSS-independent fusion from TMPRSS-mediated fusion. When tested, I did not observe fusion upon ectopic expression of these TMPRSS members, however as the subcellular localization and enzymatic activity of these TMPRSS proteases were not assessed in this thesis work, the relationship between these other TMPRSS proteases and SARS-CoV-2 fusion remain unclear, and thus future studies are warranted.

One of the most striking observations from this thesis work was that SARS-CoV-2 S could mediate TMPRSS-independent fusion in HEK293t-ACE2 cells, which I found to be sensitive to MMP inhibition. Having used MMP inhibition to tease out TMPRSS13 as a SARS-CoV-2 triggering factor, I next sought to better characterize the role of MMPs during SARS-CoV-2 S fusion. I determined that an MMP-9 inhibitor GI-X could also block cell-cell fusion, thus implicating MMP-9 as a SARS-CoV-2 fusion triggering factor. These findings are reminiscent of what Phillips et al (2017) found, as they showed that mouse coronavirus MHV-JHM.SD cell-cell fusion was not affected by TMPRSS inhibition; instead syncytium formation was eliminated by broad-spectrum MMP inhibitors (Phillips et al., 2017). However, fusion from a related mouse CoV, MHV-2, was not impacted by MMP inhibitors. Together, these data could suggest that MMPs facilitate fusion for a diversity of CoVs.

In silico protease predictions support the notion that MMP-9 specifically participates in fusion for select CoVs, as I identified putative MMP-9 cleavage sites at the S2' sites of SARS-

CoV-1, HKU1, 229E, SARS-CoV-2, and MHV-JHM.SD. In all cases, these predicted MMP-9 cleavage sites occur 2-10 residues from the canonical R|S cleavage sequence (Figure 3.3D). CoVs are known to accommodate various cleavage sites in the proximity of this canonical R|S cleavage site (T. Tang et al., 2020). For example, whereas SARS-CoV-1 S2' cleavage can be facilitated by TMPRSS2 at LKPTKR|SFIED, elastase is predicted to cleave upstream at LKPT|KRSFIED (Belouzard et al., 2010; Reinke et al., 2017). Additionally, although the precise cleavage site for cathepsin L on SARS-CoV-1 S is not known, mutagenesis analysis suggests that cathepsin L does not require the canonical R|S site (Belouzard et al., 2009). Despite accommodating S2' cleavage at different locations, it is thought that cleavage at the canonical R|S facilitates optimal fusion (Belouzard et al., 2010). In this way, MMP-9 S2' cleavage at a non-canonical site should not preclude its potential fusion triggering capacity, however it may negatively impact fusogen efficiency.

With the cell-cell fusion assays I observed that SARS-CoV-2 S could spontaneously fuse upon expression in HEK293t-ACE2 cells, whereas SARS-CoV-1 S could not. Furthermore, this fusion could be blocked by MMP-9 inhibitor GI-X. However, *in silico* analysis identified putative MMP-9 cleavage sites at the S2' site for both SARS-CoV-1 S and SARS-CoV-2 S, which does not explain the observed SARS-CoV-2-specific fusion phenotype. One possible explanation for this discrepancy is that because SARS-CoV-1 S cannot be cleaved by furin at the S1/S2 junction during S biogenesis, it is likely to be less susceptible to MMP-9 proteolysis compared to the furin-processed SARS-CoV-2 S (Figure 4.1). In support of this theory, S1/S2 cleavage has been shown to promote subsequent S2' cleavage for other CoVs (Belouzard et al., 2009; J. E. Park et al., 2016). Assuming this remains true for SARS-CoV-2 S, it is likely that S1/S2 furin cleavage is critical for efficient MMP-9-mediated S2' cleavage, and thus fusion. To

test this, I recommend adding a furin site at the S1/S2 junction for SARS-CoV-1. With the addition of a furin site, I would expect SARS-CoV-1 S to have similar sensitivity to MMP-9 inhibitors as I have shown here with SARS-CoV-2 S.

In addition to human CoVs, I identified an MMP-9 cleavage site in the S2' region of mouse CoV MHV-JHM.SD S, however no MMP-9 cleavage sites could be found in the S2' region of the related CoV MHV-2 (Figure 3.3C). Phillips et al (2017) found that MHV-JHM.SD, but not MHV-2, entry and syncytium formation were sensitive to MMP inhibition, however fusion was not attributed to a particular MMP. Given that I have identified a putative MMP-9 cleavage site in the S2' region of MHV-JHM.SD but not in MHV-2, the simplest interpretation of these data would be that MMP-9 mediates S2' cleavage to promote MHV-JHM.SD membrane fusion.

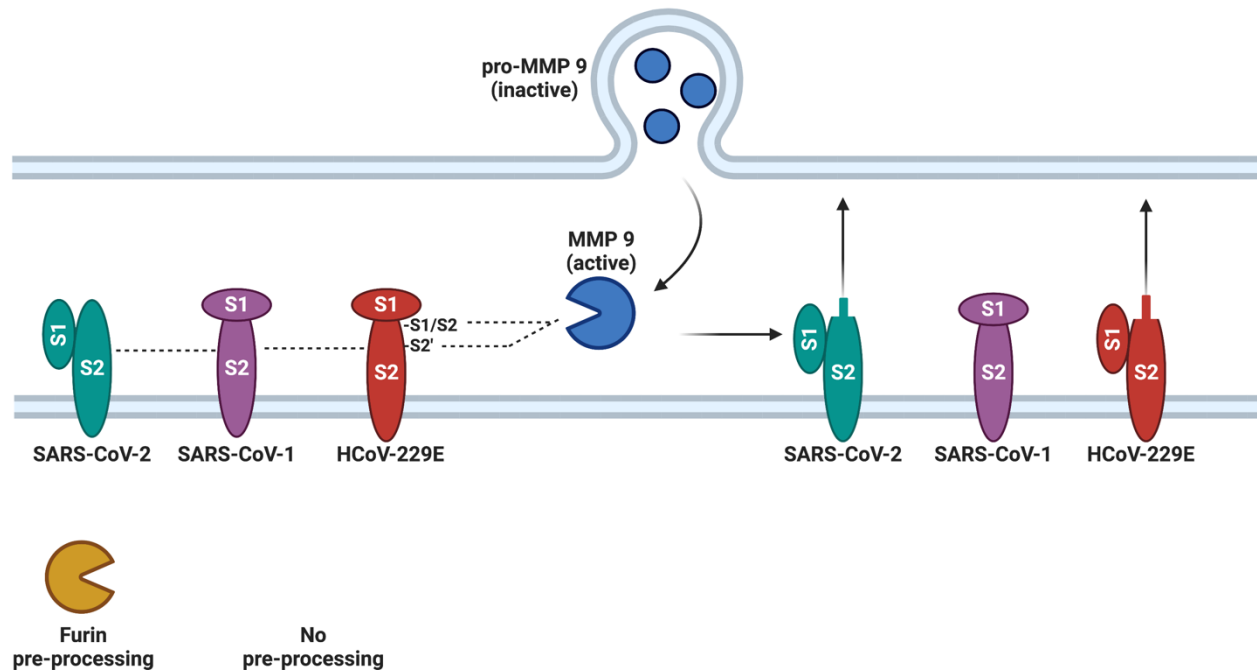


Figure 4.1 Differential furin and MMP-9 cooperativity likely impacts S fusogenic activity. Both SARS-CoV-1 S and SARS-CoV-2 S have putative MMP-9 cut sites at the S2' site, however only SARS-CoV-2 can be pre-processed by furin at the S1/S2 junction. In this way, it is possible that MMP-9 cannot efficiently cleave SARS-CoV-1 S. 229E S, like SARS-CoV-1, cannot be pre-processed by furin, however there are putative MMP-9 cleavage events at both S1/S2 and S2' of 229E S. In this way, MMP-9 may be sufficient to drive 229E S fusion. Figure was made using biorender.com

4.3 CoV pseudovirus entry is cell-type specific and is sensitive to various protease inhibitions, including MMP-9 inhibition

To determine if MMPs play a role in SARS-CoV-2 entry, we used lentiviruses pseudotyped with the S of SARS-CoV-1 or SARS-CoV-2. We found that SARS-CoV-1 and SARS-CoV-2 shared a similar cellular tropism, as Calu-3 and Huh7.5 cells were susceptible to infection, whereas HEK293t and HT1080 cells required ACE2 ectopic expression (data not shown). Despite having a similar cellular tropism, we found that SARS-CoV-1 and SARS-CoV-2 pseudoviruses used unique entry strategies in a cell-type dependent manner.

For both viruses, entry in HEK293t-ACE2 cells was predominantly skewed towards the cathepsin L-dependent pathway, as E64d significantly decreased reporter gene detection. This finding was somewhat surprising because the cell-cell fusion assay revealed that HEK293t-ACE2 cells endogenously produce an extracellular protease capable of triggering SARS-CoV-2 S fusion. For this reason, I would have expected a significant proportion of SARS-CoV-2 pseudovirus entry to occur at the cell surface, and thus be independent of cathepsin L inhibition. However, I found that E64d did not appear to impact SARS-CoV-2 pseudovirus entry to the same extent as for SARS-CoV-1 pseudoviruses. These results suggest that SARS-CoV-2 can use a cathepsin-mediated entry pathway in HEK293t-ACE2 cells but also exploits a cathepsin-independent entry route, which is less available to SARS-CoV-1 pseudoviruses (Figure 3.4A). Furthermore, although not statistically significant, MMP inhibition with Tapi-2 reduced SARS-CoV-2 pseudovirus entry, with a lesser effect on SARS-CoV-1 entry. Taken together, these findings suggest that a proportion of SARS-CoV-2 entry could be facilitated in an MMP-dependent mechanism.

However, this finding begs the question that if MMP-dependent cell-cell fusion occurs upon expression of SARS-CoV-2 S, why does it appear to play such a limited role in viral entry? Likely, the dichotomy between syncytium formation and viral entry is a result of cleavage efficiency. As previously discussed, the cell-cell fusion assay is specific to studying early entry and syncytium formation. This is because S localized on the plasma membrane can only be proteolytically processed by plasma membrane or secreted proteases and cannot be cleaved by cathepsin L. Thus, cell-cell fusion can occur even if the activating protease is extremely inefficient or present in suboptimal quantities. This is not the case during viral entry, where insufficient proteolysis at the cell surface will result in viral internalization and default to the late entry pathway, relying on cathepsin L-mediated proteolysis (Millet & Whittaker, 2018). Along these lines, it is conceivable that the protease responsible for SARS-CoV-2 cell-cell fusion is either inefficiently cleaving S or present in small quantities, thus playing a minor role in virus entry in HEK293t-ACE2.

Both SARS-CoV-1 and SARS-CoV-2 pseudovirus entry into Calu-3 cells was predominantly TMPRSS-dependent, as camostat almost completely abrogated reporter detection for both viruses. This finding complements the findings from the TMPRSS screen, as Calu-3 cells have been shown to have high TMPRSS2 and TMPRSS13 expression (Laporte et al., 2021). Furthermore, given that others have reported that Calu-3 cells have low expression of cathepsin L (Laporte et al., 2021), it makes sense that the late entry pathway could not rescue TMPRSS-inhibition (Figure 3.4B).

For pseudovirus infection of Huh7.5 cells, SARS-CoV-1 entry was predominantly via the cathepsin L-dependent pathway, whereas SARS-CoV-2 pseudovirus entry appears to be far less cathepsin L-dependent, as E64d did not significantly reduce SARS-CoV-2 pseudovirus reporter

detection (Figure 3.4C). In fact, none of the tested inhibitors significantly reduced SARS-CoV-2 pseudovirus entry. This could suggest that an additional cellular protease expressed by Huh7.5 cells is also capable of selectively activating SARS-CoV-2, circumventing dependence on cathepsin L, TMPRSS, or MMP proteases. To further characterize SARS-CoV-2 infection of Huh7.5 cells, future studies should incorporate additional broad-spectrum protease inhibitors to determine if SARS-CoV-2 entry is facilitated by a unique cellular protease.

