Identification of MMP-9 as a driving factor in SARS-CoV-2 entry

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Abstract

Since its emergence in December 2019, SARS-CoV-2 has infected over 200 million people globally. SARS-CoV-2 spike (S) decorates the viral envelope and is responsible for facilitating viral entry into the host cell. To mediate membrane fusion, S must be proteolytically cleaved. For the closely related SARS-CoV S, cleavage at the host cell surface must be facilitated by the serine protease TMPRSS2. We demonstrated that SARS-CoV-2 S can facilitate fusion independent of TMPRSS2 and sought to identify other proteases capable of driving SARS-CoV-2 S-mediated fusion. We show that the ADAMs and MMP inhibitor GI 254023X is capable of substantially reducing SARS-CoV-2 S-mediated syncytium formation. Additionally, we identified MMP-9, a protein target of GI 254023X, as a host protease capable of enhancing SARS-CoV-2 lentivirus entry in HEK293T-ACE2 cells. These results implicate ADAM and MMP proteases, in particular MMP-9, as potential antiviral drug targets against COVID-19 pathogenesis.

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List of Abbreviations

ACE2	Antiogensin-converting enzyme 2
ADAM	A disintegrin and metalloproteinase
ADAM10	A disintegrin and metalloproteinase 10
ADAM17	A disintegrin and metalloproteinase 17 (alternatively known as TACE)
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
DESC	Differentially expressed in squamous cell carcinoma
E	Envelope protein
ECM	Extracellular matrix
ERGIC	Endoplasmic reticulum-Golgi intermediate compartment
GI-X	GI 254023X
HA	Hemagglutinin
HAT	Human airway trypsin-like protease
HCoV	Human coronavirus
HR1/HR2	Heptad repeat 1/heptad repeat 2
IVA	Influenza virus A
M	Matrix protein
MERS-CoV	Middle east respiratory syndrome coronavirus
MHV	Mouse hepatitis virus
MMP	Matrix metalloproteinase
MMP-9	Matrix metalloproteinase 9 (alternatively known as Gelatinase B)
N	Nucleoprotein
nsp	Non-structural protein
NTD	N-terminal domain
RBD	Receptor binding domain
RdRp	RNA-dependent RNA polymerase
RSV	Respiratory syncytial virus
S	Spike protein
SARS-CoV	Severe acute respiratory syndrome coronavirus
TIMP	Tissue inhibitor of metalloprotease
TM	Transmembrane domain
TMPRSS	Transmembrane serine protease
TMPRSS2	Transmembrane serine protease 2
TMRPRSS13	Transmembrane serine protease 13
TTSP	Type II serine protease
VSV	Vesicular stomatitis virus

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1.0 Introduction

1.1 Human Coronaviruses: Brief History

Coronaviruses, part of the family *Coronaviridae*, are known to cause respiratory and enteric diseases. They can infect both humans and animals, and members of the *Coronaviridae* family can be subdivided into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. Alphacoronaviruses and Betacoronaviruses infect mammals, Gammacoronaviruses infect avian species, and Deltacoronaviruses infect both. To date, there are seven know coronaviruses which can infect and cause disease in humans (HCoVs). HCoV-229E and HCoV-NL63 are alphacoronaviruses while HCoV-OC43, HCoV-HKU1, Middle East Respiratory Syndrome coronavirus (MERS-CoV), Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), and SARS-CoV-2 are betacoronaviruses. All seven HCoVs were introduced to the human population through zoonotic transmission with their parental viruses likely originating in bats or rodents[1]. Of the seven human coronaviruses, HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1 are community-acquired and cause mild respiratory symptoms, fever, and fatigue in human hosts. In contrast, SARS-CoV, MERS-CoV, and SARS-CoV-2 are highly pathogenic and have been known to cause severe respiratory distress. The pathogenic HCoVs emerged in the human population through zoonotic transmission in 2003, 2012, and 2019, respectively[2,3].

In December 2019, a novel coronavirus emerged from Wuhan, Hubei Province, China, and was subsequently named SARS-CoV-2[4,5]. While the other two pathogenic coronaviruses, SARS-CoV and MERS-CoV, were quickly eliminated in humans, SARS-CoV-2 has proven to have much higher transmissibility and stability in the human population. It is the causative agent of the ongoing COVID-19 pandemic and as of June 2021, has infected over 200 million and killed over

4.5 million people globally, rendering it an urgent public health concern. Phylogenetic analysis of SARS-CoV-2 placed it in the betacoronavirus genus, which is made of up four lineages: A, B, C, an D. Like its close relative, SARS-CoV, with whom it shares 79% sequence homology, SARS-CoV-2 is a lineage B beta-CoV[6].

1.2 Coronavirus structure and life cycle

Coronaviruses are enveloped viruses with a single-stranded positive-sense RNA (+ssRNA) genome about 27 – 30 kilobases in size, the largest of all RNA viruses. Structurally, the virus is composed of the matrix protein (M), envelope protein (E), and nucleoprotein (N), which acts as a scaffold for the RNA genome. The spike protein (S) decorates the viral surface, resembling a crown, or *corona* in Latin and drives viral entry into target cells **(Schematic 1A)**[3].

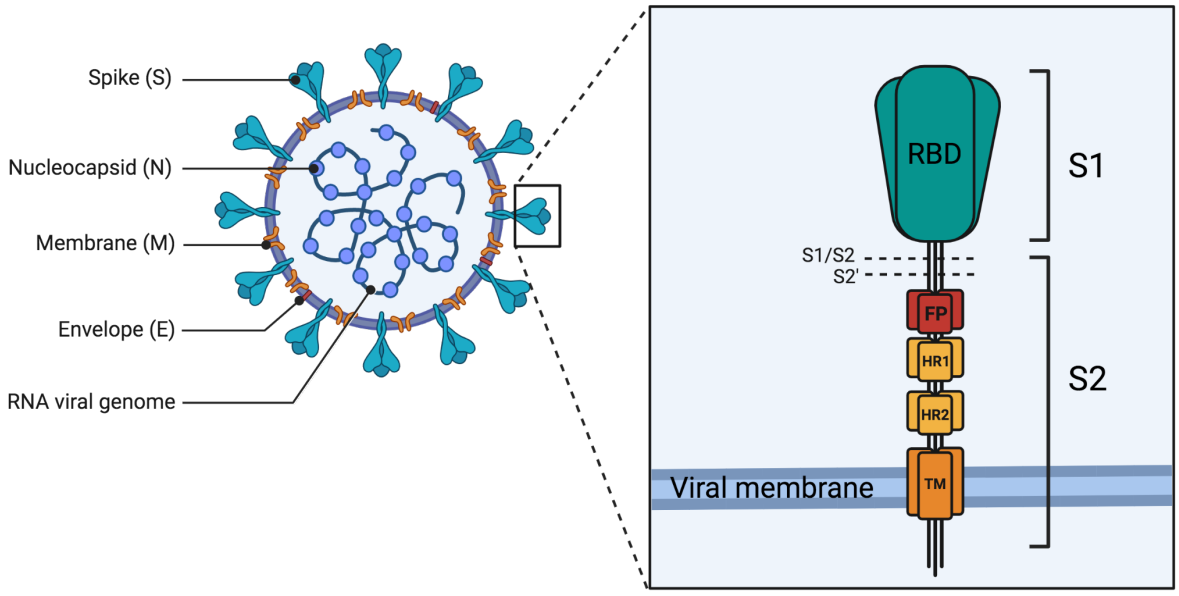
The first step of the coronavirus life cycle, entry, is initiated when the viral S protein engages the host cell receptor. S is comprised of two subunits, S1 and S2, responsible for 1) facilitating viral attachment to the host cell receptor and 2) mediating fusion between the viral and host cell membranes, respectively. S binding to the cell receptor allows it to be cleaved by host proteases at the S1/S2 junction and then at a downstream site called S2', sequentially. This can occur at the cell surface (early pathway) or in the endosome by cysteine proteases (late entry pathway). Proteolytic cleavage triggers the spike protein to undergo conformational rearrangements and facilitate fusion between the CoV and host cell membrane, releasing the viral genome into the cell[3].

Once inside the cell, +ssRNA translation of two major reading frames, ORF1a and ORF1b, containing the 16 non-structural proteins (nsps), and of the four structural proteins (E, M, N, and S) can commence. The role of nsps include RNA synthesis, proofreading, and modification as well

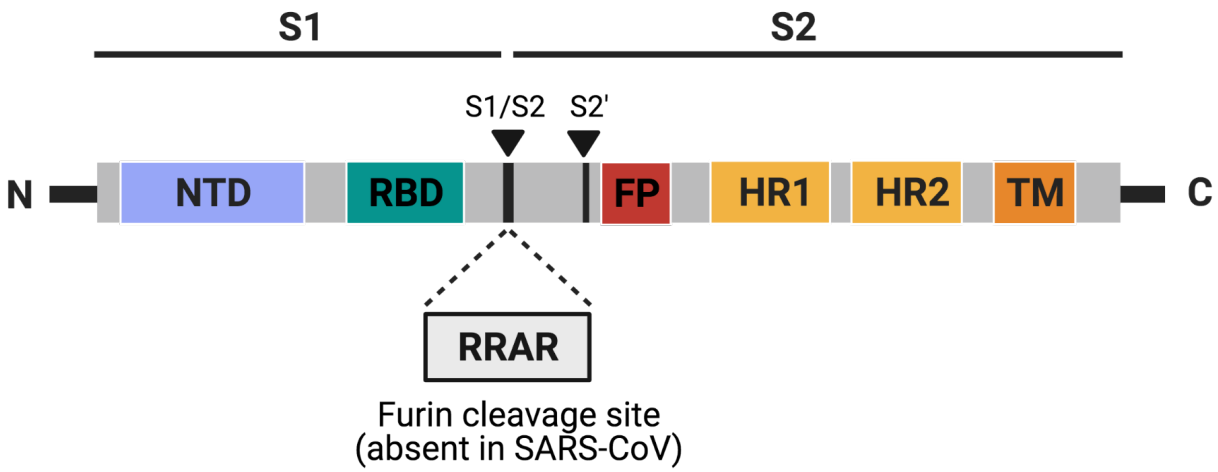
as functions to support the viral replication and transcription complex. Nsp12 encodes the RNA-dependent RNA polymerase (RdRp), a critical enzyme for coronavirus replication and a major antiviral target[7]. The RdRp inhibitor Remdesivir has been especially efficient in arresting SARS-CoV-2 RdRp activity and has been shown to improve recovery time in COVID-19 patients, though some severe adverse effects have been reported[8].

Following infection, CoV RNA replication takes place in the cell cytoplasm. It is thought that the replication of coronavirus RNA is facilitated within double-membrane vesicles originating from the endoplasmic reticulum[9]. After translation of the structural proteins E, M, N, and S, by host cell machinery, coronavirus particles assemble and bud from the endoplasmic reticulum-Golgi intermediate compartment (ERGIC)[10]. For betacoronaviruses, assembled virions have been shown to traffic through the endoplasmic reticulum and Golgi network, eventually co-localizing with LAMP1+ compartments, a marker for late endosomes and lysosomes. Infection of HeLa-mCC1a cells by mouse hepatitis virus (MHV) and Vero cells by SARS-CoV-2 revealed that betacoronaviruses have the ability to deacidify late endosomal/lysosomal compartments, deactivating the enzymes within them and exploiting these compartments for cellular release[11].

A


Spike (S)
Nucleocapsid (N)
Membrane (M)
Envelope (E)
RNA viral genome
RBD
S1
S1/S2
S2'
FP
HR1
HR2
S2
TM
Viral membrane

B


S1
S2
S1/S2
S2'
N
NTD
RBD
FP
HR1
HR2
TM
C
RRAR
Furin cleavage site
(absent in SARS-CoV)

Schematic 1. Coronavirus structure. (A) Coronavirus structure detailing the +ssRNA genome and structural proteins nucleocapsid (N), matrix (M), envelope (E), and spike (S). (B) Detailed SARS-CoV-2 S. The S1 subunit contains the N-terminal domain (NTD), and receptor binding domain (RBD). The S2 subunit contains the fusion peptide (FP), heptad repeats 1 and 2 (HRP1, HRP1) and the transmembrane domain (TM), and harbours the S2' cleavage site. SARS-CoV-2 S contains a furin cleavage site at the S1/S2 junction. Created with Biorender and adapted with permission from Rory Mulloy.

1.3 The Driver of Entry: Class I Fusion Proteins

In order to facilitate entry into host cells, viruses harbour fusion proteins on their surface. There are three types of fusion proteins harboured by viruses in order to gain entry into target cells: Class I, Class II, and Class III. The three classes differ in their structure and by their activation mechanisms. Examples of Class II and Class III fusion proteins include Zika E (family *Flaviviridae*) and vesicular stomatitis virus (VSV) glycoprotein G (family *Rhabdoviridae*), respectively. The coronavirus spike protein is a Class I fusion protein, thus class I fusion proteins will be the focus of this section.

Fusion proteins must undergo two major processes, priming and triggering, in order to complete the fusion process. Priming causes the fusion protein to go from fusion-incompetent to fusion-competent and triggering allows for the release of a fusion peptide or loop.

The Class I fusion protein structure is a trimer of heterodimers. In order to operate, Class I fusion proteins must engage their receptors and be cleaved by host cell proteases, some which are pH-dependent. After a Class I protein is triggered, the two heptad repeats interact with one another and propel the fusion protein into the host cell membrane, generating a bridge between the viral envelope and host cell membrane. The fusion protein then folds back on itself in a hairpin conformation called the six-helix bundle, connecting the viral and host cell membranes[12].

SARS-CoV-2 S is a Class I fusion protein, and thus requires binding to its host cell receptor and priming by host proteases in order to mediate fusion. Each monomer of SARS-CoV-2 S is comprised of two subunits, S1 and S2, responsible for facilitating receptor binding and membrane fusion, respectively **(Schematic 1B).** The prefusion form of S exists in a metastable state which undergoes major conformational changes after proteolytic activation by host factors, resulting in

a thermodynamically favoured postfusion conformation. Cleavage by host proteases at the S1/S2 junction induces structural rearrangement revealing the S2' site on S2. Host proteases must then cleave S2', activating the S2 subunit and allowing it to mediate fusion and take on its postfusion structure. The change from pre- to postfusion structure is an irreversible process for SARS-CoV-2 and for all Class I and Class II viral fusion proteins[13]. This is in contrast to the VSV glycoprotein, G, a Class III fusion protein, where conformation reversal from postfusion to prefusion is possible and where proteolytic processing is not required for fusion[14].

