Bedeutung der ACE2-Spaltung durch Wirtszellproteasen für die SARS-Coronavirus-Infektion

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„Ein Gelehrter in seinem Laboratorium ist nicht nur ein Techniker; er steht auch vor den Naturgesetzen wie ein Kind vor der Märchenwelt.“

Marie Curie

Inhalt

Kurzfassung

Das *severe acute respiratory syndrome* Coronavirus (SARS-CoV) ist ein hochpathogenes Virus, dessen zoonotischer Eintrag in die Bevölkerung eine substantielle Gesundheitsgefahr darstellt. Die Identifizierung von Wirtszellfaktoren, die für die SARS-CoV-Ausbreitung und Pathogenese wichtig sind, könnte neue Ansatzpunkte für die Therapie liefern. Das SARS-CoV-Oberflächenprotein Spike (S) bindet an den zellulären Rezeptor *angiotensin converting Enzyme 2* (ACE2) und vermittelt den viralen Eintritt in Zielzellen. Die Spaltung und Aktivierung des S Proteins durch Wirtszellproteasen ist für den infektiösen, S Protein-vermittelten Zelleintritt von SARS-CoV essentiell. Die Typ II Transmembranserinproteasen (TTSPs) TMPRSS2 und HAT spalten und aktivieren das S Protein, zumindest nach gerichteter Expression in Zelllinien. Ob diese Enzyme in der menschlichen Lunge, den Zielzellen der SARS-CoV-Infektion, exprimiert werden, war jedoch unklar und sollte im Rahmen der vorliegenden Arbeit untersucht werden. TMPRSS2 und HAT spalten auch den viralen Rezeptor ACE2 und es wurde postuliert, dass die ACE2-Spaltung den viralen Eintritt erhöht. Der zugrundeliegende Mechanismus war jedoch nicht bekannt und sollte innerhalb der vorliegenden Arbeit aufgeklärt werden.

Es konnte gezeigt werden, dass TMPRSS2 und HAT zusammen mit ACE2 in Epithelzellen des Respirationstrakts exprimiert werden. Die Proteasen könnten daher die Ausbreitung von SARS-CoV im Lungenepithel fördern. Weiterhin wurde eine Aminosäuresequenz in ACE2 identifiziert, die für die Prozessierung durch TMPRSS2 und HAT essentiell ist. Die funktionelle Analyse von ACE2-Mutanten zeigte, dass die Spaltung in diesem Bereich infektionsverstärkend wirkt. Immunfluoreszenz-Studien erbrachten Hinweise darauf, dass die Verstärkung der Infektion auf eine erhöhte Aufnahme von Virus-Partikeln in die Zelle zurückzuführen ist. Schließlich konnte demonstriert werden, dass TMPRSS2 und eine weitere zelluläre Protease, *A Disintegrin And Metalloproteinase* 17 (ADAM17), um die ACE2-Spaltung konkurrieren und die ADAM17-Spaltstelle in ACE2 konnte kartiert werden. Die ACE2-Spaltung durch ADAM17 war jedoch für den S Protein-getriebenen Zelleintritt verzichtbar. Zusammenfassend zeigen diese Untersuchungen, dass TMPRSS2 und HAT die SARS-CoV-Infektion durch Spaltung von S Protein und Rezeptor fördern. Die Proteasen stellen daher mögliche Angriffspunkte für die antivirale Intervention dar.

Schlagwörter: respiratorische Viren, ARDS, Influenza, SARS- CoV, ACE2, TTSPs

Abstract

The *Severe Acute Respiratory Syndrome Coronavirus* (SARS-CoV) is a highly pathogenic virus and its zoonotic entry into the human population represents a substantial health threat. The identification of host cell factors important for SARS-CoV spread and pathogenesis might yield new targets for therapy. The SARS-CoV envelope protein spike (S) binds to the cellular receptor, *Angiotensin Converting Enzyme* 2 (ACE2), and mediates viral entry into target cells. Cleavage and activation of the S protein by host cell proteases is essential for the infectious S protein-mediated entry. The type II transmembrane serine proteases (TTSPs) TMPRSS2 and HAT cleave and activate the S protein, at least upon expression in cell culture. Whether these enzymes are expressed in human lung cells, the target cells of SARS-CoV infection, was unclear and was to be investigated in the present thesis. TMPRSS2 and HAT also cleave the viral receptor ACE2 and it was postulated that ACE2 cleavage increases viral entry into host cells. However, the underlying mechanism was not known and was to be analyzed within the present study.

It could be shown that TMPRSS2, HAT and ACE2 are coexpressed in epithelial cells of the respiratory tract. Therefore, these proteases could promote the spread of SARS-CoV in lung epithelium. Furthermore, an amino acid sequence in ACE2, which is essential for the processing by TMPRSS2 and HAT, was identified. The functional analysis of ACE2 mutants demonstrated that cleavage at this site increases S protein-driven host cell entry, and immunofluorescence studies provided evidence that the augmented entry efficiency was due to increased viral particle uptake into the cell. Finally, it was demonstrated that TMPRSS2 and another cellular protease, *A Disintegrin And Metalloproteinase 17* (ADAM17), compete for ACE2 cleavage and the cleavage site for ADAM17 in ACE2 could be identified. However, ACE2 cleavage by ADAM17 was found to be dispensable for the S protein-driven cell entry. In summary, these studies indicate that TMPRSS2 and HAT promote SARS-CoV infection by cleavage of the viral S protein and its receptor. The proteases therefore constitute potential targets for antiviral intervention.

Keywords: respiratory viruses, ARDS, influenza, SARS-CoV, ACE2, TTSPs

1 Einleitung

1.1 Respiratorische Viren

Respiratorische Viren zählen zu den häufigsten Erregern von Infektionskrankheiten und sind noch heute mit einer hohen Mortalität und Morbidität assoziiert [1, 2]. Zu den gut untersuchten humanpathogenen respiratorischen Viren gehören die saisonalen Influenza-Viren, die Rhinoviren (HRV), die respiratorischen Synzytial-Viren (RSV), die respiratorischen Adenoviren und einige Coronaviren (229E, OC43, NL63, HKU1). Sie werden entweder direkt über Tröpfchen beim Niesen und Husten ausgestoßen, oder indirekt durch Schmierinfektion übertragen. Die Symptome Fieber, Schüttelfrost, Gliederschmerzen und Appetitlosigkeit können 1-10 Tage nach Kontakt mit dem jeweiligen Virus auftreten und klingen, wenn keine Komplikationen wie z.B. bakterielle Sekundärinfektion auftreten, 2-5 Tage später wieder ab [3-7]. Einige dieser Viren können schwerwiegendere Krankheiten hervorrufen oder sie infizieren bevorzugt Menschen in bestimmten Altersgruppen, wie Kinder, ältere Menschen und chronisch Kranke. Die respiratorischen Viren, allen voran die Influenza-Viren, sind damit ein ernstzunehmendes öffentliches Gesundheitsproblem. Mit einer jährlichen Sterberate von 250.000- 500.000 Menschen weltweit allein für die saisonalen Influenza Viren [8] werden die respiratorischen Viren nicht umsonst die „geheime Pandemie“ genannt.

Zu den saisonal grassierenden respiratorischen Viren kommen pandemisch auftretende Viren wie pandemische Influenza-A-Viren und das *Severe Acute Respiratory* Coronavirus (SARS-CoV) hinzu, die den Handlungsdruck auf Politik und Gesundheitsämter weiter erhöhen. So forderte die spanische Grippe, die von 1918-20 kursierte und durch ein neues Influenza-A-Virus hervorgerufen wurde, 25-50 Millionen Todesopfer, und hatte damit genauso viele Opfer wie die Pest von 1934 [9]. Die 2006 auftretende Vogelgrippe (Subtyp A/H5N1) hingegen wurden zwar nur in Einzelfällen von Mensch zu Mensch übertragen, was die Opferzahl gering hielt, Experten befürchteten allerdings eine Rekombination mit den humanen Influenza- Viren, womit eine rapide Verbreitung in der immunologisch naiven Bevölkerung möglich wäre [10]. Die 2009 auftretende Schweinegrippe (Subtyp A/H1N1) erlangte enorme öffentliche Aufmerksamkeit durch ihre Verwandtschaft mit der spanischen Grippe. Das Virus hatte die Fähigkeit zur effektiven Übertragung von Mensch zu Mensch bereits erlangt und verursachte ca. 20.000 Labor- bestätigte Tote, wobei die Dunkelziffer weit höher liegt (www.who.int/csr/don/2010_08_06/en/).

Die Zahl der Opfer der SARS-CoV Pandemie von 2002/2003 konnte durch Überwachung, Prävention sowie rechtzeitiges Erkennen der Krankheit und konsequentes Handeln auf 774 von 8098 Infizierten beschränkt werden [11]. Die MERS-CoV Epidemie, die im Januar 2012 begann, verursachte bis jetzt 145 Tote von 536 bestätigt Infizierten (http://www.who.int/csr/disease/coronavirus_infections/en/). Dabei sind Menschen mit erheblichen Vorerkrankungen wie Diabetes mellitus oder chronisch obstruktiver Lungenerkrankung, mit Immundefekten oder zurückliegendem Nierenversagen besonders betroffen [12]. Es wird daher angenommen, dass Personen mit Grunderkrankungen eine höhere Anfälligkeit für einen schweren Verlauf der MERS-CoV-Infektion haben. Da es bisher aber nicht zu einer anhaltenden Virusübertragung in der Bevölkerung gekommen ist, scheint die Übertragung des Virus von Mensch zu Mensch ineffizient zu verlaufen. Nur der enge persönliche Kontakt zwischen Patienten und Pflegepersonal bzw. Familienangehörigen ermöglicht eine Übertragung. In den letzten Wochen wurden allerdings verstärkt Fälle von Übertragungen auf medizinisches Personal gemeldet (http://www.who.int/csr/disease/coronavirus_infections/en/). Die Sorge, dass sich das Virus an den Menschen als neuen Wirt adaptiert und effizienter übertragen wird, bleibt daher bestehen. Deswegen ist es von höchster Bedeutung, die Verbreitung von humanen und tierischen respiratorischen Viren zu erforschen, um durch neue wissenschaftliche Erkenntnisse bessere Präventionsmaßnahmen und Therapien zu entwickeln.

1.2 Acute Respiratory Distress Syndrome (ARDS)

Das *Acute Respiratory Distress Syndrome* (ARDS) bezeichnet die Schädigung von Lungengewebe mit Perfusions-, Gerinnungs-, und Permeabilitätsstörungen sowie Lungenödemen [13]. Als Auslöser gelten Sepsis, Inhalation von Gasen oder Flüssigkeiten, Lungenquetschungen, Verbrennungen und schwere Grunderkrankungen wie Pneumonie und Pankreatitis. Außerdem kann ARDS durch viral bedingte Erkrankungen hervorgerufen werden. Unter den respiratorischen Viren, die ARDS auslösen, sind die pandemisch auftretenden Influenza- Viren, sowie SARS- und MERS-CoV zu nennen [14].

Trotz massiven Aufwands gibt es bis heute keine spezifische Therapie für die Behandlung von ARDS, was wiederum zu einer hohen Mortalitätsrate (40-60%) unter den Erkrankten führt [15]. Ein vielversprechender experimenteller Therapieansatz beruht auf dem Schutz des Lungenendothelzellgewebes, dem Primärziel der viralen Infektion. Die Aktivierung des Lungenepithels, als Reaktion des Körpers auf die Infektion stellt den Krankheitsbeginn dar. Die beschädigten Endothelzellen aktivieren das Entzündungssystem, die daraus folgenden Veränderungen im Koagulationssystem können zu *Diffuse Alveolar Damage* (DAD) und ARDS führen [16-18]. Erste vielversprechende Experimente an Mäusen zeigen, dass der *Angiotensin- Converting Enzyme* (ACE) Inhibitor Captopril vor experimentell induzierter Lungenschädigung schützt [15]. ACE ist Bestandteil

des Renin-Angiotensin-Systems (RAS), das den Flüssigkeits- und Elektrolythaushalt des Körpers reguliert und somit entscheidend auf den Blutdruck einwirkt. Das RAS ist in der Lunge sehr aktiv: hier wird vermehrt Angiotensin I (ANGI) gebildet, dass durch ACE in ANGII umgewandelt wird. Eine abnorme Anhäufung von ANGII verstärkt die Ausschüttung von entzündungsfördernden Zytokinen. Die Reduktion von ANGII durch die Inhibierung von ACE führt so zum Schutz des Lungengewebes [19, 20]. Auch für das ACE Homolog ACE2 konnte eine Schutzfunktion vor dem durch Chemikalien oder Sepsis induzierten ARDS nachgewiesen werden [19]. ACE2 ist ebenfalls Teil des RAS und wandelt das potentielle lungenschädigende ANGII in Angiotensin 1-7 um (Abbildung 1).

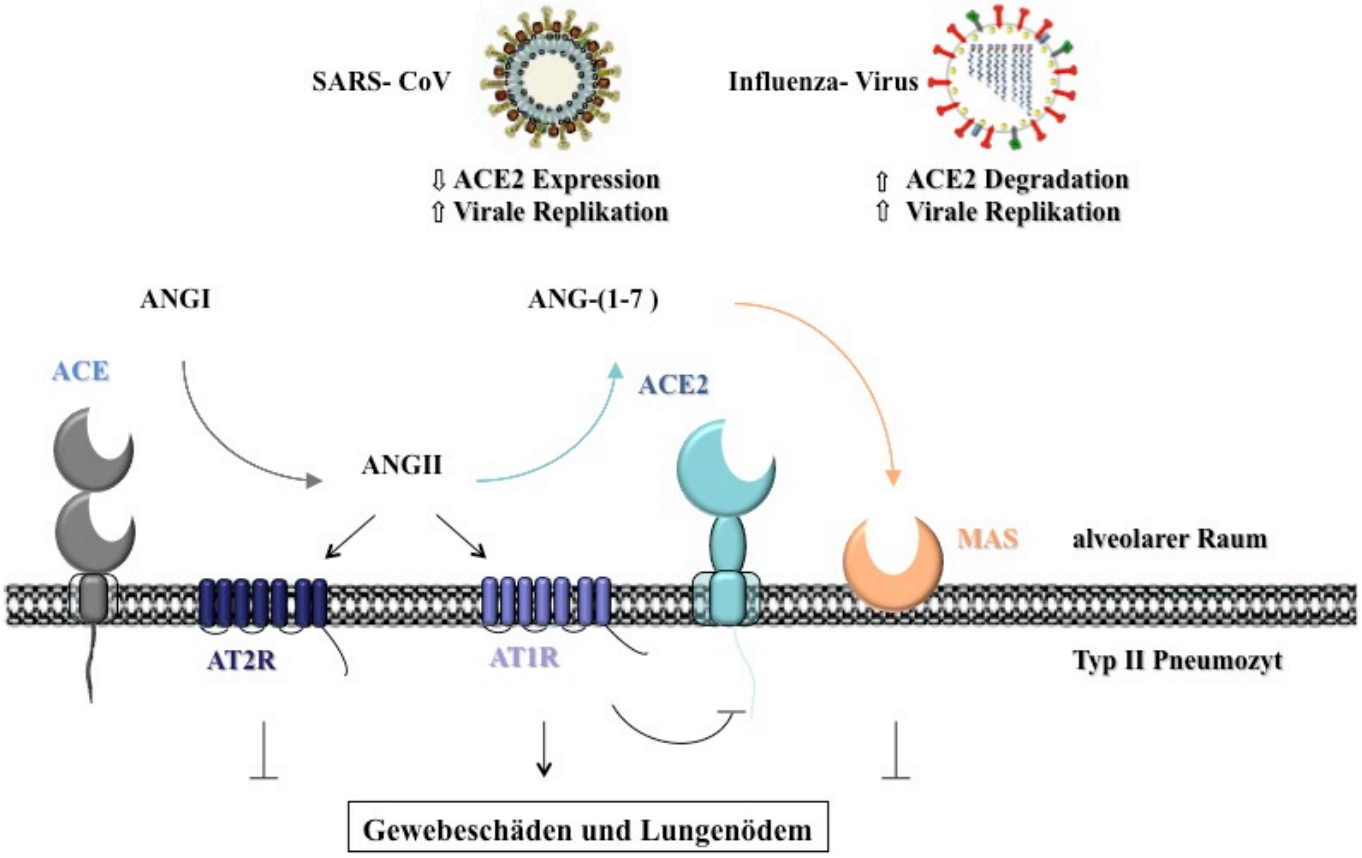

Abbildung 1: Schematische Darstellung der durch virale Infektion induzierten akuten Lungenschädigung. ACE spaltet Angiotensin I (ANGI) in Angiotensin II (ANGII). ANGII bindet entweder an den Rezeptor AT1R, was zu Gewebsschäden und Lungenödemen führen kann, oder an den Rezeptor AT2R, was die Reduktion des Gewebeschadens zur Folge hat. ACE2 inaktiviert ANGII, indem es dieses in Angiotensin 1-7 umwandelt. Das kann dann an den G-Protein gekoppelten Rezeptor Mas binden und erfüllt als Gegenspieler von AT1R ebenfalls eine gewebeschützende Funktion. SARS-CoV bindet an den Rezeptor ACE2 und reduziert die Menge von ACE2 an der Zelloberfläche durch Shedding und Internalisierung. Das Influenza A Virus H1N1 führt hingegen zum proteasomalen Abbau von ACE2.

Für eine wichtige Rolle von ACE2 im Zusammenhang mit ARDS spricht auch, dass die ARDS auslösenden Viren SARS-CoV und Influenza A (H1N1) während der Infektion ACE2 auf verschiedenen Wegen herab regulieren [21, 22]. Während der SARS-CoV Infektion wird der SARS-

CoV Rezeptor ACE2 nach Virus-Bindung internalisiert und nachfolgend die Expression herabreguliert. Das Influenza-A-Virus H1N1 (A/Puerto Rico/8/1934) hingegen verursacht die Degradation von ACE2 im Proteasom nach Neuraminidase abhängiger Spaltung. Daher ist die Erforschung der Zellbiologie von ACE2 als Teil des Renin- Angiotensin- Systems, als SARS-CoV Rezeptor und als Teil des durch Influenza und SARS-CoV induzierten ARDS von enormer Bedeutung.

1.3 Coronaviren als Auslöser respiratorischer Erkrankungen

Coronaviren sind artspezifisch und können Menschen und verschiedene andere Wirbeltiere wie Säuge- und Nagetiere, Fische und Vögel infizieren [23]. Beim Menschen lösen die humanen Coronaviren (hCoV) üblicherweise leichte, selbst-limitierende Infektionen des oberen Respirationstrakts aus: 30% der Erkältungskrankheiten schreibt man den hCoV 229E, OC43 und NL63 zu. In den letzten Jahren stellte sich aber heraus, dass hCoV auch schwerwiegendere Erkrankungen des unteren Respirationstrakts verursachen können und das vor allem bei Patienten mit unreifem oder unterdrücktem Immunsystem sowie bei Patienten mit schweren Grunderkrankungen [24, 25]. Dabei verursachen sie Bronchitis, Laryngotracheitis, Bronchiolitis und Lungenentzündung [26, 27]. Ein weiteres Coronavirus, CoV-HKU1, wurde 2005 aus einem Patienten mit akuter Lungenentzündung isoliert [28, 29]. Die genannten vier hCoV sind an den Menschen angepasst und weltweit verbreitet [30, 31]. Das Auftreten des *Severe Acute Respiratory Syndrome* Coronavirus (SARS-CoV) im Jahr 2002 und des *Middle East Respiratory Syndrome* Coronavirus (MERS-CoV) im Jahr 2012 machen deutlich, dass der Eintrag tierischer CoV, in diesem Fall aus Fledermäusen, in die humane Bevölkerung (Zoonose) ein schweres Gesundheitsrisiko darstellen kann [32-34].

1.3.1 Klassifizierung

Die Coronaviren gehören zur Familie der *Coronaviridae,* Ordnung Nidovirales, die in die Unterfamilien Coronavirinae und Torovirinae untergliedert wird [35, 36]. Die Mitglieder der Unterfamilie Coronavirinae unterteilen sich in vier Genera, die Alpha-, Beta- Gamma- und Deltacoronaviren (Abb.2). Die Alpha- und Betacoronaviren beinhalten die humanpathogenen Spezies NL63, 229E, OC43, HUK1, SARS-CoV und MERS-CoV genauso wie SARS- und MERS-CoV-ähnliche Viren der Fledermäuse. Zu den Gamma- und Deltacoronaviren gehören vorrangig die vogelpathogenen Viren (Abbildung 2).

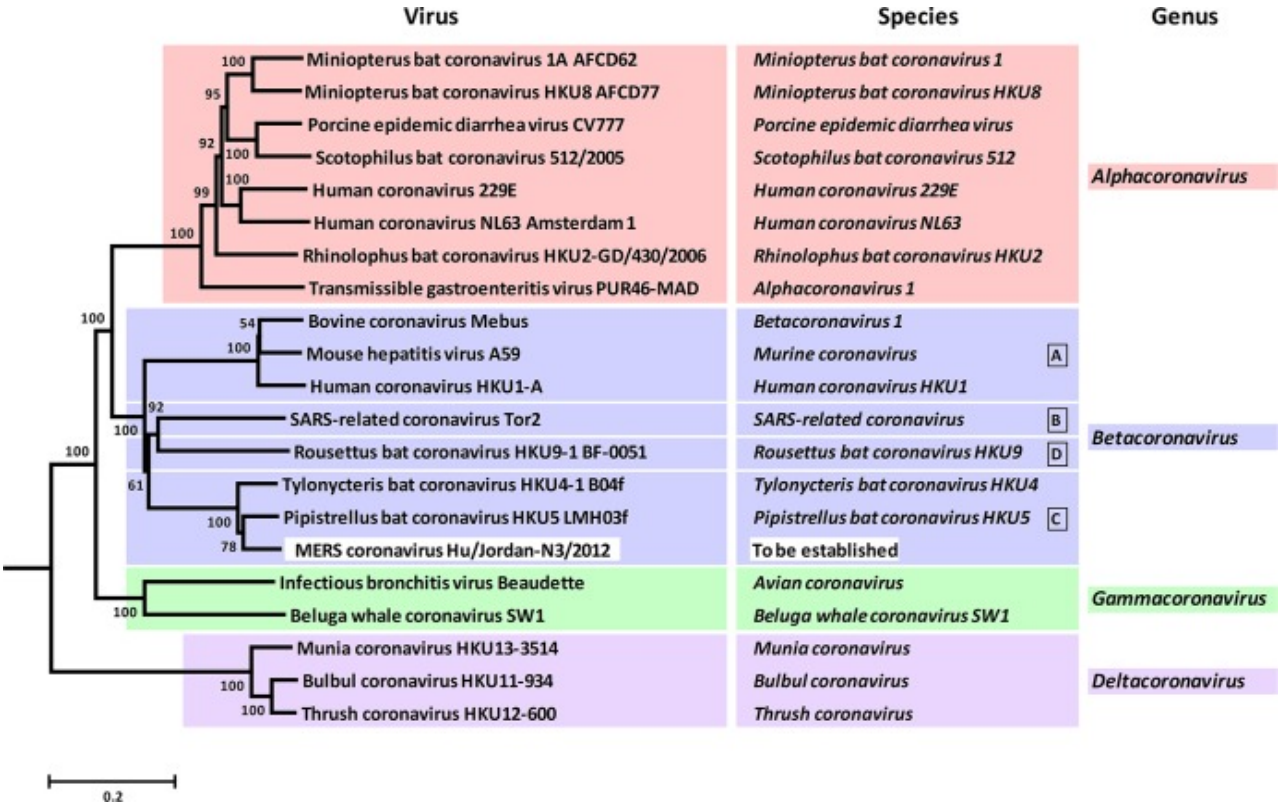

Abbildung 2: Phylogenetische Verwandtschaftsverhältnisse der Mitglieder der Coronaviren. Es ist ein *rooted neighbor-joining* Baum dargestellt, der auf den Aminosäuresequenzvergleichen der unter den Coronaviren konservierten Domänen im Replikase Polyprotein 1ab (ADRP, nsp3; Mpro, nsp5; RdRP, nsp12; Hel, nsp13; ExoN, nsp14; NendoU, nsp15; O-MT, nsp16) beruht. Der Baum zeigt vier monophyletische Cluster, entsprechend der Genera Alpha-, Beta-, Gamma- und Deltacoronaviren. Die Betacoronavirus Untergruppen (A-D) sind ebenfalls aufgezeigt (Abbildung übernommen aus: "*Middle East Respiratory Syndrome Coronavirus (MERS-CoV): Announcement of the Coronavirus Study Group*"; de Groot et al.; 2013, [37].

1.3.2 Morphologie und Genomstruktur

Coronaviren weisen einen Durchmesser von 120 bis 160 nm auf. In ihre Virushülle sind drei bis vier Membranproteine eingelagert. Das für die Namensgebung verantwortliche Glykoprotein Spike (S) (180- 220kDa) ist als Trimer in die Virusmembran eingebaut und erscheint im Elektronenmikroskop wie eine Corona (siehe Abbildung 3B). Das zweite Membranprotein, das *envelope* (E)-Protein (9-12 kDa), ist an der Partikelbildung beteiligt und wird in wesentlich geringeren Kopienzahlen als das Spike Protein in die virale Hüllmembran eingebaut. Das Membran (M)-Protein (23-35 kDa) ist ein nach innen gerichtetes Membranprotein, das die Innenseite der Virushülle auskleidet. Einige Coronaviren, im speziellen die Betacoronaviren der Gruppe A, haben ein zusätzliches Oberflächenprotein, die Hämagglutin-Esterase (HE). Im Inneren des Virions befindet sich das Nukleokapsid, dass aus dem Nukleokapsidprotein (N) (50- 60 kDa) und der viralen, genomischen

RNA gebildet wird. Das N-Protein bindet an die virale Erbinformation, eine einzelsträngige RNA mit positiver Polarität, und verknüpft sie zusätzlich mit dem M-Protein in der Virushülle (siehe Abbildung 3A) [38-41].

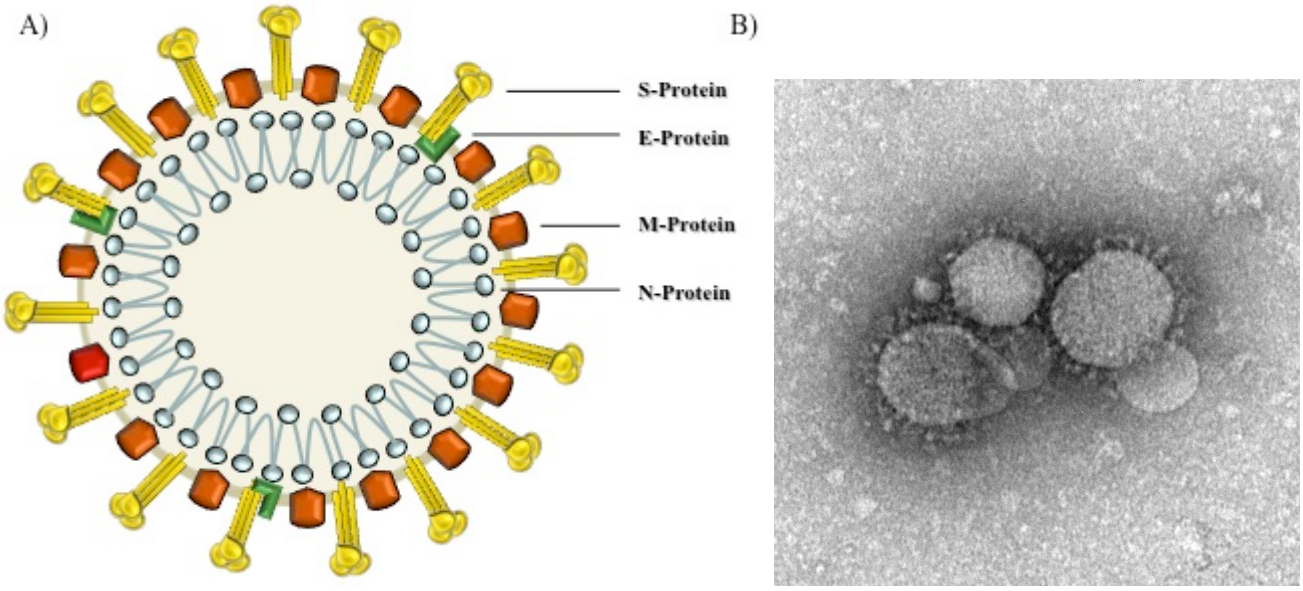

Abbildung 3: Morphologie der Coronaviren. A) Schematische Darstellung der Coronavirus-Partikel mit dem Spike (S)-Protein, dem *envelope* (E)-Protein, dem Membran (M)-Protein und dem Nukleokapsid (N)-Protein (Abbildung modifiziert nach: *„The SARS coronavirus: a postgenomic era“;* Holmes, K.V.; 2003 [42]). B) Elektronenmikroskopische Aufnahme von MERS-CoV. Die Corona wird durch die Spike-Trimere gebildet (CDC/ Maureen Metcalfe; Azaibi Tamin/2012).

