

# **Functional analysis of the MERS-coronavirus spike protein**

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*For my parents.*

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## 1. Abstract

Ten years after the outbreak of the *Severe Acute Respiratory Syndrome Coronavirus*, SARS-CoV, which caused the first pandemic of the 21<sup>st</sup> century, a novel betacoronavirus, *Middle East Respiratory Syndrome Coronavirus*, MERS-CoV, emerged in the Arabian Peninsula. Its ongoing spread poses a significant threat to public health. The spike (S) protein of coronaviruses mediates viral entry into target cells and is a key determinant of viral tropism and pathogenesis. Understanding the parameters governing MERS-CoV spike (MERS-S)-driven entry might thus yield valuable information on MERS-CoV biology and was the first goal of the present study. In order to facilitate host cell entry, coronavirus S proteins depend on activation by host cell proteases, which are potential targets for therapeutic intervention. Therefore, the second goal of the present thesis was to identify the proteases responsible for MERS-S activation. The coronavirus S protein is the major target for the neutralizing antibody response and experimental systems for MERS-S can be used as diagnostic tools. The final goal of this thesis was thus to investigate the MERS-CoV seroprevalence in Saudi Arabian individuals.

A lentiviral vector system was established that allows the analysis of MERS-S-driven host cell entry. With the help of this system, MERS-S was found to mediate entry into a broad spectrum of human cell lines, including cells from lung, kidney and colon which is in concordance with the clinical picture of MERS. Host cell entry was independent of previously described coronavirus entry receptors but was promoted by the endosomal cysteine protease cathepsin L and the transmembrane serine protease TMPRSS2. In contrast, the activity of proprotein convertases was dispensable for MERS-S protein-driven entry. Finally, neutralization of S protein-mediated entry revealed that neutralizing antibodies were absent in sera from patients of the Eastern Province of Saudi Arabia taken between 2010-2011 and 2012, indicating that MERS-CoV infections were rare events before the MERS outbreak in 2012. Collectively, these results provide important insights into the processes governing MERS-CoV entry and shed light onto MERS epidemiology. Furthermore, the demonstration that the protease inhibitor camostat, which is approved for use in humans in Japan, blocks MERS-CoV entry by inhibiting TMPRSS2, might help to establish treatment options for MERS patients.

Keywords: MERS-CoV, spike protein, neutralizing antibodies, proteolytic activation, TMPRSS2, cathepsin, proprotein convertases

## 2. Zusammenfassung

Zehn Jahre nach dem Ausbruch des *Severe Acute Respiratory Syndrome Coronavirus*, SARS-CoV, ist ein neues Betacoronavirus, das *Middle East Respiratory Syndrome Coronavirus*, MERS-CoV, auf der arabischen Halbinsel entdeckt worden. Seine anhaltende Ausbreitung stellt eine Bedrohung für die öffentliche Gesundheit dar. Das Spike (S) Protein der Coronaviren vermittelt den viralen Eintritt in Wirtszellen und bestimmt wesentlich den viralen Tropismus und die virale Pathogenese. Das Verständnis der Determinanten des MERS-CoV Spike (MERS-S)-vermittelnden Eintritts in Zellen könnte daher wichtige Einblicke in die MERS-CoV-Biologie liefern und war somit das erste Ziel dieser Arbeit. Um den Eintritt in die Zelle zu ermöglichen, muss das Coronavirus S-Protein durch Wirtszellproteasen aktiviert werden, welche potentielle Ziele für die therapeutischen Intervention darstellen. Daher sollten im zweiten Ziel dieser Arbeit Proteasen identifiziert werden, die MERS-S aktivieren. Das S-Protein ist das Hauptangriffsziel neutralisierender Antikörper und experimentelle Systeme zur S-Analyse können für die Diagnostik eingesetzt werden. Das letzte Ziel dieser Arbeit war es daher, die MERS-CoV Seroprävalenz in Saudi Arabien zu ermitteln.

Es wurde ein lentivirales Vektorensystem etabliert, welches die Analyse des MERS-S-getriebenen Zelleintritts ermöglicht. Mit Hilfe dieses Systems konnte gezeigt werden, dass MERS-S den Eintritt in ein breites Spektrum humaner Zelllinien, wie Lungen-, Nieren- und Darmzellen vermittelt, was mit der klinischen Manifestation von MERS einhergeht. Der Wirtszelleintritt war unabhängig von bereits beschriebenen Coronavirus Eintrittsrezeptoren, wurde jedoch durch die endosomale Cysteinprotease Cathepsin L und die Transmembranserinprotease TMPRSS2 gefördert. Im Gegensatz dazu war die Aktivität von Proprotein Konvertasen für den S-Protein-vermittelnden Eintritt entbehrlich. Schließlich zeigten Neutralisationstests, dass Seren von Patienten aus der östlichen Provinz Saudi Arabiens, die zwischen 2010-2011 und 2012 entnommen wurden, keine MERS-S-neutralisierenden Antikörper enthielten. Dies deutet darauf hin, dass MERS-CoV-Infektionen vor dem Ausbruch 2012 nur selten vorkamen. Die gewonnenen Ergebnisse tragen wesentlich zum Verständnis des MERS-CoV-Eintritts in Zellen bei und liefern wichtige Informationen zur MERS-CoV-Epidemiologie. Weiterhin könnte die Beobachtung, dass der Protease-Inhibitor Camostat, der für den Einsatz im Menschen zugelassen ist (in Japan), TMPRSS2 blockiert und damit den MERS-CoV Eintritt inhibiert, helfen, Behandlungsstrategien für MERS-Patienten zu etablieren.



Schlagwörter: MERS-CoV, Spike Protein, neutralisierende Antikörper, proteolytische Aktivierung, TMPRSS2, Cathepsin, Proprotein Konvertasen

### 3. Introduction

#### 3.1 Coronaviruses

Coronaviruses (CoVs) are enveloped viruses, which contain the largest linear single-stranded and positive-sense viral RNA genome known so far [Siddell *et al.*, 1983]. Although the first coronavirus, the *Infectious bronchitis virus* (IBV), was discovered in 1932 [Hudson and Beaudette 1932], the virus family *Coronaviridae* was proposed more than 30 years later after human coronaviruses were discovered in patients suffering from the common cold [Tyrrell and Bynoe 1965, Tyrrell *et al.*, 1975].

The family *coronaviridae* contains two subfamilies, *Torovirinae* and *Coronavirinae*, and within the latter, four genera have been identified based on serological and genetic analyses according to the *International Committee on Taxonomy of Viruses* (ICTV): Alpha-, beta-, gamma-, and deltacoronaviruses [[http://ictvdb.bio-mirror.cn/Ictv/fs\\_coron.htm](http://ictvdb.bio-mirror.cn/Ictv/fs_coron.htm), Cavanagh *et al.*, 1993]. A model of coronavirus evolution was established by Woo *et al.*, tracing the gene source of alpha- and betacoronavirus back to bats and the one for gamma- and deltacoronaviruses to birds [Woo *et al.*, 2012].

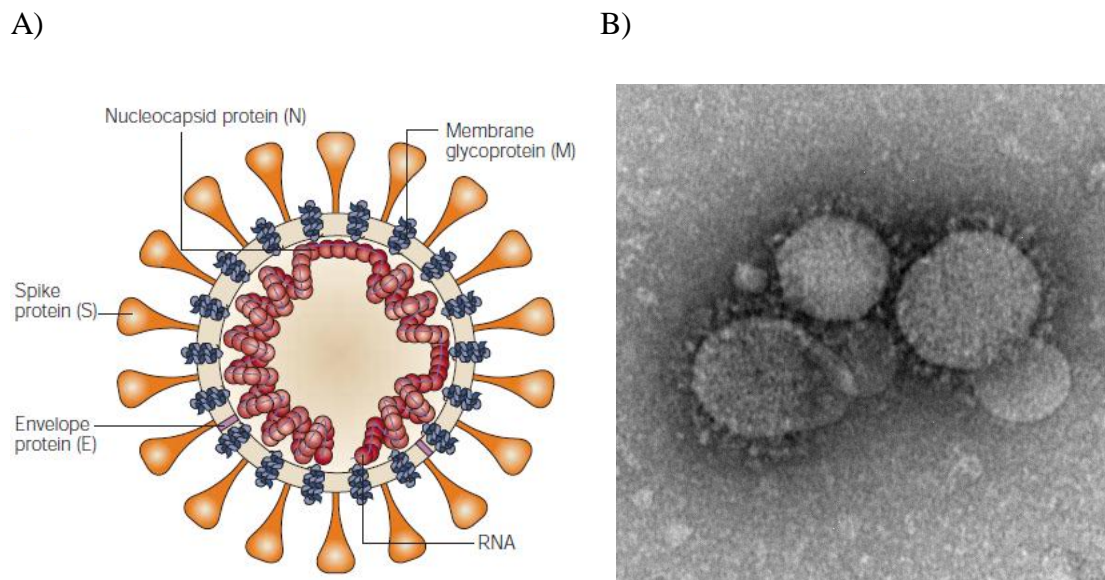
Coronaviruses mainly cause acute and self-limiting respiratory, nervous system and enteric diseases [Guy *et al.*, 2000, Perlman and Netland 2009]. Nevertheless, infection can also result in fatal systemic diseases [Herrewegh *et al.*, 1997]. Until the emergence of the SARS-CoV in 2003, human coronaviruses were known as the causative agent of 15-30% of the common cold [Holmes 2003]. The first human coronaviruses, HCoV-229E and HCoV-OC43, were identified in the 1960s [McIntosh *et al.*, 1974, Holmes 2003]. They cause comparatively mild common colds, but they can also be responsible for more serious respiratory disease in infants and immunocompromised patients [Pene *et al.*, 2003]. Subsequently, two other coronaviruses, HCoV-NL63 and CoV-HKU1, were isolated and identified to cause mild to serious lower respiratory tract infections. HCoV-NL63 was isolated from a child suffering from bronchiolitis and conjunctivitis. However, it can cause acute respiratory disease in children and immunosuppressed adults and is associated with croup [van der Hoek *et al.*, 2004, van der Hoek *et al.*, 2005]. CoV-HKU1 was isolated from a patient with pneumonia, and is associated with acute bronchiolitis and asthmatic exacerbation [Woo *et al.*, 2005, Lau *et al.*, 2006]. These four human coronaviruses are adapted to spread in the human population and circulate globally [Hendley *et al.*, 1972].

In the year 2002, the “harmless” picture of coronaviruses had to be redefined, when the causative agent of the Severe Acute Respiratory Syndrome (SARS) appeared, causing life-threatening pneumonia [Peiris *et al.*, 2003, Zhong *et al.*, 2003]. Ten years later, Zaki and colleagues isolated a so far unknown coronavirus from a male patient with acute pneumonia, which was later identified as a novel human coronavirus, termed *Middle East Respiratory Syndrome Coronavirus* (MERS-CoV) [van Boheemen *et al.*, 2012, Zaki *et al.*, 2012].

In contrast to HCoV-229E, HCoV-OC43, HCoV-NL63 and CoV-HKU1, SARS-CoV and MERS-CoV were recently transmitted from animals to humans and both can cause severe disease in infected patients [Ksiazek *et al.*, 2003, Bermingham *et al.*, 2012, Zaki *et al.*, 2012].

### 3.1.1 Morphology of Coronavirus particles

Coronavirus virions are 60 to 220 nm in diameter and have a pleomorphic, although generally spherical shape (Figure 1B) [Siddell *et al.*, 1983].



**Figure 1:** A) Schematic depiction of the coronavirus virion structure. The viral RNA is associated with the nucleocapsid protein N. The lipid bilayer includes the spike protein, the membrane protein and the envelope protein (S, M, E) (Figure was taken from [Stadler *et al.*, 2003]). B) MERS-CoV virions visualized by negative stain electron microscopy. The virions display the characteristic corona-like shape, originating from protruding spike proteins (Figure was taken from the Centers for Disease Control and Prevention (CDC) <http://www.cdc.gov/Features/novel-coronavirus/>).

The large positive-stranded RNA genome comprises about 30 kb and is associated with the N protein as a helical nucleocapsid, which in turn interacts with the viral membrane protein M [Stadler *et al.*, 2003]. The characteristic crown-like shape of the virion is conveyed by the spike (S) protein which radiates from the viral envelope and mediates receptor binding and viral entry. The minor envelope protein E is necessary for particle assembly (Figure 1A) [Stadler *et al.*, 2003].

### **3.2 Severe Acute Respiratory Syndrome (SARS)**

The Severe Acute Respiratory Syndrome (SARS) was the first pandemic disease in the 21<sup>st</sup> century. It began in Guangdong, a province in Southern China, in November 2002 where outbreaks of an atypical pneumonia were observed [Peng *et al.*, 2003, Zhong *et al.*, 2003]. The chain of transmission crossed Chinese borders in February 2003 when a sick physician travelled from Guangdong province to a hotel in Hong Kong, where he transmitted the disease to 10 other guests, who then started SARS outbreaks in various Asian countries and North America. Within weeks after this event, the disease had spread to over 29 countries, infecting 8089 and killing almost 800 people (World Health Organization; [http://www.who.int/csr/sars/country/table2003\\_09\\_23/en/](http://www.who.int/csr/sars/country/table2003_09_23/en/)). On March 13, 2003 the World Health Organisation (WHO) declared a global alert and due to containment activities the spread of SARS could finally be stopped in July 2003, although in the beginning of 2004 four new, relatively mild cases of SARS were diagnosed [Zhong *et al.*, 2003, Liang *et al.*, 2004]. A novel coronavirus, termed SARS-CoV, was identified as the causative agent of the disease in 2003 [Drosten *et al.*, 2003, Peiris *et al.*, 2003, Poutanen *et al.*, 2003]. Within weeks after the discovery of the virus, its complete genome was sequenced, revealing that SARS-CoV is not closely related to any of the previously characterized coronaviruses and forms a distinct group [Marra *et al.*, 2003, Poon *et al.*, 2003, Rota *et al.*, 2003].

The major routes of human-to-human transmission of SARS-CoV were virus-carrying droplets and direct contact with infected patients. However, alternative ways of transmission like oral and conjunctival routes were also described [Peiris *et al.*, 2003, Varia *et al.*, 2003, Loon *et al.*, 2004]. The incubation period ranged from 2 to 10 days, although in some patients disease developed after 16 days [Lee *et al.*, 2003], and the initial clinical symptoms were comparable to that seen in influenza patients, including fever, myalgia, headache and

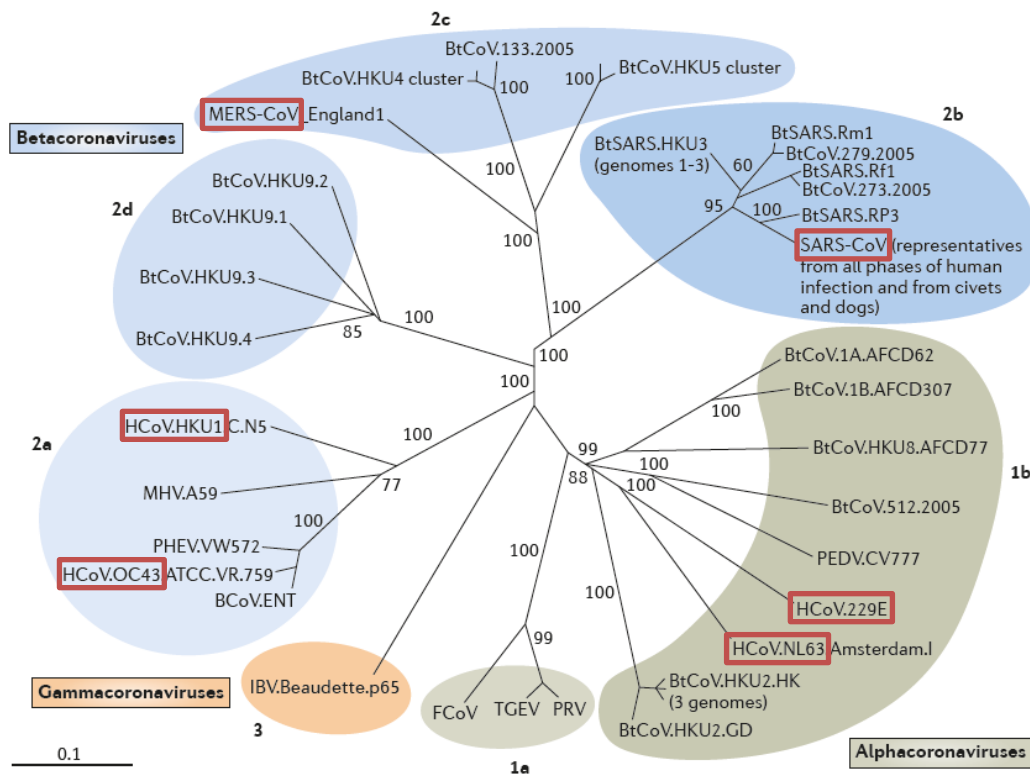
diarrhea. However, in some patients the disease developed into an atypical pneumonia which could progress into Acute Respiratory Distress Syndrome (ARDS) [Peiris *et al.*, 2003].

It was suspected early on that SARS-CoV was an animal virus, which had crossed the species barrier to infect humans [Marra *et al.*, 2003]. Indeed, samples from Himalayan palm civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*) taken in animal markets in Guangdong province contained a SARS-like coronavirus with 99% nucleotide homology to the human one [Guan *et al.*, 2003, Xu *et al.*, 2004]. In addition, SARS-CoV specific antibodies were detected in animal traders [Guan *et al.*, 2003]. However, due to the finding that farmed civet cats had a much lower SARS-CoV antibody prevalence compared to animals sold in markets, it was suggested that civets might have served as intermediate or amplification hosts and not as natural reservoir [Tu *et al.*, 2004]. In the search for the natural reservoir, bats were discussed in several publications. A novel Bat-CoV was identified in the genus *Miniopterus spp.* and its spike protein showed 41% sequence homology to the SARS-spike protein [Poon *et al.*, 2005]. Species from the genus *Rhinolophus* (horseshoe bats) demonstrated a high SARS-CoV antibody prevalence [Li *et al.*, 2005], and specifically in *Rhinolophus sinicus* a SARS-CoV-like virus was identified, which was related to the human and civet one [Lau *et al.*, 2005]. 84% of the bat sera contained neutralizing antibodies against bat-SARS-CoV [Lau *et al.*, 2005]. Additional identifications of two novel bat coronaviruses from Chinese horseshoe bats that are closely related to SARS-CoV and the ability of one of them to utilize human angiotensin- converting enzyme 2 (ACE2) as an entry receptor, supports the evidence of bats to be a potential natural reservoir for the SARS-CoV [Ge *et al.*, 2013].

### 3.3 Middle East Respiratory Syndrome (MERS)

#### 3.3.1 Epidemiology

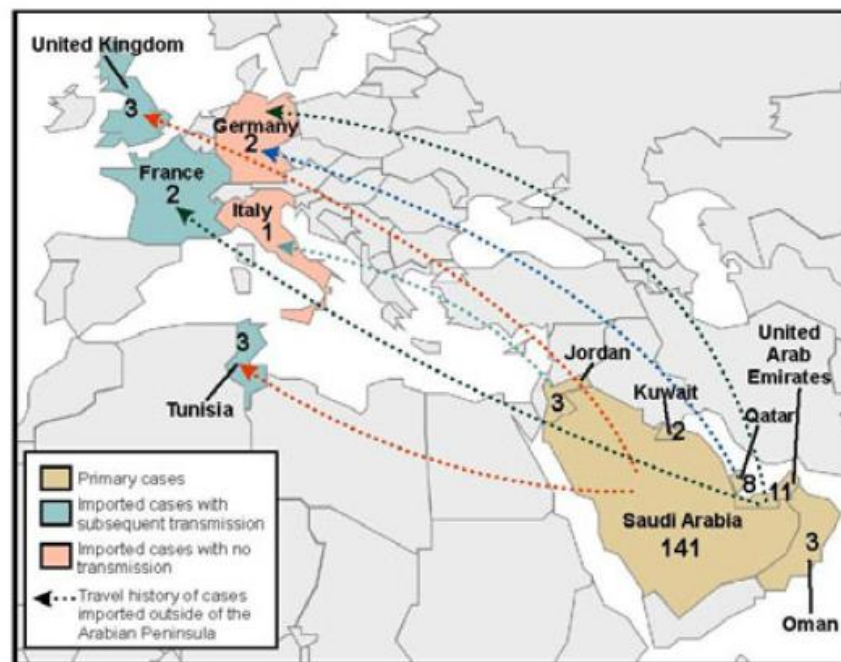
On June 13, 2012, a 60 year old man from Saudi Arabia was hospitalized, presenting fever, cough, expectorations and shortness of breath. Despite intensive medical treatment, the man died on day 11 after committal, due to progressive respiratory and renal failure. Chest radiography indicated ARDS with multiorgan dysfunction syndrome [Zaki *et al.*, 2012]. Thereupon, infection of cell cultures with serum and sputum samples from the patient was found to induce a cytopathic effect (CPE), indicating virus replication [Zaki *et al.*, 2012]. A pan-coronavirus PCR, amplifying a conserved region of the open reading frame 1b of the replicase gene of coronaviruses [Drosten *et al.*, 2003] and subsequent phylogenetic analysis revealed the presence of a novel coronavirus, named HCoV-EMC (Erasmus Medical Center), which belongs to the lineage C of the genus betacoronavirus, together with the bat CoV HKU4 and HKU5 [van Boheemen *et al.*, 2012, Zaki *et al.*, 2012] (Figure 2).



**Figure 2:** Phylogenetic tree of coronaviruses (adapted from [Graham *et al.*, 2013]). Three genera of coronaviruses and their species are shown: Alphacoronaviruses (grey), betacoronaviruses (blue) and gammacoronaviruses (orange). Deltacoronaviruses are not shown. Human coronaviruses are highlighted.

The complete genome of the novel coronavirus was determined using a combination of high-throughput techniques, including deep sequencing, cycle sequencing, rapid amplification of cDNA ends and Sanger sequencing [van Boheemen *et al.*, 2012, Zaki *et al.*, 2012]. HCoV-EMC is the first human coronavirus within this lineage and was later renamed “*Middle East Respiratory Syndrome Coronavirus*” (MERS-CoV) by the Coronavirus Study Group of the International Committee on Taxonomy of Viruses [de Groot *et al.*, 2013].

Shortly after the index case, a patient with a travel history to Saudi Arabia was transferred to a London intensive care unit, after he was hospitalized in Qatar with fever and hypoxia [Bermingham *et al.*, 2012]. Amongst other diagnostic tests, a pan-coronavirus PCR was performed, leading to a reaction product which showed a 99.5% sequence homology to the virus isolated from the patient in Saudi Arabia. Globally, from September 2012 to date (9<sup>th</sup> May 2014), 536 MERS-CoV infections resulting in 145 deaths were reported to the WHO (World Health Organization; [http://www.who.int/csr/disease/coronavirus\\_infections/MERS\\_CoV\\_Update\\_09\\_May\\_2014.pdf?ua=1](http://www.who.int/csr/disease/coronavirus_infections/MERS_CoV_Update_09_May_2014.pdf?ua=1)). The high case fatality rate of roughly 30% is exceptional among all of the known human coronaviruses [Chan *et al.*, 2013, Kindler *et al.*, 2013]. Infections were identified in Saudi Arabia [Zaki *et al.*, 2012, Memish *et al.*, 2013], Jordan [Hijawi *et al.*, 2013], Qatar [Bermingham *et al.*, 2012], Oman, Kuwait, United Kingdom [Bermingham *et al.*, 2012], Germany [Buchholz *et al.*, 2013], France [Mailles *et al.*, 2013], Tunisia [Gulland 2013], Italy [Puzelli *et al.*, 2013], Greece ([http://who.int/csr/don/2014\\_04\\_20\\_mers/en/](http://who.int/csr/don/2014_04_20_mers/en/)), Malaysia ([http://who.int/csr/don/2014\\_04\\_17\\_mers/en/](http://who.int/csr/don/2014_04_17_mers/en/)), Philippines, in the Far East ([http://who.int/csr/don/2014\\_04\\_17\\_mers/en/](http://who.int/csr/don/2014_04_17_mers/en/)) and in the United States of America ([http://who.int/csr/don/2014\\_05\\_05\\_mers/en/](http://who.int/csr/don/2014_05_05_mers/en/)). The majority of the cases occurred in the Middle East and all other cases were at least indirectly linked to this region (Figure 3). In contrast to SARS-CoV, the MERS-CoV transmission rate seems to be rather low, thus impeding pandemic viral spread [Breban *et al.*, 2013]. Human-to-human transmission was reported in household settings and health-care units, but the route of transmission has not been fully established [Assiri *et al.*, 2013, Guery *et al.*, 2013, Hijawi *et al.*, 2013, Memish *et al.*, 2013].



**Figure 3:** Geographic distribution of MERS cases. Depicted are all laboratory-confirmed MERS-CoV infections worldwide until February 1, 2014. The map is subdivided into countries in which MERS-CoV is endemic (brown), countries with imported cases with (green) and without (pink) subsequent human-to-human transmission. The travel history of the import is indicated with dotted arrows (Figure was taken from [Milne-Price *et al.*, 2014]).

### 3.3.2 Natural reservoir and intermediate host of MERS-CoV

Phylogenetic analyses showed that MERS-CoV is closely related to bat CoV-HKU4 and CoV-HKU5, and the discovery of MERS-CoV-related viruses in *Nycteris* and *Pipistrellus* bats from Africa, Europe and America provided further evidence for bats being an animal host to MERS-CoV [Annan *et al.*, 2013, Anthony *et al.*, 2013, Lau *et al.*, 2013, Memish *et al.*, 2013]. Most notably, in Egyptian tomb bats from Saudi Arabia, a 190 nucleotide fragment with 100% identity to the MERS-CoV sequence was found [Memish *et al.*, 2013]. In agreement with these findings, bat cell lines were found to be susceptible to MERS-CoV infection [Müller *et al.*, 2012]. Molecular clock analysis of unlinked isolates calculated the divergence of MERS-CoV from a common ancestor in mid-2011 [Cotten *et al.*, 2013]. However, the small size of the identified nucleotide sequences and the fact that direct contact between human and bat is rather infrequent, points towards an intermediate host, possibly



farm animals, including camels and goats, since they are used for production of meat and milk as well as for races in the Middle East. In addition, contact between camels and MERS-CoV patients has been reported [Albarrak *et al.*, 2012, Drosten *et al.*, 2013, Kupferschmidt 2013] and enteric coronaviruses were previously identified in juvenile dromedary camels [Wünschmann *et al.*, 2002]. MERS-CoV neutralizing antibodies were also found in camel sera from Jordan and the Canary islands [Reusken *et al.*, 2013]. Thereupon, studies from Egypt, Jordan, Saudi Arabia and the United Arab Emirates revealed neutralizing antibodies against MERS-CoV or a closely related virus in dromedary camels [Hemida *et al.*, 2013, Perera *et al.*, 2013, Reusken *et al.*, 2013, Alagaili *et al.*, 2014, Alexandersen *et al.*, 2014, Haagmans *et al.*, 2014, Meyer *et al.*, 2014]. However, it has to be taken into account that camel antibodies have a different configuration compared to human antibodies and might thus bind to a broader spectrum of coronaviruses [Hamers-Casterman *et al.*, 1993, Kupferschmidt 2013]. Besides this, the question unanswered is whether camels transmit MERS-CoV to humans or vice versa. Isolation of the MERS-CoV related virus from camels could shed light into this question. However, camel sera generally have a very high antibody titer, which might hamper virus isolation [Kupferschmidt 2013].

