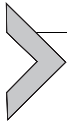

CHAPTER FOUR

Structural insights into coronavirus entry

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Abstract

Coronaviruses (CoVs) have caused outbreaks of deadly pneumonia in humans since the beginning of the 21st century. The severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in 2002 and was responsible for an epidemic that spread to five continents with a fatality rate of 10% before being contained in 2003 (with additional cases reported in 2004). The Middle-East respiratory syndrome coronavirus (MERS-CoV) emerged in the Arabian Peninsula in 2012 and has caused recurrent outbreaks in humans with a fatality rate of 35%. SARS-CoV and MERS-CoV are zoonotic viruses that crossed the species barrier using bats/palm civets and dromedary camels, respectively. No specific treatments or vaccines have been approved against any of the six human coronaviruses, highlighting the need to investigate the principles governing viral entry and cross-species transmission as well as to prepare for zoonotic outbreaks which are likely to occur due to the large reservoir of CoVs found in mammals and birds. Here, we review our understanding of the infection mechanism used by coronaviruses derived from recent structural and biochemical studies.

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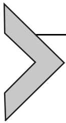
Abbreviations

3D	three dimensional
9-O-Ac-Sia	5-*N*-acetyl-9-*O*-acetyl-sialosides
ACE2	angiotensin-converting enzyme 2
APN	amino-peptidase N
CoV	Coronavirus
cryoEM	cryo-electron microscopy
DPP4	dipeptidyl peptidase 4
Fab	antigen-binding fragment of an antibody
MERS-CoV	Middle-East respiratory syndrome coronavirus
MHV	mouse hepatitis virus
PDCoV	porcine δ-CoV
PEDV	porcine epidemic diarrhea virus
SARS-CoV	severe acute respiratory syndrome coronavirus
TGEV	transmissible gastroenteritis virus



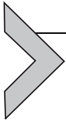
1. Introduction

Coronaviruses (CoVs) are enveloped viruses with a positive sense RNA genome, that belong to the subfamily *Coronavirinae* within the family *Coronaviridae*, which is part of the Nidovirales order. They are classified in four genera (α, β, γ, and δ) and four lineages are recognized whithin the β-CoV genus (A, B, C and D). CoVs cause a variety of respiratory and enteric diseases in mammalian and avian species. Until recently, CoVs were considered to be pathogens with a largely veterinary relevance but with limited impact on human health. However, outbreaks of severe acute respiratory syndrome (SARS) in 2002–2004 and of Middle-East respiratory syndrome (MERS) starting in 2012, with fatality rates of 10% and 35%, respectively, led CoVs to be recognized as zoonotic threats with pandemic potential. Four other CoVs are endemic in the human population and cause up to 30% of mild respiratory tract infections as well as occasional severe disease in young children, the elderly or immunocompromised individuals (Isaacs et al., 1983; Su et al., 2016). These viruses are HCoV-NL63 and HCoV-229E (α-CoVs) as well as HCoV-OC43 and HCoV-HKU1 (β-CoVs). Numerous SARS-CoV and MERS-CoV-like viruses currently circulate in bats and dromedaries making outbreaks of highly pathogenic human CoVs a global health threat (Ge et al., 2013; Haagmans et al., 2014; Hu et al., 2017; Menachery et al., 2015, 2016; Sabir et al., 2016).

The CoV virion contains at least four structural proteins: spike (S), envelope (E), membrane (M) and nucleocapsid (N). In contrast to other β-CoV lineages, lineage A CoVs also encode a hemagglutinin–esterase which serves as receptor-destroying enzyme to facilitate release of viral progeny from infected cells and escape from attachment to non-permissive host cells or decoys (Bakkers et al., 2017, 2016). S is a class 1 viral fusion protein that promotes host attachment and fusion of the viral and cellular membranes during entry (Bosch et al., 2003). As a consequence, S determines host range and cell tropism. S is also the main target of neutralizing antibodies elicited during infection and the focus of vaccine design. S is trimeric and each protomer is synthesized as a single polypeptide chain of 1100–1600 residues, depending on the CoV species. For many CoVs, S is processed by host proteases to generate two functional subunits, designated S_1 and S_2, which remain non-covalently bound in the prefusion conformation (Bosch et al., 2003). The S_1 subunit comprises the apex of the S trimer, including the receptor-binding domains, and stabilizes the prefusion state of the S_2 fusion machinery, which is anchored in the viral membrane. For all CoVs, S is further cleaved by host proteases at the so-called S_2' site located immediately upstream of the fusion peptide. This cleavage has been proposed to activate the protein for membrane fusion *via* large-scale, irreversible conformational changes (Heald-Sargent and Gallagher, 2012; Millet and Whittaker, 2015).

Although several class 1 viral fusion proteins have been extensively studied, CoV S proteins have proven reluctant to structural characterization until recently. Structural studies were largely limited to X-ray crystallographic analysis of isolated receptor-binding domains in complex with viral receptor ectodomains or neutralizing antibodies (Li et al., 2005a; Lu et al., 2013; Peng et al., 2011; Prabakaran et al., 2006; Reguera et al., 2012; Wang et al., 2013; Wong et al., 2017; Wu et al., 2009; Yu et al., 2015) and of the S_2 postfusion core (Duquerroy et al., 2005; Gao et al., 2013; Supekar et al., 2004; Xu et al., 2004a, b; Zheng et al., 2006) with the exception of two low-resolution electron microscopy reports (Beniac et al., 2006, 2007). In the past few years, however, technical advances in single-particle cryo-electron microscopy (cryoEM) (Bai et al., 2013; Brilot et al., 2012; Campbell et al., 2012, 2015; Li et al., 2013; Punjani et al., 2017; Scheres, 2012) together with the implementation of strategies for the stabilization of CoV S proteins in prefusion conformation (Pallesen et al., 2017; Walls et al., 2017a) led to a surge of structural data for multiple S ectodomain trimers. We review here our current understanding of the mechanism used by CoVs to infect host

cells based on recent structural and biochemical studies of S glycoprotein ectodomains in prefusion and postfusion states as well as complexes with known receptors or neutralizing antibodies.



2. Prefusion S architecture

CryoEM studies of the S glycoproteins of mouse hepatitis virus (MHV) and HKU1 led to the first structures at high-enough resolution to obtain an atomic model of the prefusion state (Kirchdoerfer et al., 2016; Walls et al., 2016a). These structures revealed that prefusion S ectodomains are ~160 Å-long trimers with a triangular cross-section (Fig. 1A and B).

The S_1 subunit adopts a "V" shaped architecture for β and γ CoVs (Gui et al., 2017; Kirchdoerfer et al., 2016; Shang et al., 2018a; Tortorici et al., 2019; Walls et al., 2016a; Yuan et al., 2017) (Fig. 1C), or a square-shaped organization for α- and δ-CoVs (Shang et al., 2018b; Walls et al., 2016b; Xiong et al., 2018). The S_1 subunit folds as β-rich domains designated A, B, C, D. Several α-CoVs harbor a likely duplication of their domain A at the N-terminus of the S glycoprotein (Hulswit et al., 2016; Walls et al., 2016b). This additional domain, designated domain 0, was visualized in the NL63 S structure and hypothesized to interact with heparan sulfate present at the host cell surface during viral entry (Milewska et al., 2014; Walls et al., 2016b). Domain A and domain 0 adopt a galectin-like β-sandwich fold conserved across all CoV genera (Kirchdoerfer et al., 2016; Peng et al., 2011, 2012; Walls et al., 2016a) (Fig. 1D). Domain B, which shows the highest sequence variability within CoV S_1 subunits, has a markedly different architecture between α-, β-, γ- and δ-CoVs. B domains of β-CoVs contain a β-sheet core subdomain decorated with a highly variable external subdomain mediating receptor engagement (Chen et al., 2013; Kirchdoerfer et al., 2016; Li et al., 2005b; Lu et al., 2013; Tortorici et al., 2019; Walls et al., 2016a; Wang et al., 2013) (Fig. 1E). B domains of α-, γ- and δ-CoV form a β-sandwich decorated with loops mediating receptor attachment (Reguera et al., 2012; Shang et al., 2018a, b; Walls et al., 2016b; Wong et al., 2017; Xiong et al., 2018). In the context of the S trimer, β/γ CoV B domains interact with the A and B domains of another protomer, whereas they pack against the A domain of the same protomer in α/δ-CoVs (Shang et al., 2018a; Walls et al., 2016a, b; Xiong et al., 2018).

The S_2 subunit, which is more conserved than S_1, comprises the fusion machinery and connects to the viral membrane. It is assembled from a large

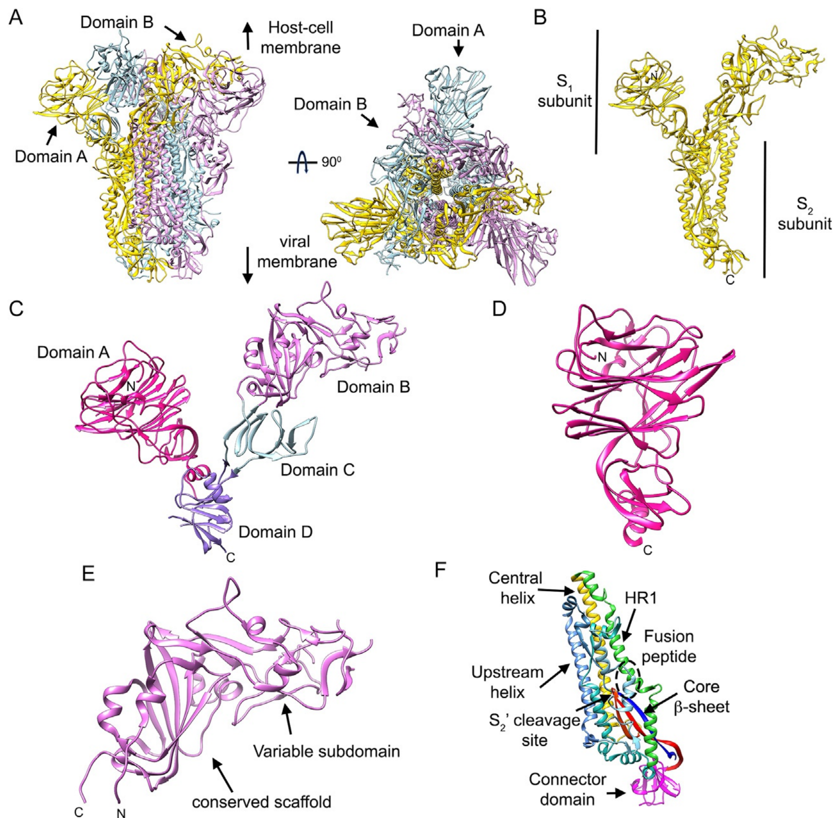
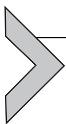