Das Genom der Coronaviren ist eine einzelsträngige, polyzistronische RNA in positiver Orientierung, die ca. 27.000 bis 31.000 Nukleotide (nt) umfasst. Damit zählt es zu den größten Genomen aller RNA- Viren. Die hohe genetische Instabilität viraler RNA-Genome wird unter anderem durch eine *Proof Reading* Fähigkeit des unter den RNA-Viren einzigartigen Exoribonukleaseproteins ExoN wieder wettgemacht [43].

Am 5´-Ende der viralen RNA befindet sich eine *Cap*- Struktur, die die Stabilität der RNA erhöht und die Initiierung der Translation im Zytoplasma der Wirtszelle ermöglicht [11]. Darauf folgt eine nichtkodierende Region (*untranslated region*, UTR), die eine kurze *Leader*-Sequenz, die für die Ribosomenbindung verantwortlich ist, enthält. Am 3´-Ende findet man eine zweite UTR und einen poly-A-Schwanz. Die Coronaviren besitzen sechs bis vierzehn offene Leserahmen (*open reading frames*, ORF). Im Falle von SARS-CoV kodieren zwei der vierzehn ORF, ORF 1a und 1b, für die Replikaseproteine, die die Replikation der viralen RNA vermitteln [44]. Sie liegen in einem überlappenden Leserahmen (ORF 1a/b) am 5´-Ende des Genoms. Die überlappende Stelle bildet eine Haarnadelstruktur, die während der Translation eine Leserasterverschiebung verursacht, die es ermöglicht, den zweiten Leserahmen (ORF 1b) ebenfalls zu translatieren [45]. Des weiteren kodiert das SARS-CoV Genom für vier Strukturproteine (S, M, E, N) (siehe Abbildung 3A) und acht akzessorische Proteine [46] .

1.3.3 Replikationszyklus

Nach der Bindung des Spike-Proteins an seinen zellulären Rezeptor erfolgt entweder die Aufnahme des Virions in Wirtszellendosomen mit anschließender Fusion der Endosomen- und Virusmembran, oder es kommt zur Fusion der viralen Membran mit der Plasmamembran der Wirtszelle [11, 47, 48]. Die Membranfusion wird durch das Spike-Protein getrieben und kann durch die Bindung von Spike an einen zellulären Rezeptor oder durch die proteolytische Spaltung des Spike-Proteins durch eine Wirtszellprotease ausgelöst werden. Das Nukleokapsid wird in das Zytoplasma der Wirtszelle entlassen, wo die virale Plus-Strang-RNA als mRNA für die Translation der Polyproteine 1a und 1b dient [47, 49] (siehe Abbildung 4). Diese werden durch Autoproteolyse in die reifen Proteine überführt, die daraufhin den Replikations-/Transkriptions-Komplex bilden. Nun wechselt der genomische RNA-Strang seine Funktion von der „mRNA“ während der Translation zur „Matrize“ für die Genomreplikation und es kommt zur Synthese der Minus-strängigen RNA, die als Template für die Transkription mehrerer kleiner subgenomischer Plus-Strang-RNAs dient, die zur Synthese aller weiteren Proteine genutzt werden. Außerdem dient die Minus-Strang RNA als Matrize für die Synthese neuer Plus-Strang-RNA-Genome. Die neuen Genome werden durch N-Proteine gebunden und die Strukturproteine M, E und S werden in die Membran des endoplasmatischen Retikulums (ER) eingebaut. Nach der Bindung des Nukleokapsids an das in der ER-Membran lokalisierte M-Protein kommt es zum Zusammenbau der Viruspartikel, die in das ER-Lumen abgeschnürt wird. Die nachfolgende Freisetzung der Partikel erfolgt über Exozytose [47] (siehe Abbildung 4).

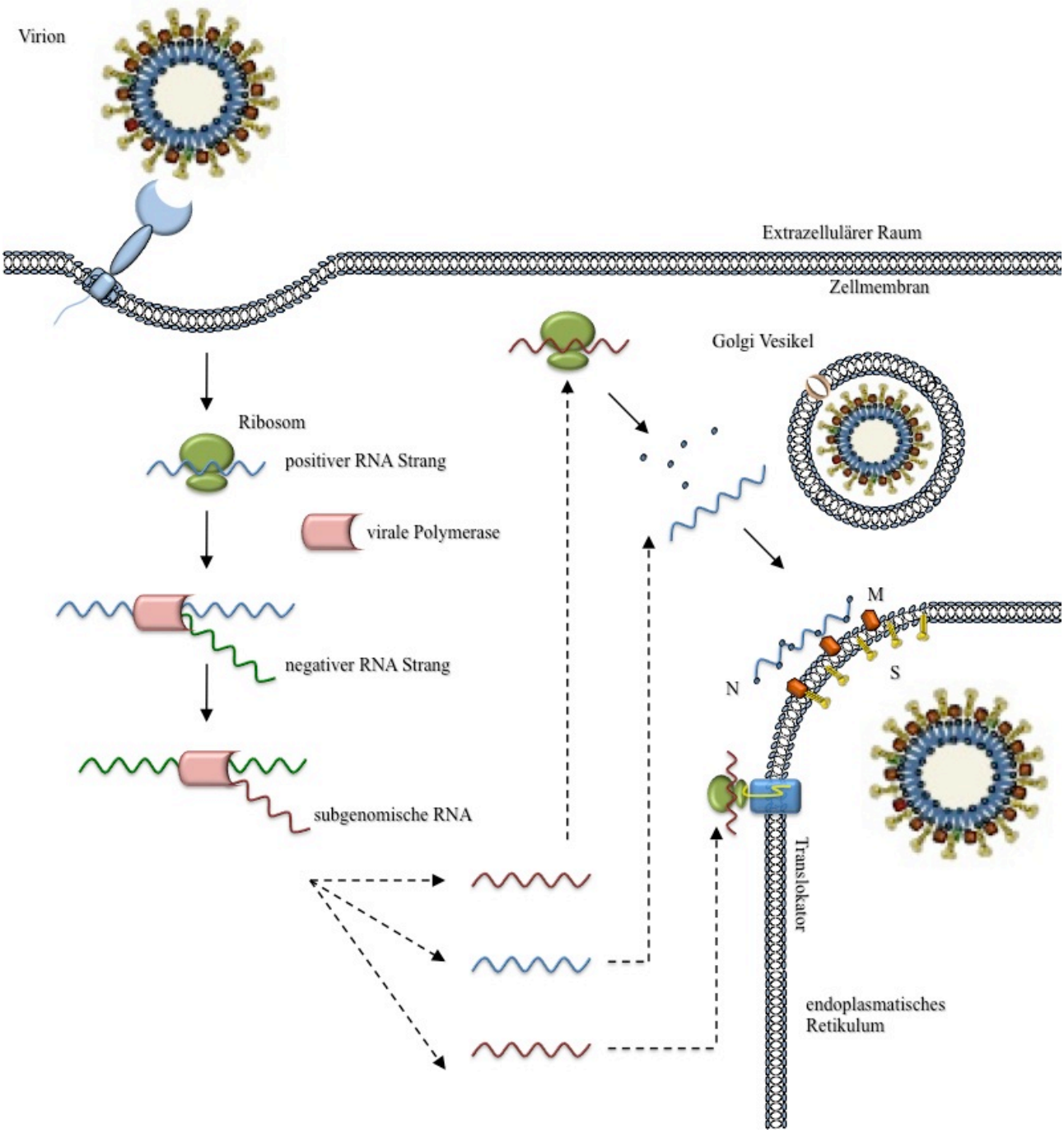

Abbildung 4: Replikationszyklus der Coronaviren. Die Spike-Protein-Bindung an einen zellulären Rezeptor führt entweder zur rezeptorvermittelten Endozytose und zur Fusion der viralen Membran mit der endosomalen Membran oder zur Fusion der viralen Membran mit der Plasmamembran der Wirtszelle. Nach der Freisetzung des RNA-Genoms werden frühe Proteine, Faktoren des Replikationskomplexes, translatiert. Daraufhin wird ein RNA-Strang mit negativer Orientierung synthetisiert, der als Vorlage für die Synthese subgenomischer RNAs und für die Synthese neuer positiv-orientierter RNA-Genome benutzt wird. Das Spike (S)- Protein und das Membran (M)-Protein werden in die Membran des endoplasmatischen Retikulum (ER) eingebaut. Nukleokapsid (N)-Proteine binden den neuen positiven RNA-Strang. Der Zusammenbau neuer Viren erfolgt am ER, wo das N-Protein den Kontakt zwischen RNA-Genom und den Oberflächenproteinen herstellt. Die Viruspartikel wird in das Lumen des ERs abgeschnürt. Mit Hilfe von Exozytose gelangt das Virus danach in den extrazellulären Raum [50].

1.4 Das SARS-Coronavirus

Im Februar 2003 meldete die *World Health Organisation* (WHO) den Ausbruch einer neuen Infektionserkrankung, die erstmals im November 2002 in der südchinesischen Provinz Guangdong auftrat [51]. Die von dem *Severe acute respiratory syndrome* (SARS) betroffenen Patienten hatten hohes Fieber, trockenen Husten, Kurzatmigkeit und Kopfschmerzen. Die meisten Patienten entwickelten eine Lungenentzündung, die in 50% der Fälle die Gabe von Sauerstoff und bei 20% der Betroffenen eine intensivmedizinische Behandlung nötig machte. Oft wurde zusätzlich eine Diarrhö mit einem großen Volumen wässerigen Stuhls beobachtet. Die Übertragung erfolgte hauptsächlich durch Tröpfcheninfektion sowie direkten Kontakt mit Körpersekreten eines SARS- Patienten (Quelle: RKI, WHO und CDC). Innerhalb eines Monats verbreitete sich das Virus nach Hong Kong, Singapur, Vietnam und Kanada. Der Indexfall war ein Mediziner, der fünf Tage nach dem Beginn der ersten Symptome nach Hong Kong reiste. Von hier breitete sich die Krankheit schnell über mehr als zwei Dutzend Länder in Nord- und Südamerika, Europa und Asien aus (http://www.who.int/csr/sars/en/). Im April 2003 wurde ein neues Coronavirus als verursachendes Agens identifiziert [33]. Das SARS-CoV ist ein umhülltes RNA-Virus mit einem Genom von 30.000 Nukleotiden. Das natürliche Reservoir des SARS-CoV ist wahrscheinlich eine Fledermaus der Familie der Hufeisennasen (Rhinolophus) [52, 53]. Es wurden viele dem humanen SARS-CoV ähnliche Viren aus Fledermäusen isoliert [54-56], diese Viren waren allerdings nicht in der Lage, den humanen SARS-CoV-Rezeptor, ACE2, zu nutzen. Daher lag der Schluss nahe, dass die Fledermaus ähnlichen SARS-CoV ein Stadium in einem Zwischenwirt hatten und sich dort an das Rezeptormolekül ACE2 angepasst haben [57]. Xing- Yi Ge und Kollegen gelang es schließlich ein SARS-CoV-ähnliches Virus aus den Hufeisennasen zu isoliert, das eine Identität von 95% zu den humanen SARS-CoV-Stämmen aufweist und in der Lage ist, das gleiche Rezeptormolekül wie das humane SARS-CoV für den Zelleintritt zu nutzen [52]. Das neu entdeckte Fledermaus Virus hat die evolutionäre Lücke geschlossen, da es nicht nur Fledermaus ACE2, sondern auch humanes ACE2 als Eintrittsrezeptor nutzen kann [52]. Diese Entdeckung legt nahe, dass ein direkter Übergang von der Fledermaus auf den Menschen möglich ist. Allerding ist auch ein zweites Szenario denkbar. Auf Tiermärkten Südchinas gesammelte und untersuchte Zibetkatzen (Paguma larvata) und Marderhunde (Nyctereutes procyonoides), die dort als Delikatesse verkauft wurden, wiesen eine Durchseuchung von 100% mit SARS-CoV ähnlichen Viren auf [58, 59]. SARS-CoV verwandte Viren aus Zibetkatzen sind zu 99% identisch mit SARS-CoV aus dem Menschen und können ebenfalls ACE2 als Rezeptor für den Zelleintritt verwenden [60]. Daher ist sowohl eine direkte Übertragung von SARS-CoV auf den Menschen möglich als auch eine indirekte Übertragung über Zwischenwirte. Ob das Virus durch Verzehr infizierter Tiere, direkten Kontakt mit dem infizierten Wirt oder über die Luft auf den Menschen übertragen wurde, ist bis jetzt noch unklar. Im Juli 2003 wurde der SARS-CoV Ausbruch als beendet erklärt. Zu diesem Zeitpunkt

waren über 8.000 Menschen erkrankt, von denen 774 verstarben. Damit ist die SARS-CoV-Infektion mit einer Letalität von 9,4% verbunden. Danach gab es nur noch vier kleinere sporadische Ausbrüche, von denen drei die Folge von Laborinfektionen waren [61, 62].

Die SARS-CoV-Pandemie ist zwar beendet, die Gefahr jedoch, die von Viren ausgeht, die als Zoonose in unsere Population gelangen, ist weiterhin präsent. Die aktuelle Epidemie, ausgelöst durch das MERS-CoV 2012/2013 [32] zeigt das eindrucksvoll, die Erforschung hat deshalb weiterhin höchste Priorität.

1.4.1 Aufbau und Funktion des SARS-CoV- Spike Proteins

Das Glykoprotein Spike (S) ist eines der vier Strukturproteine des SARS-CoV und wird in die virale Hülle eingebaut, wo es als Peplomer aus der Virushülle herausragt (Abbildung 5). Das Spike Protein ist der Schlüssel zur Zielzelle, da es mit dem zellulären Rezeptor ACE2 interagiert und den infektiösen Eintritt des Virus in die Wirtszelle vermittelt [48, 63, 64]. Das SARS-S besteht aus 1255 aa (*amino acids*) und hat eine Ähnlichkeit von 20-27% mit den Spike Proteinen anderer Coronaviren. Das N-terminale Signalpeptid (13aa) ist für die Translokation in das ER während der Translation verantwortlich. Im ER wird das Spike Protein gefaltet und mit Zuckergruppen modifiziert, außerdem lagern sich Spike-Protein Monomere im ER zu Trimeren zusammen [65, 66]. Der carboxyterminale Teil des Spike-Proteins umfasst die Transmembranregion (21aa) und den zytoplasmatischen Schwanz (39aa). Die extrazelluläre Domäne (1182aa) des Spike-Proteins besteht aus einer S1 und einer S2 Untereinheit, die die funktionellen Elemente für die Rezeptorbindung und die Fusion der viralen mit der zellulären Membran tragen. Die S1-Untereinheit beherbergt die ACE2-Rezeptorbindedomäne (RBD) (zwischen 318aa und 510aa) [57, 67, 68]. Sie besteht aus zwei Subdomänen, einer Kerndomäne und einem verlängerten *Loop [50]*. Die Kerndomäne ist zwischen den Coronaviren konserviert, interagiert aber nicht mit ACE2. Der verlängerte *Loop* ist zwischen den CoV wesentlich weniger konserviert als die Kerndomäne und vermittelt den Kontakt zwischen SARS-Spike und dem Rezeptor ACE2 [69, 70]. In der S2 Untereinheit liegen zwei fusionsaktive, α- helikale Strukturen, die *heptad repeats (*HR1 und HR2) [65] (Abbildung 5) und ein putatives Fusionspeptid [71, 72]. Damit gehört das SARS-CoV- S zu den Klasse I Fusionsproteinen. Diese werden als Vorläufer synthetisiert, die durch Proteasen der Wirtszelle in zwei Untereinheiten gespalten werden. Die proteolytische Spaltung der viralen Klasse I Fusionsproteine ist der kritische Punkt, denn hier wird der metastabile Zustand des Proteins generiert [73]. Die Oberflächenproteine des Influenza-Virus und des humanen Immundefizienz-Virus (HIV), die ebenfalls Klasse I Fusionsproteine sind, werden klassischerweise während der Virion Biogenese gespalten [74]. Zusätzlich zu dieser Spaltung ist ein weiterer Trigger nötig um das im metastabilen Zustand befindliche Fusionspeptid zu aktivieren und in die

Wirtszellmembran zu integrieren. Für Influenza- Viren ist das der sinkende pH in den Endosomen und für HIV die Rezeptorbindung. Die Spaltung des SARS-S geschieht auf oder in der neu infizierten Zelle, wodurch sich das Spike-Protein von anderen Glykoproteinen unterscheidet, denn es ist direkt nach Spaltung in seinem fusionsaktiven Zustand und dieses Fusionspeptid wird in die Wirtzellmembran integriert. Daraufhin wird die Formation eines „sechs- Helix- Bündels“ voran getrieben, in dem sich je drei HRs2 zurückfalten und antiparallel an drei HRs1 binden [75]. Ist die stabile „sechs- Helix-Bündel“ Struktur ausgebildet, verschmelzen zunächst die beiden äußeren Lipidmembranen miteinander. Nachdem auch die inneren Membranen verschmolzen sind, kommt es zu einer stetig wachsenden Fusionspore, durch die das virale Nukleokapsid schließlich in das Zytoplasma entlassen wird [70, 76].

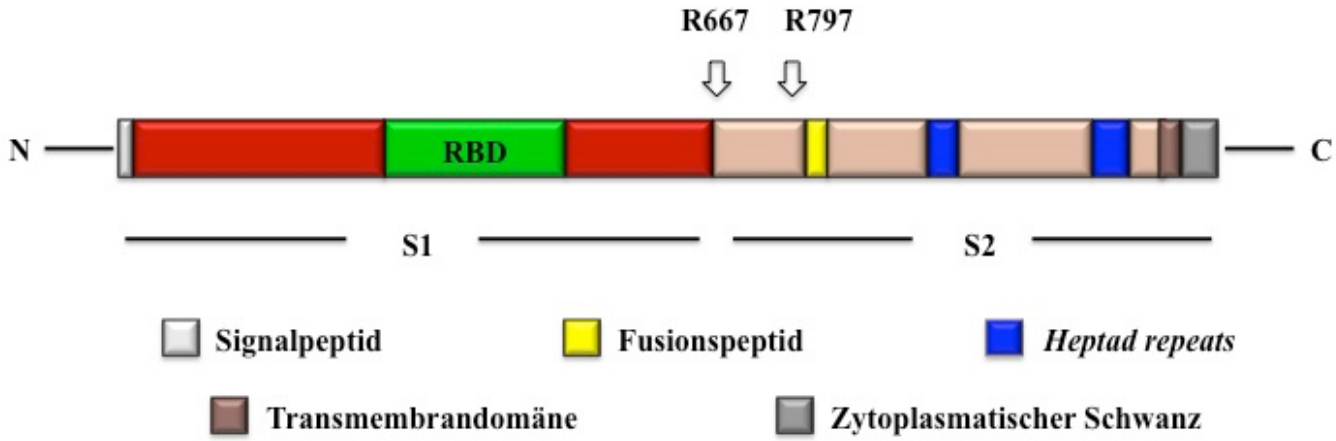

Abbildung 5: Domänen-Struktur des SARS-Coronavirus Spike-Proteins. Die S1-Untereinheit besteht aus dem Signalpeptid, das den kotranslationalen Transport des S Proteins in das ER vermittelt, und der Rezeptorbindedomäne (RBD), die die Bindung an ACE2 vermittelt. Die S2-Untereinheit beherbergt die funktionellen Elemente, die für die Membranfusion verantwortlich sind. Dazu gehören das Fusionspeptid und die *heptad repeats*. Außerdem liegt hier die Transmembrandomäne und der zytoplasmatische Schwanz des Proteins.

1.4.2 Der SARS-Coronavirus Rezeptor *Angiotensin-Converting Enzyme* 2

Im Jahr 2000 wurde das *Angiotensin- Converting Enzyme*- (ACE) Homolog ACE2 als Schlüsselprotein im Renin-Angiotensin- Systems (RAS) entdeckt [77]. Es besteht aus 805 aa und ist ein Typ I Transmembranprotein mit einer extrazellulären katalytischen Domäne. Das sich darin befindende aktive Zentrum hat eine Zink-Metallopeptidase-Aktivität. Die carboxyterminale Domäne weist eine 48%-ige Sequenzähnlichkeit zu Collectrin auf, einem nichtkatalytischen Protein, das in die Reabsorption von Aminosäuren in der Niere involviert ist [78]. Die N-Terminale Domäne von Collectrin weist eine hohe Ähnlichkeit mit somatischem ACE auf. Es liegt daher nahe, dass ACE2 als Genfusionsprodukt aus ACE und Collectrin hervorgegangen ist [79]. ACE2 wird in Herz , Niere ,Hoden , Darm, Lunge und Leber exprimiert [80].

Sowohl ACE als auch ACE2 nutzen Zinkionen, um ihre Reaktion zu katalysieren. ACE besitzt zwei Zink- bindende Domänen und wandelt Angiotensin I (AngI), das durch Renin aus Angiotensinogen entstanden ist, in Angiotensin II (AngII) um, indem es ein C- terminales Dipeptid abspaltet. ACE2 komplexiert ein Zink und wandelt das AngII in Angiotensin 1-7 um, indem es einzelne Aminosäuren abspaltet [77]. In geringerem Maße setzt es auch ANGI in Angiotensin 1-9 um.

Eine wegweisende Arbeit von Li und Kollegen zeigte, das ACE2 der funktionelle Rezeptor für das SARS-CoV ist [63]. Mit Hilfe der Rezeptor-Bindungsdomäne (RBD) in der S1-Einheit des Spike Proteins (Abbildung 5) interagiert das trimere Spike mit ACE2. Die ACE2-Spike Interaktion führt zur Endozytose der Virus-Partikel durch Internalisierung des ACE2-Virus Komplexes. Dieser Prozess ist unabhängig von der ACE2 Peptidase Aktivität, denn auch mit enzymatisch inaktivem ACE2 ist es dem SARS-CoV möglich die Zielzelle zu infizieren. Darüber hinaus wird auch die Substratbindung von ACE2 durch die Anwesenheit des Spike-Trimers nicht gestört [69]. Experimente mit ACE2 Knock out Mäusen zeigen zwar ein erhöhtes Risiko für Herzversagen oder diabetische Nephropathie, die Tiere sind jedoch vor der SARS-CoV Infektion geschützt [22, 81].

1.5 Proteolytische Aktivierung des Spike-Proteins

Die Spaltung von Klasse I Fusionsproteinen durch zelluläre Proteasen ist für die virale Infektiosität essentiell. Die Proteasen der Wirtszelle werden anhand ihres katalytischen Zentrums in sechs Gruppen eingeteilt. Die Hydrolyse der Peptidbindungen geschieht mit Hilfe der funktionellen Aminosäuren Asparaginsäure (A), Cystein (C), Glutaminsäure (G), Serin (S), Threonin (T) oder einem Metallokomplex (M; eine Aminosäure komplexiert mit z.B. Zink, Mangan oder Kobalt) [82].

Bei den meisten Viren mit Klasse I Fusionspeptiden ist die Spaltung des Oberflächenprotein nur der erste Schritt hin zur Fusionsaktivität. Ist das Glykoprotein in diesem metastabilen Zustand, ist es für den nachfolgenden, die Membranfusion auslösenden Trigger empfänglicher. Diese Triebfeder der Fusion kann wie bereits erwähnt die Bindung an den Rezeptor oder der sinkende pH- Wert im Endosomen sein [73]. Die Spaltung des Glykoproteins kann daher in der Virus produzierenden Zelle erfolgen, woraufhin es aktiv in das Virion eingebaut wird. Das SARS-CoV Spike Protein und die Spike Proteine anderer Coronaviren bilden eine Ausnahme: Bei diesen Proteinen scheint die Spaltung des Glykoproteins auszureichen, um die Membranfusion auszulösen. Dies muss allerdings nach Rezeptorbindung geschehen, sodass die Glykoproteine inaktiv in das Virion eingebaut werden müssen. Das Spike Protein kann in vitro durch unterschiedlichste Proteasen aktiviert werden. Dazu zählen lösliche Proteasen, die im extrazellulären Raum vorliegen, wie Trypsin, Thermolysin oder Elastase, oder membranständige Proteasen, wie Typ II Transmembran Serin Proteasen (TTSP), oder

endosomale Proteasen wie Cathepsin L [83-87]. Welche dieser Proteasen das SARS-CoV während der Primärinfektion in den Typ II Pneumozyten aktivieren, ist noch nicht vollständig geklärt.

1.5.1 Aktivierung durch die endosomale Protease Cathepsin L

Cathepsine sind Cysteinproteasen, die ubiquitär vorkommen und eine wichtige Rolle in diversen physiologischen wie pathologischen Prozessen spielen. Die meisten Mitglieder der Cathepsin Familie sind nur bei niedrigem pH-Wert aktiv und daher hauptsächlich in Endosomen und Lysosomen lokalisiert. Hier sind sie verantwortlich für die Degradation von Proteinen. Sie bestehen aus einer schweren und einer leichten Kette, die durch eine Disulfidbrücke miteinander verbunden sind. Cathepsine werden als inaktive Vorläufermoleküle gebildet, die während der Reifung im ER durch Abspaltung des Propeptids und späterer Autokatalyse im sauren Milieu des Endosomens aktiviert werden [88, 89]. Die Nutzung spezifischer Inhibitoren zeigte, dass die Aktivität von endosomalen Cystein-Proteasen, insbesondere von Cathepsin L, für den SARS-CoV-Eintritt in Wirtszellen essentiell ist [84, 90]. Außerdem konnte mit Hilfe von rekombinanten Proteinen gezeigt werden, dass SARS-S durch Cathepsin L gespalten wird, wohingegen die Spaltung von SARS-S in Zellkultur noch nicht nachgewiesen wurde.

Somit ergibt sich zunächst folgendes Modell für den SARS-CoV Eintritt: Das Spike-Glykoproteintrimer bindet seinen Rezeptor ACE2, das löst eine Konformationsänderung im Spike Protein aus, die die Erreichbarkeit für Wirtszellproteasen erhöht [91]. Zunächst kommt es aber zur Rezeptor-vermittelten Endozytose des Komplexes und der Ansäuerung des Endosomenmilieus. Die dadurch aktivierten Cathepsine sind nun in der Lage, das Spike Protein zu spalten, was die Integration des freigelegten Fusionspeptids in die Endosomenmembran und schlussendlich die Membranfusion nach sich zieht [47, 72, 84, 91].

Die präzise Spaltstelle, die durch Cathepsin L im Spike Protein angegriffen wird, ist bis jetzt noch nicht eindeutig identifiziert worden, denn die Erkennungssequenzen, die Cathepsine nutzen, sind relativ unspezifisch [92]. Es könnte daher sein, dass das Spike Protein nicht nur an einer sondern an mehreren Stellen gespalten wird [72, 93-95]. Die Beobachtung, dass die Cathepsin L-Aktivität für den Wirtszelleintritt verschiedener Coronaviren wichtig ist, macht sie zu einer möglichen Angriffsstelle für die antivirale Therapie [84, 96-100].

1.5.2 Aktivierung durch Type II Transmembran Serin Proteasen (TTSP)

Die humanen Type II Transmembran Serin Proteasen (TTSPs) sind eine Familie aus 19 Mitgliedern, von denen die meisten auch im respiratorischen Trakt exprimiert werden [101]. Im Gegensatz zu anderen Zelloberflächenproteinen, wie der der ADAM (*A Disintegrin And Metalloproteinase)* Familie, die nur an der Zelloberfläche agieren, werden die meisten TTSPs auch sekretiert oder in zytoplasmatische Speicherorganellen abgegeben, die Signal- basierend entlassen werden. Die TTSPs sind involviert in Homöostase, Blutgerinnung, Wundheilung, Verdauung und Immunantworten, genauso wie in Tumorinvasion und Metastasierung [101]. Mitglieder der TTSP- Familie zeigen eine konservierte Domänenstruktur (Abbildung 6). Der N-Terminus liegt zytoplasmatisch vor und interagiert mit Komponenten des Zytoskeletts und Signalmolekülen [101]. Darauf folgt die Transmembrandomäne, die das Protein in der Membran verankert und eine variable Stamm-Region, die regulatorische Funktionen, wie Enzymaktivität, Substratspezifität und Protein-Protein Interaktionen in sich vereint. Die C-terminal gelegene Protease Domäne trägt ein aktives Zentrum, das aus der katalytischen Triade Histidin, Aspartat und Serin besteht.