### **3.3.3 Clinical characteristics and drug therapy of MERS-CoV infection**

The estimated incubation period of the reported MERS cases was 5.2 days (95% CI (confidence interval) 1.9-14.7) [Assiri *et al.*, 2013]. The median age of MERS-CoV patients was 56 years [Gulland 2013, The Who Mers-Cov Research 2013, Reuss *et al.*, 2014] with the majority being male patients. Patients suffering from a MERS-CoV infection often present the following symptoms: Fever, myalgia, cough, shortness of breath, diarrhea and vomiting [Albarrak *et al.*, 2012, Danielsson *et al.*, 2012, Zaki *et al.*, 2012, Drosten *et al.*, 2013, Guery *et al.*, 2013, Guberina *et al.*, 2014]. Chest radiographies show abnormalities, as initially observed for the index patient [Zaki *et al.*, 2012]. Most cases progressed to severe pneumonia with ARDS, requiring intensive care and ventilation. Septic shock and fatal multiorgan failure were also observed in these patients. Interestingly, the majority of severe cases was connected to underlying comorbidities, such as diabetes, cancer, hypertension, obesity and chronic diseases, while absence of severe disease was frequently observed in previously healthy patients [Assiri *et al.*, 2013, The Who Mers-Cov Research 2013]. Noteworthy, the prevalence

of type II diabetes and obesity in Saudi Arabia is quite high [Al-Daghri *et al.*, 2011], which might promote disease progression.

Broad-spectrum antibiotics against bacterial superinfection and often oseltamivir as well as corticosteroids were administered to MERS patients [Albarrak *et al.*, 2012, Zaki *et al.*, 2012, Assiri *et al.*, 2013, Drosten *et al.*, 2013, Omrani *et al.*, 2013, Guberina *et al.*, 2014]. A study of Guberina *et al.* reported that treatment with corticosteroids improved the respiratory condition of a MERS patient significantly, but further investigation of this drug and its therapeutic effect is required [Guberina *et al.*, 2014]. A combination of IFN- $\alpha$ 2b and ribavirin could reduce viral replication in susceptible cell lines [Falzarano *et al.*, 2013], although their administration to patients did not prevent a fatal outcome [Al-Tawfiq *et al.*, 2014]. In accordance with the susceptibility of SARS-CoV to inhibition by IFN treatment [Cinatl *et al.*, 2003], type I and III interferon were found to inhibit MERS-CoV replication *in vitro* and *ex vivo* [de Wilde *et al.*, 2013, Kindler *et al.*, 2013, Zielecki *et al.*, 2013]. The application of high concentrations of ribavirin was effective *in vitro* and the reduction of the MERS-CoV replication and induced CPE was more efficient in combination with IFN- $\alpha$ 2b [Falzarano *et al.*, 2013]. Treatment of MERS-CoV infected Rhesus macaques with ribavirin and IFN- $\alpha$ 2b resulted in a decrease of viral replication and an improvement of the clinical picture [Falzarano *et al.*, 2013]. Furthermore, de Wilde and colleagues developed an assay to screen for antiviral compounds which inhibit MERS-CoV replication, and identified cyclosporin A (CsA) [de Wilde *et al.*, 2013]. It should be noted that CsA is an immunosuppressant, and its administration to MERS patients needs to be considered.

### **3.3.4 Animal models for MERS-CoV**

To test potential therapeutic strategies, the development of animal models for MERS-CoV infection is important. Replication of MERS-CoV in small animals, like mice, syrian hamsters and ferrets was not detectable [de Wit *et al.*, 2013, Munster *et al.*, 2013, Coleman *et al.*, 2014, Raj *et al.*, 2014, Yao *et al.*, 2014], while infection studies of Rhesus macaques showed that these nonhuman primates are susceptible to MERS-CoV infection [de Wit *et al.*, 2013, Munster *et al.*, 2013, Yao *et al.*, 2014]. The animals developed a mild-to moderate respiratory disease after inoculation. Virus replication was detected in the lower respiratory tract, predominantly in type I and II pneumocytes in the alveoli, which might explain the observed limited amount of virus shedding [de Wit *et al.*, 2013, Yao *et al.*, 2014]. A specific antibody

response in the macaques was also detected [Yao *et al.*, 2014]. However, the clinical picture in macaques does not completely present the clinical image of MERS in humans, since neither renal disease nor severe pneumonia were developed in macaques. The generation of a small-animal model is still essential because this model is more widely used in research groups and more practicable than working with nonhuman primates. Zhao and colleagues were able to develop a mouse model for MERS that expresses the human host-cell receptor DPP4. These mice can be infected by MERS-CoV and develop pneumonia [Zhao *et al.*, 2014].

### **3.3.5 Diagnostic tools**

#### **PCR**

A pan-coronavirus reverse transcriptase polymerase chain reaction (RT-PCR) assay, which was designed to amplify a highly conserved region of the RNA-dependent RNA polymerase (RdRp, encoded by the ORF1b gene), is suitable to detect MERS-CoV RNA [Drosten *et al.*, 2003, Vijgen *et al.*, 2008, Zaki *et al.*, 2012]. Additionally, a genome region upstream of the envelope gene (upE assay) and another part of ORF1b are amplified by related tests [Corman *et al.*, 2012]. In addition, confirmatory real-time RT-PCR assays amplifying sequences within ORF1a and the N gene segment are available for MERS-CoV detection [Corman *et al.*, 2012]. However, viral RNA could not be detected in all symptomatic patients [Corman *et al.*, 2012].

#### **Serology**

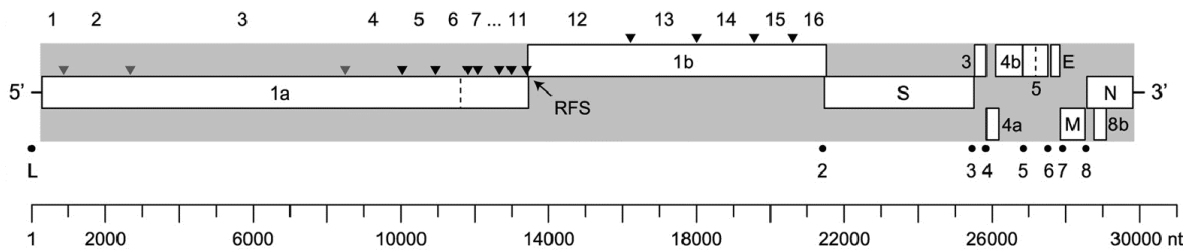
A successful screening to complement RT-PCR findings can be the evaluation with enzymatic immunoassays (EIA) using recombinant viral proteins, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) [Corman *et al.*, 2012, Reusken *et al.*, 2013]. The latter one is based on the detection of an antibody response (by immunofluorescence microscopy) in serum samples from convalescent patients which bind to viral antigens in cells infected with MERS-CoV [Corman *et al.*, 2012]. This assay has also been proved suitable for the detection of SARS-CoV-specific antibodies [Rabenau *et al.*, 2005]. However, cross reactivity with other human betacoronaviruses cannot be excluded at the moment [Blanchard *et al.*, 2011, Corman *et al.*, 2012, Chan *et al.*, 2013].

A neutralisation assay as confirmatory tests for IFA was used for example by Chan *et al.*, where inactivated patient serum is mixed with the replication-competent virus. Target cells are then inoculated with the mixture and examined for cytopathic effects [Chan *et al.*, 2013]. This assay is believed to be highly specific. Therefore, it was surprising, that high titers of MERS-CoV neutralizing antibodies were detected in the sera from SARS patients [Chan *et al.*, 2013]. Crossreactivity has previously been reported only for closely related coronaviruses [He *et al.*, 2006] and is due to sequence and structural homologies between the respective coronavirus spike proteins. Since the homology between MERS-CoV and SARS-CoV on amino acid level is low, the authors suggest that structural homologies are responsible for the crossreactivity. However, virus neutralisation assays must be carried out in biosafety level 3 laboratories. Viral vectors pseudotyped with the spike proteins of interest, mimic cellular entry of coronaviruses and can be used for the detection of neutralizing antibodies [Simmons *et al.*, 2004, Perera *et al.*, 2013, Zhao *et al.*, 2013].

### **3.4 Biology of MERS Coronavirus**

#### **3.4.1 Genome structure**

The MERS-CoV genome consists of 30119 nucleotides and comprises at least ten open reading frames (ORFs), with a high GC content of 41%, which is typical for coronaviruses [van Boheemen *et al.*, 2012]. Downstream of the 5'-UTR there are two large overlapping replicase open reading frames, ORF1a and ORF1b, which require an ribosomal frame shift to produce the polyprotein pp1ab. The polyprotein is subsequently cleaved into 15 or 16 predicted nonstructural proteins (nsps) [van Boheemen *et al.*, 2012]. Downstream of ORF1b, the genome encodes the structural proteins S (spike), E (envelope), M (membrane), and N (nucleocapsid) (ORF2,-6,-7, and -8a), which are translated via subgenomic mRNAs that contain a 5' leader sequence, identical to the genomic 5' region (Figure 4). Moreover, proteins expressed from the group-specific ORFs, ORF 3, 4a, 4b and 5 were identified. Some of them act as potent interferon antagonists, specifically the accessory proteins encoded by ORF4a, 4b, 5 as well as the structural M protein [Yang *et al.*, 2013]. Finally, it is noteworthy that the MERS-CoV genetic structure is similar to that of the bat betacoronaviruses HKU4 and HKU5 [van Boheemen *et al.*, 2012].



**Figure 4:** Genome organisation of MERS-CoV. Shown are the coding sequences and terminal untranslated regions of the genome. ORFs are depicted by rectangles. The ribosomal frameshift site (RFS) between ORF1a/ORF1b, the leader TRS (transcription-regulatory sequence) (L) and the body TRSs (numbered dots) are indicated, as well as the structural proteins S (spike), E (envelope), M (membrane), and N (nucleocapsid). Figure is derived from [van Boheemen *et al.*, 2012].

### 3.5 Viral Life Cycle of Coronaviruses

The coronavirus infection cycle begins with the binding of the spike glycoprotein to its cellular receptor, followed by a direct entry at the cell surface or fusion with the endosomal compartment after endocytosis [Belouzard *et al.*, 2012]. After that, the positive-stranded RNA genome is released into the cytoplasm of the host cell, followed by translation of the open reading frames ORF1a and ORF1b by ribosomal frameshifting into the polyprotein pp1ab [Stadler *et al.*, 2003]. This protein is cleaved autoproteolytically into several replicase proteins, which form the viral replication complex, including the viral RNA-dependent RNA polymerase, which produces a negative-stranded RNA copy of the viral genome [Stadler *et al.*, 2003]. Furthermore, a nested set of sub-genomic negative-sense RNAs is produced, serving as templates for the production of positive-stranded sub-genomic mRNAs, which are finally translated into the structural and accessory proteins [Stadler *et al.*, 2003, Hofmann and Pohlmann 2004]. In the cytoplasm, the N protein and the genomic RNA interact and form the nucleocapsid, which acquires its envelope by budding through intracellular membranes located between the Endoplasmic Reticulum (ER) and the Golgi apparatus (ER-Golgi intermediate compartment, ERGIC). The M, E and S proteins pass the ER and are transported to the budding complex, where the helical nucleocapsid associates with the M protein to

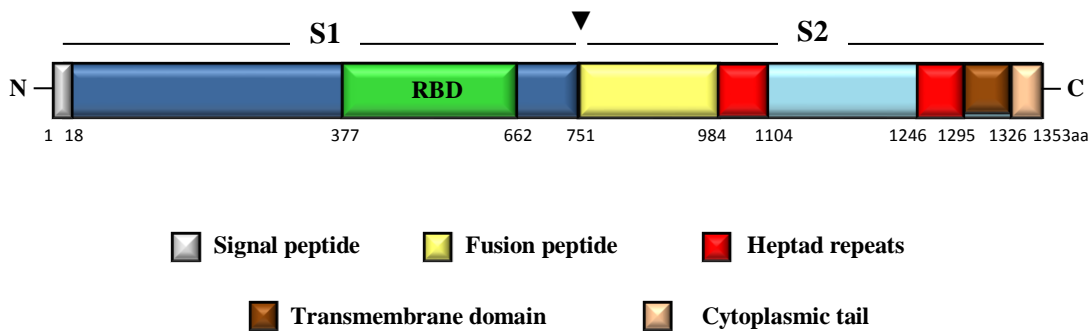
trigger assembly of the viral particle. Within the ERGIC, glycans associated with the spike protein are processed and the spike protein of some coronaviruses is cleaved [Belouzard *et al.*, 2012, Heald-Sargent and Gallagher 2012]. The final release of the particles proceeds via exocytosis at the plasma membrane [Stadler *et al.*, 2003].

### **3.6 MERS-CoV entry into target cells**

#### **3.6.1 The MERS-CoV Spike Protein**

The spike protein of coronaviruses in general not only mediates virus entry into target cells, but also plays an important role for tissue tropism and virus virulence [Belouzard *et al.*, 2012]. Synthesized in the secretory pathway of an infected cell, spike monomers are assembled into trimers and incorporated as peplomers into viral particles [Stadler *et al.*, 2003, Heald-Sargent and Gallagher 2012]. Spike proteins are type I transmembrane proteins and can be depicted as 20 nm-long projections on the viral membrane providing the corona-like shape seen in the electron microscope (Figure 1B) [Davies and Macnaughton 1979]. The coronavirus spike protein belongs to the class I viral fusion proteins, like other viral glycoproteins, including the influenza hemagglutinin and the human immunodeficiency virus envelope protein [Harrison 2005]. Fusion proteins from this group consist of trimers of hairpins containing a central  $\alpha$ -helical coiled-coiled-structure [Weissenhorn *et al.*, 2007]. They are divided into an N-terminal surface unit which harbours the receptor binding domain, called S1 for coronaviruses, as well as a C-terminal transmembrane unit, S2, which contains the fusion machinery.

The MERS-S protein consists of 1353 amino acids, with the S1 subunit ranging from residue 1 to 751 and the S2 subunit comprising the residues 752 to 1352 [Mou *et al.*, 2013]. The binding of the spike protein to the cellular receptor DPP4/CD26 is mediated by the receptor binding domain (RBD), located between the residues 358-662 of the S1 subunit [Chen *et al.*, 2013, Du *et al.*, 2013, Mou *et al.*, 2013]. The RBD consists of a core subdomain, which is highly similar to that of related coronaviruses and an external receptor binding motif that makes specific contacts to DPP4 and is not conserved between coronaviruses (Figure 5) [Jiang *et al.*, 2013, Lu *et al.*, 2013, Wang *et al.*, 2013, Zhou *et al.*, 2014]. The RBD elicits neutralizing antibodies, which block the binding of the spike protein to DPP4 [Du *et al.*, 2013, Jiang *et al.*, 2013, Mou *et al.*, 2013], thus proposing a useful vaccine candidate.

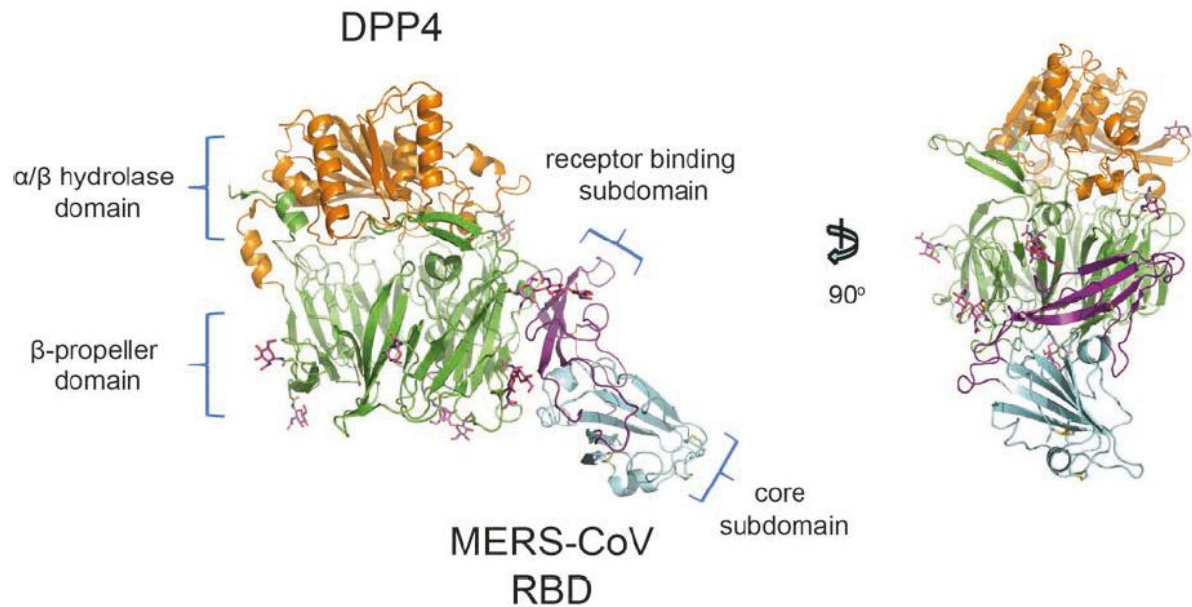


**Figure 5:** Shown is an image of the MERS-S protein, according to the predicted structures from Du *et al.* [Du *et al.*, 2013]. The signal peptide mediates the translocation of the spike protein into the ER. The RBD within the S1 unit binds to the receptor, while the S2 subunit harbours the elements of the fusion machinery. The possible proteolytic cleavage site is indicated with a rectangle.

### 3.6.2 Host cell receptor and tropism of MERS-CoV

To facilitate host cell entry, the S1 subdomain of a coronavirus spike protein has to attach to a specific cellular receptor (Figure 6). For human coronaviruses, the following receptors have been identified: Aminopeptidase N (APN), also known as CD13, serves as a receptor for HCoV-229E [Yeager *et al.*, 1992]. HCoV-NL63 as well as SARS-CoV employ angiotensin-converting enzyme 2 (ACE2) [Li *et al.*, 2003, Hofmann *et al.*, 2005]. Finally, sialic acid is engaged by HCoV-OC43 for host cell entry [Vlasak *et al.*, 1988]. Initial efforts to identify the MERS-CoV entry receptor, these well-known coronavirus receptors could be excluded [Müller *et al.*, 2012, Raj *et al.*, 2013]. Employing pull-down assays and mass spectrometric analysis of RBD binding partners, Raj and colleagues were able to identify dipeptidyl peptidase 4 (DPP4, also known as CD26) as an interaction partner of the MERS-S protein [Raj *et al.*, 2013]. Furthermore, it was shown that soluble DPP4 binds to the MERS-CoV S1 subunit, and non-susceptible cell lines gained susceptibility for S1-binding after transient expression of DPP4. Additionally, infection of susceptible cells was inhibited with a soluble form of DPP4. The same observation was made with bat DPP4, although the receptor activity of this protein was not as pronounced as that measured for human DPP4 [Raj *et al.*, 2013]. DPP4 is the third exopeptidase identified as a coronavirus receptor. It consists of a  $\alpha/\beta$ -

hydrolase domain and an extracellular domain, including a  $\beta$ -propeller domain with 8 blades that contact the RBD of the spike protein (Figure 6) [Wang *et al.*, 2013].



**Figure 6:** Crystal structure of the complex between DPP4 and the RBD of MERS-S. The receptor contains an N-terminal eight-bladed  $\beta$ -propeller domain (green) and a C-terminal  $\alpha/\beta$ -hydrolase domain (orange). The RBD of the MERS-CoV consists of a core (cyan) and a receptor-binding-subdomain, that makes the contact to DPP4 (adapted from [Wang *et al.*, 2013]).

DPP4 is a multifunctional type II transmembrane glycoprotein, which cleaves peptides and plays a role in signal transduction and glucose metabolism, cell adhesion and apoptosis, depending on its cellular localisation [Boonacker and Van Noorden 2003, Lambeir *et al.*, 2003]. Regarding the clinical picture, DPP4 plays also a role in type 2 diabetes mellitus, which is often a comorbidity in MERS-CoV infected patients, by cleaving and thereby inactivating a hormone which stimulates insulin secretion [Augustyns *et al.*, 1999]. DPP4 is highly conserved between different species and sequence comparisons revealed that DPP4 orthologs of human macaque, horse, rabbit and pig origin have no or little variation in the residues contacting the RBD of MERS-S [Bosch *et al.*, 2013, Raj *et al.*, 2013]. In contrast, the RBD contact site is not conserved in ferret DPP4 and MERS-CoV cannot use ferret DPP4 as a functional receptor for host cell entry [Bosch *et al.*, 2013, de Wit *et al.*, 2013]. DPP4 is expressed in the non-ciliated cells of cultured primary human bronchiolar epithelium and lung



tissue, as well as epithelial cells of liver, intestine, kidney, prostate, on leukocytes and in a soluble form in plasma [Boonacker and Van Noorden 2003, Raj *et al.*, 2013]. In concordance with the presence of the receptor in these cells, *in vitro* experiments revealed that MERS-CoV can infect cells in the human lower respiratory tract, kidney, intestine and liver [Chan *et al.*, 2013, Zielecki *et al.*, 2013] with predominantly non-ciliated cells being targeted in the bronchus and lung [Chan *et al.*, 2013, Kindler *et al.*, 2013]. The virus was also detected in patients' respiratory swabs, tracheal aspirate, sputum, urine and stool samples [Zaki *et al.*, 2012, Drosten *et al.*, 2013, Guery *et al.*, 2013].

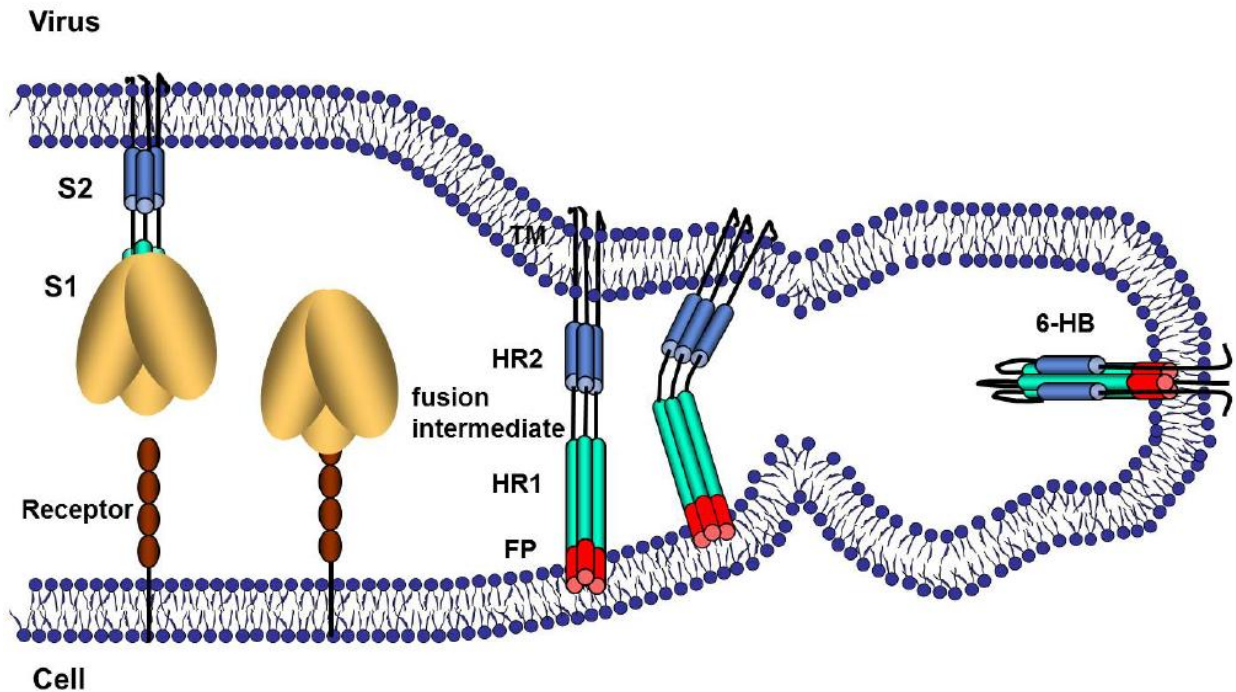
### 3.6.3 Spike-mediated membrane fusion

The binding of the viral spike protein to the cellular receptor is a prerequisite for spike protein-driven fusion between the viral and the host cell membrane [Yamada and Liu 2009]. This process can take place either at the cell surface or within the endosomal compartment after internalisation via endocytosis. To generate the fusion competent form, class I viral fusion proteins need to be proteolytically primed by host cell proteases.

There is a diversity of triggers that stimulate a viral surface glycoprotein for membrane fusion. The process can be initiated by receptor binding as it is the case for HIV gp160 and the murine leukemia virus (MLV) glycoprotein [Belouzard *et al.*, 2012, Simmons *et al.*, 2013]. Other viruses such as SARS-CoV, VSV and influenza virus require pH acidification in addition to receptor binding [White *et al.*, 1981, Simmons *et al.*, 2004, Yang *et al.*, 2004]. Furthermore, activation of the spike protein can be acquired by proteolytic cleavage [Simmons *et al.*, 2004, Watanabe *et al.*, 2008, Taguchi and Matsuyama 2009].

The coronavirus spike protein belongs to the class I viral fusion proteins, which contain conserved functional elements in their transmembrane unit for facilitating membrane fusion: A fusion peptide and two heptad repeats (HRs) are located between the surface unit and the transmembrane domain (Figure 5) [de Groot *et al.*, 1987]. If a primed class I membrane fusion protein is exposed to a trigger like low pH or receptor binding, the fusion peptide is projected towards the target cell and inserts into the cellular membrane (Figure 7). The fusion peptide is usually located at the N-terminus of the transmembrane subunit, as it is the case for HIV and likely also coronaviruses, or internally of the transmembrane protein subunit (Ebola virus glycoprotein, SARS-CoV spike) [Sainz *et al.*, 2005, Weissenhorn *et al.*, 2007, Belouzard *et al.*, 2009]. Subsequently, the two HRs fold back onto each other, thereby

forming a six-helix bundle structure (Figure 7). This conformational change relocates the N-terminal fusion peptide and thus the target cell membrane close to the transmembrane domain and thus the viral membrane, which ultimately results in membrane fusion (Figure 7) [Bosch *et al.*, 2003, Xu *et al.*, 2004].



**Figure 7:** Glycoprotein-driven membrane fusion. The membrane fusion reaction is triggered by binding of the surface unit (S1) to a cellular receptor, pH reduction and/or glycoprotein proteolysis. The membrane fusion reaction is facilitated by the transmembrane unit (S2). In the course of membrane fusion, the fusion peptide (FP) inserts into the host cell membrane (fusion-intermediate stage) and the heptad repeats (HRs) in the transmembrane unit form a the six-helix bundle (6-HB) structure. As a consequence, the viral and host cell membrane are in close proximity, facilitating their fusion. Figure was taken from [Heald-Sargent and Gallagher 2012].

