

AOP ID and Title:

AOP 422: Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption

Short Title: SARS-CoV-2 leads to intestinal barrier disruption**Authors**

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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.96	Included in OECD Work Plan

Background

AOP422 is developed as a part of the [CIAO project](#), "Modelling the Pathogenesis of COVID-19 Using the Adverse Outcome Pathway (AOP)". The overall goal is to organize and understand the vast amount of data that is constantly evolving as a result of the COVID-19 pandemic and identify uncertainties and knowledge gaps that may be missing using the AOP framework. Many AOPs were developed in the CIAO project, each AOP focusing on a specific element of the SARS-COV-2 virus responses in humans.

AOP422 focuses on the gastrointestinal disorders following SARS-CoV-2 infection of the enterocytes inducing disruption of intestinal barrier.

Summary of the AOP**Events****Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)**

Sequence	Type	Event ID	Title	Short name
1	MIE	1739	Binding to ACE2	Binding to ACE2
2	KE	1738	SARS-CoV-2 cell entry	SARS-CoV-2 cell entry
3	KE	1901	Interferon-I antiviral response, antagonized by SARS-CoV-2	IFN-I response, antagonized
4	KE	1847	Increased SARS-CoV-2 production	SARS-CoV-2 production
5	KE	1931	Intestinal barrier, disruption	Disruption of the intestinal barrier

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding to ACE2	adjacent	SARS-CoV-2 cell entry	High	High
SARS-CoV-2 cell entry	adjacent	Interferon-I antiviral response, antagonized by SARS-CoV-2		
Interferon-I antiviral response, antagonized by SARS-CoV-2	adjacent	Increased SARS-CoV-2 production		
Increased SARS-CoV-2 production	adjacent	Intestinal barrier, disruption	Low	Low

Stressors

Name	Evidence
Sars-CoV-2	

Overall Assessment of the AOP**References****Appendix 1****List of MIEs in this AOP**[Event: 1739: Binding to ACE2](#)

Short Name: Binding to ACE2

Key Event Component

Process	Object	Action
receptor binding	angiotensin-converting enzyme 2	occurrence

AOPs Including This Key Event

AOP ID and Name

Event Type

AOP422

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	MolecularInitiatingEvent
Aop:374 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on brain cells (neuronal and non-neuronal) leads to neuroinflammation resulting in encephalitis	MolecularInitiatingEvent
Aop:381 - Binding of viral S-glycoprotein to ACE2 receptor leading to dysgeusia	MolecularInitiatingEvent
Aop:385 - ACE2 binding to viral S-protein leading to microvascular disfunction via ACE2 dysregulation	MolecularInitiatingEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	MolecularInitiatingEvent
Aop:395 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on pericytes leads to disseminated intravascular coagulation resulting in cerebrovascular disease (stroke)	MolecularInitiatingEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	MolecularInitiatingEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	MolecularInitiatingEvent
Aop:426 - SARS-CoV-2 spike protein binding to ACE2 receptors expressed on pericytes leads to endothelial cell dysfunction, microvascular injury and myocardial infarction.	MolecularInitiatingEvent
Aop:427 - ACE2 downregulation following SARS-CoV-2 infection triggers dysregulation of RAAS and can lead to heart failure.	MolecularInitiatingEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	MolecularInitiatingEvent
Aop:428 - Binding of S-protein to ACE2 in enterocytes induces ACE2 dysregulation leading to gut dysbiosis	MolecularInitiatingEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	MolecularInitiatingEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	MolecularInitiatingEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	MolecularInitiatingEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Receptor recognition is an essential determinant of molecular level in this AOP. ACE2 was reported as an entry receptor for SARS-CoV-2. The viral entry process is mediated by the envelope-embedded surface-located spike (S) glycoprotein. Jun Lan and Walls, A.C et al (Nature 581, 215–220; Cell 180, 281–292) demonstrated a critical initial step of infection at the molecular level from the interaction of ACE2 and S protein. ACE2 has shown that receptor binding affinity to S protein is nM range. To elucidate the interaction between the SARS-CoV-2 RBD and ACE2 at a higher resolution, they also determined the structure of the SARS-CoV-2 RBD–ACE2 complex using X-ray crystallography. The expression and distribution of the ACE2 in human body may indicate the potential infection of SARS-CoV-2. Through the developed single-cell RNA sequencing (scRNA-Seq) technique and single-cell transcriptomes based on the public database, researchers analyzed the ACE2 RNA expression profile at single-cell resolution. High ACE2 expression was identified in type II alveolar cells (Zou, X. et al. Front. Med.2020)

SARS-CoV-2 belongs to the Coronaviridae family, which includes evolutionary related enveloped (+) strand RNA viruses of vertebrates, such as seasonal common coronaviruses, SARS-CoV and CoV-NL63, SARS-CoV (Kim Young Jun et al)

Human viruses strains	Genus	Major cell receptor	First report	Animal reservoir	Intermediate host	Pathology	Diagnostic test	Evidence
HCoV-NL63	Alphacoronavirus	ACE2	2004	Bat	Unknown	Mild respiratory tract illness	RT-PCR, IF, ELISA, WB	Strong
SARS-CoV	Betacoronavirus	ACE2	2003	Bat	Pangolin	Severe acute respiratory syndrome	RT-PCR, IF, ELISA, WB	Strong
SARS-CoV-2	Betacoronavirus	ACE2	2020	Bat	Pangolin	Severe acute respiratory syndrome	RT-PCR, IF, ELISA, WB	Strong

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
Mustela lutreola	Mustela lutreola	High	NCBI
Felis catus	Felis catus	Moderate	NCBI
Panthera tigris	Panthera tigris	Moderate	NCBI
Canis familiaris	Canis lupus familiaris	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Key Event Description

Angiotensin-converting enzyme 2 ([ACE2](#)) is an enzyme that can be found either attached to the membrane of the cells (mACE2) in many tissues and in a soluble form (sACE2).

A table on ACE2 expression levels according to tissues (*Kim et al.*)

	Sample size	ACE2 mean expression	Standard deviation of expression
Intestine	51	9.50	1.183
Kidney	129	9.20	2.410
Stomach	35	8.25	3.715
Bile duct	9	7.23	1.163
Liver	50	6.86	1.351
Oral cavity	32	6.23	1.271
Lung	110	5.83	0.710
Thyroid	59	5.65	0.646
Esophagus	11	5.31	1.552
Bladder	19	5.10	1.809
Breast	113	4.61	0.961
Uterus	25	4.37	1.125
Prostate	52	4.35	1.905

ACE2 receptors in the brain (endothelial, neuronal and glial cells):

The highest ACE2 expression level in the brain was found in the pons and medulla oblongata in the human brainstem, containing the medullary respiratory centers (Lukiw et al., 2020). High ACE2 receptor expression was also found in the amygdala, cerebral cortex and in the regions involved in cardiovascular function and central regulation of blood pressure including the sub-fornical organ, nucleus of the tractus solitarius, paraventricular nucleus, and rostral ventrolateral medulla (Gowrisankar and Clark 2016; Xia and Lazartigues 2010). The neurons and glial cells, like astrocytes and microglia also express ACE-2.

In the brain, ACE2 is expressed in endothelium and vascular smooth muscle cells (Hamming et al., 2004), as well as in neurons and glia (Gallagher et al., 2006; Matsushita et al., 2010; Gowrisankar and Clark, 2016; Xu et al., 2017; de Moraes et al., 2018) (from Murta et al., 2020). Astrocytes are the main source of angiotensinogen and express ATR1 and MasR; neurons express ATR1, ACE2, and MasR, and microglia respond to ATR1 activation (Shi et al., 2014; de Moraes et al., 2018).

ACE2 receptors in the intestines

The highest levels of ACE2 are found at the luminal surface of the enterocytes, the differentiated epithelial cells in the small intestine, lower levels in the crypt cells and in the colon (Liang et al, 2020; Hashimoto et al., 2012, Fairweather et al. 2012; Kowalczyk et al. 2008).

How it is Measured or Detected

In vitro methods supporting interaction between ACE2 and SARS-CoV-2 spike protein

Several reports using surface plasmon resonance (SPR) or biolayer interferometry binding (BLI) approaches, to study the interaction between recombinant ACE2 and S proteins have determined a dissociation constant (Kd) for SARS-CoV S and SARS-CoV-2 S as follow,

Reference	ACE2 protein	SARS-CoV S	SARS-CoV2 S	Method	Measured Kd
doi:10.1126/science.abb2507	1–615 aa	306–577 aa		SPR	325.8 nM
			1–1208 aa		14.7 nM
doi:10.1001/jama.2020.3786	19–615 aa	306–527 aa		SPR	408.7 nM
			319–541 aa		133.3 nM
Lan et al., 2020	19–615 aa	306–527 aa		SPR	31.6 nM
			319–541 aa		4.7 nM
doi:10.1016/j.cell.2020.02.058	1–614 aa	306–575 aa		BLI	1.2 nM
			328–533 aa		5 nM
doi:10.1126/science.abb2507	1–615 aa	306–577 aa		BLI	13.7 nM
			319–591 aa		34.6 nM

Pseudo typed vesicular stomatitis virus expressing SARS-CoV-2 S (VSV-SARS-S2) expression system can be used efficiently infects cell lines, with Calu-3 human lung adenocarcinoma epithelial cell line, CaCo-2 human colorectal

adenocarcinoma colon epithelial cell line and Vero African grey monkey kidney epithelial cell line being the most permissive (Hoffmann et al., 2020; Ou et al., 2020). It can be measured using a wide variety of assays targeting different biological phases of infection and altered cell membrane permeability and cell organelle signaling pathway. Other assay measured alteration in the levels of permissive cell lines all express ACE2 or hACE2-expressing 293T cell (e.g. pNUO1-hACE2, pFUSE-hlgG1-Fc2), as previously demonstrated by indirect immunofluorescence (IF) or by immunoblotting are associated with ELISA(W Tai et al., nature 2020). To prioritize the identified potential KEs for selection and to select a KE to serve as a case study, further in-silico data that ACE2 binds to SARS-CoV-2 S is necessary for virus entry. The above analysis outlined can be used evidence-based assessment of molecular evidence as a MIE.

References

- de Morais SDB, et al. Integrative Physiological Aspects of Brain RAS in Hypertension. *Curr Hypertens Rep.* 2018 Feb 26; 20(2):10.
- Gallagher PE, et al. Distinct roles for ANG II and ANG-(1-7) in the regulation of angiotensin-converting enzyme 2 in rat astrocytes. *Am J Physiol Cell Physiol.* 2006 Feb; 290(2):C420-6.
- Gowrisankar YV, Clark MA. Angiotensin II regulation of angiotensin-converting enzymes in spontaneously hypertensive rat primary astrocyte cultures. *J Neurochem.* 2016 Jul; 138(1):74-85.
- Hamming I et al. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol.* 2004 Jun;203(2):631-7.
- Jakhmola S, et al. SARS-CoV-2, an Underestimated Pathogen of the Nervous System. *SN Compr Clin Med.* 2020.
- Lukiw WJ et al. SARS-CoV-2 Infectivity and Neurological Targets in the Brain. *Cell Mol Neurobiol.* 2020 Aug 25;1-8.
- Matsushita T, et al. CSF angiotensin II and angiotensin-converting enzyme levels in anti-aquaporin-4 autoimmunity. *J Neurol Sci.* 2010 Aug 15; 295(1-2):41-5.
- Murta et al. Severe Acute Respiratory Syndrome Coronavirus 2 Impact on the Central Nervous System: Are Astrocytes and Microglia Main Players or Merely Bystanders? *ASN Neuro.* 2020. PMID: 32878468
- Shi A, et al. Isolation, purification and molecular mechanism of a peanut protein-derived ACE-inhibitory peptide. *PLoS One.* 2014; 9(10):e111188.
- Xia, H. and Lazartigues, E. Angiotensin-Converting Enzyme 2: Central Regulator for Cardiovascular Function. *Curr. Hypertens.* 2010 Rep. 12 (3), 170– 175

List of Key Events in the AOP

Event: 1738: SARS-CoV-2 cell entry

Short Name: SARS-CoV-2 cell entry

Key Event Component

Process	Object	Action
membrane fusion	transmembrane protease serine 2	occurrence
endocytosis involved in viral entry into host cell	cathepsin L1 (human)	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	KeyEvent
Aop:395 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on pericytes leads to disseminated intravascular coagulation resulting in cerebrovascular disease (stroke)	KeyEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	KeyEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	KeyEvent
Aop:426 - SARS-CoV-2 spike protein binding to ACE2 receptors expressed on pericytes leads to endothelial cell dysfunction, microvascular injury and myocardial infarction.	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	KeyEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

Organ term**Domain of Applicability****Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Manis javanica	Manis javanica	Low	NCBI
Canis familiaris	Canis lupus familiaris	Moderate	NCBI
Macaca fascicularis	Macaca fascicularis	Not Specified	NCBI
Mesocricetus auratus	Mesocricetus auratus	Not Specified	NCBI
Mustela putorius furo	Mustela putorius furo	Not Specified	NCBI
Felis catus	Felis catus	Moderate	NCBI
Mustela lutreola	Mustela lutreola	High	NCBI
Neovison vison	Neovison vison	High	NCBI
Panthera tigris	Panthera tigris	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
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All life stages High

Sex Applicability

Sex	Evidence
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Unspecific High

TMPRSS2 vertebrates (Lam et al., 2020)

NRP1 in human & rodents (but also present in monkey and other vertebrates (Lu and Meng, 2015)

The ability for SARS-CoV-2 to use multiple host pathways for viral entry, means that it is critical to map which viral entry pathway is prevalent in specific cell types. This is key for understanding coronavirus biology, but also use informed decisions to select cells for cell-based genetic and small-molecule screens and to interpret data. In fact, a combination of protease inhibitors that block both TRMPSS2 and cathepsin L is the most efficient combination to block coronavirus infection (Yamamoto et al., 2020, Shang et al., 2020, Shirato et al., 2018). In accordance, SARS-CoV-2 entry processes are highly dependent on endocytosis and endocytic maturation in cells that do not express TMPRSS2, such as VeroE6 or 293T cells (Murgolo et al., 2021, Kang et al., 2020, Mirabelli et al., 2020, Riva et al., 2020). However, even in these cells, heterologous expression of TMPRSS2 abrogates the pharmacological blockade of cathepsin inhibitors (Kawase et al., 2012, Hoffmann et al., 2020a). Treatment of SARS-CoV-2 with trypsin enables viral cell surface entry, even when TMPRSS2 is absent. Moreover, TMPRSS2 is more efficient to promote viral entry than cathepsins (Lamers et al., 2020), as when both factors are present, cathepsin inhibitors are less effective than TMPRSS2 inhibitors (Hoffmann et al., 2020b). Therefore it is critical to map which cells contain the different types of proteases.

In summary, TMPRSS2 appears to be expressed in a wide range of healthy adult organs, but in restricted cell types, including:

- AT2 and clara cells of lungs
- sinusoidal endothelium, and hepatocyte of the liver,
- endocrine cells of the prostate,
- goblet cells, and enterocytes of the small intestine,
- intercalated cells, and the proximal tubular of the kidney.
- Ciliated, secretory and suprabasal of nasal
- spermatogonial stem cells of testes
- cyto tropoblast and peri vascular cells of placenta
- The nasal epithelium expresses various combinations of factors that, in principle, could facilitate SARS-CoV-2 infection, but it also expresses robust basal levels of RFs, which may act as a strong protective barrier in this tissue.

There is a shift in TMPRSS2 regulation during nasal epithelium differentiation in young individuals that is not occurring in old individuals (Lin et al., 1999, Lucas et al., 2008, Singh et al., 2020).

Only a small minority of human respiratory and intestinal cells have genes that express both ACE2 and TMPRSS2. Amongst the ones that do, three main cell types were identified: A) lung cells called type II pneumocytes (which help maintain air sacs, known as alveoli); B) intestinal cells called enterocytes, which help the body absorb nutrients; and C) goblet cells in the nasal passage, which secrete mucus (Ziegler et al., 2020).

The clinical manifestations of COVID-19 include not only complications from acute myocardial injury, elevated liver enzymes, and acute kidney injury in patients presenting to hospitals, but also gastrointestinal symptoms in community patients experiencing milder forms of the disease (Madjid et al., 2020, Pan et al., 2020).

NRP-1:

All life stages

The expression of isoforms 1 (NRP1) and 2 (NRP2) does not seem to overlap. Isoform 1 is expressed by the blood vessels of different tissues. In the developing embryo it is found predominantly in the nervous system. In adult tissues, it is highly expressed in heart and placenta; moderately in lung, liver, skeletal muscle, kidney and pancreas; and low in adult brain. Isoform 2 is found in liver hepatocytes, kidney distal and proximal tubules. Expressed in colon and 234 other tissues with Low tissue specificity (UniProtKB).

The expression of NRP1 protein in gastric cancer was not related to gender or age (Cao et al., 2020).

Sex Applicability:**TMPRSS2:**

Androgen receptors (ARs) play a key role in the transcription of TMPRSS2 (Fig. 1). This may explain the predominance of males to COVID-19 infection, fatality, and severity because males tend to have a higher expression and activation of ARs than females, which is due to the presence of dihydrotestosterone (DHT).

