Journal Pre-proof

Coronavirus entry: how we arrived at SARS-CoV-2

Gary R Whittaker, Susan Daniel, Jean K Millet

PII: S1879-6257(21)00016-X

DOI: https://doi.org/10.1016/j.coviro.2021.02.006

Reference: COVIRO 1077

To appear in: *Current Opinion in Virology*

Please cite this article as: Whittaker GR, Daniel S, Millet JK, Coronavirus entry: how we arrived at SARS-CoV-2, *Current Opinion in Virology* (2021), doi: https://doi.org/10.1016/j.coviro.2021.02.006

Coronavirus entry: how we arrived at SARS-CoV-2

Gary R. Whittaker [1*], Susan Daniel [2] and Jean K. Millet [3]

[1] Department of Microbiology and Immunology and Master of Public Health Program, Cornell University, Ithaca, NY, USA

[2] Robert Frederick Smith School of Chemical & Biomolecular Engineering, Cornell University, Ithaca, NY, USA

[3] Université Paris-Saclay, INRAE, UVSQ, Virologie et Immunologie Moléculaires, Jouy-en-Josas, France

*Corresponding author – 618 Tower Rd, Ithaca NY 14853, USA; grw7@cornell.edu; (607) 253 4019

Abstract

Because of the COVID-19 pandemic, the novel coronavirus SARS-CoV-2 has risen to shape scientific research during 2020, with its spike (S) protein being a predominant focus. The S protein is likely the most complicated of all viral glycoproteins and is a key factor in immunological responses and virus pathogenesis. It is also the driving force dictating virus entry mechanisms, which are highly "plastic" for coronaviruses, allowing a plethora of options for different virus variants and strains in different cell types. Here we review coronavirus entry as a foundation for current work on SARS-CoV-2. We focus on the post-receptor binding events and cellular pathways that direct the membrane fusion events

necessary for genome delivery, including S proteolytic priming and activation. We also address aspects of the entry process important for virus evolution and therapeutic development.

Keywords: coronavirus entry; endocytosis; SARS-COV-2; protease; COVID-19

Historical context

Coronaviruses were first recognized in the 1930s due to outbreaks of respiratory disease in poultry [1], followed by the subsequent isolation and identification of infectious bronchitis virus (IBV) of chickens [2]. IBV became the prototype coronavirus and was later categorized as a gammacoronavirus. Other pioneering work in animal health in the 1940s and 1950s led to the identification of the etiological agent of transmissible gastroenteritis of swine (TGEV) [3], which was later classified as an alphacoronavirus, and murine hepatitis virus (MHV) [4], a prototype betacoronavirus. In humans, the first coronaviruses were discovered in the 1960s through isolation in tracheal organ cultures. Some early strains such as B814 isolated by the MRC Common Cold Unit and OC38 from the NIH Laboratory of Viral Diseases are no longer studied [5,6]. Other isolates named HCoV-OC43 and HCoV-229E (which became members of the betacoronavirus and alphacoronavirus genus respectively) are still being studied to this day. The term "coronavirus" was proposed in 1968 to group these early human strains with animal viruses such as IBV based on their shared characteristic appearance in electron microscopy images [7]. Notably, clinical isolates of coronavirus often grew poorly in cell culture and underwent selection in embryonated eggs (IBV) or mouse brain (OC43), with such laboratory-adapted variants becoming the "go to" coronaviruses for many years—but these viruses were generally understudied.

Scientific and medical interest in coronaviruses changed dramatically in 2003 with the outbreak of severe acute respiratory syndrome (SARS). The causative agent (SARS-CoV) was identified as a

betacoronavirus having an origin in bats, which emerged as a zoonotic agent via masked palm civets and raccoon dogs—linked to exposure of humans by these species in live animal markets [8,9]. The SARS-CoV outbreak was contained relatively rapidly, despite its initial global spread via travelers. However, the impact of the outbreak was significant and it stimulated a brief period of accelerated coronavirus discovery with the identification of HCoV-NL63 and HCoV-HKU1 [10,11], both of which are now considered community-acquired respiratory (CAR) coronaviruses along with HCoV-OC43 and HCoV-229E [9].

The next zoonotic coronavirus outbreak came in 2012 with Middle East respiratory syndrome (MERS), caused by another bat-origin betacoronavirus (MERS-CoV), in this case with a reservoir in camels [8,9]. MERS-CoV has continued to re-emerge at a low level since 2012, with a focus of infection in certain countries in the Middle East, with only occasional spread to other countries via travelers. In late 2019, a novel coronavirus, with similarity to SARS-CoV but much more extensive transmission (SARS-CoV-2), emerged to trigger the COVID-19 pandemic [8]. Most human coronaviruses are now considered to be bat-origin, along with the majority of animal coronaviruses of veterinary importance; however, certain betacoronaviruses (lineage A)—including mouse hepatitis virus (MHV) an important pathogenesis model—appear to have a rodent reservoir, and gammacoronaviruses and the newly identified fourth genus, deltacoronavirus, have an avian origin.

Virus entry basics

Viral receptors are key to our understanding of virus entry, and much is now known about coronaviruses in this respect. This has been reviewed recently [12], and for coronaviruses is encompassed by a subset of cell surface molecules including the exopeptidases ACE2, DPP4 and APN, CEACAMs and other non-specific attachment factors. This article is focused on post-receptor events in virus entry, and specific receptors will only be referred to in that context. Our knowledge of virus entry has a foundation in

classical cell biology, pioneered in the 1980s by Simons and Helenius among others [13]. Many of these early studies used electron microscopy combined with biochemical techniques to elucidate receptor-mediated endocytosis as a primary means for virus entry into the cell. During the 1990s and early 2000s, techniques of molecular biology were applied to dissect out specific entry routes into the cell, reviewed in [14]. However, through these periods, coronaviruses received relatively little attention.