With SARS-CoV-1 and SARS-CoV-2 pseudovirus infection of HEK293t-ACE2, Calu-3, and Huh7.5 cells only slight variations between virus entry strategies were observed, namely that SARS-CoV-2 pseudovirus entry appears to be less reliant on cathepsin L activity. MMP inhibitors Tapi-2 and GI-X did not significantly impact entry in any of these cell types. HT1080 cells have been shown to have high constitutive expression of various MMPs, including MMP-9 (Chintala et al., 1998). To find a pseudovirus entry model that recapitulates the findings from the cell-cell fusion assays, we tested pseudovirus entry in HT1080-ACE2 cells. Preliminary results obtained by C. Stewart and K. Fu found that SARS-CoV-1 pseudovirus entry was dominantly cathepsin L-dependent whereas SARS-CoV-2 pseudovirus entry was mainly MMP-dependent (Figure 3.4D). This stark distinction between SARS-CoV-1 and SARS-CoV-2 entry requirement is in agreement with the findings from the cell-cell fusion assays and further supports the notion that SARS-CoV-2, not SARS-CoV-1, can access MMPs to drive membrane fusion.

One unexpected observation from the HT1080-ACE2 infection assay was that the cathepsin L-mediated pathway was not able to rescue SARS-CoV-2 pseudovirus entry upon MMP inhibition. I expected that chemical inhibition of the early-activating proteases would decrease pseudovirus entry, but this decrease would be somewhat masked due to a redirection of the virus into the late pathway. One explanation of these data could be that cathepsin L

expression or activity is reduced in HT1080-ACE2 cells, rendering the late pathway extremely inefficient. In support of this conclusion, we have previously observed that HT1080 are refractory to cathepsin-dependent EboV pseudovirus entry (C. Stewart, unpublished data). I suspect that for both Calu-3 and HT1080-ACE2 cells, the cathepsin L pathway is extremely inefficient, explaining why blockage of early triggering proteases can sufficiently inhibit SARS-CoV-2 entry. Whereas SARS-CoV-1 pseudovirus infection of HT1080-ACE2 cells relies on cathepsin L-dependent entry, I suspect SARS-CoV-2 can circumvent this route via MMP proteolysis. However, these data convincingly show that both SARS-CoV-2 S-mediated cell-cell and virus-cell fusion can be abrogated by MMP-9 inhibition, highlighting the plasticity of SARS-CoV-2 fusion.

4.4 Chemical inhibition of MMP-9 in HUVECs does not impact 229E viral entry but abrogates virus-mediated syncytium formation

To determine the role of MMP-9 with replication-competent virus, I developed a CoV infection model using 229E infection of primary human umbilical vein endothelial cells (HUVECs). Although 229E is distantly related to SARS-CoV-2, protease prediction analysis revealed putative MMP-9 cleavage at the S2' site for both SARS-CoV-2 and 229E. Additionally, I identified a predicted MMP-9 cleavage site at the S1/S2 junction of 229E, which could reconcile the lack of a furin cleavage site (Figure 3.3D & Figure 4.1). HUVECs were chosen because, as primary endothelial cells, they have the capacity to produce several MMPs, including MMP-9 (Taraboletti et al., 2002) and there have been reports of SARS-CoV-2 infection of the endothelium (Liu et al., 2021; Puelles et al., 2020; Varga et al., 2020). In this way, I hoped 229E

infection of HUVECs would provide a suitable model to reveal the role of MMP-9 upon replication-competent CoV infection.

Here I show that 229E infection of HUVECs triggered syncytium formation, suggesting that 229E S proteolysis at both the S1/S2 and the S2' site can occur on the cell surface (Figure 3.6 & 4.1). However, I found that the establishment of viral infection was not impacted by chemical inhibition of TMPRSS or MMP-9. Instead, 229E entry was sensitive to cathepsin inhibition. Although MMP inhibition did not impact 229E entry, the addition of MMP-9 inhibitor GI-X post-infection completely abrogated syncytium formation, suggesting that although viral entry relies on endosomal cathepsin L, syncytium formation is MMP-9-dependent. Under the assumption that syncytium formation implies that there is access to a S-triggering cellular protease at the cell surface, why would this protease not participate in viral entry? This phenomenon could be explained either due to inefficient proteolysis or dynamic protease availability.

Reminiscent of my findings from the SARS-CoV-2 S cell-cell fusion and entry assay in HEK293t-ACE2 cells, it is possible that 229E S is insufficiently cleaved by MMP-9, which would result in viral endosomal internalization where fusion would occur in a cathepsin-mediated fashion. Whereas, even if MMP-9 inefficiently cleaves 229E S, plasma membrane expression of S permits more time to mediate fusion triggering with neighbouring cells.

An alternative, but not mutually exclusive, explanation for the different protease requirements for 229E entry and syncytium formation is differential protease availability pre- and post-infection. Cellular transcription and translation can be radically altered in response to viral infection (Bojkova et al., 2020; J. Chen et al., 2017; Yeo et al., 2002). It is possible that at the time of initial infection, MMP-9 is insufficiently available to drive viral entry, yet over the

course of infection, MMP-9 is upregulated, thus providing a viable mechanism to mediate cell-cell fusion and perhaps viral-cell fusion of neighbouring cells.

In support of this hypothesis, others have shown that MMP-9 tends to be maintained at low steady-state levels in various cell types, and only induced in response to a stimulus (Jang et al., 2017; Vafadari et al., 2016). MMP-9 transcription can be upregulated by inflammatory mediators, such as IL-6 (Kothari et al., 2014), which is induced in response many viral infections, including infection of HUVECs with HCoV-OC43 (Robinson et al., 2020; Velazquez-Salinas et al., 2019). However, MMP-9 has various post-transcriptional regulatory mechanisms to ensure fine-tuned protease activity. One of these regulatory mechanisms is transcriptional repression, which is facilitated by the presence of destabilizing AU-rich elements in the 3'UTR of the MMP-9 transcript (Akool et al., 2003; Huwiler et al., 2003). AREs are commonly found within highly potent mRNAs that require tightly controlled expression, such as cytokines and oncogenic mRNAs (Bakheet et al., 2006). P-bodies consist of mRNA decapping, deadenylation, and exonuclease machinery that are thought to participate in ARE-mRNA sequestration and/or turnover (Franks & Lykke-Andersen, 2007; Parker & Sheth, 2007; Sheth & Parker, 2003). Because P-bodies are constitutively present, ARE-mRNAs are normally repressed. However, upon P-body disassembly, these ARE-mRNAs are released and free to be translated (Blanco et al., 2014; Corcoran et al., 2012; Franks & Lykke-Andersen, 2007). The cellular P-body formation/disassembly status provides insight into the gene expression profile of various ARE-mRNAs, likely including MMP-9. Here, I found that 229E infection of HUVECs elicits P-body loss and results in a corresponding increase in steady-state MMP-9 abundance. This is consistent with what we previous found, as OC43-infected HUVECs had diminished P-bodies and increased steady-state ARE-mRNA levels (Robinson et al., 2020). Additionally, others have also

found that infection with hepatitis B virus and respiratory syncytial virus also increased MMP-9 synthesis, although it is unclear if P-bodies were involved (J. Chen et al., 2017; Yeo et al., 2002). Based on these data, I propose a model where the antiviral response elicited during 229E infection triggers the synthesis and secretion of these MMP-9-inducing cytokines, resulting in autocrine and paracrine MMP-9 transcription activation. This process is further exacerbated by 229E-mediated disassembly of P-bodies, which would normally buffer MMP-9 production (Figure 4.2). I provide evidence to support this hypothesis by measuring MMP-9 mRNA abundance and P-body disassembly status, however these data do not provide direct evidence of increased enzymatically active MMP-9. Downstream of post-transcriptional repression, there are additional MMP-9 regulatory mechanisms, such as pro-enzyme activation and TIMP-inhibition (Gaffney et al., 2015). Future studies should determine if 229E infection increases the production of enzymatically active MMP-9 from infected and bystander cells.

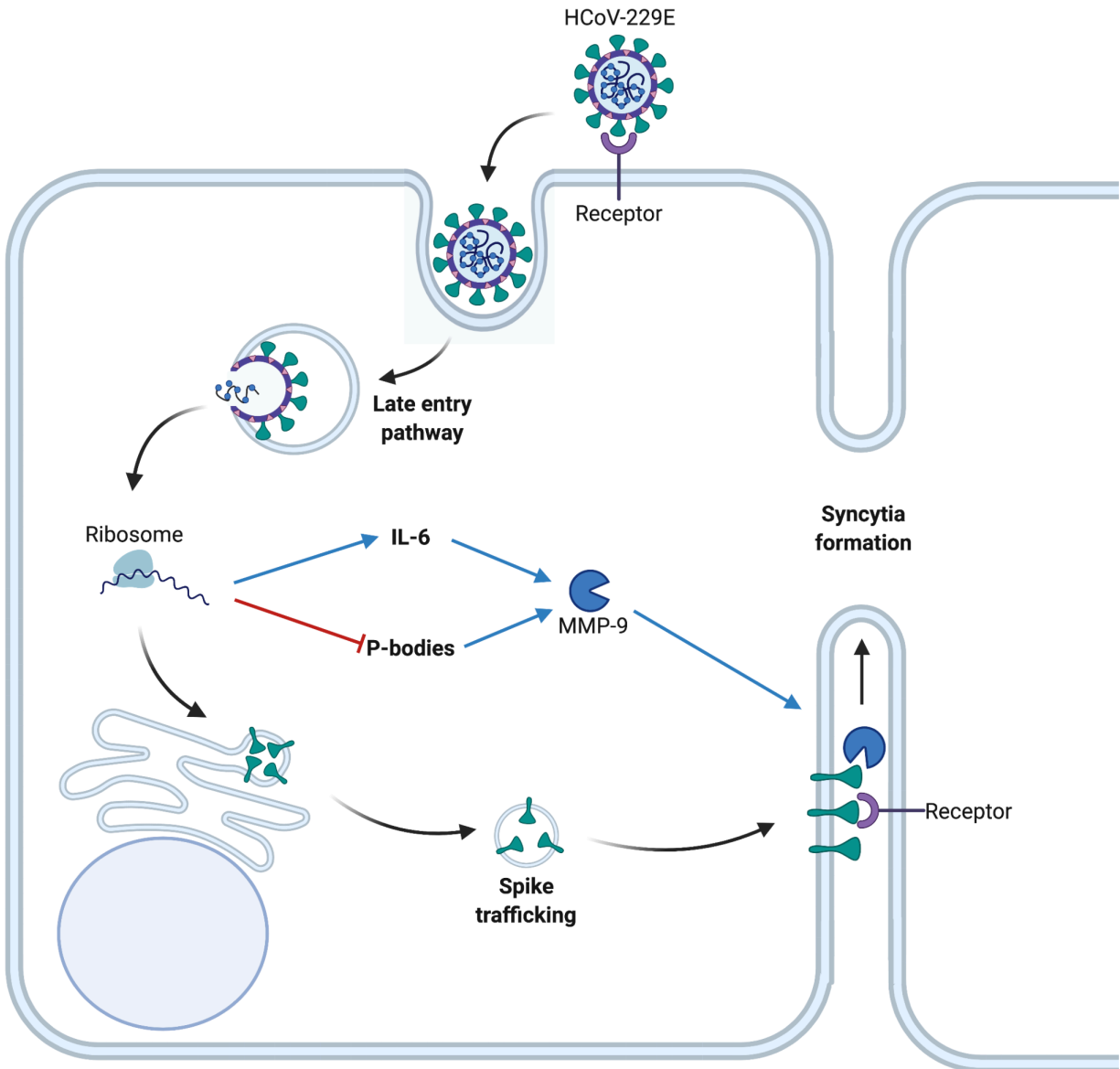


Figure 4.2 Proposed model for 229E infection and syncytium formation in HUVECs. Initial 229E entry occurs via the endocytic route due to lack of sufficient S proteolysis at the cell surface. However, during the course of infection, the HUVECs mount an antiviral response, triggering the synthesis of IL-6. Furthermore, 229E infection triggers the disassembly of P-bodies. Increased IL-6 and P-body disassembly contribute to increased MMP-9 transcription and mRNA stability, respectively. During viral assembly, a subset of 229E S is localized to the plasma membrane, where S can be cleaved at the S1/S2 and S2' with the newly produced MMP-9 mediating syncytium formation with neighbouring cells. Figure was made using biorender.com