### 3.7 Host cell proteases and enzymatic processing of viral glycoproteins

Post-translational modification of membrane and secretory proteins by endo- and carboxypeptidases is an important requirement for their biological function, for example during viral infection [Klenk and Garten 1994, White *et al.*, 2008]. Cleavage of viral class I

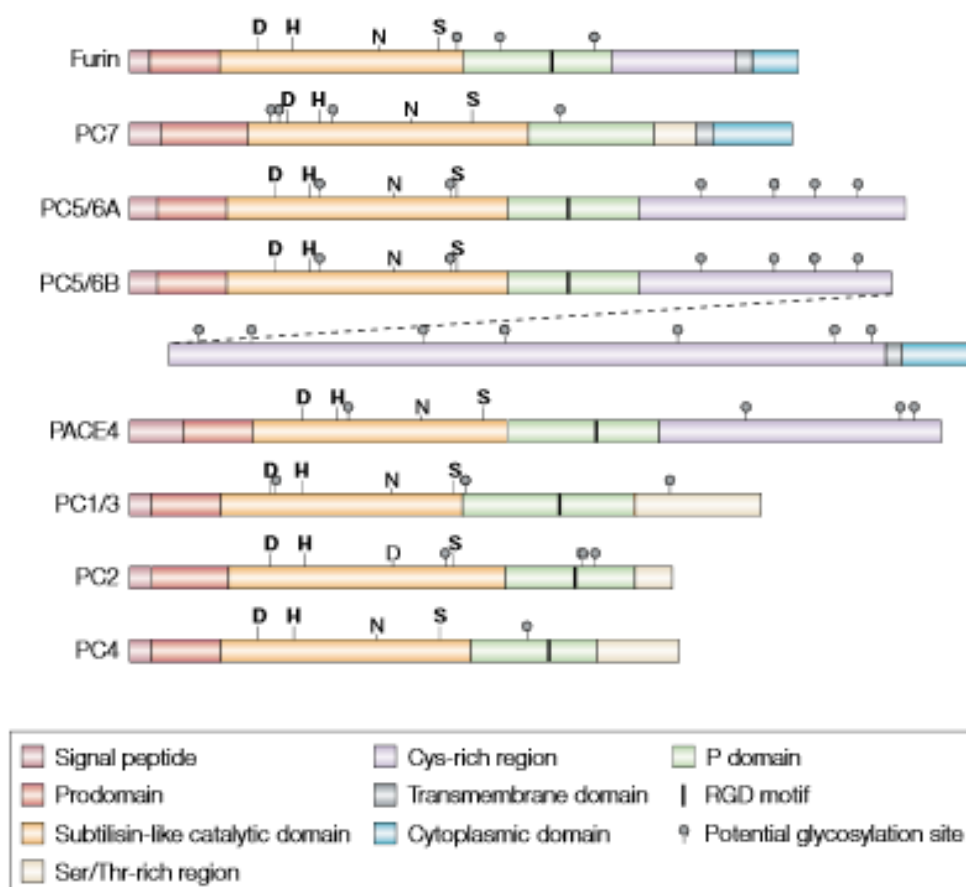
membrane fusion proteins by host cell proteases is a prerequisite for viral infectivity and can proceed at different stages of the viral replication cycle and in different cellular locations:

The HA of the influenza virus can be primed in the Golgi apparatus by subtilisin-like proteases [Stieneke-Grober *et al.*, 1992, Klenk and Garten 1994], by serine proteases in the endosomal compartment [Boycott *et al.*, 1994], at the cell surface [Böttcher *et al.*, 2006], and by soluble proteases in the extracellular compartment [Lazarowitz and Choppin 1975]. The spike protein of some coronaviruses, predominantly members of group II and III [Gallagher and Buchmeier 2001], are cleaved in the *trans*-Golgi compartments in infected cells by proprotein convertases [Yamada and Liu 2009] into the S1 and S2 subunit to become fusion competent [de Haan *et al.*, 2004]. Proteolytic processing was also demonstrated for the SARS-S protein [Wu *et al.*, 2004] but seems to be inefficient [Simmons *et al.*, 2011]. Alternatively, proteolytic activation of the spike protein can take place in endosomal compartments [Matsuyama *et al.*, 2005, Simmons *et al.*, 2005, Bosch *et al.*, 2008], which is also true for the Ebolavirus glycoprotein [Chandran *et al.*, 2005]. However, recent findings suggest that activation in endosomes can be bypassed by expression of transmembrane serine proteases which activate S proteins before they are transported into the endosomal compartment [Matsuyama *et al.*, 2010, Glowacka *et al.*, 2011, Shulla *et al.*, 2011]. When the experimental work documented in the present thesis begun, it was unknown which proteases activate the MERS-S protein and in which cellular compartments the activation reaction takes place.

### **3.7.1 Proprotein Convertases**

Proprotein convertases (PCs) constitute a mammalian enzyme family which plays a major role in sustaining cellular homeostasis and maturation processes of viral glycoproteins [Wise *et al.*, 1990, Pasquato *et al.*, 2013]. PCs are calcium-dependent serine proteases that are widely expressed and comprise nine members, one of them for example furin (Figure 8). Four PC members are attached to cellular membranes, while the others are either secreted or stored in core granules [Seidah and Prat 2012]. They exhibit a characteristic domain organization: The signal peptide is followed by an N-terminal prodomain, a structurally conserved catalytic domain, and variable C-terminal domain (Figure 8) [Pasquato *et al.*, 2013]. PCs are synthesized as zymogens and require two subsequent cleavages at the N-terminus. First, the

signal peptide is removed in the ER, followed by cleavage of the N-terminal prodomain in order to become functional. The autoproteolytic processing can occur in various sub-cellular locations, including the *trans*-Golgi network, the ER/cis Golgi, the dense core secretory granules or the cell membrane [Basak *et al.*, 1999, Toure *et al.*, 2000, Rousselet *et al.*, 2011]. Most PCs recognize the motif K/RXnK/R↓ in target proteins. PCs cleave and prime various viral glycoproteins during maturation in the secretory pathway of infected cells. Cleavage usually occurs at the N-terminus of the fusion peptide, thereby liberating the peptide from the surface unit and allowing it to insert into the target cell membrane.



**Figure 8:** Domain organisation of the proprotein convertases. Domains identified in proprotein convertases are indicated. To acquire the specific activity, the zymogen is first cleaved at the signal peptide within the ER, followed by processing of the N-terminal prodomain in an organelle-specific manner. Figure was taken from [Thomas 2002].

Many enveloped viruses depend on processing of their viral glycoproteins by cellular PCs in order to achieve the fusion-active state. The cleavage site in the hemagglutinin proteins of avian influenza viruses is a determinant of virulence. Thus, HA proteins of low pathogenic avian influenza virus strains (LPAI) contain a single basic amino acid at the cleavage site consisting of one single arginine or lysine, which are cleaved by trypsin-like endoproteases. In contrast, the HA of highly pathogenic avian influenza viruses (HPAI) contains a multibasic cleavage site, which harbors several arginine or lysine residues and is recognized by PCs, particularly by furin [Bosch *et al.*, 1981, Stieneke-Grober *et al.*, 1992, Vey *et al.*, 1992]. This diversity in cleavage sites of avian influenza viruses correlates with pathogenicity, because viral replication is restricted to the expression sites of the responsible proteases. The HIV-1 envelope glycoprotein precursor gp160 is processed by furin-like proteases into gp41 and gp120 [Hallenberger *et al.*, 1992] and so is the glycoprotein of most Ebolaviruses, which contain a consensus furin site, but cleavage was reported to be dispensable for membrane fusion [Volchkov *et al.*, 1998, Neumann *et al.*, 2002, Neumann *et al.*, 2007]. The spike proteins of certain beta- and gammacoronaviruses like MHV-A59 are also cleaved by PCs and cleavage was found to be required for cell-cell but not for virus-cell fusion [Frana *et al.*, 1985, Spaan *et al.*, 1988, de Haan *et al.*, 2004]. Other MHV strains, like MHV-2, express spike proteins that are not cleaved during biogenesis in the constitutive secretory pathway of infected cells, but are processed by endosomal proteases during viral entry into target cells [Qiu *et al.*, 2006]. One report indicates that the SARS-S protein is processed by PC in infected cells, although in an inefficient manner and that processing is required for viral spread [Bergeron *et al.*, 2005]. Regarding the MERS-S protein, it is at present not known whether it is cleaved during maturation by proprotein convertases and whether cleavage is required for viral infectivity.

### **3.7.2 Cathepsin L**

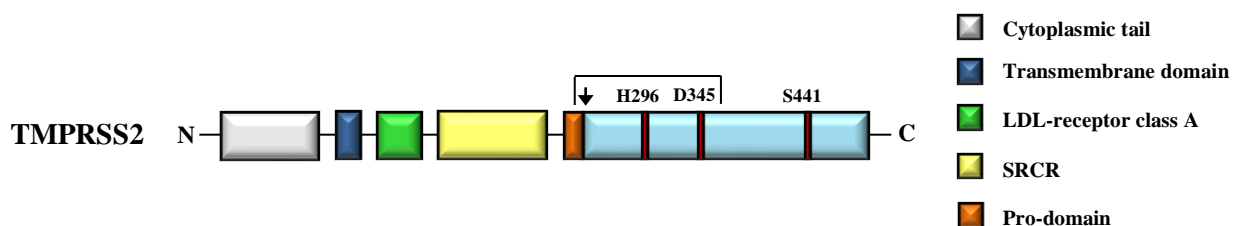
Cathepsins belong to the lysosomal cysteine proteases and are required for the degradation of polypeptides, which had previously been taken up into lysosomes [Kominami *et al.*, 1988, Stoka *et al.*, 2005]. Members are cathepsin B, C, H, F, L, K, O, S, V, X and W [Turk *et al.*, 2000, Turk *et al.*, 2001]. Some cathepsins are expressed ubiquitously (B, H, L), while the expression of others is restricted to a specific tissue [Stoka *et al.*, 2005]. Most cathepsins are

monomers and have a high sequence-and folding homology. They contain a cysteine and histidine residue in the active center and require acidic pH to gain optimal activity [Turk *et al.*, 2001]. Cathepsins are synthesized in the ER as precursor proteins (procathepsins) and are glycosylated [Kominami *et al.*, 1988]. After translocation into lysosomes, they undergo an autocatalytic cleavage. Cathepsin L (CTSL) is a lysosomal endopeptidase and belongs to the papain family. It plays a major role in antigen processing, tumour invasion and metastasis, bone resorption and degradation of proteins [Kirschke *et al.*, 1979, Kane and Gottesman 1990, Ishidoh and Kominami 1995]. CTSL also processes viral glycoproteins: Its role in viral entry and disassembly of nonenveloped reoviruses and the enveloped Ebolavirus has been well described, though the latter one depends more primarily on cathepsin B [Ebert *et al.*, 2002, Chandran *et al.*, 2005]. Simmons and colleagues showed that CTSL can cleave the SARS-S protein and that CTSL activity is required for spike protein-driven host cell entry (see Figure 10 in summary section) [Simmons *et al.*, 2005]. In the light of these data, the previously documented finding that SARS-CoV entry requires low pH [Yang *et al.*, 2004] had to be reinterpreted: The dependence on low pH does not reflect activation of the spike protein by protonation, as is the case for many other viral glycoproteins, but is due to CTSL requiring a low pH environment for its activity (Figure 10). In contrast, cellular entry of human CoV-NL63, which employs the same cellular receptor for entry as SARS-CoV, was largely independent of CTSL activity [Hofmann *et al.*, 2006, Huang *et al.*, 2006]. The site that CTSL cleaves in the SARS-S protein was mapped to the S1-S2-boundary region where the glycoproteins of other coronaviruses are cleaved by furin. However, it is currently unclear whether inactivation of this site interferes with viral infectivity [Bosch *et al.*, 2008]. Furthermore, HCoV-229E and certain MHV strains are activated by CTSL [Qiu *et al.*, 2006, Kawase *et al.*, 2009]. The role of CTSL activity in the viral entry driven by the MERS-S glycoprotein is currently not known.

### **3.7.3 Type II transmembrane serine proteases (TTSPs)**

Serine proteases encompass nearly one-third of all proteolytic enzymes known so far and have an important role in a variety of biological processes, like digestion, immunity, blood coagulation and inflammation [Choi *et al.*, 2009]. Serine proteases comprise secreted enzymes like trypsin and chymotrypsin and membrane-anchored enzymes like the type I and

type II serine proteases (TTSPs), which are subdivided into many families. Among the membrane-anchored serine proteases, type II transmembrane serine proteases have recently been shown to process viral glycoproteins (see below) [Böttcher *et al.*, 2006, Bugge *et al.*, 2009, Chaipan *et al.*, 2009, Antalis *et al.*, 2011]. TTSPs are also involved in regulatory processes like development and homeostasis, and dysregulated TTSP expression is linked to cancer development [Antalis *et al.*, 2011]. TTSPs are synthesized as single-chain zymogens and are autoproteolytically activated by cleavage in a conserved motif preceding the catalytic domain [Bugge *et al.*, 2009]. They are inserted into the plasma membrane with the transmembrane domain. The large extracellular C-terminus plays a decisive role in protein localization, activation, inhibition and substrate specificity and consists of a stem region that has regulatory functions and a serine protease domain [Hooper *et al.*, 2001, Szabo and Bugge 2008, Antalis *et al.*, 2011]. The domain structure of the stem region is variable and its composition has been used to define four TTSP subfamilies: The hepsin/TMPRSS-, matriptase-, corin- and HAT/DESC-subfamily [Bugge *et al.*, 2009, Antalis *et al.*, 2011]. One member of the hepsin/TMPRSS subfamily is the protease TMPRSS2, which contains a group A scavenger receptor domain in the stem region, followed by a single LDLRA domain (low-density lipoprotein receptor domain class A) (Figure 9) [Paoloni-Giacobino *et al.*, 1997, Antalis *et al.*, 2011].



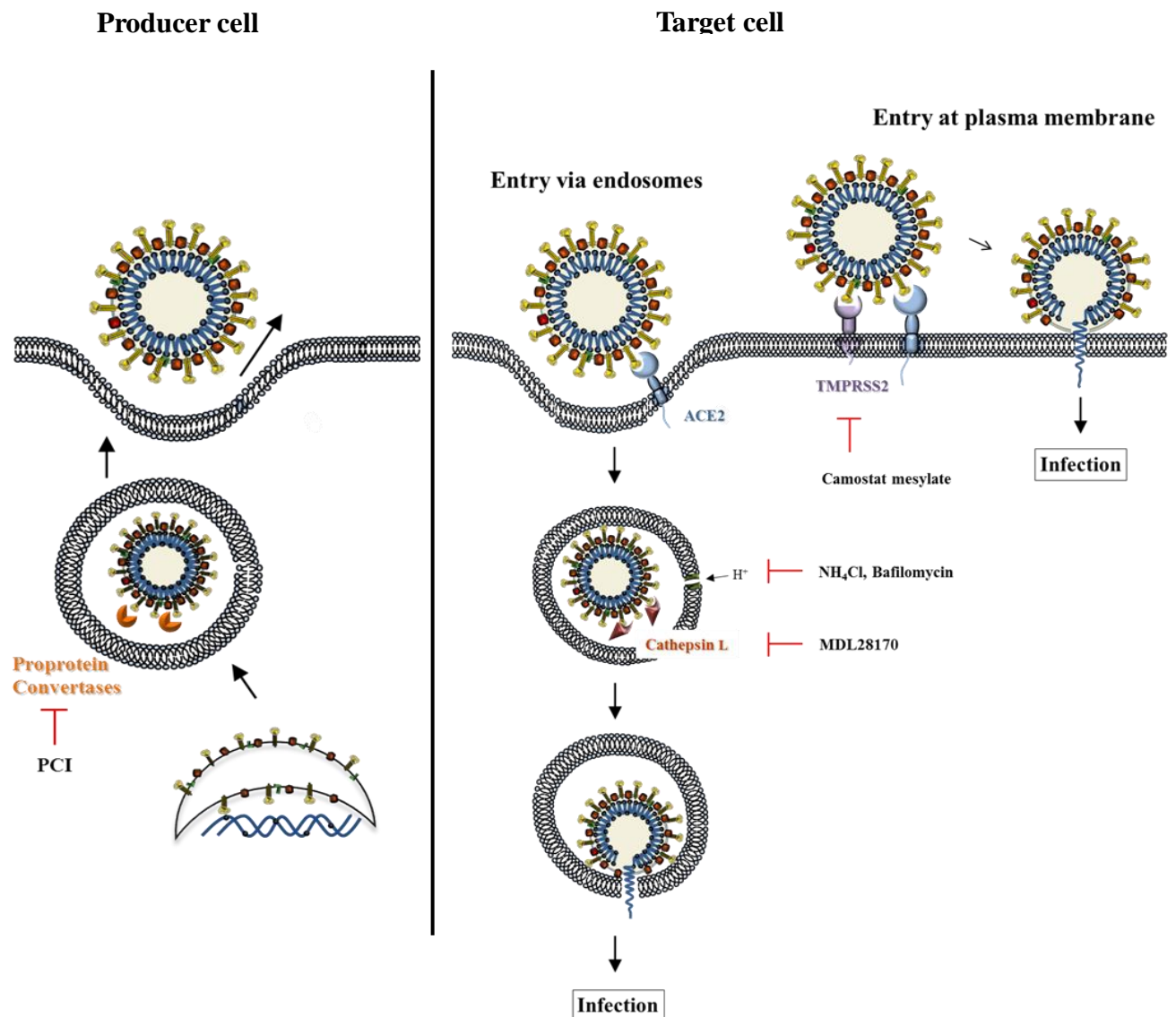
**Figure 9:** Domain organisation of the type II transmembrane serine protease family member TMPRSS2. The domain organisation is as follows: N-terminal cytoplasmic tail, transmembrane domain, stem region, including LDL-receptor class A domain and scavenger receptor cysteine-rich domain (SRCR), catalytic domain. The catalytic domain consists of a catalytic triad with a serin, histidine and aspartate residue. The cleavage site between the pro-and catalytic domain is indicated by an arrow; both domains are covalently connected by a disulphide bond.

Human TMPRSS2 is expressed to a high amount in the epithelia cells of prostate and lung, and lower levels of TMPRSS2 mRNA have been found in epithelia of breast, kidney, colon, small intestine, pancreas, ovary and stomach [Lin *et al.*, 1999, Jacquinet *et al.*, 2000] as well as in type II pneumocytes [Donaldson *et al.*, 2002]. The physiological roles of TMPRSS2 are currently not known, however studies revealed that it has an impact on epithelial sodium channel current and on the development of prostate cancer [Lin *et al.*, 1999, Lucas *et al.*, 2008]. Moreover, a secreted form of TMPRSS2 plays a role in the regulation of sperm function [Donaldson *et al.*, 2002, Chen *et al.*, 2010]. A landmark study by Böttcher and colleagues showed that TMPRSS2 activates the HA proteins of all influenza virus subtypes previously pandemic in humans [Böttcher *et al.*, 2006] and subsequent work demonstrated that also the HA of the highly pathogenic 1918 influenza virus can be activated by TMPRSS2 [Chaipan *et al.*, 2009]. In addition, it was shown that the TTSP members TMPRSS4, HAT [Böttcher *et al.*, 2006, Chaipan *et al.*, 2009], MSPL, TMPRSS13 and matriptase can activate HA proteins [Okumura *et al.*, 2010, Hamilton *et al.*, 2012, Baron *et al.*, 2013, Beaulieu *et al.*, 2013]. TMPRSS2 also activates the surface protein of human metapneumovirus [Shirogane *et al.*, 2008] and promotes CTSL-independent SARS-S glycoprotein-driven cell-cell and virus-cell fusion by cleaving the spike protein (Figure 10) [Matsuyama *et al.*, 2010, Glowacka *et al.*, 2011, Shulla *et al.*, 2011, Kawase *et al.*, 2012]. The fact that SARS-S-mediated entry can be efficiently inhibited by an HR2 peptide in TMPRSS2 positive but not in TMPRSS2 negative cells suggests that TMPRSS2 activates SARS-S at the cell surface [Matsuyama *et al.*, 2010]. In contrast, coexpression of TMPRSS2 and spike in the same cell does not lead to spike activation but instead results in shedding of the spike ectodomain into the extracellular space where it can function as an antibody decoy [Glowacka *et al.*, 2011]. Besides TMPRSS2, HAT can also activate the SARS-S protein for cell-cell fusion but not for CTSL-independent virus-cell fusion [Kam *et al.*, 2009, Bertram *et al.*, 2011]. Finally, TMPRSS4 was shown to activate SARS-S for cell-cell fusion but no evidence for spike protein processing by TMPRSS4 was obtained [Glowacka *et al.*, 2011] Which proteases activate the MERS-S protein was unknown when the studies, summarized in this thesis, were started.



## Summary

Class I viral membrane proteins are synthesized as inactive precursors and depend on cleavage by host cell proteases to acquire activity, and the responsible enzymes are targets for therapeutic intervention.



**Figure 10:** Enzymatic processing of SARS-S by host cell proteases. In the infected producer cell, the S protein is primed during maturation in the secretory pathway by proprotein convertases (left), which can be blocked by proprotein convertase inhibitor. The virus is then released by fusion of virion-containing vesicles with the plasma membrane. During entry process into the target cell (right), the S protein of SARS-CoV has to bind its cellular receptor, ACE2, which results in virion uptake into endosomes. Inside the endosome, the spike protein is activated by the pH dependent cysteine protease cathepsin L, which can be blocked by ammonium chloride, bafilomycin A1 and MDL28170. However, the spike protein can also be activated directly at the cell surface by TMPRSS2, which can in turn be inhibited by camostat mesylate.

It has been shown that coronavirus spike proteins can be activated by different enzymes and in different cellular locations: During biogenesis in the secretory pathway, by type II transmembrane serine proteases at or near the surface of host cells and by the cysteine protease CTSL upon uptake of virions into host cell endosomes (Figure 10). Which pathway is chosen by MERS-CoV for proteolytic activation is unknown.

#### 4. Aims of the study

The ongoing *Middle East Respiratory Syndrome Coronavirus* (MERS-CoV) epidemic is associated with a high case-fatality rate and poses a significant threat to public health. At present, no approved antivirals or vaccines are available to combat MERS-CoV infection and the establishment of novel antiviral strategies is an important task. The surface protein spike (S) of coronaviruses binds to host cell receptors and facilitates infectious entry of virions into target cells, and the viral and cellular components involved in the entry process are potential targets for intervention. For instance, host cell proteases, which activate S proteins, afford targets for antiviral approaches. Moreover, the S protein is the major target for neutralizing antibodies, which are a central, diagnostic marker for MERS-CoV infection and can be used to determine MERS-CoV seroprevalence of populations at risk – an important task of MERS research. Therefore, it is important to investigate how MERS-CoV S (MERS-S) facilitates host cell entry and to exploit S protein-based experimental systems for diagnostic purposes.

Three aims were defined for the present study: The first aim was to establish a vector system, which allows analysing MERS-S-driven entry in a convenient and safe fashion without the requirement to work with authentic MERS-CoV. This system was to be used to investigate key features of MERS-S-driven entry, in particular the cell tropism, pH dependence and sensitivity towards antibody-mediated neutralization.

The activation of coronavirus S proteins by host cell proteases is essential for viral infectivity. The second aim was to identify the proteases which activate MERS-S and to determine whether known proteases inhibitors can block these enzymes and thereby inhibit MERS-S-driven entry.

The third aim was to employ the vector system to determine the MERS-CoV seroprevalence in Saudi Arabian patients before the MERS outbreak in 2012. The goal of this study was to clarify whether MERS-CoV has been circulating undetected before the first MERS cases were diagnosed in 2012, which would suggest that the virus might be able to cause mild or asymptomatic infections.

5. Manuscripts

Title

**The spike-protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2 and is targeted by neutralizing antibodies**

Journal of Virology May 2013; 87(10): 5502-5511.

Title

**Inhibition of proprotein convertases abrogates processing of the MERS-coronavirus spike protein in infected cells but does not reduce viral infectivity**

Journal of Infectious Diseases July 2014. pii: jiu 407.

Title

**Lack of MERS coronavirus neutralizing antibodies in patients from Eastern Province, Saudi Arabia**

Emerging Infectious Diseases, Dec 2013; 19(12): 2034-2036

**First Manuscript**



## The Spike Protein of the Emerging Betacoronavirus EMC Uses a Novel Coronavirus Receptor for Entry, Can Be Activated by TMPRSS2, and Is Targeted by Neutralizing Antibodies

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The novel human coronavirus EMC (hCoV-EMC), which recently emerged in Saudi Arabia, is highly pathogenic and could pose a significant threat to public health. The elucidation of hCoV-EMC interactions with host cells is critical to our understanding of the pathogenesis of this virus and to the identification of targets for antiviral intervention. Here we investigated the viral and cellular determinants governing hCoV-EMC entry into host cells. We found that the spike protein of hCoV-EMC (EMC-S) is incorporated into lentiviral particles and mediates transduction of human cell lines derived from different organs, including the lungs, kidneys, and colon, as well as primary human macrophages. Expression of the known coronavirus receptors ACE2, CD13, and CEACAM1 did not facilitate EMC-S-driven transduction, suggesting that hCoV-EMC uses a novel receptor for entry. Directed protease expression and inhibition analyses revealed that TMPRSS2 and endosomal cathepsins activate EMC-S for virus-cell fusion and constitute potential targets for antiviral intervention. Finally, EMC-S-driven transduction was abrogated by serum from an hCoV-EMC-infected patient, indicating that EMC-S-specific neutralizing antibodies can be generated in patients. Collectively, our results indicate that hCoV-EMC uses a novel receptor for protease-activated entry into human cells and might be capable of extrapulmonary spread. In addition, they define TMPRSS2 and cathepsins B and L as potential targets for intervention and suggest that neutralizing antibodies contribute to the control of hCoV-EMC infection.

Human coronaviruses were long considered agents of mild respiratory disease, with the prototype viruses 229E and OC43 being responsible for up to 30% of common cold cases requiring medical attention (1, 2). However, the outbreak of the severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002–2003 abruptly changed this view. The spread of the novel virus claimed more than 700 lives, predominantly elderly and immunocompromised individuals, and caused massive economic damage (3). SARS-CoV-related viruses were detected in bats, and it is believed that these animals served as a natural reservoir (4, 5) from which the virus was transmitted via intermediate hosts, such as palm civets (6), to humans. Thus, although most human coronaviruses known today (OC43, 229E, NL63, and HKU1) circulate worldwide and cause mild respiratory disease (7), the zoonotic transmission of novel coronaviruses to humans can pose a significant threat to public health.

A novel coronavirus, termed hCoV-EMC (8), recently emerged in the Middle East, and so far 13 laboratory-confirmed cases have been reported to the WHO, including 6 from Saudi Arabia, 2 from Qatar, 2 from Jordan, and 3 from the United Kingdom (9, 10). The cases from the United Kingdom cluster within one family, with the initial individual but not the subsequent ones having a history of travel to Pakistan and Saudi Arabia (10), suggesting that human-to-human transmission occurred. Disquietingly, the new virus shares several similarities with SARS-CoV. First, hCoV-EMC appears to be highly pathogenic, with 7 of the 13 identified cases having a fatal outcome, and infection induces a severe acute respiratory disease (8, 9). Second, the virus, like SARS-CoV, belongs to the betacoronavirus genus and might have

been transmitted from bats to humans (8), as suggested by its close relatedness to the bat coronaviruses HKU4 and HKU5 and the isolation of hCoV-EMC-related viruses in bats from Ghana and Europe (11). At present, there is no evidence for efficient interindividual transmission of hCoV-EMC (9). However, a few adaptive amino acid changes might be sufficient to allow hCoV-EMC to spread rapidly within the human population, with potentially severe consequences. Therefore, it is imperative to elucidate hCoV-EMC interactions with host cells and to transform this knowledge into effective antiviral strategies.