The S1 and S2 subunits of SARS-CoV-2 S contain different domains, all of which have distinct functions. S1, which resides at the N terminus of the spike protein, contains the N-terminal domain (NTD) and receptor binding domain (RBD). The RBD binds the human host cell receptor, angiotensin converting enzyme 2 (ACE2), which is also the receptor for SARS-CoV. The RBD is able to undergo transient conformational changes from a "down" to an "up" state, where the "up" conformation makes receptor binding determinants more accessible to the host cell receptor. An early study of SARS-CoV-2 S by *Wrapp et al.* revealed that the SARS-CoV-2 RBD has 10- to 20-fold higher binding affinity for ACE2 than the SARS-CoV RBD, a potential reason for its relatively high transmissibility[15]. While the SARS-CoV-2 NTD is not as well-understood as the RBD, it is able to be neutralized by the antibodies of some COVID-19 patients[16]. Reports have also demonstrated that the NTD can bind attachment factors such as Axl, L-SIGN, or DC-SIGN, which can help facilitate viral entry[17,18]. Both of these characteristics implicate the NTD as an important antiviral target for future studies.

The S2 subunit of SARS-CoV-2, situated at the C terminus of S, contains the fusion peptide, the heptad repeats HR1 and HR2, the transmembrane domain (TM), and the cytoplasmic tail.

Triggering of the S2 subunit by proteolytic cleavage at S2' induces the release of HR1 and HR2 and the fusion peptide. Formation of the six-helix bundle then facilitates fusion of the viral membrane with the cell membrane. The TM is embedded in the viral membrane and the cytoplasmic tail of SARS-CoV-2 contains an endoplasmic reticulum retention signal, which may play a role in the incorporation of S in the viral particle during assembly, and budding[19,20].

Activation of S occurs when host proteases cleave S following its binding to the host receptor via the RBD. Proteolytic S cleavage must occur sequentially, first at the S1/S2 junction before the S2' can be made accessible to host proteases for cleavage and release of the fusion peptide. One distinct characteristic of SARS-CoV-2 is the presence of a multibasic furin cleavage motif (RRAR) at S1/S2; it is the only lineage B betacoronavirus to harbour this site[21]. Although it is not an established concept, it has been postulated that the presence of the multibasic furin cleavage site on SARS-CoV-2 S is a contributing factor to its wide tropism[22].

1.4 SARS-CoV-2 Entry: Receptor, Early, and Late Entry Pathways

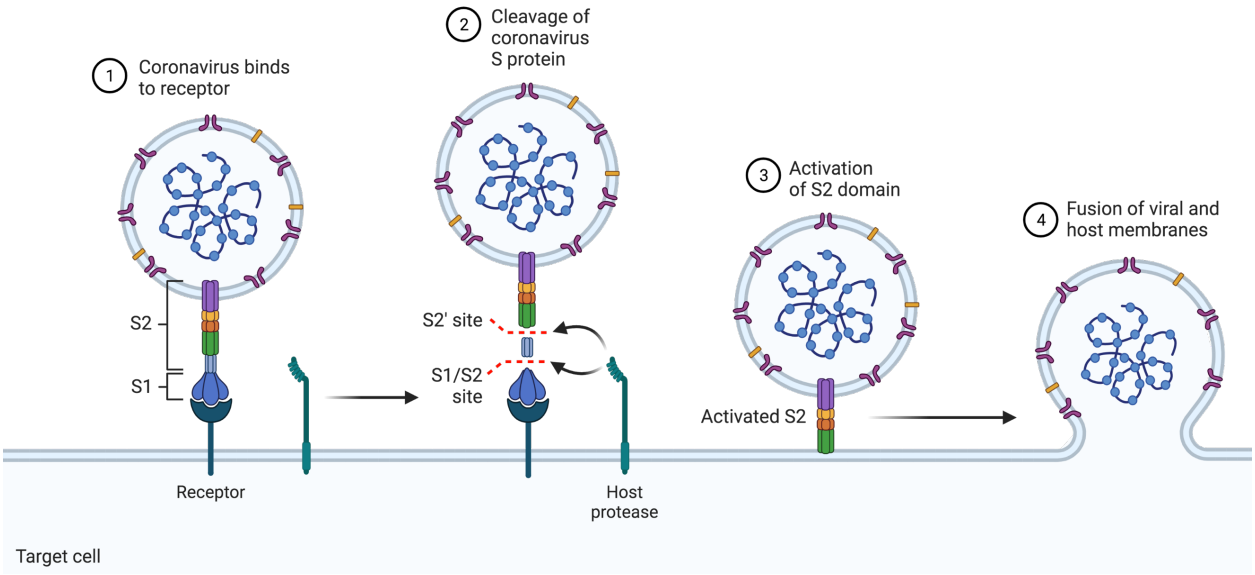
For coronaviruses, entry starts with S binding to its host cell receptor. Following receptor engagement, S is proteolytically activated by host proteases and facilitates virus-to-host membrane fusion. SARS-CoV-2 S can be activated by proteases at the cell surface (early pathway) or by proteases after internalization, within the endosome (late pathway).

The human receptor for both SARS-CoV-2 and SARS-CoV is angiotensin converting enzyme 2 (ACE2)[23]. Expressed abundantly in the lungs (in pneumocytes) and in the small intestine (in enterocytes), both target organs of SARS-CoV and SARS-CoV-2, the transmembrane protein ACE2 is a negative regulator of the angiotensin-renin pathway and plays a role in blood pressure homeostasis[24–26]. A recent study detected ACE2 mRNA in nasal epithelial cells, however the

authors note that protein expression must be confirmed, as these results conflicted with an earlier study that did not find ACE2 in the nasal passage[24,25].

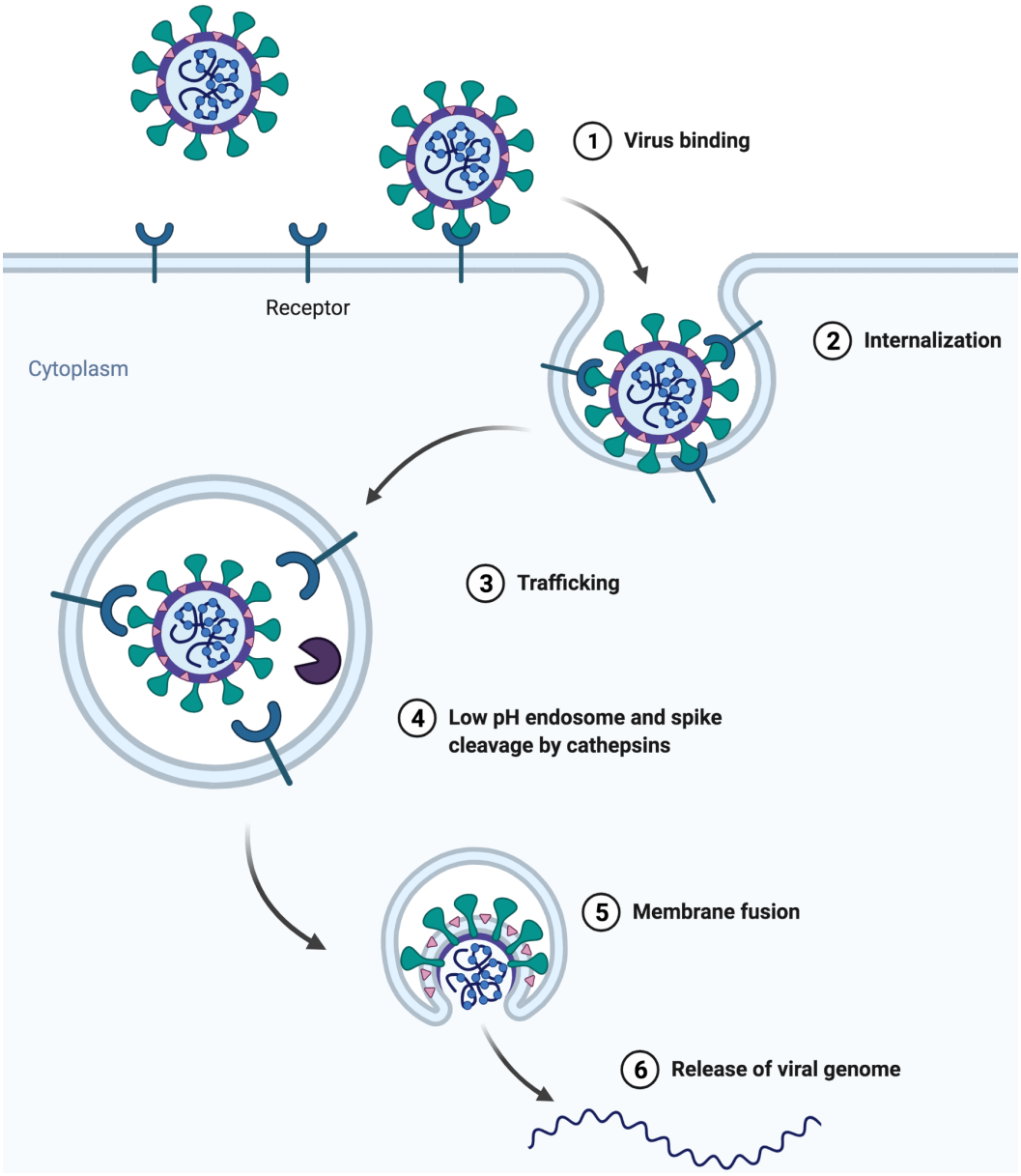
ACE2 engagement with S occurs via the RBD and is an essential part of entry because it induces structural rearrangements in S that make the S1/S2 and S2' sites more accessible for cleavage by host proteases. The ability of ACE2 to induce S structural rearrangement is what distinguishes it as the viral receptor instead of an attachment factor. Attachment factors can engage viral glycoproteins and enhance infection, but are unable to initiate the conformational change required to make the S cleavage sites accessible to host proteases. For example, attachment factors DC-SIGN and DC-SIGNR were both shown to enhance infection of SARS-CoV pseudotypes when overexpressed in target cells only when co-expressed with ACE2[27].

In the early entry pathway, S is proteolytically activated at the cell surface **(Schematic 2**). For SARS-CoV, S1/S2 and S2' cleavage are primarily mediated by proteases such as transmembrane serine protease 2 (TMPRSS2), although other trypsin-like proteases such as DESC1 and MSPL (alternatively known as TMPRSS13) have also been reported to be capable of SARS-CoV S cleavage[28]. Early studies of SARS-CoV-2 provided evidence that TMPRSS2 also cleaves SARS-CoV-2 S at S1/S2, an unsurprising observation given that SARS-CoV-2 S shares 76% amino acid sequence homology with SARS-CoV S[29]. Evidence that TMPRSS13 can activate SARS-CoV-2 S has also emerged[30,31]. The prominent role of serine proteases in S cleavage has heavily implicated them COVID-19 pathogenesis. Serine proteases have become a sought-after antiviral target in the fight against COVID-19. In particular, the pan-serine inhibitor Camostat was a potent inhibitor of SARS-CoV entry and was quickly re-purposed as an antiviral for SARS-CoV-2[23].


1 Coronavirus binds to receptor
2 Cleavage of coronavirus S protein
3 Activation of S2 domain
4 Fusion of viral and host membranes
S2
S1
S2' site
S1/S2 site
Activated S2
Receptor
Host protease
Target cell

Schematic 2. Coronavirus early entry pathway. CoVs use the S1 subunit of the spike protein (S) to bind to the receptor. S is cleaved at S1/S2, then at S2' by host surface proteases, triggering a conformational change and allowing the activated form of S2 to embed in the cell membrane and facilitate fusion of the viral and host cell membranes. Adapted from Biorender.

In the late entry pathway, coronaviruses must still bind to the host cell receptor, however, S priming does not occur at the cell surface. Instead, the virus is endocytosed. It is not until the virus reaches a late endosomal compartment that S priming and triggering occur **(Schematic 3)**. The fusion protein of some viruses such as influenza virus A (IVA) or VSV depend on a change in pH to trigger the fusion cascade. While preventing endosomal acidification has resulted in decreasing SARS-CoV and SARS-CoV-2 infection *in vitro,* S activation is not directly dependent on low pH. The use of cathepsin-specific inhibitors has revealed that within the endolysosome, SARS-CoV/SARS-CoV-2 S is cleaved by the pH-dependent cysteine proteases cathepsin L[32,33]. Notable drugs used to inhibit coronavirus entry through the late entry pathway include the antimalarial chloroquine and its derivative hydroxychloroquine sulfate (hydroxychloroquine). Chloroquine and hydroxychloroquine are lysosomotropic agents that prevent endosomal acidification, consequently disrupting the proteolytic activity of cathepsin L required for S activation[32–34]. At the beginning of the SARS-CoV-2 outbreak, hydroxychloroquine was considered a potential antiviral therapeutic and was authorized for use by the FDA. However its inability to effectively reduce SARS-CoV-2 infection *in vivo* and its adverse effects lead to the emergency authorization being revoked[35,36].


1 Virus binding
Receptor
Cytoplasm
2 Internalization
3 Trafficking
4 Low pH endosome and spike cleavage by cathepsins
5 Membrane fusion
6 Release of viral genome

Schematic 3. Coronavirus late entry pathway. CoVs use the S1 subunit of the spike protein (S) to bind to the receptor. The virus is internalized, and in the late endosome, S is cleaved at S1/S2, then at S2' by pH-dependent proteases, triggering a conformational change and allowing the activated form of S2 to embed in the endosomal membrane and facilitate fusion with the viral membrane. Adapted from Biorender.

1.5 Host proteases: Cysteine Proteases

Cathepsins are a group of proteases first studied in the context of the lysosomal pathway with a prominent role in degradation of cellular material. Since then, further studies have demonstrated that these proteases also have roles in the cytosol, nucleus, and mitochondria. They can also be secreted into the extracellular space, where they contribute to the pathogenesis of diseases like arthritis and cancer. Cathepsins are subdivided by structure and function into serine cathepsins, aspartic cathepsins, and cysteine cathepsins. The cysteine cathepsins are the largest family of the three. They are classified as C1a family proteases and encompass 11 cathepsins: B, C, G,H, K, L, O, S, W, V, and Z/X[37]. While each of these cathepsins have complex and sometimes overlapping roles in protein degradation, cell signalling, and various diseases, in the context of this thesis the focus will be on the role of cysteine cathepsins in viral entry.