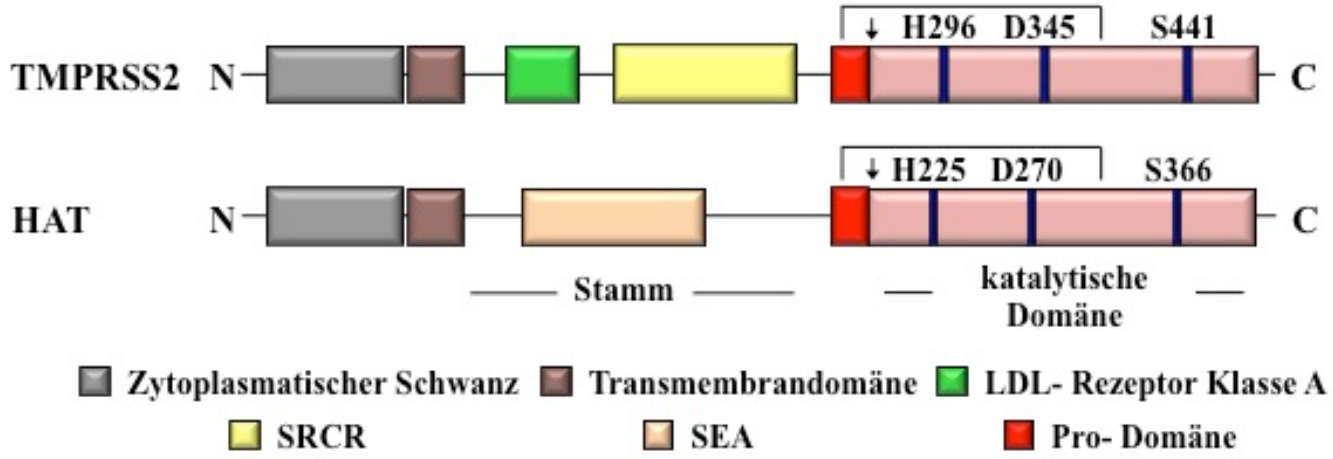

Abbildung 6: Domänen Struktur der TTSP Mitglieder TMPRSS2 und HAT. Die TTSPs enthalten eine N-terminale zytoplasmatische Domäne, eine Transmembrandomäne, eine Stamm-Region und eine katalytische Domäne. Der Stamm von TMPRSS2 trägt eine LDL- Rezeptor Klasse A Domäne und eine *scavenger receptor cysteine-rich* Domäne (SRCR), und der von HAT eine *sperm protein, enterokinase* und *agrin* (SEA) Domäne. Die katalytische Domäne beider Proteasen hat ein aktives Zentrum aus der katalytischen Triade Histidin (H), Aspartat (D) und Serin (S). Beide werden als Zymogen gebildet, das durch die autokatalytische Spaltung in die aktive Form übergeht. Die Spaltung findet nach einem Lysin oder Arginin statt, das in einem konservierten Aktivierungsmotiv liegt (durch Pfeil gekennzeichnet). Das reife Enzym wird durch eine Disulfidbrücke zusammen gehalten (Abbildung modifiziert nach *„Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research“* ; Simmons, G.; 2013[102].

Die TTSPs werden als inaktive Vorläufermoleküle (Zymogene) exprimiert, die erst durch Spaltung in der Prodomäne, zwischen Stamm und Protease Domäne, aktiviert werden. Die beiden Domänen werden im reifen Enzyme durch eine Disulfidbrücke zusammengehalten [101, 103]. Eine Rolle von TTSPs bei viralen Infektionen wurde zuerst durch Böttcher und Kollegen beschrieben, die zeigten, dass Transmembrane Protease, Serine 2 (TMPRSS2) und *Human airway trypsin-like* (HAT) das Oberflächenprotein Hämagglutinin der Influenza-A-Viren des Menschen spalten. Anschließend wurde für TMPRSS2 gezeigt, dass die Protease nach Expression in ACE2-positiven Zellen in der

Lage ist, SARS-S für die Zell-Zell- und Virus-Zellfusion zu aktivieren [87, 104, 105]. Der SARS-S-vermittelte Eintritt in Zellen, die TMPRSS2 exprimieren, hängt nicht mehr von der Aktivität von Cathepsin L ab, man geht daher davon aus, dass die Rezeptor-vermittelte Endozytose umgangen wird und die Membranfusion an der Zelloberfläche abläuft.

Die Fähigkeit von TMPRSS2 das SARS-Spike-Protein zu aktivieren ist vermutlich abhängig von der räumlichen Orientierung der beiden Proteine zueinander. Werden beide in einer Zelle koexprimiert, ist zwar Spike-Protein- Spaltung nachweisbar, diese ist allerdings nicht aktivierend [87, 104]. Nur wenn TMPRSS2 zusammen mit ACE2 auf der Zielzelle und SARS-S auf der Virusmembran bzw. auf benachbarten Zellen lokalisiert sind, findet eine aktivierende Spaltung des Spike-Proteins statt [87, 104]. Mögliche Erklärungen für diesen Befund sind, dass das Spike-Protein erst nach Rezeptorbindung in enge räumliche Nähe zur Protease gebracht wird oder dass die Rezeptorbindung Konformationsänderungen im S Protein induziert, die für die Aktivierung durch TMPRSS2 essentiell sind oder dass der Spaltung direkt die Integration des Fusionspeptids in die Wirtszellmembran folgt, sie also an der Membran stattfinden muss. Zusammenfassend zeigen diese Beobachtungen, dass neben der Spike-Protein- Aktivierung durch Cathepsin L mit der Spike-Protein- Spaltung durch TMPRSS2 ein weiterer Aktivierungsweg besteht.

Neben TMPRSS2 kann auch HAT das Spike-Protein aktivieren [85]. Allerdings spalten HAT und TMPRSS2 an unterschiedlichen Stellen. Der Aminosäurerest R667 ist wichtig für die Proteolyse durch Trypsin und HAT, nicht aber für die Spaltung durch TMPRSS2 [85, 94, 106]. Diese Unterschiede könnten erklären, warum HAT nicht aber TMPRSS2 in der Lage ist, das Spike-Protein für die Zell-Zellfusion zu aktivieren, wenn sowohl Spike als auch Protease in der gleichen Zelle exprimiert werden. Im Gegensatz dazu führt die Expression von HAT auf Zielzellen nicht zu einer Cathepsin L- unabhängigen Virus-Zellfusion [85], obwohl die S Protein- getriebene Zell-Zellfusion durch Expression von HAT auf den Zielzellen gefördert wird [107, 108]. Der Grund für diese scheinbar widersprüchlichen Beobachtungen ist gegenwärtig unklar.

Auch TMPRSS4, ein weiteres Mitglieder der TTSP-Familie, ist in der Lage SARS-S für die Zell-Zellfusion zu aktivieren. Eine TMPRSS4- vermittelte Virus-Zellfusion konnte jedoch genauso wenig nachgewiesen werden wie die Spaltung des Spike-Proteins durch TMPRSS4 [87]. Wahrscheinlich ist die durch TMPRSS4 vermittelte Spike-Protein- Spaltung ineffizient, jedoch ausreichend für die Aktivierung des Spike-Proteins im Zell-Zellfusionstests, bei dem große Oberflächen interagieren und Spike-Protein, Rezeptor und Protease stark exprimiert werden. Es ist daher zu vermuten, dass TMPRSS4 physiologisch keine wichtige Rolle bei der Aktivierung des SARS-CoV spielt.

Die Demonstration, dass SARS-CoV durch TMPRSS2 und Cathepsin L in Zellkultur aktiviert werden, zeigt, dass diese Proteasen mögliche Angriffspunkte für die antivirale Therapie darstellen.

[109, 110], Es ist allerdings unklar, ob diese Proteasen auch zur Aktivierung des Virus im infizierten Wirt beitragen. Zur Klärung dieser Frage ist es notwendig zu untersuchen, ob Proteasen und Rezeptor im Lungenepithel koexprimiert werden. Außerdem kann mit Hilfe von Inhibitoren dieser Proteasen deren Beitrag zur viralen Ausbreitung und Pathogenese im Mausmodell untersucht werden. Schließlich stehen Cathepsin L und TMPRSS2 knockout Mäuse zur Verfügung, um die Rolle dieser Proteasen in der SARS-CoV-Infektion zu charakterisieren.

1.5.3 Aktivierung durch eine unbekannte Leupeptin-sensitive Protease

Die Aktivierung von SARS-S durch Cathepsin L und TTSPs ist gut etabliert. Dennoch gibt es Hinweise darauf, dass eine weitere Protease in die SARS-S Spaltung involviert ist. Die ineffiziente Zell-Zellfusion zwischen Spike-exprimierenden 293T Zellen und Zellen, die lediglich geringe Mengen an ACE2 endogen exprimieren, kann durch Zugabe einer löslichen Protease oder durch Überexpression von ACE2 stark erhöht werden [85, 86]. Der starke Anstieg der Zell-Zellfusionsaktivität nach gerichteter Expression von ACE2 ohne Koexpression einer Protease und ohne Zugabe einer löslichen Protease warf die Frage auf, ob unter diesen Bedingungen die Aktivität einer endogen exprimierten Protease für die Spike-Protein-Aktivierung wichtig ist. Es konnte gezeigt werden, dass unter diesen Bedingungen die Zell-Zellfusion durch Zugabe von Leupeptin, einem Inhibitor von Cystein, Serin und Threonin Peptidasen reduziert wird, nicht jedoch durch einen Cathepsin B/L Inhibitor [86]. Dies legt den Schluss nahe, dass eine bisher unidentifizierte Leupeptin-sensitive Protease in der Lage ist, SARS-S für die Zell-Zellfusion zu aktivieren. Die Feststellung der Identität dieser Protease und ihrer Rolle bei der SARS-S Aktivierung sind Gegenstand der aktuellen Forschung.

1.5.4 Proteolyse von ACE2 und deren Einfluss auf die Infektion

Die Rolle der Wirtszellproteasen während des Zelleintritts von SARS-CoV geht über die Spaltung und Aktivierung des Spike-Proteins hinaus: Auch der Rezeptor ACE2 wird durch zelluläre Proteasen prozessiert [105, 111, 112]. Es ist schon länger bekannt, das ACE2 nicht nur membrangebunden an der Zelloberfläche, sondern auch löslich im extrazellulären Raum vorkommt. Lösliches ACE2 wird durch die Zink-komplexierende Metalloprotease ADAM17/TACE (*A Disintegrin And Metalloproteinase/ Tumor necrosis factor-α converting enzyme*) produziert, die ACE2 nahe der Transmembrandomäne spaltet [113]. Die Stimuli für das Abspalten der Ektodomäne (*shedding*) können Wachstumsfaktoren, Phorbol- Ester (Phorbol-12-Myristat-13-Acetat, PMA) und Calcium-Ionen-Konzentrationsänderungen sein [114]. Alle diese Faktoren führen über Proteinkinase C (PKC)-vermittelte Signaltransduktion zur ADAM17-Phosphorylierung und so zu dessen Aktivierung. Das ACE2-Substrat AngII erhöht über die Bindung an seinen Rezeptor AT1R die intrazelluläre Calcium-

Konzentration und damit die ADAM17-Aktivität. Außerdem interagiert ACE2 mit dem Calcium-bindenden Enzym Calmodulin (CaM), was mit einer Erhöhung des intrazellulären Calcium-Spiegels und einer Stimulation des *sheddings* einhergeht [115-117]. Eine Pionierarbeit von Haga und Kollegen zeigte, dass auch die Bindung von SARS-Spike an ACE2 zum ADAM17-induzierten ACE2-shedding führt und es wurde postuliert, dass dieser Prozess wichtig ist für die zelluläre Aufnahme des Virions in die Wirtszelle [111, 118]. Wird ADAM17 inhibiert, sinkt die Infektiosität des SARS-CoV und die Ausbreitung des Virus in infizierten Mäusen [112]. Der Prozess des induzierten Ektodomänen-Sheddings in der Anwesenheit des SARS-CoV reduziert die ACE2-Expression an der Zelloberfläche [22, 111, 119-122]. Ein Verringern der zellulären ACE2-Expression wurden in verschiedenen Zellkultursystemen der SARS-CoV-Infektion und in SARS-CoV-infizierten Patienten beobachtet [122]. Das könnte auch der Grund für den schweren Verlauf der SARS-Krankheit bis hin zur Entwicklung von ARDS erklären. Denn je weniger ACE2 auf der Oberfläche der Zelle umso weniger kann es seine gewebeschützenden Funktion erfüllen (1.2 Acute Respiratory Distress Syndrome (ARDS)).

ADAM17 ist nicht die einzige Protease, die ACE2 prozessieren und damit auf die SARS-CoV-Infektion einwirken kann. Die TTSPs TMPRSS2 und HAT sind ebenfalls in der Lage ACE2 zu spalten und es wurde postuliert, dass die ACE2-Spaltung den viralen Eintritt in Zielzellen fördert [105], der experimentelle Beweis für diese Hypothese steht jedoch noch aus. Ob die ACE2-Spaltung durch TTSP ebenfalls durch SARS-CoV induziert wird ist unbekannt.

Auf der Basis der oben beschriebenen Befunde ergibt sich ein erweitertes Modell für den SARS-CoV-Eintritt in Zellen: Die Bindung des Spike-Proteins an ACE2 führt zur Rekrutierung membranständiger Proteasen (ADAM17/TMPRSS2), die ACE2 prozessieren und damit die Rezeptor-vermittelte Endozytose auslösen. Ist die ACE2-prozessierende Protease in der Lage, auch das Spike-Protein zu spalten, wie im Fall von TMPRSS2, geschieht die Membranfusion direkt an der Zelloberfläche. Ist die Rezeptor-prozessierende Protease dazu nicht in der Lage, wie im Fall von ADAM17, erfolgt die endozytotische Aufnahme des Virus und die Aktivierung des Spike-Proteins durch endosomale Proteasen.

2. Zielsetzung

Das Spike (S) Protein des Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) vermittelt den viralen Eintritt in Zielzellen. Die Spaltung und Aktivierung des S Proteins durch Wirtszellproteasen ist für die virale Infektiosität essentiell. Die Typ II Transmembranserinproteasen (TTSPs) TMPRSS2 und HAT aktivieren das S Protein in Zellkulturmodellen. Ihre Bedeutung für die virale Ausbreitung im Menschen ist dagegen unklar. TMPRSS2 und HAT spalten auch den SARS-CoV-Rezeptor Angiotensin-Converting Enzyme 2 (ACE2), den das S Protein für die Anheftung und Aufnahme in Zielzellen nutzt. Es wurde postuliert, dass die ACE2-Spaltung durch TMPRSS2/HAT den viralen Eintritt in Zielzellen fördert, ein formaler Beweis dafür wurde jedoch nicht erbracht, und der molekulare Mechanismus der Eintrittsverstärkung war unbekannt.

Für die vorliegende Arbeit wurden die folgenden Ziele definiert:

Im ersten Teil der Arbeit sollte analysiert werden, ob TMPRSS2/HAT und ACE2 im menschlichen Respirationstrakt koexprimiert werden. Eine Koexpression von Protease und Rezeptor würde darauf hinweisen, dass die Proteasen die virale Ausbreitung im Wirt fördern könnten.

Im zweiten Teil der Arbeit sollte geklärt werden, über welchen Mechanismus die Expression von TMPRSS2 und HAT den SARS-CoV-Eintritt in Zielzellen verstärkt. Dazu sollte die TMPRSS2/HAT-Spaltstelle(n) in ACE2 identifiziert werden und es sollte untersucht werden, ob ACE2-Spaltung für den verstärkten SARS-CoV-Eintritt nach Protease-Expression verantwortlich ist. Weiterhin sollten funktionelle Analysen mit Hilfe von rekombinantem S Protein und S Protein-tragenden Vektoren zeigen, wie die Protease-Expression den Eintritt verstärkt. Schließlich sollte geklärt werden, ob eine weitere Protease, *A Disintegrin And Metalloproteinase* 17 (ADAM17), die ACE2 spalten kann, den S Protein-getriebenen Eintritt fördert.

3 Manuskripte

Titel

Influenza and SARS-Coronavirus Activating Proteases TMPRSS2 and HAT Are Expressed at Multiple Sites in Human Respiratory and Gastrointestinal Tracts

PLoS One, 2012; 7(4): e35876

Titel

TMPRSS2 and ADAM17 Cleave ACE2 Differentially and Only Proteolysis by TMPRSS2 Augments Entry Driven by the Severe Acute Respiratory Syndrome Coronavirus Spike Protein

Journal of Virology, 2014; ;88(2):1293-307

3.1 Erstes Manuskript

Influenza and SARS-Coronavirus Activating Proteases TMPRSS2 and HAT Are Expressed at Multiple Sites in Human Respiratory and Gastrointestinal Tracts

Die experimentelle Arbeit entstand in gleichberechtigter Kooperation mehrerer Autoren. Im Zuge meiner Doktorarbeit und für diese Publikation habe ich die Experimente zu SARS-CoV durchgeführt, während meine Kollegen das Influenza Virus bearbeitet haben.

Influenza and SARS-Coronavirus Activating Proteases TMPRSS2 and HAT Are Expressed at Multiple Sites in Human Respiratory and Gastrointestinal Tracts

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Abstract

The type II transmembrane serine proteases TMPRSS2 and HAT activate influenza viruses and the SARS-coronavirus (TMPRSS2) in cell culture and may play an important role in viral spread and pathogenesis in the infected host. However, it is at present largely unclear to what extent these proteases are expressed in viral target cells in human tissues. Here, we show that both HAT and TMPRSS2 are coexpressed with 2,6-linked sialic acids, the major receptor determinant of human influenza viruses, throughout the human respiratory tract. Similarly, coexpression of ACE2, the SARS-coronavirus receptor, and TMPRSS2 was frequently found in the upper and lower aerodigestive tract, with the exception of the vocal folds, epiglottis and trachea. Finally, activation of influenza virus was conserved between human, avian and porcine TMPRSS2, suggesting that this protease might activate influenza virus in reservoir-, intermediate- and human hosts. In sum, our results show that TMPRSS2 and HAT are expressed by important influenza and SARS-coronavirus target cells and could thus support viral spread in the human host.

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Introduction

Influenza viruses and the SARS-coronavirus (SARS-CoV) are highly transmissible respiratory viruses which pose a serious threat to human health. The yearly recurring influenza epidemics are associated with significant morbidity and mortality, particularly among the elderly, and the global spread of pandemic influenza viruses can cause millions of deaths [1]. The severe acute respiratory syndrome coronavirus (SARS-CoV), which causes a novel lung disease, SARS, emerged in 2002 and spread to 26 countries in 2003, with 774 fatal infections [2]. Both SARS-CoV and influenza viruses circulate in animal reservoirs, water fowl (influenza) and bats (SARS-CoV) [3,4]. Therefore, the identification of cellular factors essential for viral spread in animal and human cells should allow novel approaches to prevention and therapy.

The SARS-CoV spike protein (SARS-S) and the influenza virus hemagglutinin (HA) are inserted into the viral membranes and mediate host cell entry. For this, SARS-S and influenza HA bind to host cell receptors, ACE2 (SARS-CoV) [5] and 2,6-linked sialic acid on membrane proteins or lipids (human influenza viruses) [6], and mediate the fusion of the viral membrane with a host cell membrane. As a consequence, viral components are released into the host cell and can subvert the synthetic capabilities of the host cell for production and release of progeny particles.

The influenza HA and the SARS-S-protein are both synthesized as inactive precursors which transit into their active forms upon cleavage by host cell proteases. Cleavage of SARS-S and influenza HA is essential for viral infectivity and the responsible proteases are targets for antiviral intervention [7,8], but their nature is incompletely defined. Recent evidence indicates that the type II transmembrane serine proteases (TTSPs) TMPRSS2, TMPRSS4 and HAT can activate human influenza viruses for spread in protease transfected cells [4,9,10]. In addition, endogenous TMPRSS2 was shown to promote influenza virus spread in the cell lines Caco-2 and Calu-3 [11,12]. The SARS-CoV was found to be activated by cathepsin L upon viral uptake into host cell endosomes [8]. However, several recent reports demonstrated that expression of TMPRSS2 in target cells rendered cathepsin activity dispensable for infectious entry of

SARS-CoV [13–15], suggesting that both SARS-CoV and influenza viruses can exploit TTSPs to promote their spread.
Despite the intriguing findings made in cell culture, the role of TMPRSS2 and HAT in influenza virus and SARS-CoV spread and pathogenesis remains to be defined. For this, it is essential to determine the extent of TMPRSS2 and HAT expression in viral target cells in human tissues. Here, we show that TMPRSS2 and HAT are coexpressed with ACE2 and 2,6-linked sialic acids, the key receptor determinants of SARS-CoV and influenza virus, respectively, in major portions of the human respiratory tract, indicating that these proteases could support SARS-CoV and influenza virus spread in humans. In addition, we demonstrate that HA activation is conserved between human TMPRSS2 and TMPRSS2 of animal species critically involved in zoonotic transmission of influenza virus, underlining a potentially important role of this protease in the influenza virus zoonosis.

Materials and Methods

Cell culture

293T cells were obtained from the American Type Culture Collection (ATCC) and were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, and grown in a humidified atmosphere of 5% CO_2.

Cell-cell fusion assay

For analysis of cell-cell fusion, 293T effector cells seeded in 6-well plates were $CaPO_4$-transfected with either empty pcDNA plasmids or plasmids encoding SARS- S in combination with plasmid pGAL4-VP16 encoding the Herpes Simplex transactivator VP16 fused to GAL4, as described [13]. In parallel, 293T target cells were seeded in 48-well plates and transfected with plasmids encoding the indicated proteases or empty plasmid together with plasmid pGal5-luc, which encodes a promoter with five Gal4 binding sites in front of a luciferase gene. Transfected effector and target cells were mixed, incubated with trypsin or PBS and fusion was quantified by determination of luciferase activities in cell lysates 48 h after cocultivation using a commercially available kit (Promega, Madison, USA).

Production of lentiviral pseudotypes and infection experiments

For generation of lentiviral pseudoparticles, $CaPO_4$ transfections were performed as described [13]. Briefly, 293T cells were transiently cotransfected with pNL4-3 E-R- Luc [16] and expression plasmids coding for influenza virus HA and neuraminidase (NA) or vesicular stomatitis virus glycoprotein (VSV-G) [10]. For analysis of HA activation by TTSPs, expression plasmids for the indicated proteases [11,13] or empty vector were cotransfected into cells producing pseudoparticles. The culture medium was replaced at 16 h and harvested at 48 h post transfection. The supernatants were passed through 0.45 mm filters and stored at -80°C. For infection, pseudoparticles were treated with either PBS or trypsin followed by incubation with 293T target cells for three days before cells were lysed and luciferase-activities determined using a commercially available kit (Promega, Madison, USA).

Analysis of SARS-S and 1918 HA cleavage

For the detection of HA and SARS-S-cleavage in cis, 293T cells were cotransfected with plasmids encoding SARS-S [17] or 1918 HA and plasmids encoding the indicated proteases or empty vector (pcDNA). For analysis of SARS-S cleavage in trans, plasmids encoding SARS-S [17] and proteases were transfected separately into 293T cells followed by mixing of the transfected cells. Subsequently, the cells were treated with PBS or trypsin, lysed, separated via 12,5% SDS-PAGE and transferred onto nitrocellulose membranes. SARS-S was detected by staining with rabbit serum raised against the S1 subunit of SARS-S subunit [18]. For detection of HA, a mouse monoclonal antibody was used [19]. As a loading control, the stripped membranes were incubated with an anti-ß-actin antibody (Sigma, Deisenhofen, Germany). Bound antibodies were detected with HRP-coupled secondary antibodies (Dianova, Hamburg, Germany).

Immunostaining of tissue sections

Formalin fixed paraffin embedded tissue samples of a wide range of tissues from the respiratory and gastrointestinal tracts, as well as the myocardium, were obtained from the Oxford Radcliffe Biobank, with full ethical approval from the National Research and Ethics Service (Oxfordshire Research and Ethics Committee A: reference 04/Q1604/21). While all patients gave generic consent for the use of their tissue in research at the time of signing a consent form for surgery, informed consent from each patient for the use of tissue in this study was not required by the National Research and Ethics Service, because all tissue was anonymised. Tissue sections were immunostained for TMPRSS2, HAT and ACE2 or with the elderberry lectin, *Sambucus nigra*, that detects 2,6-linked sialic acids. Antigen retrieval was performed by pressure cooking in different antigen retrieval solutions. Slides were mounted in Aquatex mounting medium (Merck, UK). ACE2 immunostaining (affinity purified goat polyclonal serum, R&D Systems, Abingdon, UK) was performed and detected using a mouse anti-goat Ig (GTI-75) [20] and the Novolink™ max polymer detection system (Leica Microsystems, Newcastle, UK), as per the manufacturer's instructions after antigen retrieval in citrate pH 6.0. TMPRSS2 (mouse monoclonal antibody P5H9 A3 ascites, a generous gift from Dr J.M. Lucas, Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA, [21]) immunostaining was detected using the NovolinkTM max polymer detection system after antigen retrieval in Tris EDTA pH9.0. HAT immunostaining (mouse monoclonal antibody 337029, R&D Systems, Abingdon, UK) was performed using the Novolink™ max polymer detection system after antigen retrieval in Dako Target retrieval solution pH 6.0 (Dako, Cambridge, UK). Biotinylated elderberry lectin (Sambucus nigra) (Vector labs, Peterborough, UK) binding was detected using Streptavidin-HRP (Ar-Med Limited, Egham, UK) and detected using Dako DAB chromogen substrate (Dako, Cambridge, UK) after antigen retrieval in Tris-EDTA pH 9.0. Lung tissue was used as a positive control for TMPRSS2, ACE2 and elderberry lectin staining [11,13], while bronchus was used as a positive control for HAT immunostaining [22]. As a negative control for ACE2 immunostaining and elderberry lectin staining, normal goat polyclonal serum was substituted for the primary antibody/lectin staining step. As a negative control for TMPRSS2 and HAT immunostaining, an irrelevant mouse monoclonal (anti-ALK1 antibody, clone ALK1 [23]) was substituted for the primary antibody. Stained sections were photographed with a Nikon DS-FI1 camera with a Nikon DS-L2 control unit (Nikon UK Limited, Kingston-upon-Thames, UK) and an Olympus BX40 microscope (Olympus UK Limited, Watford, UK).

Results

Influenza viruses circulate in birds and poultry, with water fowl constituting the natural reservoir, and coinfection of swine with

different influenza viruses is believed to play an important role in the emergence of pandemic viruses [1,3]. TMPRSS2, TMPRSS4 and HAT were shown to activate influenza virus in transfected cells [4,9,10], but only for TMPRSS2 further evidence for a potential contribution to viral spread in humans was reported [11,12]. In order to assess the role of TMPRSS2 in the influenza virus zoonosis, we tested whether this protease derived from chicken and swine is able to cleave HA. In parallel, we examined if TMPRSS2 of mouse origin facilitates HA proteolysis, since mice are commonly used as a model system for influenza virus spread and pathogenesis. Finally, cleavage and activation of HA by human and mouse HAT and TMPRSS4 were also evaluated.

Western blot analysis of transfected cells revealed that TMPRSS2, TMPRSS4 and HAT of all animal species tested cleaved the HA precursor HA0 and produced HA1 cleavage fragments identical to those observed for the human enzymes (Fig. 1A). The slightly faster migration of HA1 fragments generated by TMPRSS2 compared to the other proteases is due to differential HA glycosylation [11]. Cleavage resulted in HA activation, since lentiviral vectors produced in the protease expressing cells were fully infectious in the absence of trypsin treatment (Fig. 1B). In contrast, no HA cleavage and activation was observed in cells transfected with empty vector or cells expressing human TMPRSS3 (Fig. 1A,B), which was previously demonstrated not to process HA [11]. Similarly, swine, chicken and mouse TMPRSS2 cleaved SARS-S into multiple fragments, as previously documented for human TMPRSS2 [13], although some variation in cleavage efficiency was noted (Fig. 1C). In contrast, trypsin digestion produced the S1 subunit, as expected [24,25]. Of note, SARS-S was cleaved by TMPRSS2 upon coexpression of both proteins (cis cleavage, Figure 1C) and upon mixing of SARS-S expressing cells with protease expressing cells (trans cleavage, Fig. S1), although some variability in cleavage efficiency was noted in the latter setting. In agreement with SARS- S trans cleavage, expression of TMPRSS2 in target cells (TMPRSS2 panels) endogenously expressing very low amounts of viral receptor, ACE2 [5], allowed efficient SARS-S-driven cell- cell fusion and fusion efficiency was not increased by the addition of trypsin. In contrast, SARS-S-driven fusion with control transfected cells (pcDNA panel) was inefficient and fusion efficiency was rescued by trypsin treatment (Fig. 1D). Finally, transfection of ACE2 plasmid into target cells (ACE2 panel) also boosted cell-cell fusion and fusion efficiency was only modestly increased by trypsin, in agreement with our previous finding that receptor and protease expression on target cells can both limit SARS-S-mediated cell-cell fusion [26]. In sum, these results demonstrate that cleavage-activation of influenza HA and SARS-S is conserved between human, porcine, avian and murine TMPRSS2 as well as human and murine HAT. Our observations also suggest that TMPRSS2 can support influenza virus spread in species integral to the influenza zoonosis, and that mice are suitable models to study the role of TMPRSS2, TMPRSS4 and HAT in viral spread and pathogenesis.