The interaction of the coronavirus spike (S) protein with host cell receptors and proteases is essential for the first step in coronavirus infection, i.e., viral invasion of host cells (12, 13). The binding of the S protein to host cell receptors attaches viruses to target cells and is a major determinant of the viral cell and organ tropism (14). Two receptors for human coronaviruses have been identified so far, namely, CD13 (used by hCoV-229E) (15) and ACE2 (used by SARS-CoV and hCoV-NL63) (16, 17). Moreover, sialic acid has been described as a receptor determinant of hCoV-OC43 (18), and the coronavirus murine hepatitis virus (MHV) was shown to

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engage murine but not human CEACAM1 for cellular entry (19), although it is worth noting that MHV host range mutants which employ human CEACAM1 for cellular entry have been reported (20, 21). The S proteins are synthesized as inactive precursors and transform into an active state upon proteolytic cleavage (12, 13). The activity of the pH-dependent endosomal cysteine proteases cathepsin B and, particularly, cathepsin L was found to be required for entry of SARS-CoV (22) and hCoV-229E (23) into certain host cells, and evidence for S-protein proteolysis by cathepsins was provided (22). However, recent work indicates that the type II transmembrane serine proteases (TTSPs) TMPRSS2 and HAT can cleave the SARS-CoV S protein (SARS-S) and that SARS-S processing by TMPRSS2 allows for cathepsin B/L-independent virus-cell fusion (24–28). Which receptors and proteases are used by hCoV-EMC for attachment and activation is unknown at present.

Here we investigated the viral and cellular determinants of hCoV-EMC entry into host cells. We show that the hCoV-EMC S protein (EMC-S) mediated transduction of a broad range of human target cells when the protein was incorporated into lentiviral particles. EMC-S did not engage known coronavirus receptors for cellular entry and was activated by TMPRSS2 and endosomal cathepsins. Finally, transduction was inhibited by serum from an hCoV-EMC-infected patient, indicating that S-protein-specific neutralizing antibodies are generated in the context of hCoV-EMC infection.

## MATERIALS AND METHODS

**Cell culture.** Most of the adherent cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen) supplemented with 5 to 10% fetal calf serum (FCS; Blochrom) and 1% penicillin-streptomycin sulfate (Cytogen). 293T cells genetically engineered to stably express human ACE2 (hACE2; 293T-ACE2 cells) (29) or hCD13 (293T-CD13 cells) (S. Bertram et al., submitted for publication) were maintained in DMEM supplemented with 100  $\mu$ g/ml Zeocin (Invitrogen). Caco-2 cells were cultured in DMEM-GlutaMAX medium (Invitrogen) supplemented with 10% FCS. The promonocytic cell line THP-1 was grown in RPMI 1640 medium (Gibco) supplemented with 10% FCS and antibiotics and differentiated into a macrophage-like culture by induction with phorbol myristate acetate (PMA) (20 ng/ml; Sigma) for 48 h. For generation of primary human monocytes, peripheral blood mononuclear cells were purified by Ficoll gradient centrifugation and cultured in monocyte adherence medium (RPMI 1640 medium with 7.5% human fibrin-depleted plasma) for 24 h. Thereafter, adherent cells were resuspended in monocyte differentiation medium (X-Vivo 10 medium [Lonza] supplemented with 1% human fibrin-depleted plasma and 1% penicillin-streptomycin sulfate), seeded into 96-well plates at a density of  $7.5 \times 10^4$  cells/well, and allowed to differentiate into monocyte-derived macrophages for at least 7 days before usage for transduction experiments. Microscopic inspection of cells used for transduction revealed a clear macrophage-like morphology. All cells were grown in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>.

**Plasmids and antibodies.** Expression plasmids for SARS-S, 229E-S, Zaire ebolavirus glycoprotein (EBOV-GP), vesicular stomatitis virus glycoprotein (VSV-G), and murine leukemia virus envelope protein (MLV-Env) have been described previously (30, 31). Plasmids encoding the transmembrane proteases TMPRSS2, TMPRSS4, TMPRSS3, TMPRSS6, and HAT were published earlier (24, 32–34). An expression plasmid encoding EMC-S (EMC-S FLAG) with a C-terminal FLAG tag was generated by reverse transcription, using RNAs from CoV-EMC-infected cell cultures and the oligonucleotides 2c-nhCoV-S-BamHI-F (TACGGATCCG CCACCATGATACACTCAGTGTCTTCTACTGATGT) and 2c-nhCoV-SflagC-Sall-R (AGCGTCGACTTACTTGTGCATCGTCATCCTTGTAAAT CGCCTCGTGAACATGAACCTTATGCGGC), followed by ligation of

the PCR fragment into plasmid pCG1. The resulting plasmid was used as a template for amplification of the EMC-S open reading frame without a tag, using oligonucleotides p5-BetaCoV\_Kpn (GATCGGTACCACCATG ATACACTCAGTGTCTTCTACTG) and p3-BetaCoV\_Xho (GATCCTCG AGTTAGTGAACATGAACCTTATGCGGCTCTAGG). The PCR amplicon was then inserted into the plasmid pCAGGS by using the KpnI and XhoI restriction sites. The resulting plasmid expresses EMC-S with a sequence identical to that deposited under GenBank accession number AFS88936. To generate EMC-S with a V5 tag, the C-terminal sequence of EMC-S was amplified using oligonucleotides BetaCoV\_seq 2567 (CTCA ATCATCTCCTATCATACCAGG) and p3-BetaCoV\_Xho\_V5 (GATCCT CGAGTTACGTAGAATCGAGACCGAGGAGGGTTAGGGATAGG CTTACCGTGAACATGAACCTTATGCGGCTCTAGG), followed by replacement of the corresponding sequence within the pCAGGS-EMC-S plasmid by use of SphI and XhoI. The integrity of all PCR-amplified nucleotide sequences was verified by automated sequence analysis. For generation of lentiviral virus-like particles (VLPs), plasmid p96ZM651gag-opt, encoding human immunodeficiency virus type 1 Gag (p55), was employed (35). A V5-reactive monoclonal antibody was obtained from Invitrogen: HIV Gag proteins were detected using the hybridoma 183-H12-5C cell culture supernatant (NIH AIDS Reagent Program). Secondary antibodies were purchased from Dianova. An hCoV-EMC-reactive serum was isolated from a blood sample obtained from an hCoV-EMC-infected patient treated in a hospital in Essen, Germany.

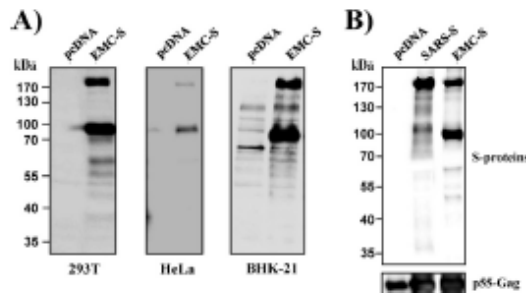
**Production and analysis of VLPs.** VLPs were generated as described previously (25). Briefly, plasmid p96ZM651gag-opt was coexpressed with either an empty plasmid as a control or plasmids encoding the S proteins of SARS-CoV and hCoV-EMC, followed by concentration of supernatants by ultrafiltration using Vivaspin centrifugal concentrators (Sartorius) and purification of VLPs by centrifugation of supernatants through a 20% sucrose cushion at  $13,000 \times g$  for 2 to 3 h at 4°C. Concentrated VLPs were treated with trypsin (100  $\mu$ g/ml) for 10 min at room temperature, followed by the addition of the same concentration of soybean trypsin inhibitor (Sigma). The samples were then analyzed by Western blotting, employing a monoclonal antibody directed against the V5 tag.

**Production and analysis of lentiviral pseudotypes.** Lentiviral pseudotypes were generated and harvested from the culture supernatants of transfected 293T cells as described previously (31, 36). The supernatants were harvested, passed through 0.45- $\mu$ m-pore-size filters, and stored at  $-80^\circ\text{C}$ . Before usage in experiments, pseudotypes were normalized for equal infectivity by transduction of Caco-2 cells with serially diluted supernatants followed by determination of luciferase activity in cell lysates according to the manufacturer's instructions (Promega). Normalization of the viral p24 capsid protein content was performed using a commercially available HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) (HIV-1 p24 antigen capture assay; Advanced BioScience Laboratories).

**Neutralization assay.** The neutralizing activity of serum obtained from an hCoV-EMC-infected patient treated in Essen, Germany, was measured by preincubation of lentiviral pseudotypes with defined serum dilutions in triplicate for 30 min at 37°C in a total volume of 50  $\mu$ l. Thereafter, the mixture was added to  $10^4$  Caco-2 cells in 96 wells for 8 h, followed by complete exchange of the culture medium. After 72 h, the reduction in transduction efficiency was measured by quantifying luciferase activities in cell lysates.

**Inhibition studies.** The inhibitors MDL28170 (Calbiochem), camostat mesylate (Toctris), leupeptin, and ammonium chloride (Sigma) were diluted in solvent as recommended by the manufacturers and used at the indicated concentrations. Within the experiments, the volume of solvent was kept constant, and solvent without inhibitor was used as a control. Target cells were pretreated with the inhibitors for 60 min at 37°C and transduced with infectivity-normalized pseudotypes in the presence of inhibitor. The inhibitor-containing medium was replaced by fresh culture medium without inhibitor at 8 h posttransduction, and transduction efficiency was measured after 72 h.

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**FIG 1** EMC-S is expressed in transfected cells and incorporated into lentiviral particles. (A) Plasmids encoding EMC-S with a C-terminal tag were transfected into 293T, HeLa, and BHK-21 cells, as indicated. The transfected cells were lysed, and the lysates were analyzed by Western blotting using tag-specific monoclonal antibodies. Cells transfected with empty plasmid (pcDNA) served as a negative control. (B) SARS-S or EMC-S with a C-terminal V5 tag was coexpressed with HIV-1 Gag protein in 293T cells. Cells expressing Gag-Pol alone were used as a control (pcDNA). At 48 h posttransfection, virus-like particles were harvested, concentrated via ultrafiltration and high-speed centrifugation through a sucrose cushion, and analyzed by Western blotting using a V5-specific antibody and a p55-Gag-reactive antibody as a loading control. The results are representative of at least three independent experiments.

**RESULTS**

**The human coronavirus EMC spike protein is expressed in transfected 293T cells and incorporated into retroviral particles.** Retroviral particles pseudotyped with S proteins (pseudotypes) have been used successfully to characterize the host cell entry of coronaviruses, including SARS-CoV (37–39). Therefore, we asked if pseudotyping can be employed to study the cellular entry driven by EMC-S. Efficient expression of viral envelope proteins in transfected cells is a prerequisite for pseudotyping. Western blot analysis of 293T, HeLa, and BHK-21 cells transfected to express EMC-S with a C-terminal antigenic tag revealed two prominent bands, of 170 and 100 kDa, and a minor band, of 60 kDa, which were not detected in control cells transfected with empty plasmid, indicating that the S protein was expressed efficiently (Fig. 1A). The detection of two bands is consistent with cleavage of the S protein (170 kDa) into an N-terminal (not detected) and a C-terminal (100 kDa) subunit by host cell proteases. In order to determine whether efficient EMC-S expression allows for virion incorporation, we coexpressed HIV-1 Gag and EMC-S in 293T cells and analyzed the S-protein incorporation into particles released from these cells. SARS-S was included as a positive control, since we and others previously showed that SARS-S is incorporated into retroviral particles (37–39). Western blot analysis of pseudoparticles released from transfected 293T cells indeed revealed efficient particle incorporation of SARS-S, and the S protein was largely uncleaved, in agreement with published data (37–39) (Fig. 1B). A signal of similar intensity was measured for EMC-S, and again, two prominent bands, of 170 and 100 kDa, were detected (Fig. 1B), indicating that EMC-S and SARS-S are incorporated into retroviral particles with similar efficiencies and that the former but not the latter S protein is efficiently processed by host cell proteases.

**The human coronavirus EMC spike protein mediates entry into cell lines derived from different human tissues.** We next

investigated if retroviral particles pseudotyped with EMC-S were able to transduce human cells. Pseudotypes bearing the G protein of VSV were used as a positive control, since VSV-G facilitates transduction of an extremely broad range of cell types. Bald particles bearing no glycoprotein were used as negative controls (pCAGGS). A vector encoding luciferase was used for pseudotyping, and transduction efficiency was determined by quantifying luciferase activities in the lysates of transduced cells. Pseudotypes bearing VSV-G were able to robustly transduce all cell types tested, while signals measured upon transduction with particles bearing no glycoprotein were within the background range (Fig. 2A), as expected. EMC-S facilitated transduction of Caco-2 (colon), HOS (osteosarcoma), Huh-7 (liver), and MRC5 (lung) cells, while RPE (retina), U373 (glioblastoma), EA-hy (endothelium), and A549 (lung) cells were not susceptible to EMC-S-mediated transduction (Fig. 2A, left panel). Transduction of 293 and 293T (kidney) cells was also observed (Fig. 2A, right panel), although some batches of these cells were not susceptible, potentially due to batch- or culture-dependent differences in the expression of the so far unknown hCoV-EMC receptor. Finally, THP-1 monocytic cells were nonsusceptible to EMC-S-driven transduction but acquired susceptibility upon PMA-induced differentiation into macrophages (Fig. 2B, left panel), suggesting that expression of the EMC-S receptor might be induced upon differentiation of monocytes into macrophages. Indeed, primary human monocyte-derived macrophages could be transduced efficiently by EMC-S-bearing pseudotypes (Fig. 2B, right panel), in agreement with robust receptor expression. In sum, EMC-S mediates transduction of a broad range of target cells, indicating that the viral receptor is expressed in different cell types and organs.

**The spike protein of the human coronavirus EMC does not use known coronavirus receptors for host cell entry.** The following proteinaceous receptors for coronavirus have been identified so far: CD13 (used by hCoV-229E and others [15]), murine CEACAM-1 (used by MHV [19]), and ACE2 (used by SARS-CoV and hCoV-NL63 [16, 17]). We investigated if EMC-S was able to employ one of these receptors for host cell entry. For this purpose, the respective receptors were transiently expressed in 293T cells and the cells subsequently transduced with pseudotypes bearing the S proteins of hCoV-229E (229E-S), MHV (MHV-S), and SARS-CoV. ACE1 was also included in this experiment due to its sequence similarity with ACE2. Expression of the respective coronavirus receptors did not modulate host cell entry driven by VSV-G (Fig. 3). In contrast, ACE2 but not ACE1 expression augmented transduction driven by SARS-S, while expression of CD13 and murine but not human CEACAM-1 facilitated entry driven by 229E-S and MHV-S, respectively (Fig. 3), in keeping with published results (15, 19). Notably, expression of none of the receptors tested augmented transduction driven by EMC-S (Fig. 3), indicating that hCoV-EMC uses a different host cell factor for cellular entry.

**Low pH and cathepsin activity are required for transduction mediated by the human coronavirus EMC spike protein.** The cellular entry of enveloped viruses requires envelope protein-driven fusion of the viral membrane with a host cell membrane. The membrane fusion reaction is energetically unfavorable and can proceed only once the envelope protein receives a trigger, either receptor engagement, low pH, or both (40, 41). In addition, many viral glycoproteins require activation by host cell proteases, including the pH-dependent endosomal cysteine proteases



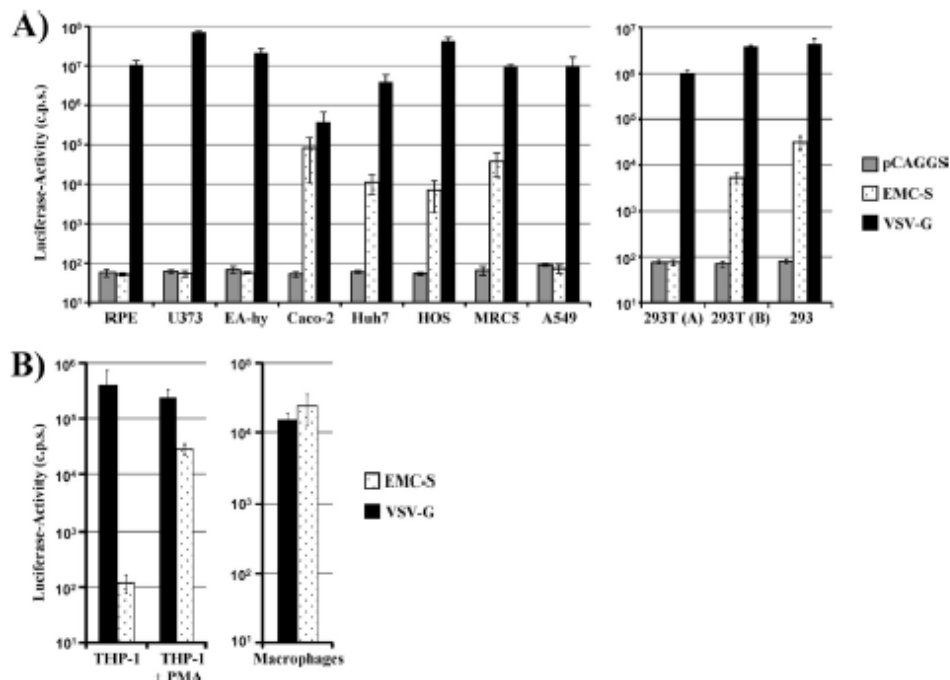


FIG 2 Lentiviral pseudotypes bearing EMC-S transduce a broad spectrum of human cells. Lentiviral pseudotypes carrying the glycoprotein of VSV or EMC as well as pseudotypes bearing no glycoprotein (pCAGGS) were normalized for equal p24 capsid protein content and used for transduction of the indicated human cell lines (A) or unstimulated and PMA-stimulated THP-1 cells and primary human monocyte-derived macrophages (B). Cells were incubated with pseudotypes for 8 h, followed by replacement of the infection medium with fresh culture medium. After 72 h, cell lysates were prepared and analyzed for luciferase activity. The results shown are representative of three (A) or two (B) experiments performed in triplicate, using two independent pseudotype preparations. Error bars indicate standard deviations (SD).

cathepsins B and L, which activate the Ebola virus glycoprotein (EBOV-GP) (42) and SARS-S (22). Entry of viruses which depend on low pH can be inhibited by lysosomotropic agents such as ammonium chloride, and these agents also block entry of viruses

which require cathepsin B/L activity for entry (22). In contrast, protease inhibitors such as MDL28170 specifically block activation of viruses which require cathepsin B/L activity for entry but do not inhibit viruses which are activated by low pH alone (22).

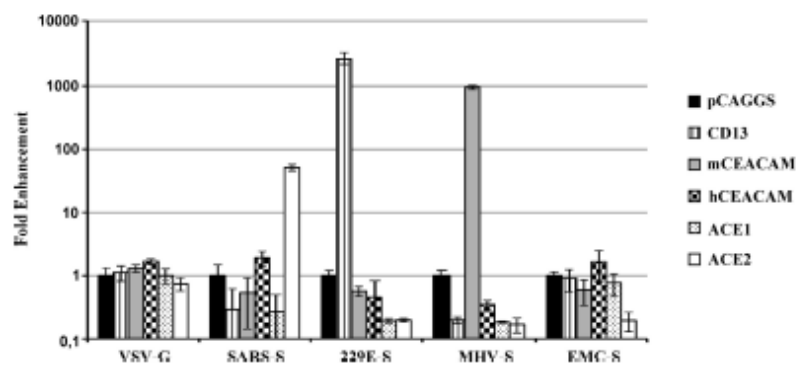


FIG 3 EMC-S does not employ previously described coronavirus receptors for entry into target cells. 293T cells were transiently transfected with empty plasmid (pCAGGS) or plasmids expressing the indicated coronavirus receptors. The cells were then transduced with infectivity-normalized pseudotypes bearing VSV-G, SARS-S, 229E-S, MHV-S, or EMC-S. Luciferase activities in cell lysates were determined at 72 h postinfection. Similar results were obtained in an independent experiment. Transduction of cells transfected with receptor-encoding plasmids is shown as fold enhancement relative to transduction of cells transfected with empty plasmid, which was set as 1.

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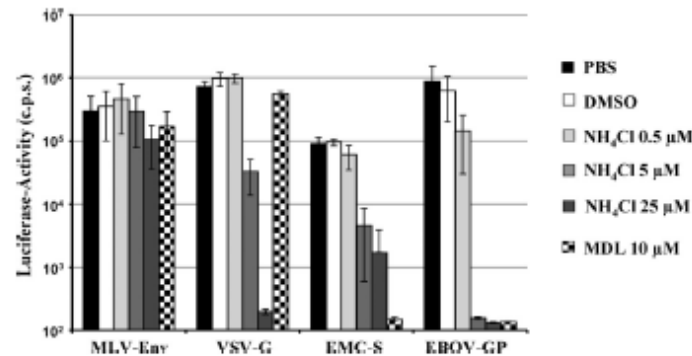


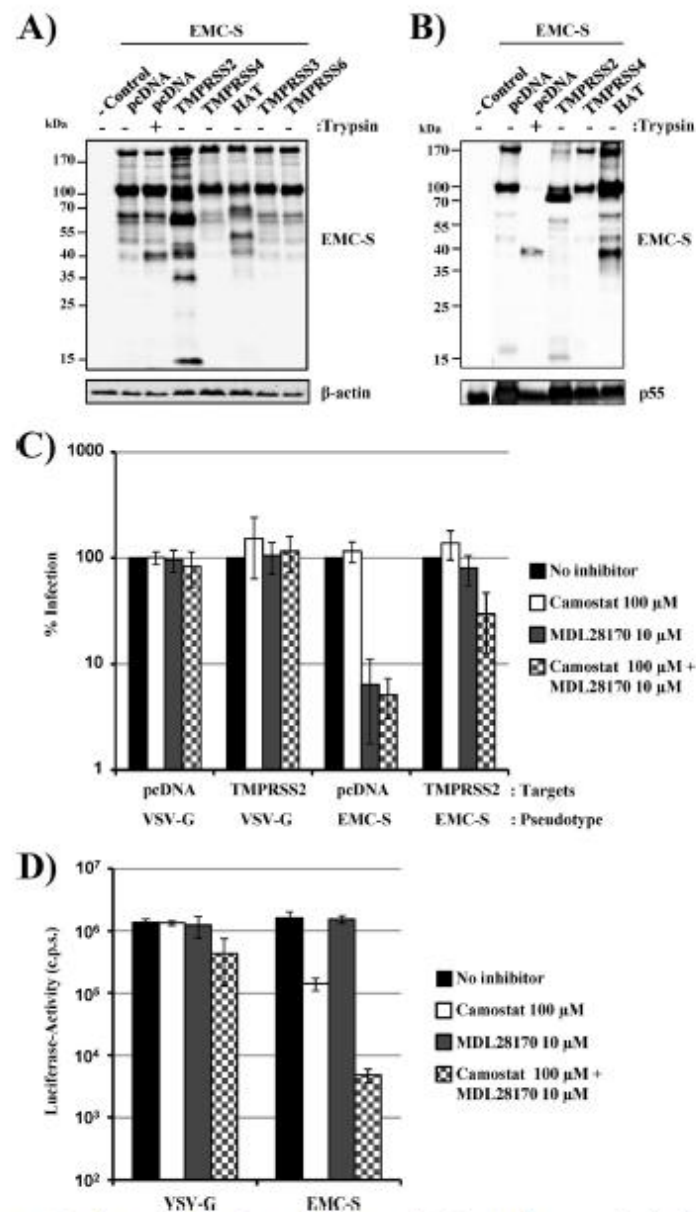
FIG 4 EMC-S-driven cellular entry depends on the activity of cathepsin B and/or L. MRC5 target cells were preincubated with the indicated concentrations of ammonium chloride, MDL28170, dimethyl sulfoxide (DMSO), or phosphate-buffered saline (PBS) for 60 min, followed by inoculation with infectivity-normalized pseudotypes bearing the glycoprotein of VSV, MLV, hCoV-EMC, or EBOV. Luciferase activity in cell lysates was measured at 72 h postinfection. The results of a representative experiment performed in triplicate are shown. Transduction efficiency is shown relative to transduction of PBS-treated cells, which was set as 100%. Error bars indicate SD. Similar results were obtained in another experiment using different pseudotype preparations.

We employed ammonium chloride and MDL28170 to determine which host cell factors trigger EMC-S for virus-cell fusion. The murine leukemia virus envelope protein (MLV-Env) is triggered by receptor engagement for membrane fusion (43, 44), and transduction mediated by MLV-Env was not blocked by any of the agents tested (Fig. 4), as expected. In contrast, VSV-G requires low pH as a trigger for membrane fusion (45), and VSV-G-driven transduction was inhibited by ammonium chloride but not by MDL28170 (Fig. 4), in keeping with published results (22). Transduction by EBOV-GP depends on cathepsin B/L activity (42) and was thus inhibited by both ammonium chloride and MDL28170 (Fig. 4), again in accordance with published data (22, 42). Finally, transduction mediated by EMC-S was also blocked by ammonium chloride and MDL28170 (Fig. 4), indicating that EMC-S, like EBOV-GP and SARS-S, depends on cathepsin B/L activity for host cell entry.

**TMPRSS2 activates the spike protein of human coronavirus EMC for cathepsin B/L-independent host cell entry.** We and others have previously shown that the TTSP TMPRSS2 cleaves SARS-S and that cleavage activates SARS-S for cathepsin B/L-independent host cell entry (25–27). We therefore analyzed if TMPRSS2 can also activate EMC-S for virus-cell fusion. For this purpose, we first asked if TMPRSS2 and other members of the TTSP family cleave EMC-S with a C-terminal antigenic tag when coexpressed in transfected 293T cells. The effect of trypsin on EMC-S was analyzed in parallel. Western blot analysis of cells transfected to express EMC-S in the absence of a protease revealed prominent bands of 170 and 100 kDa and a minor band of approximately 60 kDa (Fig. 5A), in agreement with our previous findings (Fig. 1A). Trypsin treatment produced an additional cleavage fragment of 40 kDa, indicating that a trypsin-sensitive site is located within the S2 portion of EMC-S. Coexpression of TMPRSS2 produced prominent EMC-S cleavage fragments of 90 and 58 kDa, with additional fragments of 15, 35, and 40 kDa (Fig. 1A and 5A). Expression of HAT also resulted in EMC-S proteolysis, but the cleavage fragments differed from those produced by TMPRSS2. Finally, coexpression of TMPRSS3, TMPRSS4, and TMPRSS6 did not result in EMC-S cleavage, despite robust expression of these proteases in the transfected cells, as determined

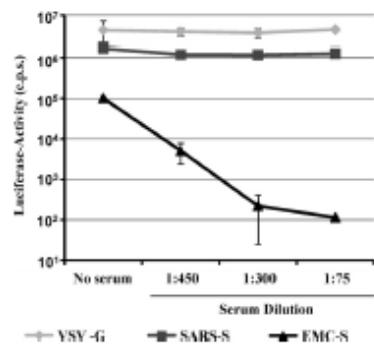
by Western blotting of epitope-tagged proteins (data not shown), indicating that these proteases do not recognize EMC-S as a substrate. Thus, EMC-S is processed by a so far unknown protease expressed in host cells and can additionally be cleaved by trypsin, TMPRSS2, or HAT. Western blot analysis of EMC-S bearing VLPs generated in the presence of trypsin, TMPRSS2, and HAT revealed that EMC-S cleavage products were incorporated into the viral membrane (Fig. 5B). Thus, the EMC-S fragments of 170 and 100 kDa detected in control VLPs were partially replaced by prominent fragments of 40 and 90 kDa in VLPs generated in cells coexpressing HAT and TMPRSS2, respectively (Fig. 5B). Similarly, a predominant fragment of 40 kDa was detected in trypsin-treated VLPs.