In the endosomal pathway, endocytosed material is trafficked into early endosomes, then late endosomes which can fuse to lysosomes forming an endolysosome. Within late endosomes resides many cysteine cathepsins. Most cysteine cathepsins, including cathepsin L and cathepsin B, are activated at acidic pH (6) and inactivated at neutral pH. These cysteine cathepsins play a critical role in facilitating the late viral entry pathway of various Class I fusion viruses. Cathepsin B has been shown to cleave and activate Ebola GP and Hendra F[38,39]. Cathepsin L can also activate Ebola GP as well as MERS-CoV S, SARS-CoV S, and SARS-CoV-2 S[32,38,40,41].

One question that has been considered is whether fusion protein triggering is truly being facilitated by direct cathepsin cleavage, or if it is simply the low pH environment that is triggering fusion. To explore this concept, one group used an inhibitor of cathepsin B/L, MDL28170, and compared its ability to inhibit viral pseudotypes harbouring SARS-CoV S or VSV G, the latter of

which requires only low pH to facilitate viral entry. They found that MDL28170 inhibited SARS-CoV pseudotype entry but not VSV, confirming that SARS-CoV S relies on pH-dependent cysteine cathepsin activity and not just low pH alone[32]. A similar study came to the same conclusion using MDL28170 to inhibit MERS-CoV infection[40]. These studies implicate cysteine proteases as promising target candidates for broad-spectrum anti-viral therapeutics.

1.6 Host Proteases: Type II Serine Proteases

Type II serine proteases (TTSPs) are a family of 17 proteases that contain a "stem domain" flanked by an N-terminal transmembrane domain, and a C-terminal extracellular domain. Within the C-terminal domain resides the serine protease domain capable of cleaving histidine, aspartic acid, and serine residues. The TTSP family is comprised of four subfamilies: human airway trypsin-like protease/differentially expressed in squamous cell carcinoma (HAT/DESC), hepsin/transmembrane serine (TMPRSS), matriptase, or corin. Each subfamily is distinguished from the others by variations in their "stem" structure. While the physiological roles of each member of the TTSP are not completely elucidated, there has been evidence of some TTSPs in blood pressure regulation (corin), digestion (enteropeptidase), hearing (TMPRSS3 and TMPRSS5), and epithelial homeostasis (HAT, HAT-like 2, and TMPRSS2)[42–44]. It is speculated that the role of most TTSPs lies in maintaining homeostasis.

In particular, transmembrane serine protease 2 (TMPRSS2) is highly expressed in the prostate and the human epithelial airway[45,46]. Physiologically, TMPRSS2 may have a minor role in regulating airway epithelial sodium channels[46]. Aberrant expression of TMPRSS2 in prostate cancer has been linked to cancer invasion, metastasis, and tumour growth[47]. In the context of viral infection, the proteolytic activity of TMPRSS2 has proven useful for activating viral

glycoproteins, priming them for entry into target cells. TMPRSS2, along with another TTSP, human airway trypsin-like protease (HAT), are both effective primers of the influenza virus A (IVA) fusion protein, hemagluttinin (HA)[48]. Furthermore, TMPRSS2 cleaves and activates the human metapneumovirus fusion protein, F[49]. As was previously mentioned, TMPRSS2 is a well-studied primer of SARS-CoV, MERS-CoV, and SARS-CoV-2 spike proteins which has made it a highly considered therapeutic target in light of COVID-19.

The proteolytic activity of TMPRSS2 extends beyond its ability to prime viral fusion proteins. It is also a regulator of ACE2, the human receptor of SARS-CoV and SARS-CoV-2. A study by *Heurich et al.* demonstrated that cleavage of ACE2 by TMPRSS2 caused in increase in SARS-CoV entry into target cells[50]. Given that TMPRSS2 is co-expressed in the nasal epithelia with ACE2, it can postulated that the impact of TMPRSS2 on SARS-CoV and SARS-CoV-2 entry is not limited to S activation; these results provide evidence that TMPRSS2 may increase the susceptibility of ACE2-expressing target cells to SARS-CoV-2 infection[24].

While TMPRSS2 is the most well-studied and most potent of the serine proteases involved in viral fusion protein activation, other TMPRSS proteins such as TMPRSS13 and TMPRSS11D have also been shown to activate and enhance SARS-CoV-2 S-mediated entry[31].

1.7 Host Proteases: ADAMs

A disintegrin and metalloproteinases (ADAMs) are a zinc-dependent protein family consisting of 25 different proteins, only 13 of which are proteolytic. Structurally, membrane bound ADAMs are comprised of a propeptide domain, furin-recognition site, metalloproteinase domain, disintegrin domain, cysteine-rich domain, transmembrane domain, and cytoplasmic

domain. Membrane-bound ADAM protein functions include regulation of cellular signals, cell-cell adhesion, and regulation of the extracellular matrix (ECM)[51].

ADAM17 was the first member of ADAMs family to be characterized for proteolytic activity. It was discovered in 1997 for cleaving TNF-a, giving rise to its alternative name TNF-a converting enzyme (TACE)[52,53]. One of the first studies characterizing ADAM17 found ADAM17 RNA in most human tissues with highest expression in heart, placental, testes, and ovary tissues. Furthermore, ACE2 shedding has been shown to be modulated by ADAM17. Investigation of the role of ADAM17-mediated ACE2 cleavage in SARS-CoV infection has produced conflicting results[50,54]. Heurich and colleagues used PMA or TAPI-0 to stimulate or inhibit ADAM17 activity, respectively. When they infected the ADAM17-stimulated or -inhibited cells with SARS-CoV lentiviral pseuodtypes, they observed no difference in viral entry[50]. These results are in contrast to an earlier report by *Haga et al*., who found that siRNA knockdown of ADAM17 reduced SARS-CoV viral entry[54].

In their viral entry assay, *Haga et al.* used ADAM10 as a control protease and demonstrated the siRNA knockdown of ADAM10 did not affect entry of SARS-CoV pseudotypes. However, the same study also demonstrated that overexpression of ADAM10 in ADAM17-knockout cells could augment SARS-CoV entry. ADAM10 is a known regulator of cell adhesion through cleavage of N- and E-cadherin, maintains the extracellular matrix through cleavage of its substrate type IV collagen, and is a potent regulator of Notch signaling[55–58]. Altogether, these conflicting results may be attributed to differences experimental design and infection conditions. Without many other studies exploring ADAM proteins in the context of coronavirus infection,

these results demonstrate a need for further investigation into the role of the ADAMs in entry for SARS-CoV and related viruses.

1.8 Host Proteases: MMPs

Matrix metalloproteinases (MMPs) are a group of proteases that belong to the same superfamily as the ADAMs (metzincins). There are 23 proteins in the MMP family, all of which are zinc-dependent proteins and can be secreted or membrane-bound. In general, their structure consists of a pro-peptide domain, metalloproteinase domain, C-terminal, and hemopexin-like domain. MMPs are secreted as inactive zymogens and activation is induced by cleavage of the pro-peptide domain by other proteases. There are four types of secreted MMPs categorized based on their substrate specificity: collagenases, gelatinases, matrylsins, and furin-activated MMPs[51]. MMPs have a wide range of physiological functions including cell proliferation, angiogenesis, cell motility, wound healing, and degradation of the ECM[51,59].

MMPs have been extensively studied in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) with several clinical studies providing evidence of increased MMP levels in ALI/ARDS patients[59,60]. ALI/ARDS are caused by a severe inflammatory response in the lungs following infection or injury. Pro-inflammatory cytokine recruitment of MMPs to the lungs has been suggested to be a contributing factor to lung damage in ALI/ARDS due to MMP destruction of the ECM. While MMP expression is maintained at low levels in most tissues, neutrophils, which are immune responders to microbial infection, have been shown to produce high levels of MMP-9[51].

MMP-9, also known as Gelatinase B, is one of the most well-studied MMPs and has an array of functions related to its enzymatic activity, including cleaving substrates gelatin and

collagen (types III, IV, and V) in the ECM. MMP-9 also plays a major role in lung disease and injury; elevated levels of MMP-9 were found in the lungs of cystic fibrosis patients and MMP-9 upregulation has been observed in response to respiratory syncytial virus (RSV) infection in pediatric patients[61,62]. Additionally, some studies have provided evidence of increased MMP-9 expression following infection by respiratory viruses such as RSV or IVA[63,64]. MMP-9-deficient mice also had reduced neutrophil migration to the lungs when compared to wild-type mice following RSV or IVA infection, and reduced lung injury was reported in MMP9-deficient mice infected with IVA[63–65]. In addition to these studies demonstrating that MMP-9 is necessary for neutrophil recruitment to the site of infection, MMP-9 release post-infection is primarily facilitated by neutrophils which store MMPs in their granules. This is in contrast to most other cell types that regulate MMP expression using transcription factors. MMP regulation occurs at the transcriptional and post-transcriptional levels, and MMP enzymatic activity can be regulated by tissue inhibitors of metalloproteases (TIMPs)[51,60].

There has been some evidence demonstrating that coronavirus infection can also stimulate MMP-9 secretion. In 2007, *Desforges et al.* demonstrated that infection of the promonocytic cell line THP-1 by the lineage B betacoronavirus HCoV-229E increased MMP9 levels[66]. Another study detected an increase in MMP-9 expression in astrocytes and microglia following HCoV-OC43 infection (lineage A betacoronavirus)[67]. Interestingly, a recent report has also provided evidence that elevated MMP-9 levels in COVID-19 patients is correlated with respiratory failure[68]. Each of these studies implicates MMP-9 as a key player in coronavirus pathogenesis leading to severe complications in infected patients.

1.9 COVID-19 and Syncytium Formation

The majority of people infected with SARS-CoV-2 exhibit manageable COVID-19 symptoms including fever, dry cough, fatigue, and anosmia. Severe COVID-19 symptoms leading to hospitalization includes the development of pneumonia or ARDS characterized by difficulty breathing and chest pain.

In severe COVID-19, the development of ARDS can be incited by excessive migration of leukocytes and cytokines (cytokine storm) to the lungs leading to their destruction. An additional hallmark of severe COVID-19 progression is the fusion of cells forming multinucleated syncytia in patient organs. Evidence of syncytium formation is found primarily in pneumocytes, however there have been some instances of syncytia in cardiomyocytes and histiocytes as well[69–71].

Following infection, SARS-CoV-2 S synthesized within the host cell can be trafficked and presented on its outer membrane. This allows S to interact with ACE2 and host proteases on neighbouring cells, triggering the same fusion cascade as described for viral-to-host membrane fusion in section 1.4, leading to cell-to-cell membrane fusion. Histological studies that found syncytia in patient pneumocytes and cardiomyoctyes also found evidence of SARS-CoV-2 S in these tissues[69,71].

Coronaviruses are not the only family of viruses where infection can result in syncytium formation. Syncytium formation can be induced following infection by measles, herpes simplex virus 1, and HIV[72–75]. Notably, respiratory syncytial virus (RSV), named for its ability to induce syncytia, is a virus that can cause syncytia in patient lungs. RSV is an enveloped virus and a member of the viral family *Pneumoviridae*. Infection by RSV is common amongst young children and can lead to the hospitalization of children and elderly people. RSV harbours two surface glycoproteins, G and F, responsible for attachment and membrane fusion, respectively. In one

study aiming to elucidate the differences between RSV G and F, the authors demonstrated syncytium formation in cells infected with viral pseudotypes harbouring only RSV F, but not after infection by viral pseudotypes harbouring only RSV G, supporting that idea that F is the fusion glycoprotein[76]. Additionally, early reports studying RSV entry demonstrated that viral pseudotypes with only RSV F were still infective, suggesting that while RSV G is the main component responsible for viral attachment to cells, F is capable of receptor binding as well[77]. Little is known about RSV receptors, but many attachment factors such as glycoaminoglycans, heparan sulfate, and CX_3CR1 have been identified[78–80]. Like the SARS-CoV-2 S protein, RSV F is a Class I fusion protein with a similar fusion mechanism. It is comprised of two subunits, F1 and F2, and is primed by furin-like proteases in producer cells at two distinct sites[81]. As with all Class I fusion proteins, RSV F must be triggered in order to facilitate membrane fusion, however, distinct triggering factors required for F-mediated membrane fusion have not yet been identified[82,83]. Aside from the activation of the fusion glycoprotein F in syncytium formation, reports have also shown that actin formation is required for RSV-induced syncytia to occur *in vitro*[84].

In the case of COVID-19, syncytium formation requires engagement between SARS-CoV-2 S and ACE2 as well as S-triggering factors such as TMPRSS2. Cell-cell fusion assays are commonly used as a method to elucidate the mechanism of viral glycoprotein facilitated fusion and to identify alternative host proteases what might be triggering factors. However, syncytium formation also relies on elements of the host cell. A study by *Sanders et al.* demonstrated that inhibitors of actin polymerization and cholesterol were able to reduce syncytium formation facilitated by SARS-CoV-2 S[85]. Another study by *Braga et al.* identified the calcium-activated ion channel and scramblase TMEM16F as another potential target for S-induced syncytium

formation[86]. Syncytia are thought to contribute to disease pathogenesis by acting as sites of viral replication and viral immune evasion. Therefore, reducing the ability of SARS-CoV-2 to form syncytia is an important therapeutic strategy in the fight against COVID-19.

2.0 Rationale and Objectives

Following infection by SARS-CoV-2, cells express S on their own membranes. S displayed on the host cell surface can bind ACE2 and be cleaved by host cell proteases on neighbouring cells, initiating cell-cell fusion and forming multinucleated syncytia. Post-mortem evidence of syncytia in patient lungs and heart have been reported following SARS-CoV-2 infection[69,71]. Syncytium formation is a hallmark of severe COVID-19 pathogenesis, contributing to organ damage and providing a place for the virus to replicate and spread amongst cells while still evading the immune system[69].