Binding of human influenza viruses to 2,6-linked sialic acids present on proteins and lipids on the host cell surface is critical for infectious viral entry into host cells [6]. We assessed whether TMPRSS2 and HAT are coexpressed with 2,6-linked sialic acid human tissues. Immunostaining demonstrated the presence of 2,6-linked sialic acids on the surface of almost all cell types (Fig. 2, 3, 4), in keeping with previous results [27–30], with the notable exception of vascular smooth muscle cells (data not shown), suggesting that expression of proteases, such as TMPRSS2 and HAT, but not 2,6-linked sialic acid is likely to be a major determinant of viral tropism. TMPRSS2 was expressed by epithelial cells at all sites examined in the aerodigestive tracts, as well as by many endothelial cells and myocytes of blood vessels, leucocytes (including alveolar macrophages) and smooth muscle cells (Fig. 2, 3, 4, table 1), indicating that TMPRSS2 could activate influenza virus in most permissive epithelia. HAT showed a distribution similar to TMPRSS2 (Fig. 2, 3, 4, table 1), but immunostaining of pneumocytes (alveolar epithelial cells) was weaker, implying low levels of protease expression at this site (Fig. 2A, C). Unlike TMPRSS2, which appeared to be expressed by the majority of type 2 pneumocytes, HAT was expressed by fewer than 50% type 2 pneumocytes, but was additionally seen to be expressed by occasional type 1 pneumocytes (Fig. 2A, C). Type 2 pneumocytes are defined by their morphology rather than a particular immunophenotype, being plump rather than flattened epithelial cells [31]. All sections were examined by an experienced consultant pathologist (ES) in order to identify the cell types that were immunopositive. The exact intensities of staining for TMPRSS2 and HAT of various epithelial types in the aero-digestive tracts are summarized in table 1. While TMPRSS2 expression by bronchial and intestinal smooth muscle cells was noted, these cells appeared negative for HAT, although some vascular smooth muscle cells were found to be positive (table 1). Interestingly, TMPRSS2 but not HAT was expressed by cardiac myocytes (Fig. 4), suggesting that influenza myocarditis might be promoted by TMPRSS2 but not HAT. Notwithstanding, our data demonstrate the potential importance of both proteases in influenza infection.

TMPRSS2 on target cells activates SARS-S on adjacent cells for cell-cell fusion and activates virion-associated SARS-S for infectious host cell entry [13–15]. A wide range of sites demonstrated coexpression of ACE2 and TMPRSS2, and could thus support SARS-CoV spread (table 1). Specifically, in the lung type 2, but not type 1 pneumocytes express both molecules, as do alveolar macrophages and the epithelial cells of intrapulmonary bronchi (Fig. 2A, B). In the upper respiratory tract, the epithelium of the bronchi, larynx, nasal mucosa and respiratory sinuses (Fig. 2 E, F) expresses both molecules, while ACE2 expression is absent from the trachea, vocal folds and epiglottis, although a previous study by Ren et al demonstrated ACE2 expression on the surface epithelium and mucus gland epithelium of trachea, similar to our findings in the larynx and bronchus [32]. This suggests that ACE2 expression may be variable but widespread in the upper airway. The epithelia of the tonsil (Fig. 3A, B) and buccal mucosa (Fig. 2E, F) express both TMPRSS2 and ACE2. Additionally, ACE2 expression by some interstitial macrophages/dendritic cells in intra-alveolar septa of the lung, adjacent to TMPRSS2-expressing type 2 pneumocytes. In the gastrointestinal tract, epithelial co-expression of TMPRSS2 and ACE2 was identified at all sites examined, namely the oesophagus, stomach, ileum and colon. The two molecules were also expressed in cardiac myocytes. Furthermore, variable expression of both molecules by endothelial cells and myoctes of blood vessels, leucocytes and smooth muscle cells was seen (Fig. 2, 3, 4, table 1). Taken together, these results suggest that TMPRSS2 could promote SARS-CoV spread in important target sites, the gastrointestinal and respiratory tracts (table 1).

Discussion

Influenza virus and SARS-CoV hijack host cell proteases to acquire infectivity and for influenza it has been shown that broad spectrum protease inhibitors have therapeutic potential [33–35]. However, the proteases responsible for viral activation in the infected host are unclear, although several candidates have been suggested [7,36]. Recent studies demonstrate that TMPRSS2 and

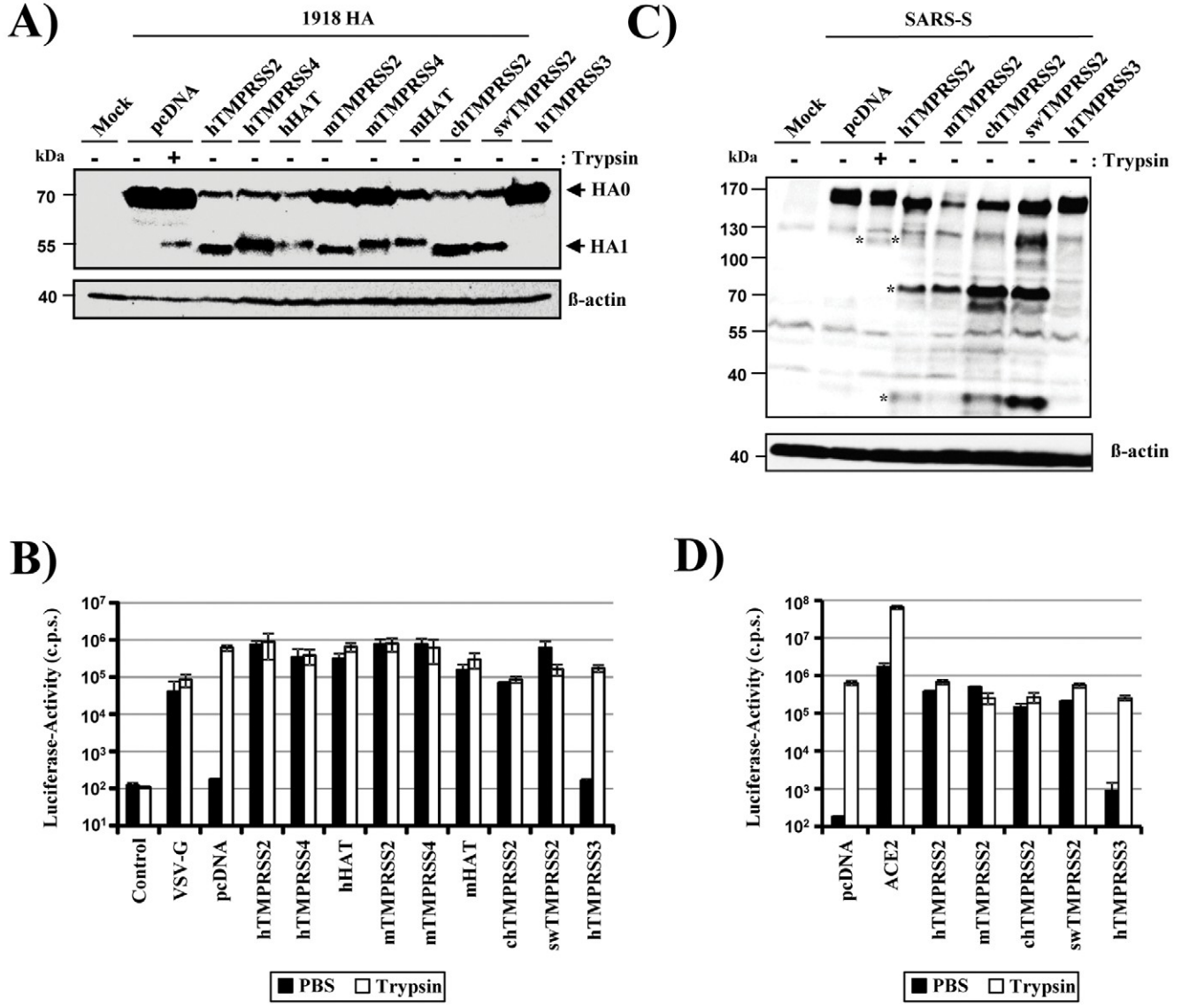

Figure 1. Proteolytic activation of influenza virus hemagglutinin and SARS spike protein is conserved between TMPRSS2 of human, porcine, avian and murine origin. (A) Expression plasmids encoding the HA of the 1918 influenza virus and the indicated proteases or empty vector (pcDNA) were transiently cotransfected into 293T cells. The cells were then treated with PBS or trypsin, and HA cleavage was detected by Western blot analysis of cell lysates using a monoclonal antibody specific for HA. Detection of ß-actin served as loading control. (B) Lentiviral reporter viruses bearing 1918 HA and NA or the VSV-G glycoproteins were generated in 293T cells coexpressing the indicated proteases or empty vector (pcDNA), treated with PBS (black bars) or trypsin (white bars), and used for infection of 293T target cells. Viruses harboring no glycoprotein were generated in parallel as control. Luciferase activities in the cell lysates were determined at 72 h post infection. The results of a representative experiment performed in triplicates are shown. Error bars indicate standard deviation (SD). Comparable results were obtained in a separate experiment. (C) To detect SARS-S cleavage in cis, expression plasmids coding for SARS-S and the indicated proteases or empty vector (pcDNA) were transiently cotransfected into 293T cells, which were then treated with trypsin or PBS. Subsequently, S-protein cleavage was detected by Western blot analysis of cell lysates using a serum specific for the S1 subunit of SARS-S. SARS-S cleavage fragments produced by trypsin and TMPRSS2 are indicated by asterisks. Detection of ß-actin served as a loading control. (D) Effector 293T cells were cotransfected with a SARS-S expression plasmid and a plasmid encoding GAL4-VP16 and mixed with target cells cotransfected with a plasmid encoding a GAL4-VP16 responsive luciferase expression cassette and an ACE2 expression plasmid or protease expression plasmid or empty plasmid. The effector and target cells were mixed, treated with PBS (black bars) or trypsin (white bars) and the luciferase activities in cell lysates quantified at 48 h after cell mixing. The results of a representative experiment performed in triplicates are shown. Error bars indicate standard deviation (SD). Similar results were observed in two independent experiments.

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HAT activate influenza virus [4,9,10] and SARS-coronavirus [13–15,37] in cell culture. We show that both proteases are expressed on receptor-positive cells throughout most of the human respiratory tract and might thus support influenza virus and SARS-CoV spread in and between individuals. In addition, influenza virus activation was conserved between TMPRSS2 orthologues of human, porcine and avian origin, suggesting that zoonotically transmitted influenza viruses may engage TMPRSS2 to facilitate their activation.

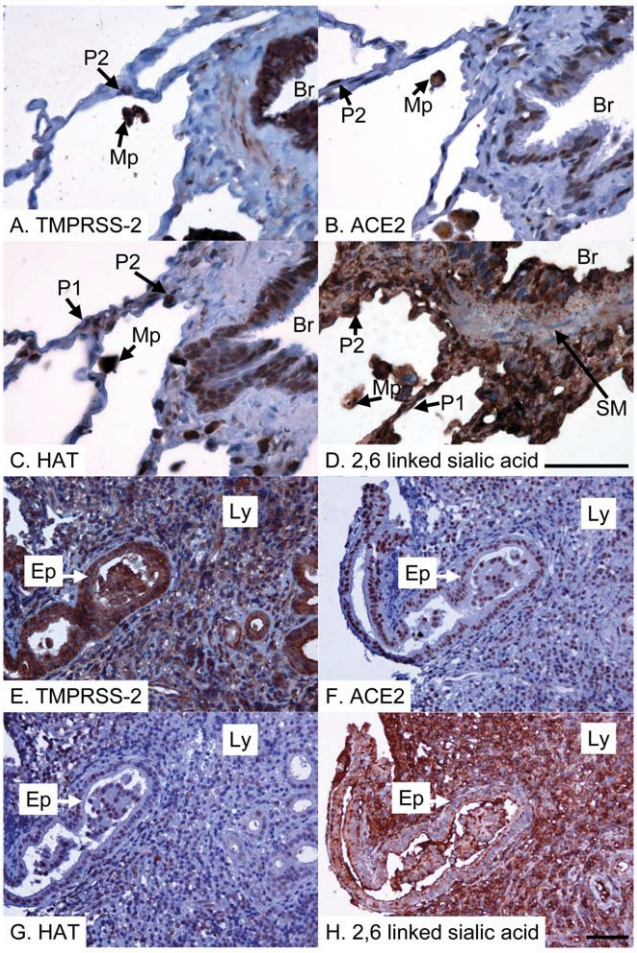

Figure 2. Pulmonary and respiratory sinus expression of SARS-CoV and influenza virus activating proteases and receptors. Lung (A–D) and sinus (E–H) tissue immunostained for TMPRSS2 (A&E), ACE2 (B&F) and HAT (C&G), or stained for 2,6-linked sialic acid (D&H; detected with elderberry (Sambucus nigra) lectin). All positive reactions are detected with the peroxidase technique (brown) and the tissue is counterstained with haematoxylin (blue). (A) There is strong positive anti-TMPRSS2 immunostaining of bronchial epithelium (lining the bronchus, marked Br), type 2 pneumocytes (P2) and alveolar macrophages (Mp). (B) There is moderately strong positive anti-ACE2 immunostaining of bronchial epithelium (lining the bronchus, marked Br), type 2 pneumocytes (P2) and alveolar macrophages (Mp). (C) There is moderately positive anti-HAT immunostaining of bronchial epithelium (lining the bronchus, marked Br) and alveolar macrophages (Mp), with weakly positive immunostaining of some type 1 (P1) and type 2 pneumocytes (P2). (D) All structures are strongly stained for 2,6-sialic acid except for smooth muscle (SM). (E) There is strong positive anti-TMPRSS2 immunostaining of sinus epithelium (Ep) and lymphoid cells (Ly). (F) There is strong positive anti-ACE2 immunostaining of sinus epithelium (Ep) and lymphoid cells (Ly). (G) There is moderately strong anti-HAT immunostaining of sinus epithelium (Ep) and occasional weakly positive immunostaining of lymphoid cells (Ly). (H) All structures are strongly stained for 2,6-sialic acid. Scale bar = 50 microns (shown in panels D and H and also pertaining to 3 preceding panels in each case).
doi:10.1371/journal.pone.0035876.g002

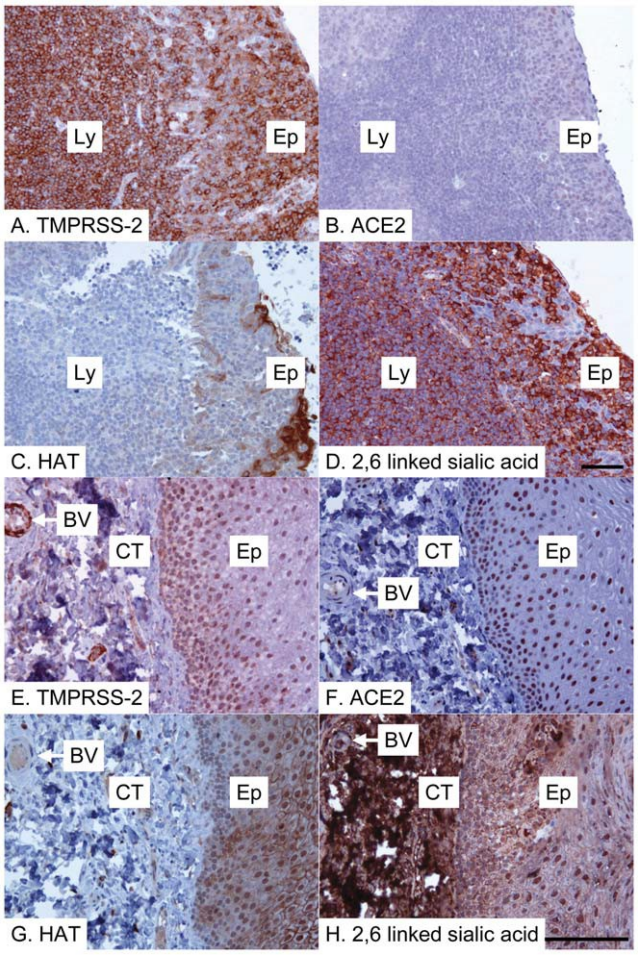

Figure 3. Tonsil and buccal mucosal expression of SARS-CoV and influenza virus activating proteases and receptors. Tonsil (A–D) and buccal mucosa (E–H) immunostained for TMPRSS2 (A&E), ACE2 (B&F) and HAT (C&G), or stained for 2,6-linked sialic acid (D&H; detected with elderberry (Sambucus nigra) lectin). All positive reactions are detected with the peroxidase technique (brown) and the tissue is counterstained with haematoxylin (blue). (A) There is strong positive anti-TMPRSS2 immunostaining of tonsillar epithelium (Ep) and lymphocytes (Ly). (B) There is weakly positive anti-ACE2 immunostaining of tonsillar epithelium (Ep), but little obvious positive immunostaining of lymphocytes (Ly). (C) There is strongly positive anti-HAT immunostaining of the basal and superficial, but not the middle, layers of tonsillar epithelium (Ep), but little obvious positive immunostaining of lymphocytes (Ly). (D) All structures are strongly stained for 2,6-sialic acid except for a few areas of cells within the tonsillar epithelium (Ep). (E) There is strong positive anti-TMPRSS2 immunostaining of buccal epithelium (Ep) and of a blood vessel (BV) in the underlying connective tissue (CT). (F) There is strong positive anti-ACE2 immunostaining of buccal epithelium (Ep) and weaker positive immunostaining of a blood vessel (BV) in the underlying connective tissue (CT). (G) There is strong positive anti-HAT immunostaining of buccal epithelium (Ep), but a blood vessel (BV) in the underlying connective tissue (CT) appears negative. (H) All structures are strongly stained for 2,6-sialic acid. Scale bar = 50 microns (shown in panels D and H and also pertaining to 3 preceding panels in each case).
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Influenza viruses usually replicate in the tracheao-bronchial epithelium [38–40]. Spread in these tissues might be supported by both TMPRSS2 and HAT, which we found to be expressed by cells positive for 2,6-linked sialic acid in the nasal and buccal mucosa as well as in the epithelium of trachea, bronchus and larynx. If infection is associated with pneumonia, a complication more frequently observed with pandemic compared to seasonal influenza viruses, viral spread to the alveolar epithelium is observed [38]. Type I pneumocytes have been suggested to be major targets of influenza virus in the alveoli [40] and were found to be positive for 2,6-linked sialic acid in this study. The protease responsible for HA activation in these cells remains to be defined,

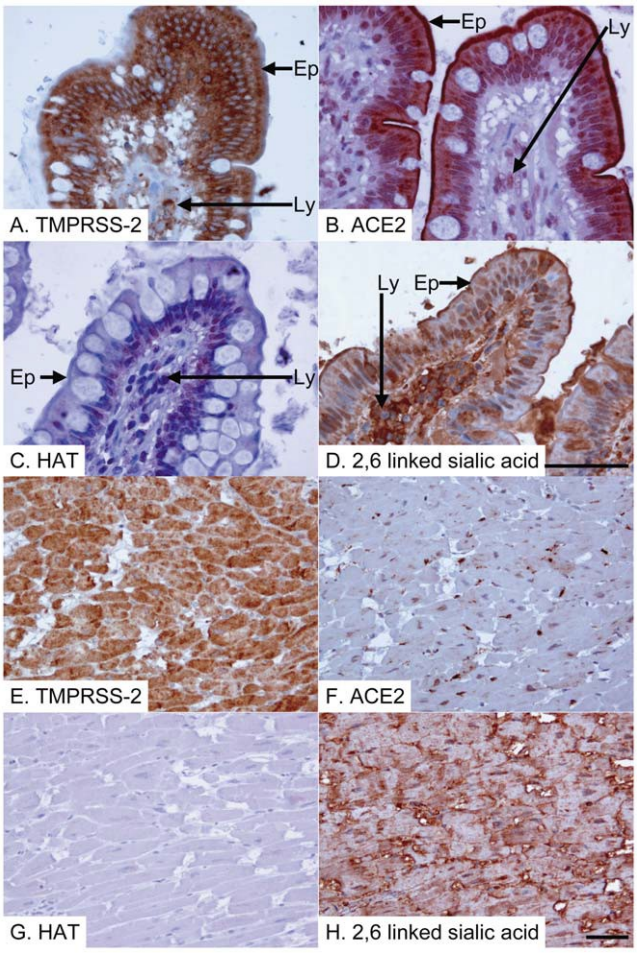

Figure 4. Ileal & myocardial expression of SARS-CoV and influenza virus activating proteases and receptors. Ileum (A–D) and myocardium (E–H) immunostained for TMPRSS2 (A&E), ACE2 (B&F) and HAT (C&G), or stained for 2,6-linked sialic acid (D&H; detected with elderberry (Sambucus nigra) lectin). All positive reactions are detected with the peroxidase technique (brown) and the tissue is counterstained with haematoxylin (blue). (A) There is strong positive anti-TMPRSS2 immunostaining of ileal epithelium (Ep) and also of lymphocytes (Ly) within the core of the villus. (B) There is strong positive anti-ACE2 immunostaining of ileal epithelium (Ep) and also of lymphocytes (Ly) within the core of the villus. (C) There is strongly positive anti-HAT immunostaining of the basal part of the ileal epithelial cells (Ep), but only weak positive immunostaining of occasional lymphocytes (Ly) within the villus core. (D) All structures are strongly stained for 2,6-sialic acid, including ileal epithelium (Ep) and lymphocytes (Ly). (E) There is strong positive anti-TMPRSS2 immunostaining of cardiac myocytes. (F) There is strong positive anti-ACE2 immunostaining of some cardiac myocytes. (G) There is no anti-HAT immunostaining of cardiac myocytes. (H) There is strong 2,6-sialic acid staining of cardiac myocytes. Scale bar = 50 microns (shown in panels D and H and also pertaining to 3 preceding panels in each case).
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since TMPRSS2 was absent from this cell type and expression of HAT was infrequent and weak. However, other studies found that type II pneumocytes are preferentially infected [41] and these cells were identified as positive for 2,6-linked sialic acid, TMPRSS2 and occasionally for HAT within the present study. The presence of cells positive for 2,6-linked sialic acid, TMPRSS2 and/or HAT was not limited to the respiratory tract, in keeping with published findings which demonstrate TMPRSS2 expression in the epithelia of several organs [21,42–45]. It is therefore tempting to speculate that TMPRSS2 and HAT might also support viral spread outside the lung and might thus contribute to complications associated with influenza infection, like gastrointestinal manifestations, myocarditis and encephalopathy [38]. In sum, TMPRSS2 and, with the exception of the alveolar epithelium, HAT could activate influenza viruses throughout the respiratory tract and might support viral spread in extra-respiratory tissues.

The mode of cleavage activation is a major virulence determinant of avian influenza viruses [11,46–48]. Viruses with a multi-basic cleavage site in HA are believed to be activated by ubiquitously expressed host cell proteases and can thus replicate systemically and cause severe disease [11]. In contrast, it has been posited that replication of viruses with a mono-basic cleavage site is confined to the aerodigestive tract, because the expression of as yet unidentified HA-activating protease(s) is limited to this organ [11]. Our results suggest that TMPRSS2 could be the elusive protease, but it remains to be demonstrated whether TMPRSS2 expression in waterfowl and poultry is indeed specific for the aerodigestive tract. HA activation was conserved between avian, porcine and human TMPRSS2, which share high sequence identity (see figure S2), indicating that TMPRSS2 might support influenza virus spread not only in the reservoir (waterfowl) and humans but also in an important intermediate host (swine).

The SARS-CoV causes a severe respiratory illness with fatal outcome in about 10% of the afflicted individuals. The metalloprotease ACE2 has been identified as the SARS-CoV receptor and expression of ACE2 on type II pneumocytes, the major viral target cells, and other pulmonary cells has been demonstrated [49–52]. A cornerstone study by Simmons and colleagues showed that infectious SARS-CoV entry into cell lines depends on the activity of endosomal cathepins, particularly cathepsin L [8], suggesting that cathepsin activity might be required for viral spread in the respiratory tract. However, several studies showed that TMPRSS2 activates SARS-CoV for cathepsin-independent host cell entry and demonstrated TMPRSS2 expression in type II pneumocytes [13–15]. In addition, a recent report indicates that HAT can promote SARS-S-driven cell-cell but not virus-cell fusion [37]. Thus, the SARS-CoV may use TTSPs in addition to or instead of cathepsins to ensure its activation in key target cells. Our analysis confirms and extends these findings by demonstrating that ACE2 and TMPRSS2 are coexpressed by cells in the nasal and buccal mucosa as well as in the epithelia of bronchus and larynx. Thus, one can speculate that, in the context of the infected host, cathepsin activity might not be essential for SARS-CoV spread in most parts of the respiratory tract. Experiments with cathepsin inhibitors and knock-out mice are required to address this possibility. The gastrointestinal tract is a well-established target of SARS-CoV [49] and it has actually been suggested that a wide range of tissues and organs can be infected by the virus [53]. Our finding that TMPRSS2 and ACE2 are coexpressed in colon and various other tissues indicates that also the extrarespiratory spread of SARS-CoV might be promoted by TMPRSS2.

Collectively, our findings are compatible with an important role of TMPRSS2 and HAT in influenza virus and of TMPRSS2 in SARS-coronavirus infection. Knock down of these proteases in primary pulmonary cells and the analysis of Tmprss2 knock-out mice would allow to precise definition of the role of these proteases in viral spread. The latter animals are available [54] and do not show an obvious phenotype, indicating that TMPRSS2 might be an attractive target for novel drugs active against respiratory viruses.

Table 1. Expression pattern of influenza virus and SARS-coronavirus activating proteases and receptors in human tissues.

Antigen/site	Cells/structure	TMPRSS2	HAT	ACE2	2,6-linked sialic acid
Lung	Alveolar Epithelium	type 2 not type 1 pneumocytes+	occasional type 2 & type 1 pneumocytes weakly+	type 2 not type 1 pneumocytes+	type 2 & type 1 pneumocytes+
	Bronchial epithelium	+	+	+	+
	Alveolar macrophages	+	+	+	+
	Other		interstitial macrophages/dendritic cells+; bronchial smooth muscle cells weakly+	some interstitial macrophages/ dendritic cells	+at all sites, except the majority of smooth muscle cells (SMC)
Bronchus & Larynx	Epithelium	+on respiratory, glandular, transitional and (weakly on) squamous epithelium	+on respiratory, transitional and squamous, but not glandular epithelium	weakly+on respiratory & transitional epithelium; strongly+on glandular epithelium	+on respiratory, glandular, transitional and squamous epithelium
Trachea	Epithelium	+on respiratory, glandular, transitional and (weakly on) squamous epithelium	+on respiratory, transitional and squamous, but not glandular epithelium	–	+on respiratory, glandular, transitional and squamous epithelium
Vocal folds & epiglottis	Squamous epithelium	weakly+	+	–	+
Buccal mucosa	Squamous epithelium	+	+	+	+
Nasal mucosa & respiratory sinuses	Epithelium	+	+respiratory & transitional epithelium	+respiratory, transitional & glandular epithelium	+
	Lymphocytes	+	Occasionally+	Variably+	–
Tonsil	Squamous epithelium	+	+	weakly+	+
	Lymphocytes	+	–	–	+
Oesophagus	Squamous epithelium	weakly+	+	weakly+	+
Stomach	Epithelium	+	+	weakly+	+
Ileum	Epithelium	+	weakly+	+	+
Colon	Epithelium	+	weakly+	+	+
Myocardium	Myocytes	+	–	+	+
Blood vessels	Endothelial cells	some weakly+	variably+	variably+	all+
	Vascular smooth muscle cells	+	variably+	variably+	–
Leucocytes	Lymphocytes	+	weakly+(negative in tonsil)	+(negative in tonsil)	+
	Other leucocytes	variably+	variably+	variably+	generally+
Smooth muscle cells	Oesophagus, stomach, intestine, bronchus	+	–	+	–

Cell types expressing TMPRSS2, ACE2, HAT, 2,6-linked sialic acid are marked+, while those that do not express these molecules are marked2. ''weakly'' refers to a low level of staining.
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Supporting Information

Figure S1 To detect SARS-S cleavage in trans, expression plasmids coding for SARS-S and the indicated proteases or empty vector (pcDNA) were separately transfected into 293T cells. Subsequently, the SARS-S and protease expressing cells were mixed, treated with trypsin or PBS and S-protein cleavage was detected by Western blot analysis using a serum specific for the S1 subunit of SARS-S. SARS-S cleavage fragments produced by trypsin and TMPRSS2 are indicated by asterisks. Detection of ß-actin served as a loading control.(TIF)

Figure S2 The amino acid sequences of TMPRSS2 of human (NCBI Reference SequenceNP_001128571.1), chicken (XM_416737.3), swine (BAF76737.1) and mouse origin (AAF97867.1) were aligned using VectorNTI. Identical amino acids are marked in black, similar amino acids are marked in grey.(TIF)

Author Contributions

Conceived and designed the experiments: EJS SP. Performed the experiments: HL SB AH SG PP. Analyzed the data: EJS SP. Contributed reagents/materials/analysis tools: JL PSN SB AH SD PP. Wrote the paper: EJS SP.

1. Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931–939.
2. Skowronski DM, Astell C, Brunham RC, Low DE, Petric M, et al. (2005) Severe acute respiratory syndrome (SARS): a year in review. Annu Rev Med 56:357–381.
3. Parrish CR, Kawaoka Y (2005) The origins of new pandemic viruses: the acquisition of new host ranges by canine parvovirus and influenza A viruses. Annu Rev Microbiol 59: 553–586.
4. Wang W, Butler EN, Veguilla V, Vassell R, Thomas JT, et al. (2008) Establishment of retroviral pseudotypes with influenza hemagglutinins from H1, H3, and H5 subtypes for sensitive and specific detection of neutralizing antibodies. J Virol Methods 153: 111–119.
5. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, et al. (2003) Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426: 450–454.
6. Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69: 531–569.
7. Bertram S, Glowacka I, Steffen I, Kuhl A, Pöhlmann S (2010) Novel insights into proteolytic cleavage of influenza virus hemagglutinin. Rev Med Virol 20:298–310.
8. Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, et al. (2005) Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc Natl Acad Sci U S A 102: 11876–11881.
9. Böttcher E, Matrosovich T, Beyerle M, Klenk HD, Garten W, et al. (2006) Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. J Virol 80: 9896–9898.
10. Chaipan C, Kobasa D, Bertram S, Glowacka I, Steffen I, et al. (2009) Proteolytic activation of the 1918 influenza virus hemagglutinin. J Virol 83: 3200–3211.
11. Bertram S, Glowacka I, Blazejewska P, Soilleux E, Allen P, et al. (2010) TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J Virol 84: 10016–10025.
12. Bottcher-Friebertshauser E, Stein DA, Klenk HD, Garten W (2011) Inhibition of influenza virus infection in human airway cell cultures by an antisense peptide-conjugated morpholino oligomer targeting the hemagglutinin-activating protease TMPRSS2. J Virol 85: 1554–1562.
13. Glowacka I, Bertram S, Muller MA, Allen P, Soilleux E, et al. (2011) Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. J Virol 85: 4122–4134.
14. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, et al. (2010) Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. J Virol 84: 12658–12664.
15. Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, et al. (2011) A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. J Virol 85: 873–882.
16. Connor RI, Chen BK, Choe S, Landau NR (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phago-cytes. Virology 206: 935–944.
17. Hofmann H, Hattermann K, Marzi A, Gramberg T, Geier M, et al. (2004) S protein of severe acute respiratory syndrome-associated coronavirus mediates entry into hepatoma cell lines and is targeted by neutralizing antibodies in infected patients. J Virol 78: 6134–6142.
18. He Y, Zhou Y, Wu H, Luo B, Chen J, et al. (2004) Identification of immunodominant sites on the spike protein of severe acute respiratory syndrome (SARS) coronavirus: implication for developing SARS diagnostics and vaccines. J Immunol 173: 4050–4057.
19. Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, et al. (2005) A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol 79: 11533–11536.
20. Tedoldi S, Paterson JC, Cordell J, Tan SY, Jones M, et al. (2006) Jaw1/LRMP, a germinal centre-associated marker for the immunohistological study of B-cell lymphomas. J Pathol 209: 454–463.
21. Lucas JM, True L, Hawley S, Matsumura M, Morrissey C, et al. (2008) The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. J Pathol 215: 118–125.
22. Takahashi M, Sano T, Yamaoka K, Kamimura T, Umemoto N, et al. (2001) Localization of human airway trypsin-like protease in the airway: an immunohistochemical study. Histochem Cell Biol 115: 181–187.
23. Pulford K, Lamant L, Morris SW, Butler LH, Wood KM, et al. (1997) Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. Blood 89: 1394–1404.
24. Bergeron E, Vincent MJ, Wickham L, Hamelin J, Basak A, et al. (2005) Implication of proprotein convertases in the processing and spread of severe acute respiratory syndrome coronavirus. Biochem Biophys Res Commun 326: 554–563.
25. Yao YX, Ren J, Heinen P, Zambon M, Jones IM (2004) Cleavage and serum reactivity of the severe acute respiratory syndrome coronavirus spike protein. J Infect Dis 190: 91–98.
26. Simmons G, Bertram S, Glowacka I, Steffen I, Chaipan C, et al. (2011) Different host cell proteases activate the SARS-coronavirus spike-protein for cell-cell and virus-cell fusion. Virology 413: 265–274.
27. Drickamer K, Taylor ME (1998) Evolving views of protein glycosylation. Trends Biochem Sci 23: 321–324.
28. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, et al. (2010) Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC Vet Res 6: 4.
29. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JS (2007) Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Respir Res 8: 73.
30. Yao L, Korteweg C, Hsueh W, Gu J (2008) Avian influenza receptor Expression in H5N1-infected and noninfected human tissues. FASEB J 22: 733–740.
31. Young B, et al. (2006) Wheater's Functional Histology: A Text and Colour Atlas.Churchill Livingstone.
32. Ren X, Glende J, Al Falah M, de V V, Schwegmann-Wessels C, et al. (2006) Analysis of ACE2 in polarized epithelial cells: surface expression and function as receptor for severe acute respiratory syndrome-associated coronavirus. J Gen Virol 87: 1691–1695.
33. Zhirnov OP, Ovcharenko AV, Bukrinskaia AG, Ursaki LP, Ivanova LA (1984) [Antiviral and therapeutic action of protease inhibitors in viral infections: experimental and clinical observations]. Vopr Virusol 29: 491–497.
34. Zhirnov OP, Ovcharenko AV, Bukrinskaya AG (1984) Suppression of influenza virus replication in infected mice by protease inhibitors. J Gen Virol 65(Pt 1):191–196.
35. Zhirnov OP, Klenk HD, Wright PF (2011) Aprotinin and similar protease inhibitors as drugs against influenza. Antiviral Res.
36. Kido H, Okumura Y, Takahashi E, Pan HY, Wang S, et al. (2012) Role of host cellular proteases in the pathogenesis of influenza and influenza-induced multiple organ failure. Biochim Biophys Acta 1824: 186–194.
37. Bertram S, Glowacka I, Muller MA, Lavender H, Gnirss K, et al. (2011) Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. J Virol 85: 13363–13372.
38. Kuiken T, Taubenberger JK (2008) Pathology of human influenza revisited.Vaccine 26 Suppl 4: D59–D66.
39. Walsh JJ, Dietlein LF, Low FN, Burch GE, Mogabgab WJ (1961) Bronchotracheal response in human influenza. Type A, Asian strain, as studied by light and electron microscopic examination of bronchoscopic biopsies. Arch Intern Med 108: 376–388.
40. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, et al. (2007) Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 171: 1215–1223.
41. Shieh WJ, Blau DM, Denison AM, Deleon-Carnes M, Adem P, et al. (2010) 2009 pandemic influenza A (H1N1): pathology and pathogeneses of 100 fatal cases in the United States. Am J Pathol 177: 166–175.
42. Jacquinet E, Rao NV, Rao GV, Hoidal JR (2000) Cloning, genomic organization, chromosomal assignment and expression of a novel mosaic serine proteinase: epitheliasin. FEBS Lett 468: 93–100.
43. Jacquinet E, Rao NV, Rao GV, Zhengming W, Albertine KH, et al. (2001) Cloning and characterization of the cDNA and gene for human epitheliasin.Eur J Biochem 268: 2687–2699.
44. Lin B, Ferguson C, White JT, Wang S, Vessella R, et al. (1999) Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res 59: 4180–4184.
45. Vaarala MH, Porvari K, Kyllonen A, Lukkarinen O, Vihko P (2001) The TMPRSS2 gene encoding transmembrane serine protease is overexpressed in a majority of prostate cancer patients: detection of mutated TMPRSS2 form in a case of aggressive disease. Int J Cancer 94: 705–710.
46. Bosch FX, Garten W, Klenk HD, Rott R (1981) Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of Avian influenza viruses. Virology 113: 725–735.
47. Horimoto T, Kawaoka Y (1994) Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J Virol 68: 3120–3128.
48. Ito T, Goto H, Yamamoto E, Tanaka H, Takeuchi M, et al. (2001) Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol 75: 4439–4443.
49. Ding Y, He L, Zhang Q, Huang Z, Che X, et al. (2004) Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. J Pathol 203: 622–630.
50. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis GJ, et al. (2004) Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol 203: 631–637.
51. Mossel EC, Wang J, Jeffers S, Edeen KE, Wang S, et al. (2008) SARS-CoV replicates in primary human alveolar type II cell cultures but not in type I-like cells. Virology 372: 127–135.
52. To KF, Lo AW (2004) Exploring the pathogenesis of severe acute respiratory syndrome (SARS): the tissue distribution of the coronavirus (SARS-CoV) and its putative receptor, angiotensin-converting enzyme 2 (ACE2). J Pathol 203: 740–743.
53. Gu J, Gong E, Zhang B, Zheng J, Gao Z, et al. (2005) Multiple organ infection and the pathogenesis of SARS. J Exp Med 202: 415–424.
54. Kim TS, Heinlein C, Hackman RC, Nelson PS (2006) Phenotypic analysis of mice lacking the Tmprss2-encoded protease. Mol Cell Biol 26: 965–975

3.2 Zeites Manuskript

TMPRSS2 and ADAM17 Cleave ACE2 Differentially and Only Proteolysis by TMPRSS2 Augments Entry Driven by the Severe Acute Respiratory Syndrome Coronavirus Spike Protein

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TMPRSS2 and ADAM17 Cleave ACE2 Differentially and Only Proteolysis by TMPRSS2 Augments Entry Driven by the Severe Acute Respiratory Syndrome Coronavirus Spike Protein

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The type II transmembrane serine proteases TMPRSS2 and HAT can cleave and activate the spike protein (S) of the severe acute respiratory syndrome coronavirus (SARS-CoV) for membrane fusion. In addition, these proteases cleave the viral receptor, the carboxypeptidase angiotensin-converting enzyme 2 (ACE2), and it was proposed that ACE2 cleavage augments viral infectivity. However, no mechanistic insights into this process were obtained and the relevance of ACE2 cleavage for SARS-CoV S protein (SARS-S) activation has not been determined. Here, we show that arginine and lysine residues within ACE2 amino acids 697 to 716 are essential for cleavage by TMPRSS2 and HAT and that ACE2 processing is required for augmentation of SARS-S-driven entry by these proteases. In contrast, ACE2 cleavage was dispensable for activation of the viral S protein. Expression of TMPRSS2 increased cellular uptake of soluble SARS-S, suggesting that protease-dependent augmentation of viral entry might be due to increased uptake of virions into target cells. Finally, TMPRSS2 was found to compete with the metalloprotease ADAM17 for ACE2 processing, but only cleavage by TMPRSS2 re- sulted in augmented SARS-S-driven entry. Collectively, our results in conjunction with those of previous studies indicate that TMPRSS2 and potentially related proteases promote SARS-CoV entry by two separate mechanisms: ACE2 cleavage, which might pro- mote viral uptake, and SARS-S cleavage, which activates the S protein for membrane fusion. These observations have interesting impli- cations for the development of novel therapeutics. In addition, they should spur efforts to determine whether receptor cleavage pro- motes entry of other coronaviruses, which use peptidases as entry receptors.

Coronaviruses are enveloped RNA viruses which cause enteric, respiratory, and central nervous system diseases in a variety of animals and humans (1). The coronaviruses NL63, 229E, and OC43 are adapted to spread in humans, and infection is usually associated with mild respiratory symptoms (2–8). In contrast, the zoonotic transmission of animal coronaviruses to humans can result in novel, severe diseases. The severe acute respiratory syndrome coronavirus (SARS-CoV), which is believed to have been transmitted from bats via an intermediate host to humans (9–11), is the causative agent of the respiratory disease SARS, which claimed more than 700 lives in 2002-2003 (12). Similarly, the recently emerged Middle East respiratory syndrome coronavirus (MERS-CoV) induces a severe, SARS-related respiratory disease, and its spread is at present responsible for 64 deaths (13, 14). The elucidation of the molecular processes underlying the spread and pathogenesis of highly pathogenic coronaviruses is required to devise effective antiviral strategies and is therefore the focus of current research efforts.

The coronavirus surface protein spike (S) mediates entry into target cells by binding to a cellular receptor and by subsequently fusing the viral envelope with a host cell membrane (15, 16). The receptor binding activity of the S proteins is located within the S1 subunit, while the S2 subunit harbors the functional elements required for membrane fusion (15, 16). The SARS-CoV S protein (SARS-S) utilizes angiotensin converting enzyme 2 (ACE2) as a receptor for host cell entry (17, 18). ACE2, a metallopeptidase, is expressed on major viral target cells, type II pneumocytes and enterocytes (19–22), and its catalytic domain binds to SARS-S with high affinity (17, 23). Binding of SARS-S to ACE2 triggers subtle conformational rearrangements in SARS-S, which are believed to increase the sensitivity of the S protein to proteolytic digest at the border between the S1 and S2 subunits (24, 25). Cleavage of the S protein by host cell proteases is essential for viral infectivity (15), and the responsible enzymes constitute potential targets for intervention.

The SARS-CoV can hijack two cellular proteolytic systems to ensure the adequate processing of its S protein. Cleavage of SARS-S can be facilitated by cathepsin L, a pH-dependent endo-/lysosomal host cell protease, upon uptake of virions into target cell endosomes (25). Alternatively, the type II transmembrane serine proteases (TTSPs) TMPRSS2 and HAT can activate SARS-S, presumably by cleavage of SARS-S at or close to the cell surface, and activation of SARS-S by TMPRSS2 allows for cathepsin L-independent cellular entry (26–28). Both TMPRSS2 and HAT are expressed in ACE2-positive cells in the human lung (27, 29), and results obtained with surrogate cell culture systems suggest that TMPRSS2 might play a significant role in SARS-CoV spread in the human respiratory tract (30). Notably, TMPRSS2 and HAT also activate influenza viruses bearing a hemagglutinin with a monobasic cleavage site (31, 32) and TMPRSS2 was shown to cleave and activate the F protein of human metapneumovirus (33), indicating that several human respiratory viruses hijack TTSPs to promote their spread.

The role of host cell proteases in SARS-CoV infection is not limited to cleavage of the S protein: two studies suggest that ACE2 is proteolytically processed by host cell proteases and that process-

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ing might play an important role in SARS-CoV entry and pathogenesis. It was shown that SARS-S binding to ACE2 triggers processing of ACE2 by a disintegrin and metallopeptidase domain 17 (ADAM17)/tumor necrosis factor u-converting enzyme (TACE), and evidence was provided that this process, which facilitates shedding of ACE2 into the extracellular space, promotes uptake of SARS-CoV into cells (34, 35). However, it is disputed whether the increased uptake translates into increased infection efficiency (34, 36). Irrespective of its role in entry, the SARS-S-induced shedding of ACE2 might be integral to the development of SARS. Thus, ACE2 expression was shown to protect against experimentally in- duced lung injury in a mouse model, and evidence for a decreased ACE2 expression in the context of SARS-CoV infection was ob- tained (37, 38). It is therefore conceivable that S protein-induced, ADAM17-mediated shedding of ACE2 might promote SARS pathogenesis. A more recent study demonstrated that ACE2 is also processed by TMPRSS2 and HAT, and it was suggested that ACE2 cleavage increases SARS-S-mediated entry (28). However, the mechanism underlying augmentation of infection is unclear and the role of ACE2 proteolysis in TMPRSS2/HAT-dependent SARS-S activation is unknown. Similarly, the potential interplay between ACE2 processing by TMPRSS2/HAT and ADAM17 and its consequences for SARS-CoV entry have not been examined. Here, we show that ACE2 proteolysis by TMPRSS2/HAT ac- counts for the ability of these proteases to augment SARS-S- driven entry but is dispensable for SARS-S activation. In addition, we provide evidence that increased SARS-S-mediated entry into TMPRSS2/HAT-expressing cells might be due to augmented viral uptake. Finally, we show that TMPRSS2 and ADAM17 compete for ACE2 cleavage and that only processing by TMPRSS2 pro- motes SARS-S-driven entry.

MATERIALS AND METHODS

Cell culture. 293T and Cos-7 cells were maintained in Dulbecco's modi- fied Eagle medium (DMEM; Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biochrom) and 1% penicillin/ streptomycin sulfate (Cytogen). The cells were grown in a humidified atmosphere at 37°C and 5% CO_2.

Plasmids. Expression plasmids for SARS-S and vesicular stomatitis virus G protein (VSV-G) as well as the plasmid used for production of the Fc-tagged S1 subunit of SARS-S have been described previously (39–41). Plasmids encoding the human transmembrane proteases TMPRSS2, TMPRSS3, TMPRSS4, TMPRSS6, and HAT, as well as murine, porcine, and avian TMPRSS2, murine TMPRSS4, and murine HAT, were also described earlier (26, 29, 42, 43). The sequences encoding the enzymatic inactive proteases TMPRSS2 and HAT with an N-terminal myc tag as well as the ACE2 mutants were generated by overlapextension PCR and in- serted into the plasmids pCAGGS (44) and pcDNA3.1 zeo, respectively. The integrity of all PCR-amplified sequences was confirmed by auto- mated sequence analysis. Finally, for generation of lentiviral pseudotypes, the vector pNL-Luc-E$^-$R$^-$ was employed (45).

Analysis of ACE2 cleavage. For the detection of ACE2 cleavage by TTSPs, 293T cells were cotransfected with an expression plasmid encoding ACE2 and either plasmids encoding the specified proteases or empty plasmid. The medium was replaced with fresh DMEM at 6 to 8 h posttransfection. At 48 h posttransfection, the cells were washed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris-HCl [pH 7.3], 150 mM NaCl). Subsequently, 2X SDS loading buffer was added and the lysates were incubated at 95°C. Thereafter, the lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes, and ACE2 was detected by staining with either a mouse anti-ACE2 antibody directed against the ectodomain (R&D Systems) at a dilution of 1:2,000 or with a rabbit anti-ACE2 serum specific for the C terminus (Abgent) at a dilution of 1:500, followed by incubation with horseradish peroxidase (HRP)-coupled anti-mouse or rabbit secondary antibodies (Dianova). As loading control, expression of ß-actin was detected by employing an anti-ß-actin antibody (Sigma). For the analysis of ADAM17-mediated ACE2 shedding, 10 μM phorbol 12- myristate 13-acetate (PMA; Sigma) was added for 16 h to cultures of ACE2-transfected 293T cells. Subsequently, the supernatants were har- vested and cleared from cell debris by centrifugation. The proteins present in cleared supernatants were precipitated as described previously (36). In brief, trichloroacetic acid (TCA) was added to supernatants, the mixtures were incubated for 30 min at 4°C, and precipitated proteins were pelleted through centrifugation at 14,000 rpm for 5 min. Subsequently, the pellet was washed twice with ice-cold acetone and then resuspended in alkaline loading buffer (50 mM Tris [pH 8], 2% SDS, 100 mM dithiothreitol [DTT], 10% glycerol, bromophenol blue) and incubated at 95°C before analysis by SDS-PAGE and immunoblotting as described above. The pro- gram ImageJ (46) was employed for signal quantification.

Analysis of SARS-S-driven host cell entry. The SARS-S-mediated host cell entry was analyzed by employing a lentiviral vector system, as described previously (40). In brief, lentiviral pseudotypes were pro- duced by cotransfecting 293T cells with the HIV-1-derived vector pNL-Luc-E-R- and expression plasmids for SARS-S, VSV-G, or empty plasmid (pcDNA). At 6 to 8 h posttransfection, the culture medium was replaced with fresh medium, and at 48 h posttransfection, the supernatants were harvested, passed through 0.45-μm-pore-size filters, aliquoted, and stored at −80°C. To analyze the impact of TMPRSS2 and HAT on SARS-S-driven transduction, plasmids encoding the receptor ACE2 or ACE2 mutants were cotransfected with plasmids encoding the indicated proteases or empty plasmid. One day prior to infection, the target cells were seeded in 96-well plates at 30,000 cells/well. Subsequently, pseudotypes were added and the cells were incubated for 6 h at 37°C. Thereafter, the medium was replaced with fresh medium. Finally, the luciferase activities in cell lysates were determined at 72 h postransduction using a commercially available kit (Promega).

Modulation of SARS-S-driven host cell entry. The cathespin B/L inhibitor MDL28170 (Calbiochem), the ADAM17 inhibitor TAPI-1 (Calbiochem), or PMA (Sigma) was diluted in solvent as recommended by the manufacturers. For the analysis of entry inhibition by MDL28170, target cells expressing ACE2 or coexpressing ACE2 and protease were incubated with inhibitor (10 μM final concentration) or solvent alone for 60 min before pseudotypes were added. After incubation at 37°C for 8 h, the supernatants were removed and fresh culture medium without inhibitor was added. Transduction efficiency was determined at 72 h after the addition of pseudotypes as described above. In order to determine the impact of TAPI-1 and PMA on SARS-S-dependent transduction, target cells were incubated with pseudotypes at 4°C for 1 h followed by removal of unbound particles by washing. Subsequently, the indicated concentra- tions of TAPI-1 or PMA (see the legend to Fig. 8) were added and the cultures incubated at 37°C for 8 h. Thereafter, the culture supernatants were removed and replaced by fresh culture medium without TAPI-1 or PMA. Transduction efficiency was determined at 72 h after the addition of pseudotypes.

ACE2 surface expression. For analysis of ACE2 surface expression, plasmids encoding the ACE2 wild type (wt) or the specified ACE2 mutants were transiently transfected into 293T cells. At 48 h posttransfection, the cells were detached and washed with fluorescence-activated cell sorter (FACS) buffer (1X PBS, 5% FCS, 2 mM EDTA) and stained with a goat anti-ACE2 antiserum (R&D Systems). After binding of the primary antibodies for 45 min at 4°C, cells were washed three times with FACS buffer and incubated for 45 min at 4°C with Cy5-coupled anti-goat secondary antibody (Dianova). After three washes with FACS buffer, the cells were fixed with 2% paraformaldehyde (PFA) and analyzed in a Becton, Dick-

inson LSR II flow cytometer. FCS Express software (De Novo Software) was employed for data analysis.

High-salt washes of ACE2 and protease-coexpressing cells. In order to prevent retention of ACE2 cleavage fragments at the cell surface, the ACE2 and protease-coexpressing cells were pelleted and incubated with high-salt buffer (0.5 M, 1 M, and 1.5 M NaCl) on ice. Subsequently, the cells were pelleted again and the supernatants were collected. The proteins present in cleared supernatants were precipitated as described previously (36), and the presence of ACE2 in cell pellets and supernatants was analyzed by Western blotting as specified above.

Cleavage of recombinant ACE2 and analysis of cleavage sites by mass spectrometry. For the analysis of cleavage of isolated ACE2, 1 μg of recombinant ACE2 (R&D Systems) was incubated with 0.2 μg of recombinant HAT (R&D Systems) in assay buffer (50 mM Tris, 0.05% [wt/vol], and Brij 35 [pH 9.5]) for 2 h at 37°C in a total volume of 25 μl. Subsequently, the reactions were stopped by the addition of SDS loading buffer, and the reaction products were analyzed by 12.5% SDS-PAGE and Western blotting. For the analysis of cleavage sites by mass spectrometry (MS), ACE2 cleavage products were separated on precast NuPAGE bis-Tris 4 to 12% gradient gels using a morpholinepropanesulfonic acid buffer system according to the manufacturer (Invitrogen). After colloidal Coomassie staining, gel bands were excised, and one part of the band was subjected to in-gel digestion with trypsin, while endoproteinase Asp-N was used for the other part. In-gel digestion and mass spectrometric analysis of the proteolytic peptides by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) were performed as described previously (43, 47). Extracted peptides were dried, redissolved in 0.5% trifluoroacetic acid/0.1% octyl-glucopyranoside, and spotted onto an AnchorChip target (Bruker Daltonics) precoated with u-cyano-4-hydroxycinnamic acid. Preparation was according to either the standard thin-layer affinity method (47) or an adapted method to improve coverage of small hydrophilic peptides. For this purpose, 0.5 μl was spotted onto the matrix surface, let dry, and washed once with ammonium dihydrogen phosphate (10 mM in 0.1% trifluoroacetic acid [TFA]).

Cellular uptake of SARS-S. 293T cells were seeded on coated coverslips, transfected with plasmids encoding ACE2 and TMPRSS2 or empty vector, and incubated with the Fc-tagged S1 subunit of SARS-S for 1 h. Incubation was performed at 4°C to allow binding but not uptake or at 37°C to allow both processes. Subsequently, the cells were prepared for confocal microscopy. For this, the cells were washed three times with PBS followed by 20 min of fixation with 4% PFA. To stop the PFA fixation, the cells were treated with 50 mM NH_4C for 10 min. Subsequently, the cells were washed three times with PBS and then permeabilized by treatment with 0.2% Triton X-100 for 15 min. Thereafter, the cells were washed again three times with PBS and then blocked with 3% bovine serum albumin (BSA). Bound SARS-S1-Fc fusion protein was detected by using a fluorescein isothiocyanate (FITC)-coupled, anti-human Ig-specific secondary antibody (Dianova), while ACE2 expression was detected by staining with a mouse anti-ACE2 antibody, followed by staining with Red-X-coupled anti-mouse secondary antibody (Dianova). Finally, the cells were washed again, the coverslips were embedded in mounting medium, and staining was analyzed by confocal microscopy employing a LSM5 Pa confocal microscope (Carl Zeiss). The FACS-based analysis of SARS-S1-Fc binding to cells was conducted as described previously (41).

Statistical analysis. Statistical significance was calculated using two-tailed Student's *t* test for independent samples.

RESULTS

TMPRSS2, HAT, and hepsin cleave ACE2. We first examined if ACE2 cleavage by TTSPs is detectable in transiently transfected 293T cells, which we routinely employ to assess SARS-S-driven cell-cell and virus-cell fusion. Indeed, coexpression of ACE2 and TMPRSS2 or HAT of human and animal origin resulted in ACE2 cleavage with a C-terminal ACE2 fragment of 13 kDa being readily detectable in cell lysates (Fig. 1A and B, middle panels). ACE2 processing into a 13-kDa C-terminal fragment was also observed for hepsin, while TMPRSS3, TMPRSS4, and TMPRSS6 did not facilitate ACE2 proteolysis (Fig. 1A and B, middle panels), despite efficient expression in transiently transfected cells (not shown). Production of the 13-kDa ACE2 fragment was dependent on the enzymatic activities of TMPRSS2 and HAT, since the fragment was not generated in cells expressing enzymatically inactive mutants of these proteases (Fig. 1C). Finally, titration experiments showed that the efficiency of ACE2 cleavage was dependent on the protease expression level. Thus, low protease expression resulted in the generation of additional cleavage products of approximately 15 to 17 kDa (Fig. 1C), which likely represent incompletely processed C-terminal fragments of ACE2. In sum, our results demonstrate that TMPRSS2 and HAT remove a short C-terminal fragment from ACE2, in keeping with previous findings (28). In addition, a new ACE2-processing TTSP, hepsin, was identified, which cleaves ACE2 in a manner similar to that observed for TMPRSS2 and HAT.