Thus far, I have relied on small molecule compounds to characterize viral requirements for fusion. Using a panel of these compounds, I provide evidence suggesting that both SARS-CoV-2 and 229E, but not SARS-CoV-1, can exploit MMP-9 to mediate fusion. Given that both broad-spectrum MMP inhibition with Tapi-2 and a more selective MMP inhibition with GI-X were phenotypically identical, this strongly suggests a target of these compounds is playing a role in fusion for select CoVs. However, small molecule inhibitors can have multiple targets or trigger pleiotropic cellular effects. For example, in addition to inhibiting MMP-9, GI-X has been shown to have inhibitory activity against related metalloproteinases (J. H. Park et al., 2020). To directly test if MMP-9 promotes S-proteolysis during 229E entry, I pre-treated 229E inoculum with soluble MMP-9 prior to infecting HUVECs. I found that treating the virus with soluble MMP-9 did not significantly increase 229E infection by measure of dsRNA (Figure 3.8A & B). Given that 229E readily infects HUVECs via a cathepsin-mediated endosomal pathway, it is possible that even with soluble MMP-9 supplementation, inefficient MMP-9 proteolysis of S prevents a detectable shift from cathepsin-mediated entry to an MMP-9-mediated entry route. By blocking the cathepsin-mediated entry route with E64d, I found that MMP-9-treated virus modestly rescued 229E infection compared to the untreated control (Figure 3.8C & D).

Although MMP-9 treatment increased 229E infection in E64d-treated HUVECs, it did not fully restore infection as expected. Given that for other CoVs it has been shown that the early entry route is supposedly 100-1000 times more efficient than endosomal uptake (Matsuyama et al., 2005), if MMP-9 can proteolytically process 229E S, I would expect MMP-9 supplementation to more strongly rescue 229E entry. This partial rescue could be explained by the temporal regulation of virus fusogen activation. Canonically, virus fusogens must be primed and undergo receptor binding before fusogen triggering (White et al., 2008) (Figure 1.5).

Because triggering induces irreversible fusogen conformational changes, it must take place in close proximity of a susceptible host cell membrane. If both S1/S2 and S2' cleavage events occur before being bound to the cellular receptor, there is a possibility that S will enter a stable 6-helix bundle conformation before engaging the target membrane, rendering the virus fusogenically inert. This occurs when SARS-CoV-1 pseudoviruses are pre-treated with trypsin, which can cleave at both S1/S2 and S2' sites (Simmons et al., 2005). However, trypsin treatment following receptor binding promotes SARS-CoV-1 pseudovirus entry (Simmons et al., 2005). Like trypsin, MMP-9 is a soluble protease, which has predicted cut sites in both the S1/S2 and the S2' regions of 229E S. Thus, it is possible that MMP-9 can only promote 229E fusion when S2' proteolysis occurs after receptor binding (Figure 4.3). Whereas 229E pre-treatment with MMP-9 prematurely drive S into a fusogenically inert conformation.

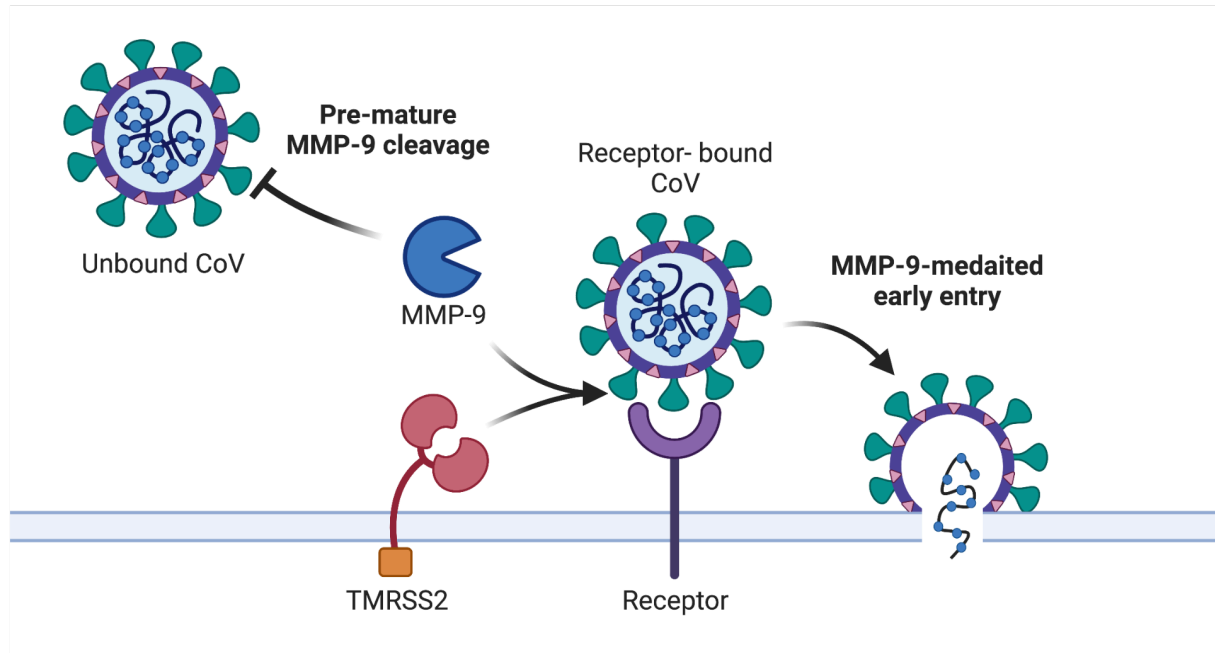


Figure 4.3 MMP-9 proteolysis could promote or prevent CoV viral fusion competency depending on timing of proteolysis. This schematic illustrates a model in which a soluble protease, such as MMP-9, could serve to promote CoV fusion via S cleaving, assuming proteolysis occurs close to the target membrane. Thus, MMP-9 could have an analogous role to a transmembrane protease, like TMRSS2. However, if MMP-9 CoV triggering occurs far from a target membrane, this could drive S into a low energy configuration, making viral fusion impossible. Figure was made using biorender.com

To determine if sMMP-9 could better rescue infection if proteolysis occurs after receptor binding, I incubated sMMP-9 and 229E with HUVECs at 4°C for 30 minutes, allowing for receptor attachment to occur, however delays virus receptor-mediated endocytosis (Chang et al., 2014). Unfortunately, HUVECs were not able to recover from low temperature treatment (data not shown). Future studies should repeat this experimental design in another susceptible cell-type than can tolerate 4°C viral binding.

4.5 Concluding remarks

CoVs comprise a diverse group of viruses that infect a wide range of animals, including humans. In humans, CoV infection is typically restricted to the respiratory tract, however for many CoVs, there have been reports of multiorgan tropism (Arbour et al., 2000; Chan et al., 2015; Gupta et al., 2020; Rosen et al., 2021). The CoV host-range and tissue tropism can be attributed to their promiscuous entry requirements. Like other enveloped viruses, to successfully infect a host cell, CoV S protein must mediate fusion between the viral and cellular membranes (White et al., 2008). For fusion to occur S must bind to the cellular receptor and be sufficiently proteolytically processed. The location of these cleavage events can influence the mechanism and efficiency of viral entry as well as facilitate viral syncytium formation, contributing to cell-to-cell spread (Belouzard et al., 2009, 2012; Millet & Whittaker, 2015). Despite the importance of host proteases to CoV infection, the diversity of protease usage has not been fully appreciated.

With the onset of the COVID-19 pandemic, various SARS-CoV-2 entry inhibitors have entered clinical trials, including hydroxychloroquine (HCQ), which blocks the cathepsin L-dependent pathway, and camostat, a TMPRSS inhibitor (National Library of Medicine - NCT04608266, 2020; T. Ou et al., 2021). In this thesis, using cell fusion assays in tandem with

pseudovirus virus infection assays, I provide the first evidence to implicate MMP-9 as a triggering factor for SARS-CoV-2 fusion. Given that MMP-9 has been shown to be increased in patients with severe COVID-19 (Iwasaki-Hozumi et al., 2021; Ueland et al., 2020), it is possible that MMP-9 may provide an alternative proteolytic option for SARS-CoV-2, allowing viral entry and syncytium formation to occur despite the use of these proposed antivirals.

Furthermore, using *in situ* protease predictions, I determined that in addition to SARS-CoV-2, endemic human CoVs 229E and HKU1, and mouse CoV MHV-JHM.SD have putative MMP-9 triggering potential. Informed by these predictions, I found that MMP-9 appears to play a limited role during 229E entry into HUVECs but is required for syncytium formation. Furthermore, I show that MMP-9 production is rapidly induced following CoV infection, suggesting that MMP-9-mediated CoV triggering could have a significant role during human CoV infection. While more research is required to fully characterize this hypothesis, this thesis work provides the first evidence implicating MMP-9 as a CoV triggering factor.

References

- Akool, E.-S., Kleinert, H., Hamada, F. M. A., Abdelwahab, M. H., Förstermann, U., Pfeilschifter, J., & Eberhardt, W. (2003). Nitric Oxide Increases the Decay of Matrix Metalloproteinase 9 mRNA by Inhibiting the Expression of mRNA-Stabilizing Factor HuR. *Molecular and Cellular Biology*, 23(14), 4901–4916. <https://doi.org/10.1128/mcb.23.14.4901-4916.2003>
- Arbour, N., Day, R., Newcombe, J., & Talbot, P. J. (2000). Neuroinvasion by Human Respiratory Coronaviruses. *Journal of Virology*. <https://doi.org/10.1128/jvi.74.19.8913-8921.2000>
- Bakheet, T., Williams, B. R. G., & Khabar, K. S. A. (2006). ARED 3.0: the large and diverse AU-rich transcriptome. *Nucleic Acids Research*. <https://doi.org/10.1093/nar/gkj052>
- Bakkers, M. J. G., Lang, Y., Feitsma, L. J., Hulswit, R. J. G., de Poot, S. A. H., van Vliet, A. L. W., Margine, I., de Groot-Mijnes, J. D. F., van Kuppeveld, F. J. M., Langereis, M. A., Huizinga, E. G., & de Groot, R. J. (2017). Betacoronavirus Adaptation to Humans Involved Progressive Loss of Hemagglutinin-Esterase Lectin Activity. *Cell Host and Microbe*. <https://doi.org/10.1016/j.chom.2017.02.008>
- Baron, J., Tarnow, C., Mayoli-Nüssle, D., Schilling, E., Meyer, D., Hammami, M., Schwalm, F., Steinmetzer, T., Guan, Y., Garten, W., Klenk, H.-D., & Böttcher-Friebertshäuser, E. (2013). Matriptase, HAT, and TMPRSS2 Activate the Hemagglutinin of H9N2 Influenza A Viruses. *Journal of Virology*. <https://doi.org/10.1128/jvi.02320-12>
- Barré, O., Dufour, A., Eckhard, U., Kappelhoff, R., Béliveau, F., Leduc, R., & Overall, C. M. (2014). Cleavage specificity analysis of six type II transmembrane serine proteases (TTSPs) using PICS with proteome-derived peptide libraries. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0105984>
- Beaudette, F., & Hudson, C. (1937). Cultivation of the virus of infectious bronchitis. *J Am Vet Med Assoc*.
- Belouzard, S., Chu, V. C., & Whittaker, G. R. (2009). Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proceedings of the National Academy of Sciences of the United States of America*, 106(14), 5871–5876. <https://doi.org/10.1073/pnas.0809524106>
- Belouzard, S., Madu, I., & Whittaker, G. R. (2010). Elastase-mediated activation of the severe acute respiratory syndrome coronavirus spike protein at discrete sites within the S2 domain. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.M110.103275>
- Belouzard, S., Millet, J. K., Licitra, B. N., & Whittaker, G. R. (2012). Mechanisms of coronavirus cell entry mediated by the viral spike protein. In *Viruses*. <https://doi.org/10.3390/v4061011>
- Benton, D. J., Wrobel, A. G., Xu, P., Roustan, C., Martin, S. R., Rosenthal, P. B., Skehel, J. J., & Gamblin, S. J. (2020). Receptor binding and priming of the spike protein of SARS-CoV-2 for membrane fusion. *Nature*, 588(7837), 327–330. <https://doi.org/10.1038/s41586-020-2772-0>
- Bertram, S., Dijkman, R., Habjan, M., Heurich, A., Gierer, S., Glowacka, I., Welsch, K., Winkler, M., Schneider, H., Hofmann-Winkler, H., Thiel, V., & Pohlmann, S. (2013a). TMPRSS2 Activates the Human Coronavirus 229E for Cathepsin-Independent Host Cell Entry and Is Expressed in Viral Target Cells in the Respiratory Epithelium. *Journal of Virology*, 87(11), 6150–6160. <https://doi.org/10.1128/jvi.03372-12>
- Bertram, S., Dijkman, R., Habjan, M., Heurich, A., Gierer, S., Glowacka, I., Welsch, K.,