As a class I fusion protein, the coronavirus spike must be proteolytically cleaved in order to facilitate fusion. The serine protease TMPRSS2 is known to be a potent activator of SARS-CoV and influenza virus A[28,48]. In the case of SARS-CoV, *in vitro* studies have shown that TMPRSS2 is required for spike activation. Conversely, early reports investigating syncytium formation mediated by SARS-CoV-2 S have shown that fusion with this virus is TMPRSS2 independent[33]. We hypothesized that a protease outside of the serine protease family could be activating SARS-CoV-2 S. Thus, the objective of this project was to identify host proteases capable of triggering SARS-CoV-2 S-mediated fusion. Identification of a such a protein (or proteins) would reveal a new class of antiviral targets and would be a factor in explaining the wide tropism of SARS-CoV-2. To investigate our objective, we used *in vitro* techniques such as cell-cell fusion assays and viral entry assays to:

1. Explore the role of other TMPRSS proteases in SARS-CoV-2 S mediated fusion.
2. Understand the importance of the S2' site in SARS-CoV-2 S activation.

3. Identify other protease families involved in SARS-CoV-2 S triggering, and test the effect of those proteases on S-mediated cell-cell fusion and viral entry.

3.0 Materials and Methods

3.1 Drugs

GI 245023X (Cedarlane) and Camostat mesylate (Cedarlane) were reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 10mM and stored at -20^{o}C.

3.2 Plasmids

pcDNA3-ADAM10-HA (Addgene plasmid #65106), pcDNA3-ADAM17-HA (Addgene plasmid #65105), pcDNA3-Delta(Pro-MP)ADAM17-HA (Addgene plasmid #65221), and pcDNA3-Delta(Pro-MP)ADAM10-HA (Addgene plasmid #65107) were gifts from Axel Ullrich. pcDNA3.1-SARS2-Spike (Addgene plasmid #145032) and pcDNA3.1-SARS-Spike were gifts from Fang Li (Addgene plasmid #145031). D614G and R815A mutations were introduced using overlapping PCR. Our ACE2 plasmid was a gift from Michael Farzan (Scripps, Florida). All the TMPRSS plasmids were part of the MISSION® TRC-Hs 3.0 (Human) library (Sigma).

Wuhan SARS-CoV-2 S cDNA (BioBasic) was codon optimized and obtained in four fragments. Fragments were amplified by PCR, assembled using Gibson Assembly, and cloned into the pCAGGS vector. Overlapping PCR was used to introduce D614G and R815A point mutations.

3.3 Antibodies

Antibodies used were: mouse MMP-9 monoclonal antibody (Thermo Fisher, #MA5-15886; 1:500 dilution in 5% BSA in TBST), mouse MMP-2 monoclonal antibody (Sigma, #MAB3308; 1:500 dilution in 5% BSA in TBST), mouse SARS-CoV/SARS-CoV-2 Spike Protein S2 monoclonal antibody (Thermo Fisher, #MA5-35946; 1:1000 dilution in 5% milk in TBST), mouse FLAG M2 monoclonal antibody (Sigma, #SLBV9325; 1:1000 dilution in 5% milk in TBST), rabbit GAPDH polyclonal antibody (Abcam, #ab9485; 1:1000 dilution in 5% milk in TBST), rabbit vinculin

monoclonal antibody (Abcam, #ab129002; 1:1000 dilution in 5% milk in TBST), mouse IgG HRP-linked antibody (NEB, #7076S; 1:10000 dilution in 5% milk or BSA in TBST), rabbit IgG HRP-linked antibody (NEB, #7074S; 1:10000 dilution in 5% milk in TBST).

3.4 MMP-9

Human recombinant MMP-9 (Cedarlane) was reconstituted using 30% glycerol in milliQ water that had been passed through a 0.22µM syringe filter (Mandel Scientific) aliquoted, and stored at -20^{o}C.

3.5 Cell lines and cell culture

Human Embryonic Kidney HEK293T (ATCC) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Wisent) modified with 10% Fetal Bovine Serum (FBS, Sigma), 0.3mg/mL L-glutamine, 100 U/L penicillin, and 100µg/mL streptomycin (Wisent). Calu-3 (Cedarlane) cells were cultured in Minimum Essential Medium (MEM, Sigma) modified with 10% FBS and 1X antibiotic-antimycotic solution (Wisent). Cells were maintained in 5% CO_2 at 100% relative humidity at 37^{o}C.

HEK293T-ACE2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Wisent) modified with 10% Fetal Bovine Serum (FBS, Sigma), 0.3mg/mL L-glutamine, 100 U/L penicillin, 100µg/mL streptomycin (Wisent), and puromycin.

3.6 Syncytium formation assays and cell overlay assays

Syncytium formation assay

HEK293T-ACE2 cells were seeded in 24-well plates and grown to 90% confluency. Using jetPRIME transfection reagent (Polyplus Transfection) and following the manufacturer's protocol, cells were co-transfected with plasmids encoding GFP, SARS-CoV-2 spike (C9 tagged), and

pCAGGS empty vector plasmids. Simultaneously, cells were treated with DMSO, 5µM of GI 245023X, 25µM of Camostat, or combination. Images were taken 24h post-transfection using the ZOE fluorescent cell imager (Bio-Rad).

Cell-cell fusion assay

HEK293T (effector) cells were seeded in 6-well plates and grown to 90% confluency before being co-transfected with plasmids encoding GFP, SARS-CoV-2 Spike (C9) and empty vector pCAGGS plasmids or GFP and pCAGGS only (negative control). To investigate the role of ADAM proteinases in S-mediated fusion, HEK293T-ACE2 (target) cells were seeded in 6-well plates and grown to 90% confluency before co-transfection with empty vector pCAGGS and ADAM10-HA, ADAM10ΔMP-HA, ADAM17-HA, or ADAM17ΔMP-HA. 24h post-transfection, effector and target cells were detached using Versene and co-cultured at a 1:1 ratio. Images were obtained 24h post-co-culture using the ZOE fluorescent cell imager (Bio-Rad). 5µM GI 245023X treatment was used as a negative control.

In the cell-cell fusion assays using Calu-3 cells, HEK293T (effector) cells were seeded in 6-well plates and grown to 90% confluency before being co-transfected with plasmids encoding GFP, SARS-CoV-2 Spike (C9) and empty vector pCAGGS plasmids or GFP and pCAGGS only (negative control). 24h post-transfection, the transfected HEK293T cells were detached using Versene and co-cultured at a 1:1 ratio with Calu-3 cells (target cells) detached by 0.05% Trypsin/0.53mM EDTA (Corning). Plates were pre-coated using poly-D-lysine hydrobromide (Millipore Sigma) according to manufacturer protocol. Cell co-cultures were treated with DMSO, 5µM of GI 245023X, 25µM of Camostat, or combination. Images were obtained 24h post-co-culture using the ZOE fluorescent cell imager (Bio-Rad).

3.7 MMP Pulldown

For immunoblots of media collected from the cell lines, HEK293T-ACE2 or Calu-3 cells were cultured in fresh DMEM or MEM (with appropriate supplements), respectively, 24h prior to media harvesting. 1mL of media was harvested when cells reached 90% confluency. For immunoblots of media collected from transfected HEK293T-ACE2 cells, cells were seeded in a 6cm plate and were either not transfected or transfected with empty vector (pCAGGS), 1µg, or 2µg of SARS-CoV-2 Spike protein. 1mL of media was collected 24h post-transfection.

Harvested media was centrifuged at 12000rpm to remove cellular debris and supplemented with 1X protease inhibitor cocktail (Cell Signalling). Samples were incubated with Gelatin Sepharose 4B beads (Cytiva) and incubated at 4^{o}C overnight on a rotator. The next day, the sample flowthrough was discarded and the beads were resuspended in lysis buffer containing 1% Triton X-100, 0.1% NP-40, 150mM NaCl, and 10mM Tris-HCl (pH 7.4) and 1X loading sample buffer with 5% β-mercaptoethanol. Samples were boiled at 95^{o}C for 10 minutes before analysis by immunoblotting.

3.8 Immunoblots

Cells were lysed in a buffer containing 1% Triton X-100, 0.1% NP-40, 150mM NaCl, and 10mM Tris-HCl (pH 7.4) with protease inhibitor cocktail (Cell Signalling). A bicinchoninic assay (BCA) was used to determine protein concentration (Thermo). Samples were boiled for 10min and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to polyvinylidene difluoride (PVDF) membranes (Bio-rad). Membranes were blocked with 5% skim milk or 5% bovine serum albumin (BSA) in 1X tris-buffered saline with Polysorbate 20 (TBST) for one hour and subsequently incubated with the appropriate primary

antibody overnight at 4°C. Membranes were then incubated then incubated horseradish peroxidase (HRP) conjugated secondary antibody for one hour at room temperature. Proteins were visualized using chemiluminescence following the manufacturer's protocol (Bio-Rad Clarity or SuperSignal West Femto ECL substrate).

3.9 Lentiviral pseudotype production

Lentivirus pseudotypes were generated by co-transfecting HEK293T cells with a retroviral vector encoding LacZ, and SARS-CoV-2 S (Wuhan) or SARS-CoV-2 S (D614G) at a 1:1:1 ratio. Transfections were performed using jetPRIME transfection reagent (Polyplus Transfection) following the manufacturer's protocol. At 24, 48, and 72h post-transfection, supernatants were collected and concentrated through a 20% (w/v) sucrose cushion by ultracentrifugation (20 000 RPM, 4°C, 1.5h, Beckman Coulter Optima XPN-100, SW32Ti rotor). The resulting pellets were resuspended in PBS, aliquoted, and stored at -80°C.

3.10 Lentivirus infections

HEK293T-ACE2 cells were seeded at 32 000 cells/well in 96-well plates that were pre-coated using poly-D-lysine hydrobromide (Millipore Sigma) according to manufacturer protocol. 24h after seeding, media was changed to serum free DMEM supplemented with 0.3mg/mL L-glutamine, 100 U/L penicillin, and 100µg/mL streptomycin (Wisent) and polybrene (Sigma). Lentivirus particles were combined with human recombinant MMP9 (BioVision) or PBS and incubated at 37°C for 30min before being added into infection wells. Serum free DMEM was replaced with complete DMEM (10% FBS, 0.3mg/mL L-glutamine, 100 U/L penicillin, and 100µg/mL streptomycin) 24h post-infection. 48h post-infection, cells were fixed with

formaldehyde and stained with LacZ staining solution with X-gal and incubated at 37^{o}C overnight. Stained/infected cells were counted manually.

4.0 Results

4.1 TMPRSS2 and TMPRSS13 enhance SARS-CoV-2 syncytium formation

To compare the protease requirement for SARS-CoV and SARS-CoV-2 S-mediated membrane fusion, we sought to first establish a fusion assay. HEK293T cells stably expressing the SARS-CoV and SARS-CoV-2 receptor, ACE2 (HEK293T-ACE2), were transfected with green-fluorescent protein (GFP) and SARS-CoV or SARS-CoV-2 S, with or without TMPRSS2 **(Figure 1, top panel)**. Consistent with previous literature, SARS-CoV was unable to confer cell fusion in the absence of TMPRSS2[87]. In the case of SARS-CoV-2 S, overexpression of TMPRSS2 enhanced syncytium formation, but was not required for cell fusion to occur. These findings indicate that a protease other than the previously identified TMPRSS2 is responsible for triggering SARS-CoV-2 S-mediated fusion, demonstrating a need to identify alternative S-triggering factors.

In previous studies aiming to elucidate proteases responsible for cleaving SARS-CoV S, the serine proteases HAT and TMPRSS13 were identified as SARS-CoV S triggering factors[28,31]. Since SARS-CoV and SARS-CoV-2 S have 76% sequence homology, there is a possibility that other serine proteases may also be able to cleave SARS-CoV-2 S[29]. Therefore, we performed a TMPRSS screen using HEK293T cells in a cell-cell overlay assay. Effector cells transfected with dsRED and SARS-CoV-2 S were co-cultured with target cells transfected with GFP, ACE2, and an empty vector, TMPRSS2, TMPRSS3, TMPRSS11A, TMPRSS11B, or TMPRSS13. Images obtained 24 hours post-transfection revealed that, besides the positive control TMPRSS2, only TMPRSS13 was able to enhance SARS-CoV-2 S-mediated fusion **(Figure 2)**. Overall, these results confirm TMPRSS2 and TMPRSS13 as proteases capable of priming SARS-CoV-2 S for membrane fusion.

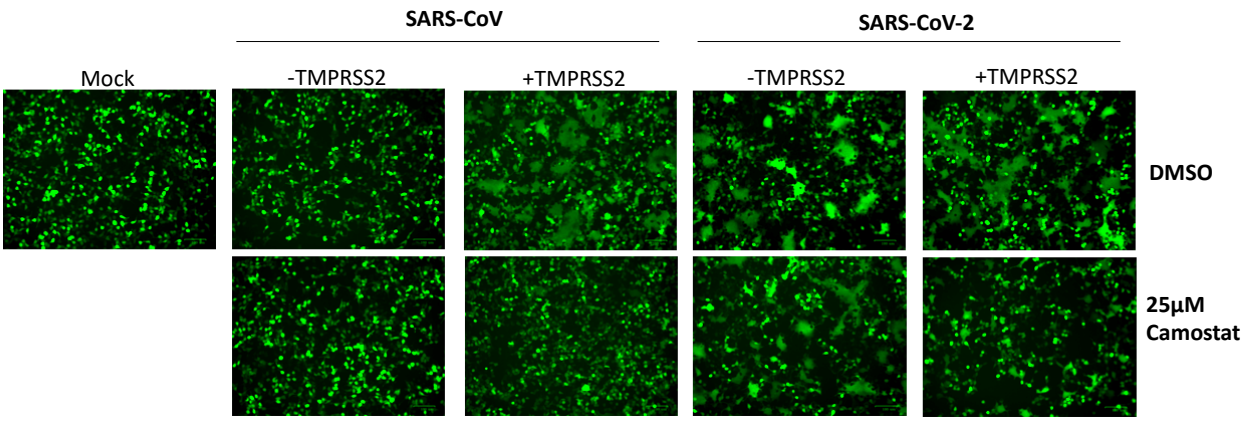

SARS-CoV
SARS-CoV-2
Mock
-TMPRSS2
+TMPRSS2
-TMPRSS2
+TMPRSS2
DMSO
25µM
Camostat

Figure 1. SARS-CoV-2 S-mediated fusion is TMPRSS-2 independent. HEK293T-ACE2 cells were co-transfected GFP and pCAGGS empty vector (mock) or with GFP and SARS-CoV S/SARS-CoV-2 S (FLAG-tagged), with or without TMPRSS2 (top panel). Cells co-transfected with the same combinations were treated with 25µM of camostat at the time of transfection. Images were obtained 24h post-transfection.