Fig. 1 CryoEM structure of the apo-HCoV-OC43 S glycoprotein. (A) Ribbon diagrams of the apo HCoV-OC43 S ectodomain trimer (PDB: 6OHW) in two orthogonal orientations, from the side (left) and from the top, looking towards the viral membrane (right). (B) Side view of one S protomer. (C) Ribbon diagram of the HCoV-OC43 S_1 subunit. (D–E) Close-up view of HCoV-OC43 domain A (D) and domain B (E). (F) Ribbon diagram of the HCoV-OC43 S_2 subunit in the prefusion conformation. The N- and C-termini are labeled in panels (B–E).

number of α-helices, an antiparallel core β-sheet, a β-rich connector domain and a stem helix leading to the heptad-repeat 2 (HR2) and the transmembrane region (Fig. 1F) (Gui et al., 2017; Kirchdoerfer et al., 2016, 2018; Pallesen et al., 2017; Shang et al., 2018a, b; Tortorici et al., 2019; Walls et al., 2016a, b, 2017b; Xiong et al., 2018; Yuan et al., 2017). Key S_2 features facilitating virus-cell fusion include the fusion peptide, two heptad repeat regions (named HR1 and HR2) and the transmembrane domain. In the prefusion S conformation, a central helix stretches along the threefold axis, perpendicular to the viral membrane, and is located downstream the HR1

motif, which folds as four consecutive α-helices (Kirchdoerfer et al., 2016; Walls et al., 2016a). Moreover, an upstream helix runs parallel to and is zipped against the central helix *via* hydrophobic contacts (Fig. 1F). The CoV S_2 subunit shares similarity with the pneumovirus/paramyxovirus F proteins—including a comparable 3D organization of the core β-sheet, the upstream helix and the central helix—suggesting an evolutionary relatedness between the viral fusion proteins of these different viruses and a conservation of their fusion mechanism (McLellan et al., 2013; Walls et al., 2016a, 2017b; Wong et al., 2016; Xu et al., 2015; Yin et al., 2006).

A conserved tryptophan-rich segment (Y(V/I)KWPW(Y/W)VWL) directly preceding the CoV S transmembrane region is crucial for proper trimerization. This segment is also required functionally for formation of a fusion pore (Schroth-Diez et al., 2000). Furthermore, transmembrane domain interactions within and possibly between S trimers have been proposed to be essential to complete the membrane fusion process (Schroth-Diez et al., 2000). The transmembrane domain is followed by an intraviral/cytoplasmic tail of variable length (36–46 residues) depending on the coronavirus species, which contains a palmitoylated cysteine-rich region (of about 18–24 residues with 7–10 cysteines) and a variable C-terminal end (Thorp et al., 2006). The cytoplasmic tail is involved in assembly, intracellular transport, cell-surface expression and cell-cell fusion (Bos et al., 1995; Bosch et al., 2005; Chang et al., 2000; Lontok et al., 2004; Petit et al., 2005; Ye et al., 2004; Youn et al., 2005). Currently, no structural information is available for any CoV full-length S, hindering our understanding of the influence of the transmembrane and cytoplasmic domains on the conformation of exposed antigenic sites, as previously studied for HIV-1 envelope (Chen et al., 2015;Dev et al., 2016).



3. Diversity of CoV receptors and entry mechanisms

CoV entry into susceptible cells is a complex process that requires the concerted action of receptor-binding and proteolytic processing of the S protein to promote virus-cell fusion (Heald-Sargent and Gallagher, 2012; Millet and Whittaker, 2015). Domain 0, domain A and/or domain B can act as receptor-binding domains and both attachment and entry receptors have been described, depending on the CoV species.

Lineage A β-CoVs attach *via* their S domain A to 5-*N*-acetyl-9-*O*-acetyl-sialosides (9-*O*-Ac-Sia) found on glycoproteins and glycolipids at the

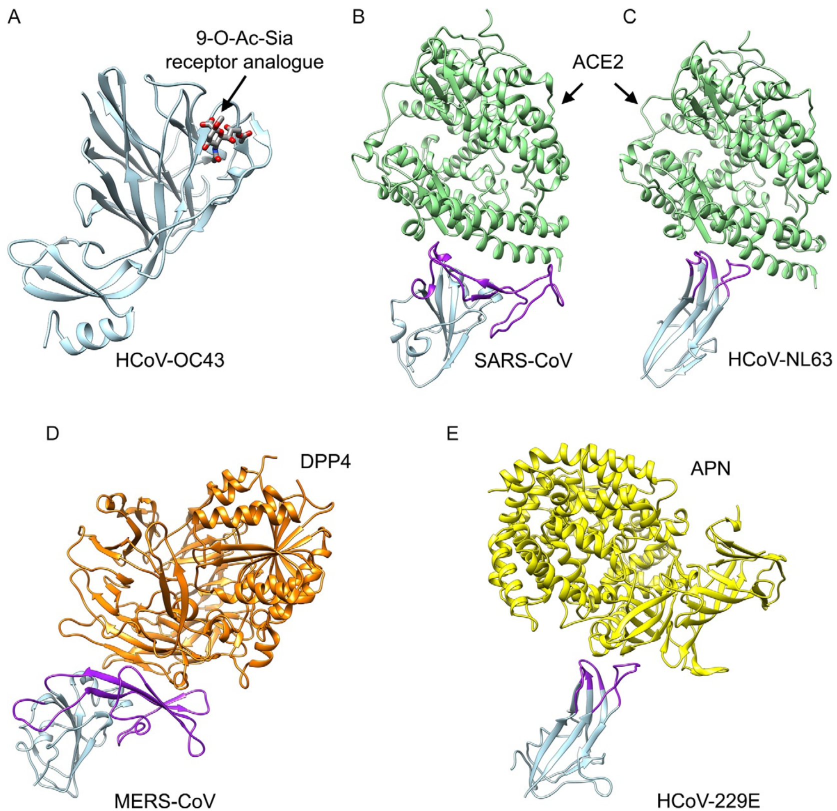


Fig. 2 Structural studies of human CoV attachment to host receptors. (A–E), Ribbon diagrams of the complex between domain A of HCoV-OC43 S with a 9-*O*-Ac-Sia receptor analogue ((A) PDB: 6NZK), or the domain B of SARS-CoV S with ACE2 ((B) PDB: 2AJF), HCoV-NL63 S with ACE2 ((C) PDB: 3KBH), MERS-CoV S with DPP4 ((D) PDB: 4L72) and HCoV-229E S with APN ((E) PDB: 6ATK). In panels (B–E), each domain B is rendered in light blue and the receptor binding-motifs are colored purple.

host cell surface to promote entry into susceptible cells (Vlasak et al., 1988). These include human CoVs OC43 and HKU1, bovine CoV (BCoV) and porcine hemagglutinating encephalomyelitis virus. We recently identified and visualized by cryoEM the HCoV-OC43 S sialoside-binding site, which is located in a groove at the surface of domain A (Fig. 2A) (Hulswit et al., 2019; Tortorici et al., 2019). This site is conserved in all other CoVs known to attach to 9-*O*-Ac-Sia (β-CoVs, lineage A) and shares architectural similarity with the ligand-binding pockets of CoV hemagglutinin-esterases and influenza virus C/D hemagglutinin-esterase-fusion glycoproteins, highlighting common structural principles of recognition (Bakkers et al., 2017, 2016;

Hulswit et al., 2019; Rosenthal et al., 1998; Tortorici et al., 2019). The current consensus in the field is that HCoV-OC43 only utilizes 9-*O*-Ac-sialosides as host receptors. In line with this statement, ligand-interacting residues were shown to be essential for S-mediated viral entry (Hulswit et al., 2019; Tortorici et al., 2019) and 9-*O*-Ac-Sia depletion from target cells resulted in severe decrease in virus infectivity (Krempl et al., 1995; Vlasak et al., 1988). Free 9-*O*-Ac-Sia, however, did not trigger S conformational changes associated with membrane fusion (Tortorici et al., 2019). This observation contrasts with data for SARS-CoV S, for which addition of the human angiotensin-converting enzyme 2 (ACE2) ectodomain (the proteinaceous receptor) promoted S refolding to the postfusion state (Song et al., 2018; Walls et al., 2019). These findings suggested that either 9-*O*-Ac-Sia-containing receptors differ from proteinaceous receptors in their mode of action, or that an interaction with a yet unidentified proteinaceous receptor is required before or after virus internalization for HCoV-OC43 entry into target cells.

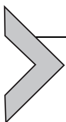
The sialoside-binding site identified in HCoV-OC43 S is not conserved among CoVs which are also known to interact with sialoglycans to initiate host cell infection but are outside of the lineage A of β-CoVs, such as MERS-CoV (β-CoV, lineage C) or infectious bronchitis virus (IBV, δ-CoV) (Li et al., 2017; Wickramasinghe et al., 2011). Some α-CoVs such as transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) use domain 0 to attach to sialoglycans, presumably to increase virus concentration at the cell surface and enhance subsequent attachment to proteinaceous receptors (Liu et al., 2015; Schwegmann-Wessels et al., 2003). Carbohydrate binding *via* this domain has been proposed to be a determinant of the TGEV enteric tropism since loss of domain 0 appears to correlate with a loss of enteric tropism for porcine respiratory coronavirus (PRCoV), the latter virus being a naturally occurring TGEV variant (Krempl et al., 1997).