- Winkler, M., Schneider, H., Hofmann-Winkler, H., Thiel, V., & Pohlmann, S. (2013b). TMPRSS2 Activates the Human Coronavirus 229E for Cathepsin-Independent Host Cell Entry and Is Expressed in Viral Target Cells in the Respiratory Epithelium. *Journal of Virology*. <https://doi.org/10.1128/jvi.03372-12>
- Blanco, F. F., Sanduja, S., Deane, N. G., Blackshear, P. J., & Dixon, D. A. (2014). Transforming Growth Factor β Regulates P-Body Formation through Induction of the mRNA Decay Factor Tristetraprolin. *Molecular and Cellular Biology*, 34(2), 180–195. <https://doi.org/10.1128/mcb.01020-13>
- Bojkova, D., Klann, K., Koch, B., Widera, M., Krause, D., Ciesek, S., Cinatl, J., & Münch, C. (2020). Proteomics of SARS-CoV-2-infected host cells reveals therapy targets. *Nature*. <https://doi.org/10.1038/s41586-020-2332-7>
- Bosch, B. J., van der Zee, R., de Haan, C. A. M., & Rottier, P. J. M. (2003). The Coronavirus Spike Protein Is a Class I Virus Fusion Protein: Structural and Functional Characterization of the Fusion Core Complex. *Journal of Virology*, 77(16), 8801–8811. <https://doi.org/10.1128/jvi.77.16.8801-8811.2003>
- Brew, K., & Nagase, H. (2010). The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. In *Biochimica et Biophysica Acta - Molecular Cell Research*. <https://doi.org/10.1016/j.bbamcr.2010.01.003>
- Buchrieser, J., Dufloo, J., Hubert, M., Monel, B., Planas, D., Rajah, M. M., Planchais, C., Porrot, F., Guivel-Benhassine, F., Van der Werf, S., Casartelli, N., Mouquet, H., Bruel, T., & Schwartz, O. (2020). Syncytia formation by SARS-CoV-2-infected cells. *The EMBO Journal*, 39(23), 1–12. <https://doi.org/10.15252/emboj.2020106267>
- Burks, J. S., Devald, B. L., Jankovsky, L. D., & Gerdes, J. C. (1980). Two coronaviruses isolated from central nervous system tissue of two multiple sclerosis patients. *Science*. <https://doi.org/10.1126/science.7403860>
- Burrage, P. S., Mix, K. S., & Brinckerhoff, C. E. (2006). Matrix metalloproteinases: Role in arthritis. In *Frontiers in Bioscience*. <https://doi.org/10.2741/1817>
- Bussani, R., Schneider, E., Zentilin, L., Collesi, C., Ali, H., Braga, L., Volpe, M. C., Colliva, A., Zanconati, F., Berlot, G., Silvestri, F., Zacchigna, S., & Giacca, M. (2020). Persistence of viral RNA, pneumocyte syncytia and thrombosis are hallmarks of advanced COVID-19 pathology. *EBioMedicine*. <https://doi.org/10.1016/j.ebiom.2020.103104>
- Carman, K. B., Calik, M., Karal, Y., Isikay, S., Kocak, O., Ozcelik, A., Yazar, A. S., Nuhoglu, C., Sag, C., Kilic, O., Dinleyici, M., Lacinel Gurlevik, S., Yimenicioglu, S., Ekici, A., Perk, P., Tosun, A., Isik, I., Yazar, C., Arslantas, D., & Dinleyici, E. C. (2019). Viral etiological causes of febrile seizures for respiratory pathogens (EFES Study). *Human Vaccines and Immunotherapeutics*. <https://doi.org/10.1080/21645515.2018.1526588>
- Cathomen, T., Naim, H. Y., & Cattaneo, R. (1998). Measles Viruses with Altered Envelope Protein Cytoplasmic Tails Gain Cell Fusion Competence. *Journal of Virology*. <https://doi.org/10.1128/jvi.72.2.1224-1234.1998>
- Chan, J. F. W., Lau, S. K. P., To, K. K. W., Cheng, V. C. C., Woo, P. C. Y., & Yue, K. Y. (2015). Middle East Respiratory syndrome coronavirus: Another zoonotic betacoronavirus causing SARS-like disease. *Clinical Microbiology Reviews*. <https://doi.org/10.1128/CMR.00102-14>
- Chandran, K., Sullivan, N. J., Felbor, U., Whelan, S. P., & Cunningham, J. M. (2005). Virology: Endosomal proteolysis of the ebola virus glycoprotein is necessary for infection. *Science*. <https://doi.org/10.1126/science.1110656>

- Chang, C. C., Wu, M., & Yuan, F. (2014). Role of specific endocytic pathways in electrotransfection of cells. *Molecular Therapy - Methods and Clinical Development*. <https://doi.org/10.1038/mtm.2014.58>
- Chen, B. (2019). Molecular Mechanism of HIV-1 Entry. In *Trends in Microbiology*. <https://doi.org/10.1016/j.tim.2019.06.002>
- Chen, J., Xu, W., Chen, Y., Xie, X., Zhang, Y., Ma, C., Yang, Q., Han, Y., Zhu, C., Xiong, Y., Wu, K., Liu, F., Liu, Y., & Wu, J. (2017). Matrix Metalloproteinase 9 Facilitates Hepatitis B Virus Replication through Binding with Type I Interferon (IFN) Receptor 1 To Repress IFN/JAK/STAT Signaling. *Journal of Virology*. <https://doi.org/10.1128/jvi.01824-16>
- Chernomordik, L. V., & Kozlov, M. M. (2008). Mechanics of membrane fusion. In *Nature Structural and Molecular Biology*. <https://doi.org/10.1038/nsmb.1455>
- Chintala, S. K., Sawaya, R., Aggarwal, B. B., Majumder, S., Giri, D. K., Kyritsis, A. P., Gokaslan, Z. L., & Rao, J. S. (1998). Induction of matrix metalloproteinase-9 requires a polymerized actin cytoskeleton in human malignant glioma cells. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.273.22.13545>
- Chung, T. W., Kim, J. R., Suh, J. Il, Lee, Y. C., Chang, Y. C., Chung, T. H., & Kim, C. H. (2004). Correlation between plasma levels of matrix metalloproteinase (MMP)-9 /MMP-2 ratio and α -fetoproteins in chronic hepatitis carrying hepatitis B virus. *Journal of Gastroenterology and Hepatology (Australia)*. <https://doi.org/10.1111/j.1440-1746.2004.03344.x>
- Ciechonska, M., Key, T., & Duncan, R. (2014). Efficient Reovirus- and Measles Virus-Mediated Pore Expansion during Syncytium Formation Is Dependent on Annexin A1 and Intracellular Calcium. *Journal of Virology*. <https://doi.org/10.1128/jvi.00121-14>
- Collier, I. E., Smith, J., Kronberger, A., Bauer, E. A., Wilhelm, S. M., Eisen, A. Z., & Goldberg, G. I. (1988). The structure of the human skin fibroblast collagenase gene. *Journal of Biological Chemistry*. [https://doi.org/10.1016/s0021-9258\(18\)38029-3](https://doi.org/10.1016/s0021-9258(18)38029-3)
- Corcoran, J. A., Khaperskyy, D. A., Johnston, B. P., King, C. A., Cyr, D. P., Olsthoorn, A. V., & McCormick, C. (2012). Kaposi's Sarcoma-Associated Herpesvirus G-Protein-Coupled Receptor Prevents AU-Rich-Element-Mediated mRNA Decay. *Journal of Virology*, 86(16), 8859–8871. <https://doi.org/10.1128/jvi.00597-12>
- Corman, Victor M., Muth, D., Niemeyer, D., & Drosten, C. (2018). Hosts and Sources of Endemic Human Coronaviruses. In *Advances in Virus Research*. <https://doi.org/10.1016/bs.aivir.2018.01.001>
- Corman, Victor Max, Baldwin, H. J., Tatenos, A. F., Zerbinati, R. M., Annan, A., Owusu, M., Nkrumah, E. E., Maganga, G. D., Oppong, S., Adu-Sarkodie, Y., Vallo, P., da Silva Filho, L. V. R. F., Leroy, E. M., Thiel, V., van der Hoek, L., Poon, L. L. M., Tschapka, M., Drosten, C., & Drexler, J. F. (2015). Evidence for an Ancestral Association of Human Coronavirus 229E with Bats. *Journal of Virology*, 89(23), 11858–11870. <https://doi.org/10.1128/jvi.01755-15>
- Corse, E., & Machamer, C. E. (2003). The cytoplasmic tails of infectious bronchitis virus E and M proteins mediate their interaction. *Virology*. [https://doi.org/10.1016/S0042-6822\(03\)00175-2](https://doi.org/10.1016/S0042-6822(03)00175-2)
- Côté, M., Zheng, Y.-M., & Liu, S.-L. (2009). Receptor Binding and Low pH Coactivate Oncogenic Retrovirus Envelope-Mediated Fusion. *Journal of Virology*. <https://doi.org/10.1128/jvi.00748-09>
- Cui, J., Li, F., & Shi, Z. L. (2019). Origin and evolution of pathogenic coronaviruses. *Nature*