Residue R621 is dispensable for cleavage of cellular ACE2. The identification of the TMPRSS2 and HAT cleavage sites in ACE2 is a prerequisite to understanding how ACE2 cleavage by these proteases impacts SARS-S-driven entry. We employed recombinant ACE2 and HAT, both of which are commercially available, to address this question. Incubation of ACE2 with HAT but not reaction buffer alone produced an 80-kDa fragment (Fig. 2A), indicating that ACE2 proteolysis by HAT in cells can be reproduced *in vitro* with recombinant proteins. To identify the cleavage site, we performed a mass spectrometric peptide mapping analysis of intact ACE2 and the 80-kDa fragment using two different proteases with a complementary cleavage specificity, trypsin and endoproteinase Asp-N. In contrast to the peptide maps of intact ACE2, no peptides matching the C-terminal sequence of amino acids 603 to 733 were detectable in the trypsin/Asp-N digests of the 80-kDa fragment (Fig. 2B). This finding, together with the observation that the Asp-N cleavage product 598-DQSIKVRISLKSAL G-612 is present in the Asp-N digest of intact ACE2 but not in that of the 80-kDa fragment (Fig. 2C), indicated that the HAT cleavage site resides in this 15-amino-acid sequence. When we screened the Asp-N digests for the corresponding candidate peptide species, we found the Asp-N cleavage product 598-DQSIKVR-604 to be present in the Asp-N digest of the 80-kDa fragment but not in that of intact ACE2 (Fig. 2D), indicating that the cleavage occurred at R604, in agreement with the preference of HAT for cleavage C-terminally of Arg (48). These results were confirmed by mass spectrometric sequencing of peptides 598-DQSIKVRISLKSALG-612 and 598-DQSIKVR-604 (data not shown). Thus, mass spectrometric analysis revealed that the 80-kDa fragment was generated upon HAT-dependent proteolysis of recombinant ACE2 at residue R604, corresponding to R621 of cellular ACE2. In order to elucidate if cellular ACE2 is also cleaved by HAT at this site or at an arginine or lysine residue located in close proximity, we generated K619A, R621A, K619A R621A, and K625A mutants. However, all ACE2 mutants were cleaved with an efficiency similar to that of the ACE2 wt in cells coexpressing HAT or TMPRSS2 (Fig. 2E). These findings indicate that the cleavage site in recombinant ACE2 might be located at R604, while the corresponding residue in cellular ACE2 and R621, as well as nearby basic residues, are dispensable for cleavage.

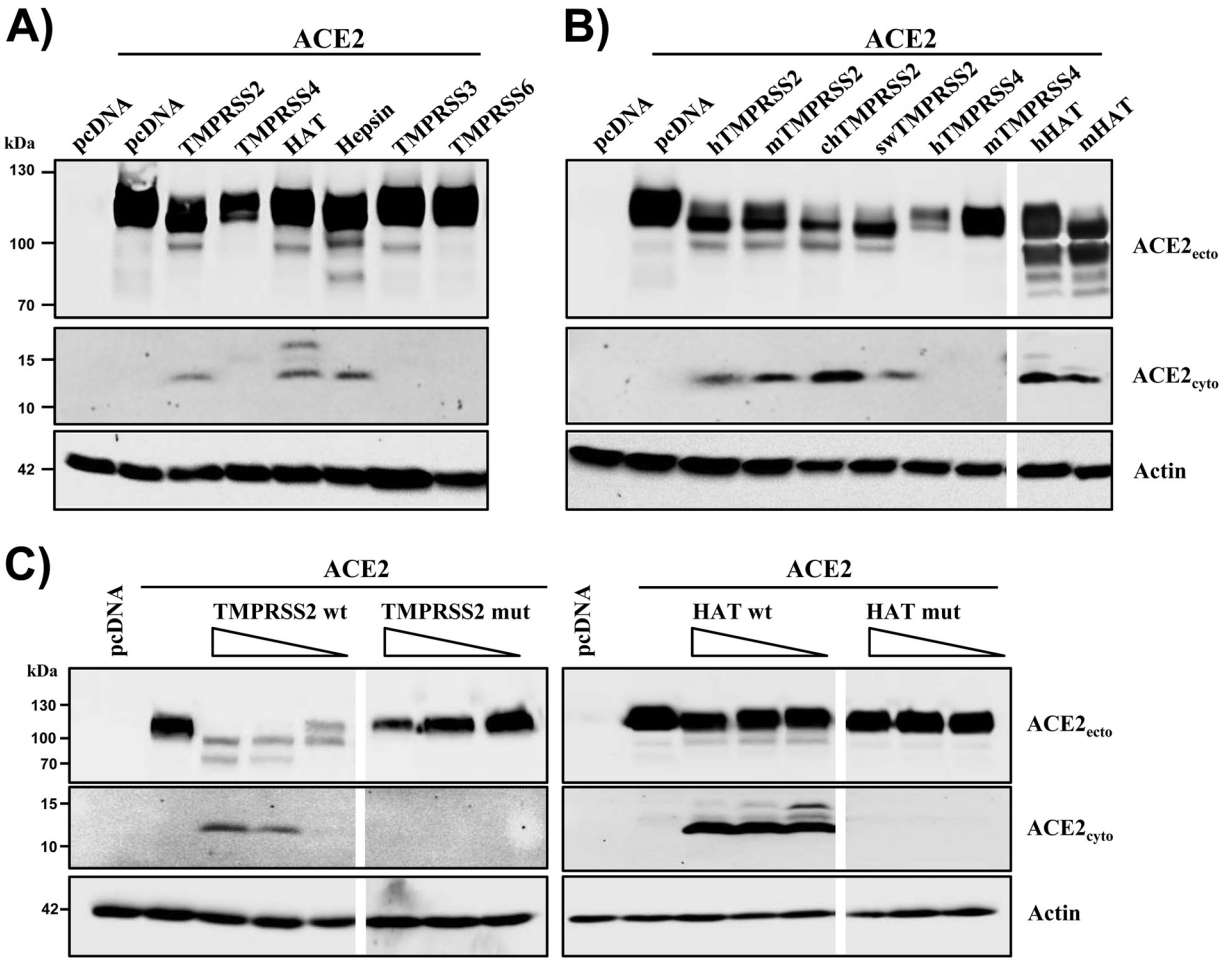

FIG 1 Cleavage of ACE2 by type II transmembrane serine proteases. (A) Plasmids encoding ACE2 and the indicated proteases were transiently cotransfected into 293T cells. Cells cotransfected with empty plasmid (pcDNA) or transfected with empty plasmid alone served as negative controls. The transfected cells were lysed and the lysates analyzed by Western blotting using an ACE2 monoclonal antibody directed against the ACE2 ectodomain (top panel) or a polyclonal antibody directed against the C terminus of ACE2 (middle panel). Detection of ß-actin in cell lysates served as a loading control (bottom panel). (B) The experiment was carried out as described for panel A, but proteases from the indicated species were analyzed. The results of two gels run in parallel are shown. (C) The experiment was carried out as described for panel A, but different amounts of plasmid encoding TMPRSS2 and HAT wild types or catalytically inactive proteases (TMPRSS2 mut, HAT mut) were cotransfected. Ecto, ectodomain; cyto, cytoplasmic domain; h, human; m, mouse; ch, chicken; sw, swine.

Arginine and lysine residues within amino acids 697 to 716 are essential for efficient ACE2 cleavage by TMPRSS2 and HAT. Since work with recombinant proteins did not allow the identifi- cation of the ACE2 site cleaved by HAT or TMPRSS2 in cells, we next employed mutagenesis to identify the cleavage site(s) of these proteases. Sequence analysis revealed the presence of five clusters of arginine and lysine residues located between R619 and the transmembrane domain of ACE2 (residues 741 to 761), which could be recognized by TTSPs (Fig. 3A). In order to assess the importance of these clusters for ACE2 cleavage by TMPRSS2 and HAT, we mutated the clustered arginine and lysine residues to alanine, resulting in mutants C0 to C4 (Fig. 3A). In addition, we combined a mutation of cluster 0, which harbors the residue (R621) cleaved in the context of recombinant proteins, with mutations of the remaining clusters, giving yield to mutants C0 + C1 to C0 + C4 (Fig. 3A). Expression of all ACE2 mutants in cell lysates was readily detected by Western blotting, although expression of mutants C2 and C0 + C2 was reduced compared to expression of the ACE wt (Fig. 3B and C, top panels). Analysis of ACE2 cleavage revealed that mutation of lysine and arginine residues in clusters 0 to 3 did not interfere with ACE2 processing by TMPRSS2 and HAT (Fig. 3B and C, bottom panels). In contrast, mutation of the arginine and lysine residues within cluster 4 markedly reduced cleavage by both TMPRSS2 and HAT (Fig. 3B, bottom panel), and cleavage was further diminished when a mutation of cluster 4 was combined with a mutation of cluster C0 (mutant C0 + C4) (Fig. 3C, bottom panel, and Fig. 3D). These results identify the arginine and lysine residues within cluster 4 (amino acids 697 to 716) as essential for ACE2 proteolysis by TMPRSS2 and HAT and suggest that cluster 0 (amino acids 619 to 625) contributes to efficient cleavage of cluster 4.

ACE2 cleavage is essential for TMPRSS2- and HAT-mediated augmentation of SARS-S-driven transduction. We next used our ACE2 mutants to investigate the role of ACE2 proteolysis in the TMPRSS2- and HAT-dependent enhancement of SARS-S-driven entry. As a prerequisite to these studies, we first assessed surface expression and receptor function of the ACE2 mutants in the absence of protease expression. Surface expression of most

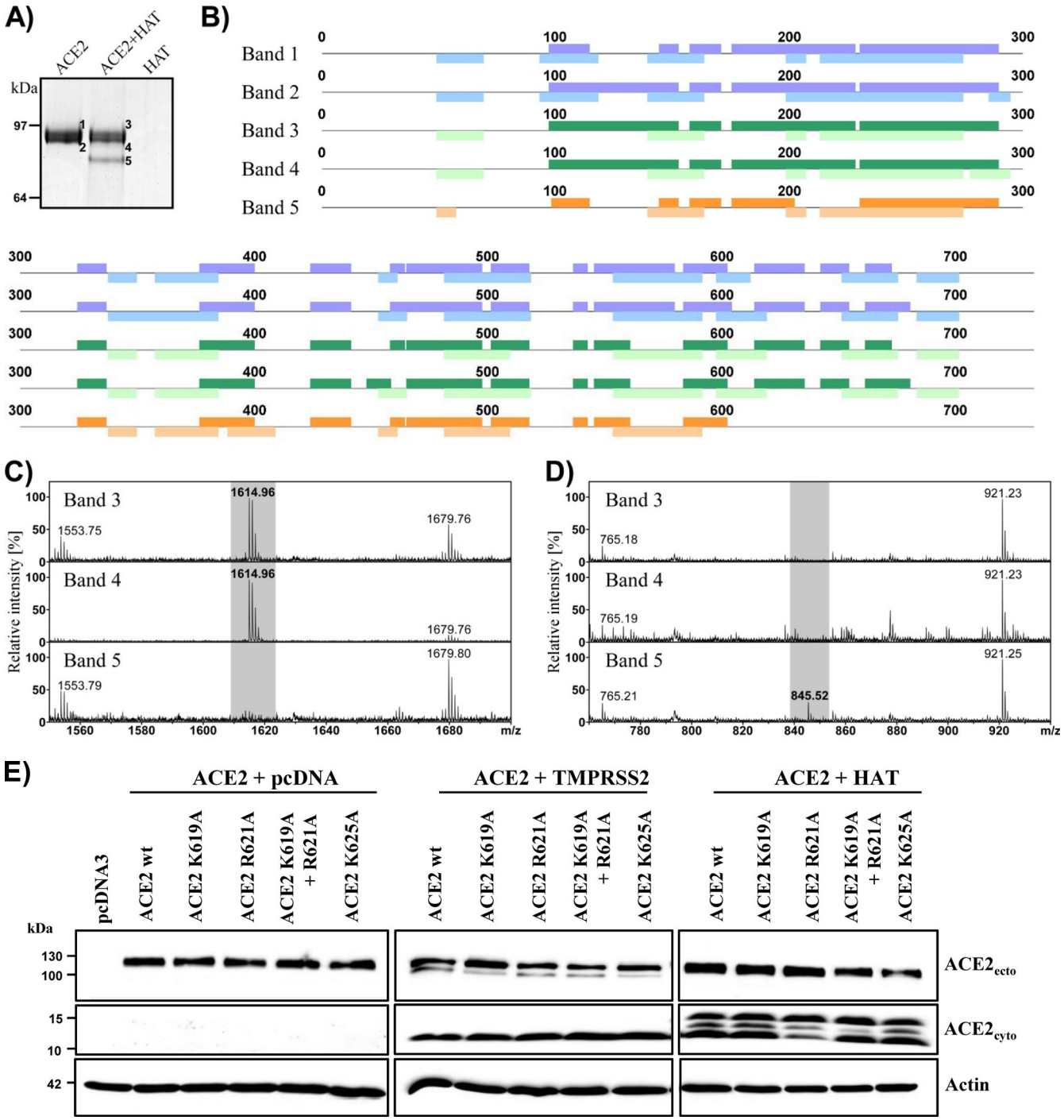

FIG 2 Residue R621 is the HAT cleavage site in recombinant ACE2 but is not essential for cleavage of cellular ACE2 by TMPRSS2 and HAT. (A) Coomassie-stained SDS-PAGE gel showing that recombinant ACE2 (apparently migrating as a double band around 90 kDa in this gel system, bands 1 to 4) is cleaved into an 80-kDa fragment (band 5) upon incubation with HAT. Note that the 80-kDa fragment does not appear as a double band, suggesting that the differences in the electrophoretic mobility of intact ACE2 may be due to ragged C-terminal sequences (e.g., absence of the 10-His tag as stated in the product information for recombinant ACE2 [R&D Systems]). (B) Bands 1 to 5 were excised and subjected to mass spectrometric peptide mapping with trypsin and endoproteinase Asp-N, respectively. Visualization of peptide assignments to the ACE2 sequence (upper bars, trypsin; lower bars, Asp-N) shows the absence of the C-terminal part (amino acids 603 to 733) in the 80-kDa fragment. (C and D) Mass spectrometric analysis of Asp-N digests of ACE2 (bands 3 and 4) and its cleavage product (band 5). In the zoomed-in mass spectra in panel C, the mass signals at *m/z* 1,614.96 (highlighted in gray) represent the Asp-N cleavage product 598-DQSIKVRISLK SALG-612 ($[M+H]^+_{calc}$ = 1,614.954). Its presence in intact ACE2 (bands 3 and 4, upper and middle panels) together with its absence in the 80-kDa fragment (band 5, lower panel) indicated that the HAT cleavage site resides within this sequence. In the zoomed-in mass spectra in panel D, the mass signal at *m/z* 845.52 (highlighted in gray) represents the Asp-N cleavage product 598-DQSIKVR-604 ($[M+H]^+_{calc}$= 845.484). Its absence in intact ACE2 (bands 3 and 4, upper and middle panels) together with its presence in the 80-kDa fragment (band 5, lower panel) revealed R604 as the HAT cleavage site. (E) Plasmids encoding the ACE2 wt or the indicated ACE2 mutants jointly with plasmids encoding TMPRSS2 or HAT were transiently cotransfected into 293T cells. The transfected cells were lysed and the lysates analyzed by Western blotting using an ACE2 monoclonal antibody directed against the ACE2 ectodomain (top panel) or a polyclonal antibody directed against the C terminus of ACE2 (middle panel). Detection of ß-actin in cell lysates served as a loading control (bottom panel). Ecto, ectodomain; cyto, cytoplasmic domain.

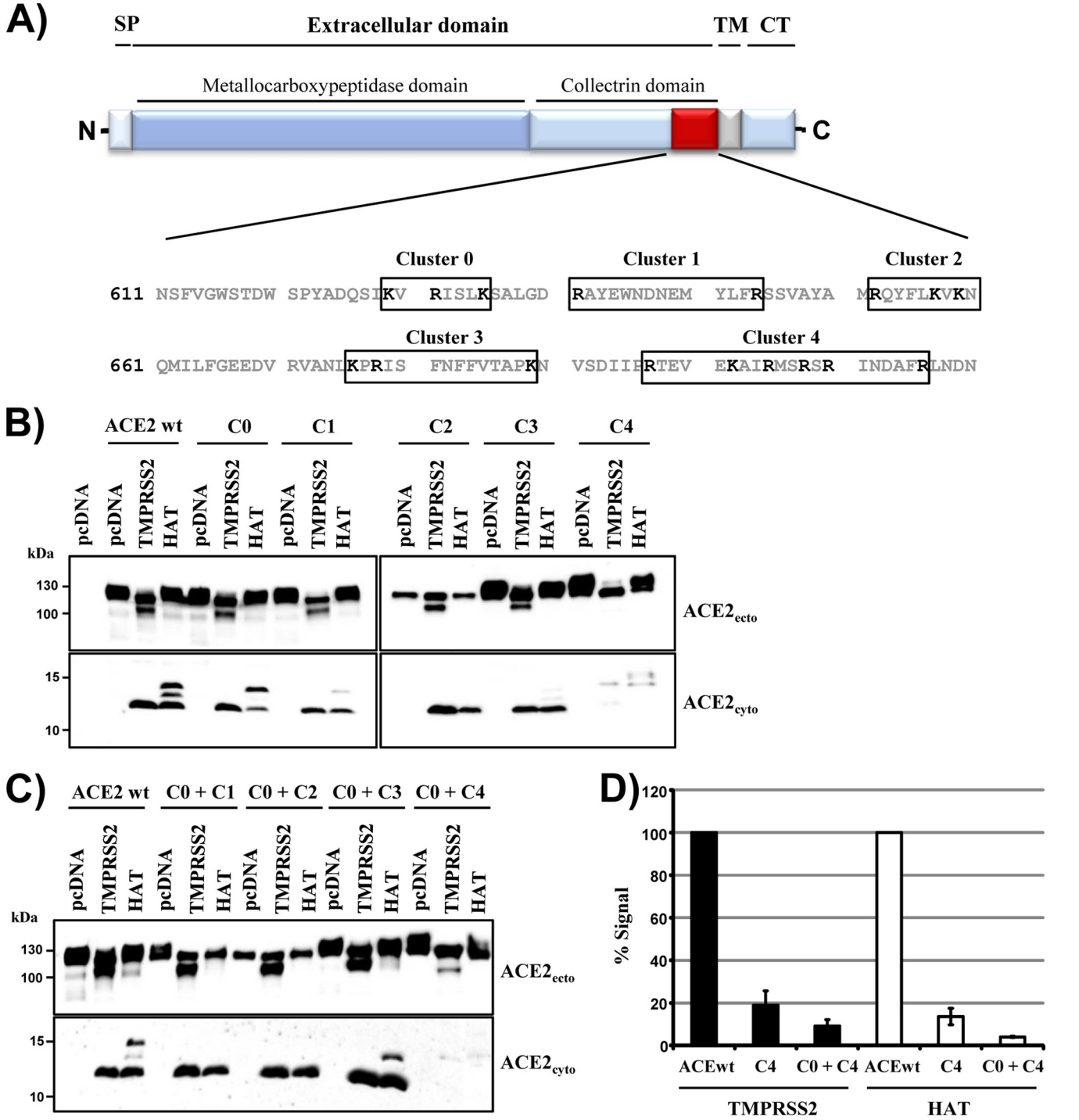

FIG 3 Arginine and lysine residues within ACE2 amino acids 697 to 716 are essential for ACE2 cleavage by TMPRSS2 and HAT. (A) The domain organization of ACE2 is depicted schematically. The membrane-proximal region of ACE2 potentially harboring the TMPRSS2 and HAT cleavage site is highlighted, and its amino acid sequence is provided. Five clusters of arginine and lysine residues were identified in the membrane-proximal sequence, and their position is indicated by boxes. The residues were mutated to alanine, resulting in ACE2 mutants C0, C1, C2, C3, and C4. In addition, mutant C0 was combined with the remaining mutants, resulting in double mutants C0 + C1, C0 + C2, C0 + C3, and C0+ C4. (B and C) Plasmids encoding the ACE2 wt or the indicated ACE2 mutants jointly with plasmids encoding TMPRSS2 or HAT or no protease (pcDNA) were transiently cotransfected into 293T cells. The transfected cells were lysed and the lysates analyzed by Western blotting using an ACE2 monoclonal antibody directed against the ACE2 ectodomain (top panels) or a polyclonal antibody directed against the C terminus of ACE2 (bottom panels). (D) The intensity of the C-terminal cleavage fragment observed upon processing of the ACE2 wt or the indicated ACE2 mutants was quantified via ImageJ software. The average signals measured upon analysis of at least three independent Western blots are shown. Error bars indicate standard errors of the mean (SEM). The signal measured upon cleavage of the ACE2 wt was set as 100%. Ecto, ectodomain; cyto, cytoplasmic domain.

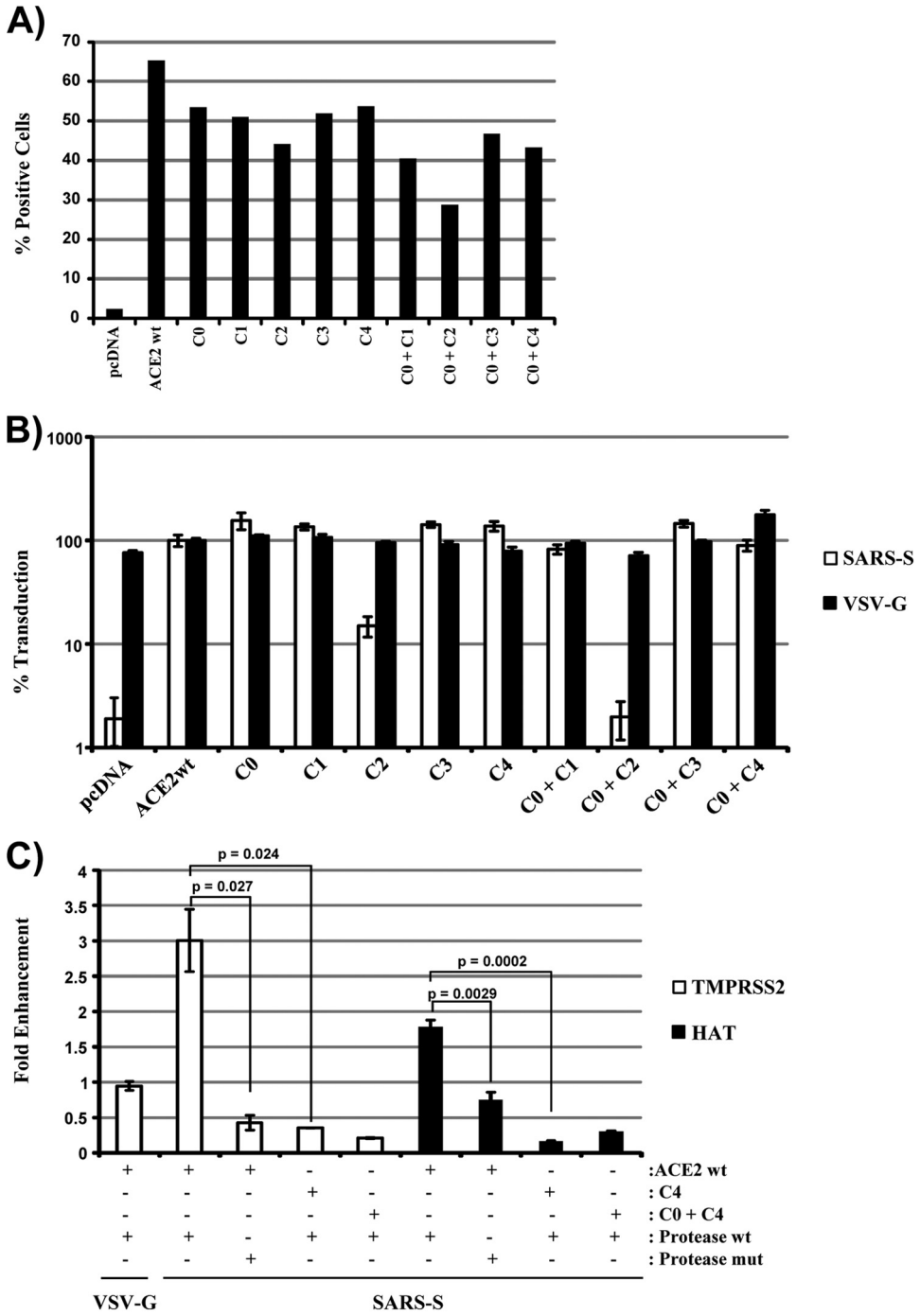

FIG 4 Arginine and lysine residues with ACE2 amino acids 697 to 716 are essential for TMPRSS2- and HAT-dependent augmentation of entry mediated by the SARS-CoV spike protein. (A) Plasmids encoding the ACE2 wt or the indicated ACE2 mutants were transiently transfected into 293T cells, and ACE2 surface expression was detected by FACS. Results of a single experiment are shown and were confirmed in two separate experiments. (B) Plasmids encoding the ACE2 wt or the indicated ACE2 mutants were transiently transfected into 293T cells and the cells transduced with a lentiviral vector pseudotyped with SARS-S. Cells transfected with empty plasmid (pcDNA) served as a negative control, while a vector pseudotyped with VSV-G served as a control for susceptibility to ACE2-independent transduction. Luciferase activities in cell lysates were determined at 72 h postransduction. The results of a representative experiment performed with triplicate samples are shown; error bars indicate standard deviations (SD). Similar results were obtained in two additional experiments. (C) The experiment was carried out as described for panel B, but transduction of target cells expressing the ACE2 wt or the indicated ACE2 mutants jointly with the indicated proteases was assessed. The results are shown as fold enhancement of transduction upon expression of the proteases TMPRSS2 and HAT. The results represent the averages from two to six independent experiments, and error bars indicate SEM.

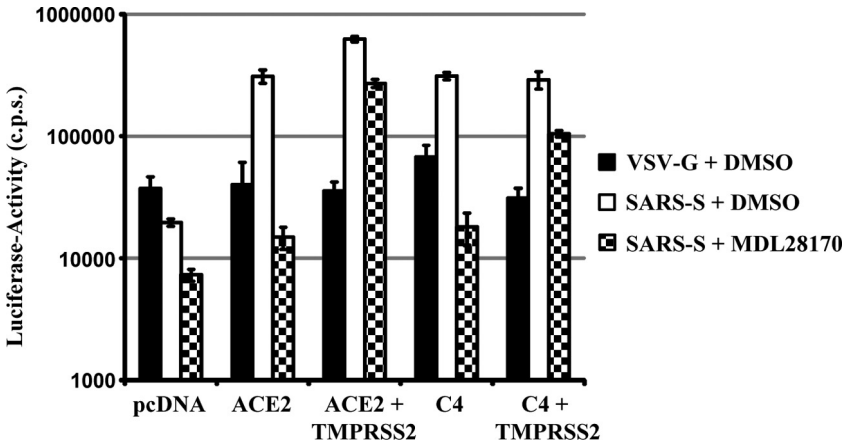

FIG 5 ACE2 cleavage is dispensable for SARS-S activation by TMPRSS2. Plasmids encoding the ACE2 wt or ACE2 mutant C4 were transiently cotransfected into 293T cells with either plasmid encoding TMPRSS2 or empty plasmid. Subsequently, the cells were incubated with the cathepsin B/L inhibitor MDL28170 or an equal volume of dimethyl sulfoxide (DMSO) and transduced with pseudotypes bearing the indicated glycoproteins. Luciferase activities in cell lysates were determined at 72 h posttransfection. The results of a representative experiment performed with triplicate samples are shown, and error bars indicate SD. Comparable results were obtained in two separate experiments.

ACE2 mutants was robust, although mutation of one and particularly two arginine and lysine clusters invariably caused a modest decrease in ACE2 expression levels (Fig. 4A). A more notable reduction in ACE2 surface expression was measured upon mutation of cluster 2 (mutants C2 and C0 + C2) (Fig. 4A), in keeping with the reduced expression of these mutants in cell lysates (Fig. 3B and C).

The receptor activity of the ACE2 mutants mirrored their expression profile: most ACE2 mutants analyzed, including the protease-resistant mutants C4 and C0 + C4, supported transduction by SARS-S-bearing pseudotypes with an efficiency similar to that of the ACE2 wt (Fig. 4B). In contrast, a 5- and 50-fold-reduced transduction efficiency was measured for mutants C2 and C0 + C2, respectively (Fig. 4B).

In order to investigate protease-dependent enhancement of infectivity, the ACE2 wt or ACE2 mutants C4 and C0 + C4 were coexpressed with TMPRSS2 or HAT, and the efficiency of SARS-S-mediated transduction was examined. Coexpression of the ACE2 wt with TMPRSS2 and to a lesser degree with HAT augmented SARS-S- but not VSV-G-driven entry (Fig. 4C), as expected (28). In addition, augmentation of entry was dependent on the enzymatic activity of the proteases (Fig. 4C), again in keeping with published results (28). In contrast, expression of TMPRSS2 and HAT did not increase SARS-S-mediated entry into cells expressing the cleavage-resistant ACE2 mutants C4 and C0 + C4 (Fig. 4C), demonstrating that ACE2 proteolysis is essential for the protease-dependent augmentation of SARS-S-driven cellular entry.