CoVs exploit a limited variety of proteinaceous receptors compared with the large number and diversity of viral species. All CoVs known to engage proteinaceous receptors do so using domain B with the exception of MHV, which binds CEACAM1a using domain A (Dveksler et al., 1991; Peng et al., 2011; Williams et al., 1991). Remarkably, viruses from different genera, such as HCoV-NL63 (α-CoV) and SARS-CoV (β-CoV), can recognize the same region of ACE2 (entry receptor) using structurally distinct B domains (Hofmann et al., 2005; Li et al., 2005a, 2003; Wu et al., 2009). Many α-CoVs, including HCoV-229E, TGEV and PRCV, as well as porcine δ-CoV (PDCoV) utilize aminopeptidase N (APN) as entry receptor (Delmas et al., 1992; Delmas et al., 1993; Li et al., 2018; Reguera et al., 2012;

Wong et al., 2017; Yeager et al., 1992) whereas MERS-CoV uses dipeptidyl peptidase 4 (DPP4) (Lu et al., 2013; Raj et al., 2013; Wang et al., 2013). Crystal structures of SARS-CoV, HCoV-NL63, MERS-CoV, HCoV-229E B domains in complex with their cognate receptors provided atomic details of the interacting-interface and identified key residues for cross-species transmission and infection (Fig. 2) (Li et al., 2005a; Lu et al., 2013; Wang et al., 2013; Wong et al., 2017; Wu et al., 2009). This information will be useful to guide the development of therapeutics and vaccines against human CoVs.

Recent cryoEM studies revealed that MERS-CoV S and SARS-CoV S can adopt open and closed conformations in which the receptor binding site of domain B is exposed and occluded, respectively (Gui et al., 2017; Kirchdoerfer et al., 2018; Pallesen et al., 2017; Song et al., 2018; Walls et al., 2019; Yuan et al., 2017). In contrast, the MHV, HCoV-NL63, HCoV-HKU1, PDCoV, IBV and HCoV-OC43 S glycoproteins appear to only adopt a closed conformation (Kirchdoerfer et al., 2016; Shang et al., 2018a, b; Tortorici et al., 2019; Walls et al., 2016a, b; Xiong et al., 2018) and unknown trigger(s), besides proteolytic activation, might be necessary for these viruses to expose their receptor-binding motifs for recognition to occur. These findings suggest that CoVs have evolved a fine-tuned mechanism to balance masking of the receptor-binding motifs, putatively to avoid neutralization by the host humoral immune response, and their necessary exposure to enable receptor recognition and infection of host cells (Walls et al., 2016b, 2019; Wong et al., 2017).

Upon host recognition, CoVs are internalized *via* receptor-mediated clathrin-dependent, caveolin-dependent or other uptake pathways (Burkard et al., 2014; Eifart et al., 2007; Inoue et al., 2007; Nomura et al., 2004). For instance, both clathrin-dependent and clathrin/caveolae-independent entry pathways have been reported for SARS-CoV (Inoue et al., 2007; Wang et al., 2008). Feline infectious peritonitis virus was suggested to enter host cells *via* a clathrin/caveolin-independent internalization route (Regan et al., 2008; Van Hamme et al., 2008) whereas a caveolin-dependent endocytic uptake has been suggested for HCoV-229E and HCoV-OC43 (Nomura et al., 2004; Owczarek et al., 2018).



4. S proteolytic cleavage

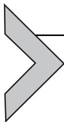
Several reports have demonstrated the key role of proteolytic processing of CoV S for cell-cell fusion activity and/or virus entry into host cells using experiments of inhibition of intracellular proteases

(Burkard et al., 2014; Frana et al., 1985; Simmons et al., 2005; Yamada and Liu, 2009) and/or substitutions of residues at the S_1/S_2 or S_2' cleavage sites (Belouzard et al., 2009; Li et al., 2015; Millet and Whittaker, 2014; Wicht et al., 2014; Yang et al., 2015).

Prior to and/or after uptake of the virion by a host cell, the S protein is proteolytically processed by host proteases at one or two cleavage sites and both receptor-binding and proteolytic processing act in synergy to induce large-scale S conformational changes promoting CoV entry. One of the cleavage sites is located at the boundary between the S_1 and S_2 subunits (S_1/S_2 cleavage site), whereas the other is located immediately upstream of the fusion peptide (S_2' cleavage site), reviewed in (Millet and Whittaker, 2015). Cleavage at the S_1/S_2 site can occur upon viral egress, such as for MHV (Frana et al., 1985), or upon encounter with a target cell, such as for SARS-CoV (Belouzard et al., 2009; Bosch et al., 2008; Shulla et al., 2011), to yield two non-covalently associated subunits. This first cleavage event, along with binding to the host receptor, promotes further cleavage at the S_2' site for SARS-CoV S (Belouzard et al., 2009) and MERS-CoV S (Millet and Whittaker, 2014;Park et al., 2016). Proteolysis at the conserved S_2' site is essential for fusion activation of all characterized CoV S proteins, and it can occur at the host membrane or in internal cellular compartments of the target cell (Belouzard et al., 2009; Burkard et al., 2014; Millet and Whittaker, 2015; Park et al., 2016).

Cleavage at the MERS-CoV S_1/S_2 site by furin during viral egress enables subsequent exposure of the S_2' site upon binding to the host receptor and a second cleavage step by serine proteases anchored in the membrane of the target cells, eventually leading to fusion at the cytoplasmic membrane (early entry) (Park et al., 2016). Conversely, MERS-CoV budding with uncleaved S glycoproteins traffic to the endosomes of target cells where cathepsin L or other proteases promote membrane fusion (late entry) (Park et al., 2016). The former mechanism has been proposed to be the route of MERS-CoV entry into cell types relevant to lung infection, and therefore a significant determinant of MERS-CoV virulence (Park et al., 2016). Moreover, tetraspanin CD9 has been implicated in clustering DPP4 and transmembrane serine proteases to promote early entry of MERS-CoV (Earnest et al., 2017, 2015). PEDV, which replicates in the epithelial cells of the small intestine, undergoes S proteolytic activation by trypsin, which is highly abundant in the intestinal lumen (Wicht et al., 2014). The critical importance of cleavage at the S_1/S_2 site was also exemplified in studies with the MERS-CoV-related bat coronavirus HKU4. Although HKU4

S recognizes human DPP4, *in vitro* infectivity assays revealed that entry into human cells required addition of exogenous trypsin, suggesting proteolytic activation of this bat virus did not occur in human cells (Wang et al., 2014). In line with these findings, various DPP4 mammalian orthologues, with variable binding affinities for the MERS-CoV S receptor-binding domain, were shown to support virus or pseudovirus entry into target cells in the presence of an activating protease (Barlan et al., 2014). These results collectively illustrate how specific S proteolytic cleavage participates in determining the intracellular site of fusion and also viral tropism and pathogenesis of CoVs. Therefore, the zoonotic potential of CoVs is not only determined by receptor engagement, but also by proteolytic processing of the S protein required for fusion activation.



5. Mechanism of fusion activation

We showed that *in vitro* trypsin cleavage of MHV, SARS-CoV and MERS-CoV S, under limited proteolysis conditions, recapitulated fusion activation by inducing the pre-to postfusion transition (Walls et al., 2017b). The cryoEM structure of the MHV S_2 subunit ectodomain trimer revealed that membrane fusion involves large-scale S conformational changes that are reminiscent of the ones described for other class 1 fusion proteins, including the pneumovirus/paramyxovirus F glycoproteins (Fig. 3) (McLellan et al., 2011; Swanson et al., 2010, 2011; Walls et al., 2016b; Yin et al., 2005). These experiments also demonstrated that (i) the S_1 subunits stabilize the S_2 fusion machinery in the spring-loaded, metastable prefusion state before initiation of infection; and (ii) postfusion S is the ground state of the fusion reaction. Similarly to the organization of influenza virus hemagglutinins (Xiong et al., 2013), domain B interacts with the HR1-central helix hairpin in prefusion closed S structures likely to stabilize S_2 in the spring-loaded prefusion state. This interaction appears to coordinate receptor engagement with fusion. Upon receptor binding and proteolytic cleavage at the S_1/S_2 and S_2' sites, the S_1 crown is likely shed (as observed for MERS-CoV S by Yuan et al., 2017) to facilitate a conformational change of S_2, which involves projection of the fusion peptide to a distance of ~100 Å and its insertion into the target membrane (Fig. 3) (Walls et al., 2016a, 2017b). The free energy released upon S_2 refolding from the prefusion to the postfusion state is believed to bring the viral and host membranes in close proximity and promote membrane merger (Harrison, 2008).