- Reviews Microbiology*, 17(3), 181–192. <https://doi.org/10.1038/s41579-018-0118-9>
- De Wit, E., Van Doremalen, N., Falzarano, D., & Munster, V. J. (2016). SARS and MERS: Recent insights into emerging coronaviruses. *Nature Reviews Microbiology*, 14(8), 523–534. <https://doi.org/10.1038/nrmicro.2016.81>
- Desforges, M., Milette, T. C., Gagnon, M., & Talbot, P. J. (2007). Activation of human monocytes after infection by human coronavirus 229E. *Virus Research*. <https://doi.org/10.1016/j.virusres.2007.06.016>
- Ding, X. wei, Sun, X., Shen, X. fang, Lu, Y., Wang, J. qiang, Sun, Z. rong, Miao, C. hong, & Chen, J. wei. (2019). Propofol attenuates TNF- α -induced MMP-9 expression in human cerebral microvascular endothelial cells by inhibiting Ca²⁺/CAMK II/ERK/NF- κ B signaling pathway. *Acta Pharmacologica Sinica*. <https://doi.org/10.1038/s41401-019-0258-0>
- Dominguez, S. R., Robinson, C. C., & Holmes, K. V. (2009). Detection of four human coronaviruses in respiratory infections in children: A one-year study in Colorado. *Journal of Medical Virology*. <https://doi.org/10.1002/jmv.21541>
- Drosten, C., Günther, S., Preiser, W., van der Werf, S., Brodt, H.-R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R. A. M., Berger, A., Burguière, A.-M., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J.-C., Müller, S., ... Doerr, H. W. (2003). Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome. *New England Journal of Medicine*. <https://doi.org/10.1056/nejmoa030747>
- Eckhard, U., Huesgen, P. F., Schilling, O., Bellac, C. L., Butler, G. S., Cox, J. H., Dufour, A., Goebeler, V., Kappelhoff, R., auf dem Keller, U., Klein, T., Lange, P. F., Marino, G., Morrison, C. J., Prudova, A., Rodriguez, D., Starr, A. E., Wang, Y., & Overall, C. M. (2016). Active site specificity profiling of the matrix metalloproteinase family: Proteomic identification of 4300 cleavage sites by nine MMPs explored with structural and synthetic peptide cleavage analyses. *Matrix Biology*, 49(2016), 37–60. <https://doi.org/10.1016/j.matbio.2015.09.003>
- Enjuanes, L., Almazán, F., Sola, I., & Zuñiga, S. (2006). Biochemical aspects of coronavirus replication and virus-host interaction. In *Annual Review of Microbiology*. <https://doi.org/10.1146/annurev.micro.60.080805.142157>
- Epand, R. M. (2003). Fusion peptides and the mechanism of viral fusion. In *Biochimica et Biophysica Acta - Biomembranes*. [https://doi.org/10.1016/S0005-2736\(03\)00169-X](https://doi.org/10.1016/S0005-2736(03)00169-X)
- Finberg, K. E., Heeney, M. M., Campagna, D. R., Aydinok, Y., Pearson, H. A., Hartman, K. R., Mayo, M. M., Samuel, S. M., Strouse, J. J., Markianos, K., Andrews, N. C., & Fleming, M. D. (2008). Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nature Genetics*. <https://doi.org/10.1038/ng.130>
- Franks, T. M., & Lykke-Andersen, J. (2007). TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. *Genes and Development*. <https://doi.org/10.1101/gad.1494707>
- Fung, T. S., & Liu, D. X. (2019). Human coronavirus: Host-pathogen interaction. In *Annual Review of Microbiology*. <https://doi.org/10.1146/annurev-micro-020518-115759>
- Gaffney, J., Solomonov, I., Zehorai, E., & Sagi, I. (2015). Multilevel regulation of matrix metalloproteinases in tissue homeostasis indicates their molecular specificity in vivo. In *Matrix Biology*. <https://doi.org/10.1016/j.matbio.2015.01.012>
- Gaudin, Y. (2000). Reversibility in fusion protein conformational changes. The intriguing case of

- rhabdovirus-induced membrane fusion. In *Sub-cellular biochemistry*.
https://doi.org/10.1007/0-306-46824-7_10
- Gierer, S., Bertram, S., Kaup, F., Wrensch, F., Heurich, A., Kramer-Kuhl, A., Welsch, K., Winkler, M., Meyer, B., Drosten, C., Dittmer, U., von Hahn, T., Simmons, G., Hofmann, H., & Pohlmann, S. (2013). The Spike Protein of the Emerging Betacoronavirus EMC Uses a Novel Coronavirus Receptor for Entry, Can Be Activated by TMPRSS2, and Is Targeted by Neutralizing Antibodies. *Journal of Virology*. <https://doi.org/10.1128/jvi.00128-13>
- Glowacka, I., Bertram, S., Muller, M. A., Allen, P., Soilleux, E., Pfefferle, S., Steffen, I., Tsegaye, T. S., He, Y., Gnirss, K., Niemeyer, D., Schneider, H., Drosten, C., & Pohlmann, S. (2011). Evidence that TMPRSS2 Activates the Severe Acute Respiratory Syndrome Coronavirus Spike Protein for Membrane Fusion and Reduces Viral Control by the Humoral Immune Response. *Journal of Virology*, 85(9), 4122–4134.
<https://doi.org/10.1128/jvi.02232-10>
- Grant, O. C., Montgomery, D., Ito, K., & Woods, R. J. (2020). Analysis of the SARS-CoV-2 spike protein glycan shield reveals implications for immune recognition. *Scientific Reports*. <https://doi.org/10.1038/s41598-020-71748-7>
- Guan, Y., Zheng, B. J., He, Y. Q., Liu, X. L., Zhuang, Z. X., Cheung, C. L., Luo, S. W., Li, P. H., Zhang, L. J., Guan, Y. J., Butt, K. M., Wong, K. L., Chan, K. W., Lim, W., Shortridge, K. F., Yuen, K. Y., Peiris, J. S. M., & Poon, L. L. M. (2003). Isolation and characterization of viruses related to the SARS coronavirus from animals in Southern China. *Science*. <https://doi.org/10.1126/science.1087139>
- Gupta, A., Madhavan, M. V., Sehgal, K., Nair, N., Mahajan, S., Sehrawat, T. S., Bikkdeli, B., Ahluwalia, N., Ausiello, J. C., Wan, E. Y., Freedberg, D. E., Kirtane, A. J., Parikh, S. A., Maurer, M. S., Nordvig, A. S., Accili, D., Bathon, J. M., Mohan, S., Bauer, K. A., ... Landry, D. W. (2020). Extrapulmonary manifestations of COVID-19. *Nature Medicine*, 26(7), 1017–1032. <https://doi.org/10.1038/s41591-020-0968-3>
- Hamre, D., & Procknow, J. J. (1966). A New Virus Isolated from the Human Respiratory Tract. *Proceedings of the Society for Experimental Biology and Medicine*. <https://doi.org/10.3181/00379727-121-30734>
- Han, J., Pluhackova, K., & Böckmann, R. A. (2017). The multifaceted role of SNARE proteins in membrane fusion. In *Frontiers in Physiology*. <https://doi.org/10.3389/fphys.2017.00005>
- Higuchi, H., Bronk, S. F., Bateman, A., Harrington, K., Vile, R. G., & Gores, G. J. (2000). Viral fusogenic membrane glycoprotein expression causes syncytia formation with bioenergetic cell death: Implications for gene therapy. *Cancer Research*.
- Hoffmann, M., Kleine-Weber, H., & Pöhlmann, S. (2020). A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Molecular Cell*. <https://doi.org/10.1016/j.molcel.2020.04.022>
- Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N. H., Nitsche, A., Müller, M. A., Drosten, C., & Pöhlmann, S. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*, 181(2), 271-280.e8.
<https://doi.org/10.1016/j.cell.2020.02.052>
- Hsieh, P.-K., Chang, S. C., Huang, C.-C., Lee, T.-T., Hsiao, C.-W., Kou, Y.-H., Chen, I.-Y., Chang, C.-K., Huang, T.-H., & Chang, M.-F. (2005). Assembly of Severe Acute Respiratory Syndrome Coronavirus RNA Packaging Signal into Virus-Like Particles Is Nucleocapsid Dependent. *Journal of Virology*. <https://doi.org/10.1128/jvi.79.22.13848->

13855.2005

- Huang, I. C., Bosch, B. J., Li, F., Li, W., Kyoung, H. L., Ghiran, S., Vasilieva, N., Dermody, T. S., Harrison, S. C., Dormitzer, P. R., Farzan, M., Rottier, P. J. M., & Choe, H. (2006). SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.M508381200>
- Hui, L., Nie, Y., Li, S., Guo, M., Yang, W., Huang, R., Chen, J., Liu, Y., Lu, X., Chen, Z., Yang, Q., & Wu, Y. (2020). Matrix metalloproteinase 9 facilitates Zika virus invasion of the testis by modulating the integrity of the blood-testis barrier. *PLoS Pathogens*. <https://doi.org/10.1371/journal.ppat.1008509>
- Huwiler, A., Akool, E. S., Aschrafi, A., Hamada, F. M. A., Pfeilschifter, J., & Eberhardt, W. (2003). ATP Potentiates Interleukin-1 β -induced MMP-9 Expression in Mesangial Cells via Recruitment of the ELAV Protein HuR. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.M305722200>
- Inoue, Y., Tanaka, N., Tanaka, Y., Inoue, S., Morita, K., Zhuang, M., Hattori, T., & Sugamura, K. (2007). Clathrin-Dependent Entry of Severe Acute Respiratory Syndrome Coronavirus into Target Cells Expressing ACE2 with the Cytoplasmic Tail Deleted. *Journal of Virology*. <https://doi.org/10.1128/jvi.00253-07>
- International Committee on Taxonomy of Viruses. (2012). Virus Taxonomy: Classification and Nomenclature of Viruses. In *Ninth Report of the International Committee on Taxonomy of Viruses*.
- Iwasaki-Hozumi, H., Chagan-Yasutan, H., Ashino, Y., & Hattori, T. (2021). Blood levels of Galectin-9, an immuno-regulating molecule, reflects the severity for the acute and chronic infectious diseases. In *Biomolecules*. <https://doi.org/10.3390/biom11030430>
- Jackson, H. W., Defamie, V., Waterhouse, P., & Khokha, R. (2017). TIMPs: Versatile extracellular regulators in cancer. In *Nature Reviews Cancer*. <https://doi.org/10.1038/nrc.2016.115>
- Jahn, R., Lang, T., & Südhof, T. C. (2003). Membrane fusion. In *Cell*. [https://doi.org/10.1016/S0092-8674\(03\)00112-0](https://doi.org/10.1016/S0092-8674(03)00112-0)
- Jang, J., Jung, Y., Kim, Y., Jho, E. H., & Yoon, Y. (2017). LPS-induced inflammatory response is suppressed by Wnt inhibitors, Dickkopf-1 and LGK974. *Scientific Reports*. <https://doi.org/10.1038/srep41612>
- Jeffers, S. A., Tusell, S. M., Gillim-Ross, L., Hemmila, E. M., Achenbach, J. E., Babcock, G. J., Thomas, W. D., Thackray, L. B., Young, M. D., Mason, R. J., Ambrosino, D. M., Wentworth, D. E., DeMartini, J. C., & Holmes, K. V. (2004). CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proceedings of the National Academy of Sciences of the United States of America*. <https://doi.org/10.1073/pnas.0403812101>
- Johnson, B. A., Xie, X., Bailey, A. L., Kalveram, B., Lokugamage, K. G., Muruato, A., Zou, J., Zhang, X., Juelich, T., Smith, J. K., Zhang, L., Bopp, N., Schindewolf, C., Vu, M., Vanderheiden, A., Winkler, E. S., Swetnam, D., Plante, J. A., Aguilar, P., ... Menachery, V. D. (2021). Loss of furin cleavage site attenuates SARS-CoV-2 pathogenesis. *Nature*, August 2020. <https://doi.org/10.1038/s41586-021-03237-4>
- Kamentsky, L., Jones, T. R., Fraser, A., Bray, M. A., Logan, D. J., Madden, K. L., Ljosa, V., Rueden, C., Eliceiri, K. W., & Carpenter, A. E. (2011). Improved structure, function and compatibility for cellprofiler: Modular high-throughput image analysis software. *Bioinformatics*. <https://doi.org/10.1093/bioinformatics/btr095>