In order to assess if cleavage results in EMC-S activation, we performed inhibition analyses with MDL28170, an inhibitor of cathepsin B/L (22), and camostat mesylate (camostat), a serine protease inhibitor active against TMPRSS2 (28). VSV-G-dependent transduction of 293T cells transfected with empty plasmid or a TMPRSS2 expression plasmid was not inhibited by MDL28170, camostat, or a combination of both inhibitors (Fig. 5C), as expected. Similarly, camostat treatment of target cells transfected with empty plasmid had little effect on EMC-S-mediated transduction, although a slight reduction in transduction efficiency was observed. In contrast, incubation of empty plasmid-transfected target cells with MDL28170 alone markedly reduced transduction efficiency, and inhibition was not increased by simultaneous treatment with camostat (Fig. 5C). Notably, inhibition by MDL28170 was fully rescued by expression of TMPRSS2, but entry remained sensitive to inhibition by camostat (Fig. 5C), indicating that TMPRSS2 can activate EMC-S for cathepsin B/L-independent entry. The activation of EMC-S by TMPRSS2 in transfected cells raised the question of which activation pathway dominates in target cells that are naturally susceptible to EMC-S-mediated transduction. We selected Caco-2 cells to address this question, since these cells express endogenous TMPRSS2 (32) and are susceptible to EMC-S-driven transduction (Fig. 2A). None of the inhibitors tested reduced VSV-G-mediated transduction of Caco-2 cells, although a modest reduction was observed when a combination of MDL28170 and camostat was tested (Fig. 5D). Incubation of



**FIG 5** TMPRSS2 activates EMC-S for cathepsin B/L-independent entry into target cells. (A) 293T cells were transfected with empty plasmid (–control) or cotransfected with plasmids encoding EMC-S and the indicated proteases. Forty-eight hours after transfection, cells were either left untreated or treated with trypsin, and lysates were analyzed by Western blotting using a V5 antibody or a  $\beta$ -actin antibody as a loading control. The results are representative of three independent experiments with different plasmid preparations. (B) Incorporation of cleaved EMC-S forms into lentiviral particles was assessed by coexpression of EMC-S (with a C-terminal V5 tag) with the indicated proteases and HIV-1 Gag. Virus-like particles were concentrated from the culture supernatants and analyzed by Western blotting using a V5 antibody, with a p55-Gag antibody used as a loading control. The experiment shown is representative of three independent experiments. (C) 293T cells were transiently transfected with empty plasmid (pcDNA) or a plasmid encoding TMPRSS2 and used as target cells for transduction by lentiviral vectors bearing EMC-S or VSV-G (as a control). Prior to infection, target cells were incubated with the indicated protease inhibitors. Transduction of untreated cells was set as 100%. Error bars indicate standard errors of the means (SEM). (D) Caco-2 cells, which express endogenous TMPRSS2, were incubated with DMSO or the indicated protease inhibitors, followed by transduction with lentiviral pseudotypes bearing EMC-S or VSV-G (as a control). At 72 h postinfection, luciferase activities in cell lysates were measured. The results of a representative experiment performed in triplicate are shown; error bars indicate SD. Comparable results were observed in two independent experiments.

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**FIG 6** Host cell entry driven by EMC-S is neutralized by serum from an hCoV-EMC-infected patient. Pseudotypes bearing the glycoproteins of VSV, SARS-CoV, and hCoV-EMC, normalized for equal infectivity, were preincubated with the indicated serum dilutions in triplicate for 30 min at room temperature. Thereafter, the mixtures were added to 293T-ACE2 cells for 8 h, followed by replacement of the infection medium by fresh culture medium. Luciferase activities were determined after 72 h postinfection. The results shown are representative of three independent experiments. Error bars indicate SD.

Caco-2 cells with camostat reduced EMC-S-mediated transduction about 10-fold, while MDL28170 treatment had no effect, indicating that TMPRSS2-dependent activation prevails over cathepsin B/L-mediated activation in Caco-2 cells (Fig. 5D). However, a combination of camostat and MDL28170 was required to reduce transduction efficiency to background levels (Fig. 5D), indicating that both TMPRSS2 and cathepsin B/L can activate EMC-S for entry into naturally susceptible cells.

**The serum from a human coronavirus EMC-infected patient contains neutralizing antibodies.** The generation of neutralizing antibodies might be important for the control of hCoV-EMC infection, and an efficient method for the detection of such antibodies could be highly useful for diagnostic efforts. Therefore, we tested if EMC-S-mediated transduction could be blocked by serum obtained from an hCoV-EMC-infected patient who survived the disease. The patient serum did not diminish VSV-G- or SARS-S-mediated transduction of 293T-ACE2 cells but reduced EMC-S-mediated transduction up to 1,000-fold, in a concentration-dependent manner (Fig. 6). Thus, neutralizing antibodies are generated in infected, surviving patients and can be detected in a highly efficient and quantitative fashion in the pseudotype transduction system.

## DISCUSSION

The constantly increasing exploitation of natural resources, personal mobility, and climate change facilitate the zoonotic transmission of novel viruses to humans (46), with potentially dramatic consequences, as exemplified by the SARS pandemic. The outbreak of hCoV-EMC in Saudi Arabia raises concerns that a SARS-like pandemic might be about to unfold (47). Assessment of the pandemic potential and the development of specific antiviral measures require an understanding of hCoV-EMC interactions with host cells. Employing lentiviral pseudoparticles, we investigated the viral and cellular factors contributing to hCoV-EMC entry into host cells. We showed that a broad panel of human cell lines is susceptible to EMC-S-driven transduction, suggesting that

the viral receptor is broadly expressed. The nature of the receptor remains to be explored, since none of the known coronavirus receptors augmented EMC-S-dependent transduction. Inhibitors of cathepsins B and L and TMPRSS2 blocked EMC-S-dependent virus-cell fusion, indicating that EMC-S can use redundant proteolytic pathways to ensure its activation. Finally, serum from an hCoV-EMC-infected patient inhibited EMC-S- but not VSV-G- or SARS-S-driven transduction, indicating that S-protein-specific neutralizing antibodies are generated in patients and might contribute to control of hCoV-EMC infection. Our results reveal important insights into the molecular processes underlying hCoV-EMC entry into host cells and define targets for intervention.

The incorporation of foreign glycoproteins into lentiviral particles, termed pseudotyping, offers an efficient means of studying host cell entry of highly pathogenic viruses without the need for a high level of biocontainment. Pseudotyping has been used successfully in the past to study the host cell entry of coronaviruses, including SARS-CoV (37, 39), and proved to be a suitable tool to analyze the entry of hCoV-EMC. Thus, EMC-S was efficiently incorporated into lentiviral particles, and pseudotypes bearing EMC-S were able to readily transduce a panel of human cell lines, including cells derived from the colon, liver, kidney, and lung. Whether these cell lines are also susceptible to infection by authentic hCoV-EMC and adequately mimic infection of primary cells remains to be determined. The susceptibility of lung-derived cell lines to EMC-S-mediated transduction was expected, since the virus causes severe respiratory disease and was isolated from sputum (8). The susceptibility of intestine- and kidney-derived cell lines is notable because these organs are also targeted by SARS-CoV (48, 49). The invasion of the intestines by SARS-CoV might account for the diarrhea frequently observed in SARS cases (50), while viral spread to the kidney might promote renal failure (51), a symptom occasionally associated with SARS-CoV infection. Five of the nine hCoV-EMC-infected patients documented up to December 2012 developed renal failure (9), and it is thus conceivable that hCoV-EMC, like SARS-CoV, is capable of extrapulmonary spread, which might account for certain aspects of the disease presentation.

The observation that SARS-CoV and hCoV-EMC both infect certain kidney- and colon-derived cell lines (8, 17, 52, 53) indicates that these viruses might use the same receptor for host cell entry, i.e., ACE2. Alternatively, hCoV-EMC might have adapted to use one of the other previously identified coronavirus receptor proteins, i.e., CD13 (15) or CEACAM-1 (19). However, directed expression of these receptors did not augment EMC-S-driven transduction of target cells, while transduction mediated by control S proteins was enhanced, demonstrating that EMC-S did not adapt to use any of the known coronavirus receptors. This conclusion is in keeping with results reported by Müller and colleagues, who recently demonstrated that hCoV-EMC does not use ACE2 for entry (53). Which cellular receptor is bound by EMC-S and whether binding is mediated by a receptor binding site proposed on the basis of bioinformatic analysis (54) remain to be determined. As was done for the identification of the SARS-CoV receptor ACE2 (17), coimmunoprecipitation followed by mass spectrometric identification of binding partners might be a suitable way to identify the elusive hCoV-EMC receptor. The observation that THP-1 cells acquire susceptibility to EMC-S-driven transduction upon differentiation into macrophages, most likely because of up-regulation of receptor expression, might aid in these endeavors.

The coronavirus S proteins characterized so far are class I membrane fusion proteins. A characteristic of class I membrane fusion proteins is their need for proteolytic activation (40). Thus, the proteins are synthesized as inactive precursors which transit into an active state only upon proteolysis. A seminal study showed that SARS-S, like EBOV-GP, is activated by the pH-dependent endosomal cysteine proteases cathepsins B and L during uptake of virions into target cells (22). Inhibitors of cathepsins B and L, such as MDL28170, are available, and their development as therapeutics for coronavirus and filovirus infection has been proposed (22, 55). However, recent studies demonstrated that the type II transmembrane serine proteases TMPRSS2 and HAT, which were initially shown to activate influenza viruses (56), also activate SARS-S and that activation by TMPRSS2 renders SARS-S-mediated entry cathepsin B/L independent (24–28). TMPRSS2 and HAT are coexpressed with ACE2 in human respiratory epithelium (57), and TMPRSS2 was shown to contribute to SARS-S activation in cultured human airway-derived cells (28), indicating that TMPRSS2-mediated SARS-S activation might be operative *in vivo*.

In contrast to SARS-S, EMC-S is efficiently cleaved in transfected cells, and likely also in infected cells. The nature of the responsible proteolytic activity and the biological relevance of cleavage are unknown at present. Although EMC-S is cleaved in transfected cells and processed S protein is incorporated into virions, further processing by host cell proteases during viral entry is required for EMC-S activation. A similar observation has been documented for EBOV-GP, which is processed by furin in infected cells (58) but requires cathepsin activity in target cells in order to facilitate infectious entry (42). EMC-S can be activated by cathepsin B/L, as demonstrated by inhibition of EMC-S-dependent transduction by the cathepsin B/L inhibitor MDL28170. In addition, EMC-S can be processed and activated by TMPRSS2 for cathepsin B/L-independent entry. Thus, EMC-S, like SARS-S, is activated by endosomal cathepsins and type II transmembrane serine proteases in cell culture, suggesting that the activities of both proteases must be blocked in patients in order to efficiently suppress viral replication.

Coronavirus S proteins are major targets for neutralizing antibodies (37, 39, 59, 60), and the observation that serum from an hCoV-EMC-infected patient specifically neutralized EMC-S but not VSV-G or SARS-S is in keeping with this notion. Notably, work published during the revision of the present article demonstrated that the patient serum we used also neutralizes authentic hCoV-EMC (61), indicating that the pseudoparticle system adequately mirrors neutralization of authentic hCoV-EMC. Whether the neutralizing antibodies contributed to control of hCoV-EMC infection or were produced largely after the viral spread subsided is at present unknown. Regardless of the role of neutralizing antibodies, the easy detection of neutralizing activity present in human serum by the vector systems used here suggests that this tool might prove useful in determining the frequency of hCoV-EMC infection in patients and the potential cross-reactivity of serum with other coronavirus S proteins. Our initial analysis demonstrates that antibodies generated in hCoV-EMC-infected patients do not cross-neutralize SARS-S. Whether the same is true for the more closely related S proteins of HKU4 and HKU5 remains to be investigated.

In sum, the present study provides important insights into hCoV-EMC cell tropism and should contribute to our under-

standing of the pathogenesis of this virus. In addition, it demonstrates that hCoV-EMC uses a novel receptor for cellular entry, and it defines host cell enzymes important for activation of EMC-S, which might constitute targets for intervention. Finally, the study identifies pseudotypes as a valuable tool for the detection of neutralizing antibodies directed against EMC-S, a finding that has important implications for diagnostic approaches and efforts to characterize the humoral immune response in hCoV-EMC-infected patients.

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**Second Manuscript**



**Inhibition of proprotein convertases abrogates processing of the MERS-coronavirus spike protein in infected cells but does not reduce viral infectivity**

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Running title: Role of proprotein convertases in MERS-coronavirus infection

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Middle East respiratory syndrome coronavirus (MERS-CoV) infection is associated with a high-case fatality rate and the potential pandemic spread of the virus is a public health concern. The spike protein of MERS-CoV (MERS-S) facilitates viral entry into host cells, which depends on activation of MERS-S by cellular proteases. Proteolytic activation of MERS-S during viral uptake into target cells has been demonstrated. However, it is unclear whether MERS-S is also cleaved during S protein synthesis in infected cells and whether cleavage is required for MERS-CoV infectivity. Here, we show that MERS-S is processed by proprotein convertases in MERS-S transfected and MERS-CoV infected cells and that several RXXR motifs located at the border between the surface and transmembrane subunit of MERS-S are required for efficient proteolysis. However, blockade of proprotein convertases did not impact MERS-S-dependent transduction of target cells expressing high amounts of the viral receptor, DPP4, and did not modulate MERS-CoV infectivity. These results show that MERS-S is a substrate for proprotein convertases and demonstrate that processing by these enzymes is dispensable for S protein activation. Efforts to inhibit MERS-CoV infection by targeting host cell proteases should therefore focus on enzymes which process MERS-S during viral uptake into target cells.

**SIGNIFICANCE**

The spread of the emerging Middle East respiratory syndrome coronavirus (MERS-CoV) caused 80 deaths so far and the identification of novel antiviral strategies is an important task. The viral infectivity depends on proteolytic activation of the spike protein by host cell proteases and the responsible enzymes constitute potential targets for intervention. We and others could previously show that the spike protein can be cleaved and activated during viral entry. In the present study we demonstrate that the spike protein is also cleaved during biosynthesis in infected cells and we identify proprotein convertases as the responsible enzymes. However, processing of the spike protein by these proteases was found to be dispensable for viral infectivity. These results provide valuable insights into MERS-CoV interactions with host cells and suggest that, unlike for other viral infections, proprotein convertase activity is not a suitable target for antiviral strategies.

## INTRODUCTION

The emergence and subsequent pandemic spread of the severe acute respiratory syndrome coronavirus in 2002/2003 caused almost 800 deaths and wrecked enormous economic havoc (1, 2). The virus was transmitted from bats, potentially via intermediate hosts, to humans demonstrating that the zoonotic transmission of novel coronaviruses from animal reservoirs to humans can pose a significant threat to public health (3, 4). A similar outbreak scenario unfolded in 2012 when a novel coronavirus, initially named coronavirus EMC and now termed Middle East respiratory syndrome coronavirus (MERS-CoV) (5), was detected in a patient from Jordan hospitalized with a severe and ultimately fatal pneumonia (6). Subsequently, the virus spread within the Middle East and, due to travel activity, occasionally to Europe (7-10). The outbreak so far entailed 189 laboratory confirmed infections and 82 deaths (as of March 12, 2014) (11) and adaptation of the virus to more efficient human-to-human spread is a public health concern (12). Therefore, it is imperative to identify and explore novel targets for antiviral therapy.

The viral surface protein spike (S), a type I transmembrane protein synthesized in the constitutive secretory pathway of infected cells, mediates coronavirus entry into target cells (13, 14). For this, the MERS-CoV spike protein (MERS-S) binds to its receptor dipeptidylpeptidase (DPP4, CD26) on the surface of target cells (15, 16) and drives fusion of the viral envelope with a target cell membrane, which allows delivery of viral proteins and RNA into the host cell cytoplasm, the site of viral replication. However, MERS-S and other coronavirus S proteins are synthesized as inactive precursors in infected cells and only acquire the ability to drive membrane fusion upon processing into the surface unit (S1) and the transmembrane unit (S2) by host cell proteases (17, 18). The activity of the responsible proteases is essential for viral infectivity, which makes these enzymes potential targets for antiviral intervention. In fact, all viral glycoproteins with a domain organization comparable to that of MERS-S, which are collectively termed class I

membrane fusion proteins, depend on proteolytic cleavage to unfold their fusogenic potential (19). Specifically, cleavage can prime viral glycoproteins for a subsequent trigger of the membrane fusion reaction, like low pH, as is the case for the influenza virus hemagglutinin (20). Alternatively, cleavage can directly activate glycoproteins for the membrane fusion reaction, as is suspected for MERS-S and other coronavirus S proteins (18).

It has been demonstrated that the cysteine protease cathepsin L (17, 21), the type II transmembrane serine protease TMPRSS2 (17, 21, 22) and a so far unidentified enzyme (21) can activate MERS-S during viral uptake into target cells. Cathepsin L and TMPRSS2 are also hijacked by other coronaviruses for viral activation (23-27) and TMPRSS2 has recently been shown to be essential for activation of H1N1 influenza viruses in mice (28). However, priming of viral glycoproteins, including priming of the S protein of certain strains of the coronavirus infectious bronchitis virus (IBV) (29), may also proceed in the constitutive secretory pathway of infected cells and is often accomplished by furin and other proprotein convertases (30-33). Whether MERS-S is also cleaved during the passage of the secretory pathway and whether these cleavage events contribute to S protein activation is at present unknown.

Here, we show that proprotein convertases process MERS-S in transfected and infected cells and we demonstrate that the integrity of several RXXR motifs located at the border of the S1 and S2 subunit is required for S protein processing. Similarly, treatment of MERS-CoV infected cells with a proprotein convertase inhibitor abrogated S protein cleavage but did not alter viral infectivity, indicating that S protein processing in infected cells is dispensable for MERS-S activation.

## MATERIAL AND METHODS

**Plasmids.** Expression plasmids encoding MERS-S (17), vesicular stomatitis virus glycoprotein (VSV-G) (34), Zaire ebolavirus glycoprotein (EBOV-GP) (34), Lassa virus glycoprotein (LASV-GPC) (34), DPP4 (16) and TMPRSS2 (24) have been described previously. Plasmids pGAL4-VI16 (35), pGAL5-luc (35), pNL4-3-Luc-R'E- (36), p96ZM651gag-opt (37) as well as plasmids encoding MERS-S (17) and EBOV-GP (38) with C-terminal V5 tag have also been described. A construct expressing LASV-GPC with a C-terminal V5 tag was generated by PCR-based mutagenesis using the primers 5'-GCGCCCGGGGATGGGACAAATAGTGACATTC-3' and 5'-CGCGCTAGCCCTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTCTCTTCCATTTACAGG-3' and inserted into plasmid pCAGGS via the NheI and SmaI restriction sites. Mutagenesis of MERS-S was performed by overlap extension polymerase chain reaction (PCR), resulting in MERS-S potential cleavage site mutants (PCM) 1 (626-AXXA-629), PCM2 (691-AXXA-694), PCM3 (748-AXXA-751) and PCM4 (884-AXXA-887) containing a V5 tag. For the generation of these mutants the following oligonucleotides were used as inner primers: 5'-GCACAGCTGTAGGTGTTGCACAGCAGGCCTTTGTTTATGATGCG-3' and 5'-GGTACGCATCATAAACAAGGCCTGCTGTGCAACACCTACAGCTGTGC-3' (mutant PCM1), 5'-CCATGTCTCAATACTCCGCTTCTACGGCATCAATGC-3' and 5'-CGCCGTTTAAGCATTGATGCCGTAGAAGCGGAGTATTGAGACATGG-3' (mutant PCM2), 5'-CCTAGTACTCTCACACCTGCCAGTGTGGCCTCTGTTCCAGG-3' and 5'-GCCAAGCGCATTTCACCTGGAACAGAGGCCACACTGGCAGGTGTGAGAGTACTAGG-3' (mutant PCM3) and 5'-CCTGTTTCTATATCTACTGGCAGTGCTAGTGCAGCTAGTGC-3' and 5'-GCAAATCCTCAATAGCACTAGCTGCACTAGCACTGCCAGTAGATATAGAAACAGG-3' (mutant PCM4). Oligonucleotides 5'-GATCGGTACCACCACCATGATACACT-3' and 5'-GCTG

GACCTTGCTGCATGCAATCATCGTAACCTTGCATATAACCAGG-3' served as outer primers. The PCR products were cloned into pCAGGS via KpnI and SphI restriction sites. The integrity of all PCR amplified sequences was confirmed by automated sequence analysis.

**Cell culture.** 293T cells were propagated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS, PAN-Biotech), penicillin and streptomycin. Caco-2 cells were cultured in DMEM-GlutaMAX medium (Invitrogen) supplemented with 10% FBS, penicillin and streptomycin. Vero B4 cells were cultured in DMEM supplemented with 5% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids (all from Life Technologies) and antibiotics as stated above. All cell lines were grown in humidified atmosphere at 37°C and 5% CO<sub>2</sub>.

**Analysis of MERS-S expression and cleavage.** For the detection of the expression of MERS-S wt and MERS-S mutants in transfected cells, 293T cells were calcium phosphate transfected with the respective plasmids encoding S proteins with C-terminal V5 antigenic tag. For analysis of MERS-S processing by TMPRSS2, a TMPRSS2 expression plasmid was cotransfected. The transfection medium was replaced at 8 h post-transfection with fresh culture medium and cells were harvested at 48 h post-transfection. The harvested cells were either treated with trypsin (Sigma, 25 µg/ml final concentration) or PBS for 10 min and then lysed in sodium dodecyl sulfate (SDS) loading buffer. For immunoblotting, the lysates were separated by SDS-gel electrophoresis and transferred onto nitrocellulose membranes (Hartenstein). MERS-S expression was detected using a monoclonal antibody directed against the V5 tag (Invitrogen) or a polyclonal antibody directed against the S2 subunit of the MERS-S protein (Sino Biological Inc.). For detection of MERS-S protein in infected cells, Caco2 and VeroB4 cells were infected with

MERS-CoV (human betacoronavirus 2c EMC/2012) at an MOI of 0.01 and 5, respectively. At 24 h post infection, the cells were harvested and treated with NuPAGE LDS sample buffer (Invitrogen), boiled for 20 min at 95 °C and analyzed by Western blot as described above. As a loading control the membranes were incubated with anti- $\beta$ -actin antibody (Sigma).

**Analysis of MERS-S incorporation into virus-like particles.** For the production of virus-like particles (VLPs), 293T cells were cotransfected with the HIV-1 Gag (p55)-encoding plasmid p96ZM651gag-opt and plasmids encoding MERS-S wt or MERS-S mutants. Cotransfection of empty plasmid served as negative control. The transfection medium (i.e. culture medium after addition of transfection reagents) was replaced by fresh culture medium at 8 h post transfection and supernatants containing VLPs were harvested at 48 h post transfection. The supernatants were sterile-filtered using 0.45  $\mu$ m pore-size filters (Sartorius) and concentrated by ultracentrifugation through a 20% sucrose cushion at 20,000 g for 2 h at 4°C. Pellets were resuspended in TNE buffer (0.01 M Tris-HCl, pH 7.4; 0.15 M NaCl; 2 mM EDTA), 2  $\times$  SDS loading buffer was added and the samples analyzed by Western blot for expression of MERS-S, as described above, and for expression of p55 Gag employing supernatants of hybridoma 182-H12-5C (AIDS Reagent Program, Division of AIDS, NIAID, NIH), which produces an antibody directed against the p24-capsid protein.

**Cell-cell fusion assay.** The cell-cell fusion assay was carried out as described before (24). In brief, 293T effector cells were seeded in six-well plates and calcium phosphate transfected with either empty plasmid as negative control or plasmids encoding MERS-S wt and MERS-S mutants in combination with plasmid pGAL4-VP16, which encodes the herpes simplex virus VP16 transactivator fused to the DNA binding domain of the yeast transcription factor GAL4. In



parallel, 293T target cells were seeded in 48-well plates and cotransfected with either empty plasmid or expression plasmids coding for DPP4 and/or TMPRSS2 together with plasmid pGAL5-luc, which encodes the luciferase reporter gene under the control of a promoter containing five GAL4 binding sites. At 8 h post transfection the transfection medium was replaced by fresh culture medium. At 24 h post transfection, effector cells were detached, diluted in fresh medium and added to the target cells. At 8 h after cell-mixing, medium supplemented with trypsin (Sigma) was added to the cultures at a final concentration of 100 ng/ $\mu$ l. At 48 h after cocultivation, cell-cell fusion was quantified by determination of luciferase activities in cell lysates, using a commercially available kit (PJK).

**MERS-S-driven transduction.** A lentiviral vector was pseudotyped with MERS-S as described before (17). Briefly, 293T cells were calcium phosphate transfected with the HIV-1 derived vector pNL4-3-Luc-RE<sup>+</sup> (36) and expression plasmid encoding MERS-S wt or mutants, LASV-GPC, EBOV-GP or VSV-G. Pseudotypes bearing no glycoprotein served a negative control. The transfection medium was replaced by fresh culture medium at 8 h post transfection and culture supernatants were harvested at 48 h post transfection. The supernatants were passed through 0.45  $\mu$ m pore-size filters, aliquotted and stored at -80°C. For transduction, 293T cells transfected with DPP4 expression plasmid or Caco-2 cells were seeded in 96-well plates and incubated with equal volumes of vector containing supernatants. Normalization of supernatants for equal viral p24-capsid protein content was performed using a commercially available HIV-1 p24-ELISA (Advanced BioScience Laboratories). At 8 h post transduction, transduction medium (culture medium containing vector) was removed and replaced by fresh culture medium. At 72 h after addition of supernatants to target cells, luciferase activities in cell lysates were determined with a commercially available kit (Promega) according to the manufacturer's instructions.

**Cathepsin bypass.** 293T cells were transfected with expression plasmids for DPP4 or/and TMPRSS2 and seeded into 96-well plates. Prior to transduction, cells were pretreated with DMSO or the cathepsin B/L inhibitor MDL28170 (Calbiochem) for 60 min at 37°C. Thereafter, the cells were transduced with equal volumes of pseudotypes in the presence of inhibitor at a final concentration of 10  $\mu$ M. At 8 h post transduction the medium was replaced by fresh culture medium without inhibitor and transduction efficiency was measured after 72 h by determining luciferase activities in cell lysates.

**Inhibition of proprotein convertases.** In order to assess the role of proprotein convertase activity in MERS-S processing in transfected 293T cells, the cells were transfected with plasmids encoding MERS-S, EBOV-GP and LASV-GPC or empty plasmid. The medium was changed at 6 h post transfection and proprotein convertase inhibitor (Merck) was added to the fresh medium at the indicated concentrations. Medium was replaced again at 32 h post transfection and inhibitor was replenished. Cells were lysed at 48 h post transfection and cleavage of viral glycoproteins was detected by Western blot, employing a monoclonal antibody recognizing the V5 tag. As a loading control, an anti- $\beta$ -actin antibody was used.