Regulation of COVID-19 severity and fatality by sex hormones. Females have aromatase, the enzyme that converts androgen substrates into estrogen. On the other hand, males have steroid 5α reductase, the enzyme that is responsible for the conversion of testosterone into dihydrotestosterone (DHT). In case of males, DHT activates androgen receptor (AR) that binds to the androgen response element (ARE) present in the promoter of TMPRSS2 gene, leading to its transcription. This ultimately results into enhanced processing of viral spike protein for greater entry and spread of SARS-CoV-2 into host cells. On the other hand, in females, estrogen activates estrogen receptor (ER), which binds to the estrogen response element (ERE) present in the promoter of eNOS gene to drive its transcription and catalyze the formation of nitric oxide (NO) from L-arginine. This NO is involved in vasodilation as well as inhibition of viral replication.

NRP-1:

For more information difference of NRP1 expression between male and female see <https://www.proteinatlas.org/ENSG00000099250-NRP1/tissue>.

The expression of NRP1 protein in gastric cancer was not related to gender, age. The expression of NRP1 protein in gastric cancer is closely correlated to clinical stage, tumor size, TNM stage, differentiation, and lymph node metastasis (Cao et al., 2020).

SARS-CoV-2 Spike protein co-opts VEGF-A/Neuropilin-1 receptor signalling to induce analgesia had same results on both male and female rodents (Moutal et al., 2020).

Key Event Description

Coronavirus is recognized by the binding of S protein on the viral surface and angiotensin-converting enzyme 2 (ACE2) receptor on the cellular membrane, followed by viral entry via processing of S protein by transmembrane serine protease 2 (TMPRSS2) (Hoffmann et al., 2020b). ACE2 is expressed on epithelial cells of the lung and intestine, and also can be found in the heart, kidney, adipose, and male and female reproductive tissues (Lukassen et al., 2020, Lamers et al., 2020, Chen et al., 2020, Jing et al., 2020, Subramanian et al., 2020).

SARS-CoV-2 is an enveloped virus characterized by displaying spike proteins at the viral surface (Juraszek et al., 2021). Spike is critical for viral entry (Hoffmann et al., 2020b) and is the primary target of vaccines and therapeutic strategies, as this protein is the immunodominant target for antibodies (Yuan et al., 2020, Ju et al., 2020, Robbiani et al., 2020, Premkumar et al., 2020, Liu et al., 2020). Spike is composed of S1 and S2 subdomains. S1 contains the N-terminal (NTD) and receptor-binding (RBD) domains, and the S2 contains the fusion peptide (FP), heptad repeat 1 (HR1) and HR2, the transmembrane (TM) and cytoplasmic domains (CD) (Lan et al., 2020). S1 leads to the recognition of the angiotensin-converting enzyme 2 (ACE2) receptor and S2 is involved in membrane fusion (Hoffmann et al., 2020b, Letko et al., 2020, Shang et al., 2020).

Upon binding to ACE2, the spike protein needs to be activated (or primed) through proteolytic cleavage (by a host protease) to allow membrane fusion. Fusion is a key step in viral entry as it is the way to release SARS-CoV-2 genetic material inside the cell. Cleavage happens between its spike's S1 and S2 domains, liberating S2 that inserts its N-terminal domain into a host cell membrane and mediates membrane fusion (Millet and Whittaker, 2018). Many proteases were identified to activate coronaviruses including furin, cathepsin L, trypsin-like serine proteases TMPRSS2, TMPRSS4, TMPRSS11, and human airway trypsin-like protease (HATs). These may operate at four different stages of the [virus infection cycle](#): (a) pro-protein convertases (e.g., furin) during virus packaging in virus-producing cells, (b) extracellular proteases (e.g., elastase) after virus release into extracellular space, (c) cell surface proteases [e.g., type II transmembrane serine protease (TMPRSS2)] after virus attachment to virus-targeting cells, and (d) lysosomal proteases (e.g., cathepsin L) after virus endocytosis in virus-targeting cells (Li, 2016). SARS-CoV-2 lipidic envelope may fuse with two distinct membrane types, depending on the host protease(s) responsible for cleaving the spike protein: (i) cell surface following activation by serine proteases such as TMPRSS2 and furin (Hoffmann et al., 2020b); or (ii) endocytic pathway within the endosomal-lysosomal compartments including processing by lysosomal cathepsin L (Yang and Shen, 2020). These flexibility for host cell factors mediating viral entry, highlights that the availability of factors existing in a cell type dictates the mechanism of viral entry (Kawase et al., 2012). When TMPRSS2 (or other serine proteases such as TMPRSS4 (Zang et al., 2020) or human airway trypsin-like protease [HAT] (Bestle et al., 2020a)) is expressed, fusion of the virus with the cell surface membrane is preferred (Shirato et al., 2018), while in their absence, the virus can penetrate the cell by endocytosis (Kawase et al., 2012). A third factor has also been shown to facilitate SARS-CoV-2 entry in cells that have ACE2 and even promote, although to very low levels, SARS-CoV-2 entry in cells that lack ACE2 and TMPRSS2 which is the neuropilin-1 (NRP-1) (Cantuti-Castelvetri et al., 2020). This key event deals with SARS-CoV-2 entry in host cells and is divided in three categories: TMPRSS2, cathepsin L and NRP-1.

TMPRSS2 Spike cleavage:

TMPRSS2 (transmembrane serine protease 2, (<https://www.ncbi.nlm.nih.gov/gene/7113>) is a cell-surface protease (Hartenian et al., 2020) that facilitates entry of viruses into host cells by proteolytically cleaving and activating viral envelope glycoproteins. Viruses found to use this protein for cell entry include Influenza virus and the human coronaviruses HCoV-229E, MERS-CoV, SARS-CoV and SARS-CoV-2 (COVID-19 virus).

TMPRSS2 is a membrane bound serine protease also known as epitheliasin. TMPRSS2 belongs to the S1A class of serine proteases alongside proteins such as factor Xa and trypsin. Whilst there is evidence that TMPRSS2 autoclaves to generate a secreted protease, its physiological function has not been clearly identified. However, it is known to play a crucial role in facilitating entry of coronavirus particles into cells by cleaving the spike protein (Huggins, 2020).

After ACE2 receptor binding, SARS-CoV-2 S proteins can be subsequently cleaved and activated by host cell-surface protease at the S1/S2 and S2' sites, generating the subunits S1 and S2 that remain non-covalently linked. Cleavage leads to activation of the S2 domain that drives fusion of the viral and host membranes (Hartenian et al., 2020, Walls et al., 2016). For other coronaviruses, processing of spike was proposed to be sequential with S1/S2 cleavage preceding that of S2. Cleavage at S1/S2 may be crucial for inducing conformational changes required for receptor binding or exposure of the S2 site to host proteases.

The S1/S2 site of SARS-CoV-2 S protein contains an insertion of four amino acids providing a minimal furin cleavage site (RRAR685↓) (that is absent in SARS-CoV). Interestingly, the furin cleavage site has been implicated in increased viral pathogenesis (Bestle et al., 2020b, Huggins, 2020). Processing of the spike protein by furin at the S1/S2 cleavage site is thought to occur following viral replication in the endoplasmic reticulum Golgi intermediate compartment (ERGIC) (Hasan et al., 2020). The spike S2' cleavage site of SARS-CoV-2 possesses a paired dibasic motif with a single KR segment (KR815↓) (as SARS-CoV) that is recognized by trypsin-like serine proteases such as TMPRSS2. **The current data support a model for SARS-CoV-2 entry in which furin-mediated cleavage at the S1/S2 site pre-primers spike during biogenesis, facilitating the activation for membrane fusion by a second cleavage event at S2' by TMPRSS2 following ACE2 binding** (Bestle et al., 2020b, Johnson et al., 2020).

Virus	S1/S2 site	S2' site
SARS-CoV-2	TNSP RRAR SVSA	PSKPS KR SFIEDL
SARS-CoV	S---LLR STS	PLKPT KR SFIEDL

Camostat mesylate, an inhibitor of TMPRSS2, blocks SARS-CoV-2 infection of lung cells like Calu-3 cells but not Huh7.5 and Vero E6 cells. Cell entry was assessed using a viral isolate and viral pseudotypes (artificial viruses) expressing the COVID-19 spike (S) protein. The ability of the viral pseudotypes (expressing S protein from SARS-CoV and SARS-CoV-2) to enter human and animal cell lines was demonstrated, showing that SARS-CoV-2 can enter similar cell lines as SARS-CoV. Amino acid analysis and cell culture experiments showed that, like SARS-CoV, SARS-CoV-2 spike protein binds to human and bat angiotensin-converting enzyme 2 (ACE2) and uses a cellular protease TMPRSS2 for priming. Priming activates the spike protein to facilitate viral fusion and entry into cells. Cell culture experiments were performed using immortalized cell lines and primary human lung cells (Hoffmann et al., 2020b, Rahman et al., 2020).

Spike binding to neuropilin-1:

Neuropilin-1 (NRP1) is a transmembrane glycoprotein that serves as a cell surface receptor for semaphorins and various ligands involved in angiogenesis in vertebrates. NRP1 is expressed in neurons, blood vessels (endothelial cells), immune cells and many other cell types in the mammalian body (maternal fetal interface) and binds a range of structurally and functionally diverse extracellular ligands to modulate organ development and function (Raimondi et al., 2016). NRP1 is well described as a co-receptor for members of the class 3 semaphorins (SEMA3) or vascular endothelial growth factors (VEGFs) (Gelfand et al., 2014). Structurally, NRP1 comprises seven sub-domains, of which the first five are extracellular; two CUB domains (a1 and a2), two coagulation factor V/VIII domains (FV/VIII; b1 and b2) and a meprin, A5 μ -phosphatase domain (MAM; c). NRP1 contains only a short cytosolic tail with a PDZ-binding domain lacking internal signaling activity. The different ligand families bind to different sites of NRP1; SEMA3A binding requires the first three sub-domains of NRP1 (a1, a2, and b1), whereas binding of VEGF-A requires the b1 and b2 domains (Muhl et al., 2017). Additional studies conducted by means of in silico computational technology to identify and validate inhibitors of the interaction between NRP1 and SARS-CoV-2 Spike protein are reported in (Perez-Miller et al., 2020). Represents a schematic picture of VEGF-A triggered phosphorylation of VEGF-R2. Screening of NRP-1/VEGF-A165 inhibitors by in-cell Western (Perez-Miller et al., 2020).v NRP1 acts as a co-receptor for SARS-CoV-2.

NRP1 is a receptor for furin-cleaved SARS-CoV-2 spike peptide (Cantuti-Castelvetri et al., 2020, Daly et al., 2020, Johnson et al., 2020). Blockade of NRP1 reduces infectivity and entry, and alteration of the furin site leads to loss of NRP1 dependence, reduced replication in Calu3, augmented disease in a hamster pathogenesis disease model (Johnson et al., 2020). In fact, a small sequence of amino acids was found that appeared to mimic a protein sequence found in human proteins that interact with NRP1. The spike protein of SARS-CoV-2 binding with NRP1 aids viral infection of human cells. This was confirmed by applying a range of structural and biochemical approaches to establish that the spike protein of SARS-CoV-2 does indeed bind to NRP1. The host protease furin cleaves the full-length precursor S glycoprotein into two associated polypeptides: S1 and S2. Cleavage of S generates a polybasic RRAR C-terminal sequence on S1, which conforms to a C-end rule (CendR) motif that binds to cell surface neuropilin-1 (NRP1) and neuropilin-2 (NRP2) receptors. It was reported that the S1 CendR motif directly bound NRP1 by X-ray crystallography and biochemical approaches. Blocking this interaction using RNAi or selective inhibitors reduced SARS-CoV-2 entry and infectivity in cell culture (Daly et al., 2020).

NRP1, known to bind furin-cleaved substrates, significantly potentiates SARS-CoV-2 infectivity, which was revealed by a monoclonal blocking antibody against NRP1. It was found that a SARS-CoV-2 mutant with an altered furin cleavage site did not depend on NRP1 for infectivity. Pathological analysis of olfactory epithelium obtained from human COVID-19 autopsies revealed that SARS-CoV-2 infected NRP1-positive cells faced the nasal cavity (Cantuti-Castelvetri et al., 2020). Furthermore, it has been found that NRP1 is a new potential SARS-CoV-2 infection mediator implicated in the neurologic features and central nervous system involvement of COVID-19. Preclinical studies have suggested that NRP1, a transmembrane receptor that lacks a cytosolic protein kinase domain and exhibits high expression in the respiratory and olfactory epithelium, may also be implicated in COVID-19 by enhancing the entry of SARS-CoV-2 into the brain through the olfactory epithelium. NRP1 is also expressed in the CNS, including olfactory-related regions such as the olfactory tubercles and paraolfactory gyri. Supporting the potential role of NRP1 as an additional SARS-CoV-2 infection mediator implicated in the neurologic manifestations of COVID-19. Accordingly, the neurotropism of SARS-CoV-2 via NRP1-expressing cells in the CNS merits further investigation (Davies et al., 2020).

Up-regulation of NRP1 protein in diabetic kidney cells hints at its importance in a population at risk of severe COVID-19. Involvement of NRP-1 in immune function is compelling, given the role of an exaggerated immune response in disease severity and deaths due to COVID-19. NRP-1 has been suggested to be an immune checkpoint of T cell memory. It is unknown whether involvement and up-regulation of NRP-1 in COVID-19 may translate into disease outcome and long-term consequences, including possible immune dysfunction (Mayi et al., 2021).

The main feature of NRP1 co-receptor is to form complexes with multiple other receptors. Hence, there is a competition between receptors to complex with NRP-1, which may determine their abilities both quantitatively and qualitatively to transduce signals. It is tempting to hypothesize that the occupancy of NRP-1 with one receptor may thus decrease its availability for virus entry. Recent proteomics work has shown that NRP-1 can form a complex with the $\alpha 7$ nicotinic receptor in mice. Both receptors are expressed in the human nasal and pulmonary epithelium (Mayi et al., 2021).

NRP1, is highly expressed in the respiratory and olfactory epithelium; it is also expressed in the CNS, including olfactory related regions such as the olfactory tubercles and paraolfactory gyri (Davies et al., 2020).

More information on tissue distribution and protein expression of NRP1 can be found in <https://www.proteinatlas.org/ENSG0000000992.50-NRP1>

Spike entry via lysosomal cathepsins and endocytosis:

Evidence shows the role of TMPRSS2 and other serine proteases in activating the coronavirus spike protein for plasma membrane fusion. However, studies using various cell culture systems showed that SARS-CoV2 could enter cells via an alternative endosomal-lysosomal pathway. Evidence came from studies demonstrating that lysosomotropic agents reduced SARS-CoV replication in cells lacking TMPRSS2 and other studies, using highly potent and specific small-molecule cathepsin inhibitors, to understand the role of cathepsins in processing and activating the spike for membrane fusion, mainly of cathepsin L (one of the 11 cathepsins) (Rossi et al., 2004, Simmons et al., 2005). SARS-CoV-2 and other coronaviruses can establish infection through endosomal entry in commonly used in vitro cell culture systems. Of relevance, inhibitors of the endosomal pathway, as the cathepsin inhibitor Z-FA-FMK and PIKfyve inhibitor apilimod, blocked viral entry in Huh7.5 and Vero E6 cells but not Calu-3 cells.

Viral entry leads to delivery of virion proteins and translation of viral proteins immediately:

Coronavirus is a class of viruses that have single-stranded positive-sense RNA genomes in their envelopes [Kim D, et al., 2020]. The virus contains a 29.7 kB positive-sense RNA genome flanked by 5' and 3' untranslated regions of 265 and 342 nucleotides, respectively that contain cis-acting secondary RNA structures essential for RNA synthesis [Huston N. C. et al., 2021]. The genome just prior to the 5' end contains the transcriptional regulatory sequence leader (TRS-L) [Budzylowicz C.J., et al., 1985]. The SARS-CoV genome is polycistronic and contains 14 open reading frames (ORFs) that are expressed by poorly understood mechanisms [Snijder E. J., et al., 2003]. Preceding each ORF there are other TRSs called the body TRS (TRS B). The 5' two-thirds of the genome contains two large, overlapping, nonstructural ORFs and the 3' third contains the remainder ORFs [Di H., et al., 2018]. Genome expression starts with the translation of two large ORFs of the 5' two-thirds: ORF1a of 4382 amino acids and ORF1ab of 7073 amino acid that occurs via a programmed (-1) ribosomal frameshifting [Snijder E. J., et al., 2003], yielding pp1a and pp1ab. These two polyproteins are cleaved into 16 subunits by two viral proteinases encoded by ORF1a, nsp3, and nsp5 that contain a papain-like protease domain and a 3C-like protease domain [Sacco M. D. et al., 2020]. The processing products are a group of replicative enzymes, named nsp1-nsp16, that assemble into a viral replication and transcription complex (RTC) associated with membranes of endoplasmic reticulum (ER) with the help of various membrane-associated viral proteins [Klein S., et al., 2021, Snijder E. J., et al., 2020, V'kovski P., et al., 2021]. This association leads to replication factories or organelles, that are originate new membranous structures that are observed by electron microscopy. They are a feature of all coronaviridae and the site of viral replication and transcription hidden from innate immune molecules.