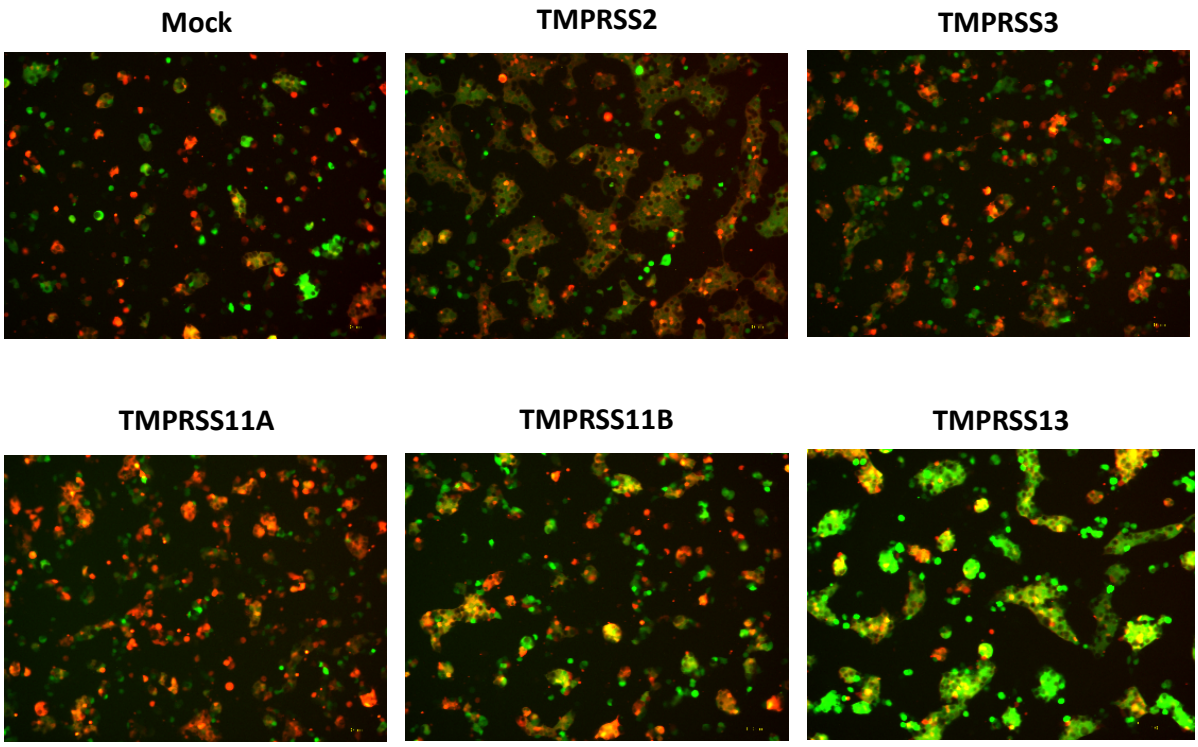

Mock
TMPRSS2
TMPRSS3
TMPRSS11A
TMPRSS11B
TMPRSS13

Figure 2. TMPRSS13 enhances SARS-CoV-2 S-mediated syncytium formation. HEK293T effector cells were co-transfected with dsRED and SARS-CoV-2 S (FLAG-tagged) and co-cultured with HEK293T target cells co-transfected with GFP, ACE2, and TMPRSS2/3/11A/11B/13. Images were obtained 24h post-transfection.

4.2 Serine proteases are not required for SARS-CoV-2 S-mediated fusion

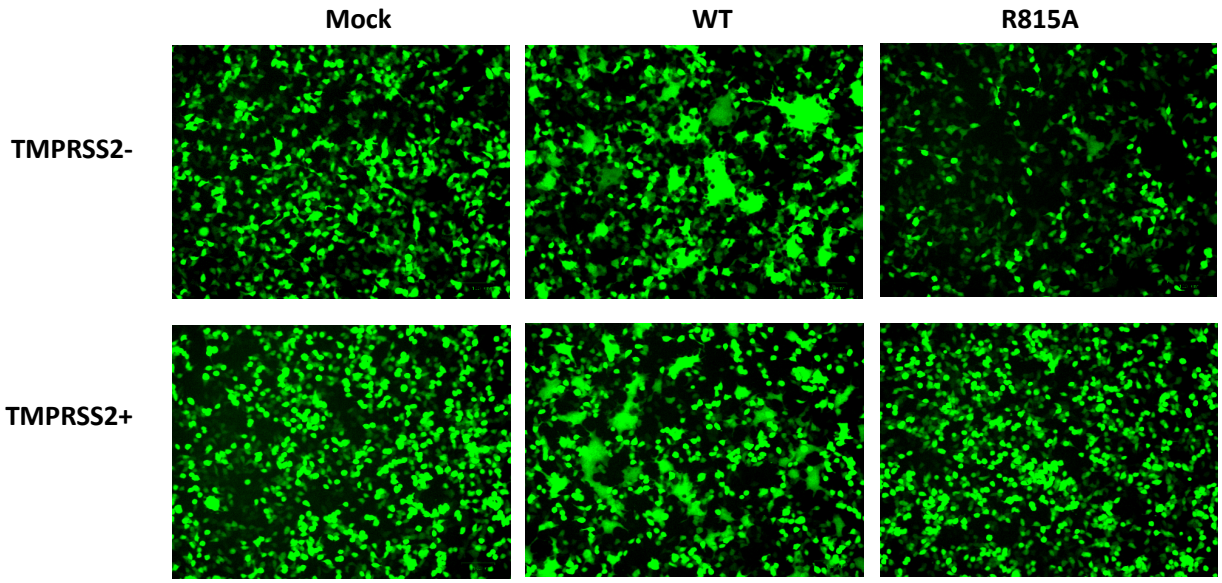
The ability for SARS-CoV-2 to form syncytia in HEK293T-ACE2 cells without serine protease overexpression is a quality that makes it distinct from SARS-CoV. To rule out the possibility that the HEK293T-ACE2 cells may be expressing alternative serine proteases at levels sufficient for cleavage and activation of SARS-CoV-2 S, we treated cells with the pan-serine protease inhibitor, Camostat, in a syncytium formation assay **(Figure 1, bottom panel)**. As expected, Camostat-treated cells overexpressing SARS-CoV S were unable to form syncytia even when TMPRSS2 was overexpressed. Conversely, Camostat treatment in SARS-CoV-2 S transfected cells was able to reduce fusion but not completely inhibit it. These results provide evidence that SARS-CoV-2 S activation is not exclusive to serine proteases.

4.3 The S2' site is required for SARS-CoV-2 syncytium formation

To further investigate the SARS-CoV-2 priming and triggering mechanism, we explored the role of the SARS-CoV-2 cleavage sites. Since the S1/S2 site is already cleaved within the producer cell by furin, we sought to elucidate the role of SARS-CoV-2 S2' cleavage by mutating the SARS-CoV-2 S protein at position 815 from an arginine to an alanine (R815A). Flag-tagged R815A and wild type (WT) SARS-CoV-2 S were co-expressed with GFP and an empty vector or TMPRSS2 in HEK293T-ACE2 cells **(Figure 3A).** 24h post-transfection, syncytium formation was evident in cells expressing WT SARS-CoV-2 S, but R815A-transfected cells showed no fusion, demonstrating that cleavage of the S2' site is essential for spike activation and fusion activity. Cell lysates were collected and resolved by SDS-PAGE to ensure sufficient R815A expression and to further elucidate R815A cleavage **(Figure 3B).** In samples transfected with WT SARS-CoV-2, two bands at 250kDa and 100kDa represent the uncleaved S0 and furin-cleaved S2 subunit,

respectively. R815A expression was confirmed by presence of the S0 band. Consistent with previous reports, R815A was not cleaved even at the S1/S2 junction, demonstrating that the presence of R815 is not only important for S-mediated fusion, but for the overall conformation and proper processing of SARS-CoV-2 S[19].

(A)


Mock
WT
R815A
TMPRSS2-
TMPRSS2+

(B)

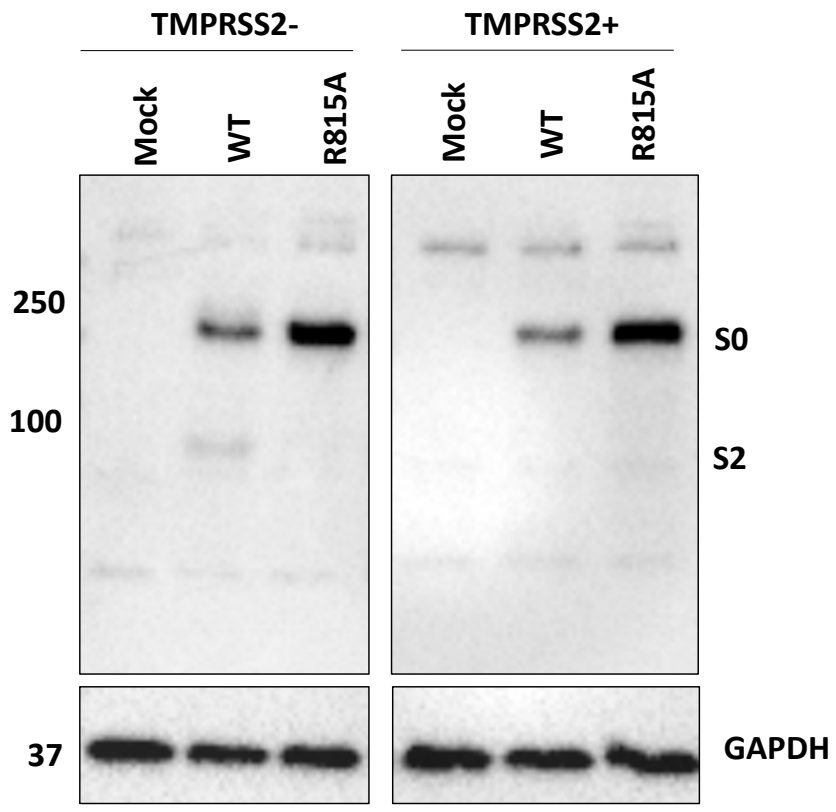
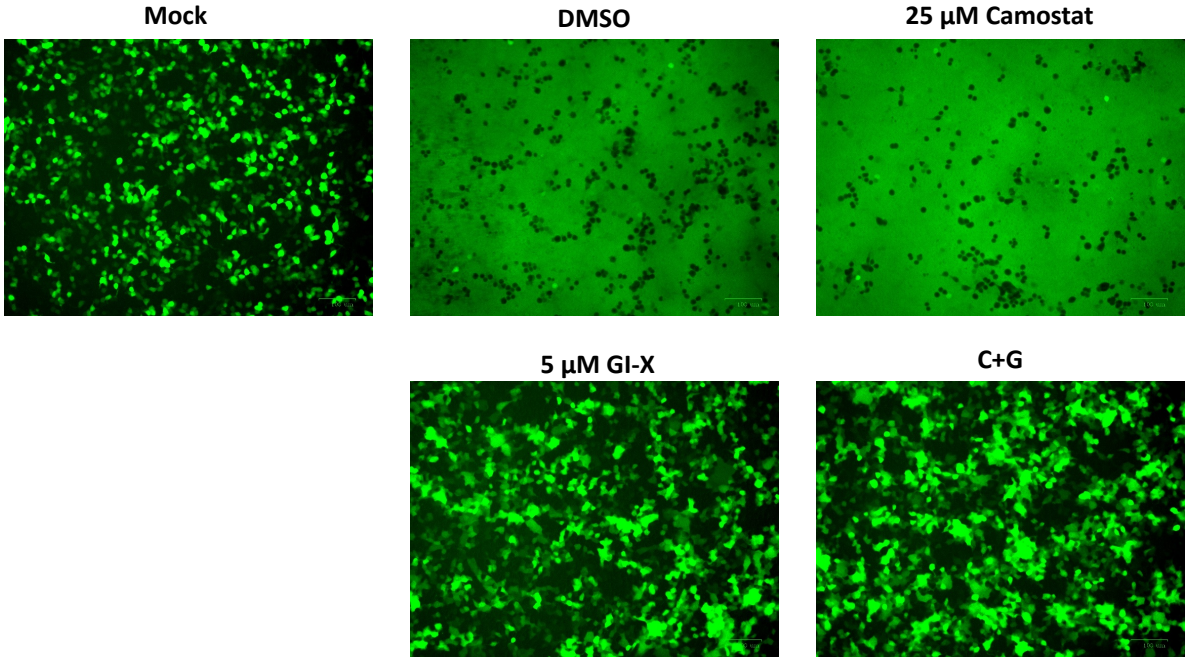

TMPRSS2-
TMPRSS2+
Mock
WT
R815A
Mock
WT
R815A
250
100
37
S0
S2
GAPDH

Figure 3. The SARS-CoV-2 S2' mutant R815A is fusion-deficient and is not cleaved at S1/S2. (A) HEK293T-ACE2 cells were co-transfected with GFP, wild type (WT) SARS-CoV-2 spike (FLAG), or R815A SARS-CoV-2 spike (FLAG-tagged), with or without TMPRSS2. Images were obtained 24h post-transfection. (B) Cell lysates were collected and resolved on SDS-PAGE. Samples were probed using FLAG or GAPDH antibodies.

4.4 The ADAM and MMP inhibitor GI 254023X reduces SARS-CoV-2 S-mediated syncytium formation in HEK293T cells

Having confirmed that an intact S2' site is essential for SARS-CoV-2 S-mediated syncytium formation and that serine proteases are not the only family of proteins involved in SARS-CoV-2 S triggering, we then returned to the task of identifying alternative S-triggering factors. In a syncytium formation assay, HEK293T-ACE2 cells co-transfected with GFP and SARS-CoV-2 S were treated with Camostat, the ADAM and MMP inhibitor GI 254023X (GI-X), or a combination of both. Images obtained 24h post-transfection revealed that Camostat was unable to inhibit syncytium formation, validating the results found in **Figure 1A**, and confirming that fusion mediated by SARS-CoV-2 S can be independent of serine proteases**.** Interestingly, GI-X markedly reduced the amount of syncytia able to form, both alone and in combination with Camostat **(Figure 4A),** implicating that the GI-X protein targets (ADAMs and MMPs) could be potential SARS-CoV-2 S activators. Cells were lysed, proteins were deglycosylated with PNGase F, and analyzed using SDS-PAGE. While samples treated with GI-X showed an overall decrease in spike expression, no drastic changes in processing were observed.