To assess whether proprotein convertase activity is required for MERS-S-driven cell-cell fusion, 293T target cells, expressing MERS-S or no glycoprotein as well as effector cells transfected to express DPP4 and/or TMPRSS2 were incubated with 1  $\mu$ M proprotein convertase inhibitor. One day after transfection, the cells were mixed to allow cell-cell fusion and medium was supplemented with proprotein convertase inhibitor at 1  $\mu$ M final concentration. Cell-cell fusion was quantified by determination of luciferase activity in cell lysates at 48h after co-cultivation, using a commercially available kit (PJK).

For analysis of the importance of proprotein convertase activity for MERS-S-driven virus-cell fusion, vectors bearing MERS-S, LASV-GPC, EBOV-GP or VSV-G or no glycoprotein were generated in the presence or absence of 1  $\mu$ M proprotein convertase inhibitor. As target cells, 293T cells, transfected with DPP4 encoding plasmid or empty plasmid, were seeded into 96 well plates and pretreated with 0.5  $\mu$ M proprotein convertase inhibitor for 60 min at 37°C. The cells were then incubated with pseudotypes for 8 h, followed by replacement of infection medium with culture medium containing inhibitor. Transduction efficiency was measured after 72 h by determining luciferase activities in cell lysates.

To determine the role of proprotein convertase activity in infection with authentic MERS-CoV, Caco-2 cells seeded in 24-well plates were incubated with DMSO or rising concentrations of proprotein convertase inhibitor for 1 h at 37°C and were then inoculated with MERS-CoV (MOI of 0.01 and 0.001 in quadruplicates) in the presence of inhibitor. After an incubation of 30 min at 4°C, the cells were washed and again incubated in culture medium with the inhibitor. At 24 h post infection, the cells were washed, harvested and the pellet was lysed with RIPA lysis buffer, supplemented with 4xNuPAGE (Invitrogen) and boiled for 20 min at 95°C. S protein expression in lysates was detected by Western blot, employing a polyclonal antibody directed against the S2 subunit of MERS-S (Sino Biological Inc.). In parallel, for quantification of viral RNA, 50  $\mu$ l of the cell supernatant was dissolved in RAV1 buffer (Macherey-Nagel) for RNA extraction followed by qRT-PCR analysis using the upE assay as previously described (39). Quantification of infectious particles was done by plaque assay using Vero B4 cells as published (7). Briefly, 10-fold dilutions of supernatants were tested in duplicates using cell monolayers of Vero B4 cells. After 1 h of virus adsorption cells were washed and overlaid with a 1.2% avicel resin. After 3 days the plates were fixed with 7% paraformaldehyde and stained with crystal violet solution. To investigate the influence of PCI on the formation of MERS-CoV induced

cytopathogenic effects Vero B4 cells were infected at an MOI of 0.1 and fixed with 7% paraformaldehyde after 42 h post infection. MERS-CoV antigen detection was performed by incubation with a MERS patient serum as described (40). Bound antibodies were detected with a cyanine 2-labeled goat-anti human immunoglobulin G secondary antibody (Dianova). Nuclei were stained by mounting the slides with DAPI containing ProLong Gold antifade mounting medium (Life technologies).

## RESULTS

**MERS-S is proteolytically processed in transfected and infected cells.** In order to investigate MERS-S cleavage in virus producing cells, we determined whether the S protein is cleaved in transfected and infected cells. Western blot analysis of S protein transfected 293T cells and MERS-CoV infected Vero B4 cells with an antibody specific to the S2 subunit of MERS-S revealed two prominent S protein bands with molecular weights of 170 kDa and 90 kDa (Fig. 1), in keeping with our previous results (17). The 170 kDa band corresponds to uncleaved MERS-S while the presence of the 90 kDa indicates efficient processing of MERS-S into an N-terminal S1 subunit (not detected) and a C-terminal S2 subunit (Fig. 1).

**Several RXXR motifs located at the border of the S1 and S2 subunits are required for processing of MERS-S.** Inspection of the sequences located at the border of the S1 and S2 subunits of MERS-S revealed the presence of four RXXR sequences (Fig. 2A), which might represent recognition sites for proprotein convertases (33). In order to determine the importance of these motifs for MERS-S cleavage and activation, we changed the arginine residues in 626-RXXR-629, 691-RXXR-694, 748-RXXR-751 and 884-RXXR-887 to alanine residues, creating the potential cleavage site mutants PCM1, PCM2, PCM3 and PCM4 (Fig. 2A). All S protein mutants were expressed with the same efficiency as wt MERS-S in transfected cells (Fig. 2B). The only exception was PCM2, for which consistently no expression was detected (not shown). Importantly, mutation of potential cleavage sites 3 and 4 alone impacted S protein processing: The presence of the 90 kDa band was reduced in cells expressing PCM3 and a band with a molecular weight slightly higher than 90 kDa was observed upon expression of PCM4 (Fig. 2B). Processing of PCM1 was comparable to that seen for MERS-S wt. However, combined mutation of potential cleavage sites 1 and 3 (PCM1 + PCM3) abrogated S protein processing (Fig. 2B),

suggesting that also potential cleavage site 1 contributes to S protein cleavage in this experimental setting. Similar effects were seen when potential cleavage sites 3 and 4 were simultaneously altered (PCM3 + PCM4) or when all three potential cleavage sites were mutated in parallel (PCM1 + PCM3 + PCM4). Collectively, these results indicate that MERS-S is cleaved in transfected cells and that cleavage is reduced or altered upon mutation of potential cleavage sites 1, 3 and 4.

**Mutation of 748-RXXR-751 (PCM3) and 884-RXXR-887 (PCM4) reduces MERS-S-driven cell-cell and virus-cell fusion.** Next, we investigated whether the integrity of potential cleavage sites 1, 3 and 4 is required for S protein-driven cell-cell and virus-cell fusion. For analysis of cell-cell fusion, we employed a previously described assay (24), which is based on directed expression of S protein and receptor in effector and target 293T cells, respectively. Expression of MERS-S in effector cells allowed inefficient fusion with control transfected 293T target cells, which are known to express low amounts of endogenous DPP4 (41), and the efficiency of cell-cell fusion was markedly increased when target cells were transfected with DPP4 encoding plasmid (Fig. 3A). In contrast, cell-cell fusion driven by PCM1, PCM3 and PCM4 was inefficient (PCM1) or absent (PCM3, PCM4), irrespective of expression of DPP4 (Fig. 3A). Similar results were obtained when virus-cell fusion was analyzed employing a previously described lentiviral vector (17) and DPP4-transfected 293T cells as targets, although the most severe defects were observed for PCM1 and PCM4 while PCM3 retained substantial activity (Fig. 3B). Finally, S protein mutants in which more than one potential cleavage site was altered showed a phenotype comparable to that observed for PCM1 and PCM4, suggesting that potential cleavage sites 1, 3 and 4 are all required for full S protein activity in cell-cell and virus-cell fusion reactions, although the relative contribution of single sites varies.

In order to define the nature of the defects associated with mutation of potential cleavage sites, we selected PCM3 and PCM4 for detailed analysis, since both mutants showed altered S protein cleavage (Fig. 2B) but differed in their capacity to drive virus-cells fusion: PCM3 exhibited substantial activity while PCM4 was largely inactive (Fig. 3B). We first examined whether the reduced activities of PCM3 and PCM4 in the virus-cell fusion assay were due to reduced incorporation of the respective S proteins in lentiviral particles. Western blot analysis of particles concentrated through a sucrose cushion revealed that particle incorporation of both mutants was comparable to that detected for MERS-S wt (Fig. 3C). Of note, the processing defects observed for cell-associated S proteins (Fig. 2B) were even more pronounced for virion-associated S proteins: PCM3 was mainly uncleaved and the cleavage product of PCM4, although prominent, had a slightly higher molecular weight than the cleavage product obtained for MERS-S wt (Fig. 3C). Transduction of the colon carcinoma cell line Caco-2, which expresses endogenous DPP4 (42), showed that PCM3 is able to drive virus-cell fusion while PCM4 is largely defective (Fig. 3D). These results confirm the observations made with 293T target cells and demonstrate that defects of PCM3 and PCM4 in virus-cell fusion are not due to reduced particle incorporation of these S protein mutants.

**Mutation of 748-RXXR-751 (PCM3) and 884-RXXR-887 (PCM4) is compatible with MERS-S processing by trypsin and TMPRSS2.** In order to assess whether mutation of potential cleavage sites 3 and 4 interferes with S protein activation pathways operative once mature S protein is presented on the surface of cells or virions, we assessed S protein activation by trypsin and TMPRSS2. Proteolysis of MERS-S wt upon addition of trypsin or coexpression of TMPRSS2 was readily detectable (Fig. 4A), as expected (17). In keeping with this finding, cell-cell fusion driven by MERS-S wt was promoted by directed expression of DPP4 or TMPRSS2 on target cells and by trypsin treatment (Fig. 4B). Thus, receptor and protease expression can both

limit MERS-S-driven cell-cell fusion, as previously reported for SARS-S (43). Finally, the cathepsin B/L inhibitor MDL28170 reduced MERS-S-driven fusion of virions with control transfected cells and this effect was rescued by expression of TMPRSS2, as expected from previous work (17). PCM3 was efficiently cleaved (Fig. 4A) and activated by trypsin and TMPRSS2 for cell-cell fusion (Fig. 4B). Moreover, fusion of virions with control transfected cells was blocked by the cathepsin B/L inhibitor and this effect was not seen when TMPRSS2 expressing target cells were examined (Fig. 4C), indicating that the potential cleavage site 3 is dispensable for S protein activation by trypsin, TMPRSS2 and cathepsin B/L. In contrast, PCM4 failed to drive cell-cell and virus-cell fusion under all conditions tested (Fig. 4B and not shown), despite efficient processing by both trypsin and TMPRSS2 (Fig. 4A) - although it should be noted that one cleavage fragment observed upon TMPRSS2-mediated proteolysis of MERS-S wt was either weak or not detected upon cleavage of PCM4. In sum, these results demonstrate that the reduced capacity of PCM3 and PCM4 to drive cell-cell and virus-cell fusion is not due to limited cleavability by trypsin and, in case of PCM3, the trypsin-like protease TMPRSS2.

**MERS-S is processed by proprotein convertases.** We next sought to identify the proteases responsible for MERS-S cleavage in virus producing cells. For this, we investigated whether S protein cleavage can be blocked by a cell-permeable tripeptide derivative containing an Arg-X-Arg motive, which is known to inhibit several proprotein convertases, including furin (44). Treatment with this proprotein convertase inhibitor (PCI) reduced the production of the 90 kDa band in cells transfected to express MERS-S (Fig. 5). Moreover, the presence of PCI diminished processing of the Zaire ebolavirus glycoprotein (EBOV-GP) into GP1 and GP2 (Fig. 5), which depends on the activity of the proprotein convertase furin (45). In contrast, processing of the glycoprotein of Lassa virus (LASV-GPC), which is mediated by the proprotein convertase SKI-1/S1P (46), was not suppressed (Fig. 5). This finding is in keeping with the known differences in



substrate specificity (and thus inhibitor sensitivity) of SKI-I/SIP compared to proprotein convertases with specificity for basic amino acids, like furin (47). Collectively, these results show that the activity of proprotein convertases is essential for MERS-S processing in transfected cells.

**Proprotein convertase activity is not essential for efficient MERS-S-driven cell-cell and virus-cell fusion.** The results obtained so far were compatible with the concept that cleavage of MERS-S by proprotein convertases depends on the integrity of PCS1, 3 and 4 and is required for S protein activity in cell-cell and virus-cell fusion reactions. Unexpectedly, however, the continuous presence of PCI in target and effector cell cultures, prior, during and after mixing had no appreciable effect on MERS-S-mediated cell-cell fusion (Fig. 6A). Next, we asked whether MERS-S processing by proprotein convertases is required for MERS-S-mediated virus-cell fusion. Addition of PCI to cells producing MERS-S harboring lentiviral particles reduced transduction of 293T target cells roughly 100-fold (Fig. 6B). In contrast, infectivity of particles bearing LASV-GPC or EBOV-GP was not affected (Fig. 6B), in keeping with the findings that processing of LASV-GPC is not inhibited by PCI (Fig. 5) and that processing of EBOV-GP by proprotein convertases is dispensable for GP-driven virus-cell fusion (48, 49). Notably, no inhibitory effect was detected when 293T cells transfected to express DPP4 were chosen as targets (Fig. 6B), indicating that S protein processing in virus producing cells might be dispensable for infectivity when target cells express robust amounts of DPP4. In sum, these results suggest that proprotein convertase activity is largely dispensable for MERS-S-driven cell-cell and virus-cell fusion, at least when target cells produce high levels of DPP4.

**Proprotein convertase activity is not required for MERS-CoV infectivity.** The lack of a prominent inhibitory effect of PCI on MERS-S-driven cell-cell and virus-cell fusion might be due to high levels of directed MERS-S and DPP4 expression in these experimental systems. Therefore, we assessed whether PCI inhibits MERS-CoV spread in target cells expressing

endogenous DPP4. PCI treatment of Caco-2 cells infected with MERS-CoV did not inhibit total MERS-S expression but reduced S protein cleavage efficiently and in a concentration dependent manner (Fig. 7A), confirming that MERS-S is a substrate of proprotein convertases in infected cells. However, PCI treatment did not reduce MERS-CoV propagation, as determined by the amount of viral RNA (Fig. 7B, left panel) or infectious units (Fig. 7B, right panel) present in culture supernatants. Similarly, treatment of MERS-CoV infected cultures had little if any effect on the formation of cytopathic effects (CPE) as demonstrated by comparable destruction of the cell monolayer (Fig. 7C). Thus, processing of MERS-S by proprotein convertases is dispensable for MERS-CoV infectivity in cell cultures.

## DISCUSSION

The processing of the glycoproteins of HIV (30) and highly pathogenic avian influenza viruses (31) by proprotein convertases is essential for viral infectivity. As a consequence, antiviral strategies aiming at the inhibition of these enzymes are being developed (44). Our results add MERS-S to the list of proprotein convertase substrates and show that several RXXR motifs within MERS-S are required for efficient S protein processing. However, activity of proprotein convertases was dispensable for infectivity of MERS-CoV, indicating that in the context of MERS-CoV infection proprotein convertases do not constitute targets for antiviral intervention. Finally, we observed that mutation of RXXR motifs within MERS-S not only compromises proteolytic processing but also reduces S protein-driven cell-cell and virus-cell fusion. Why intact RXXR motifs were required for the latter processes remains to be clarified.

The recent finding that TMPRSS2 expression primes influenza virus HA in cell culture (50-52) and is required for spread and pathogenesis of H1N1 influenza virus in mice (28) showed that influenza viruses might depend on the activity of a single protease for amplification in the infected host. These findings, jointly with the observations that TMPRSS2 also activates MERS-CoV (17, 21, 22) and other coronaviruses (23, 25, 53) and that knock-out of TMPRSS2 has no phenotype in the absence of infection (54), defined TMPRSS2 as a prime target for therapeutic intervention. However, TMPRSS2 might not be the only cellular protease which constitutes a potential therapeutic target in the context of coronavirus infection. Thus, it is at present unclear whether cleavage of MERS-S during biogenesis in infected cells is required for subsequent cleavage and activation of the S protein by TMPRSS2 during viral uptake into target cells. Such a scenario would not be unprecedented given that entry mediated by the glycoproteins of certain bunyaviruses depends on glycoprotein processing during synthesis in the constitutive secretory pathway and, as suggested by a recent report, during viral uptake into target cells (55).

Alternatively, it is conceivable that processing of MERS-S in infected cells is dispensable for virus-cell but required for cell-cell fusion, which results in formation of syncytia, a feature of MERS-CoV pathogenesis (6).

We found that MERS-S is efficiently cleaved in S protein transfected 293T cells and MERS-CoV infected Vero B4 and Caco-2 cells and inhibition experiments showed that proprotein convertase activity is essential for MERS-S cleavage. The finding that MERS-S is efficiently cleaved in transfected or MERS-CoV infected cells is in keeping with several studies examining MERS-S expression for other purposes (21, 56, 57). One report showed that MERS-S is not cleaved in transfected cells (22) but a MERS-S variant with a truncated cytoplasmic tail was examined and it is conceivable that this deletion interfered with S protein exposure to or recognition by proprotein convertases. Alternatively, the cells used for S protein expression might have produced only low amounts of proprotein convertases. Robust S protein processing by proprotein convertases has also been reported for mouse hepatitis virus strain A59 (58, 59) and IBV (29, 60) while cleavage of SARS-S by these proteases is inefficient (43, 61, 62). Thus, MERS-S belongs to the subgroup of coronavirus S proteins, which are substrates of proprotein convertases, and MERS-S processing in different cellular systems, including the lung cell line Calu-3, is robust.

Most proprotein convertases cleave the following motif: (R/K)-2nX-R↓, with n standing for 0-3 amino acids (47). The finding that RXXR motifs located at the predicted border between S1 and S2 subunit are important for MERS-S processing by proprotein convertases might therefore not be unexpected. Nevertheless, it is noteworthy that at least two RXXR motifs had to be mutated in parallel to markedly reduce S protein processing. This suggests that the processing enzyme(s) can recognize more than one cleavage site. Indeed, the S2 fragment produced upon cleavage of PCM4 showed a slightly increased molecular weight relative to the fragment

generated from MERS-S wt, which would be in keeping with usage of an upstream cleavage site, most likely potential cleavage site 3. Alternatively, the integrity of the RXXR motifs located at the border of the S1 and S2 subunit might be required to present a single cleavage site in a protease sensitive configuration.

Processing by proprotein convertases is required for full activity of certain MHV and IBV S proteins in cell-cell and virus-cell fusion reactions, although inactivation of the respective cleavage sites does not abrogate viral infectivity (29, 58-60). Similarly, mutation of furin cleavage sites in the glycoproteins of ebolaviruses is compatible with robust viral spread in vitro and in vivo (48, 49). In contrast, mutation of the furin cleavage site in the HIV envelope protein renders virions non-infectious and it has been suggested that processing of SARS-S by proprotein convertases, although being inefficient, is still required for full viral spread and for the establishment of cytopathic effects in infected cultures (61). The present study provides evidence that efficient blockade of MERS-S processing by PCI has no appreciable effects on S protein activity in cell-cell fusion assay and does not modulate MERS-CoV spread in susceptible cells. In contrast, inhibition of MERS-S processing markedly reduced MERS-S-driven fusion of virions with 293T cells expressing low amounts of endogenous DPP4. It is therefore conceivable that processing of MERS-S by proprotein convertases is required for optimal spread of MERS-CoV in target cells expressing low levels of DPP4. Such a scenario would be in keeping with the observation that inefficient SARS-S-driven cell-cell fusion due to limited receptor expression can be rescued by directed expression of S protein activating proteases and vice versa (43).

The mutation of the RXXR motifs at the border between the S1 and S2 subunit markedly reduced S protein processing by proprotein convertases and S protein activity in cell-cell and virus-cell fusion assays. However, inhibition studies with PCI revealed that these phenotypes were not linked, raising the question why the RXXR motifs are essential for S protein function.

We suspected that these motifs might be necessary for efficient receptor binding, due to a potential impact on S protein folding, but studies conducted with soluble DPP4 fused to the Fc portion of human immunoglobulin did not support this hypothesis (not shown). Alternatively, the integrity of the RXXR sequences might be required for S protein activation. The finding that both PCM3 and PCM4 are cleaved efficiently by trypsin and TMPRSS2 seems to argue against this possibility. However, it is conceivable that fusion of MERS-S-expressing cells with 293T target cells might depend on the activity of an endogenously expressed protease, which cleaves MERS-S at RXXR motifs. Similarly, the markedly reduced capacity of PCM1 and PCM4 to drive fusion of virions with 293T cells might be the consequence of inefficient activation by cathepsin L, the protease which facilitates MERS-S-dependent transduction and MERS-CoV infection of 293T cells (17, 21). In this regard, it is noteworthy that PCM1 and PCM3 showed differential activity in cell-cell and virus-cell fusion assays, which would be in keeping with a differential protease-dependence of these processes. Collectively, the role of RXXR motifs in MERS-S activity requires further investigation and one focus should be on proteolytic activation.

In sum, our results identify MERS-S as a substrate of proprotein convertases but indicate that the activity of these enzymes is dispensable for MERS-CoV spread in target cell lines and potentially also in the infected human host. Furthermore, our findings suggest that the mode of S protein activation might depend on the level of receptor expression, a scenario that warrants further analyses.

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**FIGURE LEGENDS**

**FIG 1** MERS-S is cleaved in transfected and infected cells. 293T cells were transfected with a plasmid encoding the MERS-S protein or with empty plasmid (pcDNA). Vero B4 cells were either infected with MERS-CoV at an MOI of 5 or mock infected. Subsequently, the cells were lysed and analyzed by Western blot employing a polyclonal antibody directed against MERS-S. A  $\beta$ -actin antibody served as a loading control. Similar results were obtained in two separate experiments.

**FIG 2** RXXR motifs located at the border between S1 and S2 are required for efficient MERS-S processing. A) The domain organization of the MERS-S protein is schematically depicted. The MERS-S sequence at the border between S1 and S2 subunit is shown. RXXR motifs, which constitute potential cleavage sites are highlighted and the start of the S2 subunit is underlined. The mutations introduced into the potential cleavage sites in MERS-S are shown. PCM, potential cleavage site mutant; SP, signal peptide; RBD, receptor binding domain; TM, transmembrane domain; CT, cytoplasmic tail. B) 293T cells were transfected with expression plasmids coding for MERS-S wt and the indicated MERS-S mutants equipped with a C-terminal V5 tag. Transfection of empty plasmid (pcDNA) served as negative control. Expression of S proteins in cell lysates was determined by Western blot, using a V5 tag-specific monoclonal antibody. Expression of  $\beta$ -actin in cell lysates was assessed as a loading control. The results shown are representative for at least three independent experiments.

**FIG 3** Mutation of 748-RXXR-751 (PCM3) and 884-RXXR-887 (PCM4) reduces MERS-S-driven cell-cell and virus-cell fusion. A) 293T effector cells were co-transfected with pGAL4-

VP16 expression plasmid and plasmids encoding MERS-S wt or MERS-S mutants PCM1, PCM3 and PCM4. Cells transfected with empty plasmid (pcDNA) served as negative control. These effector cells were mixed with target cells, co-transfected with plasmid encoding the receptor DPP4 or empty plasmid, together with a plasmid encoding luciferase under the control of a promoter with five GAL4 binding sites. Cell-cell fusion was quantified by determination of luciferase activities in cell lysates. The results of a representative experiment performed with triplicate samples are shown. Error bars indicate standard deviation (SD). Similar results were obtained in two separate experiments. B) 293T cells transfected with DPP4 expression plasmid were transduced with pseudotypes bearing either no glycoprotein (pcDNA), the glycoprotein of vesicular stomatitis virus (VSV-G), MERS-S wt or the indicated MERS-S mutants. Luciferase activities in cell lysates were measured at 72 h post transduction. The results of a representative experiment performed with triplicate samples are shown and were confirmed in two separate experiments. Error bars indicate SD. C) 293T cells were cotransfected with an expression plasmid encoding HIV-1 p55 Gag and plasmids coding MERS-S wt or the indicated MERS-S mutants equipped with a C-terminal V5 tag. Transfection of empty plasmid (pcDNA) served as negative control. Subsequently, virus-like particles (VLPs) were harvested and concentrated by ultracentrifugation through a sucrose cushion. The presence of p55 Gag and MERS-S in concentrated VLPs was analyzed by Western blot employing anti-V5 and anti-p55 Gag antibodies. Comparable results were obtained in two independent experiments. D) The experiment was carried out as described for panel B but Caco-2 cells were used as target cells. The results of representative experiments performed with triplicate samples are shown. Similar results were obtained in a separate experiment.

FIG 4 Mutation of 748-RXXR-751 (PCM3) and 884-RXXR-887 (PCM4) is compatible with S protein processing by trypsin and TMPRSS2. A) Plasmids encoding MERS-S wt or the indicated MERS-S mutants were co-transfected into 293T cells with plasmid encoding TMPRSS2 or empty plasmid (pcDNA). Subsequently, the cells were treated with trypsin or PBS and S protein expression in cell lysates was analyzed by Western blot, using anti-V5 antibody. Detection of  $\beta$ -actin served as a loading control. MERS-S cleavage fragments generated by TMPRSS2 are pointed out by asterisks. Comparable observations were made in three independent experiments. B) The cell-cell fusion assay was carried out as described for figure 3A but target cells transfected to express DPP4, TMPRSS2 or both DPP4 and TMPRSS2 were used. Cells transfected with empty plasmid (pcDNA) served as control. The effector/target cells mixtures were treated with trypsin or PBS and cell-cell fusion was quantified by determination of luciferase activities in cell lysates. The results of a representative experiment performed with triplicate samples are shown and were confirmed in two separate experiments. Error bars indicate standard deviation (SD). Tryp., trypsin. C) Target 293T cells were transfected to express DPP4, TMPRSS2 or both DPP4 and TMPRSS2. Cells transfected with empty plasmid (pcDNA) served as negative control. The cells were incubated with the cathepsin B/L inhibitor MDL28170 for 1 h and then transduced with pseudotypes bearing MERS-S wt, PCM3 or VSV-G. Luciferase activities in cell lysates were measured at 72 h post transduction. The results of a representative experiment performed with triplicate samples are shown. Error bars indicate SD and comparable results were obtained in two independent experiments.

FIG 5 MERS-S is cleaved by proprotein convertases. 293T cells were transfected with expression plasmids encoding MERS-S, Zaire ebolavirus glycoprotein (EBOV-GP) or Lassa virus glycoprotein (LASV-GPC), all equipped with a C-terminal V5 tag. Cells transfected with empty



plasmid (pcDNA) served as negative control. Subsequently, cells were incubated with the indicated concentrations of the proprotein convertase inhibitor (PCI). At 48 h post transfection glycoprotein expression was analyzed by Western blot, using the V5 antibody. Detection of  $\beta$ -actin served as loading control. The results shown are representative of three independent experiments.

FIG 6 Proprotein convertases activity is dispensable for MERS-S-driven cell-cell and virus-cell fusion. A) The cell-cell fusion assay was carried out as described for figure 3A except that target cells transfected to express DPP4, TMPRSS2 or both DPP4 and TMPRSS2 were used. Both effector and target cells were incubated with 1  $\mu$ M of proprotein convertase inhibitor (PCI) as indicated and one day later mixed for co-cultivation. The effector/target cells mixtures were incubated with PBS or PCI and cell-cell fusion was quantified by determination of luciferase activities in cell lysates. The results of a representative experiment performed with triplicates samples are shown. Error bars indicate standard deviation (SD). Two separate experiments yielded similar results. B) Lentiviral pseudotypes carrying MERS-S, Lassa virus glycoprotein (LASV-GPC), Zaire ebolavirus glycoprotein (EBOV-GP) or the glycoprotein of vesicular stomatitis virus (VSV-G) as well as pseudotypes bearing no viral glycoprotein (pcDNA) were generated in the presence or absence of PCI (1  $\mu$ M). Subsequently, target 293T cells transfected with empty plasmid or DPP4 expression plasmid were pre-incubated with DMSO, PBS or PCI at a final concentration of 0.5  $\mu$ M, for 30-60 min followed by transduction with the above specified pseudotypes. At 72 h post transduction, luciferase activities in cell lysates were measured. The average of five to seven independent experiments performed with triplicate samples is shown. Transduction with pseudotypes bearing VSV-G in the

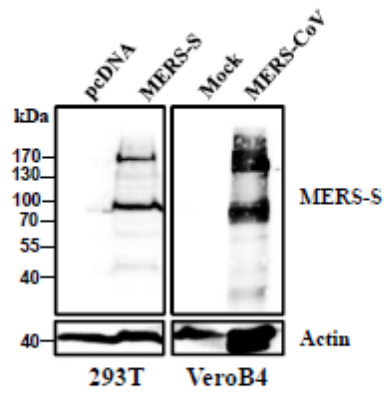
absence of inhibitor was set as 100%. Error bars indicate standard error of the mean (SEM).