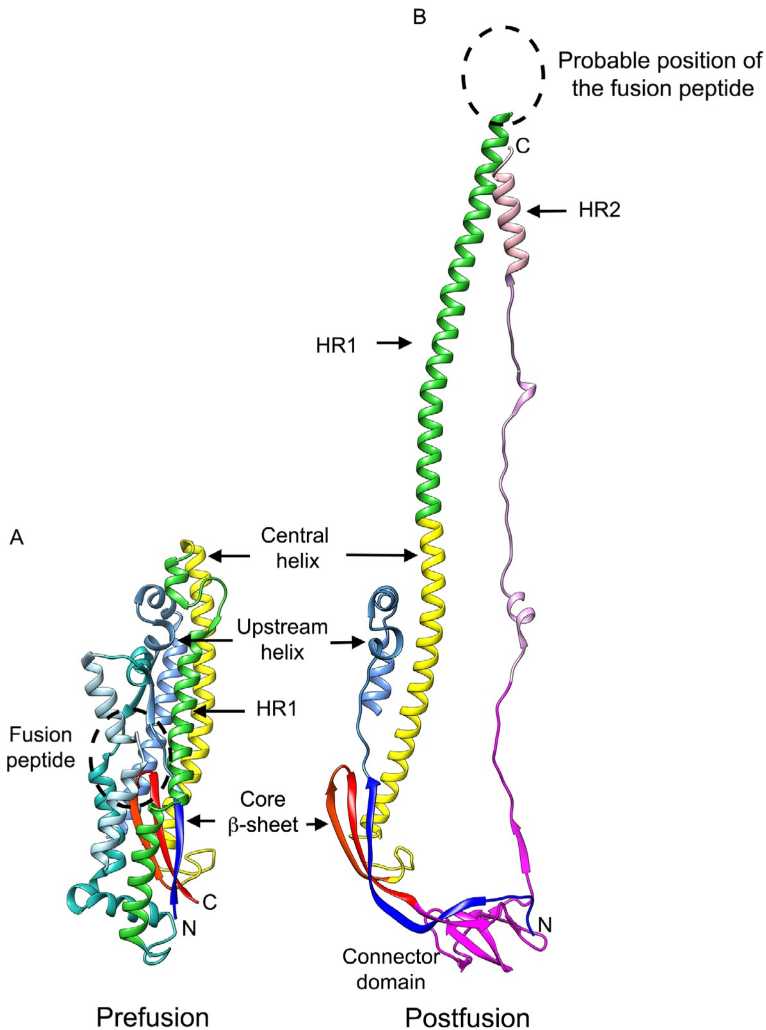


Fig. 3 CoV S conformational changes driving the fusion reaction. (A), Ribbon diagram of the MHV S_2 subunit in the prefusion conformation, PDB: 3JCL. (B), Ribbon diagram of the MHV S_2 subunit in the postfusion conformation, PDB: 6B3O. The prefusion to postfusion transition involves a "jack-knife" refolding of the HR1 helices and intervening regions into a single continuous helix appended to the central helix. The connector domain and HR2 in the prefusion structure and the fusion peptide in the postfusion structure of MHV were not resolved and are therefore not shown.

Recent structural work comparing recombinant S proteins from SARS-CoV and MERS-CoV in isolation and in complex with their cognate receptors or neutralizing antibodies suggested an activation mechanism for coronavirus fusion (Gui et al., 2017; Kirchdoerfer et al., 2018;

Pallesen et al., 2017; Song et al., 2018; Walls et al., 2019; Yuan et al., 2017). Specifically, SARS-CoV and MERS-CoV S structures in complex with neutralizing antibodies isolated from survivors showed both antibodies competitively blocked receptor interaction, in agreement with previous surface plasmon resonance data (Corti et al., 2015; Rockx et al., 2008; Traggiai et al., 2004; Walls et al., 2019). The anti-SARS-CoV S230 antibody, however, functionally mimicked the receptor by promoting S fusogenic conformational rearrangements through a molecular ratcheting mechanism (Walls et al., 2019) (Fig. 4). These observations suggested that upon receptor recognition, bound B domains are locked in the open state, thereby releasing the constraints imposed on the HR1-central helix hairpin, allowing refolding of the S_2 fusion machinery and membrane fusion to occur (Pallesen et al., 2017; Song et al., 2018; Walls et al., 2019; Yuan et al., 2017) (Fig. 4). Proteolytic activation is likely required to ensure that S glycoproteins will work in synergy, with proper spatial and temporal coordination, to drive fusion of the viral and host membranes.

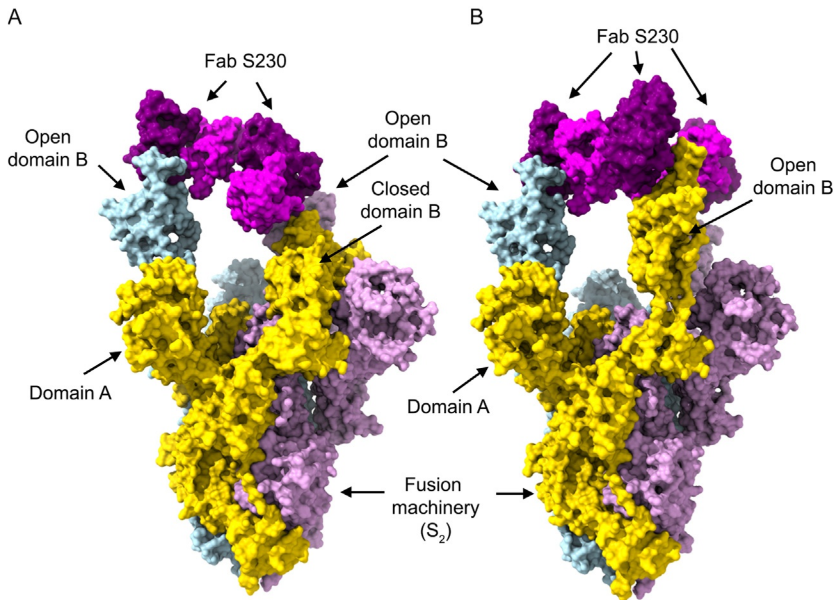


Fig. 4 CryoEM structures of the SARS-CoV S glycoprotein in complex with the S230 neutralizing antibody. (A–B), Molecular surface representation of a complex with one open, one partially open, and one closed B domain, PDB: 6NB6 (left) and with three open B domains that do not follow threefold symmetry, PDB: 6NB7 (right). The structures are rendered with different colors for each S protomer (light blue, plum and gold) and the S230 Fab heavy (dark magenta) and light (magenta) chains (only the variable domains are shown).

6. Epitope masking and glycan shielding

A deep knowledge of the organization and chemical composition of carbohydrates obstructing the surface of CoV S glycoproteins is key for understanding accessibility to neutralizing antibodies and for guiding the rational development of subunit vaccines and therapeutics. S glycoproteins feature ∼20–35 predicted N-linked oligosaccharides per protomer. A cryoEM structure of the HCoV-NL63 S ectodomain allowed to visualize for the first time the extensive N-linked glycans covering the surface of a CoV S trimer (Walls et al., 2016b) (Fig. 5). A subsequent study revealed that numerous glycosylation sites are strictly or topologically conserved between PDCoV S and HCoV-NL63 S although the two glycoproteins share only

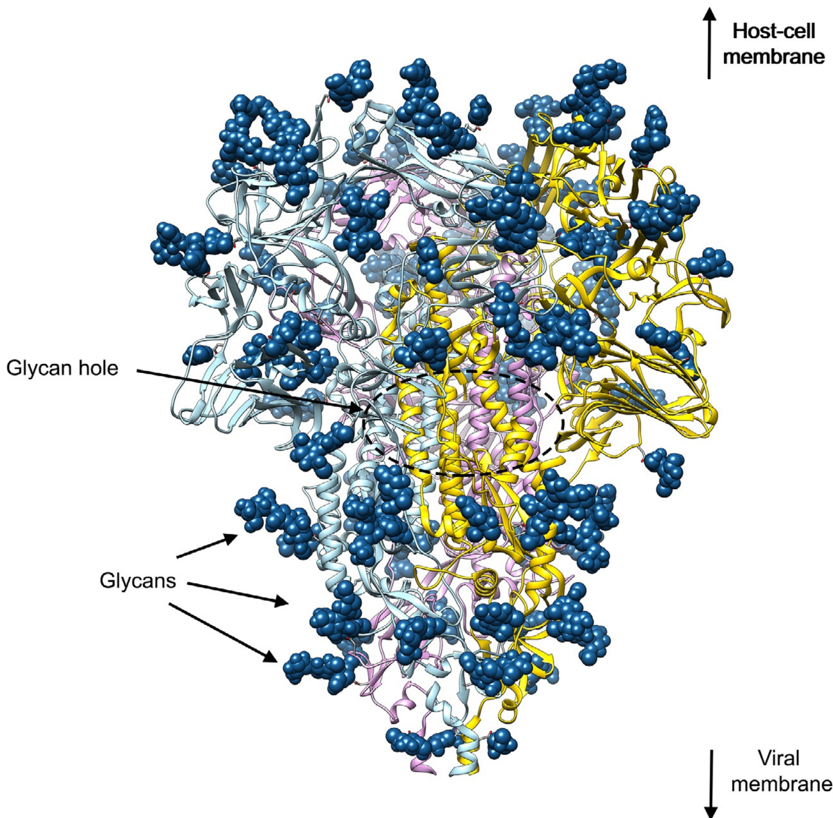
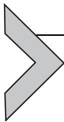


Fig. 5 Organization of the HCoV-NL63 S glycan shield. Ribbon representation of the S ectodomain trimer with N-linked glycans rendered as dark-blue spheres, PDB: 5SZS.

43% amino acid sequence identity and the two viruses belong to different genera infecting different hosts (Xiong et al., 2018). This observation suggested that all CoVs face similar immune pressure in their respective hosts, and that the areas that are masked by the conserved glycans might be key to the function of S. Based on the information gained from the HCoV-NL63 S structure, in which a glycan participates to masking the receptor-binding loops, it was proposed that the S glycan shield is involved in immune evasion, similarly to the well-characterized HIV-1 envelope trimer (Walls et al., 2016b).

Comparison of the N-linked oligosaccharides of full-length MERS-CoV S derived from virions produced in African green monkey VeroE6 cells, or of a purified MERS-CoV S ectodomain recombinantly produced in HEK293F cells, revealed an extensive overlap of glycan composition, including the presence of hybrid and complex glycans (Walls et al., 2019). Processed oligosaccharides were also observed decorating S trimers at the surface of authentic SARS-CoV virions (Krokhin et al., 2003; Ritchie et al., 2010). These data indicated that at least a fraction of the MERS-CoV and SARS-CoV virions produced in a cell are exposed to the glycan-processing enzymes residing in the Golgi apparatus during assembly and budding, in contrast with previous models of CoV budding (Ng et al., 2003; Stertz et al., 2007).