- Kawase, M., Shirato, K., Matsuyama, S., & Taguchi, F. (2009). Protease-Mediated Entry via the Endosome of Human Coronavirus 229E. *Journal of Virology*. <https://doi.org/10.1128/jvi.01933-08>
- Kessenbrock, K., Plaks, V., & Werb, Z. (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. In *Cell*. <https://doi.org/10.1016/j.cell.2010.03.015>
- Kido, H., & Okumura, Y. (2008). MSPL/TMPRSS13. In *Frontiers in Bioscience*. <https://doi.org/10.2741/2717>
- Kim, D., Lee, J. Y., Yang, J. S., Kim, J. W., Kim, V. N., & Chang, H. (2020). The Architecture of SARS-CoV-2 Transcriptome. *Cell*, 181(4), 914-921.e10. <https://doi.org/10.1016/j.cell.2020.04.011>
- Kima, I. S., Jenni, S., Stanifer, M. L., Roth, E., Whelan, S. P. J., Van Oijen, A. M., & Harrison, S. C. (2017). Mechanism of membrane fusion induced by vesicular stomatitis virus G protein. *Proceedings of the National Academy of Sciences of the United States of America*. <https://doi.org/10.1073/pnas.1618883114>
- Klein, S., Cortese, M., Winter, S. L., Wachsmuth-Melm, M., Neufeldt, C. J., Cerikan, B., Stanifer, M. L., Boulant, S., Bartenschlager, R., & Chlanda, P. (2020). SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *Nature Communications*. <https://doi.org/10.1038/s41467-020-19619-7>
- Knoops, K., Kikkert, M., Van Den Worm, S. H. E., Zevenhoven-Dobbe, J. C., Van Der Meer, Y., Koster, A. J., Mommaas, A. M., & Snijder, E. J. (2008). SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biology*. <https://doi.org/10.1371/journal.pbio.0060226>
- Kothari, P., Pestana, R., Mesraoua, R., Elchaki, R., Khan, K. M. F., Dannenberg, A. J., & Falcone, D. J. (2014). IL-6-Mediated Induction of Matrix Metalloproteinase-9 Is Modulated by JAK-Dependent IL-10 Expression in Macrophages. *The Journal of Immunology*. <https://doi.org/10.4049/jimmunol.1301906>
- Kyrieleis, O. J. P., Huber, R., Ong, E., Oehler, R., Hunter, M., Madison, E. L., & Jacob, U. (2007). Crystal structure of the catalytic domain of DESC1, a new member of the type II transmembrane serine proteinase family. *FEBS Journal*. <https://doi.org/10.1111/j.1742-4658.2007.05756.x>
- Lankelma, J. M., Voorend, D. M., Barwari, T., Koetsveld, J., Van der Spek, A. H., De Porto, A. P. N. A., Van Rooijen, G., & Van Noorden, C. J. F. (2010). Cathepsin L, target in cancer treatment? In *Life Sciences*. <https://doi.org/10.1016/j.lfs.2009.11.016>
- Laporte, M., Raeymaekers, V., van Berwaer, R., Vandeput, J., Marchand-Casas, I., Thibaut, H.-J., Van Looveren, D., Martens, K., Hoffmann, M., Maes, P., Pöhlmann, S., Naesens, L., & Stevaert, A. (2021). The SARS-CoV-2 and other human coronavirus spike proteins are fine-tuned towards temperature and proteases of the human airways. *PLoS Pathogens*, 17(4), 1–29. <https://doi.org/10.1101/2020.11.09.374603>
- Le Boeuf, F., Gebremeskel, S., McMullen, N., He, H., Greenshields, A. L., Hoskin, D. W., Bell, J. C., Johnston, B., Pan, C., & Duncan, R. (2017). Reovirus FAST Protein Enhances Vesicular Stomatitis Virus Oncolytic Virotherapy in Primary and Metastatic Tumor Models. *Molecular Therapy - Oncolytics*, 6(September), 80–89. <https://doi.org/10.1016/j.omto.2017.08.001>
- Leroy, H., Han, M., Woottum, M., Bracq, L., Bouchet, J., Xie, M., & Benichou, S. (2020). Virus-mediated cell-cell fusion. In *International Journal of Molecular Sciences*. <https://doi.org/10.3390/ijms21249644>

- Lester, S. N., & Li, K. (2014). Toll-like receptors in antiviral innate immunity. In *Journal of Molecular Biology*. <https://doi.org/10.1016/j.jmb.2013.11.024>
- Li, F. (2016). Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annual Review of Virology*, 3, 237–261. <https://doi.org/10.1146/annurev-virology-110615-042301>
- Li, Wendong, Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., Wang, H., Crameri, G., Hu, Z., Zhang, H., Zhang, J., McEachern, J., Field, H., Daszak, P., Eaton, B. T., Zhang, S., & Wang, L. F. (2005). Bats are natural reservoirs of SARS-like coronaviruses. *Science*. <https://doi.org/10.1126/science.1118391>
- Li, Wenhui, Moore, M. J., Vasllieva, N., Sui, J., Wong, S. K., Berne, M. A., Somasundaran, M., Sullivan, J. L., Luzuriaga, K., Greeneugh, T. C., Choe, H., & Farzan, M. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*. <https://doi.org/10.1038/nature02145>
- Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., & Shafie, S. (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature*. <https://doi.org/10.1038/284067a0>
- Liu, J., Li, Y., Liu, Q., Yao, Q., Wang, X., Zhang, H., Chen, R., Ren, L., Min, J., Deng, F., Yan, B., Liu, L., Hu, Z., Wang, M., & Zhou, Y. (2021). SARS-CoV-2 cell tropism and multiorgan infection. In *Cell Discovery*. <https://doi.org/10.1038/s41421-021-00249-2>
- Lokman, S. M., Rasheduzzaman, M., Salauddin, A., Barua, R., Tanzina, A. Y., Rumi, M. H., Hossain, M. I., Siddiki, A. M. A. M. Z., Mannan, A., & Hasan, M. M. (2020). Exploring the genomic and proteomic variations of SARS-CoV-2 spike glycoprotein: A computational biology approach. *Infection, Genetics and Evolution*. <https://doi.org/10.1016/j.meegid.2020.104389>
- Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., Bi, Y., Ma, X., Zhan, F., Wang, L., Hu, T., Zhou, H., Hu, Z., Zhou, W., Zhao, L., ... Tan, W. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *The Lancet*. [https://doi.org/10.1016/S0140-6736\(20\)30251-8](https://doi.org/10.1016/S0140-6736(20)30251-8)
- Madsen, D. H., Szabo, R., Molinolo, A. A., & Bugge, T. H. (2014). TMPRSS13 deficiency impairs stratum corneum formation and epidermal barrier acquisition. *Biochemical Journal*. <https://doi.org/10.1042/BJ20140337>
- Manicone, A. M., & McGuire, J. K. (2008). Matrix metalloproteinases as modulators of inflammation. In *Seminars in Cell and Developmental Biology*. <https://doi.org/10.1016/j.semcdb.2007.07.003>
- Marzi, A., Gramberg, T., Simmons, G., Möller, P., Rennekamp, A. J., Krumbiegel, M., Geier, M., Eisemann, J., Turza, N., Saunier, B., Steinkasserer, A., Becker, S., Bates, P., Hofmann, H., & Pöhlmann, S. (2004). DC-SIGN and DC-SIGNR Interact with the Glycoprotein of Marburg Virus and the S Protein of Severe Acute Respiratory Syndrome Coronavirus. *Journal of Virology*. <https://doi.org/10.1128/jvi.78.21.12090-12095.2004>
- Matsuyama, S., Nagata, N., Shirato, K., Kawase, M., Takeda, M., & Taguchi, F. (2010). Efficient Activation of the Severe Acute Respiratory Syndrome Coronavirus Spike Protein by the Transmembrane Protease TMPRSS2. *Journal of Virology*. <https://doi.org/10.1128/jvi.01542-10>
- Matsuyama, S., Ujike, M., Morikawa, S., Tashiro, M., & Taguchi, F. (2005). Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proceedings of the National Academy of Sciences of the United States of America*.

- <https://doi.org/10.1073/pnas.0503203102>
- McNew, J. A., Weber, T., Engelman, D. M., Söllner, T. H., & Rothman, J. E. (1999). The length of the flexible SNAREpin juxtamembrane region is a critical determinant of SNARE-dependent fusion. *Molecular Cell*. [https://doi.org/10.1016/S1097-2765\(00\)80343-3](https://doi.org/10.1016/S1097-2765(00)80343-3)
- Millet, J. K., & Whittaker, G. R. (2015). Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. *Virus Research*, 202, 120–134. <https://doi.org/10.1016/j.virusres.2014.11.021>
- Millet, J. K., & Whittaker, G. R. (2018). Physiological and molecular triggers for SARS-CoV membrane fusion and entry into host cells. *Virology*. <https://doi.org/10.1016/j.virol.2017.12.015>
- Mondal, S., Adhikari, N., Banerjee, S., Amin, S. A., & Jha, T. (2020). Matrix metalloproteinase-9 (MMP-9) and its inhibitors in cancer: A minireview. In *European Journal of Medicinal Chemistry*. <https://doi.org/10.1016/j.ejmech.2020.112260>
- Nagase, H., Visse, R., & Murphy, G. (2006). Structure and function of matrix metalloproteinases and TIMPs. In *Cardiovascular Research*. <https://doi.org/10.1016/j.cardiores.2005.12.002>
- National Library of Medicine - NCT04608266. (2020). CAMOVID : evaluation of Efficacy and Safety of Camostat Mesylate for the Treatment of SARS-CoV-2 Infection - COVID-19 in Ambulatory Adult Patients. <https://Clinicaltrials.Gov/Show/NCT04608266>.
- Nedialkova, D. D., Gorbalenya, A. E., & Snijder, E. J. (2010). Arterivirus Nsp1 modulates the accumulation of minus-strand templates to control the relative abundance of viral mRNAs. *PLoS Pathogens*. <https://doi.org/10.1371/journal.ppat.1000772>
- Ou, T., Mou, H., Zhang, L., Ojha, A., Choe, H., & Farzan, M. (2021). Hydroxychloroquine-mediated inhibition of SARS-CoV-2 entry is attenuated by TMPRSS2. *PLoS Pathogens*. <https://doi.org/10.1371/journal.ppat.1009212>
- Ou, X., Liu, Y., Lei, X., Li, P., Mi, D., Ren, L., Guo, L., Guo, R., Chen, T., Hu, J., Xiang, Z., Mu, Z., Chen, X., Chen, J., Hu, K., Jin, Q., Wang, J., & Qian, Z. (2020). Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nature Communications*. <https://doi.org/10.1038/s41467-020-15562-9>
- Pager, C. T., & Dutch, R. E. (2005). Cathepsin L Is Involved in Proteolytic Processing of the Hendra Virus Fusion Protein. *Journal of Virology*. <https://doi.org/10.1128/jvi.79.20.12714-12720.2005>
- Pallesen, J., Wang, N., Corbett, K. S., Wrapp, D., Kirchdoerfer, R. N., Turner, H. L., Cottrell, C. A., Becker, M. M., Wang, L., Shi, W., Kong, W. P., Andres, E. L., Kettenbach, A. N., Denison, M. R., Chappell, J. D., Graham, B. S., Ward, A. B., & McLellan, J. S. (2017). Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. *Proceedings of the National Academy of Sciences of the United States of America*. <https://doi.org/10.1073/pnas.1707304114>
- Park, J. E., Li, K., Barlan, A., Fehr, A. R., Perlman, S., McCray, P. B., & Gallagher, T. (2016). Proteolytic processing of middle east respiratory syndrome coronavirus spikes expands virus tropism. *Proceedings of the National Academy of Sciences of the United States of America*. <https://doi.org/10.1073/pnas.1608147113>
- Park, J. H., Choi, J. Y., Jo, C., & Koh, Y. H. (2020). Involvement of ADAM10 in acrolein-induced astrocytic inflammation. *Toxicology Letters*. <https://doi.org/10.1016/j.toxlet.2019.10.005>
- Parker, R., & Sheth, U. (2007). P Bodies and the Control of mRNA Translation and Degradation. In *Molecular Cell*. <https://doi.org/10.1016/j.molcel.2007.02.011>