How it is Measured or Detected

SARS-CoV2 entry can be determined by many different ways:

- 1) quantitative RT-PCR specific to the subgenomic mRNA of the N transcript, in cells manipulated with host factors that express of not TMPRSS2, cathepsinL, neuropilin-1, hACE2 [Glowacka I, et al. (2011)], or exogenous addition of HAT or furin.
- 2) using spike-pseudotyped viral particles expressing GFP/luciferase/βgalactosidase and comparing with vesicular stomatitis virus G pseudotyped particles expressing the same reporter analysed in manipulated cultured with cell lines, followed by determining fluorescence, bioluminescence, luciferase activity in cell lysates [Hoffmann M, et al. (2020)].

TMPRSS2:

TMPRSS2 gene expression can be measured by RNAseq and microarray (Baughn et al., 2020).

Expression levels of TMPRSS2 can be measured by RNA in situ hybridization (RNA-ISH) (Qiao et al., 2020)

NRP-1:

Several methods have been identified in the literature for measuring and detecting NRP1 receptor binding. Briefly described:

1. X-ray crystallography and biochemical approaches help to show that the S1 CendR motif directly bound NRP1 (1). Binding of the S1 fragment to NRP1 was assessed and ability of SARS-CoV-2 to use NRP1 to infect cells was measured in angiotensin-converting enzyme-2 (ACE-2)-expressing cell lines by knocking out NRP1 expression, blocking NRP1 with 3 different anti-NRP1 monoclonal antibodies, or using NRP1 small molecule antagonists (Centers for Disease Control and Prevention, 2020, Daly et al., 2020).

Key findings (Centers for Disease Control and Prevention, 2020, Daly et al., 2020):

- The S1 fragment of the cleaved SARS-CoV-2 spike protein binds to the cell surface receptor neuropilin-1 (NRP1).
- SARS-CoV-2 utilizes NRP1 for cell entry as evidenced by decreased infectivity of cells in the presence of: NRP1 deletion (p <0.01). Three different anti-NRP1 monoclonal antibodies (p <0.001). Selective NRP1 antagonist, EG00229 (p <0.01).
- 2. Cell lines were modified to express ACE2 and TMPRSS2, the two known SARS-CoV-2 host factors, and NRP1 to assess the contribution of NRP1 to infection. Autopsy specimens from multiple airway sites were stained with antibodies against SARS-CoV-2 proteins, ACE2, and NRP1, to visualize co-localization of proteins (6, 15).

Key findings (Cantuti-Castelvetri et al., 2020, Centers for Disease Control and Prevention, 2020):

- Infectivity of cells expressing angiotensin converting enzyme-2 (ACE2, receptor for SARS-CoV-2), transmembrane protease serine-2 (TSS2, primes the Spike [S] protein), and neuropilin-1 (NRP1) with pseudovirus expressing the SARS-CoV-2 S1 protein was approximately 3-fold higher than in cells expressing either ACE2 or TSS2 alone (p<0.05).
- Analysis of autopsy tissue from COVID-19 patients showed co-localization of the SARS-CoV-2 spike (S) protein and NRP1 in olfactory and respiratory epithelium.

Virtual screen of nearly 0.5 million compounds against the NRP-1 CendR site, resulting in nearly 1,000 hits. A pharmacophore model was derived from the identified ligands, considering both steric and electronic requirements. Preparation of receptor protein and grid for virtual screening, docking of known NRP-1 targeting compounds, ELISA based NRP1-VEGF-A165 protein binding assay; more details on methodology in the referenced paper (Perez-Miller et al., 2020)

References

- BAUGHN, L. B., SHARMA, N., ELHAIK, E., SEKULIC, A., BRYCE, A. H. & FONSECA, R. 2020. Targeting TMPRSS2 in SARS-CoV-2 Infection. *Mayo Clin Proc*, 95, 1989-1999.
- BESTLE, D., HEINDL, M. R., LIMBURG, H., VAN LAM VAN, T., PILGRAM, O., MOULTON, H., STEIN, D. A., HARDES, K., EICKMANN, M., DOLNIK, O., ROHDE, C., KLENK, H. D., GARTEN, W., STEINMETZER, T. & BÖTTCHER-FRIEBERTSHÄUSER, E. 2020a. TMPRSS2 and furin are both essential for proteolytic activation of SARS-CoV-2 in human airway cells. *Life Science Alliance*, 3.
- BESTLE, D., HEINDL, M. R., LIMBURG, H., VAN LAM VAN, T., PILGRAM, O., MOULTON, H., STEIN, D. A., HARDES, K., EICKMANN, M., DOLNIK, O., ROHDE, C., KLENK, H. D., GARTEN, W., STEINMETZER, T. & BOTTCHER-FRIEBERTSHAUSER, E. 2020b. TMPRSS2 and furin are both essential for proteolytic activation of SARS-CoV-2 in human airway cells. *Life Sci Alliance*, 3.
- BUDZILOWICZ, C.J., WILCZYNSKI, S.P., AND WEISS, S.R. (1985). Three intergenic regions of coronavirus mouse hepatitis virus strain A59 genome RNA contain a common nucleotide sequence that is homologous to the 3' end of the viral mRNA leader sequence. *J Virol* 53, 834-840.
- CANTUTI-CASTELVETRI, L., OJHA, R., PEDRO, L. D., DJANNATIAN, M., FRANZ, J., KUIVANEN, S., VAN DER MEER, F., KALLIO, K., KAYA, T., ANASTASINA, M., SMURA, T., LEVANOV, L., SZIROVICZA, L., TOBI, A., KALLIO-KOKKO, H., OSTERLUND, P., JOENSUU, M., MEUNIER, F. A., BUTCHER, S. J., WINKLER, M. S., MOLLENHAUER, B.,

- HELENIUS, A., GOKCE, O., TEESALU, T., HEPOJOKI, J., VAPALAHTI, O., STADELMANN, C., BALISTRERI, G. & SIMONS, M. 2020. Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity. *Science*, 370, 856-860.
- CAO, H., LI, Y., HUANG, L., BAI, B. & XU, Z. 2020. Clinicopathological Significance of Neuropilin 1 Expression in Gastric Cancer: A Meta-Analysis. *Dis Markers*, 2020, 4763492.
- CENTERS FOR DISEASE CONTROL AND PREVENTION, U. S. D. O. H. A. H. S. 2020. Covid-19 Science Update 2020.
- CHEN, L., LI, X., CHEN, M., FENG, Y. & XIONG, C. 2020. The ACE2 expression in human heart indicates new potential mechanism of heart injury among patients infected with SARS-CoV-2. *Cardiovasc Res*, 116, 1097-1100.
- DALY, J. L., SIMONETTI, B., KLEIN, K., CHEN, K. E., WILLIAMSON, M. K., ANTON-PLAGARO, C., SHOEMARK, D. K., SIMON-GRACIA, L., BAUER, M., HOLLANDI, R., GREBER, U. F., HORVATH, P., SESSIONS, R. B., HELENIUS, A., HISCOX, J. A., TEESALU, T., MATTHEWS, D. A., DAVIDSON, A. D., COLLINS, B. M., CULLEN, P. J. & YAMAUCHI, Y. 2020. Neuropilin-1 is a host factor for SARS-CoV-2 infection. *Science*, 370, 861-865.
- DAVIES, J., RANDEVA, H. S., CHATHA, K., HALL, M., SPANDIDOS, D. A., KARTERIS, E. & KYROU, I. 2020. Neuropilin1 as a new potential SARSCoV2 infection mediator implicated in the neurologic features and central nervous system involvement of COVID19. *Mol Med Rep*, 22, 4221-4226.
- DI, H., MCINTYRE, A.A., AND BRINTON, M.A. (2018). New insights about the regulation of Nidovirus subgenomic mRNA synthesis. *Virology* 517, 38-43.
- GELFAND, M. V., HAGAN, N., TATA, A., OH, W. J., LACOSTE, B., KANG, K. T., KOPYCINSKA, J., BISCHOFF, J., WANG, J. H. & GU, C. 2014. Neuropilin-1 functions as a VEGFR2 co-receptor to guide developmental angiogenesis independent of ligand binding. *Elife*, 3, e03720.
- HARTENIAN, E., NANDAKUMAR, D., LARI, A., LY, M., TUCKER, J. M. & GLAUNSINGER, B. A. 2020. The molecular virology of coronaviruses. *J Biol Chem*, 295, 12910-12934.
- HASAN, A., PARAY, B. A., HUSSAIN, A., QADIR, F. A., ATTAR, F., AZIZ, F. M., SHARIFI, M., DERAKHSHANKHAH, H., RASTI, B., MEHRABI, M., SHAHPASAND, K., SABOURY, A. A. & FALAHATI, M. 2020. A review on the cleavage priming of the spike protein on coronavirus by angiotensin-converting enzyme-2 and furin. *J Biomol Struct Dyn*, 1-9.
- HOFFMANN, M., KLEINE-WEBER, H. & POHLMANN, S. 2020a. A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol Cell*, 78, 779-784 e5.
- HOFFMANN, M., KLEINE-WEBER, H., SCHROEDER, S., KRUGER, N., HERRLER, T., ERICHSEN, S., SCHIERGENS, T. S., HERRLER, G., WU, N. H., NITSCHKE, A., MULLER, M. A., DROSTEN, C. & POHLMANN, S. 2020b. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*, 181, 271-280 e8.
- HUGGINS, D. J. 2020. Structural analysis of experimental drugs binding to the SARS-CoV-2 target TMPRSS2. *J Mol Graph Model*, 100, 107710.
- HUSTON, N.C., WAN, H., STRINE, M.S., DE CESARIS ARAUJO TAVARES, R., WILEN, C.B., AND PYLE, A.M. (2021). Comprehensive in vivo secondary structure of the SARS-CoV-2 genome reveals novel regulatory motifs and mechanisms. *Mol Cell* 81, 584-598 e585.
- JING, Y., RUN-QIAN, L., HAO-RAN, W., HAO-RAN, C., YA-BIN, L., YANG, G. & FEI, C. 2020. Potential influence of COVID-19/ACE2 on the female reproductive system. *Mol Hum Reprod*, 26, 367-373.
- JOHNSON, B. A., XIE, X., 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., SWETNAM, D., PLANTE, J. A., AGUILAR, P., PLANTE, K. S., LEE, B., WEAVER, S. C., SUTHAR, M. S., ROUTH, A. L., REN, P., KU, Z., AN, Z., DEBBINK, K., SHI, P. Y., FREIBERG, A. N. & MENACHERY, V. D. 2020. Furin Cleavage Site Is Key to SARS-CoV-2 Pathogenesis. *bioRxiv*.
- JU, B., ZHANG, Q., GE, J., WANG, R., SUN, J., GE, X., YU, J., SHAN, S., ZHOU, B., SONG, S., TANG, X., YU, J., LAN, J., YUAN, J., WANG, H., ZHAO, J., ZHANG, S., WANG, Y., SHI, X., LIU, L., ZHAO, J., WANG, X., ZHANG, Z. & ZHANG, L. 2020. Human neutralizing antibodies elicited by SARS-CoV-2 infection. *Nature*, 584, 115-119.
- JURASZEK, J., RUTTEN, L., BLOKLAND, S., BOUCHIER, P., VOORZAAT, R., RITSCHER, T., BAKKERS, M. J. G., RENAULT, L. L. R. & LANGEDIJK, J. P. M. 2021. Stabilizing the closed SARS-CoV-2 spike trimer. *Nat Commun*, 12, 244.
- KANG, Y.-L., CHOU, Y.-Y., ROTHLAUF, P. W., LIU, Z., SOH, T. K., CURETON, D., CASE, J. B., CHEN, R. E., DIAMOND, M. S., WHELAN, S. P. J. & KIRCHHAUSEN, T. 2020. Inhibition of PIKfyve kinase prevents infection by Zaire ebolavirus and SARS-CoV-2. *bioRxiv*, 2020.04.21.053058.
- KAWASE, M., SHIRATO, K., VAN DER HOEK, L., TAGUCHI, F. & MATSUYAMA, S. 2012. Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. *J Virol*, 86, 6537-45.
- KIM, D., LEE, J.Y., YANG, J.S., KIM, J.W., KIM, V.N., AND CHANG, H. (2020). The Architecture of SARS-CoV-2 Transcriptome. *Cell* 181, 914-921 e910.
- KLEIN, S., CORTESE, M., WINTER, S.L., WACHSMUTH-MELM, M., NEUFELDT, C.J., CERIKAN, B., STANIFER, M.L., BOULANT, S., BARTENSCHLAGER, R., AND CHLANDA, P. (2020). SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *Nat Commun* 11, 5885.
- LAM, S. D., BORDIN, N., WAMAN, V. P., SCHOLLES, H. M., ASHFORD, P., SEN, N., VAN DORP, L., RAUER, C., DAWSON, N. L., PANG, C. S. M., ABBASIAN, M., SILLITOE, I., EDWARDS, S. J. L., FRATERNALI, F., LEES, J. G., SANTINI, J. M. & ORENGO, C. A. 2020. SARS-CoV-2 spike protein predicted to form complexes with host receptor protein orthologues from a broad range of mammals. *Sci Rep*, 10, 16471.
- LAMERS, M. M., BEUMER, J., VAN DER VAART, J., KNOOPS, K., PUSCHHOF, J., BREUGEM, T. I., RAVELLI, R. B. G., PAUL VAN SCHAYCK, J., MYKYTYN, A. Z., DUIMEL, H. Q., VAN DONSELAAR, E., RIESEBOSCH, S., KUIJPERS, H. J. H., SCHIPPER, D., VAN DE WETERING, W. J., DE GRAAF, M., KOOPMANS, M., CUPPEN, E., PETERS, P. J., HAAGMANS, B. L. & CLEVERS, H. 2020. SARS-CoV-2 productively infects human gut enterocytes. *Science*, 369, 50-54.
- LAN, J., GE, J., YU, J., SHAN, S., ZHOU, H., FAN, S., ZHANG, Q., SHI, X., WANG, Q., ZHANG, L. & WANG, X. 2020. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature*, 581, 215-220.
- LETKO, M., MARZI, A. & MUNSTER, V. 2020. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol*, 5, 562-569.
- LI, F. 2016. Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annu Rev Virol*, 3, 237-261.
- LIN, B., FERGUSON, C., WHITE, J. T., WANG, S., VESSELLA, R., TRUE, L. D., HOOD, L. & NELSON, P. S. 1999. Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. *Cancer Res*, 59, 4180-4.
- LIU, L., WANG, P., NAIR, M. S., YU, J., RAPP, M., WANG, Q., LUO, Y., CHAN, J. F., SAHI, V., FIGUEROA, A., GUO, X. V., CERUTTI, G., BIMELA, J., GORMAN, J., ZHOU, T., CHEN, Z., YUEN, K. Y., KWONG, P. D., SODROSKI, J. G., YIN, M. T., SHENG, Z., HUANG, Y., SHAPIRO, L. & HO, D. D. 2020. Potent neutralizing antibodies against multiple epitopes on SARS-CoV-2 spike. *Nature*, 584, 450-456.
- LU, Y. & MENG, Y. G. 2015. Quantitation of Circulating Neuropilin-1 in Human, Monkey, Mouse, and Rat Sera by ELISA. *Methods Mol Biol*, 1332, 39-48.
- LUCAS, J. M., TRUE, L., HAWLEY, S., MATSUMURA, M., MORRISSEY, C., VESSELLA, R. & NELSON, P. S. 2008. The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. *J Pathol*, 215, 118-25.
- LUKASSEN, S., CHUA, R. L., TREFZER, T., KAHN, N. C., SCHNEIDER, M. A., MULEY, T., WINTER, H., MEISTER, M., VEITH, C., BOOTS, A. W., HENNIG, B. P., KREUTER, M., CONRAD, C. & EILS, R. 2020. SARS-CoV-2 receptor ACE2 and TMPRSS2 are primarily expressed in bronchial transient secretory cells. *EMBO J*, 39, e105114.
- MADJID, M., SAFAVI-NAEINI, P., SOLOMON, S. D. & VARDENY, O. 2020. Potential Effects of Coronaviruses on the Cardiovascular System: A Review. *JAMA Cardiol*, 5, 831-840.
- MAYI, B. S., LEIBOWITZ, J. A., WOODS, A. T., AMMON, K. A., LIU, A. E. & RAJA, A. 2021. The role of Neuropilin-1 in COVID-19. *PLoS Pathog*, 17, e1009153.
- MILLET, J. K. & WHITTAKER, G. R. 2018. Physiological and molecular triggers for SARS-CoV membrane fusion and entry into host cells. *Virology*, 517, 3-8.
- MIRABELLI, C., WOTRING, J. W., ZHANG, C. J., MCCARTY, S. M., FURSMIDT, R., FRUM, T., KADAMBI, N. S., AMIN, A. T., O'MEARA, T. R., PRETTO, C. D., SPENCE, J. R., HUANG, J., ALYSANDRATOS, K. D., KOTTON, D. N., HANDELMAN, S. K., WOBUS, C. E., WEATHERWAX, K. J., MASHOUR, G. A., O'MEARA, M. J. & SEXTON, J. Z. 2020. Morphological Cell Profiling of SARS-CoV-2 Infection Identifies Drug Repurposing Candidates for COVID-19. *bioRxiv*, 2020.05.27.117184.