A common feature observed in the glycosylation patterns of S glycoproteins is the presence of less densely glycosylated regions surrounding the S_1/S_2 cleavage site and the conserved fusion peptide, near the S_2' cleavage site, probably to allow access to activating host proteases and for membrane fusion to take place (Walls et al., 2016b; Walls et al., 2019) (Fig. 5). These "glycan holes" could be targeted for epitope-focused immunogen design or new therapeutic development against CoV, as supported by the identification of a neutralization epitope within a comparable breach of the HIV-1 envelope glycan shield (McCoy et al., 2016).



7. Concluding remarks

Recent structural and functional characterization of CoV S glycoproteins provided insights into the mechanism used by these viruses to infect host cells and suggested possible strategies for rational design of vaccines and therapeutics. Introducing stabilizing mutations, which prevent the prefusion to postfusion S transition, led to the elicitation of improved neutralization titers in mice and will be a key tool for the design of subunit

vaccines against CoVs (Kirchdoerfer et al., 2018; Pallesen et al., 2017). Furthermore, the exposure of the fusion peptide at the surface of prefusion S trimers (Walls et al., 2016a) and its conservation among CoVs indicate it might be an attractive target for broad inhibition of CoV entry. Major antigenic determinants of MHV and SARS-CoV S overlap with the fusion peptide region (Daniel et al., 1993; Zhang et al., 2004) and binding of neutralizing antibodies to this site could putatively prevent fusogenic conformational changes, as proposed for influenza virus hemagglutinin or HIV envelope (Corti et al., 2011; Kong et al., 2016; Lang et al., 2017). Finally, masking strain-specific antigenic regions *via* engineering of additional N-linked glycosylation sites, as implemented for the MERS-CoV domain B (Du et al., 2016), bears the promise of focusing the immune response on highly conserved epitopes and eliciting broadly neutralizing antibodies against CoVs.

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References

Bai, X.C., Fernandez, I.S., McMullan, G., Scheres, S.H., 2013. Ribosome structures to near-atomic resolution from thirty thousand cryo-EM particles. elife 2, e00461.

Bakkers, M.J., Lang, Y., Feitsma, L.J., Hulswit, R.J., de Poot, S.A., van Vliet, A.L., Margine, I., de Groot-Mijnes, J.D., van Kuppeveld, F.J., Langereis, M.A., Huizinga, E.G., de Groot, R.J., 2017. Betacoronavirus adaptation to humans involved progressive loss of hemagglutinin-esterase lectin activity. Cell Host Microbe 21, 356.

Bakkers, M.J., Zeng, Q., Feitsma, L.J., Hulswit, R.J., Li, Z., Westerbeke, A., van Kuppeveld, F.J., Boons, G.J., Langereis, M.A., Huizinga, E.G., de Groot, R.J., 2016. Coronavirus receptor switch explained from the stereochemistry of protein-carbohydrate interactions and a single mutation. Proc. Natl. Acad. Sci. U. S. A. 113, E3111.

Barlan, A., Zhao, J., Sarkar, M.K., Li, K., McCray, P.B., Perlman, S., Gallagher, T., 2014. Receptor variation and susceptibility to Middle East respiratory syndrome coronavirus infection. J. Virol. 88, 4953.

Belouzard, S., Chu, V.C., Whittaker, G.R., 2009. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. Proc. Natl. Acad. Sci. U. S. A. 106, 5871.

Beniac, D.R., Andonov, A., Grudeski, E., Booth, T.F., 2006. Architecture of the SARS coronavirus prefusion spike. Nat. Struct. Mol. Biol. 13, 751.

Beniac, D.R., deVarennes, S.L., Andonov, A., He, R., Booth, T.F., 2007. Conformational reorganization of the SARS coronavirus spike following receptor binding: implications for membrane fusion. PLoS One 2, e1082.

Bos, E.C., Heijnen, L., Spaan, W.J., 1995. Site directed mutagenesis of the murine coronavirus spike protein. Effects on fusion. Adv. Exp. Med. Biol. 380, 283.

Bosch, B.J., Bartelink, W., Rottier, P.J., 2008. Cathepsin L functionally cleaves the severe acute respiratory syndrome coronavirus class I fusion protein upstream of rather than adjacent to the fusion peptide. J. Virol. 82, 8887.

Bosch, B.J., de Haan, C.A., Smits, S.L., Rottier, P.J., 2005. Spike protein assembly into the coronavirion: exploring the limits of its sequence requirements. Virology 334, 306.

Bosch, B.J., van der Zee, R., de Haan, C.A., Rottier, P.J., 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J. Virol. 77, 8801.

Brilot, A.F., Chen, J.Z., Cheng, A., Pan, J., Harrison, S.C., Potter, C.S., Carragher, B., Henderson, R., Grigorieff, N., 2012. Beam-induced motion of vitrified specimen on holey carbon film. J. Struct. Biol. 177, 630.

Burkard, C., Verheije, M.H., Wicht, O., van Kasteren, S.I., van Kuppeveld, F.J., Haagmans, B.L., Pelkmans, L., Rottier, P.J., Bosch, B.J., de Haan, C.A., 2014. Coronavirus cell entry occurs through the endo-/lysosomal pathway in a proteolysis-dependent manner. PLoS Pathog. 10, e1004502.

Campbell, M.G., Cheng, A., Brilot, A.F., Moeller, A., Lyumkis, D., Veesler, D., Pan, J., Harrison, S.C., Potter, C.S., Carragher, B., Grigorieff, N., 2012. Movies of ice-embedded particles enhance resolution in electron cryo-microscopy. Structure 20, 1823.

Campbell, M.G., Veesler, D., Cheng, A., Potter, C.S., Carragher, B., 2015. 2.8 Å resolution reconstruction of the Thermoplasma acidophilum 20S proteasome using cryo-electron microscopy. Elife 4. https://doi.org/10.7554/eLife.06380.

Chang, K.W., Sheng, Y., Gombold, J.L., 2000. Coronavirus-induced membrane fusion requires the cysteine-rich domain in the spike protein. Virology 269, 212.

Chen, J., Kovacs, J.M., Peng, H., Rits-Volloch, S., Lu, J., Park, D., Zablowsky, E., Seaman, M.S., Chen, B., 2015. HIV-1 envelope. Effect of the cytoplasmic domain on antigenic characteristics of HIV-1 envelope glycoprotein. Science 349, 191.

Chen, Y., Rajashankar, K.R., Yang, Y., Agnihothram, S.S., Liu, C., Lin, Y.L., Baric, R.S., Li, F., 2013. Crystal structure of the receptor-binding domain from newly emerged Middle East respiratory syndrome coronavirus. J. Virol. 87, 10777.

Corti, D., Voss, J., Gamblin, S.J., Codoni, G., Macagno, A., Jarrossay, D., Vachieri, S.G., Pinna, D., Minola, A., Vanzetta, F., Silacci, C., Fernandez-Rodriguez, B.M., Agatic, G., Bianchi, S., Giacchetto-Sasselli, I., Calder, L., Sallusto, F., Collins, P., Haire, L.F., Temperton, N., Langedijk, J.P., Skehel, J.J., Lanzavecchia, A., 2011. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science 333, 850.

Corti, D., Zhao, J., Pedotti, M., Simonelli, L., Agnihothram, S., Fett, C., Fernandez-Rodriguez, B., Foglierini, M., Agatic, G., Vanzetta, F., Gopal, R., Langrish, C.J., Barrett, N.A., Sallusto, F., Baric, R.S., Varani, L., Zambon, M., Perlman, S., Lanzavecchia, A., 2015. Prophylactic and postexposure efficacy of a potent human monoclonal antibody against MERS coronavirus. Proc. Natl. Acad. Sci. U. S. A. 112, 10473.

Daniel, C., Anderson, R., Buchmeier, M.J., Fleming, J.O., Spaan, W.J., Wege, H., Talbot, P.J., 1993. Identification of an immunodominant linear neutralization domain on the S2 portion of the murine coronavirus spike glycoprotein and evidence that it forms part of complex tridimensional structure. J. Virol. 67, 1185.

Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjöström, H., Norén, O., Laude, H., 1992. Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. Nature 357, 417.

Delmas, B., Gelfi, J., Sjöström, H., Noren, O., Laude, H., 1993. Further characterization of aminopeptidase-N as a receptor for coronaviruses. Adv. Exp. Med. Biol. 342, 293.

Dev, J., Park, D., Fu, Q., Chen, J., Ha, H.J., Ghantous, F., Herrmann, T., Chang, W., Liu, Z., Frey, G., Seaman, M.S., Chen, B., Chou, J.J., 2016. Structural basis for membrane anchoring of HIV-1 envelope spike. Science 353, 172.

Du, L., Tai, W., Yang, Y., Zhao, G., Zhu, Q., Sun, S., Liu, C., Tao, X., Tseng, C.K., Perlman, S., Jiang, S., Zhou, Y., Li, F., 2016. Introduction of neutralizing immunogenicity index to the rational design of MERS coronavirus subunit vaccines. Nat. Commun. 7, 13473.

Duquerroy, S., Vigouroux, A., Rottier, P.J., Rey, F.A., Bosch, B.J., 2005. Central ions and lateral asparagine/glutamine zippers stabilize the post-fusion hairpin conformation of the SARS coronavirus spike glycoprotein. Virology 335, 276.

Dveksler, G.S., Pensiero, M.N., Cardellichio, C.B., Williams, R.K., Jiang, G.S., Holmes, K.V., Dieffenbach, C.W., 1991. Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. J. Virol. 65, 6881.

Earnest, J.T., Hantak, M.P., Li, K., McCray, P.B., Perlman, S., Gallagher, T., 2017. The tetraspanin CD9 facilitates MERS-coronavirus entry by scaffolding host cell receptors and proteases. PLoS Pathog. 13, e1006546.

Earnest, J.T., Hantak, M.P., Park, J.E., Gallagher, T., 2015. Coronavirus and influenza virus proteolytic priming takes place in tetraspanin-enriched membrane microdomains. J. Virol. 89, 6093.