Coronaviruses engage their receptor through their prominent surface glycoprotein (spike or S) [15]. Compared to the glycoproteins of most other viruses, S is large and complex. It has been grouped as a class I fusion protein based on its helical heptad repeats, but differs from most class I fusion proteins in several key ways. Its proteolytic activation occurs via two sequential cleavage events, in many but not all coronaviruses [15]. The first cleavage occurs at the boundary of the S1 and S2 domains (S1/S2) and can be considered a dispensable "priming" event that typically occurs in during S protein biogenesis and virus assembly. The second cleavage (S2') is the critical "activating" event for membrane fusion, as it liberates what is formally an internal fusion peptide within the S2 domain [16,17]. These cleavage events control much of virus entry and cell tropism; the CoV S is remarkably "plastic" in its ability to take advantage of differential protease expression and activation in different cells and tissues. Our knowledge of S function was transformed in 2016 with the cryo-EM structure of MHV S [18], which paved the way for a "structural era" of CoV entry [19].

However, during this time, a true understanding of the cell biological aspects of CoV entry has remained sparse and specific entry pathways have continued to be elusive, in line with the highly plastic nature of the viral S protein. Some notable insights include a study based on RNAi-mediated knock-down of endocytosis-associated proteins and pharmacological inhibitors, in which MHV entry was demonstrated to be dependent on clathrin-mediated endocytosis (CME) [20]. Viral fusion events were less associated with early endosomal marker (RAB5) but occurred more readily in vesicles containing late endosomal (RAB7) and lysosomal (LAMP1) markers indicating a "late" endosomal entry pathway. Another

important achievement was the realization that coronavirus receptors are clustered into cellular membrane microdomains along with their activating proteases [21]. Tetraspanins, as their name implies are membrane proteins with four transmembrane spans. Expressed by eukaryotes, they contain two extracellular loops and play a central role in maintaining the architecture of cellular membranes. Studies on MERS-CoV have shown that the tetraspanin CD9 played a critical role in partitioning membrane microdomains that concentrate DPP4 receptors and S-activating membrane proteases (TMPRSS2). As such, tetraspanins are considered to be critical host factors that determine the route of entry of coronaviruses into host cells. In addition, the concept of "early" and "late" entry pathways [21] appears to coincide well with a novel feature of the CoV fusion peptide; *i.e*. that it binds calcium [22], a feature that may control its activity and fusion from either the cell surface or endosomal calcium stores. The molecular organization of the novel fusion peptide is an area of active investigation; the finding of two distinct subdomains (FP1 and FP2) is being explored in relation to differences in calcium binding between different coronaviruses [23], with recent molecular dynamics simulations confirming a critical role for calcium in FP1-membrane interactions [24]. Another regulatory feature is that the S2' recognition site can be cryptic [25,26], with small differences in the specific cleavage site likely affecting the composition and activity of the fusion peptide.

Coronavirus entry pathways: a plurality of options

As a prototypical class I fusion protein, the HA of influenza virus requires a protease priming event *and* low pH for activation of the glycoprotein fusion machinery. However, the role of pH in S activation is a more indirect one that aligns with its proteolytic activation by various host proteases, some of which are pH sensitive and located in distinct cellular compartments. A general theme has emerged for the fusion-activating S2' site, which is that trypsin-like and type II serine proteases cleave at the cell surface, whereas cysteine-type cathepsin proteases cleave in intracellular compartments. This possibility of multiple activation triggers sets up the concept of "early" and "late" entry pathways, that coincide with

fusion at the plasma membrane surface (or immediately upon endocytosis) or within a more mature endosomal membrane compartment.

This dual pathway theme reconciles many confounding reports that showed in some cases a clear lack of dependence on pH in entry, and in others, effective inhibition of entry by lysosomotropic agents. After the SARS-CoV outbreak in 2003, early electron micrographs appeared to show direct plasma membrane entry of SARS-CoV into Vero cells [27]. However this was contrasted by other studies that showed SARS-CoV pseudovirion entry could be inhibited by lysosomotropic agents [28], indicating dependence on pH and thus a fusion pathway through an endosome. However this group reported that S proteins expressed at the cell surface could fuse readily with adjacent plasma membranes at neutral pH when exposed to trypsin [29], supporting a direct plasma membrane fusion pathway too and a first hint at the possibility of dual entry pathways. Later studies of feline coronavirus (FCoV) fusion to supported bilayers using single particle tracking showed that S-mediated membrane fusion of pseudovirions required protease treatment and an acidic environment to fuse, but the rate dependence on pH was negligible [30]. MHV was also found to be less sensitive to endosomal pH than influenza [20]. These later studies suggested an indirect role for pH in entry, pointing to its role being more critical for protease activity for S cleavage and endosomal maturation than its interaction with the S protein itself. As such, earlier observations of entry inhibition by lysosomotropic agents are likely an outcome of a reduction in cathepsin activity at higher pH and inhibition of S cleavage when the virus takes the late entry pathway.

As early as 2005, the hypothesis of dual entry pathways was articulated by Matsuyama *et al.* [31], where the authors observed for SARS-CoV that the local protease environment influenced its entry pathway, in particular, the view that proteases produced in the lungs by inflammatory cells (such as elastase) could lead to many-fold more efficient infection and the associated severe lung damage observed in patients. Since that report, a number of other papers have supported this notion of pathway flexibility based on protease availability in the cellular environment, focusing on proteases present in the respiratory tract.

Kam *et al.* [32], first pointed out that transmembrane serine protease (TMPRSS) localized in the human airway can cleave SARS-CoV S. In an important follow up to this paper, Shulla *et al.* [33] showed a critical requirement that both the virus receptor (ACE2) and TMPRSS2 must be in the *same* cell plasma membrane (co-planar) for infection by SARS-CoV in the early entry pathway. The mutational study by Burkard and colleagues on MHV entry also showed that the S2' protease recognition sequence found in coronavirus spike proteins were critical determinants governing the "early" or "late" site of intracellular fusion [20]. This dual entry pathway theme extends to MERS-CoV [34] and further expands to SARS-CoV-2 in recent work (see below).