- MOUTAL, A., MARTIN, L. F., BOINON, L., GOMEZ, K., RAN, D., ZHOU, Y., STRATTON, H. J., CAI, S., LUO, S., GONZALEZ, K. B., PEREZ-MILLER, S., PATWARDHAN, A., IBRAHIM, M. M. & KHANNA, R. 2020. SARS-CoV-2 Spike protein co-opts VEGF-A/Neuropilin-1 receptor signaling to induce analgesia. *bioRxiv*.
- MUHL, L., FOLESTAD, E. B., GLADH, H., WANG, Y., MOESSINGER, C., JAKOBSSON, L. & ERIKSSON, U. 2017. Neuropilin 1 binds PDGF-D and is a co-receptor in PDGF-D-PDGFRbeta signaling. *J Cell Sci*, 130, 1365-1378.
- MUKHERJEE, S. & PAHAN, K. 2021. Is COVID-19 Gender-sensitive? *J Neuroimmune Pharmacol*, 16, 38-47.
- MURGOLO, N., THERIEN, A. G., HOWELL, B., KLEIN, D., KOEPLINGER, K., LIEBERMAN, L. A., ADAM, G. C., FLYNN, J., MCKENNA, P., SWAMINATHAN, G., HAZUDA, D. J. & OLSEN, D. B. 2021. SARS-CoV-2 tropism, entry, replication, and propagation: Considerations for drug discovery and development. *PLoS Pathog*, 17, e1009225.
- PAN, X. W., XU, D., ZHANG, H., ZHOU, W., WANG, L. H. & CUI, X. G. 2020. Identification of a potential mechanism of acute kidney injury during the COVID-19 outbreak: a study based on single-cell transcriptome analysis. *Intensive Care Med*, 46, 1114-1116.
- PEREZ-MILLER, S., PATEK, M., MOUTAL, A., CABEL, C. R., THORNE, C. A., CAMPOS, S. K. & KHANNA, R. 2020. In silico identification and validation of inhibitors of the interaction between neuropilin receptor 1 and SARS-CoV-2 Spike protein. *bioRxiv*.
- PREMKUMAR, L., SEGOVIA-CHUMBEZ, B., JADI, R., MARTINEZ, D. R., RAUT, R., MARKMANN, A., CORNABY, C., BARTELT, L., WEISS, S., PARK, Y., EDWARDS, C. E., WEIMER, E., SCHERER, E. M., ROUPHAEL, N., EDUPUGANTI, S., WEISKOPF, D., TSE, L. V., HOU, Y. J., MARGOLIS, D., SETTE, A., COLLINS, M. H., SCHMITZ, J., BARIC, R. S. & DE SILVA, A. M. 2020. The receptor binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Sci Immunol*, 5.
- QIAO, Y., WANG, X. M., MANNAN, R., PITCHIAYA, S., ZHANG, Y., WOTRING, J. W., XIAO, L., ROBINSON, D. R., WU, Y. M., TIEN, J. C., CAO, X., SIMKO, S. A., APEL, I. J., BAWA, P., KREGEL, S., NARAYANAN, S. P., RASKIND, G., ELLISON, S. J., PAROLIA, A., ZELENKA-WANG, S., MCMURRY, L., SU, F., WANG, R., CHENG, Y., DELEKTA, A. D., MEI, Z., PRETTO, C. D., WANG, S., MEHRA, R., SEXTON, J. Z. & CHINNAIYAN, A. M. 2020. Targeting transcriptional regulation of SARS-CoV-2 entry factors ACE2 and TMPRSS2. *Proc Natl Acad Sci U S A*.
- RAHMAN, N., BASHARAT, Z., YOUSUF, M., CASTALDO, G., RASTRELLI, L. & KHAN, H. 2020. Virtual Screening of Natural Products against Type II Transmembrane Serine Protease (TMPRSS2), the Priming Agent of Coronavirus 2 (SARS-CoV-2). *Molecules*, 25.
- RAIMONDI, C., BRASH, J. T., FANTIN, A. & RUHRBERG, C. 2016. NRP1 function and targeting in neurovascular development and eye disease. *Prog Retin Eye Res*, 52, 64-83.
- RIVA, L., YUAN, S., YIN, X., MARTIN-SANCHO, L., MATSUNAGA, N., BURGSTALLER-MUEHLBACHER, S., PACHE, L., DE JESUS, P. P., HULL, M. V., CHANG, M., CHAN, J. F.-W., CAO, J., POON, V. K.-M., HERBERT, K., NGUYEN, T.-T., PU, Y., NGUYEN, C., RUBANOV, A., MARTINEZ-SOBRIDO, L., LIU, W.-C., MIORIN, L., WHITE, K. M., JOHNSON, J. R., BENNER, C., SUN, R., SCHULTZ, P. G., SU, A., GARCIA-SASTRE, A., CHATTERJEE, A. K., YUEN, K.-Y. & CHANDA, S. K. 2020. A Large-scale Drug Repositioning Survey for SARS-CoV-2 Antivirals. *bioRxiv*, 2020.04.16.044016.
- ROBBIANI, D. F., GAEBLER, C., MUECKSCH, F., LORENZI, J. C. C., WANG, Z., CHO, A., AGUDELO, M., BARNES, C. O., GAZUMYAN, A., FINKIN, S., HAGGLOF, T., OLIVEIRA, T. Y., VIANI, C., HURLEY, A., HOFFMANN, H. H., MILLARD, K. G., KOST, R. G., CIPOLLA, M., GORDON, K., BIANCHINI, F., CHEN, S. T., RAMOS, V., PATEL, R., DIZON, J., SHIMELIOVICH, I., MENDOZA, P., HARTWEGER, H., NOGUEIRA, L., PACK, M., HOROWITZ, J., SCHMIDT, F., WEISBLUM, Y., MICHAELIDIS, E., ASHBROOK, A. W., WALTARI, E., PAK, J. E., HUEY-TUBMAN, K. E., KORANDA, N., HOFFMAN, P. R., WEST, A. P., JR., RICE, C. M., HATZIOANNOU, T., BJORKMAN, P. J., BIENIASZ, P. D., CASKEY, M. & NUSSENZWEIG, M. C. 2020. Convergent antibody responses to SARS-CoV-2 in convalescent individuals. *Nature*, 584, 437-442.
- ROSSI, A., DEVERAUX, Q., TURK, B. & SALI, A. 2004. Comprehensive search for cysteine cathepsins in the human genome. *Biol Chem*, 385, 363-72.
- SACCO, M.D., MA, C., LAGARIAS, P., GAO, A., TOWNSEND, J.A., MENG, X., DUBE, P., ZHANG, X., HU, Y., KITAMURA, N., *et al.* (2020). Structure and inhibition of the SARS-CoV-2 main protease reveal strategy for developing dual inhibitors against M(pro) and cathepsin L. *Sci Adv* 6(50):eabe0751.
- SHANG, J., WAN, Y., LUO, C., YE, G., GENG, Q., AUERBACH, A. & LI, F. 2020. Cell entry mechanisms of SARS-CoV-2. *Proc Natl Acad Sci U S A*, 117, 11727-11734.
- SHIRATO, K., KAWASE, M. & MATSUYAMA, S. 2018. Wild-type human coronaviruses prefer cell-surface TMPRSS2 to endosomal cathepsins for cell entry. *Virology*, 517, 9-15.
- 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. *Proc Natl Acad Sci U S A*, 102, 11876-81.
- SINGH, M., BANSAL, V. & FESCHOTTE, C. 2020. A single-cell RNA expression map of human coronavirus entry factors. *bioRxiv*.
- SNIJDER, E.J., BREDENBEEK, P.J., DOBBE, J.C., THIEL, V., ZIEBUHR, J., POON, L.L.M., GUAN, Y., ROZANOV, M., SPAAN, W.J.M., AND GORBALENYA, A.E. (2003). Unique and Conserved Features of Genome and Proteome of SARS-coronavirus, an Early Split-off From the Coronavirus Group 2 Lineage. *Journal of Molecular Biology* 331, 991-1004.
- SNIJDER, E.J., LIMPENS, R., DE WILDE, A.H., DE JONG, A.W.M., ZEVENHOVEN-DOBBE, J.C., MAIER, H.J., FAAS, F., KOSTER, A.J., AND BARCENA, M. (2020). A unifying structural and functional model of the coronavirus replication organelle: Tracking down RNA synthesis. *PLoS Biol* 18, e3000715.
- SUBRAMANIAN, A., VERNON, K. A., SLYPER, M., WALDMAN, J., LUECKEN, M. D., GOSIK, K., DUBINSKY, D., CUOCO, M. S., KELLER, K., PURNELL, J., NGUYEN, L., DIONNE, D., ROZENBLATT-ROSEN, O., WEINS, A., REGEV, A. & GREKA, A. 2020. RAAS blockade, kidney disease, and expression of ACE2, the entry receptor for SARS-CoV-2, in kidney epithelial and endothelial cells.
- UNIPROT KB - O14786 (NRP1_HUMAN)
- V'KOVSKI, P., KRATZEL, A., STEINER, S., STALDER, H., AND THIEL, V. (2021). Coronavirus biology and replication: implications for SARS-CoV-2. *Nat Rev Microbiol*. 19, 155–170.
- WALLS, A. C., TORTORICI, M. A., BOSCH, B. J., FRENZ, B., ROTTIER, P. J. M., DIMAIO, F., REY, F. A. & VEESLER, D. 2016. Cryo-electron microscopy structure of a coronavirus spike glycoprotein trimer. *Nature*, 531, 114-117.
- WANG, Y., LIU, M. & GAO, J. 2020. Enhanced receptor binding of SARS-CoV-2 through networks of hydrogen-bonding and hydrophobic interactions. *Proc Natl Acad Sci U S A*, 117, 13967-13974.
- YAMAMOTO, M., KISO, M., SAKAI-TAGAWA, Y., IWATSUKI-HORIMOTO, K., IMAI, M., TAKEDA, M., KINOSHITA, N., OHMAGARI, N., GOHDA, J., SEMBA, K., MATSUDA, Z., KAWAGUCHI, Y., KAWAOKA, Y. & INOUE, J. I. 2020. The Anticoagulant Nafamostat Potently Inhibits SARS-CoV-2 S Protein-Mediated Fusion in a Cell Fusion Assay System and Viral Infection In Vitro in a Cell-Type-Dependent Manner. *Viruses*, 12.
- YANG, N. & SHEN, H.-M. 2020. Targeting the Endocytic Pathway and Autophagy Process as a Novel Therapeutic Strategy in COVID-19. *International Journal of Biological Sciences*, 16, 1724-1731.
- YUAN, M., WU, N. C., ZHU, X., LEE, C. D., SO, R. T. Y., LV, H., MOK, C. K. P. & WILSON, I. A. 2020. A highly conserved cryptic epitope in the receptor binding domains of SARS-CoV-2 and SARS-CoV. *Science*, 368, 630-633.
- ZANG, R., GOMEZ CASTRO, M. F., MCCUNE, B. T., ZENG, Q., ROTHLAUF, P. W., SONNEK, N. M., LIU, Z., BRULLOIS, K. F., WANG, X., GREENBERG, H. B., DIAMOND, M. S., CIORBA, M. A., WHELAN, S. P. J. & DING, S. 2020. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. *Sci Immunol*, 5.
- ZIEGLER, C. G. K., ALLON, S. J., NYQUIST, S. K., MBANO, I. M., MIAO, V. N., TZOUANAS, C. N., CAO, Y., YOUSIF, A. S., BALS, J., HAUSER, B. M., FELDMAN, J., MUUS, C., WADSWORTH, M. H., 2ND, KAZER, S. W., HUGHES, T. K., DORAN, B., GATTER, G. J., VUKOVIC, M., TALIAFERRO, F., MEAD, B. E., GUO, Z., WANG, J. P., GRAS, D., PLAISANT, M., ANSARI, M., ANGELIDIS, I., ADLER, H., SUCRE, J. M. S., TAYLOR, C. J., LIN, B., WAGHRAY, A., MITSIALIS, V., DWYER, D. F., BUCHHEIT, K. M., BOYCE, J. A., BARRETT, N. A., LAIDLAW, T. M., CARROLL, S. L., COLONNA, L., TKACHEV, V., PETERSON, C. W., YU, A., ZHENG, H. B., GIDEON, H. P., WINCHELL, C. G., LIN, P. L., BINGLE, C. D., SNAPPER, S. B., KROPSKI, J. A., THEIS, F. J., SCHILLER, H. B., ZARAGOSI, L. E., BARBRY, P., LESLIE, A., KIEM, H. P., FLYNN, J. L., FORTUNE, S. M., BERGER, B., FINBERG, R. W., KEAN, L. S., GARBER, M., SCHMIDT, A. G., LINGWOOD, D., SHALEK, A. K., ORDOVAS-MONTANES, J., LUNG-NETWORK@HUMANCELLATLAS.ORG, H. C. A. L. B. N. E. A. & NETWORK, H. C. A. L. B. 2020. SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues. *Cell*, 181, 1016-1035 e19.

Short Name: IFN-I response, antagonized**Key Event Component**

Process	Object	Action
type I interferon signaling pathway	interferon alpha	decreased
type I interferon signaling pathway	interferon beta	decreased
cellular response to exogenous dsRNA	RNA viral genome	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	KeyEvent

Stressors

Name
Stressor:624 SARS-CoV-2

Biological Context**Level of Biological Organization**

Cellular

Cell term**Cell term**

cell

Organ term**Organ term**

organ

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI
mink	Mustela lutreola	High	NCBI
cat	Felis catus	High	NCBI
rhesus macaque	Macaca mulatta	High	NCBI
dog	Canis lupus familiaris	Moderate	NCBI
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Broad mammalian host range based on spike protein tropism for and binding to ACE2 (Conceicao et al. 2020; Wu et al. 2020) and cross-species ACE2 structural analysis (Damas et al. 2020). Some literature found on non-human hosts indicates that NSPs and accessory proteins can interact in a similar manner with bird (chicken) and other mammal proteins in the IFN-I pathway (Moustaqil et al. 2021; Rui et al. 2021).

Key Event Description

SARS-CoV-2 is an enveloped virus with a single-stranded RNA genome of ~30 kb, sequence orientation in a 5' to 3' direction typical of positive sense and reflective of the resulting mRNA (doi:<https://doi.org/10.1016/j.cell.2020.04.01>). The SARS-CoV-2 genome contains a 5'-untranslated region (UTR; 265 bp), [ORF1ab](#) (21,289 bp) holding two overlapping open reading frames (13,217 bp and 21,289 bp, respectively) that encode two polyproteins (Kim et al. 2020; O'Leary et al. 2020). Viral transcription and replication is explained in depth in [KE1847](#). Briefly, the first event upon cell entry is the primary translation of the ORF1a and ORF1b genomic RNA to produce non-structural proteins (NSPs). The ORF1a produces polypeptide 1a (pp1a, 440–500 kDa) that is cleaved into NSP-1 through NSP-11. A -1-ribosome frameshift occurs immediately upstream of the ORF1a stop codon, to allow translation through ORF1b, yielding 740–810 kDa polypeptide pp1ab, which is cleaved into 15 NSPs (duplications of NSP1-11 and five additional proteins, NSP12-16). Viral proteases NSP3 and NSP5 cleave the polypeptides through domains

functioning as a papain-like protease and a 3C-like protease, respectively (doi:<https://doi.org/10.1016/j.cell.2020.04.01>). The NSPs, structural proteins, and accessory proteins are encoded by 10 ORFs in the SARS-CoV-2 RNA genome. They may have multiple functions during viral replication as well as in evasion of the host innate immune response, thus augmenting viral replication and spread (Amor et al. 2020). Extensive protein-protein interaction (Gordon et al. 2020) and viral protein-host RNA interaction networks have been demonstrated between the viral NSPs and accessory proteins and host molecules.

This key event is focused on the specific viral:host protein interactions within the infected cell that are involved in the [IFN-I antiviral response pathways](#). IFN-I is the main component of the innate immune system that is suppressed by the SARS-CoV-2 coronavirus early in infection. The primary form of host intracellular virus surveillance detects viral components to induce an immediate systemic type I interferon (IFN) response. Cellular RNA sensors called pattern recognition receptors (PRRs) such as RIG-I, MDA5 and LGP2 detect the presence of viral RNAs and promote nuclear translocation of the transcription factor IRF3, leading to transcription, translation, and secretion of IFN- α and IFN- β . This in turn leads to interaction with the IFN receptor (IFNAR), phosphorylation of STAT1 and 2, and transcription and translation of hundreds of antiviral genes (Quarleri and Delpino, 2021).