- Perfettini, J. L., Castedo, M., Roumier, T., Andreau, K., Nardacci, R., Piacentini, M., & Kroemer, G. (2005). Mechanisms of apoptosis induction by the HIV-1 envelope. In *Cell Death and Differentiation*. <https://doi.org/10.1038/sj.cdd.4401584>
- Petersen, E., Koopmans, M., Go, U., Hamer, D. H., Petrosillo, N., Castelli, F., Storgaard, M., Al Khalili, S., & Simonsen, L. (2020). Comparing SARS-CoV-2 with SARS-CoV and influenza pandemics. In *The Lancet Infectious Diseases*. [https://doi.org/10.1016/S1473-3099\(20\)30484-9](https://doi.org/10.1016/S1473-3099(20)30484-9)
- Petrany, M. J., & Millay, D. P. (2019). Cell Fusion: Merging Membranes and Making Muscle. In *Trends in Cell Biology*. <https://doi.org/10.1016/j.tcb.2019.09.002>
- Phillips, J. M., Gallagher, T., & Weiss, S. R. (2017). Neurovirulent Murine Coronavirus JHM.SD Uses Cellular Zinc Metalloproteases for Virus Entry and Cell-Cell Fusion. *Journal of Virology*. <https://doi.org/10.1128/jvi.01564-16>
- Puelles, V. G., Lütgehetmann, M., Lindenmeyer, M. T., Sperhake, J. P., Wong, M. N., Allweiss, L., Chilla, S., Heinemann, A., Wanner, N., Liu, S., Braun, F., Lu, S., Pfefferle, S., Schröder, A. S., Edler, C., Gross, O., Glatzel, M., Wichmann, D., Wiech, T., ... Huber, T. B. (2020). Multiorgan and Renal Tropism of SARS-CoV-2. *New England Journal of Medicine*. <https://doi.org/10.1056/nejmc2011400>
- Qi, M.-Y., Wang, Z.-Z., Zhang, Z., Shao, Q., Zeng, A., Li, X.-Q., Li, W.-Q., Wang, C., Tian, F.-J., Li, Q., Zou, J., Qin, Y.-W., Brewer, G., Huang, S., & Jing, Q. (2012). AU-Rich-Element-Dependent Translation Repression Requires the Cooperation of Tristetraprolin and RCK/P54. *Molecular and Cellular Biology*. <https://doi.org/10.1128/mcb.05340-11>
- Raj, V. S., Mou, H., Smits, S. L., Dekkers, D. H. W., Müller, M. A., Dijkman, R., Muth, D., Demmers, J. A. A., Zaki, A., Fouchier, R. A. M., Thiel, V., Drosten, C., Rottier, P. J. M., Osterhaus, A. D. M. E., Bosch, B. J., & Haagmans, B. L. (2013). Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature*, 495(7440), 251–254. <https://doi.org/10.1038/nature12005>
- Regan, A. D., & Whittaker, G. R. (2008). Utilization of DC-SIGN for Entry of Feline Coronaviruses into Host Cells. *Journal of Virology*. <https://doi.org/10.1128/jvi.01094-08>
- Reineke, L. C., & Lloyd, R. E. (2013). Diversion of stress granules and P-bodies during viral infection. In *Virology*. <https://doi.org/10.1016/j.virol.2012.11.017>
- Reinke, L. M., Spiegel, M., Plegge, T., Hartleib, A., Nehlmeier, I., Gierer, S., Hoffmann, M., Hofmann-Winkler, H., Winkler, M., & Pöhlmann, S. (2017). Different residues in the SARS-CoV spike protein determine cleavage and activation by the host cell protease TMPRSS2. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0179177>
- Reusken, C. B. E. M., Haagmans, B. L., Müller, M. A., Gutierrez, C., Godeke, G. J., Meyer, B., Muth, D., Raj, V. S., Vries, L. S. De, Corman, V. M., Drexler, J. F., Smits, S. L., El Tahir, Y. E., De Sousa, R., van Beek, J., Nowotny, N., van Maanen, K., Hidalgo-Hermoso, E., Bosch, B. J., ... Koopmans, M. P. G. (2013). Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: A comparative serological study. *The Lancet Infectious Diseases*. [https://doi.org/10.1016/S1473-3099\(13\)70164-6](https://doi.org/10.1016/S1473-3099(13)70164-6)
- Robinson, C.-A., Kleer, M., Mulloy, R. P. ., Castle, E. L., Boudreau, B. Q., & Corcoran, J. A. (2020). Human coronaviruses disassemble processing bodies. *BioRxiv*.
- Rockx, B., Kuiken, T., Herfst, S., Bestebroer, T., Lamers, M. M., Munnink, B. B. O., De Meulder, D., Van Amerongen, G., Van Den Brand, J., Okba, N. M. A., Schipper, D., Van Run, P., Leijten, L., Sikkema, R., Verschoor, E., Verstrepen, B., Bogers, W., Langermans, J., Langermans, J., ... Haagmans, B. L. (2020). Comparative pathogenesis of COVID-19,

- MERS, and SARS in a nonhuman primate model. *Science*.
<https://doi.org/10.1126/science.abb7314>
- Rosen, H. R., O'Connell, C., Nadim, M. K., DeClerck, B., Sheibani, S., DePasquale, E., Sanossian, N., Blodget, E., & Angell, T. (2021). Extrapulmonary manifestations of severe acute respiratory syndrome coronavirus-2 infection. In *Journal of Medical Virology*.
<https://doi.org/10.1002/jmv.26595>
- Ruch, T. R., & Machamer, C. E. (2012). The coronavirus E protein: Assembly and beyond. In *Viruses*. <https://doi.org/10.3390/v4030363>
- Schoeman, D., & Fielding, B. C. (2019). Coronavirus envelope protein: Current knowledge. In *Virology Journal*. <https://doi.org/10.1186/s12985-019-1182-0>
- Scott, H. S., Kudoh, J., Wattenhofer, M., Shibuya, K., Berry, A., Chrast, R., Guipponi, M., Wang, J., Kawasaki, K., Asakawa, S., Minoshima, S., Younus, F., Mehdi, S. Q., Radhakrishna, U., Papasavvas, M. P., Gehrig, C., Rossier, C., Korostishevsky, M., Gal, A., ... Antonarakis, S. E. (2001). Insertion of β -satellite repeats identifies a transmembrane protease causing both congenital and childhood onset autosomal recessive deafness. *Nature Genetics*. <https://doi.org/10.1038/83768>
- Sheth, U., & Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science*, 300(5620), 805–808. <https://doi.org/10.1126/science.1082320>
- Shi, Y., Barton, K., De Maria, A., Petrash, J. M., Shiels, A., & Bassnett, S. (2009). The stratified syncytium of the vertebrate lens. *Journal of Cell Science*. <https://doi.org/10.1242/jcs.045203>
- Shimada, R., Kiso, M., & Saga, Y. (2019). ES-mediated chimera analysis revealed requirement of DDX6 for NANOS2 localization and function in mouse germ cells. *Scientific Reports*.
<https://doi.org/10.1038/s41598-018-36502-0>
- Shirato, K., Kawase, M., & Matsuyama, S. (2018). Wild-type human coronaviruses prefer cell-surface TMPRSS2 to endosomal cathepsins for cell entry. *Virology*.
<https://doi.org/10.1016/j.virol.2017.11.012>
- Shirato, K., Matsuyama, S., Ujike, M., & Taguchi, F. (2011). Role of Proteases in the Release of Porcine Epidemic Diarrhea Virus from Infected Cells. *Journal of Virology*.
<https://doi.org/10.1128/jvi.00464-11>
- Shulla, A., Heald-Sargent, T., Subramanya, G., Zhao, J., Perlman, S., & Gallagher, T. (2011). A Transmembrane Serine Protease Is Linked to the Severe Acute Respiratory Syndrome Coronavirus Receptor and Activates Virus Entry. *Journal of Virology*.
<https://doi.org/10.1128/jvi.02062-10>
- Simmons, G., Gosalia, D. N., Rennekamp, A. J., Reeves, J. D., Diamond, S. L., & Bates, P. (2005). Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. *Proceedings of the National Academy of Sciences of the United States of America*.
<https://doi.org/10.1073/pnas.0505577102>
- Siu, Y. L., Teoh, K. T., Lo, J., Chan, C. M., Kien, F., Escriou, N., Tsao, S. W., Nicholls, J. M., Altmeyer, R., Peiris, J. S. M., Bruzzone, R., & Nal, B. (2008). The M, E, and N Structural Proteins of the Severe Acute Respiratory Syndrome Coronavirus Are Required for Efficient Assembly, Trafficking, and Release of Virus-Like Particles. *Journal of Virology*.
<https://doi.org/10.1128/jvi.01052-08>
- Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. *Journal of General Virology*, 94(PART10), 2141–2163.
<https://doi.org/10.1099/vir.0.056341-0>
- Sodroski, J., Goh, W. C., Rosen, C., Campbell, K., & Haseltine, W. A. (1986). Role of the

- HTLV-III/LAV envelope in syncytium formation and cytopathicity. *Nature*.
<https://doi.org/10.1038/322470a0>
- Sola, I., Mateos-Gomez, P. A., Almazan, F., Zuñiga, S., & Enjuanes, L. (2011). RNA-RNA and RNA-protein interactions in coronavirus replication and transcription. In *RNA Biology*.
<https://doi.org/10.4161/hv.8.2.14991>
- Stewart, J. N., Mounir, S., & Talbot, P. J. (1992). Human coronavirus gene expression in the brains of multiple sclerosis patients. *Virology*. [https://doi.org/10.1016/0042-6822\(92\)90220-J](https://doi.org/10.1016/0042-6822(92)90220-J)
- Szabo, R., & Bugge, T. H. (2008). Type II transmembrane serine proteases in development and disease. In *International Journal of Biochemistry and Cell Biology*.
<https://doi.org/10.1016/j.biocel.2007.11.013>
- Tang, T., Bidon, M., Jaimes, J. A., Whittaker, G. R., & Daniel, S. (2020). Coronavirus membrane fusion mechanism offers a potential target for antiviral development. *Antiviral Research*, 178(March), 104792. <https://doi.org/10.1016/j.antiviral.2020.104792>
- Tang, Y., Liu, J., Zhang, D., Xu, Z., Ji, J., & Wen, C. (2020). Cytokine Storm in COVID-19: The Current Evidence and Treatment Strategies. *Frontiers in Immunology*, 11(July), 1–13.
<https://doi.org/10.3389/fimmu.2020.01708>
- Taraboletti, G., D'Ascenzo, S., Borsotti, P., Giavazzi, R., Pavan, A., & Dolo, V. (2002). Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *American Journal of Pathology*.
[https://doi.org/10.1016/S0002-9440\(10\)64887-0](https://doi.org/10.1016/S0002-9440(10)64887-0)
- Thépaut, M., Luczkowiak, J., Vivès, C., Labiod, N., Bally, I., Lasala, F., Grimoire, Y., Fenel, D., Sattin, S., Thielens, N., Schoehn, G., Bernardi, A., Delgado, R., & Fieschi, F. (2020). DC/L-SIGN recognition of spike glycoprotein promotes SARS-CoV-2 trans-infection and can be inhibited by a glycomimetic antagonist. *BioRxiv*.
- To, K. F., Tong, J. H. M., Chan, P. K. S., Au, F. W. L., Chim, S. S. C., Chan, K. C. A., Cheugn, J. L. K., Liu, E. Y. M., Tse, G. M. K., Lo, A. W. I., Lo, Y. M. D., & Ng, H. K. (2004). Tissue and cellular tropism of the coronavirus associated with severe acute respiratory syndrome: An in-situ hybridization study of fatal cases. *Journal of Pathology*.
<https://doi.org/10.1002/path.1510>
- Tyrrell, D. A., & Bynoe, M. L. (1966). Cultivation of viruses from a high proportion of patients with colds. *Lancet*. [https://doi.org/10.1016/s0140-6736\(66\)92364-6](https://doi.org/10.1016/s0140-6736(66)92364-6)
- Ueland, T., Holter, J. C., Holten, A. R., Müller, K. E., Lind, A., Bekken, G. K., Dudman, S., Aukrust, P., Dyrhol-Riise, A. M., & Heggelund, L. (2020). Distinct and early increase in circulating MMP-9 in COVID-19 patients with respiratory failure: MMP-9 and respiratory failure in COVID-19. *Journal of Infection*, 81(3), e41–e43.
<https://doi.org/10.1016/j.jinf.2020.06.061>
- V'kovski, P., Kratzel, A., Steiner, S., Stalder, H., & Thiel, V. (2021). Coronavirus biology and replication: implications for SARS-CoV-2. In *Nature Reviews Microbiology*.
<https://doi.org/10.1038/s41579-020-00468-6>
- Vaarala, M. H., Porvari, K. S., Kellokumpu, S., Kyllönen, A. P., & Vihko, P. T. (2001). Expression of transmembrane serine protease TMPRSS2 in mouse and human tissues. *Journal of Pathology*. [https://doi.org/10.1002/1096-9896\(2000\)9999:9999::AID-PATH743>3.0.CO;2-T](https://doi.org/10.1002/1096-9896(2000)9999:9999::AID-PATH743>3.0.CO;2-T)
- Vafadari, B., Salamian, A., & Kaczmarek, L. (2016). MMP-9 in translation: from molecule to brain physiology, pathology, and therapy. In *Journal of Neurochemistry*.