Signaling events in coronavirus entry

Coronavirus entry is highly integrated with downstream signaling events, which is currently an area of active interest. A recent study on the early events of infection of HCoV-NL63 in LLC-Mk2 and primary human airway epithelial (HAE) cells has shed light on post-receptor binding events of coronavirus entry [35]. Following binding to cell-surface heparan sulfate and the virus' cognate receptor, ACE2, HCoV-NL63 virions were found to internalize through clathrin coated pits. Viral entry was sensitive to dynamin blockers indicative that it was dependent on proper scission of clathrin coated vesicles from the plasma membrane. The entry process generally followed what was described for other coronaviruses such as MHV [20]. Some differences were observed in the entry pathways used by HCoV-NL63 virions in LLC-Mk2 cells compared to entry in HAE cells, with a strict dependence for endocytosis for the former cells and the possibility of an alternative, earlier entry route for the latter. The authors suggested that the availability of TMPRSS2 protease at the surface of HAE cells could prime the spike protein for fusion before internalization, however virus-cell fusion still required endocytosis and acidification of endosomes in these cells. Rearrangements of filamentous actin were found to be important to allow virus-carrying endosomes to pass through the cellular cortex. Knowledge of later events along the endocytosis route has also been obtained by work on SARS-CoV showing that the actin-binding protein

ezrin could interact directly with the C-terminal domain of its spike protein at a post-fusion stage [36]. Functionally, ezrin was found to inhibit SARS-CoV entry and infection, possibly by hampering fusion pore opening and trapping of incoming particles within the intracellular network of filamentous actin. These findings echo the previously identified negative regulatory role of the actin cytoskeleton, as entry at the plasma membrane may lead to trapping of viruses in cortical actin, as shown elegantly by Marsh and Bron, for the model alphavirus Semliki Forest virus (SFV) [37].

Due to its role in endosomal acidification, vacuolar-type H^+ ATPase (v-ATPase) has been established as a necessary component for the endosomal route of entry that coronaviruses undertake [20]. However, the v-ATPase is not the only ATPase implicated in coronavirus entry processes, as it was shown that the Na^+/K^+-ATPase (sodium-potassium pump) also played an important regulatory role in virus entry and signaling, albeit through a very different mechanism [38]. Inhibiting Na^+/K^+-ATPase expression or activity, in particular the ATP1A1 α subunit, potently decreased infection by several coronaviruses including MHV. Inhibition using cardiotonic steroids ouabain and bufalin was shown to block infection at an early stage during viral internalization and inhibited viral fusion. In addition to its ion-exchange function, Na^+/K^+-ATPase is also known to participate in signal transduction, and it was demonstrated that ouabain induces a conformation change in the α subunit which activates phosphorylation of bound Src protein resulting in recruitment of additional signaling factors and downstream signaling events. This signaling pathway plays a critical role in the early stage inhibition of coronavirus entry by cardiotonic steroids, which is thought to occur upstream of the inhibition by classical CME inhibitors [38].

In addition to the study of signaling events directly involved in coronavirus host cell binding and internalization, early signaling pathways implicated in host innate immune responses have also been an area of active investigation. Virus entry into host cells often triggers detection by innate immune sensors that detect pathogen associated molecular patterns (PAMPs). Such sensing can occur very early on during the course of infection, including during endocytosis. In coronaviruses, this has been well

documented with SARS-CoV *in vivo* [39,40]. These studies highlighted the importance of adaptor proteins such as MyD88 and TRIF in regulating the mounting of an effective host innate immune response against infections. Notably, TRIF, a signaling adaptor for TLR3, an endosomal double stranded RNA sensor, was demonstrated to be critical to mount a protective innate immune response to SARS-CoV infection [40].

Among the various IFN-stimulated genes (ISG) expressed during the course of a viral infection the IFN-induced transmembrane (IFITM) family of proteins, which are located in endosomes, have been implicated in the restriction of a broad spectrum of enveloped viruses, including coronaviruses [41]. In contrast, it was demonstrated that for HCoV-OC43, IFITM2 and IFITM3 enhance viral entry [42]. This unexpected finding challenged the notion that the function of IFITMs is limited to that of restriction factors, but they can actually positively regulate viral entry in certain circumstances [43]. In a more recent but similarly unexpected twist, it was shown that the ISG lymphocyte antigen 6 complex, locus E (LY6E), a known proviral factor for several viruses actually restricts infection of a range of coronaviruses including HCoV-229E, MERS-CoV, and SARS-CoV-2 [44]. Mechanistically, it is thought that LY6E functions by interfering with spike-mediated membrane fusion.

Recent advances in understanding the entry mechanisms of SARS-CoV-2

Unlike many of the so-called community-acquired respiratory (CAR) CoVs [9], SARS-CoV-2—as with the zoonotic SARS-CoV and MERS-CoV—is readily isolatable in cell culture [9], which has greatly facilitated the study of its entry process compared to the historical CAR CoVs. Vero E6 (primate kidney) and Calu-3 (human lung epithelial) cells have emerged as the standard cell lines for entry and infection studies, along with Caco-2 cells (human intestinal epithelial). These cell lines are used in part because of the expression of what has rapidly became established as the SARS-CoV-2 receptor (ACE2) [45-47], which has been extensively studied in the context of predicted "spill-over" from animal species [48].