Eifart, P., Ludwig, K., Bottcher, C., de Haan, C.A., Rottier, P.J., Korte, T., Herrmann, A., 2007. Role of endocytosis and low pH in murine hepatitis virus strain A59 cell entry. J. Virol. 81, 10758.

Frana, M.F., Behnke, J.N., Sturman, L.S., Holmes, K.V., 1985. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: host-dependent differences in proteolytic cleavage and cell fusion. J. Virol. 56, 912.

Gao, J., Lu, G., Qi, J., Li, Y., Wu, Y., Deng, Y., Geng, H., Li, H., Wang, Q., Xiao, H., Tan, W., Yan, J., Gao, G.F., 2013. Structure of the fusion core and inhibition of fusion by a heptad repeat peptide derived from the S protein of Middle East respiratory syndrome coronavirus. J. Virol. 87, 13134.

Ge, X.Y., Li, J.L., Yang, X.L., Chmura, A.A., Zhu, G., Epstein, J.H., Mazet, J.K., Hu, B., Zhang, W., Peng, C., Zhang, Y.J., Luo, C.M., Tan, B., Wang, N., Zhu, Y., Crameri, G., Zhang, S.Y., Wang, L.F., Daszak, P., Shi, Z.L., 2013. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature 503, 535.

Gui, M., Song, W., Zhou, H., Xu, J., Chen, S., Xiang, Y., Wang, X., 2017. Cryo-electron microscopy structures of the SARS-CoV spike glycoprotein reveal a prerequisite conformational state for receptor binding. Cell Res. 27, 119.

Haagmans, B.L., Al Dhahiry, S.H., Reusken, C.B., Raj, V.S., Galiano, M., Myers, R., Godeke, G.J., Jonges, M., Farag, E., Diab, A., Ghobashy, H., Alhajri, F., Al-Thani, M., Al-Marri, S.A., Al Romaihi, H.E., Al Khal, A., Bermingham, A., Osterhaus, A.D., AlHajri, M.M., Koopmans, M.P., 2014. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. Lancet Infect. Dis. 14, 140.

Harrison, S.C., 2008. Viral membrane fusion. Nat. Struct. Mol. Biol. 15, 690.

Heald-Sargent, T., Gallagher, T., 2012. Ready, set, fuse! The coronavirus spike protein and acquisition of fusion competence. Viruses 4, 557.

Hofmann, H., Pyrc, K., van der Hoek, L., Geier, M., Berkhout, B., Pohlmann, S., 2005. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proc. Natl. Acad. Sci. U. S. A. 102, 7988.

Hu, B., Zeng, L.P., Yang, X.L., Ge, X.Y., Zhang, W., Li, B., Xie, J.Z., Shen, X.R., Zhang, Y.Z., Wang, N., Luo, D.S., Zheng, X.S., Wang, M.N., Daszak, P., Wang, L.F., Cui, J., Shi, Z.L., 2017. Discovery of a rich gene pool of bat SARS-related

coronaviruses provides new insights into the origin of SARS coronavirus. PLoS Pathog. 13, e1006698.

Hulswit, R.J., de Haan, C.A., Bosch, B.J., 2016. Coronavirus spike protein and tropism changes. Adv. Virus Res. 96, 29.

Hulswit, R.J.G., Lang, Y., Bakkers, M.J.G., Li, W., Li, Z., Schouten, A., Ophorst, B., van Kuppeveld, F.J.M., Boons, G.J., Bosch, B.J., Huizinga, E.G., de Groot, R.J., 2019. Human coronaviruses OC43 and HKU1 bind to 9-O-acetylated sialic acids via a conserved receptor-binding site in spike protein domain A. Proc. Natl. Acad. Sci. U. S. A. 116, 2681.

Inoue, Y., Tanaka, N., Tanaka, Y., Inoue, S., Morita, K., Zhuang, M., Hattori, T., Sugamura, K., 2007. Clathrin-dependent entry of severe acute respiratory syndrome coronavirus into target cells expressing ACE2 with the cytoplasmic tail deleted. J. Virol. 81, 8722.

Isaacs, D., Flowers, D., Clarke, J.R., Valman, H.B., MacNaughton, M.R., 1983. Epidemiology of coronavirus respiratory infections. Arch. Dis. Child. 58, 500.

Kirchdoerfer, R.N., Cottrell, C.A., Wang, N., Pallesen, J., Yassine, H.M., Turner, H.L., Corbett, K.S., Graham, B.S., McLellan, J.S., Ward, A.B., 2016. Pre-fusion structure of a human coronavirus spike protein. Nature 531, 118.

Kirchdoerfer, R.N., Wang, N., Pallesen, J., Wrapp, D., Turner, H.L., Cottrell, C.A., Corbett, K.S., Graham, B.S., McLellan, J.S., Ward, A.B., 2018. Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis. Sci. Rep. 8, 15701.

Kong, R., Xu, K., Zhou, T., Acharya, P., Lemmin, T., Liu, K., Ozorowski, G., Soto, C., Taft, J.D., Bailer, R.T., Cale, E.M., Chen, L., Choi, C.W., Chuang, G.Y., Doria-Rose, N.A., Druz, A., Georgiev, I.S., Gorman, J., Huang, J., Joyce, M.G., Louder, M.K., Ma, X., McKee, K., O'Dell, S., Pancera, M., Yang, Y., Blanchard, S.C., Mothes, W., Burton, D.R., Koff, W.C., Connors, M., Ward, A.B., Kwong, P.D., Mascola, J.R., 2016. Fusion peptide of HIV-1 as a site of vulnerability to neutralizing antibody. Science 352, 828.

Krempl, C., Schultze, B., Herrler, G., 1995. Analysis of cellular receptors for human coronavirus OC43. Adv. Exp. Med. Biol. 380, 371.

Krempl, C., Schultze, B., Laude, H., Herrler, G., 1997. Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus. J. Virol. 71, 3285.

Krokhin, O., Li, Y., Andonov, A., Feldmann, H., Flick, R., Jones, S., Stroeher, U., Bastien, N., Dasuri, K.V., Cheng, K., Simonsen, J.N., Perreault, H., Wilkins, J., Ens, W., Plummer, F., Standing, K.G., 2003. Mass spectrometric characterization of proteins from the SARS virus: a preliminary report. Mol. Cell. Proteomics 2, 346.

Lang, S., Xie, J., Zhu, X., Wu, N.C., Lerner, R.A., Wilson, I.A., 2017. Antibody 27F3 broadly targets influenza A group 1 and 2 hemagglutinins through a further variation in VH1-69 antibody orientation on the HA stem. Cell Rep. 20, 2935.

Li, F., Li, W., Farzan, M., Harrison, S.C., 2005a. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science 309, 1864.

Li, W., Hulswit, R.J.G., Kenney, S.P., Widjaja, I., Jung, K., Alhamo, M.A., van Dieren, B., van Kuppeveld, F.J.M., Saif, L.J., Bosch, B.J., 2018. Broad receptor engagement of an emerging global coronavirus may potentiate its diverse cross-species transmissibility. Proc. Natl. Acad. Sci. U. S. A. 115, E5135.

Li, W., Hulswit, R.J.G., Widjaja, I., Raj, V.S., McBride, R., Peng, W., Widagdo, W., Tortorici, M.A., van Dieren, B., Lang, Y., van Lent, J.W.M., Paulson, J.C., de Haan, C.A.M., de Groot, R.J., van Kuppeveld, F.J.M., Haagmans, B.L., Bosch, B.J., 2017. Identification of sialic acid-binding function for the Middle East respiratory syndrome coronavirus spike glycoprotein. Proc. Natl. Acad. Sci. U. S. A. 114, E8508.

Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426, 450.

Li, W., Wicht, O., van Kuppeveld, F.J., He, Q., Rottier, P.J., Bosch, B.J., 2015. A single point mutation creating a furin cleavage site in the spike protein renders porcine epidemic diarrhea coronavirus trypsin independent for cell entry and fusion. J. Virol. 89, 8077.

Li, W., Zhang, C., Sui, J., Kuhn, J.H., Moore, M.J., Luo, S., Wong, S.K., Huang, I.C., Xu, K., Vasilieva, N., Murakami, A., He, Y., Marasco, W.A., Guan, Y., Choe, H., Farzan, M., 2005b. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. EMBO J. 24, 1634.

Li, X., Mooney, P., Zheng, S., Booth, C.R., Braunfeld, M.B., Gubbens, S., Agard, D.A., Cheng, Y., 2013. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. Nat. Methods 10, 584.

Liu, C., Tang, J., Ma, Y., Liang, X., Yang, Y., Peng, G., Qi, Q., Jiang, S., Li, J., Du, L., Li, F., 2015. Receptor usage and cell entry of porcine epidemic diarrhea coronavirus. J. Virol. 89, 6121.

Lontok, E., Corse, E., Machamer, C.E., 2004. Intracellular targeting signals contribute to localization of coronavirus spike proteins near the virus assembly site. J. Virol. 78, 5913.

Lu, G., Hu, Y., Wang, Q., Qi, J., Gao, F., Li, Y., Zhang, Y., Zhang, W., Yuan, Y., Bao, J., Zhang, B., Shi, Y., Yan, J., Gao, G.F., 2013. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature 500, 227.

McCoy, L.E., van Gils, M.J., Ozorowski, G., Messmer, T., Briney, B., Voss, J.E., Kulp, D.W., Macauley, M.S., Sok, D., Pauthner, M., Menis, S., Cottrell, C.A., Torres, J.L., Hsueh, J., Schief, W.R., Wilson, I.A., Ward, A.B., Sanders, R.W., Burton, D.R., 2016. Holes in the glycan shield of the native HIV envelope are a target of trimer-elicited neutralizing antibodies. Cell Rep. 16, 2327.

McLellan, J.S., Chen, M., Leung, S., Graepel, K.W., Du, X., Yang, Y., Zhou, T., Baxa, U., Yasuda, E., Beaumont, T., Kumar, A., Modjarrad, K., Zheng, Z., Zhao, M., Xia, N., Kwong, P.D., Graham, B.S., 2013. Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. Science 340, 1113.