(A)


Mock
DMSO
25 µM Camostat
5 µM GI-X
C+G

(B)

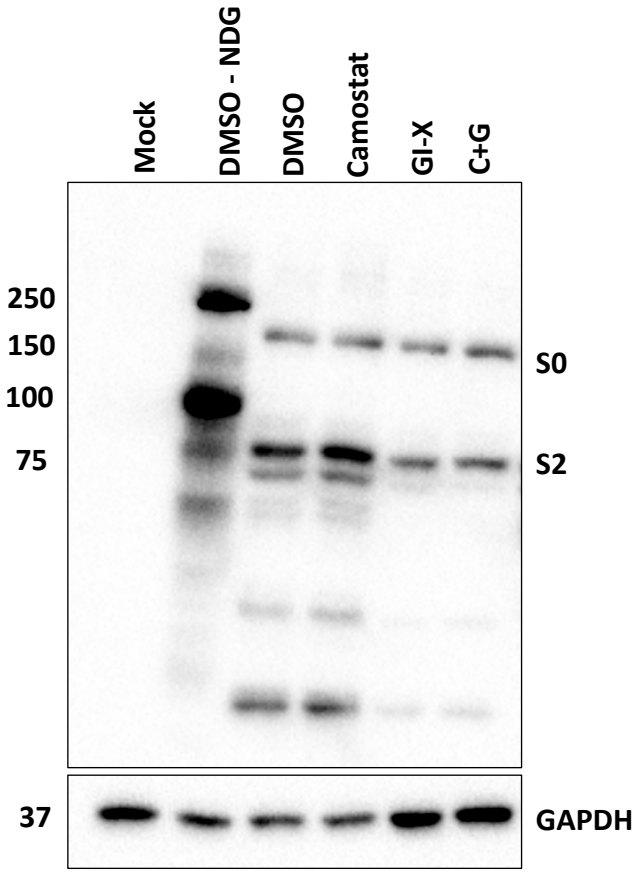

Mock
DMSO - NDG
DMSO
Camostat
GI-X
C+G
250
150
100
75
S0
S2
37
GAPDH

Figure 4. GI 254023X reduces SARS-CoV-2 S-mediated syncytium formation in HEK293T-ACE2 cells. (A) HEK293T-ACE2 cells were co-transfected with GFP and pCAGGS empty vector (mock) or GFP and SARS-CoV-2 S (C9 tagged). Cells were treated with 25μM of camostat, 5μM of GI 254023X (GI-X), or combination at the time of transfection. Images were obtained 24h post-transfection. (B) Cells were lysed and proteins were deglycosylated with PNGase F and resolved by SDS-PAGE. Western blots were probed using S2 or GAPDH antibodies. NDG = non-deglycosylated control.

4.5 GI 254023X and Camostat inhibit syncytium formation in Calu-3 cells

Because SARS-CoV-2 primarily targets the lungs, we next sought to validate our findings with the lung carcinoma cell line Calu-3. This provides a more relevant model of study since these cells endogenously express ACE2 and TMPRSS2, providing conditions that better emulate a patient lung. HEK293T cells transfected with GFP and SARS-CoV-2 S were co-cultured with Calu-3 cells for 24h under treatment with Camostat, GI-X, or a combination of both. Syncytium formation was evident in samples treated with Camostat or GI-X alone, but was extensively reduced in samples that received the combination treatment **(Figure 5)**. This suggests that both serine proteases and metalloproteases are capable of SARS-CoV-2 S activation, and that their activation mechanisms may be complementary to one another.

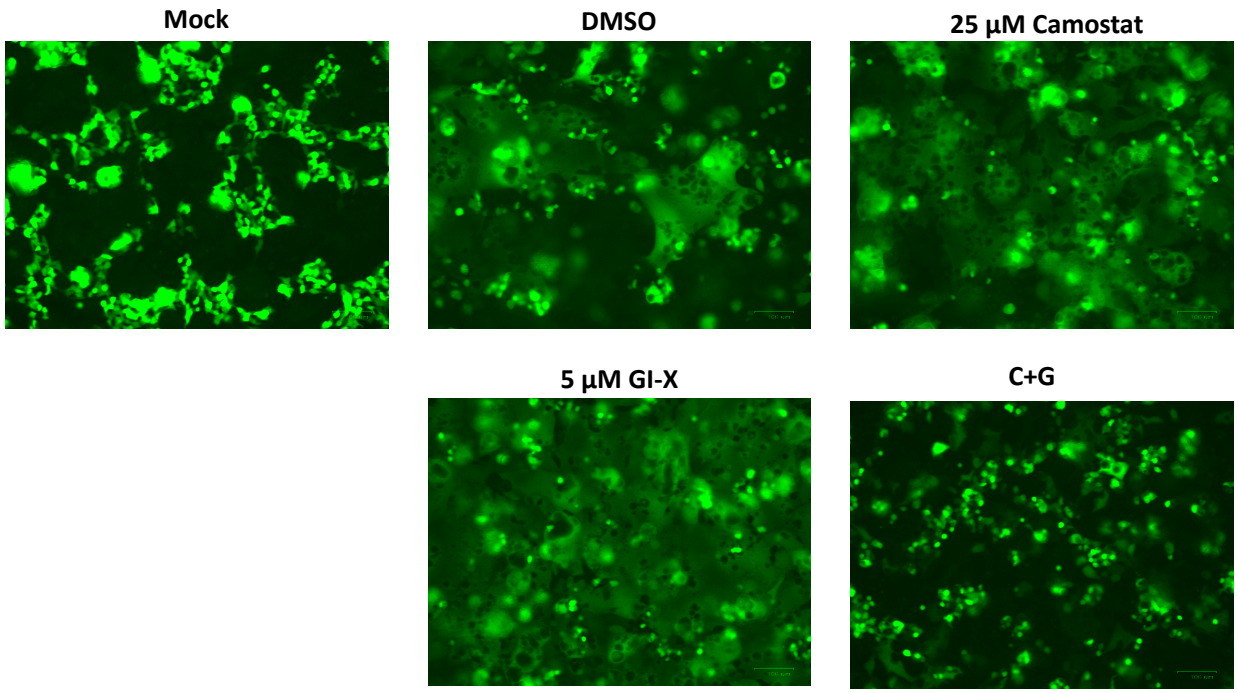
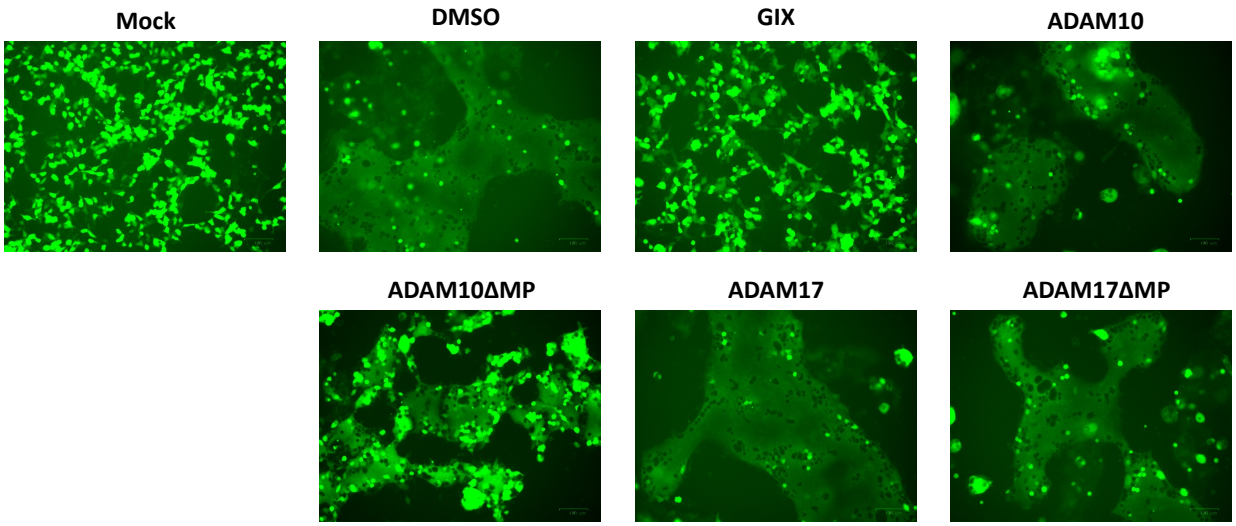

Mock
DMSO
25 µM Camostat
5 µM GI-X
C+G

Figure 5. A camostat and GI 254023X combination treatment is required to reduce SARS-CoV-2 S-mediated syncytium formation in Calu-3 and HEK293T cells. HEK293T effector cells were co-transfected with GFP and empty vector pCAGGS or SARS-CoV-2 S (C9-tagged) and co-cultured with Calu-3 cells. Overlaid populations were treated with DMSO (vehicle control), 25µM of camostat, 5µM GI-X, combination at the time of co-culture. Images were obtained 24h post-overlay.

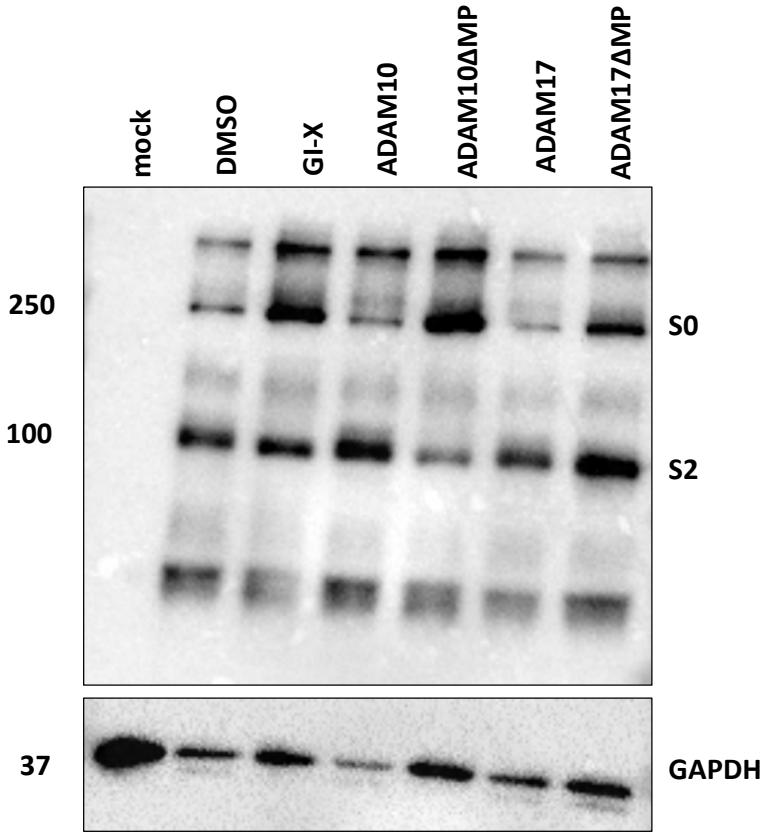
4.6 ADAM10ΔMP affects syncytium formation mediated by SARS-CoV-2 S

To investigate the role of ADAM10 in SARS-CoV-2 S activation, we decided to use a cell-cell overlay assay. Additionally, we decided to investigate ADAM17 in this experiment since it had been previously explored in SARS-CoV entry with conflicting results[50,54]. Effector HEK293T cells transfected with GFP and SARS-CoV-2 S were co-cultured with HEK293T-ACE2 target cells transfected with empty vector pCAGGS, ADAM10-HA, protease deficient ADAM10 (ADAM10ΔMP-HA), ADAM17-HA, or protease deficient ADAM17 (ADAM17ΔMP-HA). Images taken 24h post-co-culture revealed no major differences in syncytium formation in conditions where target cells were overexpressing ADAM10, ADAM17, and ADAM17ΔMP. When target cells were overexpressing ADAM10ΔMP, cell-cell fusion was still evident but an increased amount of non-fused cells was observed **(Figure 6A)**. Cell lysates resolved by SDS-PAGE confirmed expression of the ADAM plasmids **(Figure 6C)** and demonstrated no difference in SARS-CoV-2 S cleavage **(Figure 6B)**. Taken together, these results suggest that ADAM17 does not seem to influence SARS-CoV-2 S-mediated cell-cell fusion and that ADAM10 only slightly influences SARS-CoV-2 S-mediated fusion. Additionally, it does not seem that either of these proteases is capable of directly cleaving SARS-CoV-2 S to induce fusion.

(A)


Mock
DMSO
GIX
ADAM10
ADAM10ΔMP
ADAM17
ADAM17ΔMP

(B)


mock
DMSO
GI-X
ADAM10
ADAM10ΔMP
ADAM17
ADAM17ΔMP
250
100
37
S0
S2
GAPDH

(C)

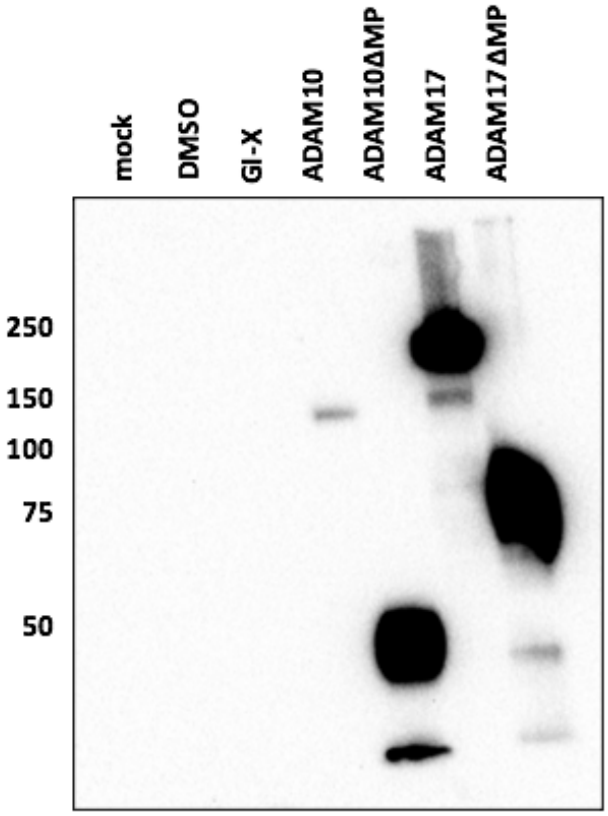

mock
DMSO
GI-X
ADAM10
ADAM10ΔMP
ADAM17
ADAM17ΔMP
250
150
100
75
50

Figure 6. SARS-CoV-2 S-mediated fusion is unchanged by the overexpression of ADAM10, ADAM10ΔMP, ADAM17, and ADAM17ΔMP. (A) Effector HEK293T cells co-transfected with GFP and SARS-CoV-2 S were co-cultured with target HEK293T cells transfected with empty vector pCAGGS, ADAM10, ADAM10ΔMP, ADAM17, or ADAM17ΔMP. Cells were treated with DMSO (vehicle control) or 5μM of GI-X as a positive control. Images were obtained 24h post-co-culture. Cell lysates were collected and analyzed by SDS-PAGE, probing for (B) S2 and GAPDH, as well as HA (C).