The cell lines differ, however, in the expression of the proteases needed for coronavirus S fusion activation and this aspect of virus entry swiftly became a focus of early work on this newly emerging virus. Cell biological studies also rapidly incorporated sequence data showing the presence of a furin-like cleavage site at the S1/S2 interface—a site notably missing from SARS-CoV and related lineage B betacoronaviruses [49,50]. TMPRSS2 quickly became established as a critical activating protease [51] and can play a major role in directing the route of virus entry [52], although other TTSPs are also likely involved [53]. TTSPs are presumed to act at the fusion peptide-proximal S2' site. TMPRSS2 is expressed in Calu3 (and Caco-2) cells and data show that it can work effectively to activate virus entry in these cells following the priming event at the S1/S2 site. In contrast, Vero E6 cells (and engineered cells such as 293T/ACE2) do not express TMPRSS2 or related TTSPs, and so in this case virus entry is cathepsin-dependent—presumably occurring though endosomal compartments [54]. As such, the SARS-CoV-2 entry pathway broadly mirrors that of SARS-CoV, with the caveat that SARS-CoV S does not appear to have an equivalent "priming" requirement at S1/S2. SARS-CoV-2 entry also fits well with the "early" and "late" pathway model proposed by Gallagher. Entry specifically via CME has been proposed as a route of internalization of SARS-CoV-2 in 293T/ACE2 cells [55]; however, as discussed by the authors there are conflicting reports regarding specific endocytosis pathways for SARS-CoV and for coronaviruses in general, and so data need to interpreted cautiously. Another key set of findings comes from a CRISPR screen where *RAB7A* and genes involved in cholesterol biogenesis, among others, were identified as critical components of the SARS-CoV-2 entry pathway [56], indicating a key role for modified late endosomes—in this case using a human lung A549 cell line expressing ACE2. While many such key findings will continue to emerge, it is always important to remember that the specific route of SARS-CoV-2 entry may be highly dependent on the cell type being infected [57], based on the highly plastic nature of the viral spike protein.

Virus entry inhibitors as coronavirus therapeutics: application to COVID-19

Development of novel therapeutic strategies can often follow an understanding of virus entry pathways. As with many viruses, specific inhibition of the receptor interaction, along with less specific inhibition of membrane fusion events are logical points in virus entry to target and there are examples of each which have been studied for COVID-19 [58-61]. While these remain promising approaches, it is the inhibition of S protein cleavage-activation that is closest to therapeutic use in humans. Following the early demonstration that camostat mesylate (clinically approved in Japan for pancreatitis) inhibits TMPRSS2-mediated SARS-CoV-2 entry in Calu3 cells [45], this drug is now in clinical trials for COVID-19. Other proteases inhibitor possibilities include cathepsin and furin inhibitors, but the plasticity of S activation is in part due to redundancy in the activating protease and so overly specific drugs are likely to be unsuccessful; camostat and the related FDA-approved nafamostat [62] inhibit a range of TTSPs in addition to TMPRSS2 and so provide a solid platform for further drug discovery. Despite initial claims, chloroquine (which raises the low pH of endocytic compartments, and can effectively block virus entry), has not proven effective at treating COVID-19.

Inhibition of signaling events in virus entry provide another rich source of therapeutic discovery. Endosomes are becoming recognized as calcium stores and modulation of calcium channels such as TPC2 using tetranidine and associated channel-modulating PIKfyve inhibitors [54,63] have been shown to be inhibitory to SARS-CoV infection, as have a selection of calcium channel blockers. As we learn more about SARS-CoV-2 infection, more candidate therapeutics will almost certainly emerge that target virus entry.

Perspectives

As discussed in this article, the coronavirus S protein is remarkably plastic, allowing a plurality of options for entry into host cells that incorporate an overlapping triad of factors: receptor binding, protease cleavage, and ions enabling membrane fusion (Figure 1). In the context of an emerging virus such as

SARS-CoV-2, while changes in receptor binding and membrane fusion clearly play their part, it seems to be the priming and activation of S through host cell proteases that drives the process of virus evolution and adaptation. This is perhaps most strikingly demonstrated by the findings from many research labs that SARS-CoV-2 rapidly adapts to growth in Vero cells via small deletions in its S1/S2 priming site, with one outcome being a reduction of virus transmission in animal models [64]. While coronaviruses have always adapted to cell culture, and there are several examples where this has occurred by selecting alternative proteases for virus entry, the rapidity of selection seen for SARS-CoV-2 is unprecedented—and also in line with certain sequences derived from non-respiratory tissues from autopsies [65], leading to questions about the relevance of cell or tissue-type selection of novel variants along with utilization of their cognate proteases in the context of viral pathogenesis [66].

It is now three decades since the term Emerging Virus was coined by Stephen Morse. In his classic text [67], coronaviruses—while mentioned—are certainly not one of the featured pathogens. In the intervening time and especially during 2020, coronaviruses have emerged as our most prominent public health threat. While much remains to be learned about these viruses, it is hoped that the systematic analysis of their biology since being discovered almost 90 years ago will provide a solid foundation for the much-needed resurgence of coronavirus research that will undoubtedly occur in years to come.

Conflict of interest

The authors declare no conflict of interest

Acknowledgements

Work in the authors labs (GRW and SD) is funded by the National Institute of Health research grant R01AI35270 and Fast Grant, Mercatus Center. We would like to thank all members of the Daniel and Whittaker groups for continued helpful input.