At two tailed students t-test was applied to assess statistical significance.

**FIG 7 Processing of MERS-S by proprotein convertases is dispensable for MERS-CoV infectivity.** Caco-2 cells were incubated with the indicated concentrations of proprotein convertase inhibitor (PCI) for 1 h and then inoculated with MERS-CoV at an MOI of 0.01 and 0.001 for 30 min at 4°C. Thereafter, virus was removed, inhibitor replenished and cells were lysed at 24 h post infection for S protein detection by Western blot analysis (A, shown only MOI 0.01). In parallel, viral genomic RNA copies (B, left panel) or infectious MERS-CoV particles (B, right panel) in supernatants of MERS-CoV infected Caco-2 cells (with and without PCI treatment) were determined by qRT-PCR (upE assay) or plaque assay. The experiment was performed in quadruplicates and repeated twice with two different MOIs giving comparable results. Shown is one representative experiment (MOI 0.001). C) Cytopathogenic effects of MERS-CoV (MOI of 0.1) infected Vero B4 cells at 42 h post infection in the presence and absence of PCI. Viral antigen was detected with a MERS patient serum (1:100 diluted) followed by a cyanine 2-labeled goat-anti human immunoglobulin G. Nuclei were stained with DAPI-containing mounting medium. Samples were analyzed by immunofluorescence microscopy (Zeiss) and pictures were taken at the same microscopic settings at a magnification of 200-fold. Similar results were obtained in two independent experiments performed with duplicate samples.

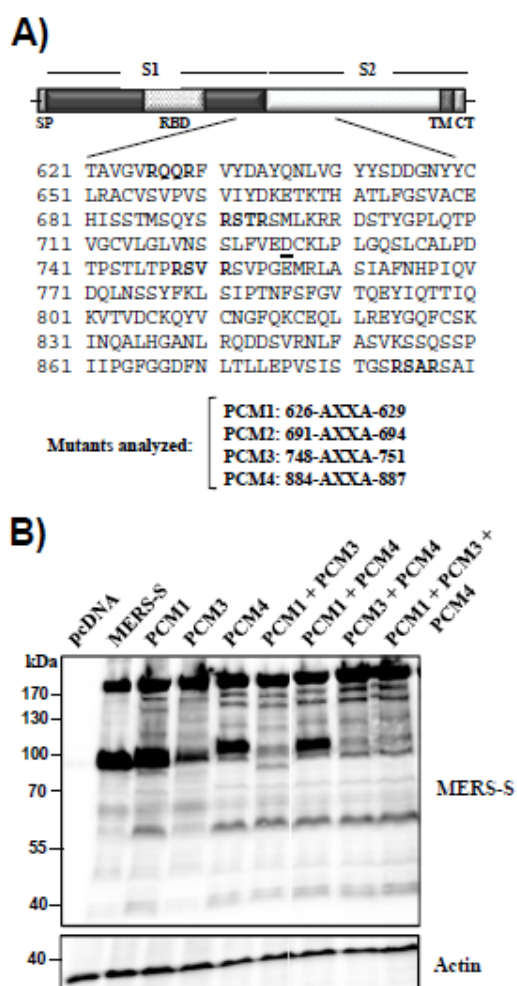
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Figure 1



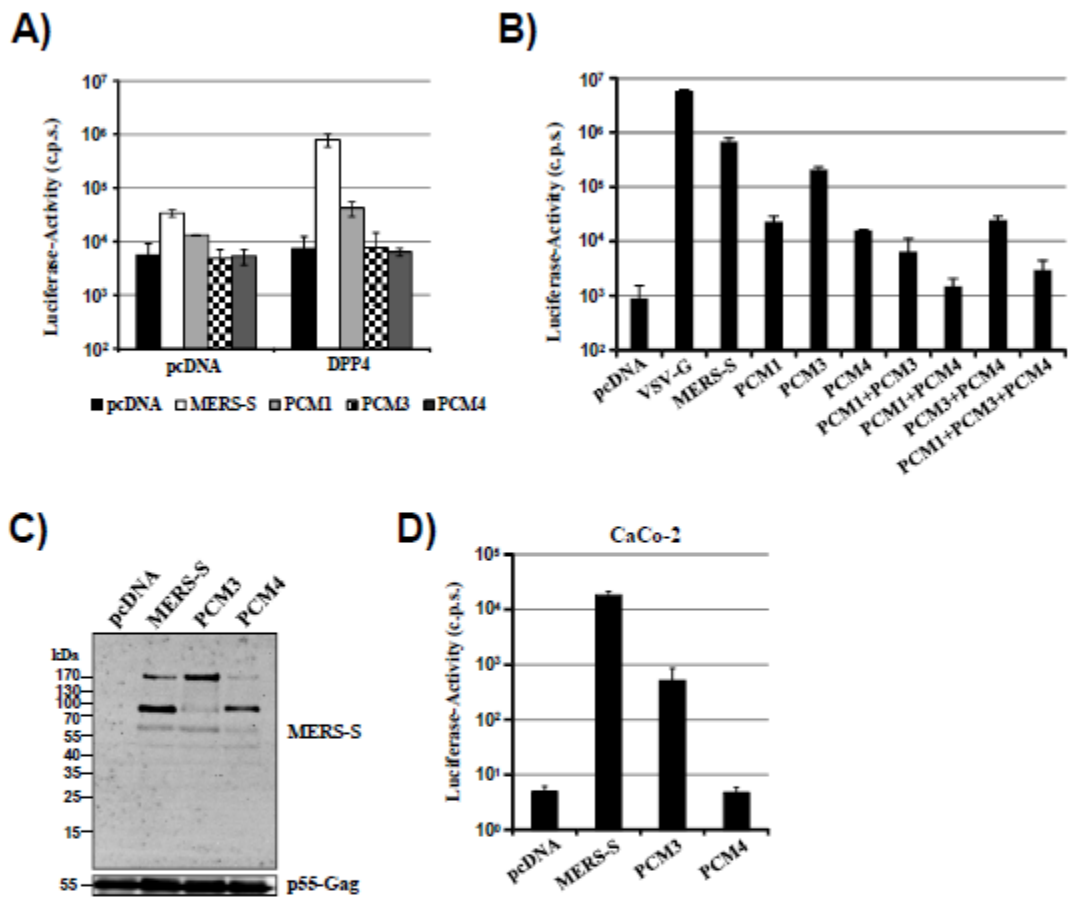
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Figure 2



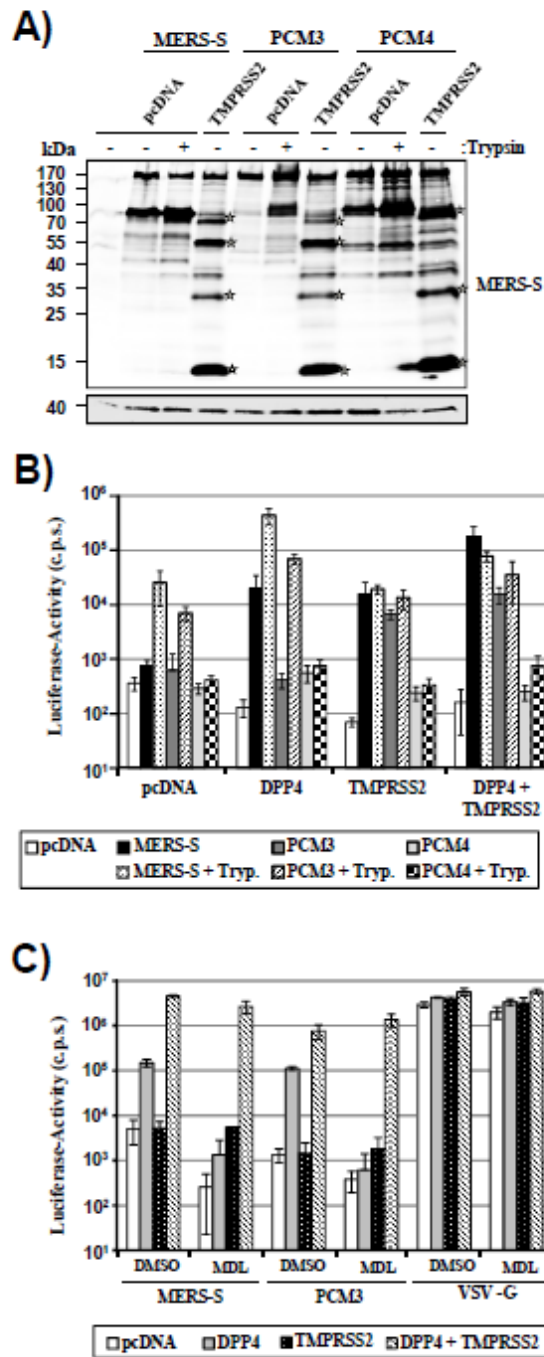
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Figure 3



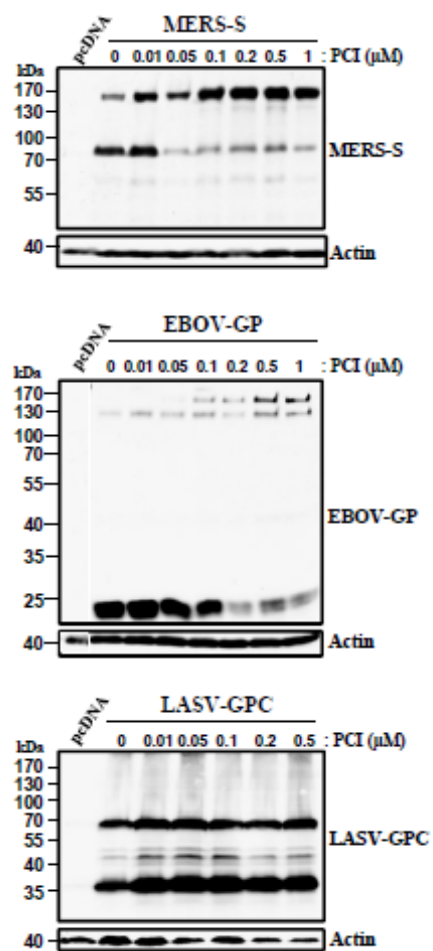
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Figure 4



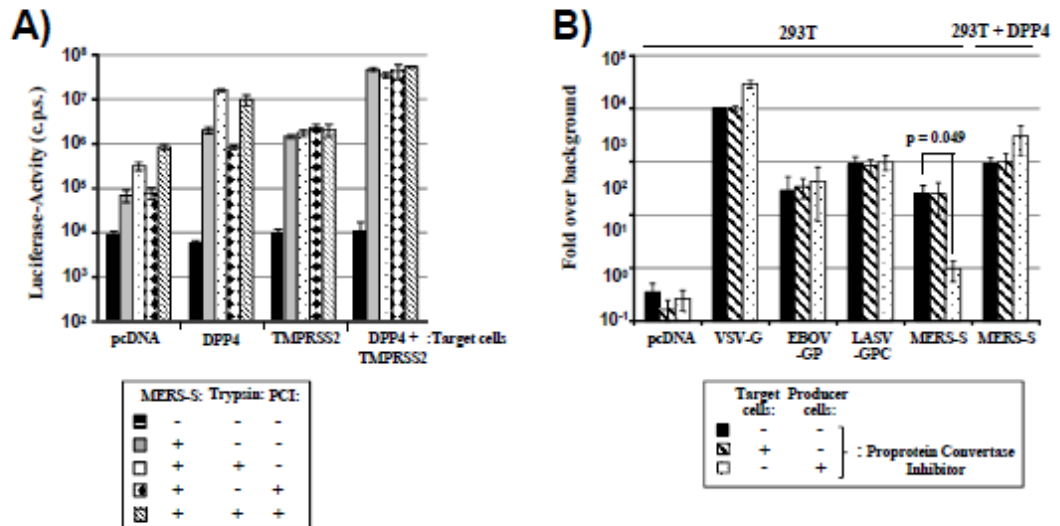
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Figure 5



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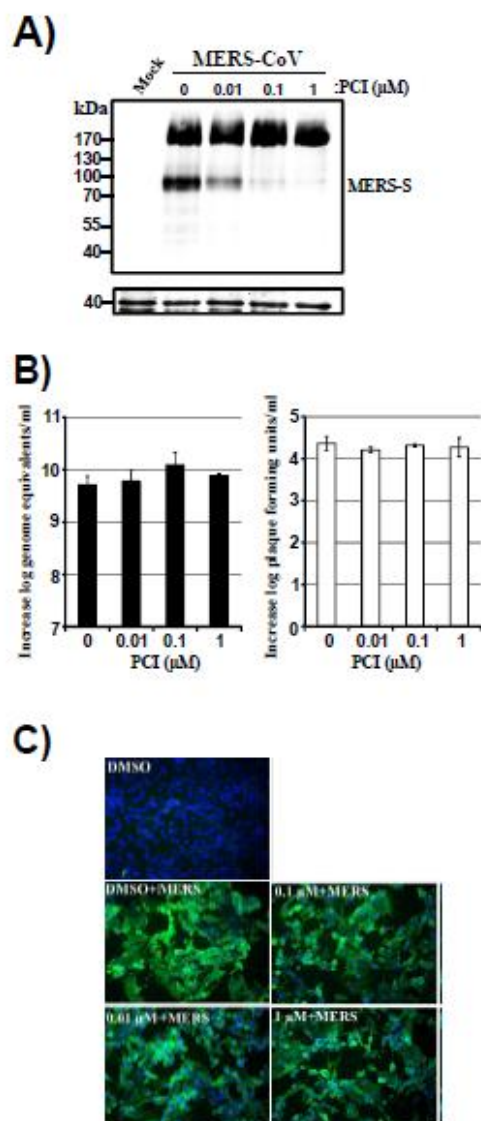
Figure 6





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Figure 7



**Third Manuscript**

## DISPATCHES

# Lack of MERS Coronavirus Neutralizing Antibodies in Humans, Eastern Province, Saudi Arabia

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We used a lentiviral vector bearing the viral spike protein to detect neutralizing antibodies against Middle East respiratory syndrome coronavirus (MERS-CoV) in persons from the Eastern Province of Saudi Arabia. None of the 268 samples tested displayed neutralizing activity, which suggests that MERS-CoV infections in humans are infrequent in this province.

The emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV, formerly termed EMC coronavirus [1]) could pose a serious threat to public health (2). As of September 2013, a total of 108 laboratory-confirmed infections (with 50 deaths) caused by MERS-CoV have been reported to the World Health Organization (WHO), most from Saudi Arabia (3), but data are limited on MERS-CoV seroprevalence in humans (4).

We recently developed a lentiviral vector system to study host cell entry mediated by the spike protein of MERS-CoV (MERS-S) (5). This system mimics key aspects of MERS-CoV cellular entry and enables sensitive and quantitative detection of neutralizing antibodies, which are known to be generated in infected patients (5). We used this system to determine the presence of MERS-CoV neutralizing antibodies in serum and plasma samples obtained from patients at King Fahd Hospital of the

University in Alkhobar, Saudi Arabia. The hospital is a referral hospital that serves the Eastern Province of Saudi Arabia, including the Dammam and Alhasa governorates, from which several MERS cases were reported, according to the Ministry of Health of Saudi Arabia and a recent study (6); no MERS patients were seen at King Fahd Hospital. Blood collection for this study was approved by the University of Dammam ethics committee, and informed, written consent for participation was received for all study participants.

## The Study

Two collections of patient samples were analyzed. The first collection consisted of 158 serum samples taken from children hospitalized for lower respiratory tract infections during May 2010–May 2011. The samples came from 77 female and 81 male patients with a median age of 11.6 months (range 7.3 months to 9 years). The second sample collection consisted of 110 plasma samples from men with a median age of 28 years (range 19–52 years) who donated blood at the hospital during December 2012.

Analysis of MERS-S–driven transduction of target cells revealed that none of the samples investigated contained neutralizing antibodies against MERS-S (Figure 1). As a control, a subset of the samples was analyzed for inhibition of cellular entry mediated by the G protein of vesicular stomatitis virus (VSV-G), an animal virus that does not circulate in Saudi Arabia, and the spike protein of the human coronavirus NL63 (NL63-S), a globally circulating coronavirus. None of the samples robustly inhibited VSV-G–dependent entry, whereas most samples markedly reduced entry-driven by NL63-S (Figure 1), as expected (7). Experiments using serum samples of known neutralizing capacity confirmed that our neutralization experiments were sensitive and specific (Figure 2). Thus, serum samples obtained from a patient infected with MERS-CoV potentially inhibited MERS-S but not VSV-G– or NL63-S–driven entry, whereas the reverse observation was made with serum samples reactive against NL63-S (Figure 2). In sum, none of the samples from children with respiratory infections and none of the samples from healthy adult men showed detectable amounts of MERS-S–neutralizing antibodies, but most neutralized NL63-S–driven host cell entry.

## Conclusions

Our results suggest that the estimated MERS-CoV seroprevalence in the area served by King Fahd Hospital was <2.3% in children during 2010–2011 and <3.3% in male adults in 2012 (upper limits of the 95% CIs for 0/158 and 0/110, respectively, by Fisher exact test). Our analysis of samples from children might have underestimated seroprevalence

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<sup>1</sup>These authors contributed equally to this article.

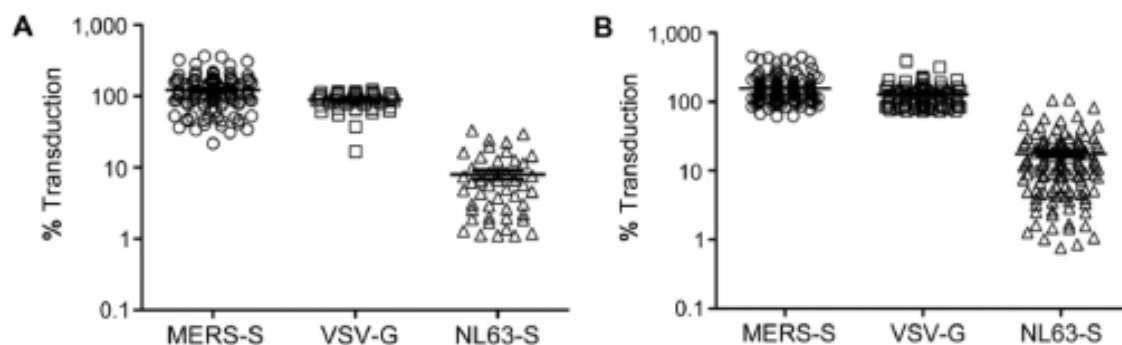


Figure 1. Neutralizing activity of serum and plasma samples obtained from patients at King Fahd Hospital of the University in Alkhobar, Saudi Arabia. A) Lentiviral vectors encoding luciferase and bearing the indicated viral glycoproteins were incubated with 1:20 dilutions of plasma from healthy adults, obtained during December 2012, and then added to target cells. Transduction efficiency was measured by quantification of luciferase activities in cell lysates and is shown relative to transduction of cells in the absence of serum, which was set at 100%. All 110 plasma samples available were tested for neutralization of Middle East respiratory syndrome coronavirus spike protein (MERS-S)-dependent transduction; subsets were also tested for neutralization of transduction driven by the G protein of vesicular stomatitis virus (VSV-G) (46/110) and the S protein of human coronavirus NL63 (NL63-S) (46/110). B) Analysis conducted as described for panel A using 158 serum samples from children with lower respiratory tract infections, obtained during May 2010–May 2011. All samples were analyzed for neutralization of MERS-S-mediated transduction; subsets were also tested for neutralization of transduction driven by VSV-G (76/158) and NL63-S (123/158). Horizontal lines indicate mean  $\pm$  SEM.

because if they were hospitalized for MERS-CoV infection, a virus-specific antibody response might have developed after sample collection. Moreover, although infection of young children has been reported (8), the average age of MERS patients is 50 years. Our findings using samples from adult men argue against the extensive spread of MERS-CoV within this group in the Eastern Province of Saudi Arabia during 2012, which is noteworthy given recent reports of asymptomatic MERS-CoV infections (9,10).

We cannot rule out that other diagnostic methods that are not limited to detection of neutralizing antibodies might have identified positive samples in our collection. Future analyses are required to determine MERS-CoV seroprevalence in larger patient collectives and in animal species, such as dromedary camels, that could transmit the virus to humans (4,11).

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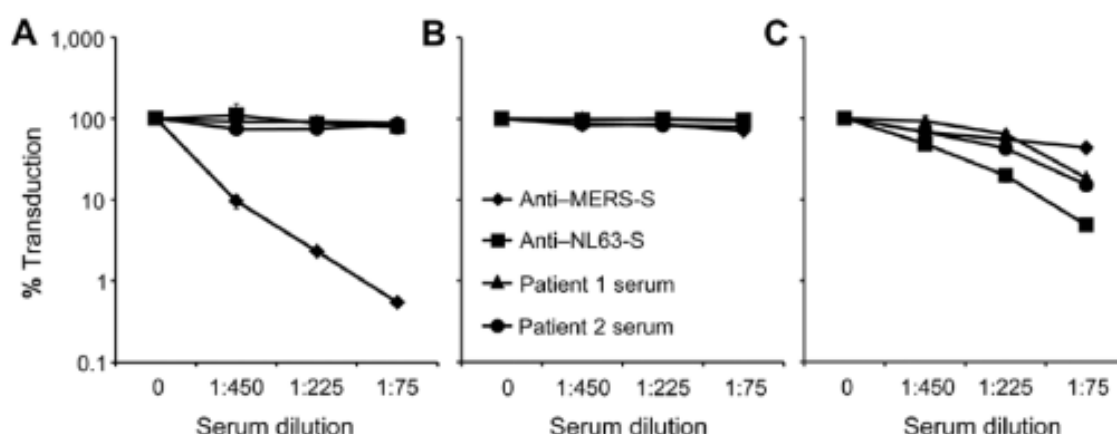


Figure 2. Analysis of serum samples with known neutralizing activity. Neutralization of transduction driven by the Middle East respiratory syndrome coronavirus spike protein (MERS-S) (A), G protein of vesicular stomatitis virus (B), and S protein of human coronavirus NL63 (NL63-S) (C) were determined as described for Figure 1, except that serum with known reactivity to MERS-S and NL63-S and serum from 2 patients at King Fahd Hospital of the University in Alkhobar, Saudi Arabia, that neutralized NL63-S-mediated transduction (Figure 1, panel A) were analyzed. Transduction of target cells in the absence of serum was set at 100%.

## DISPATCHES

Ms Gierer is a third-year PhD candidate in the Georg-August University School of Science of the University of Göttingen, Göttingen, Germany. Her research is focused on coronavirus interactions with host cells, particularly at the stage of viral entry.

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## 6. DISCUSSION

### 6.1 The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2, and is targeted by neutralizing antibodies.

The novel *Middle East Respiratory Syndrome Coronavirus* (MERS-CoV), formerly known as hCoV-EMC, with its high mortality rate, displays a significant threat to public health. In order to develop antiviral agents, viral and host cell interdependency has to be elucidated. Coronavirus entry is mediated by the spike (S) protein, which is a major determinant of the viral cell tropism [Kuo *et al.*, 2000]. The binding of the S protein to its receptor is the first step of viral invasion [Belouzard *et al.*, 2012]. Receptors, that are engaged by other human coronaviruses are CD13 (hCoV-229E), ACE2 (SARS-CoV, hCoV-NL63) and sialic acid (hCoV-OC43) [Vlasak *et al.*, 1988, Yeager *et al.*, 1992, Li *et al.*, 2003]. Which receptor is used by the MERS-CoV S protein (MERS-S) for entry was unknown. Coronavirus S proteins depend on proteolytic activation by host cell proteases to gain their fusogenic potential [Harrison 2005]. The S proteins of hCoV-229E and SARS-CoV can be activated by the pH-dependent cysteine protease cathepsin L (CTSL) [Simmons *et al.*, 2005, Kawase *et al.*, 2009] and the type II transmembrane serine protease TMPRSS2 [Matsuyama *et al.*, 2010]. The proteases which activate MERS-S were unknown.

Employing a lentiviral pseudotyping system, it could be demonstrated that the MERS-S protein does not engage known human coronavirus receptors, but facilitates entry into a broad spectrum of human cell lines, suggesting that the MERS-CoV receptor molecule is broadly expressed in human tissue. Furthermore, it could be shown that the S protein is activated by cathepsin L and TMPRSS2. Finally, a pseudotyping system was used to demonstrate the presence of neutralizing antibodies in a serum of a convalescent patient.

Previous studies have shown that the host cell entry of coronaviruses can be studied with the help of retroviral vectors, pseudotyped with coronaviral S proteins [Hofmann *et al.*, 2004, Moore *et al.*, 2004, Simmons *et al.*, 2004]. Therefore, lentiviral pseudotypes were employed in the present study to analyze MERS-S-driven host cell entry. Efficient expression of the S protein and incorporation into lentiviral pseudotypes was verified by Western blot analyses. Thereby, two prominent bands could be detected, one corresponding to the precursor S protein and one to a cleavage product, which is in concordance with the proteolytic processing

of some coronavirus S protein during biogenesis [de Haan *et al.*, 2004]. If this cleavage pattern is also detectable in MERS-CoV infected cells, and if the processing is relevant for viral infectivity was investigated in subsequent studies (see below). MERS-S-bearing vectors transduced a broad spectrum of human cell lines, including Caco-2 cells (colon), HOS (osteosarcoma), Huh-7 (liver), MRC5 (fetal lung fibroblasts) and 293T (kidney) as well as PMA-induced macrophages. Cells derived from the retina (RPE), glioblastoma (U373), endothelium (EA-hy) and from lung (A549, alveolar basal epithelial cells) were not susceptible. These findings suggest a broad expression of the receptor which is used by MERS-CoV for entry, and provides insights into the organ and tissue tropism of MERS-CoV. Moreover, these findings might explain MERS-CoV replication in multiple organ systems, which might in turn correlate with the high mortality rate that is connected with MERS-CoV infection. Independent studies confirmed the results discussed above and found that non-ciliated epithelium cells and type II pneumocytes are the major targets for MERS-CoV [Chan *et al.*, 2013, Kindler *et al.*, 2013, Raj *et al.*, 2013, Zielecki *et al.*, 2013]. Of note, MERS-CoV was shown to replicate in cells of the lower respiratory tract, while those from the upper respiratory tract were not susceptible [Chan *et al.*, 2013]. The observed MERS-CoV cell tropism of the respiratory tract correlates with the clinical picture, patients present, especially the severe pneumonia [Zaki *et al.*, 2012, Drosten *et al.*, 2013]. Renal failure as a frequent clinical symptom in MERS patients [Bermingham *et al.*, 2012] is in agreement with the susceptibility of kidney cells to S protein-driven entry [Bermingham *et al.*, 2012]. Interestingly, both SARS-CoV and MERS-CoV exhibit a similar organ tropism (lung, colon and kidney), which would suggest the use of a common cellular receptor for entry: The ACE2 molecule. However, the present study showed that lentiviral pseudotypes bearing MERS-S were not able to transduce cell lines expressing ACE2 or any of the other known coronavirus receptors, indicating that MERS-CoV utilizes a novel receptor, which is in concordance with other studies [Müller *et al.*, 2012]. Subsequently, Raj and colleagues could identify the receptor of question as the exopeptidase dipeptidyl peptidase 4 (DPP4 or CD26) [Raj *et al.*, 2013]. DPP4 expression correlates with the tissue tropism of MERS-CoV [Boonacker and Van Noorden 2003, Raj *et al.*, 2013]. However, this correlation is not absolute, thus suggesting the possibility of an alternative receptor [Zhao *et al.*, 2013].

