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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.

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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 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; that is, 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].

Because of 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••,46••,47••], which has been extensively studied in the context of predicted 'spillover' 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 293 T/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 be 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

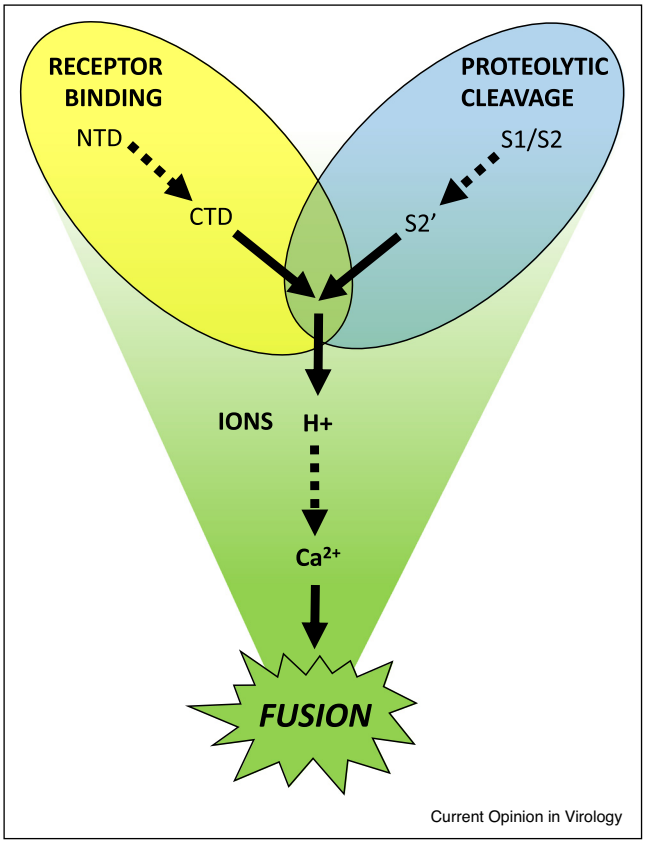
block virus entry), has not proven effective at treating COVID-19.

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].

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/subcellular 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.

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 statement

Nothing declared.

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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.

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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.

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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.

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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.

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