References

[1] Schalk AF, Hawn MC: An Apparently New Respiratory Disease of Baby Chicks. *J Am Vet Med Assoc* 1931, 78:413-420.
[2] Beaudette FR, Hudson CB: Cultivation of the virus of infectious bronchitis. *J Am Vet Med Assoc* 1937, 90:51–58.
[3] Doyle LP, Hutchings LM: A transmissible gastroenteritis in pigs. *J Am Vet Med Assoc* 1946, 108:257-259.
[4] Gledhill AW, Andrewes CH: A Hepatitis Virus of Mice. *Br J Exp Pathol* 1951, 32:559-568.
[5] Tyrrell DA, Bynoe ML: Cultivation of a Novel Type of Common-Cold Virus in Organ Cultures. *Br Med J* 1965, 1:1467-1470.
[6] McIntosh K, Becker WB, Chanock RM: Growth in suckling-mouse brain of "IBV-like" viruses from patients with upper respiratory tract disease. *Proceedings of the National Academy of Sciences* 1967, 58:2268-2273.
[7] Almeida JD, Berry DM, Cunningham CH, Hamre D, Hofstad MS, Mallucci L, McIntosh K, Tyrrell DAJ: Virology: Coronaviruses. *Nature* 1968, 220:650-650.
[8] Perlman S: Another Decade, Another Coronavirus. *New England Journal of Medicine* 2020, 382:760-762.
[9] McIntosh K, Perlman S: 157 - Coronaviruses, Including Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*. Edited by Bennett JE, Dolin R, Blaser MJ: W.B. Saunders; 2015:1928-1936.e1922.
[10] van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B: Identification of a new human coronavirus. *Nat Med* 2004, 10:368-373.
[11] Woo PCY, Lau SKP, Chu C-m, Chan K-h, Tsoi H-w, Huang Y, Wong BHL, Poon RWS, Cai JJ, Luk W-k, et al.: Characterization and Complete Genome Sequence of a Novel Coronavirus, Coronavirus HKU1, from Patients with Pneumonia. *The Journal of Virology* 2005, 79:884-895.
[12] Millet JK, Jaimes JA, Whittaker GR: Molecular diversity of coronavirus host cell entry receptors. *FEMS Microbiol Rev* 2020. fuaa057, https://doi.org/10.1093/femsre/fuaa057.
[13] Simons K, Garoff H, Helenius A: How an Animal Virus Gets into and out of Its Host Cell. *Scientific American* 1982, 246:58-69.
[14] Sieczkarski SB, Whittaker GR: Dissecting virus entry via endocytosis. *Journal of General Virology* 2002, 83:1535-1545.
[15] Heald-Sargent T, Gallagher T: Ready, Set, Fuse! The Coronavirus Spike Protein and Acquisition of Fusion Competence. *Viruses* 2012, 4:557-580.

[16] * Madu IG, Roth SL, Belouzard S, Whittaker GR: Characterization of a Highly Conserved Domain within the Severe Acute Respiratory Syndrome Coronavirus Spike Protein S2 Domain with Characteristics of a Viral Fusion Peptide. *Journal of Virology* 2009, 83:7411-7421. *Based on sequence conservation across the Coronaviridae, the authors identified the bona fide fusion peptide (FP) of the SARS-CoV spike protein, which was a novel viral FP with an "internal" loop-like structure containing key hydrophobic residues, but also conserved charged residues.*
[17] * Belouzard S, Chu VC, Whittaker GR: Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proceedings of the National Academy of Sciences* 2009, 106:5871–5876. *Using IBV and as initial bioinformatic model, the authors demonstrated the unusual capacity of the coronavirus (SARS-CoV) spike protein to be proteolytically cleaved at two distinct sites, to drive the process of membrane fusion.*
[18] * Walls AC, Tortorici MA, Bosch B-J, Frenz B, Rottier PJM, DiMaio F, Rey FA, Veesler D: Cryo-electron microscopy structure of a coronavirus spike glycoprotein trimer. *Nature* 2016, 531:114-117. *This study broke through the limitations of x-ray crystallography with regard to the complex glycoprotein that comprises the coronavirus spike protein. The authors utilized cryo-EM to solve the structure of MHV spike and paved the way for a new era in our understanding of spike protein structure and function, and rationally designed coronavirus vaccines and therapeutic antibodies.*
[19] Li F: Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annu Rev Virol* 2016, 3:237-261.
[20] ** Burkard C, Verheije MH, Wicht O, van Kasteren SI, van Kuppeveld FJ, Haagmans BL, Pelkmans L, Rottier PJM, Bosch BJ, de Haan CAM: Coronavirus Cell Entry Occurs through the Endo-/Lysosomal Pathway in a Proteolysis-Dependent Manner. *PLoS Pathog* 2014, 10:e1004502. *In this highly important contribution to the field, the entry pathway of the model betacoronavirus MHV was investigated in great detail using a combination of screens based on RNAi-mediated knock down of endocytosis-associated proteins and pharmacological inhibitors. Fluorescence microscopy analyses revealed that MHV uses clathrin-mediated endocytosis and late endosome-to-lysosome trafficking for viral fusion to occur. Lysosomal proteases were found to be important to allow MHV fusion. Mutational analyses at the S2' cleavage site demonstrated the importance of protease recognition motifs in determining the entry pathway of coronaviruses.*
[21] Hantak MP, Qing E, Earnest JT, Gallagher T: Tetraspanins: Architects of viral entry and exit platforms. *J Virol* 2018. Mar 5;93(6):e01429-17.
[22] Lai AL, Millet JK, Daniel S, Freed JH, Whittaker GR: The SARS-CoV Fusion Peptide Forms an Extended Bipartite Fusion Platform that Perturbs Membrane Order in a Calcium-Dependent Manner. *Journal of Molecular Biology* 2017, 429:3875-3892.
[23] * Straus MR, Tang T, Lai AL, Flegel A, Bidon M, Freed JH, Daniel S, Whittaker GR: Ca(2+) Ions Promote Fusion of Middle East Respiratory Syndrome Coronavirus with Host Cells and Increase Infectivity. *J Virol* 2020, 94. Jun 16;94(13):e00426-20. *The role of calcium interaction with charged residues in the MERS-CoV spike protein fusion peptide was addressed in this paper, which showed differences in likely stoichiomery between this virus (with one predicted Ca^{2+} ion bound) and SARS-CoV (with two predicted Ca^{2+} ions bound).*
[24] Khelashvili G, Plante A, Doktorova M, Weinstein H: Ca2+-dependent mechanism of membrane insertion and destabilization by the SARS-CoV-2 fusion peptide. *bioRxiv* 2020. doi: https://doi.org/10.1101/2020.12.03.410472
[25] Belouzard S, Madu I, Whittaker GR: Elastase-mediated activation of the SARS coronavirus spike protein at discrete sites within the S2 domain. *Journal of Biological Chemistry* 2010, 285:22758-22763.