McLellan, J.S., Yang, Y., Graham, B.S., Kwong, P.D., 2011. Structure of respiratory syncytial virus fusion glycoprotein in the postfusion conformation reveals preservation of neutralizing epitopes. J. Virol. 85, 7788.

Menachery, V.D., Yount, B.L., Debbink, K., Agnihothram, S., Gralinski, L.E., Plante, J.A., Graham, R.L., Scobey, T., Ge, X.Y., Donaldson, E.F., Randell, S.H., Lanzavecchia, A., Marasco, W.A., Shi, Z.L., Baric, R.S., 2015. A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. Nat. Med. 21, 1508.

Menachery, V.D., Yount, B.L., Sims, A.C., Debbink, K., Agnihothram, S.S., Gralinski, L.E., Graham, R.L., Scobey, T., Plante, J.A., Royal, S.R., Swanstrom, J., Sheahan, T.P., Pickles, R.J., Corti, D., Randell, S.H., Lanzavecchia, A., Marasco, W.A., Baric, R.S., 2016. SARS-like WIV1-CoV poised for human emergence. Proc. Natl. Acad. Sci. U. S. A. 113, 3048.

Milewska, A., Zarebski, M., Nowak, P., Stozek, K., Potempa, J., Pyrc, K., 2014. Human coronavirus NL63 utilizes heparan sulfate proteoglycans for attachment to target cells. J. Virol. 88, 13221.

Millet, J.K., Whittaker, G.R., 2014. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. Proc. Natl. Acad. Sci. U. S. A. 111, 15214.

Millet, J.K., Whittaker, G.R., 2015. Host cell proteases: critical determinants of coronavirus tropism and pathogenesis. Virus Res. 202, 120.

Ng, M.L., Tan, S.H., See, E.E., Ooi, E.E., Ling, A.E., 2003. Proliferative growth of SARS coronavirus in Vero E6 cells. J. Gen. Virol. 84, 3291.
Nomura, R., Kiyota, A., Suzaki, E., Kataoka, K., Ohe, Y., Miyamoto, K., Senda, T., Fujimoto, T., 2004. Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. J. Virol. 78, 8701.
Owczarek, K., Szczepanski, A., Milewska, A., Baster, Z., Rajfur, Z., Sarna, M., Pyrc, K., 2018. Early events during human coronavirus OC43 entry to the cell. Sci. Rep. 8, 7124.
Pallesen, J., Wang, N., Corbett, K.S., Wrapp, D., Kirchdoerfer, R.N., Turner, H.L., Cottrell, C.A., Becker, M.M., Wang, L., Shi, W., Kong, W.P., Andres, E.L., Kettenbach, A.N., Denison, M.R., Chappell, J.D., Graham, B.S., Ward, A.B., McLellan, J.S., 2017. Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. Proc. Natl. Acad. Sci. U. S. A. 114, E7348.
Park, J.E., Li, K., Barlan, A., Fehr, A.R., Perlman, S., McCray, P.B., Gallagher, T., 2016. Proteolytic processing of Middle East respiratory syndrome coronavirus spikes expands virus tropism. Proc. Natl. Acad. Sci. U. S. A. 113, 12262.
Peng, G., Sun, D., Rajashankar, K.R., Qian, Z., Holmes, K.V., Li, F., 2011. Crystal structure of mouse coronavirus receptor-binding domain complexed with its murine receptor. Proc. Natl. Acad. Sci. U. S. A. 108, 10696.
Peng, G., Xu, L., Lin, Y.L., Chen, L., Pasquarella, J.R., Holmes, K.V., Li, F., 2012. Crystal structure of bovine coronavirus spike protein lectin domain. J. Biol. Chem. 287, 41931.
Petit, C.M., Melancon, J.M., Chouljenko, V.N., Colgrove, R., Farzan, M., Knipe, D.M., Kousoulas, K.G., 2005. Genetic analysis of the SARS-coronavirus spike glycoprotein functional domains involved in cell-surface expression and cell-to-cell fusion. Virology 341, 215.
Prabakaran, P., Gan, J., Feng, Y., Zhu, Z., Choudhry, V., Xiao, X., Ji, X., Dimitrov, D.S., 2006. Structure of severe acute respiratory syndrome coronavirus receptor-binding domain complexed with neutralizing antibody. J. Biol. Chem. 281, 15829.
Punjani, A., Rubinstein, J.L., Fleet, D.J., Brubaker, M.A., 2017. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods 14, 290.
Raj, V.S., Mou, H., Smits, S.L., Dekkers, D.H., Muller, M.A., Dijkman, R., Muth, D., Demmers, J.A., Zaki, A., Fouchier, R.A., Thiel, V., Drosten, C., Rottier, P.J., Osterhaus, A.D., Bosch, B.J., Haagmans, B.L., 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature 495, 251.
Regan, A.D., Shraybman, R., Cohen, R.D., Whittaker, G.R., 2008. Differential role for low pH and cathepsin-mediated cleavage of the viral spike protein during entry of serotype II feline coronaviruses. Vet. Microbiol. 132, 235.
Reguera, J., Santiago, C., Mudgal, G., Ordoño, D., Enjuanes, L., Casasnovas, J.M., 2012. Structural bases of coronavirus attachment to host aminopeptidase N and its inhibition by neutralizing antibodies. PLoS Pathog. 8, e1002859.
Ritchie, G., Harvey, D.J., Feldmann, F., Stroeher, U., Feldmann, H., Royle, L., Dwek, R.A., Rudd, P.M., 2010. Identification of N-linked carbohydrates from severe acute respiratory syndrome (SARS) spike glycoprotein. Virology 399, 257.
Rockx, B., Corti, D., Donaldson, E., Sheahan, T., Stadler, K., Lanzavecchia, A., Baric, R., 2008. Structural basis for potent cross-neutralizing human monoclonal antibody protection against lethal human and zoonotic severe acute respiratory syndrome coronavirus challenge. J. Virol. 82, 3220.
Rosenthal, P.B., Zhang, X., Formanowski, F., Fitz, W., Wong, C.H., Meier-Ewert, H., Skehel, J.J., Wiley, D.C., 1998. Structure of the haemagglutinin-esterase-fusion glycoprotein of influenza C virus. Nature 396, 92.
Sabir, J.S., Lam, T.T., Ahmed, M.M., Li, L., Shen, Y., Abo-Aba, S.E., Qureshi, M.I., Abu-Zeid, M., Zhang, Y., Khiyami, M.A., Alharbi, N.S., Hajrah, N.H., Sabir, M.J., Mutwakil, M.H., Kabli, S.A., Alsulaimany, F.A., Obaid, A.Y., Zhou, B.,

Smith, D.K., Holmes, E.C., Zhu, H., Guan, Y., 2016. Co-circulation of three camel coronavirus species and recombination of MERS-CoVs in Saudi Arabia. Science 351, 81.

Scheres, S.H., 2012. RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol. 180, 519.

Schroth-Diez, B., Ludwig, K., Baljinnyam, B., Kozerski, C., Huang, Q., Herrmann, A., 2000. The role of the transmembrane and of the intraviral domain of glycoproteins in membrane fusion of enveloped viruses. Biosci. Rep. 20, 571.

Schwegmann-Wessels, C., Zimmer, G., Schröder, B., Breves, G., Herrler, G., 2003. Binding of transmissible gastroenteritis coronavirus to brush border membrane sialoglycoproteins. J. Virol. 77, 11846.

Shang, J., Zheng, Y., Yang, Y., Liu, C., Geng, Q., Luo, C., Zhang, W., Li, F., 2018a. Cryo-EM structure of infectious bronchitis coronavirus spike protein reveals structural and functional evolution of coronavirus spike proteins. PLoS Pathog. 14, e1007009.

Shang, J., Zheng, Y., Yang, Y., Liu, C., Geng, Q., Tai, W., Du, L., Zhou, Y., Zhang, W., Li, F., 2018b. Cryo-electron microscopy structure of porcine deltacoronavirus spike protein in the prefusion state. J. Virol. 92 (4), pii: e01556-17. https://doi.org/10.1128/JVI.01556-17.

Shulla, A., Heald-Sargent, T., Subramanya, G., Zhao, J., Perlman, S., Gallagher, T., 2011. A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. J. Virol. 85, 873.

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.

Song, W., Gui, M., Wang, X., Xiang, Y., 2018. Cryo-EM structure of the SARS coronavirus spike glycoprotein in complex with its host cell receptor ACE2. PLoS Pathog. 14, e1007236.

Stertz, S., Reichelt, M., Spiegel, M., Kuri, T., Martínez-Sobrido, L., García-Sastre, A., Weber, F., Kochs, G., 2007. The intracellular sites of early replication and budding of SARS-coronavirus. Virology 361, 304.

Su, S., Wong, G., Shi, W., Liu, J., Lai, A.C.K., Zhou, J., Liu, W., Bi, Y., Gao, G.F., 2016. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. Trends Microbiol. 24, 490.

Supekar, V.M., Bruckmann, C., Ingallinella, P., Bianchi, E., Pessi, A., Carfí, A., 2004. Structure of a proteolytically resistant core from the severe acute respiratory syndrome coronavirus S2 fusion protein. Proc. Natl. Acad. Sci. U. S. A. 101, 17958.

Swanson, K., Wen, X., Leser, G.P., Paterson, R.G., Lamb, R.A., Jardetzky, T.S., 2010. Structure of the Newcastle disease virus F protein in the post-fusion conformation. Virology 402, 372.

Swanson, K.A., Settembre, E.C., Shaw, C.A., Dey, A.K., Rappuoli, R., Mandl, C.W., Dormitzer, P.R., Carfi, A., 2011. Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. Proc. Natl. Acad. Sci. U. S. A. 108, 9619.

Thorp, E.B., Boscarino, J.A., Logan, H.L., Goletz, J.T., Gallagher, T.M., 2006. Palmitoylations on murine coronavirus spike proteins are essential for virion assembly and infectivity. J. Virol. 80, 1280.