Interactions between SARS-CoV-2 proteins and human RNAs thwart the IFN response upon infection: NSP1 binds to 40S ribosomal RNA in the mRNA entry channel of the ribosome to inhibit host mRNA translation; NSP8 and NSP9 displace signal recognition particle proteins (SRP54, 27 and 19) to bind to the 7SL RNA and block protein trafficking to the cell membrane (Banerjee et al. 2020; Gordon et al. 2020). Xia et al. (2020) found that NSP6 and NSP13 antagonize IFN-I production via distinct mechanisms: NSP6 binds TANK binding kinase 1 (TBK1) to suppress interferon regulatory factor 3 (IRF3) phosphorylation, and NSP13 binds and blocks TBK1 phosphorylation. NSP14 induces lysosomal degradation of type I IFNAR to prevent STAT activation (Hayn et al. 2021). ORF6 hijacks KPNA2 to block IRF3, and Nup98/RAE1 to block STAT nuclear import, to silence IFN-I gene expression (Xia and Shi, 2020). ORF7a suppresses STAT2 phosphorylation and ORF7b suppresses STAT1 and STAT2 phosphorylation to block ISGF3 complex formation with IRF9 (Xia and Shi, 2020). ORF8 interacts and downregulates MHC-I (Zhang et al 2020), and has been reported to block INF β expression, but the mechanism has not been identified (Rashid et al. 2021; Li et al. 2020). ORF9b antagonizes Type I Interferons by targeting multiple components of RIG-I/MDA-5-MAVS, TOMM70, NEMO and cGAS-STING signalling (Han et al. 2020; Jiang et al. 2020; Wu et al. 2021; Gordon et al 2020).

Following is a table of the current state of knowledge of SARS-CoV-2 protein putative functions in relation to IFN-I antiviral response antagonism.

Gene	Protein	Function	Role in early innate immune evasion
ORF1a	NSP1	NSP1 antagonizes interferon induction to suppress host antiviral response.	DNA Polymerase Alpha Complex: Regulates the activation of IFN-I through cytosolic RNA-DNA synthesis (POLA1/2-PRIM1/2) and primes DNA replication in the nucleus (Gordon et al. 2020; Chaudhuri et al. 2020). Can also inhibit host gene expression by binding to ribosomes and modifying host mRNAs (Shi et al. 2020; Schubert et al. 2020; Thoms et al. 2020).
	NSP2	While not essential for viral replication, deletion of NSP2 diminishes viral growth and RNA synthesis	Translation repression through binding GIGYF2 and EIF4E2 (4EHP) (Gupta et al. 2021)
	NSP3	Papain-like protease (Plpro); Cleaves the ORF1a and 1ab polypeptides	Suppresses IFN-I: Cleaves IRF3 (Moustaqil et al. 2021); binds/cleaves ISG15 (Rui et al. 2021; Shin et al. 2020; Liu et al. 2021; Klemm et al. 2020)
	NSP5	3C-like protease (3CLpro); Cleaves the ORF1a and 1ab polypeptides	Binds STING (Rui et al. 2021)
	NSP6	Limits autophagosome expansion	Suppresses IFN-I expression: Binds TBK-1 to suppress IRF3 phosphorylation (Xia et al. 2020; Quarleri and Delpino, 2021)
	NSP7	In complex with NSP8 forms primase as part of multimeric RNA-dependent RNA replicase (RdRp)	
	NSP8	Replication complex with NSP7, NSP9 and NSP12	Binds SRP72/54/19 (Gordon et al. 2020) and 7SL RNA to block IFN membrane transport (Banerjee et al. 2020)
	NSP9	Replication complex with NSP7, NSP8 and NSP12	Binds SRP and 7SL RNA with NSP8 to block IFN membrane transport (Banerjee et al. 2020)
ORF1b	NSP13	Helicase and triphosphatase that initiates the first step in viral mRNA capping.	Binds TBK1 (Xia et al. 2020)
	NSP14		Induces lysosomal degradation of IFNAR1 (Hayn et al. 2021)
ORF2	Spike (S)	ACE2 interaction, cell entry	
ORF3a	ORF3a	Interacts with M, S, E and 7a; form viroporins; immune evasion	Binds STING (Rui et al 2021)
ORF4	Envelope (E)	Viral assembly and budding	
ORF5	Membrane (M)	Viral assembly	Interacts with RIG-I and MAVS sensors of viral RNA (Fu et al 2020)
ORF6	ORF6	Viral pathogenesis and virulence; interacts with ORF8; promotes RNA polymerase activity	Hijacks the nuclear importin Karyopherin α 2 (KPNA2) to block IRF3 (Xia and Shi, 2020) and Nup98/RAE1 to block STAT nuclear import (Miorin et al. 2020; Kato et al. 2020), leading to the silence of downstream ISGs
ORF7a	ORF7a	Interacts with S, ORF3a; immune evasion	Suppresses STAT2 phosphorylation to block IFN-I response (Xia and Shi, 2020).

ORF7b	ORF7b	Structural component of virion	Suppresses STAT1 and STAT2 phosphorylation to block IFN-I response (Xia and Shi, 2020)
ORF8	ORF8	Immune evasion	Interacts and downregulates MHC-I (Zhang et al. 2020). May inhibit type I interferon (IFN- β) and interferon-stimulated response element (ISRE) (Rashid et al. 2020; Li et al. 2020)
ORF9	Nucleocapsid (N)	Stabilizes viral RNA	Attenuates stress granule formation: G3BP1/2 (Chen et al. 2020; Cascarina et al. 2020); G3BP1 also interacts with RIG-I (Kim et al. 2019) and STAT1/2 (Mu et al. 2020)
ORF9b	ORF9b	Immune evasion	Membrane protein antagonizes Type I Interferons by targeting multiple components of RIG-I/MDA-5-MAVS, TOMM70, NEMO, and cGAS-STING signaling pathways (Fu et al. 2020; Chen et al. 2020; Han et al. 2020; Jiang et al. 2020; Wu et al. 2021; Gordon et al. 2020)

How it is Measured or Detected

Detection of IFN-I suppression involves measuring gene promoter/transcription activation (luciferase assays), gene up/down regulation (quantitative PCR), protein-protein interaction (immunoprecipitation, immunoblotting) or in-situ co-location of viral and host proteins (immunofluorescent or confocal microscopy) in cell culture. Examples of methods used include the following:

Interferon I decrease (Xia et al. 2020):

- IFN-I production and signaling luciferase reporter assays
- Co-immunoprecipitation and western blot
- Indirect immunofluorescence assays
- DNA assembly and RNA transcription of a luciferase replicon for SARS-CoV-2
- Replicon RNA electroporation and luciferase reporter assay

SARS-CoV-2 ORF9b inhibits RIG-I-MAVS antiviral signaling (Wu et al. 2021)

- Viral- and host-protein-specific antibodies
- Immunoprecipitation
- Immunofluorescent microscopy
- Dual-luciferase reporter assays
- Fluorescence quantification immunoblotting

SARS-CoV-2-Human Protein-Protein Interaction Map (Gordon et al. 2020)

- Cloning and expression of viral proteins via plasmid transfection into HEK293T cell line
- Protein affinity purification using MagStrep beads with detection by anti-strep western blot of cell lysate
- Global analysis of SARS-CoV-2 host interacting proteins using affinity purification-mass spectrometry

References

- Amor et al. 2020. Innate immunity during SARS-CoV-2: evasion strategies and activation trigger hypoxia and vascular damage. *Clinical and Experimental Immunology*, 202: 193–209. doi: 10.1111/cei.13523
- Andres et al. 2020. SARS-CoV-2 ORF9c Is a Membrane-Associated Protein that Suppresses Antiviral Responses in Cells. *bioRxiv preprint* doi: <https://doi.org/10.1101/2020.08.18.256776>
- Banerjee et al. 2020. SARS-CoV-2 disrupts splicing, translation, and protein trafficking to suppress host defenses. *Cell* 183, 1325–1339. <https://doi.org/10.1016/j.cell.2020.10.004>
- Cascarina and Ross, 2020. A proposed role for the SARS-CoV-2 nucleocapsid protein in the formation and regulation of biomolecular condensates. *The FASEB Journal*, 34:9832–9842. DOI: 10.1096/fj.202001351
- Chaudhuri, A. 2021. Comparative analysis of non-structural protein 1 of SARS-CoV2 with SARS-CoV1 and MERS-CoV: An in-silico study. *Journal of Molecular Structure*, Volume 1243, 130854, <https://doi.org/10.1016/j.molstruc.2021.130854>.
- Chen et al. 2021. SARS-CoV-2 Nucleocapsid Protein Interacts with RIG-I and Represses RIG-Mediated IFN- β Production. *Viruses*. 13(1):47. <https://doi.org/10.3390/v13010047>
- Conceicao et al. 2020. The SARS-CoV-2 Spike protein has a broad tropism for mammalian ACE2 proteins. *PLoS Biol* 18(12): e3001016. <https://doi.org/10.1371/journal.pbio.3001016>
- Damas et al. 2020. Broad host range of SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in vertebrates. *PNAS* vol. 117 no. 36:22311–22322 [www.pnas.org/cgi/doi/10.1073/pnas.2010146117](https://doi.org/10.1073/pnas.2010146117)
- Fu et al. 2021. SARS-CoV-2 membrane glycoprotein M antagonizes the MAVS-mediated innate antiviral response. *Cell Mol Immunol* 18: 613–620. <https://doi.org/10.1038/s41423-020-00571-x>
- Gordon et al. 2020. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 483:459–473. <https://doi.org/10.1038/s41586-020-2286-9>
- Gupta et al. 2021. CryoEM and AI reveal a structure of SARS-CoV-2 Nsp2, a multifunctional protein involved in key host processes. *bioRxiv* 2021.05.10.443524; doi: <https://doi.org/10.1101/2021.05.10.443524>
- Han et al. 2020. SARS-CoV-2 ORF9b Antagonizes Type I and III Interferons by Targeting Multiple Components of RIG-I/MDA-5-MAVS, TLR3-TRIF, and cGAS-STING Signaling Pathways. *bioRxiv* <https://doi.org/10.1101/2020.08.16.252973>
- Hayn et al. 2021. Systematic functional analysis of SARS-CoV-2 proteins uncovers viral innate immune antagonists and remaining vulnerabilities. *Cell Reports* 35, 109126. <https://doi.org/10.1016/j.celrep.2021.109126>
- Jiang et al. 2020. SARS-CoV-2 Orf9b suppresses type I interferon responses by targeting TOM70. *Cellular & Molecular Immunology* 17:998–1000; <https://doi.org/10.1038/s41423-020-0514-8>
- Kato et al. 2021. Overexpression of SARS-CoV-2 protein ORF6 dislocates RAE1 and NUP98 from the nuclear pore complex. *Biochemical and Biophysical Research Communications* 536:59–66 <https://doi.org/10.1016/j.bbrc.2020.11.115>
- Kim et al. 2019. The stress granule protein G3BP1 binds viral dsRNA and RIG-I to enhance interferon- β response. *J. Biol. Chem.* 294(16): 6430–6438. DOI 10.1074/jbc.RA118.005868
- Kim et al. 2020. The Architecture of SARS-CoV-2 Transcriptome. *Cell* 181, 914–921. <https://doi.org/10.1016/j.cell.2020.04.011>
- Li et al. 2020. The ORF6, ORF8 and nucleocapsid proteins of SARS-CoV-2 inhibit type I interferon signaling pathway. *Virus Research* vol. 286. <https://doi.org/10.1016/j.virusres.2020.198074>

- Liu et al. 2021. ISG15-dependent activation of the sensor MDA5 is antagonized by the SARS-CoV-2 papain-like protease to evade host innate immunity. *Nature Microbiol* 6: 467–478. <https://doi.org/10.1038/s41564-021-00884-1>
- Moustaqil et al. 2021. SARS-CoV-2 proteases PLpro and 3CLpro cleave IRF3 and critical modulators of inflammatory pathways (NLRP12 and TAB1): implications for disease presentation across species, *Emerging Microbes & Infections*, 10:1, 178-195. <https://doi.org/10.1080/22221751.2020.1870414>
- Mu et al. 2020. SARS-CoV-2 N protein antagonizes type I interferon signaling by suppressing phosphorylation and nuclear translocation of STAT1 and STAT2. *Cell Discov* 6, 65. <https://doi.org/10.1038/s41421-020-00208-3>
- O'Leary et al. 2020 Unpacking Pandora from Its Box: Deciphering the Molecular Basis of the SARS-CoV-2 Coronavirus. *Int. J. Mol. Sci.* 2021, 22, 386. <https://doi.org/10.3390/ijms22010386>
- Quarleri and Delpino, 2020. Type I and III IFN-mediated antiviral actions counteracted by SARS-CoV-2 proteins and host inherited factors. *Cytokine & Growth Factor Reviews*, 58: 55-65. <https://doi.org/10.1016/j.cytogfr.2021.01.003>
- Rashid et al. The ORF8 protein of SARS-CoV-2 induced endoplasmic reticulum stress and mediated immune evasion by antagonizing production of interferon beta. *Virus Research* 296, 198350. <https://doi.org/10.1016/j.virusres.2021.198350>
- Ren et al. 2020. The ORF3a protein of SARS-CoV-2 induces apoptosis in cells. *Cellular & Molecular Immunology* 17:881–883; <https://doi.org/10.1038/s41423-020-0485-9>
- Rui et al. 2021. Unique and complementary suppression of cGAS-STING and RNA sensing-triggered innate immune responses by SARS-CoV-2 proteins. *Sig Transduct Target Ther* 6, 123. <https://doi.org/10.1038/s41392-021-00515-5>
- Schubert et al. 2020. SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation. *Nature Structural & Molecular Bio.* 27:959-966. <https://doi.org/10.1038/s41594-020-0511-8>
- Shin et al. 2020. Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity. *Nature* 587: 657–662. <https://doi.org/10.1038/s41586-020-2601-5>
- Thoms et al. 2020. Structural basis for translational shutdown and immune evasion by the Nsp1 protein of SARS-CoV-2. *Science* 369(6508): 1249-1255. DOI: 10.1126/science.abc8665
- Wu et al. 2021. SARS-CoV-2 ORF9b inhibits RIG-I-MAVS antiviral signaling by interrupting K63-linked ubiquitination of NEMO. *Cell Reports* 34, 108761. <https://doi.org/10.1016/j.celrep.2021.108761>
- Wu et al. 2020. Broad host range of SARS-CoV-2 and the molecular basis for SARS-CoV-2 binding to cat ACE2. *Cell Discovery* 6:68. <https://doi.org/10.1038/s41421-020-00210-9>
- Xia et al. 2020. Evasion of Type I Interferon by SARS-CoV-2. *Cell Reports* 33, 108234. <https://doi.org/10.1016/j.celrep.2020.108234>
- Xia and Shi, 2020. Antagonism of Type I Interferon by Severe Acute Respiratory Syndrome Coronavirus 2. *Journal of Interferon & Cytokine Research* v.40, no. 12 DOI:10.1089/jir.2020.0214
- Zhang et al. 2020. The ORF8 Protein of SARS-CoV-2 Mediates Immune Evasion through Potently Downregulating MHC-I. *bioRxiv preprint doi: https://doi.org/10.1101/2020.05.24.111823*

Event: 1847: Increased SARS-CoV-2 production

Short Name: SARS-CoV-2 production

Key Event Component

Process	Object	Action
viral RNA genome replication	viral RNA-directed RNA polymerase complex	increased
positive stranded viral RNA replication	viral RNA-directed RNA polymerase complex	increased
viral RNA genome packaging	viral assembly compartment	increased
mRNA transcription	ssRNA viral genome	increased
viral translation	ssRNA viral genome	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	KeyEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	KeyEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI
Mustela putorius furo	Mustela putorius furo	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Broad mammalian host range has been demonstrated based on spike protein tropism for and binding to ACE2 [Conceicao *et al.* 2020; Wu *et al.* 2020] and cross-species ACE2 structural analysis [Damas *et al.* 2020]. No literature has been found on primary translation and molecular interactions of nsps in non-human host cells, but it is assumed to occur if the virus replicates in other species.

Very broad mammalian tropism: human, bat, cat, dog, civet, ferret, horse, pig, sheep, goat, water buffalo, cattle, rabbit, hamster, mouse

Key Event Description

This KE1847 "Increase coronavirus production" deals with how the genome of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is translated, replicated, and transcribed in detail, and how the genomic RNA (gRNA) is packaged, and the virions are assembled and released from the cell.