[26] Bonnin A, Danneels A, Dubuisson J, Goffard A, Belouzard S: HCoV-229E spike protein fusion activation by trypsin-like serine proteases is mediated by proteolytic processing in the S2' region. *Journal of General Virology* 2018. 99:908-912.
[27] Ng ML, Tan SH, See EE, Ooi EE, Ling AE: Early events of SARS coronavirus infection in vero cells. *Journal of Medical Virology* 2003, 71:323-331.
[28] Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, Bates P: Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. *Proceedings of the National Academy of Sciences* 2005, 102:11876-11881.
[29] Simmons G, Reeves JD, Rennekamp AJ, Amberg SM, Piefer AJ, Bates P: Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proceedings of the National Academy of Sciences* 2004, 101:4240-4245.
[30] Costello DA, Millet JK, Hsia C-Y, Whittaker GR, Daniel S: Single particle assay of coronavirus membrane fusion with proteinaceous receptor-embedded supported bilayers. *Biomaterials* 2013, 34:7895–7904.
[31] * Matsuyama S, Ujike M, Morikawa S, Tashiro M, Taguchi F: Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proceedings of the National Academy of Sciences* 2005, 102:12543-12547. *This paper first articulated the possibility that SARS-CoV can enter cells by two distinct pathways, which depends on the availability of proteases in the local cellular environmnet. It also highlights that proteases present from inflammatory cells in the lungs enhance cell infection and severe lung pathology.*
[32] Kam Y-W, Okumura Y, Kido H, Ng LFP, Bruzzone R, Altmeyer R: Cleavage of the SARS Coronavirus Spike Glycoprotein by Airway Proteases Enhances Virus Entry into Human Bronchial Epithelial Cells In Vitro. *PLoS ONE* 2009, 4:e7870.
[33] * Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T: A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J Virol* 2011, 85:873-882. *This key paper definitively locates ACE-2 receptor and TMPRSS2 in the host cell plasma membrane together as the necessary arrangement of these factors for the early entry pathway.*
[34] * Qing E, Hantak MP, Galpalli GG, Gallagher T: Evaluating MERS-CoV Entry Pathways. In *MERS Coronavirus: Methods and Protocols*. Edited by Vijay R: Springer US; 2020:9-20. *This chapter popularizes the dual entry pathway paradigm and provides clear protocols for assessing early or late entry pathways of coronoaviruses. It concisely organizes the virus-cell entry factors, entry inhibitors, and viral determinants that specify cell entry route.*
[35] **. Milewska A, Nowak P, Owczarek K, Szczepanski A, Zarebski M, Hoang A, Berniak K, Wojarski J, Zeglen S, Baster Z, et al.: Entry of Human Coronavirus NL63 into the Cell. *J Virol* 2018, 92. 92. e01933-17. *This work from the Pyrc group is a comprehensive and detailed study of the entry pathways of HCoV-NL63 in LLC-MK2 cells, as well as in ex vivo three dimensional trancheobrionchial tissues. The authors employed advanced fluorescence microscopy to analyze each step in host cell entry and reveal that HCoV-NL63 enters cells mainly through clathrin-mediated endocytosis, with a role for TMPRSS2 protease during early stages and actin remodelling during later steps.*
[36] Millet JK, Kien F, Cheung C-Y, Siu Y-L, Chan W-L, Li H, Leung H-L, Jaume M, Bruzzone R, Malik Peiris JS, et al.: Ezrin Interacts with the SARS Coronavirus Spike Protein and Restrains Infection at the Entry Stage. *PLoS ONE* 2012, 7:e49566.
[37] Marsh M, Bron R: SFV infection in CHO cells: cell-type specific restrictions to productive virus entry at the cell surface. *Journal of Cell Science* 1997, 110:95-103.