Tortorici, M.A., Walls, A.C., Lang, Y., Wang, C., Li, Z., Koerhuis, D., Boons, G.J., Bosch, B.J., Rey, F.A., de Groot, R.J., Veesler, D., 2019. Structural basis for human coronavirus attachment to sialic acid receptors. Nat. Struct. Mol. Biol. 26, 481.

Traggiai, E., Becker, S., Subbarao, K., Kolesnikova, L., Uematsu, Y., Gismondo, M.R., Murphy, B.R., Rappuoli, R., Lanzavecchia, A., 2004. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. Nat. Med. 10, 871.

Van Hamme, E., Dewerchin, H.L., Cornelissen, E., Verhasselt, B., Nauwynck, H.J., 2008. Clathrin- and caveolae-independent entry of feline infectious peritonitis virus in monocytes depends on dynamin. J. Gen. Virol. 89, 2147.
Vlasak, R., Luytjes, W., Spaan, W., Palese, P., 1988. Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. Proc. Natl. Acad. Sci. U. S. A. 85, 4526.
Walls, A., Tortorici, M.A., Bosch, B.J., Frenz, B., Rottier, P.J., DiMaio, F., Rey, F.A., Veesler, D., 2017a. Crucial steps in the structure determination of a coronavirus spike glycoprotein using cryo-electron microscopy. Protein Sci. 26, 113.
Walls, A.C., Tortorici, M.A., Bosch, B.J., Frenz, B., Rottier, P.J.M., DiMaio, F., Rey, F.A., Veesler, D., 2016a. Cryo-electron microscopy structure of a coronavirus spike glycoprotein trimer. Nature 531, 114.
Walls, A.C., Tortorici, M.A., Frenz, B., Snijder, J., Li, W., Rey, F.A., DiMaio, F., Bosch, B.J., Veesler, D., 2016b. Glycan shield and epitope masking of a coronavirus spike protein observed by cryo-electron microscopy. Nat. Struct. Mol. Biol. 23, 899.
Walls, A.C., Tortorici, M.A., Snijder, J., Xiong, X., Bosch, B.J., Rey, F.A., Veesler, D., 2017b. Tectonic conformational changes of a coronavirus spike glycoprotein promote membrane fusion. Proc. Natl. Acad. Sci. U. S. A. 114, 11157.
Walls, A.C., Xiong, X., Park, Y.J., Tortorici, M.A., Snijder, J., Quispe, J., Cameroni, E., Gopal, R., Dai, M., Lanzavecchia, A., Zambon, M., Rey, F.A., Corti, D., Veesler, D., 2019. Unexpected receptor functional mimicry elucidates activation of coronavirus fusion. Cell 176, 1026.
Wang, N., Shi, X., Jiang, L., Zhang, S., Wang, D., Tong, P., Guo, D., Fu, L., Cui, Y., Liu, X., Arledge, K.C., Chen, Y.H., Zhang, L., Wang, X., 2013. Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. Cell Res. 23, 986.
Wang, Q., Qi, J., Yuan, Y., Xuan, Y., Han, P., Wan, Y., Ji, W., Li, Y., Wu, Y., Wang, J., Iwamoto, A., Woo, P.C., Yuen, K.Y., Yan, J., Lu, G., Gao, G.F., 2014. Bat origins of MERS-CoV supported by bat coronavirus HKU4 usage of human receptor CD26. Cell Host Microbe 16, 328.
Wang, S., Guo, F., Liu, K., Wang, H., Rao, S., Yang, P., Jiang, C., 2008. Endocytosis of the receptor-binding domain of SARS-CoV spike protein together with virus receptor ACE2. Virus Res. 136, 8.
Wicht, O., Li, W., Willems, L., Meuleman, T.J., Wubbolts, R.W., van Kuppeveld, F.J., Rottier, P.J., Bosch, B.J., 2014. Proteolytic activation of the porcine epidemic diarrhea coronavirus spike fusion protein by trypsin in cell culture. J. Virol. 88, 7952.
Wickramasinghe, I.N., de Vries, R.P., Grone, A., de Haan, C.A., Verheije, M.H., 2011. Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. J. Virol. 85, 8903.
Williams, R.K., Jiang, G.S., Holmes, K.V., 1991. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc. Natl. Acad. Sci. U. S. A. 88, 5533.
Wong, A.H.M., Tomlinson, A.C.A., Zhou, D., Satkunarajah, M., Chen, K., Sharon, C., Desforges, M., Talbot, P.J., Rini, J.M., 2017. Receptor-binding loops in alphacoronavirus adaptation and evolution. Nat. Commun. 8, 1735.
Wong, J.J., Paterson, R.G., Lamb, R.A., Jardetzky, T.S., 2016. Structure and stabilization of the Hendra virus F glycoprotein in its prefusion form. Proc. Natl. Acad. Sci. U. S. A. 113, 1056.
Wu, K., Li, W., Peng, G., Li, F., 2009. Crystal structure of NL63 respiratory coronavirus receptor-binding domain complexed with its human receptor. Proc. Natl. Acad. Sci. U. S. A. 106, 19970.
Xiong, X., Coombs, P.J., Martin, S.R., Liu, J., Xiao, H., McCauley, J.W., Locher, K., Walker, P.A., Collins, P.J., Kawaoka, Y., Skehel, J.J., Gamblin, S.J., 2013. Receptor binding by a ferret-transmissible H5 avian influenza virus. Nature 497, 392.

Xiong, X., Tortorici, M.A., Snijder, J., Yoshioka, C., Walls, A.C., Li, W., McGuire, A.T., Rey, F.A., Bosch, B.J., Veesler, D., 2018. Glycan shield and fusion activation of a deltacoronavirus spike glycoprotein fine-tuned for enteric infections. J. Virol. 92 (4), pii: e01628-17. https://doi.org/10.1128/JVI.01628-17.

Xu, K., Chan, Y.P., Bradel-Tretheway, B., Akyol-Ataman, Z., Zhu, Y., Dutta, S., Yan, L., Feng, Y., Wang, L.F., Skiniotis, G., Lee, B., Zhou, Z.H., Broder, C.C., Aguilar, H.C., Nikolov, D.B., 2015. Crystal structure of the pre-fusion nipah virus fusion glycoprotein reveals a novel hexamer-of-trimers assembly. PLoS Pathog. 11, e1005322.

Xu, Y., Liu, Y., Lou, Z., Qin, L., Li, X., Bai, Z., Pang, H., Tien, P., Gao, G.F., Rao, Z., 2004a. Structural basis for coronavirus-mediated membrane fusion. Crystal structure of mouse hepatitis virus spike protein fusion core. J. Biol. Chem. 279, 30514.

Xu, Y., Su, N., Qin, L., Bai, Z., Gao, G.F., Rao, Z., 2004b. Crystallization and preliminary crystallographic analysis of the heptad-repeat complex of SARS coronavirus spike protein. Acta Crystallogr. D Biol. Crystallogr. 60, 2377.

Yamada, Y., Liu, D.X., 2009. Proteolytic activation of the spike protein at a novel RRRR/S motif is implicated in furin-dependent entry, syncytium formation, and infectivity of coronavirus infectious bronchitis virus in cultured cells. J. Virol. 83, 8744.

Yang, Y., Liu, C., Du, L., Jiang, S., Shi, Z., Baric, R.S., Li, F., 2015. Two mutations were critical for bat-to-human transmission of Middle East respiratory syndrome coronavirus. J. Virol. 89, 9119.

Ye, R., Montalto-Morrison, C., Masters, P.S., 2004. Genetic analysis of determinants for spike glycoprotein assembly into murine coronavirus virions: distinct roles for charge-rich and cysteine-rich regions of the endodomain. J. Virol. 78, 9904.

Yeager, C.L., Ashmun, R.A., Williams, R.K., Cardellichio, C.B., Shapiro, L.H., Look, A.T., Holmes, K.V., 1992. Human aminopeptidase N is a receptor for human coronavirus 229E. Nature 357, 420.

Yin, H.S., Paterson, R.G., Wen, X., Lamb, R.A., Jardetzky, T.S., 2005. Structure of the uncleaved ectodomain of the paramyxovirus (hPIV3) fusion protein. Proc. Natl. Acad. Sci. U. S. A. 102, 9288.

Yin, H.S., Wen, X., Paterson, R.G., Lamb, R.A., Jardetzky, T.S., 2006. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. Nature 439, 38.

Youn, S., Collisson, E.W., Machamer, C.E., 2005. Contribution of trafficking signals in the cytoplasmic tail of the infectious bronchitis virus spike protein to virus infection. J. Virol. 79, 13209.

Yu, X., Zhang, S., Jiang, L., Cui, Y., Li, D., Wang, D., Wang, N., Fu, L., Shi, X., Li, Z., Zhang, L., Wang, X., 2015. Structural basis for the neutralization of MERS-CoV by a human monoclonal antibody MERS-27. Sci. Rep. 5, 13133.

Yuan, Y., Cao, D., Zhang, Y., Ma, J., Qi, J., Wang, Q., Lu, G., Wu, Y., Yan, J., Shi, Y., Zhang, X., Gao, G.F., 2017. Cryo-EM structures of MERS-CoV and SARS-CoV spike glycoproteins reveal the dynamic receptor binding domains. Nat. Commun. 8, 15092.

Zhang, H., Wang, G., Li, J., Nie, Y., Shi, X., Lian, G., Wang, W., Yin, X., Zhao, Y., Qu, X., Ding, M., Deng, H., 2004. Identification of an antigenic determinant on the S2 domain of the severe acute respiratory syndrome coronavirus spike glycoprotein capable of inducing neutralizing antibodies. J. Virol. 78, 6938.

Zheng, Q., Deng, Y., Liu, J., van der Hoek, L., Berkhout, B., Lu, M., 2006. Core structure of S2 from the human coronavirus NL63 spike glycoprotein. Biochemistry 45, 15205.