Coronavirus is a class of viruses that have single-stranded positive-sense RNA genomes in their envelopes [D. Kim *et al.*]. The virus contains a 29.7 kB positive-sense RNA genome flanked by 5' and 3' untranslated regions of 265 and 342 nucleotides, respectively [E. J. Snijder *et al.*] that contain cis-acting secondary RNA structures essential for RNA synthesis [N. C. Huston *et al.*]. The genome just prior to the 5' end contains the transcriptional regulatory sequence leader (TRS-L) [C.J. Budzilowicx *et al.*]. The SARS-CoV genome is polycistronic and contains 14 open reading frames (ORFs) that are expressed by poorly understood mechanisms [E. J. Snijder *et al.*]. Preceding each ORF there are other TRSs called the body TRS (TRS B). The 5' two-thirds of the genome contains two large, overlapping, nonstructural ORFs and the 3' third contains the remainder ORFs [H. Di *et al.*]. Genome expression starts with the translation of two large ORFs of the 5' two-thirds: ORF1a of 4382 amino acids and ORF1ab of 7073 amino acid that occurs via a programmed (- 1) ribosomal frameshifting [E. J. Snider *et al.*], yielding pp1a and pp1ab. These two polypeptides are cleaved into 16 subunits by two viral proteinases encoded by ORF1a, nsp3, and nsp5 that contain a papain-like protease domain and a 3C-like protease domain [M. D. Sacco *et al.*]. The processing products are a group of replicative enzymes, named nsp1-nsp16, that assemble into a viral replication and transcription complex (RTC) associated with membranes of endoplasmic reticulum (ER) with the help of various membrane-associated viral proteins [S. Klein *et al.*, E. J. Snijder *et al.*, P. V'Kovski, *et al.*]. Besides replication, which yields the positive-sense gRNA, the replicase also mediates transcription leading to the synthesis of a nested set of subgenomic (sg) mRNAs to express all ORFs downstream of ORF1b that encode structural and accessory viral proteins. These localize to the 3' one-third of the genome, as stated above, and result in a 3' coterminal nested set of 7–9 mRNAs that share ~70–90 nucleotide (nt) in the 5' leader and that is identical to the 5' end of the genome [P. Liu, and J. Leibowitz]. sgRNAs encode conserved structural proteins (spike protein [S], envelope protein [E], membrane protein [M], and nucleocapsid protein [N]), and several accessory proteins. SARS-CoV-2 is known to have at least six accessory proteins (3a, 6, 7a, 7b, 8, and 10). Overall the virus is predicted to express 29 proteins [D. Kim *et al.*]. The gRNA is packaged by the structural proteins to assemble progeny virions.

Viral translation:

SARS-CoV-2 is an enveloped virus with a single-stranded RNA genome of ~30 kb, sequence orientation in a 5' to 3' direction typical of positive sense and reflective of the resulting mRNA [D. Kim *et al.*]. The SARS-CoV-2 genome contains a 5'-untranslated region (UTR; 265 bp), ORF1ab (21,289 bp) holding two overlapping open reading frames (13,217 bp and 21,289 bp, respectively) that encode two polyproteins [D. Kim *et al.*]. Other elements of the genome include are shown below [V. B. O'Leary *et al.*]. **The first event upon cell entry is the primary translation of the ORF1a and ORF1b gRNA to produce non-structural proteins (nsps).**

This is completely dependent on the translation machinery of the host cell. Due to fewer rare "slow-codons", SARS-CoV-2 may have a higher protein translational rate, and therefore higher infectivity compared to other coronavirus groups [V. B. O'Leary *et al.*]. The ORF1a produces polypeptide 1a (pp1a, 440–500 kDa) that is cleaved into nsp-1 through nsp-11. A -1 ribosome frameshift occurs immediately upstream of the ORF1a stop codon, to allow translation through ORF1b, yielding 740–810 kDa polypeptide pp1ab, which is cleaved into 15 nsps [D. Kim *et al.*]. Two overlapping ORFs, ORF1a and ORF1b, generate continuous polypeptides, which are cleaved into a total of 16 so-called nsps [Y Finkel *et al.*]. Functionally, there are five proteins from pp1ab (nsp-12 through nsp-16) as nsp-1-11 are duplications of the proteins in pp1a due to the ORF overlap. The pp1a is approximately 1.4–2.2 times more expressed than pp1ab. After translation, the polyproteins are cleaved by viral proteases nsp3 and nsp5. Nsp5 protease can be referred to as 3C-like protease (3CL^{pro}) or as main protease (M^{pro}), as it cleaves the majority of the polyprotein cleavage sites. [H.A. Hussein *et al.*] Nsp1 cleavage is quick and nsp1 associates with host cell ribosomes and results in host cellular shutdown, suppressing host gene expression [M. Thoms *et al.*]. Fifteen proteins, nsp2–16 constitute the viral RTC. They are targeted to defined subcellular locations and establish a network with host cell factors. Nsp2–11 remodel host membrane architecture, mediate host immune evasion and provide cofactors for replication, whilst nsp12–16 contain the core enzymatic functions involved in RNA synthesis, modification and proofreading [P. V'Kovski *et al.*]. nsp-7 and nsp-8 form a complex priming the RNA-dependent RNA polymerase (RdRp or RTC) - nsp-12. nsp14 provides a 3'–5' exonuclease activity providing RNA proofreading function. Nsp-10 composes the RNA capping machinery nsp-9. nsp13 provides the RNA 5'-triphosphatase activity. Nsp-14 is a N7-methyltransferase and nsp-16 the 2'-O-methyltransferase. Many of the nsps have multiple functions and many viral proteins are involved in innate immunity inhibition. Nsp-3 is involved in vesicle formation along with nsp-4 and nsp-6 where viral replication occurs. Interactions between SARS-CoV-2 proteins and human RNAs thwart the IFN response upon infection: nsp-16 binds to U1 and U2 splicing RNAs to suppress global mRNA splicing; nsp-1 binds to 40S ribosomal RNA in the mRNA entry channel of the ribosome to inhibit host mRNA translation; nsp-8 and nsp-9 bind to the 7SL RNA to block protein trafficking to the cell membrane [A. K. Banerjee *et al.*]. Xia *et al.* [H. Xia *et al.*] found that nsp-6 and nsp-13 antagonize IFN- β production via distinct mechanisms: nsp-6 binds TANK binding kinase 1 (TBK1) to suppress interferon regulatory factor 3 (IRF3) phosphorylation, and nsp-13 binds and blocks TBK1 phosphorylation.

Viral transcription and replication:

Viral transcription and replication occur at the viral replication organelle (RO) [E. J. Snijder *et al.*]. The RO is specifically formed during infection by reshaping ER and other membranes, giving rise to small spherular invaginations, and large vesiculotubular clusters, consisting of single- and/or double-membrane vesicles (DMV), convoluted membranes (CM) and double-membrane spherules invaginating from the ER [S. Klein *et al.*, E. J. Snijder *et al.*]. There is some evidence that DMV accommodate viral replication which is based on radiolabelling viral RNA with nucleoside precursor ([5-³H]uridine) and detection by EM autoradiography [E. J. Snijder *et al.*].

Viral replicative proteins and specific host factors are recruited to ROs [E. J. Snijder *et al.*]. RNA viral genome is transcribed into messenger RNA by the viral RTC [P. Ahlquist *et al.*]. Viral RTC act in combination with other viral and host factors involved in selecting template RNAs, elongating RNA synthesis, differentiating genomic RNA replication from mRNA transcription, modifying product RNAs with 5' caps or 3' polyadenylate [P. Ahlquist *et al.*]. Positive-sense (messenger-sense) RNA viruses replicate their genomes through negative-strand RNA intermediates [M. Schwartz *et al.*]. The intermediates comprise full-length negative-sense complementary copies of the genome, which functions as templates for the generation of new positive-sense gRNA, and a nested set of sg mRNAs that lead to the expression of proteins encoded in all ORFs downstream of ORF1b. The transcription of coronaviruses is a discontinuous process that produces nested 3' and 5' co-terminal sgRNAs. Of note, the synthesis of sg mRNAs is not exclusive to the order *Nidovirales* but a discontinuous minus-strand synthesis strategy to produce a nested set of 3' co-terminal sg mRNAs with a common 5' leader in infected cells are unique features of the *coronaviruses* and *arteriviruses* [W. A. Miller and G. Koev.]. Of note, the produced genomic RNA represents a small fraction of the total vRNA [N. S. Ogando *et al.*].

The discontinuous minus-strand synthesis of a set of nested sg mRNAs happens during the synthesis of the negative-strand RNA, by an interruption mechanism of the RTC as it reads the

TRS-B preceding each gene in the 3' one-third of the viral genome [I. Sola, F. Almazan *et al.*, I. Sola, J. L. Moreno, *et al.*]. The synthesis of the negative-strand RNA stops and is re-initiated at the TRS-L of the genome sequence close from the 5' end of the genome [H. Di *et al.*]. Therefore, the mechanism by which the leader sequence is added to the 5' end requires that the RTC switches template by a jumping mechanism. This interruption process involves the interaction between complementary TRSs of the nascent negative-strand RNA TRS-B and the positive-strand gRNA at the positive-sense TRS-L. The TRS-B site has a 7-8 bp conserved core sequence (CS) that facilitates RTC template switching as it hybridizes with a near complementary CS in the TRS-L [I. Sola, F. Almazan *et al.*, I. Sola, J. L. Moreno, *et al.*]. Upon re-initiation of RNA synthesis at the TRS-L region, a negative-strand copy of the leader sequence is added to the nascent RNA to complete the synthesis of negative-strand sgRNAs. This means that all sg mRNAs as well as the genomic RNA share a common 5' sequence, named leader sequence [X. Zhang *et al.*]. This programmed template switching leads to the generation of sg mRNAs with identical 5' and 3' sequences, but alternative central regions corresponding to the beginning of each structural ORF [I. Sola *et al.* 2015, S. G. Sawicki *et al.*, Y. Yang *et al.*]. Of note, the existence of TRSs also raises the possibility that these sites are at the highest risk of recombining through TRS-B mediated template switching [Y. Yang]. The set of sg mRNAs is then translated to yield 29 identified different proteins [F. Wu *et al.*], although many papers have identified additional ORFs [D. Kim *et al.*, Y. Finkel *et al.*, A. Vandelli *et al.*]. The translation of the linear single-stranded RNA conducts to the generation of the following proteome: 4 are structural proteins, S, N, M, and E; 16 proteins nsp: the first 11 are encoded in ORF1a whereas the last 5 are encoded in ORF1ab. In addition, 9 accessory proteins named ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10 have been identified [F. Wu *et al.*]. At the beginning of infection, there is the predominant expression of the nsp that result from ORF1a and ORF1ab, however, at 5 hpi, the proteins encoded by the 5' last third are found in higher amounts, and the nucleoprotein is the protein found in higher levels [Y. Finkel *et al.*].

Viral assembly:

The final step of viral production requires virion assembly and this process is not well explored for SARS-CoV-2. For example, the role of the structural proteins of SARS-CoV-2 in virus assembly and budding in not known. In general, all beta-coronavirus structural proteins assemble at the endoplasmic reticulum (ER)-to-Golgi compartment [J. R. Cohen *et al.*, A. Perrier *et al.*] and viral assembly requires two steps: Genome packaging which is a process in which the SARS-CoV-2 gRNA must be coated by the viral protein nucleoprotein (N) protein, forming viral ribonucleoprotein (vRNPs) complexes, before being selectively packaged into progeny virions [P. V'Kovski *et al.*], a step in which vRNPs bud into the lumen of the ER and the ER-Golgi intermediate compartment (ERGIC) [N. S. Ogando *et al.*]. This results in viral particles enveloped with host membranes containing viral M, E, and S transmembrane structural proteins that need to be released from the cell.

SARS-CoV-2 gRNA packaging involves the N protein. The N protein of human coronaviruses is highly expressed in infected cells. It is considered a multifunctional protein, promoting efficient sub-genomic viral RNA transcription, viral replication, virion assembly, and interacting with multiple host proteins [P. V'Kovski *et al.*, D. E. Gordon *et al.*, R. McBride, and M. van Zyl, B. C.]. In relation to transcription and replication, the N protein could provide a cooperative mechanism to increase protein and RNA concentrations at specific localizations S. Alberti, and S. Carra, S. F. Banani *et al.*, and this way organize viral transcription. Five studies have shown that N protein undergoes liquid-liquid phase separation (LLPS) *in vitro* [A. Savastano *et al.*, H. Chen *et al.*, C. Iserman *et al.*, T. M. Perdikari *et al.*, J. Cubuk *et al.*], dependent on its C-terminal domain (CTD) [H. Chen *et al.*]. It has been hypothesised that N could be involved in replication close to the ER and in packaging of gRNA into vRNPs near the ERGIC where genome assembly is thought to take place [A. Savastano *et al.*], but so far this is still speculative. Phosphorylation of N could adjust the physical properties of condensates differentially in ways that could accommodate the two different functions of N: transcription and progeny genome assembly [A. Savastano *et al.*, C. Iserman *et al.*, C. R. Carlson *et al.*].

The ERGIC constitutes the main assembly site of coronaviruses [S. Klein *et al.*, E. J. Snijder *et al.*, L. Mendonca *et al.*] and budding events have been seen by EM studies. For SARS-CoV-2, virus-budding was mainly clustered in regions with a high vesicle density and close to ER- and Golgi-like membrane arrangements [S. Klein *et al.*, E. J. Snijder *et al.*, L. Mendonca *et al.*]. The ectodomain of S trimers were found facing the ERGIC lumen and not induce membrane curvature on its own, therefore proposing that vRNPs and spike trimers [S. Klein *et al.*].

Finally, it has been shown that SARS-CoV-2 virions de novo formed traffic to lysosomes for unconventional egress by Arf8b-dependent lysosomal exocytosis [S. Ghosh *et al.*]. This process results in lysosome deacidification, inactivation of lysosomal degradation enzymes, and disruption of antigen presentation [S. Ghosh *et al.*].

How it is Measured or Detected

Viral translation:

SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation [Schubert *et al.* 2020]

- Sucrose pelleting binding assay to verify Nsp1–40S complex formation
- In vivo translation assay
- Transient expression of FLAG-Nsp1 in HeLa cells and puromycin incorporation assay

SARS-CoV-2 disrupts splicing, translation, and protein trafficking [Banerjee *et al.* 2020]

- SARS-CoV-2 viral protein binding to RNA
- Interferon stimulation experiments
- Splicing assessment experiments
- IRF7-GFP splicing reporter, 5EU RNA labeling, capture of biotinylated 5EU labeled RNA

Membrane SUNSET assay for transport of plasma membrane proteins to the cell surface

Viral transcription:

The mRNA transcripts are detected by the real-time reverse transcription-PCR (RT-PCR) assay. Several methods targeting the mRNA transcripts have been developed, which includes the RT-PCR assays targeting RdRp/helicase (Hel), spike (S), and nucleocapsid (N) genes of SARS-CoV-2 [Chan *et al.*]. RT-PCR assays detecting SARS-CoV-2 RNA in saliva include quantitative RT-PCR (RT-qPCR), direct RT-qPCR, reverse transcription-loop-mediated isothermal amplification (RT-LAMP) [Nagura-Ikeda M, *et al.*]. The viral mRNAs are reverse-transcribed with RT, followed by the amplification by PCR.

Viral replication:

viral replication is measured by RT-qPCR in infected cells, formation of liquid organelles is assessed in vitro reconstitution systems and in infected cells. Labelling by radioactive nucleosides.

Viral production:

Plaque assays, infectivity assays, RT-qPCR to detect viral RNA in released virions, sequencing to detect mutations in the genome, electron microscopy.

References

1. D. Kim *et al.*, The Architecture of SARS-CoV-2 Transcriptome. *Cell* **181**, 914-921 e910 (2020).
2. E. J. Snijder *et al.*, Unique and Conserved Features of Genome and Proteome of SARS-coronavirus, an Early Split-off From the Coronavirus Group 2 Lineage. *Journal of Molecular Biology* **331**, 991-1004 (2003).
3. N. C. Huston *et al.*, Comprehensive in vivo secondary structure of the SARS-CoV-2 genome reveals novel regulatory motifs and mechanisms. *Mol Cell* **81**, 584-598 e585 (2021).
4. C. J. Budzylowicz, S. P. Wilczynski, S. R. Weiss, Three intergenic regions of coronavirus mouse hepatitis virus strain A59 genome RNA contain a common nucleotide sequence that is homologous to the 3' end of the viral mRNA leader sequence. *J Virol* **53**, 834-840 (1985).
5. H. Di, A. A. McIntyre, M. A. Brinton, New insights about the regulation of Nidovirus subgenomic mRNA synthesis. *Virology* **517**, 38-43 (2018).
6. M. D. Sacco *et al.*, Structure and inhibition of the SARS-CoV-2 main protease reveal strategy for developing dual inhibitors against M(pro) and cathepsin L. *Sci Adv* **6**, (2020).
7. S. Klein *et al.*, SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *BioRxiv*, (2020).
8. E. J. Snijder *et al.*, A unifying structural and functional model of the coronavirus replication organelle: Tracking down RNA synthesis. *PLoS Biol* **18**, e3000715 (2020).
9. P. V'Kovski, A. Kratzel, S. Steiner, H. Stalder, V. Thiel, Coronavirus biology and replication: implications for SARS-CoV-2. *Nat Rev Microbiol*, (2020).
10. P. Liu, J. Leibowitz, in *Molecular Biology of the SARS-Coronavirus*. (2010), chap. Chapter 4, pp. 47-61.