[38] Burkard C, Verheije MH, Haagmans BL, van Kuppeveld FJ, Rottier PJ, Bosch BJ, de Haan CA: ATP1A1-mediated Src signaling inhibits coronavirus entry into host cells. *J Virol* 2015 89:4434-3348.
[39] Sheahan T, Morrison TE, Funkhouser W, Uematsu S, Akira S, Baric RS, Heise MT: MyD88 is required for protection from lethal infection with a mouse-adapted SARS-CoV. *PLoS Pathog* 2008, 4:e1000240.
[40] Totura AL, Whitmore A, Agnihothram S, Schafer A, Katze MG, Heise MT, Baric RS: Toll-Like Receptor 3 Signaling via TRIF Contributes to a Protective Innate Immune Response to Severe Acute Respiratory Syndrome Coronavirus Infection. *mBio* 2015, 6:e00638-00615.
[41] Bertram S, Dijkman R, Habjan M, Heurich A, Gierer S, Glowacka I, Welsch K, Winkler M, Schneider H, Hofmann-Winkler H, et al.: TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. *Journal of Virology* 2013, 87:6150-6160.
[42] Zhao X, Guo F, Liu F, Cuconati A, Chang J, Block TM, Guo JT: Interferon induction of IFITM proteins promotes infection by human coronavirus OC43. *Proc Natl Acad Sci U S A* 2014, 111:6756-6761.
[43] Zhao X, Sehgal M, Hou Z, Cheng J, Shu S, Wu S, Guo F, Le Marchand SJ, Lin H, Chang J, et al.: Identification of Residues Controlling Restriction versus Enhancing Activities of IFITM Proteins on Entry of Human Coronaviruses. *J Virol* 2018, 92 92. e01535-17.
[44] ** Pfaender S, Mar KB, Michailidis E, Kratzel A, Boys IN, V'Kovski P, Fan W, Kelly JN, Hirt D, Ebert N, et al.: LY6E impairs coronavirus fusion and confers immune control of viral disease. *Nat Microbiol* 2020. 5:1330-1339 *In this groundbreaking collaborative study, the interferon-stimulated gene (ISG) product lymphocyte antigen 6 complex, locus E (LY6E) was found to restrict infection by several coronaviruses, including highly pathogenic ones such as MERS-CoV, SARS-CoV, and SARS-CoV-2. This was a surprise since LY6E was previously known to enhance infection by several other enveloped viruses such as influenza virus, flaviruses, and HIV-1. Functional analyses revealed that in the case of coronaviruses, LY6E impairs S-mediated viral fusion. In vivo mice studies with MHV showed that LY6E is an essential immune protective factor that plays a major role in controling coronavirus pathogenesis.*
[45] **. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, et al.: SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020, 181:271-280 e278. *This study illustrated the prompt attention given to the rapidly escalating public health problem of COVID-19 and was followed up by other key papers from the group. In this first study, the authors demonstrated the key roles of ACE2 and TMPRSS2 for entry of both SARS-CoV and SARS-CoV-2 into cells. Using a VSV-pseudotyping system, the authors screened several cell types, with Vero, Caco-2 and Calu-3 cells being the most permissive for SARS-CoV-2, along with Vero-TMPRSS2 cells. The protease inhibitor camostat was shown to significantly reduce SARS-CoV-2 infection of Calu-3 cells, as measured by qPCR, paving the way for clinical trials in humans*.
[46] **. Shang J, Wan Y, Luo C, Ye G, Geng Q, Auerbach A, Li F: Cell entry mechanisms of SARS-CoV-2. *Proceedings of the National Academy of Sciences* 2020 117: 11727-11734. *Another early study on SARS-CoV-2 entry used retrovirus pseudoparticles and biochemical assays to document key aspects of spike conformational flexibity and ACE2 binding, and provided functional evidence for furin-mediated spike priming.*
[47] ** Letko M, Marzi A, Munster V: Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nature Microbiology* 2020, 5:562-569. *This study approached SARS-CoV-2 from the persective of prediction of zoonitic spread and the use of human*

receptors. They screened lineage B coronaviruses in high throughput using a VSV-pseudotyping system to show that host protease processing is a significant barrier to "spillover", and confirmed that hACE2 is the receptor for SARS-CoV-2.
[48] Damas J, Hughes GM, Keough KC, Painter CA, Persky NS, Corbo M, Hiller M, Koepfli KP, Pfenning AR, Zhao H, et al.: 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, 117:22311-22322.
[49] Jaimes JA, Andre NM, Chappie JS, Millet JK, Whittaker GR: Phylogenetic Analysis and Structural Modeling of SARS-CoV-2 Spike Protein Reveals an Evolutionary Distinct and Proteolytically Sensitive Activation Loop. *J Mol Biol* 2020 432:3309-3325.
[50] * Coutard B, Valle C, de Lamballerie X, Canard B, Seidah NG, Decroly E: The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Res* 2020, 176:104742. *With the release of of the SARS-CoV-2 genomic sequnce, many research groups rapidly recognized the presence of a novel four amino acid insert at the critical spike S1/S2 priming positon, which contained an additional two arginine residues to allow recognition by the ubiquitous protease furin. The authors were the first to document this finding, which integrated their expertise with proteases to include the possibility of spike priming by other members of the furin-like proprotein convertase (PC) family.*
[51] Matsuyama S, Nao N, Shirato K, Kawase M, Saito S, Takayama I, Nagata N, Sekizuka T, Katoh H, Kato F, et al.: Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. *Proc Natl Acad Sci U S A* 2020, 117:7001-7003.
[52] Ou T, Mou H, Zhang L, Ojha A, Choe H, Farzan M: Hydroxychloroquine-mediated inhibition of SARS-CoV-2 entry is attenuated by TMPRSS2. *PLoS Pathog* 2021, 17:e1009212.
[53] * Zang R, Gomez Castro MF, McCune BT, Zeng Q, Rothlauf PW, Sonnek NM, Liu Z, Brulois KF, Wang X, Greenberg HB, et al.: TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. *Sci Immunol* 2020,5: eabc3582. *While TMPRSS2 has risen to prominence as a critical protease for entry into respiratory epithelial cells, it is likely that other TTSPs can also serve this function. The study from Zang et al demonstrates that the related protease TMPRSS4 can also promote SARS-CoV-2 infection, in this case in ACE2+ mature enterocytes in human small intestinal enteroids. The authors also demonstrate inactivation of virus by simulated human colonic fluid.*
[54] ** Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, Guo L, Guo R, Chen T, Hu J, et al.: Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun* 2020, 11:1620. *This manuscript focuses on 293/hACE2 cells and documents SARS-CoV-2 entry using a lentiviral pseudotyping system. As such, entry is mainly occurring via the endocytic route—with key roles for the late endosomal components PIKfyve, TPC2 and cathepsin L. The authors also show limited cross neutralization with SARS-CoV.*
[55] Bayati A, Kumar R, Francis V, McPherson PS: SARS-CoV-2 infects cells following viral entry via clathrin-mediated endocytosis. *J Biol Chem* 2021:100306.
[56] Daniloski Z, Jordan TX, Wessels HH, Hoagland DA, Kasela S, Legut M, Maniatis S, Mimitou EP, Lu L, Geller E, et al.: Identification of Required Host Factors for SARS-CoV-2 Infection in Human Cells. *Cell* 2020.
[57] *. Tang T, Jaimes JA, Bidon MK, Straus MR, Daniel S, Whittaker GR: Proteolytic Activation of SARS-CoV-2 Spike at the S1/S2 Boundary: Potential Role of Proteases beyond Furin. *ACS Infect Dis* 2021. Jan 12;acsinfecdis.0c00701. *This work highlights the importance of SARS-CoV-2 spike protein "priming" for viral entry. Inhibition of cleavage by a proprotein convertase inhibitor led to greatly increased infectivity of Vero E6 cells, but not of Calu-3 cells and indicated that other furin-related*