For entry into target cells, the membrane of enveloped viruses has to fuse with the host cell membrane. To unfold its fusogenic potential, the viral glycoprotein needs to be triggered, either by receptor engagement, as for the murine leukemia virus envelope protein (MLV-Env)

[McClure *et al.*, 1988], low pH (vesicular stomatitis virus glycoprotein, VSV-G) [Superti *et al.*, 1987], or both (SARS-S, influenza virus HA) [White *et al.*, 2008]. Alternatively, cleavage by host cell proteases might suffice to trigger the membrane fusion reaction as has been suggested for coronavirus S proteins [Simmons *et al.*, 2004]. It could be shown that the pH-dependent endosomal cysteine proteases cathepsin B and L activate the Ebolavirus glycoprotein [Chandran *et al.*, 2005] and that cathepsin L activates the SARS-S protein [Simmons *et al.*, 2005]. These endosomal proteases depend on low pH, which can be inhibited by lysosomotropic agents such as ammonium chloride. Direct inhibition of both endosomal proteases can be facilitated by MDL28170 [Simmons *et al.*, 2005]. To identify host cell enzymes which are responsible for the activation of MERS-S, both inhibitors were employed and were shown to block MERS-S-mediated transduction. This indicates that the processing of MERS-S, like SARS-S, requires low pH and the activity of the endosomal cysteine proteases cathepsin B and L. Two studies confirmed these findings and discovered that cathepsin L is the major protease responsible for activation of MERS-S [Qian *et al.*, 2013, Shirato *et al.*, 2013].

The influenza virus hemagglutinin [Böttcher *et al.*, 2006] and SARS-S can hijack TMPRSS2 for cellular entry. In the latter case, glycoprotein activation by TMPRSS2 allows for cathepsin-B/L-independent entry [Matsuyama *et al.*, 2010, Glowacka *et al.*, 2011, Shulla *et al.*, 2011]. Since the entry strategies of MERS-CoV seem to be similar to that of SARS-CoV, it appeared to be likely that MERS-S could also be activated by TMPRSS2 and potentially other type II transmembrane serine proteases (TTSPs). Indeed, Western blot analyses showed that MERS-S is processed by trypsin, TMPRSS2 and HAT, while the TTSPs TMPRSS3, TMPRSS4 and TMPRSS6 failed to cleave the S protein. The same observation was made when S-bearing virus-like particles were generated in the presence of these proteases. Based on these findings, inhibitors were used to investigate if the TTSPs-mediated processing of MERS-S would result in S protein activation. MDL28170 was used to inhibit cathepsin B/L and camostat mesylate to inhibit TMPRSS2 [Kawase *et al.*, 2012]. It could be shown that MDL28170 blocked entry into 293T cells and that this blockade was rescued by the expression of TMPRSS2, indicating that MERS-S can use TMPRSS2 for viral entry in a cathepsin B/L-independent manner. Moreover, infection experiments using Caco-2 cells, which endogenously express TMPRSS2 [Bertram *et al.*, 2010], revealed that MERS-S activation by TMPRSS2 (as assayed by camostat treatment) dominates over activation by



cathepsin B/L. Similar results were obtained with other TMPRSS2-expressing cells (Vero cells, Calu-3 cells) [Shirato *et al.*, 2013].

TMPRSS2 is expressed in the human respiratory epithelium together with the MERS-S receptor DPP4 [Boonacker and Van Noorden 2003, Raj *et al.*, 2013] and the SARS-S receptor ACE2 [Bertram *et al.*, 2012, Raj *et al.*, 2013]. Notably, infection of TMPRSS2 expressing VeroB4 cells induced the formation of large syncytia [Qian *et al.*, 2013, Shirato *et al.*, 2013], illustrating the high membrane fusion potential of MERS-S. Thus, inhibitors targeting proteases required for viral entry exhibit therapeutic potential to suppress viral replication, which has already been suggested for infection with filoviruses and other coronaviruses [Simmons *et al.*, 2005, Zhou and Simmons 2012]. However, the biological relevance of TMPRSS2-dependent MERS-CoV glycoprotein activation awaits further investigation and TMPRSS2-knockout mice [Hatesuer *et al.*, 2013] might be suitable tools, since Adenoviral vector-hDPP4-transduced mice were recently shown to support MERS-CoV spread [Zhao *et al.*, 2014].

The coronavirus S protein is the major target for neutralizing antibodies of the human immune response [Hofmann *et al.*, 2004], which in turn might contribute to the control of coronavirus infection. Therefore, the pseudotyping system might be useful for diagnostic purposes. Employing the pseudotyping system, neutralizing antibodies were found to be generated in a convalescent patient. The same finding was obtained with authentic MERS-CoV [Corman *et al.*, 2012, Buchholz *et al.*, 2013, Chan *et al.*, 2013, Aburizaiza *et al.*, 2014]. Furthermore, the experiments of the present study show that there is no cross-reactivity of MERS-S-neutralizing antibodies against the glycoprotein of SARS-CoV. Interestingly, Chan and colleagues found significant titers of MERS-CoV reactive antibodies in sera from convalescent SARS patients [Chan *et al.*, 2013], suggesting that antibodies directed against SARS-S might also neutralize MERS-S. A putative cross-reactive capability with the highly related HKU4 and HKU5 still has to be investigated.

In summary, the present study provided insights into the cell tropism of MERS-CoV and identified host cell proteases which can activate MERS-S. On the basis of these findings, therapeutic strategies targeting these proteases could be developed. Blockade of TMPRSS2 might be particularly attractive, since genetic knock-out of *tmprss2* in mice has no phenotype [Kim *et al.*, 2006]. Finally, the findings demonstrated that the pseudotyping system is suitable to detect neutralizing antibodies against MERS-CoV and could be developed as a diagnostic tool.

## **6.2 Inhibition of proprotein convertases abrogates processing of the MERS-coronavirus spike protein in infected cells but does not reduce viral infectivity**

The coronavirus spike (S) glycoprotein, including the MERS-CoV spike protein (MERS-S), depends on proteolytically processing by host cell proteases to obtain its fusogenic potential [Gierer *et al.*, 2013, Simmons *et al.*, 2013]. Proteolytic activation can occur at different stages of the coronavirus infection cycle: During S protein biosynthesis in the secretory pathway of infected cells and during entry into target cells, either at the cell surface or within the acidic environment of the endosome [Heald-Sargent and Gallagher 2012]. It could be shown that MERS-S can be activated both at the host cell surface by the transmembrane serine protease TMPRSS2, and following internalization of virions into endosomes by the pH-dependent cysteine protease cathepsin L [Gierer *et al.*, 2013, Qian *et al.*, 2013, Shirato *et al.*, 2013]. Furthermore, it was demonstrated that MERS-S is cleaved in transfected cells [Gierer *et al.*, 2013, Shirato *et al.*, 2013], but whether this is also the case in infected cells remained to be determined. In addition, the biological relevance of this cleavage process was unknown and the responsible enzyme remained to be identified.

The present study shows that MERS-S is also cleaved in infected cells and that proprotein convertases are responsible for the cleavage, which requires the presence of several RXXR motifs located at the border between the S1 and S2 subunit. However, processing of MERS-S by proprotein convertases was not required for viral infectivity.

It was previously demonstrated that the S protein of MERS-CoV and other coronaviruses can be activated by TMPRSS2 and cathepsin L [Simmons *et al.*, 2005, Matsuyama *et al.*, 2010, Glowacka *et al.*, 2011, Shulla *et al.*, 2011, Kawase *et al.*, 2012, Bertram *et al.*, 2013, Gierer *et al.*, 2013, Qian *et al.*, 2013, Shirato *et al.*, 2013]. However, it is conceivable that MERS-S requires an initial cleavage process during biogenesis for subsequent activation by TMPRSS2 or cathepsin L for infectious viral entry into target cells. Evidence for a two-step cleavage process, the first in the secretory pathway, the second during viral uptake, has been presented for certain bunyavirus glycoproteins [Hofmann *et al.*, 2013]. Alternatively, it is possible that cleavage during biogenesis in infected cells is necessary for cell-cell fusion but not for virus-cell fusion, which would result in the formation of syncytia, a common feature of MERS-CoV pathogenesis [Zaki *et al.*, 2012]. For instance, cleavage of the coronavirus MHV-A59 spike protein at a furin consensus sequence is required for cell-cell but not virus-cell fusion [Frana *et al.*, 1985, Spaan *et al.*, 1988, de Haan *et al.*, 2004].

Western blot analysis revealed that MERS-S is cleaved in virus producing cells. Two prominent glycoprotein signals were detected in lysates from S protein transfected 293T cells and MERS-CoV infected Vero B4 and Caco-2 cells, indicating that MERS-S is efficiently cleaved in various cell types during biogenesis in the secretory pathway. This observation is in concordance with other studies [Gierer *et al.*, 2013, Shirato *et al.*, 2013, Song *et al.*, 2013, Alagaili *et al.*, 2014]. One group did not observe cleavage products upon MERS-S expression [Qian *et al.*, 2013]. A possible explanation could be that they analyzed an S protein with a truncated cytoplasmatic tail and this deletion might alter S protein folding and proteolytic processing.

Shown in Western blot analyses, the employment of a proprotein convertase inhibitor (PCI) revealed that the protease responsible for MERS-S processing was found to be a member of the proprotein convertase family. This finding is not unprecedented, since proprotein convertases are also responsible for the processing of the spike protein of MHV-A59 [Frana *et al.*, 1985, de Haan *et al.*, 2004] and IBV [Cavanagh *et al.*, 1986, Yamada and Liu 2009]. The SARS-CoV spike protein is inefficiently cleaved by proprotein convertases, but cleavage seems to be required for viral spread and for the formation of cytopathic effects [Bergeron *et al.*, 2005, Follis *et al.*, 2006, Simmons *et al.*, 2011]. The spike protein of MHV type 2 is not cleaved by proprotein convertases and needs to be activated by cathepsin inside endosomal vesicles [Qiu *et al.*, 2006].

The consensus motif of most proprotein convertases is (R/K)-2nX-R↓ [Seidah and Prat 2012], and inspection of the MERS-S sequence revealed four minimal furin cleavage motifs (RXXX) at the border between the S1 and S2 subunit, that can possibly be engaged by proprotein convertases [Pasquato *et al.*, 2013]. To investigate the role of these motifs in MERS-S processing, critical amino acids were exchanged from arginine to alanine residues (resulting in potential cleavage site mutants, PCM) and the corresponding MERS-S mutants were analyzed for cleavability. The majority of the mutant spike proteins were efficiently expressed and the mutation of two potential cleavage sites (3 and 4) had an impact on S protein processing. However, combined mutation of two potential cleavage sites was required to completely abrogate cleavage of transfected S protein. This observation suggests that proprotein convertases might be able to engage more than one cleavage motif in the spike protein and that blockade of one motif results in the cleavage of another. An indication for that is the observation that the molecular weight of the resulting cleavage product S2 of PCM4 was slightly increased. In this case, the protease might use potential cleavage site 3,

instead of the mutated potential cleavage site 4. However, it is also conceivable that all three RXXR motifs are needed to maintain the structure of the cleavage site.

Many different type I transmembrane glycoproteins depend on processing of their viral glycoproteins by cellular PCs in order to achieve the fusion-active state, for example the HA of the highly pathogenic avian influenza viruses (HPAI) [Bosch *et al.*, 1981, Stieneke-Grober *et al.*, 1992, Vey *et al.*, 1992] or the HIV-1 envelope glycoprotein [Hallenberger *et al.*, 1992]. For other glycoproteins, however, processing by proprotein convertases has diverse consequences. For example, the MHV-A59-S is also cleaved by PCs and cleavage was found to be required for cell-cell but not for virus-cell fusion [Frana *et al.*, 1985, Spaan *et al.*, 1988, de Haan *et al.*, 2004], while MHV-2-S is not cleaved during biogenesis in the constitutive secretory pathway of infected cells [Qiu *et al.*, 2006]. Moreover, the ability of the Ebolavirus glycoprotein to facilitate viral entry was not affected by the inactivation of the furin cleavage site [Neumann *et al.*, 2002].

Functional analysis of MERS-S mutants revealed that the inactivation of three RXXR motifs reduced MERS-CoV spike-driven cell-cell and virus-cell fusion, respectively, although the mutant proteins were efficiently expressed and incorporated into virus-like particles. In contrast, the mutants were still proteolytically processed by trypsin and TMPRSS2.

These observations revealed that the cleavage of MERS-S and the ability of MERS-S to drive cell-cell and virus-cell fusion depends on the integrity of all 3 predicted cleavage sites. It was therefore unexpected that the inhibition of proprotein convertase by a proprotein convertase inhibitor had no effect on MERS-driven cell-cell and virus-cell fusion when target cells expressing high amounts of DPP4 were used. However, reduced virus-cell fusion was observed when 293T target cells with only a low endogenous expression of DPP4 were employed. Thus, a high amount of DPP4 on target cells might compensate for absence of MERS-S processing by proprotein convertases. Processing of MERS-S by proprotein convertases could therefore be necessary for optimal spread in cell lines which express only a low level of DPP4. This scenario is reminiscent of SARS-S-driven cell-cell fusion, which is ineffective when receptor expression is low but can be restored by expression of S protein-activating proteases [Simmons *et al.*, 2011]. This possibility should be further investigated. Alternatively, secondary effects on S protein processing or maturation due to inactivation of RXXR motifs could also be an explanation for the reduced MERS-S-driven cell-cell and virus-cell fusion. Finally, endogenously DPP4 expressing Caco-2 cells were treated with the proprotein convertase inhibitor and infected with authentic MERS-CoV. Confirming that

MERS-S is indeed a substrate for proprotein convertases, inhibition of cleavage by PCI administration was observed. However, amplification of MERS-CoV in cell culture was not affected by the inhibitor, as the amount of viral RNA and the titer of released infectious particles was not reduced, and there was only an insignificant change in cytopathic effects. The activity of proprotein convertase is therefore dispensable for MERS-CoV infectivity in cell culture. Therefore, the massive syncytia formation, in MERS cytopathology might be rather due to activity of proteases other than proprotein convertases, e.g. that of TTSPs [Qian *et al.*, 2013, Shirato *et al.*, 2013].

It was shown that RXXR motifs are essential for proprotein convertase-mediated processing, and mutated RXXR motifs result in reduced cell-cell and virus-cell fusion, however, inhibition studies with PCI revealed that these phenotypes were not linked. The possibility that the RXXR motifs might be necessary for appropriate spike protein folding and efficient receptor binding was excluded by FACS-based analyses of S protein binding to soluble DPP4. The RXXR motif might not only serve as target sites for proprotein convertases but could also be recognized by other proteases, which activate the spike protein for membrane fusion, a possibility that would explain the reduction of MERS-S-dependent membrane fusion activity when the recognition motifs are mutated. A candidate protease recognizing RXXR motifs is cathepsin L, since virus-cell fusion of PCM1 and PCM4 was, compared to the wild type, reduced in 293T cells. This hypothesis needs further investigation and the protease(s) in question have to be identified.

In summary, it could be shown that the MERS-CoV spike protein is a substrate for proprotein convertases, which cleaves MERS-S in the secretory pathway of infected cells. However, MERS-S cleavage by proprotein convertases was found to be dispensable for viral activation and spread in host cells. The development of therapeutic agents should therefore focus on inhibition of protease activating MERS-S during viral entry: TMPRSS2 and cathepsin L.

### 6.3 Lack of MERS coronavirus neutralizing antibodies in patients from Eastern Province, Saudi Arabia

The emergence of the *Middle East Respiratory Syndrome Coronavirus* proved to be a threat to human health that should not be underestimated. As of May 9<sup>th</sup>, 2014, 536 cases and 145 deaths have been reported ([http://www.who.int/csr/disease/coronavirus\\_infections/MERS\\_CoV\\_Update\\_09\\_May\\_2014.pdf?ua=1](http://www.who.int/csr/disease/coronavirus_infections/MERS_CoV_Update_09_May_2014.pdf?ua=1)), with the majority of infections occurring in Saudi Arabia (World Health Organization; [http://who.int/csr/don/2014\\_04\\_24\\_mers/en/](http://who.int/csr/don/2014_04_24_mers/en/)). When this study was started, it was unclear whether the virus is circulating in an animal host and is subsequently introduced into the human population, or whether it circulates continuously in humans [Cauchemez *et al.*, 2013].

Due to limited surveillance data it cannot be ruled out that a significant number of MERS cases has gone undetected because of mild progression or because of continuous, asymptomatic circulation in humans [Memish *et al.*, 2013, Omrani *et al.*, 2013, Penttinen *et al.*, 2013]. The detection of neutralizing antibodies can be used to estimate the prevalence of MERS-CoV infection in high-risk areas. Moreover, evidence for zoonotic transmission might be obtained upon comparison of antibody prevalence in humans who were exposed or not exposed to animals.

To detect neutralizing antibodies in patient sera, a lentiviral pseudotyping system has proved useful. Lentiviral pseudotypes are env-defective, single-cycle virions, complemented with a viral envelope protein of choice, e.g. the coronavirus spike protein. Upon successful transduction, a reporter gene is expressed in the target cell, which enables quantification of glycoprotein-mediated entry. However, as the particles lack the genetic information for an envelope protein, infectious progeny virions will not be released from the transduced cell; thus no high biosafety level is required when operating with this diagnostic tool. This system has previously been used to detect neutralizing antibodies in sera obtained from SARS-CoV and hCoV-NL63 patients [Hofmann *et al.*, 2004, Hofmann *et al.*, 2005] and also proved suitable to verify a neutralizing antibody response after MERS-CoV infection in the present study.

To receive information about the MERS-CoV seroprevalence, samples were obtained from the geographic area where most MERS-CoV cases occurred or were linked to: Saudi Arabia. The patient samples were obtained from the King Fahd Hospital of the University in

Alkhobar, Eastern Province, Saudi Arabia, in which no MERS patients had been attended to during the time of sample collection. The samples included 158 serum samples taken from children (age range: 7.3 months to 9 years), who presented with lower respiratory tract infection, between May 2010 and May 2011, as well as 110 plasma samples collected from healthy adult blood donors in December 2012.

Transduction analyses of target cells with MERS-S-bearing pseudotypes revealed that none of the patient samples tested contained neutralizing antibodies. As a negative control, a subset of the samples was investigated for inhibition of entry driven by the glycoprotein of the vesicular stomatitis virus (VSV-G), which is an animal virus that does not circulate in Saudi Arabia. As expected, none of the samples inhibited entry driven by VSV-G. As positive control, pseudotypes bearing the spike protein of the globally circulating human coronavirus NL63 were used and found to be frequently inhibited by the patient samples. Finally, in order to prove the sensitivity and specificity of the neutralization assay and to exclude cross-reactivity, a MERS-CoV-reactive serum of known neutralizing capacity was used and was found to robustly inhibit MERS-S-driven entry in a concentration-dependent manner. On the basis of the data obtained with controls, it can be concluded that the pseudotyping system was able to detect MERS-S-neutralizing antibodies with high specificity and sensitivity but failed to detect such antibodies in the patient samples tested.

From the 268 serum samples analysed, the estimated seroprevalence of MERS-CoV distribution in King Fahd Hospital patients using Fisher's exact test was <2.3% among children (2010-2011) and 3.3% among adults (2012) (upper limits of the 95% confidence intervals for 0/158 and 0/110). The low seroprevalence in samples derived from children obtained between 2010 and 2011 could be explained by molecular clock analyses, which estimated that the most recent ancestor of MERS-CoV existed in the middle of 2011 [Cotten *et al.*, 2013, Drosten *et al.*, 2013, Cotten *et al.*, 2014]. However, it should be noted that although infections of children with MERS-CoV have been reported [Hui *et al.*, 2014, Memish *et al.*, 2014, [http://www.recombinomics.com/News/05161301/MERS\\_CoV\\_Children\\_KSA.html](http://www.recombinomics.com/News/05161301/MERS_CoV_Children_KSA.html)], the average age of infected patients is ~50 years ([http://www.who.int/csr/disease/coronavirus\\_infections/update\\_20130920/en/](http://www.who.int/csr/disease/coronavirus_infections/update_20130920/en/)). In parallel, the missing detection of neutralizing antibodies in the adult group argues against an asymptomatic spread of MERS-CoV infection in the Eastern Province, although there have been severe case reports from this geographic region [Memish *et al.*, 2013, Omrani *et al.*, 2013].

Other studies assessing the seroprevalence of residents of the Middle East obtained findings in accordance with our data: No neutralizing antibodies were detectable in healthy blood donors and individuals which were possibly exposed [Chan *et al.*, 2013, Aburizaiza *et al.*, 2014]. Also, antibodies directed against MERS-S could not be found in samples from pilgrims returning from the Hajj in 2012 and 2013 [Al-Tawfiq and Memish 2012, Gautret *et al.*, 2014].

However, it has to be mentioned that other diagnostic tools besides neutralization assays should be employed to analyse the samples tested in the present study in order to exclude the possibility of false-negative results. These tools could include PCR-based approaches targeting specific fragments within the MERS-CoV genome, as well as immunoassays measuring the presence of MERS-CoV-specific antibodies irrespective of their capacity to neutralize [Corman *et al.*, 2012, Reusken *et al.*, 2013].

Some studies focused on the seroprevalence in dromedary camels, the animal that is believed to be the intermediate host [Albarrak *et al.*, 2012, Drosten *et al.*, 2013, Kupferschmidt 2013] and found a neutralizing antibody response against MERS-CoV or a closely related virus in sera from these animals [Hemida *et al.*, 2013, Perera *et al.*, 2013, Reusken *et al.*, 2013, Woo *et al.*, 2014]. High antibody titers were even detected in samples collected in the years 1992, 2003 and 2005 [Alagaili *et al.*, 2014, Alexandersen *et al.*, 2014, Meyer *et al.*, 2014], indicating that dromedary camels have been frequently infected since decades and that a sporadic transmission from animals to humans might have been going on for years. The first virological confirmation of MERS-CoV infection in dromedaries that was linked to a human case was made by Haagmans and colleagues [Haagmans *et al.*, 2014]. However, the route of transmission and the possibility of a third source of transmission has to be investigated, and transmission experiments in animal models could be useful to shed light onto those questions [Nishiura *et al.*, 2014].

Taken together, the findings of the study and work done by others suggest that MERS-CoV might not have circulated before 2012 in the area from which MERS cases are now constantly reported.





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## 8. Appendix

### List of abbreviations

%	Percentage
A	Alanin
ACE2	Angiotensin-converting enzyme 2
APN	Aminopeptidase N
ARDS	Acute respiratory distress syndrome
Bat-CoV	Bat coronavirus
CDC	Centers for Disease Control and Prevention
CoV	Coronavirus
CPE	Cytopathic effect
CTSL	Cathepsin L
DPP4	Dipeptidyl peptidase 4
E	Envelope
EIA	Enzymatic immunoassays
ELISA	Enzyme-linked-immunosorbent assay
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum-Golgi apparatus intermediate compartment
<i>et al.</i>	lat.: <i>et alteri</i>
FP	Fusion peptide
GC	Guanine-cytosine
GP	Glycoprotein
HA	Hemagglutinin
HAT	Human airway trypsin-like protease

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HB	Helix bundle
HCoV-EMC	Human coronavirus Erasmus Medical Center
HIV	Human immunodeficiency virus
HPAI	High pathogenic avian influenza
HR	Heptad repeats
IFA	Immunofluorescent antibody
IFN	Interferon
IgG	Immunoglobulin G
K	Lysin
Kb	Kilo bases
LDLRA	Low-density lipoprotein receptor domain class A
LPAI	Low pathogenic avian influenza
M	Membrane
MERS-CoV	Middle East Respiratory Syndrome-Coronavirus
MHV	Mouse hepatitis coronavirus
MLV	Murine leukemia virus
mRNA	Messenger ribonucleic acid
MSPL	Mosaic serine protease large form
N	Nucleocapsid
NH <sub>4</sub> Cl	Ammonium chloride
nsps	Nonstructural proteins
nm	Nanomolar
ORF	Open reading frame
PC	Proprotein convertase
PCR	Polymerase-chain-reaction
pH	Potentia Hyrogenii

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pp	Polyproteins
R	Arginine
RBD	Receptor binding domain
RBM	Receptor binding motif
RdRp	RNA-dependant RNA polymerase
RFS	Ribosomal frameshift site
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase-polymerase-chain-reaction
S	Spike
SARS-CoV	Severe Acute Respiratory Syndrome-Coronavirus
SRCR	Scavenger receptor cysteine- rich domain
TM	Transmembrane domain
TRS	Transcription- regulatory sequences
TTSP	Type II transmembrane serine protease
upE	Upstream of the envelope gene
UTR	Untranslated region
VSG-G	Vesicular stomatitis virus glycoprotein
WHO	World Health Organization

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- 03/14-17/2012                      22nd annual Meeting of the Society for Virology (Essen, Germany)
- Poster “**TMPRSS2 and HAT activate the spike protein of human coronavirus 229E for cathepsin- independent host cell entry**”
- 03/06-09/2013                      23rd Annual Meeting of the Society for Virology (Kiel, Germany)
- Poster “**The spike-protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2 and is targeted by neutralizing antibodies**”
- 09/19-20/2013                      National Symposium on Zoonoses Research 2013 (Berlin, Germany)
- Oral presentation “**Analysis of the proteolytic activation of the MERS-coronavirus spike-protein**”

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**List of Publications*****First author***

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**Gierer, S.**, S. Bertram, F. Kaup, F. Wrensch, A. Heurich, A. Kramer-Kuhl, K. Welsch, M. Winkler, B. Meyer, C. Drosten, U. Dittmer, T. von Hahn, G. Simmons, H. Hofmann and S. Pohlmann (2013). "The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2, and is targeted by neutralizing antibodies." J Virol **87**(10): 5502-5511.

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Bertram, S., R. Dijkman, M. Habjan, A. Heurich, **S. Gierer**, I. Glowacka, K. Welsch, M. Winkler, H. Schneider, H. Hofmann-Winkler, V. Thiel and S. Pohlmann (2013). "TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium." J Virol **87**(11): 6150-6160.

Heurich, A., H. Hofmann-Winkler, **S. Gierer**, T. Liepold, O. Jahn and S. Pohlmann (2014). "TMPRSS2 and ADAM17 cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the severe acute respiratory syndrome coronavirus spike protein." J Virol **88**(2): 1293-1307.

**Reviews**

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Simmons, G., P. Zmora, **S. Gierer**, A. Heurich and S. Pohlmann (2013). "Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research." Antiviral Res **100**(3): 605-614.