11. V. B. O'Leary, O. J. Dolly, C. Hoschl, M. Cerna, S. V. Ovsepan, Unpacking Pandora From Its Box: Deciphering the Molecular Basis of the SARS-CoV-2 Coronavirus. *Int J Mol Sci* **22**, (2020).
12. Y. Finkel *et al.*, The coding capacity of SARS-CoV-2. *Nature* **589**, 125-130 (2021).
13. H. A. Hussein, R. Y. A. Hassan, M. Chino, F. Febbraio, Point-of-Care Diagnostics of COVID-19: From Current Work to Future Perspectives. *Sensors (Basel)* **20**, (2020).
14. M. Thoms *et al.*, Structural basis for translational shutdown and immune evasion by the Nsp1 protein of SARS-CoV-2. *Science* **369**, 1249-1255 (2020).
15. A. K. Banerjee *et al.*, SARS-CoV-2 Disrupts Splicing, Translation, and Protein Trafficking to Suppress Host Defenses. *Cell* **183**, 1325-1339 e1321 (2020).
16. H. Xia *et al.*, Evasion of Type I Interferon by SARS-CoV-2. *Cell Rep* **33**, 108234 (2020).
17. P. Ahlquist, RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science* **296**, 1270-1273 (2002).
18. M. Schwartz *et al.*, A Positive-Strand RNA Virus Replication Complex Parallels Form and Function of Retrovirus Capsids. *Molecular Cell* **9**, 505-514 (2002).
19. W. A. Miller, G. Koev, Synthesis of subgenomic RNAs by positive-strand RNA viruses. *Virology* **273**, 1-8 (2000).
20. N. S. Ogando *et al.*, SARS-coronavirus-2 replication in Vero E6 cells: replication kinetics, rapid adaptation and cytopathology. *J Gen Virol* **101**, 925-940 (2020).
21. I. Sola, F. Almazan, S. Zuniga, L. Enjuanes, Continuous and Discontinuous RNA Synthesis in Coronaviruses. *Annu Rev Virol* **2**, 265-288 (2015).
22. I. Sola, J. L. Moreno, S. Zuniga, S. Alonso, L. Enjuanes, Role of nucleotides immediately flanking the transcription-regulating sequence core in coronavirus subgenomic mRNA synthesis. *J Virol* **79**, 2506-2516 (2005).
23. X. Zhang, C. L. Liao, M. M. Lai, Coronavirus leader RNA regulates and initiates subgenomic mRNA transcription both in trans and in cis. *J Virol* **68**, 4738-4746 (1994).
24. S. G. Sawicki, D. L. Sawicki, S. G. Siddell, A contemporary view of coronavirus transcription. *J Virol* **81**, 20-29 (2007).
25. Y. Yang, W. Yan, B. Hall, X. Jiang, Characterizing transcriptional regulatory sequences in coronaviruses and their role in recombination. *bioRxiv*, (2020).
26. F. Wu *et al.*, A new coronavirus associated with human respiratory disease in China. *Nature* **579**, 265-269 (2020).
27. A. Vandelli *et al.*, Structural analysis of SARS-CoV-2 genome and predictions of the human interactome. *Nucleic Acids Res* **48**, 11270-11283 (2020).
28. J. R. Cohen, L. D. Lin, C. E. Machamer, Identification of a Golgi complex-targeting signal in the cytoplasmic tail of the severe acute respiratory syndrome coronavirus envelope protein. *J Virol* **85**, 5794-5803 (2011).
29. A. Perrier *et al.*, The C-terminal domain of the MERS coronavirus M protein contains a trans-Golgi network localization signal. *J Biol Chem* **294**, 14406-14421 (2019).
30. D. E. Gordon *et al.*, A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* **583**, 459-468 (2020).
31. R. McBride, M. van Zyl, B. C. Fielding, The coronavirus nucleocapsid is a multifunctional protein. *Viruses* **6**, 2991-3018 (2014).
32. S. Alberti, S. Carra, Quality Control of Membraneless Organelles. *Journal of Molecular Biology* **430**, 4711-4729 (2018).
33. S. F. Banani, H. O. Lee, A. A. Hyman, M. K. Rosen, Biomolecular condensates: organizers of cellular biochemistry. *Nature Reviews Molecular Cell Biology* **18**, 285-298 (2017).
34. A. Savastano, A. I. de Opakua, M. Rankovic, M. Zweckstetter, Nucleocapsid protein of SARS-CoV-2 phase separates into RNA-rich polymerase-containing condensates. (2020).
35. H. Chen *et al.*, Liquid-liquid phase separation by SARS-CoV-2 nucleocapsid protein and RNA. *Cell Res*, (2020).
36. C. Iserman *et al.*, Specific viral RNA drives the SARS CoV-2 nucleocapsid to phase separate. *bioRxiv*, (2020).
37. T. M. Perdikari *et al.*, SARS-CoV-2 nucleocapsid protein undergoes liquid-liquid phase separation stimulated by RNA and partitions into phases of human ribonucleoproteins. *bioRxiv*, (2020).
38. J. Cubuk *et al.*, The SARS-CoV-2 nucleocapsid protein is dynamic, disordered, and phase separates with RNA. *bioRxiv*, (2020).
39. C. Iserman *et al.* (Cold Spring Harbor Laboratory, 2020).
40. C. R. Carlson *et al.*, Phosphoregulation of phase separation by the SARS-CoV-2 N protein suggests biophysical basis for its dual functions. *Molecular Cell*, (2020).
41. L. Mendonca *et al.*, SARS-CoV-2 Assembly and Egress Pathway Revealed by Correlative Multi-modal Multi-scale Cryo-imaging. *bioRxiv*, (2020).
42. S. Ghosh *et al.*, beta-Coronaviruses Use Lysosomes for Egress Instead of the Biosynthetic Secretory Pathway. *Cell* **183**, 1520-1535 e1514 (2020).
43. Schubert, K., Karousis, E.D., Jomaa, A. *et al.* SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation. *Nat Struct Mol Biol* **27**, 959-966 (2020).
44. Chan, Jasper Fuk-Woo *et al.* Improved Molecular Diagnosis of COVID-19 by the Novel, Highly Sensitive and Specific COVID-19-RdRp/Hel Real-Time Reverse Transcription-PCR Assay Validated *In Vitro* and with Clinical Specimens. *J Clin Microbiol.* 2020;58(5):e00310-20. doi:10.1128/JCM.00310-20
45. Nagura-Ikeda M, Imai K, Tabata S, *et al.* Clinical Evaluation of Self-Collected Saliva by Quantitative Reverse Transcription-PCR (RT-qPCR), Direct RT-qPCR, Reverse Transcription-Loop-Mediated Isothermal Amplification, and a Rapid Antigen Test To Diagnose COVID-19. *J Clin Microbiol.* 2020;58(9):e01438-20. doi:10.1128/JCM.01438-20
46. Conceicao C, Thakur N, Human S, Kelly JT, Logan L, Bialy D, *et al.* (2020) The SARS-CoV-2 Spike protein has a broad tropism for mammalian ACE2 proteins. *PLoS Biol* 18(12): e3001016. <https://doi.org/10.1371/journal.pbio.3001016>
47. Damas J, Hughes GM, Keough KC, Painter CA, Persky NS, Corbo M, Hiller M, Koepfli KP, Pfenning AR, Zhao H, Genereux DP, Swofford R, Pollard KS, Ryder OA, Nweeia MT, Lindblad-Toh K, Teeling EC, Karlsson EK, Lewin HA. Broad host range of SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in vertebrates. *Proc Natl Acad Sci U S A.* 2020 Sep 8;117(36):22311-22322. doi: 10.1073/pnas.2010146117. Epub 2020 Aug 21. PMID: 32826334; PMCID: PMC7486773.

Event: 1931: Intestinal barrier, disruption

Short Name: Disruption of the intestinal barrier

Key Event Component

Process	Object	Action
	barrier epithelial cell	disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent

Stressors**Name**

Sars-CoV-2

Biological Context**Level of Biological Organization**

Organ

Organ term**Organ term**

intestine

Evidence for Perturbation by Stressor**Sars-CoV-2**

Evidence: High levels of plasma markers of intestinal hyperpermeability and microbial translocation in moderate and severe COVID-19 patients ^{9,10}.

Domain of Applicability**Sex Applicability****Sex Evidence**

Male

Female

Key Event Description

A proper definition (and related ontology) of the intestinal barrier and permeability would benefit the understanding of this biological event central in many diseases. However, it is generally accepted that the intestinal barrier is a multilayer system encompassing :

- a chemical barrier able to detoxify bacterial endotoxins,
- a mucus layer providing a physical barrier against bacteria,
- an one-cell-thick epithelial layer which physical barrier function is ensured by epithelial cell integrity and by tight junction proteins (occludins, claudins and zonulins), adherence junctions and desmosomes ^{2,4,5}
- the cellular immune system present in the lamina propria underlying the epithelial cell layer
- the antibacterial proteins secreted by the specialized intestinal epithelial cells or the Paneth cells.

Together with the chemical barrier of the mucosal layer and the cellular immune system, the intestinal epithelial cell layer has actually two **barrier** functions:¹⁻³

- It acts as a **physical** barrier against external factors (pathogens, toxins),
- It acts as a **selective** barrier by regulating the absorption of essential dietary nutrients and ions, meaning their transport from the lumen into the blood.

Intestinal permeability⁶ describes the movement of molecules across the intestinal barrier from the lumen to the blood (Figure 1), and as such, is the measurable feature of the intestinal barrier.

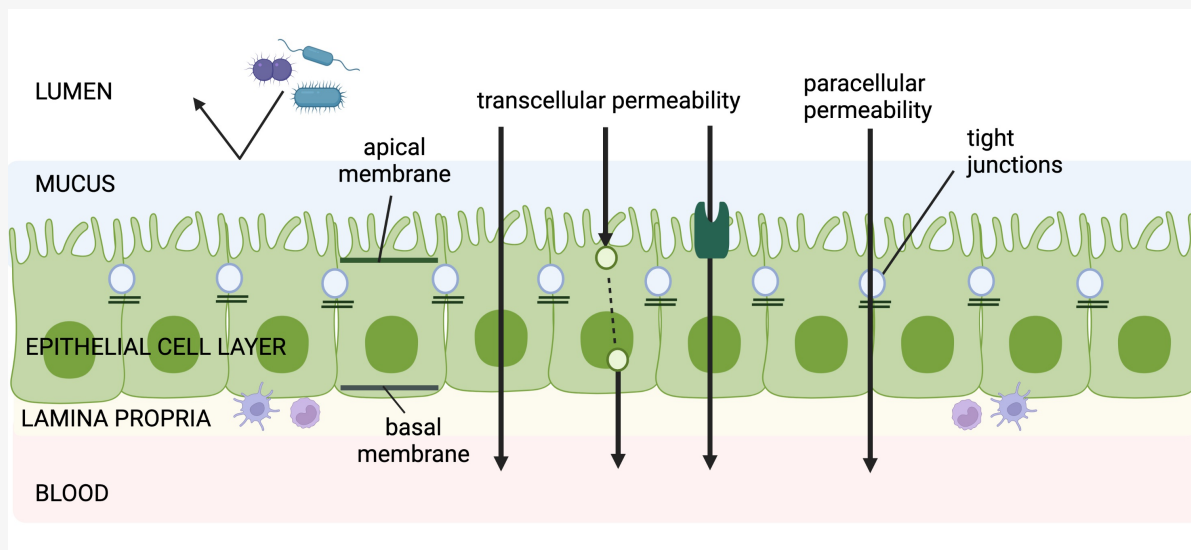


Figure 1. Created with Biorender.com

Molecules can cross the epithelium via paracellular or transcellular route. Transcellular permeability encompasses passive diffusion from the apical to the basal side (from the lumen to the blood), vesicle-mediated transcytosis and uptake mediated by a membrane receptor. Paracellular permeability is regulated by the tight junctions between adjacent cells and by the integrity of the epithelium.

Alteration or disruption of one or more layers of the intestinal barrier leads to increased intestinal permeability, also called intestinal hyperpermeability or "leaky gut", enhancing the transport of pathogens, toxins (such as lipopolysaccharides), undigested nutrients and the translocation of bacteria of the gut microbiota from the intestinal lumen into the systemic circulation³.

How it is Measured or Detected

The definition of intestinal permeability being relatively broad includes altered paracellular route, regulated by TJ proteins, transcellular routes involving membrane transporters and channels, and endocytic mechanisms. Paracellular intestinal permeability can be assessed *in vivo* via different molecules and via putative blood biomarkers and *ex vivo* in Ussing chambers combining electrophysiology and probes of different molecular sizes. The latter is still the gold standard technique for assessing the epithelial barrier function, whereas *in vivo* techniques are also broadly used despite limitations (doi: [10.3389/fnut.2021.717925](https://doi.org/10.3389/fnut.2021.717925)).

In humans.

Virtually all *in vivo* methods to assess paracellular intestinal permeability rely on the urinary excretion of orally ingested probes. Several markers, including different sizes of PEG, ⁵¹CrEDTA, and especially sugars have been used, each with advantages and disadvantages (doi: [10.3389/fnut.2021.717925](https://doi.org/10.3389/fnut.2021.717925)). **Intestinal Permeability Assessment** (IPA) directly measures the ability of two non-metabolized sugar molecules (lactulose and mannitol) to permeate the small intestinal barrier by paracellular passage (sign of perturbed TJ-lactulose) or by transcellular passage (giving information of the whole epithelial absorptive area-mannitol), respectively. The patient drinks a premeasured amount of those sugars and 6h after, the ratio of Lactulose/Mannitol levels is measured in the urine ¹¹.

Levels in plasma/serum or in feces of:

- Markers of epithelial cell damage, such as intestinal fatty acid binding protein (FABP)
- Markers of tight junction alterations, such as zonulin levels (doi: [10.1080/21688370.2016.1251384](https://doi.org/10.1080/21688370.2016.1251384))
- Microbial translocation, such as peptidoglycans and lipopolysaccharides (LPS) and gut microbiota alteration.

In vitro systems¹²

Trans epithelial electrical resistance (**TEER**) or the Lucifer Yellow (**LY**) leakage assay are techniques to measure barrier integrity and permeability of a cell layer¹³. Caco-2 cells are human epithelial colorectal adenocarcinoma cells with a structure and function similar to the differentiated small intestinal epithelial cells (e.g. exhibit microvilli). Caco-2 cells can be plated in wells as monolayers^{14,11}. Other cell lines can be used, such as intestinal epithelial cells (IEC) or primary epithelial cells from human intestinal biopsies¹². Co-culturing of enterocyte-like cells with immune cells in three-dimensional structure and within a microfluidic gut-on-chip has been shown to reflect better the physiology of the gut epithelium. Epi-IntestinalTM is an example of 3D human primary cell-based organotypic small intestinal model which allows evaluation of TEER and LY leakage assay (doi: [10.1007/s11095-018-2362-0](https://doi.org/10.1007/s11095-018-2362-0)).

In vivo system

In mice, one way to study intestinal paracellular permeability is by measuring the ability of fluorescein isothiocyanate (**FITC**)-dextran to cross from the lumen into the blood. After gavaging mice with FITC-dextran, the concentrations are measured in collected serum samples (doi: [10.3791/57032](https://doi.org/10.3791/57032)).

References

1. Chelakkot, C., Ghim, J. & Ryu, S. H. Mechanisms regulating intestinal barrier integrity and its pathological implications. *Exp. Mol. Med.* **50**, (2018).
2. Groschwitz, K. R. & Hogan, S. P. Intestinal barrier function: Molecular regulation and disease pathogenesis. *J. Allergy Clin. Immunol.* **124**, 3–20 (2009).
3. Ghosh, S. S., Wang, J., Yannie, P. J. & Ghosh, S. Intestinal barrier dysfunction, LPS translocation, and disease development. *J. Endocr. Soc.* **4**, 1–15 (2020).
4. Sturgeon, C. & Fasano, A. Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. *Tissue Barriers* **4**, 1–19 (2016).
5. Sturgeon, C., Lan, J. & Fasano, A. Zonulin transgenic mice show altered gut permeability and increased morbidity/mortality in the DSS colitis model. *Ann N Y Acad Sci* **1397**, 130–142 (2017).
6. Bischoff, S. C. *et al.* Intestinal permeability - a new target for disease prevention and therapy. *BMC Gastroenterol.* **14**, 1–25 (2014).
7. Qiu, W. *et al.* PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. *J. Clin. Invest.* **121**, 1722–1732 (2011).
8. Hering, N. A., Fromm, M. & Schulzke, J. D. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. *J. Physiol.* **590**, 1035–1044 (2012).
9. Giron, L. B. *et al.* Plasma Markers of Disrupted Gut Permeability in Severe COVID-19 Patients. *medRxiv* 2020.11.13.20231209 (2021).
10. Prasad, R. *et al.* Plasma microbiome in COVID-19 subjects: an indicator of gut barrier defects and dysbiosis Ram. *BioRxiv* (2021).
11. Aguirre Valadez, J. M. *et al.* Intestinal permeability in a patient with liver cirrhosis. *Ther. Clin. Risk Manag.* **12**, 1729–1748 (2016).
12. Fedi, A. *et al.* In vitro models replicating the human intestinal epithelium for absorption and metabolism studies: A systematic review. *J. Control. Release* **335**, 247–268 (2021).
13. Lea, T. Epithelial Cell Models; General Introduction. in *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (eds. Verhoeckx, K. et al.) 95–102 (Springer International Publishing, 2015). doi:10.1007/978-3-319-16104-4_9
14. Li, B. R. *et al.* In Vitro and In Vivo Approaches to Determine Intestinal Epithelial Cell Permeability. *J. Vis. Exp.* 1–6 (2018). doi:10.3791/57032
15. Ayeahunie, S. *et al.* Human Primary Cell-Based Organotypic Microtissues for Modeling Small Intestinal Drug Absorption Seyoum. *Pharm. Res.* **35**, 72 (2019).