- <https://doi.org/10.1111/jnc.13415>
- Vale-Costa, S., & Amorim, M. J. (2016). Recycling endosomes and viral infection. In *Viruses*. <https://doi.org/10.3390/v8030064>
- Van Der Hoek, L., Pyrc, K., Jebbink, M. F., Vermeulen-Oost, W., Berkhout, R. J. M., Wolthers, K. C., Wertheim-Van Dillen, P. M. E., Kaandorp, J., Spaargaren, J., & Berkhout, B. (2004). Identification of a new human coronavirus. *Nature Medicine*. <https://doi.org/10.1038/nm1024>
- Varga, Z., Flammer, A. J., Steiger, P., Haberecker, M., Andermatt, R., Zinkernagel, A. S., Mehra, M. R., Schuepbach, R. A., Ruschitzka, F., & Moch, H. (2020). Endothelial cell infection and endotheliitis in COVID-19. In *The Lancet*. [https://doi.org/10.1016/S0140-6736\(20\)30937-5](https://doi.org/10.1016/S0140-6736(20)30937-5)
- Velazquez-Salinas, L., Verdugo-Rodriguez, A., Rodriguez, L. L., & Borca, M. V. (2019). The role of interleukin 6 during viral infections. In *Frontiers in Microbiology*. <https://doi.org/10.3389/fmicb.2019.01057>
- Vijgen, L., Keyaerts, E., Moës, E., Thoelen, I., Wollants, E., Lemey, P., Vandamme, A.-M., & Van Ranst, M. (2005). Complete Genomic Sequence of Human Coronavirus OC43: Molecular Clock Analysis Suggests a Relatively Recent Zoonotic Coronavirus Transmission Event. *Journal of Virology*. <https://doi.org/10.1128/jvi.79.3.1595-1604.2005>
- Wang, H., Yang, P., Liu, K., Guo, F., Zhang, Y., Zhang, G., & Jiang, C. (2008). SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. *Cell Research*. <https://doi.org/10.1038/cr.2008.15>
- Wang, P., Dai, J., Bai, F., Kong, K.-F., Wong, S. J., Montgomery, R. R., Madri, J. A., & Fikrig, E. (2008). Matrix Metalloproteinase 9 Facilitates West Nile Virus Entry into the Brain. *Journal of Virology*. <https://doi.org/10.1128/jvi.00314-08>
- Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., & Wiley, D. C. (1997). Atomic structure of the ectodomain from HIV-1 gp41. *Nature*. <https://doi.org/10.1038/387426a0>
- White, J. M., Delos, S. E., Brecher, M., & Schornberg, K. (2008). Structures and mechanisms of viral membrane fusion proteins: Multiple variations on a common theme. In *Critical Reviews in Biochemistry and Molecular Biology*. <https://doi.org/10.1080/10409230802058320>
- White, J. M., & Whittaker, G. R. (2016). Fusion of Enveloped Viruses in Endosomes. In *Traffic*. <https://doi.org/10.1111/tra.12389>
- Wölfel, R., Corman, V. M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M. A., Niemeyer, D., Jones, T. C., Vollmar, P., Rothe, C., Hoelscher, M., Bleicker, T., Brünink, S., Schneider, J., Ehmman, R., Zwirgmaier, K., Drosten, C., & Wendtner, C. (2020). Virological assessment of hospitalized patients with COVID-2019. *Nature*. <https://doi.org/10.1038/s41586-020-2196-x>
- Woo, P. C. Y., Lau, S. K. P., Chu, C., Chan, K., Tsoi, H., Huang, Y., Wong, B. H. L., Poon, R. W. S., Cai, J. J., Luk, W., Poon, L. L. M., Wong, S. S. Y., Guan, Y., Peiris, J. S. M., & Yuen, K. (2005). Characterization and Complete Genome Sequence of a Novel Coronavirus, Coronavirus HKU1, from Patients with Pneumonia. *Journal of Virology*, 79(2), 884–895. <https://doi.org/10.1128/jvi.79.2.884-895.2005>
- Woo, P. C. Y., Lau, S. K. P., Huang, Y., & Yuen, K. Y. (2009). Coronavirus diversity, phylogeny and interspecies jumping. In *Experimental Biology and Medicine*. <https://doi.org/10.3181/0903-MR-94>
- Wu, Z., Harrich, D., Li, Z., Hu, D., & Li, D. (2020). The unique features of SARS-CoV-2

- transmission: Comparison with SARS-CoV, MERS-CoV and 2009 H1N1 pandemic influenza virus. *Reviews in Medical Virology*, August, 1–11.
<https://doi.org/10.1002/rmv.2171>
- Yeo, S. J., Yun, Y. J., Lyu, M. A., Woo, S. Y., Woo, E. R., Kim, S. J., Lee, H. J., Park, H. K., & Kook, Y. H. (2002). Respiratory syncytial virus infection induces matrix metalloproteinase-9 expression in epithelial cells. *Archives of Virology*. <https://doi.org/10.1007/s705-002-8316-1>
- Yoon, T. Y., & Munson, M. (2018). SNARE complex assembly and disassembly. In *Current Biology*. <https://doi.org/10.1016/j.cub.2018.01.005>
- Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. M. E., & Fouchier, R. A. M. (2012). Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *New England Journal of Medicine*. <https://doi.org/10.1056/nejmoa1211721>
- Zhivotovsky, B., & Kroemer, G. (2004). Apoptosis and genomic instability. In *Nature Reviews Molecular Cell Biology*. <https://doi.org/10.1038/nrm1443>
- Zhou, P., Yang, X. Lou, Wang, X. G., Hu, B., Zhang, L., Zhang, W., Si, H. R., Zhu, Y., Li, B., Huang, C. L., Chen, H. D., Chen, J., Luo, Y., Guo, H., Jiang, R. Di, Liu, M. Q., Chen, Y., Shen, X. R., Wang, X., ... Shi, Z. L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, 579(7798), 270–273.
<https://doi.org/10.1038/s41586-020-2012-7>
- Zhou, W., Wang, W., Wang, H., Lu, R., & Tan, W. (2013). First infection by all four non-severe acute respiratory syndrome human coronaviruses takes place during childhood. *BMC Infectious Diseases*. <https://doi.org/10.1186/1471-2334-13-433>
- Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G. F., & Tan, W. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *New England Journal of Medicine*. <https://doi.org/10.1056/nejmoa2001017>
- Zmora, P., Blazejewska, P., Moldenhauer, A.-S., Welsch, K., Nehlmeier, I., Wu, Q., Schneider, H., Pohlmann, S., & Bertram, S. (2014). DESC1 and MSPL Activate Influenza A Viruses and Emerging Coronaviruses for Host Cell Entry. *Journal of Virology*.
<https://doi.org/10.1128/jvi.01427-14>
- Zou, L., Ruan, F., Huang, M., Liang, L., Huang, H., Hong, Z., Yu, J., Kang, M., Song, Y., Xia, J., Guo, Q., Song, T., He, J., Yen, H.-L., Peiris, M., & Wu, J. (2020). SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. *New England Journal of Medicine*. <https://doi.org/10.1056/nejmc2001737>
- Zumla, A., Chan, J. F. W., Azhar, E. I., Hui, D. S. C., & Yuen, K. Y. (2016). Coronaviruses-drug discovery and therapeutic options. In *Nature Reviews Drug Discovery*.
<https://doi.org/10.1038/nrd.2015.37>

Contribution of collaborators

This thesis work contains intellectual contribution from the entire Côté lab and Corcoran lab. Additionally, some of the data included in this thesis work consists of a portion of a manuscript in preparation, with co-first authors including, Alex Phan, Kathy Fu, Geneviève Laroche, and myself. Because of this, some of the work shown here was of equal or entire contribution to other members of the Côté lab. In these cases, contribution is listed in the figure captions. In short, the pseudovirus infection assays were completed in equal contributions with Kathy Fu, with the exception of the HT1080-ACE2 pseudovirus infection, which was completed exclusively by Dr. Corina Stewart and Kathy Fu. Additionally, the single population cell-fusion assay was contributed by Alex Phan and Kathy Fu. Lastly, the CellProfiler pipeline used to identify infected cells and processing bodies was developed by Mariel Kleer.

Curriculum Vitae

Academic Background

MSc Student in Biochemistry, Microbiology and Immunology
University of Ottawa Aug. 2019 – Aug. 2021 (expected)

Bachelor of Science, with Honours in Microbiology and Immunology
Dalhousie University Sept. 2015 – June 2019

Publications & Pre-prints

Fu, K*, Laroche, G*, **Mulloy, R. P***, Phan, A*, Stewart, C. M., Hagan, M., Fink, C., Prévost, J., Beaudoin-Bussi res, G., Yockell-Lelievre, J., Stanford, W. L., Gigu re, P., Dekaban, G., Finzi, A., Dikeakos, J.D., Kobasa, D., C  t  , M. Identification of host proteases required for SARS-CoV-2 Spike-mediated membrane fusion. Manuscript in preparation.

Rosin, N. L*, Jaffer, A., Sinha, S., **Mulloy, R. P.**, Robinson, C., Labit, E., Almeida L. G., Dufour, A., Corcoran, J. A., Yipp, B., Bieraskie, J. (2021). SARS-CoV-2 infection of circulating immune cells is not responsible for virus dissemination in severe COVID-19 patients. BioRxiv pre-print server. doi: 10.1101/2021.01.19.427282

Robinson, C*, Kleer, M*, **Mulloy, R. P***, Castle, E. L., Boudreau B. B., Corcoran, J. A. (2020). Human coronaviruses disassembly processing bodies. BioRxiv pre-print server. doi: 10.1101/2020.11.08.372995

Bo, Y*, Qiu, S*, **Mulloy, R. P.**, C  t  , M. (2020) Filoviruses use the HOPS complex and UVRAG to traffic to Niemann-Pick C1 compartments during viral entry. Journal of Virology. 94(16). doi:10.1128/JVI.01002-20

Awards

Ontario Graduate Scholarship, University of Ottawa	2020
University of Ottawa Admissions Scholarship, University of Ottawa	2019
Award for Outstanding Academic Achievement, Dalhousie University	2015-2019
Undergraduate Entrance and In-Course Scholarship, Dalhousie University	2015-2019

Research Experience

MSc Student – M. C  t   Lab/J. Corcoran
University of Ottawa/ University of Calgary Aug. 2019 – Present

Honours Student
Dalhousie University 2018 - 2019

Trainee Mentorship

Direct supervisor to Mathias Ntivuguruzwa, 2020