proteases may be acting to cleave at S1/S2. This work also raises the possibility that SARS-CoV-2 could have originated from an unknown ancestor bat virus with a robust furin cleavage site.
[58] Outlaw VK, Bovier FT, Mears MC, Cajimat MN, Zhu Y, Lin MJ, Addetia A, Lieberman NAP, Peddu V, Xie X, et al.: Inhibition of Coronavirus Entry In Vitro and Ex Vivo by a Lipid-Conjugated Peptide Derived from the SARS-CoV-2 Spike Glycoprotein HRC Domain. *mBio* 2020, 11.01935-20.
[59] Cao L, Goreshnik I, Coventry B, Case JB, Miller L, Kozodoy L, Chen RE, Carter L, Walls AC, Park YJ, et al.: De novo design of picomolar SARS-CoV-2 miniprotein inhibitors. *Science* 2020, 370:426-431.
[60] Walser M, Rothenberger S, Hurdiss DL, Schlegel A, Calabro V, Fontaine S, Villemagne D, Paladino M, Hospodarsch T, Neculcea A, et al.: Highly potent anti-SARS-CoV-2 multi-DARPin therapeutic candidates. *bioRxiv* 2020 doi: https://doi.org/10.1101/2020.08.25.256339 .
[61] Tang T, Bidon M, Jaimes JA, Whittaker GR, Daniel S: Coronavirus membrane fusion mechanism offers a potential target for antiviral development. *Antiviral Res* 2020, 178:104792.
[62] Yamamoto M, Kiso M, Sakai-Tagawa Y, Iwatsuki-Horimoto K, Imai M, Takeda M, Kinoshita N, Ohmagari N, Gohda J, Semba K, et al.: 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* 2020, 12 Jun 10;12(6):629. doi: 10.3390/v12060629.
[63] Galindo I, Garaigorta U, Lasala F, Cuesta-Geijo MA, Bueno P, Gil C, Delgado R, Gastaminza P, Alonso C: Antiviral drugs targeting endosomal membrane proteins inhibit distant animal and human pathogenic viruses. *Antiviral Res* 2020:104990.
[64] Johnson BA, Xie X, Bailey AL, Kalveram B, Lokugamage KG, Muruato A, Zou J, Zhang X, Juelich T, Smith JK, et al.: Loss of furin cleavage site attenuates SARS-CoV-2 pathogenesis. *Nature* 2021. Jan 25. doi: 10.1038/s41586-021-03237-4.
[65] ** Peacock TP, Goldhill DH, Zhou J, Baillon L, Frise R, Swann OC, Kugathasan R, Penn R, Brown JC, Sanchez-David RY, et al.: The furin cleavage site of SARS-CoV-2 spike protein is a key determinant for transmission due to enhanced replication in airway cells. *bioRxiv* 2020.
doi: https://doi.org/10.1101/2020.09.30.318311 *One intriguing aspect of SARS-CoV-2 lies in the apparent genetic "instability" of the spike S1/S2 region and the rapid structural loss of the "priming" loop, which may factor heavily into the evolution of this zoonotic virus. Several groups have observed this and published the effects on virus infection and spread. This recent study shows the impact of S1/S2 deletions on transmission model in ferrets and extends to human autopsy samples, which have a low level of equivalent mutations in certain non-respiratory tissues.*
[66] Fuentes-Prior P: Priming of SARS-CoV-2 S protein by several membrane-bound serine proteinases could explain enhanced viral infectivity and systemic COVID-19 infection. *J Biol Chem* 2020. *J Biol Chem* 2020. Dec 2;jbc.REV120.015980
[67] Morse SS: *Emerging Viruses*: Oxford University Press; 1996.

Figures

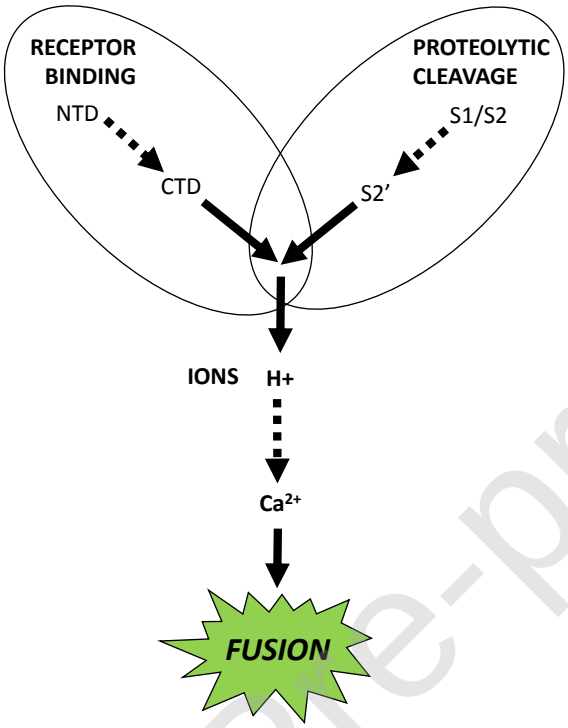

Figure 1. A coronavirus entry triad. Coronavirus host cell entry is determined by a triad of factors: receptor binding and protease cleavage work in concert with the ionic environment of the cell/sub-cellular compartment to facilitate membrane fusion. Coronavirus spike proteins are extremely "plastic" and can respond to a variety of cues encountered during virion entry enabling the use of either the "early" or "late" pathway, depending on the host cell type and microenvironmental conditions. NTD = N-terminal domain of S1, CTD = C-terminal domain of S1.