ACE2 proteolysis by TMPRSS2 is not required for SARS-S-activation. The expression of TMPRSS2 in viral target cells can increase entry efficiency (Fig. 4C) (28) and allows for cathepsin L-independent SARS-S activation (26–28). The first phenotype, increased infection, is due to ACE2 cleavage, as demonstrated above. The second phenotype, cathepsin L independence, has so far been linked to SARS-S cleavage but could also depend on ACE2 proteolysis. To address this question, we asked whether TMPRSS2 expression also facilitates cathepsin L-independent entry into target cells expressing the cleavage-resistant ACE2 mutant C4. For this, the ACE2 wt or ACE2 mutant C4 were expressed in 293T cells, and the cells were treated with PBS or cathepsin B/L inhibitor MDL28170 and transduced by pseudotypes bearing VSV-G or SARS-S. All cells were readily transduced by pseudotypes bearing VSV-G (Fig. 5). Transduction facilitated by SARS-S was profoundly augmented upon expression of the ACE2 wt, and a further increase was observed upon coexpression of TMPRSS2, in accord with our previous observations (Fig. 4C). Transduction of cells expressing ACE2 alone was markedly reduced upon pretreatment with MDL28170 and transduction efficiency was rescued upon coexpression of TMPRSS2, in agreement with published data (26–28). Notably, the same effects were observed for cells expressing ACE2 mutant C4 in conjunction with TMPRSS2 (Fig. 5), demonstrating that ACE2 cleavage is not required for TMPRSS2-mediated, cathepsin L-independent entry into target cells.

TMPRSS2 increases cellular uptake of SARS-S. The results obtained so far demonstrated that ACE2 cleavage is essential for augmentation of SARS-S-dependent entry by TMPRSS2 and HAT expression. In order to obtain insights into the underlying mechanism, we first investigated whether expression of TMPRSS2 facilitates SARS-S binding to cells. The soluble S1 subunit of SARS-S fused to human Fc (SARS-S1-Fc) bound efficiently to ACE2-expressing cells, while binding to TMPRSS2-positive cells was within the background range (Fig. 6A), and comparable results were obtained upon analysis of SARS-S1-Fc binding by immunofluorescence (data not shown). These results suggest that increased SARS-S binding does not account for the ability of TMPRSS2 to augment SARS-S-driven entry. Next, we assessed whether protease expression facilitates SARS-S uptake into target cells. For this, we incubated cells expressing ACE2 or coexpressing ACE2 and TMPRSS2 with SARS-S1-Fc and examined the cellular localization of ACE2 and SARS-S1-Fc by confocal microscopy. When 293T cells were incubated with SARS-S1-Fc at 4°C, bound SARS-S1-Fc and ACE2 colocalized at or close to the cell surface, and no difference was observed upon coexpression of TMPRSS2 (Fig. 6B). Similarly, both SARS-S1-Fc and ACE2 colocalized at the cell surface when cells were incubated at 37°C (Fig. 6B). Interestingly,

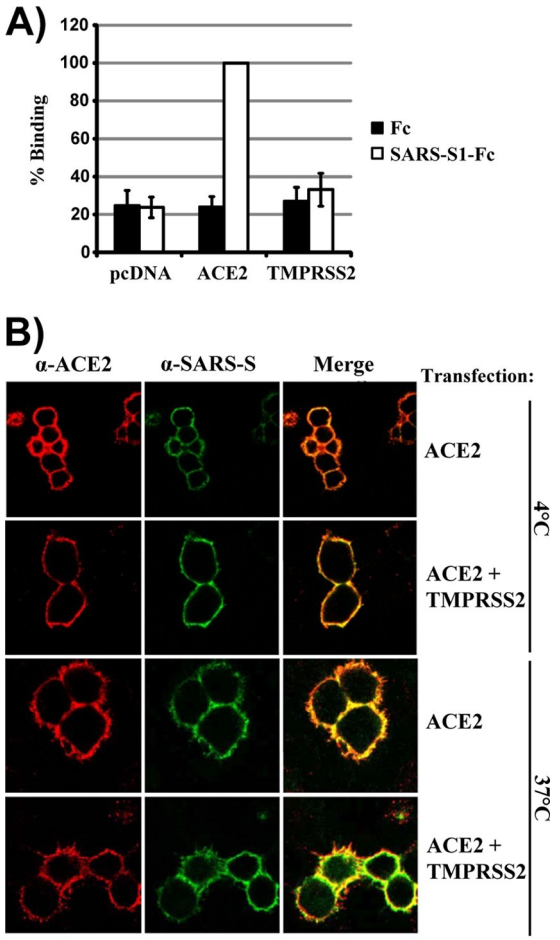

FIG 6 TMPRSS2 increases uptake of SARS-S into ACE2-expressing cells. (A) Plasmids encoding the ACE2 wt or TMPRSS2 or empty plasmid (pcDNA) were transiently transfected into 293T cells and the cells incubated with the Fc-tagged S1 subunit of SARS-S (SARS-S1-Fc) for 1 h at 4°C. As a negative control, cells were incubated with the Fc portion alone (Fc). Subsequently, the cells were washed, and the amount of bound proteins was detected by FACS analysis. The geometric mean channel fluorescence was measured, and the signal obtained for SARS-S1-Fc binding to ACE2-transfected cells was set as 100%. The averages from three independent experiments are shown, and error bars indicate SEM. (B) Plasmids encoding ACE2 and either TMPRSS2 or no protease were transiently cotransfected into 293T cells. The transfected cells were incubated with SARS-S1-Fc for 1 h at 4°C or 37°C. Subsequently, the cells were washed, and ACE2 and SARS-S1-Fc were detected by immunofluorescence staining and confocal microscopy. The results are representative of those of three separate experiments. u, anti.

a slightly different result was obtained with cells coexpressing ACE2 and TMPRSS2. Again, colocalization of SARS-S1-Fc and ACE2 at the cell surface was detectable, but a substantial part of the SARS-S1 signal was now localized inside the cell, just beneath the plasma membrane, consistent with more efficient cellular uptake of SARS-S under these conditions. In contrast, an increase in SARS-S1-Fc uptake was not observed upon coexpression of TMPRSS2 and the cleavage-resistant ACE2 mutant C4 (data not shown). Thus, TMPRSS2 expression increases uptake of SARS-S1-Fc and potentially authentic SARS-CoV, which might account for increased SARS-S-driven entry into TMPRSS2-positive cells.

TMPRSS2 expression inhibits SARS-S shedding by ADAM17. It has been proposed that SARS-S binding to ACE2 induces ACE2 shedding by ADAM17, which in turn increases cellular uptake of SARS-CoV (34, 35). In the light of these findings, we thought to clarify whether TMPRSS2 and ADAM17 modulate SARS-CoV entry via similar mechanisms. For this, we first asked whether TMPRSS2, like ADAM17, facilitates ACE2 shedding. PMA is known to induce ACE2 shedding in an ADAM17-dependent fashion (49) and was used as positive control. Indeed, PMA treatment of ACE2-expressing cells induced ACE2 release into the supernatant in a concentration-dependent fashion (Fig. 7A). In contrast, efficient ACE2 release from cells coexpressing ACE2 and TMPRSS2 was only observed upon treatment with the highest concentration of PMA examined (Fig. 7A). Similarly, constitutive shedding of ACE2, which is known to be partially ADAM17 dependent (49, 50), was suppressed by TMPRSS2 expression (Fig. 7A, lanes without PMA). Finally, the failure of TMPRSS2-expressing cells to release ACE2 into the supernatant was not due to retention of cleaved ACE2 at the cell surface, as determined by high-salt washes, and suppression of ACE2 shedding was dependent on the enzymatic activity of TMPRSS2 (Fig. 7B). These observations indicate that TMPRSS2 does not facilitate ACE2 shedding and even interferes with ACE2 shedding by ADAM17.

Arginine and lysine residues within amino acids 652 to 659 are essential for ACE2 shedding by ADAM17. The failure of TMPRSS2 to shed ACE2 suggests that TMPRSS2 and ADAM17 cleave ACE2 at different sites. To explore this possibility, we investigated the ADAM17 cleavage site employing our set of ACE2 mutants. The analysis of cell lysates for expression (Fig. 8, top panel) and TMPRSS2-mediated cleavage (Fig. 8, middle panel) of the ACE2 wt and mutants yielded results comparable to the ones we had obtained previously (Fig. 3), with a short C-terminal cleavage product being readily detectable for all ACE2 variants tested except mutant C4. In contrast, no C-terminal cleavage product was observed for cellular lysates upon PMA treatment of cells expressing the ACE2 wt and C0 to C3 (Fig. 8, middle panel), in keeping with the published observation that the cleavage product is instable and not readily detectable by immunoblotting (34, 51). A weak signal was consistently observed for mutant C4, suggesting that the amino acid changes introduced into this mutant stabilized the cleavage product. Finally, a prominent ACE2 signal was detected for supernatants of PMA-treated cells expressing the ACE2 wt and all mutants except C2 (Fig. 8, bottom panel), indicating that PMA treatment had induced ACE2 shedding and that shedding was inhibited by the changes introduced into mutant C2. Thus, arginine and lysine residues within amino acids 652 to 659 are critical for ADAM17-dependent ACE2 shedding.

Modulation of ADAM17 activity does not impact SARS-S-driven entry. Having demonstrated that different determinants in ACE2 control cleavage by TMPRSS2/HAT and ADAM17, we sought to further investigate the role of ACE2 processing by ADAM17 in SARS-S-driven entry. Our previous work indicated that ADAM17 activity is not required for SARS-S-mediated transduction or for spread of authentic SARS-CoV (36), findings that contrast with those of previous studies (34, 35). However, the activation of ADAM17 by PMA and potentially also by SARS-S is a fast, transient process (52), which might have been missed in our previous study, since cells were incubated for prolonged times with an excess of free virus in the presence and absence of ADAM17 inhibitor (36). To address this possibility, we investigated the role of ADAM17 in SARS-S-driven entry under conditions of synchronized infection in the absence of cell-free virus.

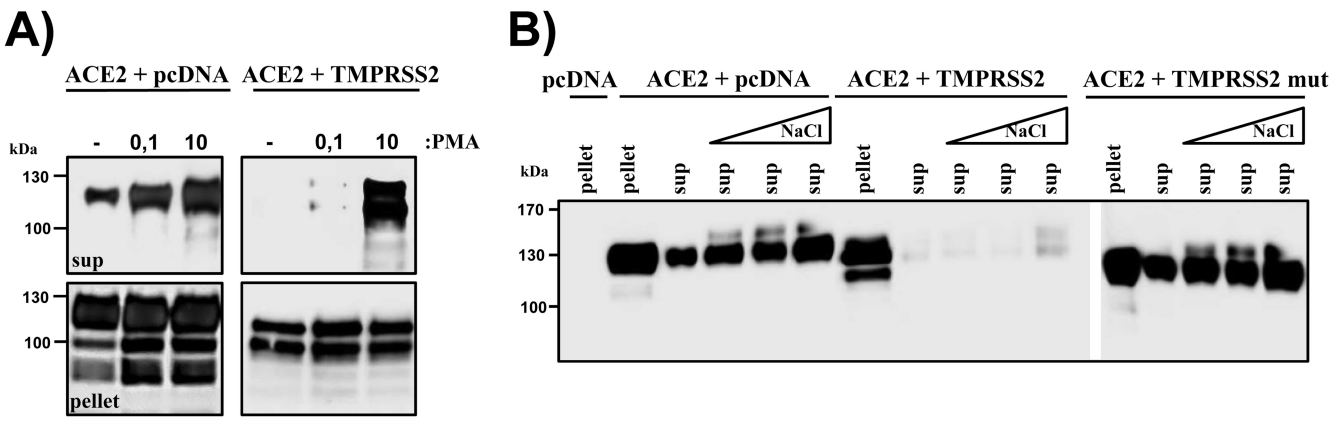

FIG 7 TMPRSS2 suppresses ACE2 shedding by ADAM17. (A) 293T cells were transiently cotransfected with plasmid encoding ACE2 and either empty plasmid (left panels) or plasmid encoding TMPRSS2 (right panels). Subsequently, cells were incubated with medium or medium supplemented with the indicated amounts of PMA, and the presence of ACE2 in culture supernatants (sup; top panel) or cell pellets (pellet; bottom panel) was examined by Western blotting employing an antibody directed against the ACE2 ectodomain. (B) 293T cells were transiently cotransfected with plasmids encoding ACE2 and either the TMPRSS2 wt or catalytically inactive TMPRSS2 (mut) or empty plasmid (pcDNA). Subsequently, supernatants were harvested and proteins precipitated (sup). The cells were pelleted, washed with buffer containing different concentrations of NaCl, and pelleted again, and proteins present in supernatants were precipitated (sup + NaCl). The presence of ACE2 in cell lysates or supernatants was analyzed by Western blotting as described for panel A.

For this, we incubated ACE2 wt-expressing 293T cells with SARS-S-bearing pseudotypes at 4°C, conditions which allow particle binding but prevent uptake. Subsequently, unbound particles were removed by washing and the cells were incubated with PMA or TAPI-1, which activates and represses ADAM17 activity, respectively. Thereafter, the compounds were removed and the transduction efficiency was quantified. Neither PMA nor TAPI-1 treatment appreciably modulated SARS-S-driven transduction under these conditions (Fig. 8B), indicating that ADAM17 activity is not required for S-protein-mediated transduction, at least in the experimental setup chosen here.

DISCUSSION

TMPRSS2 and HAT, members of the TTSP family, cleave and activate SARS-S for host cell entry (26–28, 43, 53). A recent study indicated that TMPRSS2 and HAT also process the SARS-CoV receptor ACE2 and that expression of these proteases increases viral entry into host cells (28). However, the molecular mechanisms underlying protease-augmented cellular entry and the potential contribution of ACE2 cleavage to SARS-S activation were unknown. Here, we show that arginine and lysine residues within ACE2 amino acids 697 to 716 are essential for ACE2 cleavage by TMPRSS2 and HAT and that ACE2 processing is required for augmentation of SARS-S-driven entry but not for SARS-S activation. Moreover, we demonstrate that ADAM17, an ACE2 sheddase, requires arginine and lysine residues within ACE2 amino acids 652 to 659 for receptor cleavage and competes with TMPRSS2 for ACE2 processing. However, ADAM17 activity did not modulate SARS-S-driven entry. In sum, these results and previously published work (26–28) indicate that TMPRSS2 facilitates SARS-CoV infection via two independent mechanisms, cleavage of ACE2, which might promote viral uptake, and cleavage of SARS-S, which activates the S protein for membrane fusion.

Several coronaviruses use peptidases as receptors for host cell entry: the novel coronavirus MERS binds to CD26 (54), most alphacoronaviruses use CD13, and SARS-CoV and the human coronavirus NL63 engage the carboxypeptidase ACE2 (17, 41). ACE2 is an integral component of the renin-angiotensin system (RAS), which controls blood pressure as well as fluid and salt balance (55). In addition, ACE2 expression protects against acute respiratory distress syndrome (37, 38). ACE2 exerts its regulatory activities by facilitating the generation of the heptapeptide Ang 1-7 (55), which modulates RAS activity by signaling via the G-protein-coupled receptor MAS (56). Thus, ACE2 is intimately involved in several physiological and pathophysiological processes, and cellular factors modulating ACE2 expression, receptor function, and enzymatic activity might afford novel strategies for therapeutic intervention.

TMPRSS2 and HAT processed ACE2 in an identical fashion, with a short C-terminal ACE2 fragment of approximately 13 kDa being consistently detectable in lysates of cells coexpressing ACE2 and protease but not ACE2 alone. Additional fragments of slightly higher molecular weights were observed upon expression of small amounts of protease and likely constitute cleavage intermediates. Shulla and colleagues previously reported identical processing of ACE2 by TMPRSS2 and HAT but noted the production of a 20-kDa fragment (28). The reasons for this discrepancy are at present unclear but might relate to batch-specific differences in the 293T cells employed, the use of antigenically tagged ACE2 in the published but not the present study, and most importantly, the amount of ACE2 and protease expressed. The production of the 13-kDa ACE2 cleavage fragment was also observed upon expression of animal orthologs of TMPRSS2 and HAT, indicating that ACE2 cleavage might be conserved between humans and animals. Finally, human hepsin, a TTSP expressed in kidney, pancreas, lung, and other tissues (57, 58), was found to process ACE2 and SARS-S (not shown). These observations demonstrate that all SARS-S-processing TTSPs identified so far also cleave ACE2 and raise the question how ACE2 cleavage impacts SARS-S-driven entry.

Shulla and colleagues suggested that ACE2 cleavage is required for TMPRSS2 and HAT-mediated augmentation of SARS-S- driven entry (28), but formal proof was lacking. In addition, it was not investigated whether ACE2 cleavage is required for

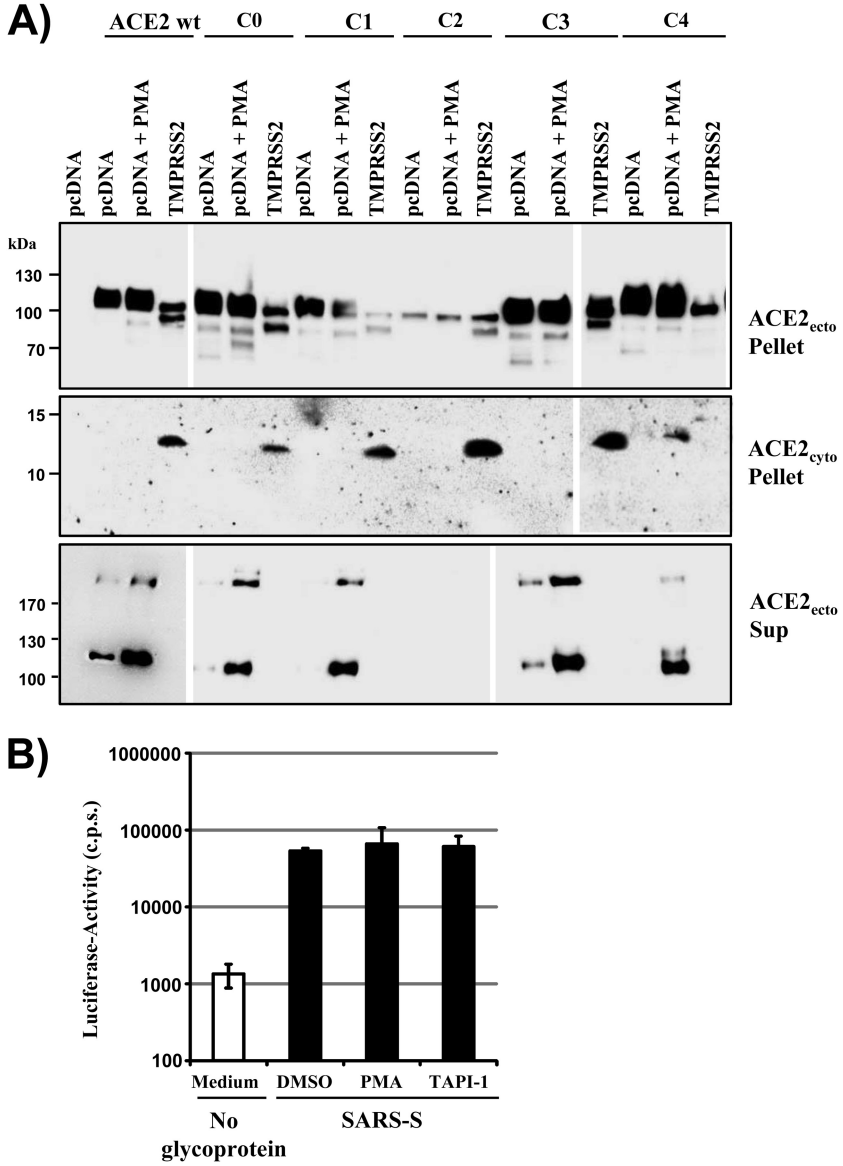

FIG 8 Arginine and lysine residues within ACE2 amino acids 652 to 659 are essential for ACE2 shedding by ADAM17. (A) 293T cells were transiently cotransfected with plasmids encoding the ACE2 wt or the indicated ACE2 mutants jointly with plasmid encoding TMPRSS2 or empty plasmid (pcDNA). The cells cotransfected with empty plasmid were cultivated in either regular medium or medium supplemented with PMA. Subsequently, ACE2 levels in cell lysates were detected by Western blotting using an ACE2 monoclonal antibody directed against the ACE2 ectodomain (top panel) or a polyclonal antibody directed against the C terminus of ACE2 (middle panel). In parallel, the presence of ACE2 in culture supernatants (Sup) was determined using an antibody against the ACE2 ectodomain (bottom panel). (B) A plasmid encoding the ACE2 wt was transiently transfected into 293T cells, and the cells were incubated with a lentiviral vector pseudotyped with SARS-S for 1 h at 4°C. A lentiviral vector bearing no glycoprotein (pcDNA) was included as a negative control. Thereafter, the cells were washed and incubated with medium containing 10 ЈLM PMA or 50 ЈLM TAPI-1 or an equal volume of DMSO at 37°C for 8 h. Cells exposed to bald vector (pcDNA) were incubated in medium alone. Subsequently, the media were replaced and the cells maintained in culture medium without inhibitor. Luciferase activities in cell lysates were determined at 72 h postransduction. The results of a representative experiment carried out with triplicate samples are shown; error bars indicate SD. Comparable results were obtained in two separate experiments.

TMPRSS2-dependent activation of SARS-S for cathepsin L-independent entry. Answering these questions requires the identification of the protease cleavage site in ACE2. ACE2 cleavage by HAT was readily detectable upon analysis of recombinant proteins and mass spectrometric analysis identified R621 as the cleavage site. However, mutation of this residue in the context of cellular ACE2 did not interfere with processing by TMPRSS2 and HAT. Differences in the folding and/or accessibility to cleavage between the recombinant and cellular proteins might account for these differential results. Instead, arginine and lysine residues within ACE2 amino acids 697 to 716 were critical for ACE2 cleavage by TMPRSS2 and HAT, as demonstrated by mutagenic analysis of potential cleavage sites in the membrane-proximal region of ACE2. Residual ACE2 cleavage, occurring after mutation of arginine and lysine residues within 697 to 716 (ACE2 mutant C4), was largely abrogated when R621 was also mutated, suggesting a mi-

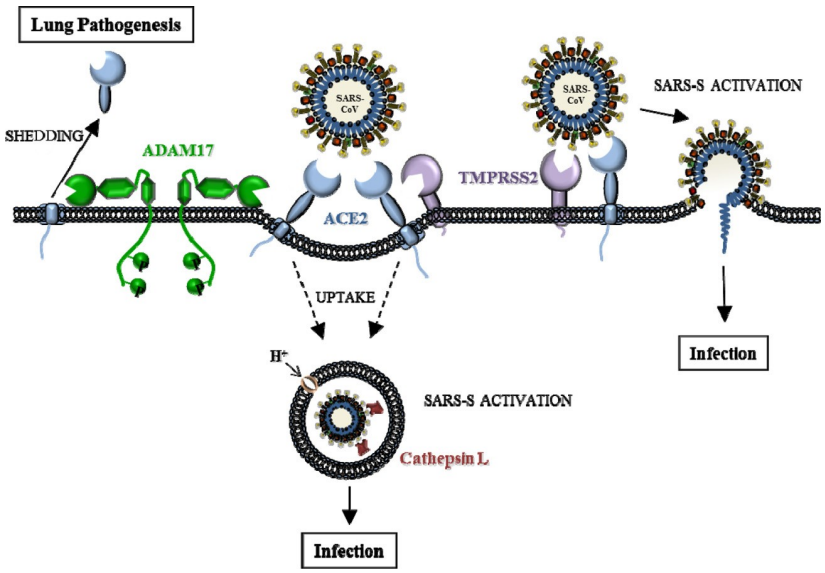

FIG 9 Role of host cell proteases in the cellular entry of SARS-CoV. Host cell entry of SARS-CoV can proceed via two distinct routes, dependent on the availability of cellular proteases required for activation of SARS-S. The first route is taken if no SARS-S-activating proteases are expressed at the cell surface. Upon binding of virion-associated SARS-S to ACE2, virions are taken up into endosomes, where SARS-S is cleaved and activated by the pH-dependent cysteine protease cathepsin L. The second route of activation can be pursued if the SARS-S-activating protease TMPRSS2 is coexpressed with ACE2 on the surface of target cells. Binding to ACE2 and processing by TMPRSS2 are believed to allow fusion at the cell surface or upon uptake into cellular vesicles but before transport of virions into host cell endosomes. Uptake can be enhanced upon SARS-S activation of ADAM17, which cleaves ACE2, resulting in shedding of ACE2 in culture supernatants. Since normal expression of ACE2 protects from lung injury and ACE2 levels are known to be reduced in SARS-CoV infection, the ADAM17-dependent ACE2 shedding is believed to promote lung pathogenesis. The present study suggests that the cellular uptake of SARS-CoV can also be augmented upon ACE2 cleavage by TMPRSS2. Since the entry-promoting function of ADAM17 is not undisputed and increased uptake upon ACE2 cleavage by TMPRSS2 has only been demonstrated with soluble SARS-S1, the respective arrows are shown in dashed lines.

nor role of this residue in ACE2 processing. Collectively, these results demonstrate that arginine and lysine residues within ACE2 amino acids 697 to 716 are essential for ACE2 cleavage and, based on the molecular weight of the C-terminal cleavage product, might represent the actual cleavage site.

The cleavage-resistant ACE2 mutant C4 was robustly ex- pressed and facilitated SARS-S-driven entry into target cells with efficiency similar to that of the ACE2 wt. Therefore, mutant C4 was used as a tool to investigate the role of ACE2 cleavage in SARS-S-driven entry. The analysis of TMPRSS2/HAT-dependent augmentation of SARS-S-mediated entry clearly revealed that ACE2 processing is a prerequisite to this process. Thus, expression of these proteases augmented entry into target cells expressing the ACE2 wt but not ACE2 mutant C4. In contrast, TMPRSS2 facilitated efficient cathepsin L-independent entry into cells expressing the ACE2 wt and mutant C4, demonstrating that ACE2 cleavage is dispensable for SARS-S activation. This observation is noteworthy, since ACE2 binding is believed to trigger conformational changes in SARS-S which alter the susceptibility of SARS-S to proteolytic activation by trypsin (25, 59). Thus, one could have assumed that cleavage by TMPRSS2 slightly alters ACE2 conformation and that only SARS-S bound to cleaved ACE2 is appropriately presented for processing by TMPRSS2, a possibility disproved by the present study. In sum, our observations indicate that TMPRSS2 and HAT impact SARS-S-driven entry via two independent mechanisms: ACE2 cleavage by these proteases increases entry efficiency, while SARS-S cleavage by TMPRSS2 activates the S protein for cathepsin L-independent host cell entry (Fig. 9).

How does ACE2 cleavage by TMPRSS2 and HAT augment SARS-S-driven entry? We did not observe substantial differences in SARS-S binding to cells expressing ACE2 or coexpressing ACE2 and TMPRSS2, demonstrating that augmentation of entry is not due to increased capture of SARS-S. Instead, we found that cells coexpressing TMPRSS2 and ACE2 internalize SARS-S more efficiently than cells expressing ACE2 alone, indicating that TMPRSS2 might promote particle uptake into receptor-positive cells. Particles taken up via cleaved ACE2 might then enter the cathepsin L-dependent pathway and fuse with the endosomal membrane (Fig. 9). Alternatively, they might be activated by TMPRSS2 and fuse with a vesicle membrane immediately after particle uptake. The molecular mechanism responsible for increased uptake is unclear at present, but we speculate that the ACE2 cleavage fragment harbors signals for internalization which might be parasitized by SARS-CoV to promote particle uptake into target cells.

The involvement of proteases in SARS-S entry is not limited to TTSPs and cathepsin L. ACE2 is cleaved by the metalloprotease ADAM17, which results in shedding of the ACE2 ectodomain (49–51). Haga and colleagues provided evidence that SARS-S also stimulates ADAM17-dependent ACE2 cleavage and that cleavage promotes uptake of authentic, infectious SARS-CoV into target cells (34, 35) (Fig. 9). In contrast, our previous analysis failed to detect evidence for an important contribution of ADAM17 to SARS-S-mediated cellular entry (36). In the present study, we revisited the role of ADAM17 in SARS-S-mediated entry.