4.8 MMP-9 expression in HEK293T-ACE2 cells

Another protein target of GI-X is the soluble protein MMP-9. To explore the possibility that inhibition of MMP-9 by GI-X leads to reduced syncytium formation, we first had to validate the MMP-9 was being expressed in HEK293T-ACE2 cells. Additionally, previous studies have reported that viral infection by IVA, RSV, or HCoV-229E stimulated an increase in MMP-9 expression *in vitro*[63,64,66]. Therefore, we sought to determine whether enrichment in MMP-9 expression would occur in response to cellular contact with SARS-CoV-2 S. To explore this, HEK293T-ACE2 cells were not transfected (NT), transfected with empty vector pCAGGS, 1µg of SARS-CoV-2 S, or 2µg of SARS-CoV-2 S. 24 hours post-transfection, cell lysates were collected before analysis by SDS-PAGE. **Figure 7A** shows confirmation of S expression 24 hours post-transfection.

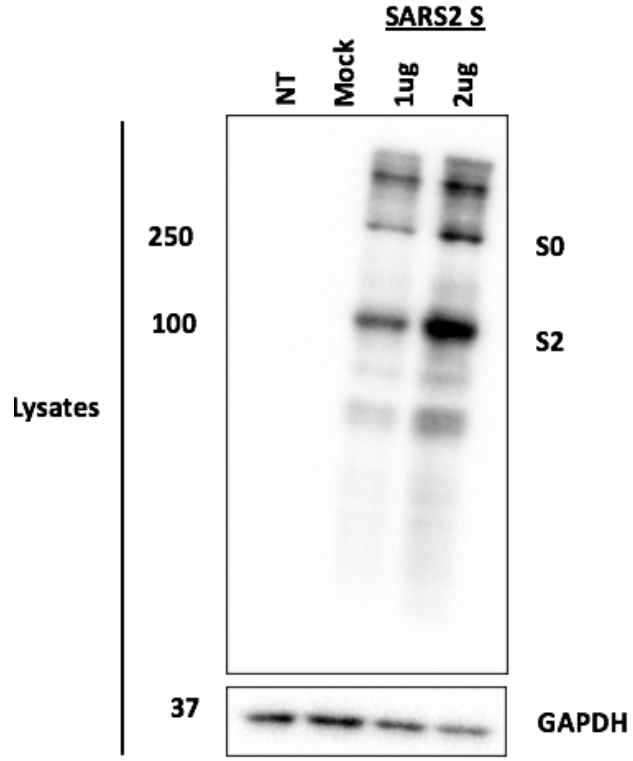
MMP-9 is first expressed as a zymogen with a molecular weight of 92kDa. Cleavage of pro-MMP-9 generates an 82kDa version of active MMP-9. Further cleavage of MMP-9 can lead to a post-activated form (65kDa) and an inactivated form (50-60kDa)[88]. MMP-9 blotting of the cell lysates revealed multiple bands **(data not shown**), making it difficult to discern which bands were MMP-9 and which bands were background bands. Furthermore, intracellular MMP-9 expression is not a direct demonstration of how much MMP-9 is secreted into the surrounding environment. Therefore, we investigated the levels of MMP-9 secretion following transfection of SARS-CoV-2 S using Gelatinase Sepharose 4B beads to pull down MMP-9 **(Figure 7B and C)**.

This experiment was performed three times. In the first two biological replicates, SARS-CoV-2 S-transfected HEK293T-ACE2 cells did not seem to secrete more MMP-9 than non-transfected or mock transfected cells **(Figure 7B)**. However, the third time the experiment was

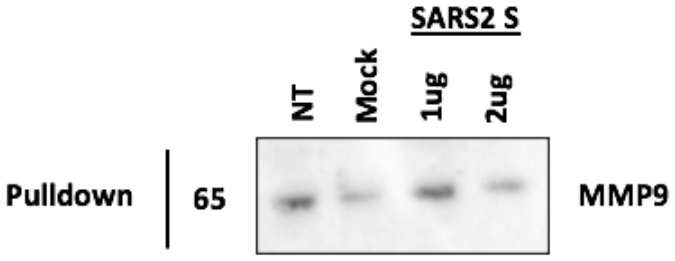
performed, there was an increase in MMP-9 secretion in cells that were transfected with SARS-CoV-2 S as compared to the non-transfected or mock transfected cells **(Figure 7C)**. As a control to ensure that this MMP-9 enrichment was not simply due to a higher amount of beads added to the samples, we also probed for MMP-2. MMP-2 is also a gelatinase and was pulled down by the Gelatin Sepharose 4B beads at the same time as MMP-9. Our MMP-2 blot showed no difference in MMP-2 secretion regardless of transfection.

Figures 7B and **7C** demonstrate inconsistent results regarding enrichment in MMP-9 secretion following SARS-CoV-2 S transfection. Nonetheless, the presence of MMP-9 in each experiment validates that MMP-9 is indeed secreted by HEK293T-ACE2 cells and that GI-X reduction of cell-cell fusion may be attributed to inhibition of MMP-9.

(A)


SARS2 S
NT
Mock
1ug
2ug
250
100
S0
S2
Lysates
37
GAPDH

(B)


SARS2 S
NT
Mock
1ug
2ug
Pulldown
65
MMP9

(C)

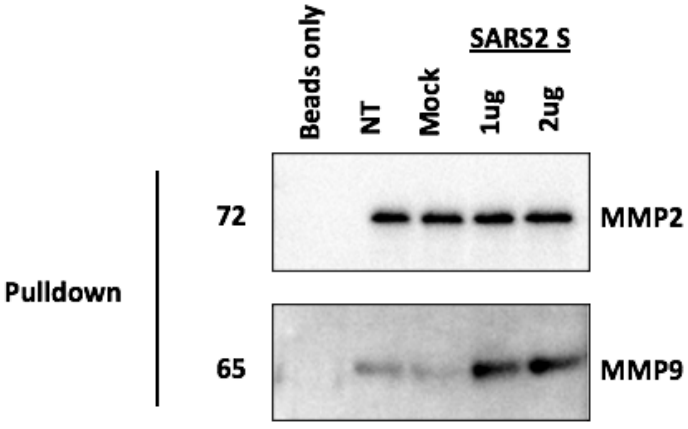

Beads only
SARS2 S
NT
Mock
1ug
2ug
72
MMP2
Pulldown
65
MMP9

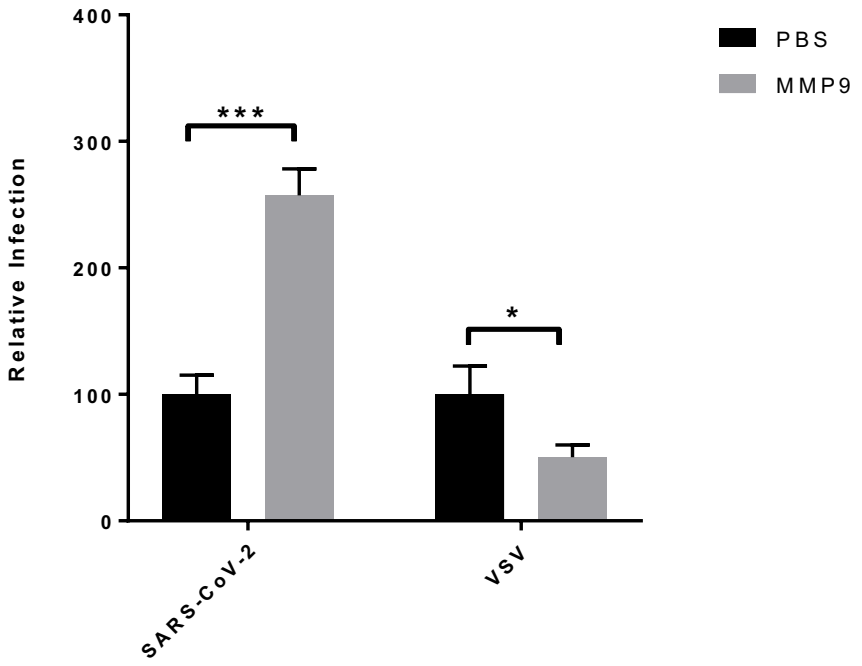
Figure 7. MMP-9 is secreted from HEK293T-ACE2 cells. HEK293T-ACE2 cells were either not transfected (NT), transfected with empty vector pCAGGS (mock), 1µg of SARS-CoV-2 S, or 2µg of SARS-CoV-2 S (C9 tagged). (A) 24h after transfection, cell lysates were collected and analyzed by SDS-PAGE probing for S2 and GAPDH. (B) SDS-PAGE was used to detect MMP-9 pulled down by Gelatin Sepharose 4B beads from the transfected cell media. (C) SDS-PAGE was used to detect MMP-9 and MMP-2 pulled down by Gelatin Sepharose 4B beads from the transfected cell media.

4.9 MMP-9 enhances infection by SARS-CoV-2 lentiviral pseudotypes in HEK293T-ACE2 cells

With the secretion of MMP-9 validated in HEK293T-ACE2 cells, we then sought to explore the potential role of MMP-9 in SARS-CoV-2 entry. To do this, we used a lentiviral pseudotype system encoding lacZ and harbouring SARS-CoV-2 S or VSV G, the latter of which does not require proteolytic cleavage to facilitate entry. HEK293T-ACE2 cells were infected using SARS-CoV-2 or VSV lentiviral pseudotypes, with or without the addition of recombinant human MMP-9. SARS-CoV-2 pseudotype entry increased by at least two-fold with the addition of MMP-9, while VSV pseudotype entry decreased **(Figure 8A)**. The decrease in VSV entry may be attributed to MMP-9 digestion of the VSV glycoprotein, G. These results suggest a role for MMP-9 in enhancing SARS-CoV-2 entry.

The D614G spike mutation is the most widespread variant globally and has been associated with higher transmissibility and higher viral load in SARS-CoV-2 infected patients[89–91]. Therefore, it is necessary to extend novel findings to ensure their relevance with D614G. HEK293T-ACE2 cells were infected with lentiviral pseudotypes harbouring D614G S or VSV G with or without addition of recombinant human MMP-9. Similar to WT SARS-CoV-2 S, SARS-CoV-2 D614G pseudotype entry increased by two-fold in the presence of MMP-9, while VSV pseudotype entry decreased **(Figure 8B).** Taken together, these results reiterate the role of MMP-9 in SARS-CoV-2 D614G entry, and suggests that MMP-9 may remain relevant for future SARS-CoV-2 variants.

(A)


400
300
200
100
0
Relative Infection
***
*
PBS
MMP9
SARS-CoV-2
VSV

(B)

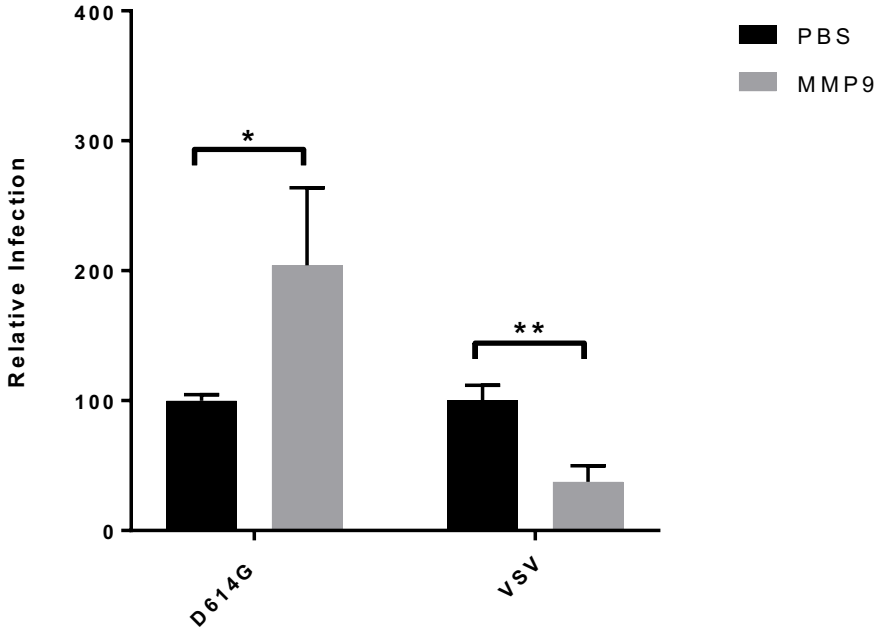

400
300
200
100
0
Relative Infection
*
**
PBS
MMP9
D614G
VSV

Figure 8. MMP-9 enhances SARS-CoV-2 lentiviral pseudotype infection in HEK293T-ACE2 cells. HEK293T-ACE2 cells were infected with (A) SARS-CoV-2 Wuhan or (B) SARS-CoV-2 D614G and VSV lentiviral pseudotypes in the presence or absence of human recombinant MMP-9. Data is presented as mean ± SD and is representative of three independent experiments. Statistical analysis was performed using an unpaired t-test. Statistical significance was determined using the Holm-Šídák method where *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

5.0 Discussion

5.1 Prelude

Infection by human coronaviruses can cause an array of symptoms, from that of the common cold to severe respiratory disease, as well as enteric disease. The pathogenic coronaviruses, SARS-CoV, MERS-CoV, and now SARS-CoV-2, are amongst those that lead to acute respiratory distress syndrome (ARDS). SARS-CoV and MERS-CoV outbreaks have reached epidemic status, while the highly transmissible SARS-CoV-2 rapidly reached pandemic status since its emergence in December 2019, inciting a state of international emergency. The rapid response to the outbreak of SARS-CoV-2 quickly identified it as a lineage B betacoronavirus and that its receptor is ACE2 which is recognized by the S protein[6].