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2056: Binding to ACE2 leads to SARS-CoV-2 cell entry

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	High
SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	adjacent		
SARS-CoV-2 infection leading to hyperinflammation	adjacent		
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent	High	High
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Moderate
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	High	Moderate
Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability**Sex Evidence**

Unspecific High

Key Event Relationship Description

This KER deals with the evidence supporting the individual weight that the surface protein of SARS-CoV-2 spike needs to bind:ACE2, and of being cleaved in two different sites, for viral entry to occur. Viral entry is essential for initiating a cascade of events leading to COVID19.

Quantitative Understanding of the Linkage**Known modulating factors**

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Chemicals (weak evidence)	PFAS (PFOS, PFOA, PFNA, PFHxS, and GenX)	Short-term (10 days), high dose (20 mg/kg/day) exposure to PFOA leads to about 1.6 fold upregulation of the pulmonary mRNA level of <i>Ace2</i> and to about 1.5 upregulation of the pulmonary mRNA level of <i>Tmprss2</i> in CD1 mice. [1] Long-term (12 weeks) of an environmentally relevant PFAS mixture (PFOS, PFOA, PFNA, PFHxS, and GenX; each in 2 mg/l concentration) exposure leads to downregulation of pulmonary mRNA expression of <i>Ace2</i> 2.5-fold in C57BL/6 J male mice. A similar decreasing trend was observed in PFAS-exposed male mice for <i>Tmprss2</i> . [2]	1) doi: 10.1016/j.toxrep.2021.11.014 2) doi: 10.1016/j.taap.2022.116284
Sex (strong evidence)	female sex (XX chromosomes) Male sex (XY chromosomes)	ACE2 localizes to the X sex chromosome and displays a sex-dependent expression profile with higher expression in female than in male tissues. [1,2] Estradiol inhibits TMPRSS2, needed to facilitate SARS-CoV-2 entry into the cell. [3] Estrogen therapy has been shown to mitigate endoplasmic reticulum stress induced by SARS-CoV-2 invasion through activation of cellular unfold protein response and regulation of inositol triphosphate (IP3) and phospholipase C. [4] Different studies have also illustrated that estradiol increases the expression of ADAM17, leading to high-circulating sACE2 potentially neutralizing SARS-CoV-2 and preventing its binding to mACE2. [5] Thus, Estradiol might reduce SARS-CoV-2 infectivity through modulation of cellular ACE2/TMPRSS2/ADAM17 axis expression. Androgen receptors (ARs) play a key role in increasing transcription of TMPRSS2. This may explain the predominance of males to COVID-19 fatality and severity. [6]	1) doi: 10.1177/1933719115597760 2) doi: 10.1016/j.mce.2015.11.004 3) doi: 10.1007/s11033-021-06390-1 4) doi: 10.1016/j.mehy.2020.110148 5) doi: 10.2217/pgs-2020-0092 6) doi: 10.1073/pnas.2021450118
Age	Young/old people	ACE2 protein expression is increased with aging in several tissues [1], including lungs and particularly in patients requiring mechanical ventilation [2]. During aging, telomere dysfunction activates a DNA damage response leading to higher ACE2 expression. Thus, telomere shortening could contribute to make elderly more susceptible to SARS-CoV-2 infection [3].	1) doi: 10.1016/j.exger.2021.111507 2) doi: 10.1371/journal.pone.0247060 3) doi: 10.15252/embr.202153658
Dyslipidemia and obesity	Lipids	Lipids, as important structural components of cellular and sub-cellular membranes, are crucial in the infection process [1]. Changes in intracellular cholesterol alter cell membrane composition, impacting structures such as lipid rafts, which accommodate many cell-surface receptors [2], including ACE2 and TMPRSS2 [3, 4]. In an in vitro study, the depletion of membrane-bound cholesterol in ACE2-expressing cells led to a reduced infectivity of SARS-CoV [3]. In vitro, higher cellular cholesterol increased uptake of SARS-CoV-2 S protein; this effect was decreased with Methyl-beta-cyclodextrin, a compound which extracts cholesterol from cell membranes [5]. HDL scavenger receptor B type 1 (SR-B1), a receptor found in pulmonary and many other cells, could facilitate ACE2-dependent entry of SARS-CoV-2 [6]. ACE2 is highly expressed in adipose tissue, thus excess adiposity may drive more infection [8]. Obese patients have more adipose tissue and therefore more ACE2-expressing cells [9]. SARS-CoV-2 dysregulates lipid metabolism in the host and the effect of such dysregulated lipogenesis on the regulation of ACE2, specifically in obesity [10]. Lung epithelial cells infected with SARS-CoV-2 showed upregulation of genes associated with lipid metabolism [11], including the SOC3 gene. A mouse model of diet-induced obesity showed higher <i>Ace2</i> expression in the lungs, which negatively correlated with the expression sterol response element binding proteins 1 and 2 (SREBP) genes. Suppression of <i>Srebp1</i> showed a significant increase in <i>Ace2</i> expression in the lung. Lipids, including fatty acids, could interact directly with SARS-CoV-2 influencing spike configuration and modifying the affinity for ACE2 and thus its infectivity [12]. The dysregulated lipogenesis and the subsequently high ACE2 expression in obese patients might be one mechanism underlying the increased risk for severe complications [10].	1) doi: 10.1001/jama.2020.12839 2) doi: 10.3389/fcell.2020.618296 3) doi: 10.1016/j.bbrc.2008.02.023 4) doi: 10.1096/fj.202000654R 5) doi: 10.1101/2020.05.09.086249 6) doi: 10.1038/s42255-020-00324-0 7) doi: 10.1016/j.lbalip.2020.158849 8) doi: 10.1016/j.obmed.2020.100283 9) doi: 10.3390/jms21103544 10) doi: 10.1101/2020.04.16.20068528
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References

COVID19 References related to CNS:

de Morais SDB, et al. Integrative Physiological Aspects of Brain RAS in Hypertension. *Curr Hypertens Rep.* 2018 Feb 26; 20(2):10.

Gallagher PE, et al. Distinct roles for ANG II and ANG-(1-7) in the regulation of angiotensin-converting enzyme 2 in rat astrocytes. *Am J Physiol Cell Physiol.* 2006 Feb; 290(2):C420-6.

Gowrisankar YV, Clark MA. Angiotensin II regulation of angiotensin-converting enzymes in spontaneously hypertensive rat primary astrocyte cultures. *J Neurochem.* 2016 Jul; 138(1):74-85.

Hamming I et al. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol.* 2004 Jun;203(2):631-7.

Jakhmola S, et al. SARS-CoV-2, an Underestimated Pathogen of the Nervous System. *SN Compr Clin Med.* 2020.

Lukiw WJ et al. SARS-CoV-2 Infectivity and Neurological Targets in the Brain. *Cell Mol Neurobiol.* 2020 Aug 25;1-8.

Matsushita T, et al. CSF angiotensin II and angiotensin-converting enzyme levels in anti-aquaporin-4 autoimmunity. *J Neurol Sci.* 2010 Aug 15; 295(1-2):41-5.

Murta et al. Severe Acute Respiratory Syndrome Coronavirus 2 Impact on the Central Nervous System: Are Astrocytes and Microglia Main Players or Merely Bystanders? *ASN Neuro.* 2020. PMID: 32878468

Shi A, et al. Isolation, purification and molecular mechanism of a peanut protein-derived ACE-inhibitory peptide. *PLoS One.* 2014; 9(10):e111188.

Xia, H. and Lazartigues, E. Angiotensin-Converting Enzyme 2: Central Regulator for Cardiovascular Function. *Curr. Hypertens.* 2010 Rep. 12 (3), 170– 175

Relationship: 2496: SARS-CoV-2 cell entry leads to IFN-I response, antagonized

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Not Specified
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent		
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Moderate
Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

It has been shown that in human populations males are more likely to suffer severe infections and deaths due to COVID-19 than females. However, in the viral entry and infection phase, one study found that women of working age had higher infection rates than men, but the suggested cause was higher contact rates among women (Doerre and Doblhammer, 2022 <https://doi.org/10.1371/journal.pone.0268119>). Contact rate increase is an important transmission factor but would not constitute a gender-based biological difference in viral entry or IFN-I pathway antagonism. A biological basis for females having higher levels of Type I IFN has been proposed concerning Toll-like receptor (TLR) 7. TLR7 is expressed in plasmacytoid dendritic cells (pDCs), an immune cell type that on infection with SARS-CoV-2 migrates from peripheral blood into the respiratory tract epithelium. TLR7 stimulates higher IFN-I production in pDCs in women than in men (Van der Sluis et al. 2022 <https://doi.org/10.1016/j.celrep.2022.111148>). It is proposed that this is due to the TLR7 gene being on the X chromosome, and that X inactivation in males is incomplete regarding the TLR7 gene, creating a double gene-dose effect in females (Spiering and de Vries, 2021 <https://doi.org/10.3389/fimmu.2021.756262>). In a mouse SARS-CoV model, XY males had more adverse outcomes than XX females and XXY males (Gadi et al. 2020 <https://doi.org/10.3389%2Ffimmu.2020.02147>). Additionally, loss-of-function TLR7 mutations have been identified that are associated with increased COVID-19 severity (Szeto et al. 2021 <https://doi.org/10.1159/000518471>). However, these results focus on disease outcome as the endpoint, where factors beyond the initial antiviral response could be involved. Also note that the nasal and upper respiratory tract (URT) epithelial cells express ACE2 receptors for SARS-CoV-2 entry while the pDCs do not, relying on viral endocytosis (Van der Sluis et al. 2022). There is not a clear picture in the literature of the timing of pDC arrival in the epithelium after exposure, and the role of TLR7 in sex differences is currently hypothetical (Spiering and de Vries, 2021).

In a large study modelling URT viral load dynamics drawn from measurements in 605 human subjects, variations over 5 orders of magnitude in URT viral load from the time of symptom onset was not explained by age, sex, or severity of illness. Additionally, these variables did not explain modelling results concerning control of viral load by immune responses in the early (innate) or late (adaptive) phases (Challenger et al. 2022 <https://doi.org/10.1186/s12916-021-02220-0>). Other sources also support that rate of infection and measured viral load does not differ by gender (e.g., Arnold et al. 2022 <https://doi.org/10.1186/s13293-022-00417-3>; Qi et al. 2021 <https://doi.org/10.1186/s13293-021-00410-2>; Cheemarla et al. 2021 <https://doi.org/10.1084/jem.20210583>). Therefore, evidence exists that the components of cell entry and the early antiviral response are not influenced by gender specific differences such as sex hormone levels or sex chromosomes to the extent of affecting viral load.

Key Event Relationship Description

Upon entry of a virus into the host cell (KE1738), the virus is unpackaged from the structural nucleocapsid (N), envelope (E), and membrane (M) proteins. The viral RNA is detected by Pattern Recognition Receptor (PRR) proteins including RIG-I and MDA5 but the M proteins can interact with these PRRs directly, and block this initial host reaction (<https://doi.org/10.1038/s41423-020-00571-x>). The viral genomic RNA can then be translated directly at the host ribosomes. The viral proteins may be processed through cleavage by viral protease enzymes. This releases a repertoire of non-structural proteins (NSPs) and accessory open reading frame (ORF) proteins that has evolved, for example in the SARS-CoV-2 virus, to bind and block the proteins in the interferon I (IFN-I) antiviral cascade (KE1901). The timely production of type I IFN by host cells is critical for limiting viral replication and promoting antiviral immunity (10.1126/science.abm8108).

Evidence Supporting this KER

Biological Plausibility

Biological plausibility comes from several studies indicating that if IFN is administered just before or upon exposure, viral production is reduced or eliminated (DOI 10.1007/s10517-021-05330-0; <https://doi.org/10.1038/s41467-021-27318-0>; doi.org/10.1016/j.immuni.2021.01.017), which supports the linkage that viral entry causes IFN antagonism. Also, it has been found that some people have developed autoimmunity toward their own IFN proteins (<https://doi.org/10.1084/jem.20210554>; <https://doi.org/10.1084/jem.20211211>). They produce autoantibodies that block even exogenously administered IFN, and this has resulted in more severe disease in these patients.

Empirical Evidence

There are studies with empirical evidence in support of temporal concordance, showing that interferon expression is delayed by SARS-CoV-2 compared to other viruses like influenza, which is also described as an untuned or imbalanced response between interferons being initially low and inflammatory cytokines elevated in moderate to severe cases ([10.1016/j.cell.2020.04.026](https://doi.org/10.1016/j.cell.2020.04.026); <https://doi.org/10.1038/s41590-020-00840-x>; <https://www.science.org/doi/10.1126/science.abc6027>; <https://doi.org/10.1038/s41467-021-27318-0>; <https://doi.org/10.1038/s41598-021-95197-y>).

Uncertainties and Inconsistencies

There are uncertainties based on differing disease outcomes, especially associated with timing of administering IFN. Effectiveness of IFN treatment is still uncertain due to some studies evaluating IFN along with other drugs (DOI: 10.1002/jmv.27317). In the small intestine of infected hamsters, a mild antiviral gene signature was observed coinciding with a low-level inflammatory response and low replication similar to some human cases (<https://doi.org/10.1016/j.immuni.2021.01.017>), in contrast to the robust replication seen in human small intestinal organoids (<https://doi.org/10.1126/science.abc1669>) and in severely ill patients. See also <https://doi.org/10.1186/s12943-021-01363-1>.

Quantitative Understanding of the Linkage

Response-response relationship

Viral exposure dose threshold to result in IFN antagonism.

Time-scale

The viral entry MIE and early KEs coincide with the time from exposure to symptoms, within which are the latent period, or time from exposure to infectiousness, and the serial interval, or the time interval between the onset of symptoms in the primary (infector) and secondary case (infectee). Viral entry leading to antagonism of the IFN response occurs during the latent period of the disease prior to symptom onset. Latent period calculation is based on serial interval and median pre-symptomatic infectious period: Serial interval 5.2 days (Rai et al. 2021) – 2.5 days pre-symptom infectious period (Byrne et al. 2020) $\approx$ 2.7 days. The latent period was longer in asymptomatic cases (4-9 days); pre-symptomatic transmission occurs from about 3 days after exposure to symptom onset at about day 5-7, viral load peaks from about day 5-7 to day 9-11, and the host can remain infectious to symptom clearance or death (Byrne et al. 2020). As noted, IFN administered prior to exposure or within the latent period window can stop replication. However, IFN administered too late, in the inflammatory stage (post-symptom onset), led to long-lasting harm and worsened disease outcome (DOI: 10.1002/jmv.27317). In a study using a primary nasal cell model (differentiated at air-liquid interface), the virus did not proliferate beyond the limit of assay detection if treated with IFN beta or lambda 16 hours prior to infection, and virus was significantly reduced in cultures treated 6h post-infection compared to untreated cultures. Treatments 24h post infection were not significantly different from untreated controls for either type of IFN (<https://doi.org/10.1038/s41467-021-27318-0>).

Known modulating factors

- Autoimmunity as noted above (patients with autoantibodies that attack IFN).
- Factors affecting entry (TMPRSS2 or neuropilin) - therapeutics?
- Androgen signaling leads to increased TMPRSS2 production (<https://doi.org/10.1016/j.mce.2009.12.022>) in males, and therefore potentially enhanced viral entry.
- Weakened IFN antiviral response in the elderly (doi: [10.3390/cells10030708](https://doi.org/10.3390/cells10030708)).
- Environmental exposures to aryl hydrocarbons and dioxins may block IFN production (doi: [10.1016/j.cotox.2018.01.004](https://doi.org/10.1016/j.cotox.2018.01.004)).

Known Feedforward/Feedback loops influencing this KER

Initial exposure to the virus is exogenous, but disease progression results from viral proliferation and release from the initial site of infection to spread to adjacent cells and eventually distal tissues.

Relationship: 2497: IFN-I response, antagonized leads to SARS-CoV-2 production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Not Specified
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent		
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Moderate
Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	adjacent	High	Moderate

Relationship: 2546: SARS-CoV-2 production leads to Disruption of the intestinal barrier

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent	Low	Low

Evidence Supporting this KER

Biological Plausibility

Within the host cell, the new virions are assembled ([KE1847](#)) and to release viral particles, the virus promotes host lysis, leading to cell death and compromising the integrity of the epithelial monolayer. The intestinal barrier is ensured by the integrity of the monolayer epithelium (via cell integrity and tight junctions (TJ)/adherens proteins), together with the chemical barrier, the mucosal layer and the cellular immune system located in the lamina propria (KE1931). Alternatively, TJs might be altered following SARS-CoV-2 infection enhancing paracellular permeability. In addition, the mucus layer and/or the cellular immune system might be perturbed.

Uncertainties and Inconsistencies

While the biological plausibility was high, currently, there is not enough evidence to support that enterocyte massive cell death following SARS-CoV-2 infection occurs systematically. Number of cases showing histomorphologic changes due intestinal infection by SARS-CoV-2 is still limited.

Quantitative Understanding of the Linkage

Known modulating factors

10.20944/preprints202206.0121.v1 (p.26-27)