The coronavirus spike protein, S, is located on the viral membrane surface. The virus is comprised of three other structural proteins: nucleoprotein (N), matrix (M), and envelope (E). Spike is made up of two subunits: S1 and S2. S1 is responsible for attachment of the virus to the host cell receptor and S2 is responsible for mediating fusion of the viral envelope to the host cell envelope[92]. As a class I fusion protein, S must be proteolytically cleaved in order to facilitate membrane fusion. Different coronaviruses require binding to different host receptors and cleavage at the S1/S2 junction and then at the S2' site by different host proteases. In the early entry pathway, serine proteases can cleave the coronavirus S. In the late entry pathway, S cleavage is facilitated by cysteine proteases. In the early entry pathway, the serine protease TMPRSS2 is best known for enhancing coronavirus entry by cleaving the spike protein. TMPRSS2 cleaves the spike protein of HCoV-229E, HCoV-NL63, MERS-CoV, and SARS-CoV[40,93–95]. The role of serine proteases in SARS-CoV-2 S activation for the surface entry pathway has been well-

studied, but fewer investigations have been done on other protease families. A few studies have explored the role of proteins in the ADAM family in SARS-CoV entry with contradictory results, demonstrating a need for validation of the conditions and methods used to study this family of proteins and for further exploration of the ADAMs in coronavirus entry[50,54].

MMP-9 is a gelatinase known for its role in digesting of almost all of the components of the ECM[51]. It is secreted at baseline levels in most cell types and studies investigating MMP-9 in response to infection have demonstrated an enhancement in its secretion following infection by RSV and IVA[63,64]. With emerging data illustrating a positive relationship between patients with high MMP-9 levels and severe COVID-19 disease, there were grounds to explore MMP-9 in the context of SARS-CoV-2 infection[68].

5.2 Serine Proteases

In this study, we first investigated the role of serine proteases as triggering factors for SARS-CoV-2 S using syncytium formation assays. In agreement with previous studies, we found enhanced SARS-CoV-2 S-mediated fusion when the spike protein was co-expressed with TMPRSS2 and TMPRSS13[23,31,96]. Our results, along with others that have obtained similar findings, demonstrate that TMPRSS2 is not the only serine protease capable of SARS-CoV-2 S activation. Further exploration into the serine protease family may reveal additional serine proteases involved in S-mediated fusion; TMPRSS11D has also been identified as a serine protease capable of activating SARS-CoV-2 S[96].

5.3 Processing at S2'

We also confirmed that the S2' site of SARS-CoV-2 S must be intact in order for S-mediated fusion to occur. Our mutant, R815A, was not only unable to be cleaved at the S2' site, but the

switch from arginine to alanine altered the overall conformation of S to the extent that it could not be properly processed even at S1/S2. These results corroborate with a study by *Nguyen et al.* However, to more effectively study the necessity of S2' in SARS-CoV-2 S-mediated fusion, it may be of interest to develop a point mutation that prevents SARS-CoV-2 S2' cleavage but does not interfere with the conformational structure of spike as a whole and allows for processing at S1/S2 by furin-like proteases[19].

5.4 ADAM Proteins

The use of Camostat in our syncytium formation assays allowed us to validate that, unlike for SARS-CoV, for SARS-CoV-2 the family of proteins capable of S activation is not exclusive to serine proteases. During the course of this investigation, reports by *Ou et al.* and *Nguyen et al.* also showed serine protease-independent syncytium formation, confirming the validity of our results[19,33]. Our finding that GI 254023X inhibits syncytium formation in HEK293T-ACE2 cells and Calu-3 cells (when used in combination with Camostat) implicates roles for ADAM and MMP family proteins in SARS-CoV-2 fusion and entry **(Figures 4 and 5)**. There has been growing interest in the effects of proteins of the ADAM family for viral infection. ADAM9 has been implicated as an important susceptibility factor for encephalomyocarditis virus (EMCV)[97]. It was also recently found that ADAM17 is required for classical swine fever virus (CSFV) entry[98]. While studies examining the role of ADAM17 and ADAM10 in coronavirus entry remain conflicting, these reports point to an emerging importance of ADAM family proteins in viral entry that require further elucidation[50,54]. Therefore, we sought to explore the role of ADAM10 and ADAM17 in SARS-CoV-2 S activation. Our findings demonstrated that ADAM17 did not have an effect on fusion while the protease deficient ADAM10 seemed to reduce fusion slightly, although neither

protease seemed to affect S processing **(Figure 7).** Further studies must be conducted to investigate the potential role of ADAMs in virally induced cell-cell fusion.

Furthermore, it may be prudent to explore the role of ADAM family proteins in COVID-19 pathogenesis. *Haga et al.* administered the ADAM17 inhibitor TAPI-2 to mice before challenging them with replicative SARS-CoV. Three days after challenge, mice pre-treated with TAPI-2 three times had a lower viral load in their lung lavage than control mice, implicating a role for ADAM17 in SARS-CoV pathogenesis[99]. To our knowledge, similar studies have not been conducted using SARS-CoV-2, but follow-up studies may seek to explore ADAM17 as an important host factor in SARS-CoV-2 entry and a potential therapeutic target in COVID-19 pathogenesis.

5.5 MMP-9

In **Figure 4A**, the reduction in cell-cell fusion due to GI-X treatment points to a serine-protease independent process for SARS-CoV-2 S activation. The overlay of S-expressing HEK293T cells with Calu-3 cells which are known to express TMPRSS2 **(Figure 5**) showed that in these conditions, both camostat and GI-X were necessary for syncytium formation to be inhibited. This demonstrates that the effect of serine proteases and MMPs on S-mediated syncytium formation are compensatory. Additionally, a cell-cell fusion model occurs at neutral pH, ruling out the possibility that pH-dependent lysosomal enzymes such as cathepsin L may be activating spike in this scenario. Finally, infection of HEK293T-ACE2 cells with lentivirus particles harbouring ancestral (Wuhan) SARS-CoV-2 S or D614G S was enhanced by addition of human recombinant MMP-9, identifying MMP-9 as a key protease in SARS-CoV-2 entry **(Figure 8)**.

The mechanism by which MMP-9 operates to enhance SARS-CoV-2 lentiviral infection remains to be elucidated. These data, along with enzymatic activity of MMP-9 present the

possibility that MMP-9 may be cleaving and activating SARS-CoV-2 S in a similar fashion to other host proteases such as TMPRSS2 or cathepsin L. In one study, *Phillips et al.* investigated the entry and cell-cell fusion mechanisms of JHM.SD (formerly MHV4), a neurovirulent strain of the murine coronavirus, mouse hepatitis virus (MHV). In neurons, the JHM.SD receptor, CEACAM1a, is expressed at extremely low levels, yet JHM.SD is able to efficiently spread and cause cell-cell fusion throughout the brain. JHM.SD spike can even facilitate cell-cell fusion when effector cells are overlaid with non-permissive cells[100,101]. Therefore, the authors hypothesized that JHM.SD may have evolved to become more sensitive to cleavage by host proteases. In this study, JHM.SD infection was only slightly inhibited by pre-treating cells with camostat (serine protease inhibitor) or E64 (cysteine protease inhibitor) prior to infection. Importantly, the metalloproteinase inhibitor batimastat was able to decrease JHM.SD infection to a greater extent than either camostat or E64, suggesting JHM.SD sensitivity to MMPs. Additionally, the authors infected cells with JHM.SD and replaced the infection media with media containing inhibitors five hours post-infection to allow for spike synthesis and syncytium formation. Remarkably, camostat alone was unable to inhibit syncytium formation, while batimastat treatment led to an obvious reduction in cell-cell fusion. Although the authors used an infection model instead of transfection to obtain spike expression on the cell surface, their results are similar to that of our syncytium formation assay in **Figure 4A**, where GI-X but not camostat was able to reduce cell-cell fusion. Furthermore, our western blots in **Figure 4B** also showed that GI-X treatment did not inhibit S1/S2 cleavage, as demonstrated by the lack of a stronger S0 band at 250kDa. *Phillips et al.* obtained similar results when they analysed their JHM.SD spike-expressing cells post-infection; S1/S2 cleavage was not inhibited by batimastat treatment. Therefore, similarly to *Phillips et al.,* our data suggests that

S1/S2 cleavage is unaffected by MMP inhibition. The enhancement of infection when MMP-9 was added to SARS-CoV-2 lentivirus infection experiments singles out MMP-9 as a potential driver of entry **(Figure 8)**. Whether or not MMP-9 (or other MMPs not explored in this thesis) cleaves the S2' site of SARS-CoV-2 is unknown. Many studies have cited difficulty in detection of S2' via immunoblotting. Therefore, to elucidate the mechanism of MMP-9 cleavage of spike at S2', alternative methods must be explored.

In investigating MMP-9, we attempted to see if MMP-9 expression could be enriched by stimulating cells with SARS-CoV-2 S. Previous studies have provided evidence of increased MMP-9 expression after infection by RSV, IVA, HCoV-OC43, and HCoV-229E [63,64,66,67]. Upon transfecting HEK293T-ACE2 cells with empty vector pCAGGS or SARS-CoV-2 S and using gelatin Sepharose 4B beads to pull down MMPs, our western blots demonstrated conflicting results with regards to enrichment in MMP-9 expression after transfection with SARS-CoV-2 S **(Figures 7B/C)**. Further experiments must be conducted in order to draw a definitive conclusion as to whether or not MMP-9 is enriched following stimulation of cells with SARS-CoV-2 S.

Interestingly, we pulled down the post-activated form of MMP-9, as indicated by 65kDa band in each sample. The MMP-9 zymogen can be cleaved by proteases such as other MMPs or stromelysin to generate its active form[88,102–104]. Cleavage by stromelysin can generate MMP-9 in its active form as well as in the post-activated form, which contains similar enzymatic activity to its active form[88]. Although protease inhibitor was used after cell media was harvested, it is still possible that MMP-9 may have been cleaved by other proteases, generating an abundance of post-activated MMP-9 compared to its other forms. Furthermore, it is possible that pro-MMP-9 (92kDa) and active MMP-9 (82kDa) were present in the media samples, but that SDS-PAGE was

not sensitive enough to detect them as evidenced by the fact that even the post-active MMP-9 bands were relatively weak. Regardless, these results confirmed the secretion of MMP-9 in HEK293T-ACE2 cells, implicating that it may have a role in SARS-CoV-2 S-mediated syncytium formation and entry.

MMP-9 is known for digesting almost all components of the ECM, and many studies have demonstrated that MMP-9 can destroy the lungs leading to ALI or ARDS[59,60]. Furthermore, since MMP-9 is a soluble protein and is secreted in different cell types within the body, its ability to enhance infection is a contributing factor to explaining the wide tropism of SARS-CoV-2. Our results, along with recent reports of high MMP-9 levels being correlated with severe COVID-19, implicate MMP-9 as a potential major contributor to COVID-19 pathogenesis[68]. In this study, we demonstrate that MMP activity can compensate for serine protease activity in syncytium formation. The use of broad-spectrum MMP inhibitors have already been shown to inhibit syncytia induced by RSV and coronaviruses *in vitro*[19,105,106]. Given that syncytium formation is a hallmark of severe COVID-19 pathogenesis, further investigation of MMP inhibition may be prudent[69]. In particular, we identify MMP-9 as a driver of SARS-CoV-2 entry although its mechanism of infection enhancement remains to be elucidated.

6.0 Conclusion

SARS-CoV-2 has been a threat to global health since its emergence in December 2019. Compared to its closest human infecting relative, SARS-CoV, SARS-CoV-2 has higher transmissibility and broader tropism[30]. Its mechanism of entry is facilitated by the spike protein which resides on the viral membrane.

In this study, we found that SARS-CoV-2 S-mediated fusion can be serine protease independent. Using syncytium formation assays, we identified the ADAM and MMP inhibitor GI 254023X as a compound capable of potently reducing SARS-CoV-2 S-mediated cell-cell fusion in HEK293T-ACE2 cells. Inhibition of virally induced syncytium formation is important for the reduction of COVID-19 pathogenesis as the formation of syncytia allows viruses to replicate and spread throughout organ tissue while simultaneously evading the host immune response. Syncytium formation is a hallmark of severe COVID-19 progression[69]. Therefore, the ability to inhibit syncytium formation after viral infection may substantially reduce disease severity.

We also explored several protein targets of GI 254023X: ADAM10, ADAM17, and MMP-9. Our results implicated a potential, minor role for ADAM10 in S-mediated fusion, but no such role for ADAM17. Significantly, our findings determined that MMP-9 was capable of markedly enhancing infection of SARS-CoV-2 lentiviral pseudotypes in HEK293T-ACE2 cells. MMP-9 has an established impact on acute lung injury and acute respiratory distress syndrome following lung infection, and high MMP-9 expression is correlated with increased disease severity in COVID-19 patients[51,59,60]. Therefore, these results recapitulate the importance of investigating MMP-9 in the context of viral infection, particularly infection by SARS-CoV-2.

Overall, our data have pointed to ADAM and MMP family proteins as important host factors in SARS-CoV-2 S-mediated fusion. In particular, we identified MMP-9 as a major driver in SARS-CoV-2 infection and therefore as a promising antiviral drug target. Future studies may aim to further elucidate the mechanism of MMP-9 in enhancing entry and explore other MMPs as potential antiviral candidates.

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Contributions of Collaborators

Kathy Fu and Rory Mulloy generated the SARS-CoV-2 S constructs (WT, R815A). Dr. Geneviève Laroche generated the D614G SARS-CoV-2 S construct. Kathy Fu and Rory Mulloy produced the lentivirus particles and the performed syncytium formation assay in Figure 1 and the overlay assay in Figure 2. Alain David ran and transferred the final MMP-9 blot in Figure 7C.