We first asked whether TMPRSS2/HAT and ADAM17 cleave ACE2 at overlapping sites, which would suggest that the two proteases might regulate SARS-S-driven entry in a similar fashion. However, the comparison of ACE2 cleavage products revealed

striking differences. The ACE2 ectodomain was shed into culture supernatants upon cleavage by ADAM17 but not TMPRSS2, although we cannot formally exclude the possibility that TMPRSS2-expressing cells released into the culture supernatants an ACE2 cleavage product which was unstable and/or not detectable with the particular antibody used. In contrast, a C-terminal, intracellular cleavage fragment was observed only upon ACE2 processing by TMPRSS2, not ADAM17. The latter finding is in agreement with previous reports suggesting that the intracellular portion released from ACE2 upon cleavage by ADAM17 is unstable (34, 51, 58). The reason for the differential shedding of the ACE2 ectodomain by ADAM17 and TMPRSS2 is at present unknown. However, the differential fate of the ACE2 cleavage products clearly indicated that these proteases cleave ACE2 at different sites. Indeed, mutagenic analysis revealed that arginine and lysine residues within ACE2 amino acids 652 to 659 are essential for ACE2 shedding by ADAM17 but do not impact ACE2 processing by TMPRSS2/HAT. Previous studies suggested that ADAM17 cleaves ACE2 at residues 708 to 709 (60) or 716 to 741 (51), respectively. Therefore, residues 652 to 659 identified in our study might not constitute the cleavage site itself but might determine whether a downstream cleavage site is recognized by ADAM17. Such a scenario is supported by previous studies suggesting that the structure of the juxtamembrane region might be more important for shedding than the sequence of the actual cleavage site (49, 61, 62).

The ACE2 mutant resistant to ADAM17 cleavage might be useful to further dissect the role of ADAM17 in SARS-S entry, although the reduced expression or stability and receptor function of this mutant will likely complicate such endeavors. In order to commence such analyses, we first addressed if a role of ADAM17 in SARS-S-driven entry can be detected under optimized conditions, in which particles are bound to cells, unbound particles are removed, and ADAM17 is immediately activated or inhibited. However, modulation of ADAM17 activity did not impact SARS-S-driven transduction, arguing against a contribution of this protease to SARS-S-mediated entry, at least under the conditions examined. On the other hand, the ACE2 mutant resistant to processing by ADAM17 failed to robustly facilitate SARS-S-driven entry even when similar amounts of ACE2 mutant and wt protein were expressed on the cell surface (due to titration of the plasmids used for transfection [data not shown]), and the nature of this defect requires further investigation.

Our study provides evidence that cleavage of ACE2 by TMPRSS2, HAT, and potentially other TTSPs could increase up- take of viral particles and is essential for protease-mediated aug- mentation of SARS-S-driven entry. In contrast, ACE2 processing by TMPRSS2 is dispensable for activation of SARS-S for cathepsin L-independent entry. Although some of these results await con- firmation with authentic SARS-CoV, the identification of the ACE2 site(s) controlling cleavage by TTSPs and ADAM17 reveals important insights into ACE2 biology and might afford novel therapeutic strategies for treatment of lung disease. Finally, our results should stimulate efforts to determine whether receptor proteolysis impacts infection by hCoV-229E and MERS-CoV, which, like SARS-CoV, use peptidases as receptors (54, 63) and are activated by TTSPs (64, 65).

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REFERENCES

1. Holmes KV. 2001. Coronaviruses, p 1187–1203. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed). 2001. Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
2. Bradburne AF, Bynoe ML, Tyrrell DA. 1967. Effects of a "new" human respiratory virus in volunteers. Br. Med. J. 3:767–769. http://dx.doi.org/10.1136/bmj.3.5568.767.
3. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B. 2004. Identification of a new human coronavirus. Nat. Med. 10:368–373. http://dx.doi.org/10.1038/nm1024.
4. Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, Wong BH, Poon RW, Cai JJ, Luk WK, Poon LL, Wong SS, Guan Y, Peiris JS, Yuen KY. 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J. Virol. 79:884–895. http://dx.doi.org/10.1128/JVI.79.2.884-895.2005.
5. Chiu SS, Chan KH, Chu KW, Kwan SW, Guan Y, Poon LL, Peiris JS. 2005. Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China. Clin. Infect. Dis. 40:1721–1729. http://dx.doi.org/10.1086/430301.
6. Gorse GJ, O'Connor TZ, Hall SL, Vitale JN, Nichol KL. 2009. Human coronavirus and acute respiratory illness in older adults with chronic obstructive pulmonary disease. J. Infect. Dis. 199:847–857. http://dx.doi.org/10.1086/597122.
7. Jean A, Quach C, Yung A, Semret M. 2013. Severity and outcome associated with human coronavirus OC43 infections among children. Pediatr. Infect. Dis. J. 32:325–329. http://dx.doi.org/10.1097/INF.0b013e3182812787.
8. Jevšnik M, Uršič T, Zigon N, Lusa L, Krivec U, Petrovec M. 2012. Coronavirus infections in hospitalized pediatric patients with acute respiratory tract disease. BMC Infect. Dis. 12:365. http://dx.doi.org/10.1186/1471-2334-12-365.
9. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo SW, Li PH, Zhang LJ, Guan YJ, Butt KM, Wong KL, Chan KW, Lim W, Shortridge KF, Yuen KY, Peiris JS, Poon LL. 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science 302:276–278. http://dx.doi.org/10.1126/science.1087139.
10. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan KH, Yuen KY. 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc. Natl. Acad. Sci. U. S. A. 102:14040–14045. http://dx.doi.org/10.1073/pnas.0506735102.
11. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, Daszak P, Eaton BT, Zhang S, Wang LF. 2005. Bats are natural reservoirs of SARS-like coronaviruses. Science 310:676–679. http://dx.doi.org/10.1126/science.1118391.
12. Peiris JS, Guan Y, Yuen KY. 2004. Severe acute respiratory syndrome. Nat. Med. 10:S88–S97. http://dx.doi.org/10.1038/nm1143.
13. Zaki AM, van BS, Bestebroer TM, Osterhaus AD, Fouchier RA. 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. 367:1814–1820. http://dx.doi.org/10.1056/NEJMoa1211721.
14. World Health Organization. 4 November 2013. Middle East respiratory syndrome coronavirus (MERS-CoV)— update. http://www.who.int/csr/don/2013_11_04/en/index.html.
15. Gallagher TM, Buchmeier MJ. 2001. Coronavirus spike proteins in viral entry and pathogenesis. Virology 279:371–374. http://dx.doi.org/10.1006/viro.2000.0757.
16. Hofmann H, Pöhlmann S. 2004. Cellular entry of the SARS coronavirus. Trends Microbiol. 12:466–472. http://dx.doi.org/10.1016/j.tim.2004.08.008.
17. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426:450–454. http://dx.doi.org/10.1038/nature02145.
18. Wang P, Chen J, Zheng A, Nie Y, Shi X, Wang W, Wang G, Luo M, Liu

H, Tan L, Song X, Wang Z, Yin X, Qu X, Wang X, Qing T, Ding M, Deng H. 2004. Expression cloning of functional receptor used by SARS coronavirus. Biochem. Biophys. Res. Commun. 315:439 – 444. http://dx.doi.org/10.1016/j.bbrc.2004.01.076.

19. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis GJ, van Goor H. 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus: a first step in understanding SARS pathogenesis. J. Pathol. 203:631– 637. http://dx.doi.org/10.1002/path.1570.

20. Mossel EC, Wang J, Jeffers S, Edeen KE, Wang S, Cosgrove GP, Funk CJ, Manzer R, Miura TA, Pearson LD, Holmes KV, Mason RJ. 2008. SARS-CoV replicates in primary human alveolar type II cell cultures but not in type I-like cells. Virology 372:127–135. http://dx.doi.org/10.1016/j.virol.2007.09.045.

21. To KF, Lo AW. 2004. Exploring the pathogenesis of severe acute respiratory syndrome (SARS): the tissue distribution of the coronavirus (SARS-CoV) and its putative receptor, angiotensin-converting enzyme 2 (ACE2). J. Pathol. 203:740 –743. http://dx.doi.org/10.1002/path.1597.

22. To KF, Tong JH, Chan PK, Au FW, Chim SS, Chan KC, Cheung JL, Liu EY, Tse GM, Lo AW, Lo YM, Ng HK. 2004. Tissue and cellular tropism of the coronavirus associated with severe acute respiratory syndrome: an in-situ hybridization study of fatal cases. J. Pathol. 202:157–163. http://dx.doi.org/10.1002/path.1510.

23. Wong SK, Li W, Moore MJ, Choe H, Farzan M. 2004. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. J. Biol. Chem. 279:3197–3201. http://dx.doi.org/10.1074/jbc.C300520200.

24. Li F, Berardi M, Li W, Farzan M, Dormitzer PR, Harrison SC. 2006. Conformational states of the severe acute respiratory syndrome coronavirus spike protein ectodomain. J. Virol. 80:6794 – 6800. http://dx.doi.org/10.1128/JVI.02744-05.

25. Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, Bates P. 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc. Natl. Acad. Sci. U. S. A. 102:11876 – 11881. http://dx.doi.org/10.1073/pnas.0505577102.

26. Glowacka I, Bertram S, Muller MA, Allen P, Soilleux E, Pfefferle S, Steffen I, Tsegaye TS, He Y, Gnirss K, Niemeyer D, Schneider H, Drosten C, Pöhlmann S. 2011. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. J. Virol. 85:4122– 4134. http://dx.doi.org/10.1128/JVI.02232-10.

27. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, Taguchi F. 2010. Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. J. Virol. 84:12658 –12664. http://dx.doi.org/10.1128/JVI.01542-10.

28. Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T. 2011. A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. J. Virol. 85:873– 882. http://dx.doi.org/10.1128/JVI.02062-10.

29. Bertram S, Heurich A, Lavender H, Gierer S, Danisch S, Perin P, Lucas JM, Nelson PS, Pöhlmann S, Soilleux EJ. 2012. Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. PLoS One 7:e35876. http://dx.doi.org/10.1371/journal.pone.0035876.

30. Kawase M, Shirato K, van der Hoek L, Taguchi F, Matsuyama S. 2012. Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. J. Virol. 86:6537– 6545. http://dx.doi.org/10.1128/JVI.00094-12.

31. Böttcher E, Matrosovich T, Beyerle M, Klenk HD, Garten W, Matrosovich M. 2006. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. J. Virol. 80: 9896 –9898. http://dx.doi.org/10.1128/JVI.01118-06.

32. Chaipan C, Kobasa D, Bertram S, Glowacka I, Steffen I, Tsegaye TS, Takeda M, Bugge TH, Kim S, Park Y, Marzi A, Pöhlmann S. 2009. Proteolytic activation of the 1918 influenza virus hemagglutinin. J. Virol. 83:3200 –3211. http://dx.doi.org/10.1128/JVI.02205-08.

33. Shirogane Y, Takeda M, Iwasaki M, Ishiguro N, Takeuchi H, Nakatsu Y, Tahara M, Kikuta H, Yanagi Y. 2008. Efficient multiplication of human metapneumovirus in Vero cells expressing the transmembrane serine protease TMPRSS2. J. Virol. 82:8942– 8946. http://dx.doi.org/10.1128/JVI.00676-08.

34. Haga S, Yamamoto N, Nakai-Murakami C, Osawa Y, Tokunaga K, Sata T, Yamamoto N, Sasazuki T, Ishizaka Y. 2008. Modulation of TNF-alpha-converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF-alpha production and facilitates viral entry. Proc. Natl. Acad. Sci. U. S. A. 105:7809 –7814. http://dx.doi.org/10.1073/pnas.0711241105.

35. Haga S, Nagata N, Okamura T, Yamamoto N, Sata T, Yamamoto N, Sasazuki T, Ishizaka Y. 2010. TACE antagonists blocking ACE2 shedding caused by the spike protein of SARS-CoV are candidate antiviral compounds. Antiviral Res. 85:551–555. http://dx.doi.org/10.1016/j.antiviral.2009.12.001.

36. Glowacka I, Bertram S, Herzog P, Pfefferle S, Steffen I, Muench MO, Simmons G, Hofmann H, Kuri T, Weber F, Eichler J, Drosten C, Pöhlmann S. 2010. Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63. J. Virol. 84:1198 –1205. http://dx.doi.org/10.1128/JVI.01248-09.

37. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, Yang P, Sarao R, Wada T, Leong-Poi H, Crackower MA, Fukamizu A, Hui CC, Hein L, Uhlig S, Slutsky AS, Jiang C, Penninger JM. 2005. Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature 436:112–116. http://dx.doi.org/10.1038/nature03712.

38. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, Huan Y, Yang P, Zhang Y, Deng W, Bao L, Zhang B, Liu G, Wang Z, Chappell M, Liu Y, Zheng D, Leibbrandt A, Wada T, Slutsky AS, Liu D, Qin C, Jiang C, Penninger JM. 2005. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. Nat. Med. 11:875– 879. http://dx.doi.org/10.1038/nm1267.

39. Hofmann H, Hattermann K, Marzi A, Gramberg T, Geier M, Krumbiegel M, Kuate S, Uberla K, Niedrig M, Pöhlmann S. 2004. S protein of severe acute respiratory syndrome-associated coronavirus mediates entry into hepatoma cell lines and is targeted by neutralizing antibodies in infected patients. J. Virol. 78:6134 – 6142. http://dx.doi.org/10.1128/JVI.78.12.6134-6142.2004.

40. Simmons G, Reeves JD, Grogan CC, Vandenberghe LH, Baribaud F, Whitbeck JC, Burke E, Buchmeier MJ, Soilleux EJ, Riley JL, Doms RW, Bates P, Pöhlmann S. 2003. DC-SIGN and DC-SIGNR bind ebola glycoproteins and enhance infection of macrophages and endothelial cells. Virology 305:115–123. http://dx.doi.org/10.1006/viro.2002.1730.

41. Hofmann H, Pyrc K, van der Hoek L, Geier M, Berkhout B, Pöhlmann S. 2005. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proc. Natl. Acad. Sci. U. S. A. 102:7988 –7993. http://dx.doi.org/10.1073/pnas.0409465102.

42. Bertram S, Glowacka I, Blazejewska P, Soilleux E, Allen P, Danisch S, Steffen I, Choi SY, Park Y, Schneider H, Schughart K, Pöhlmann S. 2010. TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J. Virol. 84:10016 –10025. http://dx.doi.org/10.1128/JVI.00239-10.

43. Bertram S, Glowacka I, Muller MA, Lavender H, Gnirss K, Nehlmeier I, Niemeyer D, He Y, Simmons G, Drosten C, Soilleux EJ, Jahn O, Steffen I, Pöhlmann S. 2011. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. J. Virol. 85:13363–13372. http://dx.doi.org/10.1128/JVI.05300-11.

44. Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199. http://dx.doi.org/10.1016/0378-1119(91)90434-D.

45. Connor RI, Chen BK, Choe S, Landau NR. 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206:935–944. http://dx.doi.org/10.1006/viro.1995.1016.

46. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671– 675. http://dx.doi.org/10.1038/nmeth.2089.

47. Jahn O, Hesse D, Reinelt M, Kratzin HD. 2006. Technical innovations for the automated identification of gel-separated proteins by MALDI-TOF mass spectrometry. Anal. Bioanal. Chem. 386:92–103. http://dx.doi.org/10.1007/s00216-006-0592-1.

48. Yasuoka S, Ohnishi T, Kawano S, Tsuchihashi S, Ogawara M, Masuda K, Yamaoka K, Takahashi M, Sano T. 1997. Purification, characterization, and localization of a novel trypsin-like protease found in the human airway. Am. J. Respir. Cell Mol. Biol. 16:300 –308. http://dx.doi.org/10.1165/ajrcmb.16.3.9070615.

49. Lambert DW, Yarski M, Warner FJ, Thornhill P, Parkin ET, Smith AI, Hooper NM, Turner AJ. 2005. Tumor necrosis factor-alpha convertase

(ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2). J. Biol. Chem. 280:30113–30119. http://dx.doi.org/10.1074/jbc.M505111200.

50. Iwata M, Silva Enciso JE, Greenberg BH. 2009. Selective and specific regulation of ectodomain shedding of angiotensin-converting enzyme 2 by tumor necrosis factor alpha-converting enzyme. Am. J. Physiol. Cell Physiol. 297:C1318–C1329. http://dx.doi.org/10.1152/ajpcell.00036.2009.
51. Jia HP, Look DC, Tan P, Shi L, Hickey M, Gakhar L, Chappell MC, Wohlford-Lenane C, McCray PB, Jr. 2009. Ectodomain shedding of angiotensin converting enzyme 2 in human airway epithelia. Am. J. Physiol. Lung Cell. Mol. Physiol. 297:L84–L96. http://dx.doi.org/10.1152/ajplung.00071.2009.
52. Le Gall SM, Maretzky T, Issuree PD, Niu XD, Reiss K, Saftig P, Khokha R, Lundell D, Blobel CP. 2010. ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site. J. Cell Sci. 123:3913–3922. http://dx.doi.org/10.1242/jcs.069997.
53. Simmons G, Zmora P, Gierer S, Heurich A, Pöhlmann S. 2013. Proteolytic activation of the SARS-coronavirus spike protein: Cutting enzymes at the cutting edge of antiviral research. Antiviral Res. 100:605–614. http://dx.doi.org/10.1016/j.antiviral.2013.09.028.
54. Raj VS, Mou H, Smits SL, Dekkers DH, Muller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL. 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature 495:251–254. http://dx.doi.org/10.1038/nature12005.
55. Kuba K, Imai Y, Ohto-Nakanishi T, Penninger JM. 2010. Trilogy of ACE2: a peptidase in the renin-angiotensin system, a SARS receptor, and a partner for amino acid transporters. Pharmacol. Ther. 128:119–128. http://dx.doi.org/10.1016/j.pharmthera.2010.06.003.
56. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de B, Heringer-Walther IS, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T. 2003. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc. Natl. Acad. Sci. U. S. A. 100:8258–8263. http://dx.doi.org/10.1073/pnas.1432869100.
57. Leytus SP, Loeb KR, Hagen FS, Kurachi K, Davie EW. 1988. A novel trypsin-like serine protease (hepsin) with a putative transmembrane domain expressed by human liver and hepatoma cells. Biochemistry 27:1067–1074. http://dx.doi.org/10.1021/bi00403a032.
58. Tsuji A, Torres-Rosado A, Arai T, Le Beau MM, Lemons RS, Chou SH, Kurachi K. 1991. Hepsin, a cell membrane-associated protease: characterization, tissue distribution, and gene localization. J. Biol. Chem. 266:16948–16953.
59. Matsuyama S, Ujike M, Morikawa S, Tashiro M, Taguchi F. 2005. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. Proc. Natl. Acad. Sci. U. S. A. 102:12543–12547. http://dx.doi.org/10.1073/pnas.0503203102.
60. Lai ZW, Hanchapola I, Steer DL, Smith AI. 2011. Angiotensin-converting enzyme 2 ectodomain shedding cleavage-site identification: determinants and constraints. Biochemistry 50:5182–5194. http://dx.doi.org/10.1021/bi200525y.
61. Ehlers MR, Schwager SL, Scholle RR, Manji GA, Brandt WF, Riordan JF. 1996. Proteolytic release of membrane-bound angiotensin-converting enzyme: role of the juxtamembrane stalk sequence. Biochemistry 35:9549–9559. http://dx.doi.org/10.1021/bi9602425.
62. Sadhukhan R, Santhamma KR, Reddy P, Peschon JJ, Black RA, Sen I. 1999. Unaltered cleavage and secretion of angiotensin-converting enzyme in tumor necrosis factor-alpha-converting enzyme-deficient mice. J. Biol. Chem. 274:10511–10516. http://dx.doi.org/10.1074/jbc.274.15.10511.
63. Delmas B, Gelfi J, L'Haridon R, Vogel LK, Sjostrom H, Noren O, Laude H. 1992. Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. Nature 357:417–420. http://dx.doi.org/10.1038/357417a0.
64. Bertram S, Dijkman R, Habjan M, Heurich A, Gierer S, Glowacka I, Welsch K, Winkler M, Schneider H, Hofmann-Winkler H, Thiel V, Pöhlmann S. 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. 87:6150–6160. http://dx.doi.org/10.1128/JVI.03372-12.
65. Gierer S, Bertram S, Kaup F, Wrensch F, Heurich A, Kramer-Kuhl A, Welsch K, Winkler M, Meyer B, Drosten C, Dittmer U, von HT, Simmons G, Hofmann H, Pöhlmann S. 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. 87:5502–5511. http://dx.doi.org/10.1128/JVI.00128-13.

4 Diskussion

Das SARS-CoV rekrutiert Wirtszellproteasen, um seine Infektiosität sicherzustellen. TMPRSS2 und HAT, Mitglieder der TTSP-Familie, spalten und aktivieren das Glykoprotein Spike (S) des SARS-CoV und ermöglichen damit den infektiösen Eintritt des Virus in Wirtszellen [85, 87, 104, 123, 124]. Neben der aktivierenden Glykoproteinspaltung wurde auch die Spaltung des SARS-CoV Rezeptors ACE2 dokumentiert, und es wurde postuliert, dass dieser Prozess die Effizienz des Zelleintritts steigert [105, 111]. Allerdings war unklar, ob TMPRSS2 und HAT in ACE2-positiven Zielzellen in der menschlichen Lunge exprimiert werden und es war nicht bekannt, wie die Protease-Expression den S Protein-getriebenen Zelleintritt verstärkt. In der vorliegenden Arbeit konnte gezeigt werden, dass TMPRSS2 und HAT in ACE2-positiven Zellen des Respirationstrakts exprimiert werden, die Proteasen könnten daher die virale Ausbreitung in und zwischen Menschen fördern. Zusätzlich konnte demonstriert werden, dass die Spaltung des SARS-CoV-Spike-Proteins (SARS-S) durch TMPRSS2 zwischen den Spezies Maus, Schwein, Vogel und Mensch konserviert ist, was das große zoonotische Potential des Virus unterstreicht. Weiterhin konnte demonstriert werden, dass auch der SARS-CoV-Rezeptor ACE2 durch TMPRSS2 und HAT proteolytisch prozessiert wird und dass Arginin- und Lysin-Reste zwischen Aminosäuren 697 und 716 in ACE2 für die Spaltung durch diese Proteasen essentiell sind. Mit Hilfe von ACE2-Mutanten konnte gezeigt werden, dass die ACE2-Spaltung für die Protease-vermittelte Verstärkung des viralen Eintritts essentiell ist und Immunfluoreszenz-Analysen lieferten Hinweise darauf, dass die Verstärkung des Eintritts auf eine erhöhte Aufnahme von Virus-Partikeln in die Zelle zurückzuführen sein könnte. Zusammenfassend weisen die Ergebnisse darauf hin, dass TMPRSS2 und verwandte Enzyme in viralen Zielzellen exprimiert werden und die virale Ausbreitung im Wirt fördern, in dem sie das S Protein und seinen Rezeptor, ACE2, proteolytisch prozessieren.

SARS-Coronavirus Activating Proteases TMPRSS2 and HAT Are Expressed at Multiple Sites in Human Respiratory and Gastrointestinal Tracts

Das SARS-CoV repliziert tief im Respirationstrakt im Epithel der Lungenbläschen. Diese werden ausgekleidet durch Typ I und Typ II Pneumozyten, die den ACE2-Rezeptor tragen [80, 114, 125-129]. Die Expression des Rezeptors ist ebenso Voraussetzung für die erfolgreiche Infektion wie die Expression S Protein-aktivierender Wirtszellproteasen. Die erste Protease, die als Aktivator für SARS-S identifiziert wurde, ist die endosomale Cysteinprotease Cathepsin L [84]. Weitere Arbeiten

zeigten, dass auch die membranständige Serin-Proteasen TMPRSS2 und HAT in der Lage sind, das SARS-CoV S-Protein zu aktivieren [85, 87, 104, 105], und dass die Expression von TMPRSS2 den Cathepsin L-unabhängigen SARS-S-getriebenen Wirtszelleintritt erlaubt [87]. Diese Daten wurden jedoch ausschließlich in Zellkultur erhalten und die Bedeutung von TMPRSS2 und HAT für die virale Ausbreitung im Menschen war unklar. Immunohistochemische Studien zeigten, dass ACE2 gemeinsam mit TMPRSS2 und HAT in der Mund- und Nasenschleimhaut, im Epithel der Bronchien und des Kehlkopfes und in den Typ II Pneumozyten der Lungenbläschen exprimiert werden. TMPRSS2 und HAT könnten daher die virale Ausbreitung in der Lunge fördern. Neben dem Respirationstrakt sind auch der Gastrointestinal-Trakt sowie Teile der Niere, Neuronen im Gehirn und verschiedenen Gewebemakrophagen Ziel der SARS-CoV-Infektion [127, 130]. In der vorliegenden Studie konnten TMPRSS2 und HAT gemeinsam mit ACE2 in den Mandeln, der Speiseröhre, dem Magen, in Teilen des Dünndarms und im Dickdarm nachgewiesen werden. Zusätzlich werden ACE2 und TMPRSS2 im Herzmuskelgewebe koexprimiert. Diese Befunde weisen darauf hin, dass TTSPs die SARS-CoV-Verbreitung in diesen Organsystemen unterstützen könnten. Inhibitoren von Cathepsin L und TMPRSS2/HAT sind daher Kandidaten für die antivirale Therapie. Die Beobachtung, dass *tmprss2*-negative Mäuse im Gegensatz zu Cathepsin-negativen Mäusen im Vergleich zu ihrem Wildtyp-Mäusen keinen Phänotyp zeigen [109] und sich zusätzlich als resistent gegen die Infektionen mit H1N1-Influenzaviren erwiesen haben [131-133], ist im Hinblick auf die Entwicklung von TMPRSS2-blockierenden Therapeutika sehr interessant.

TMPRSS2 and ADAM17 Cleave ACE2 Differentially and Only Proteolysis by TMPRSS2 Augments Entry Driven by the Severe Acute Respiratory Syndrome Coronavirus Spike Protein

Zahlreiche Coronaviren nutzen Transmembran-Ektopeptidasen als Rezeptoren für den infektiösen Eintritt in die Wirtszelle. Das neue Coronavirus MERS-CoV verwendet DPP4/CD26 [134], die meisten Alphacoronaviren APN/CD13 und SARS-CoV sowie NL63 binden an ACE2 als Rezeptor [63, 135]. Alle diese Rezeptoren haben Gemeinsamkeiten, die sie zu attraktiven Zielen für Viren machen. Sie alle haben die Fähigkeit bioaktive Peptide zu generieren, die die Gefäßpermeabilität beeinflussen, sie alle haben Einfluss auf die Cytokin-Ausschüttung, auf die Chemokin-Prozessierung, auf die Degradation der extrazellulären Matrix und auf intrazelluläre Siganlübertragungsprozesse [136-138]. Inwieweit diese Eigenschaften den Coronaviren während des Infektionsprozesses zugute kommen, und ob sie dabei lediglich die abundante Expression auf endothelialen und epithelialen Geweben nutzen oder ob ihre biologischen Eigenschaften für die Infektion eine Rolle spielen, ist noch unklar.

Die Expressionsstärke und Funktion von Transmembranproteinen wird auf verschiedenen Wegen kontrolliert, durch Modulation der Genexpression, durch Abspaltung der Ektodomäne (*shedding*), durch Internalisierung und durch Clusterbildung in Lipid-Mikrodomänen [139]. In der vorliegenden Arbeit wurde untersucht, wie Wirtszellproteasen, die ACE2 spalten, den SARS-S-getriebenen Eintritt in Zellen beeinflussen. Es konnte zunächst gezeigt werden, dass sowohl TMPRSS2 als auch HAT ACE2 proteolytisch prozessieren, und dabei Spaltprodukte mit vergleichbarem Molekulargewicht generieren, die durch Einsatz spezifischer Antikörper dem extrazellulären Anteil bzw. dem mit der Zellmembran assoziierten C-terminalen Fragment zugeordnet werden konnten. Beide Proteasen scheinen ACE2 daher an einer ähnlichen oder identischen Stelle nahe der Transmembrandomäne zu spalten. Wird die Koexpression der jeweiligen Protease auf ein Minimum reduziert, können weitere Spaltintermediate nachgewiesen werden, die auf eine unvollständige Spaltung innerhalb einer Spaltkaskade hinweisen. Die Spaltung durch TMPRSS2 ist zwischen den Spezies Mensch, Maus, Schwein und Huhn und die Spaltung durch HAT zwischen Mensch und Maus konserviert.

Zusätzlich zu TMPRSS2 und HAT konnte die ACE2-Spaltung auch für Hepsin und Matriptase (nicht gezeigt) nachgewiesen werden, die auch SARS-S spalten [85]. Die daraus resultierende Frage ist: wie beeinflusst die ACE2-Spaltung den SARS-S getriebenen Zelleintritt? Shulla und Kollegen dokumentierten Hinweise darauf, dass die Prozessierung von ACE2 durch TMPRSS2 und HAT den SARS-S-getriebenen Zelleintritt verstärkt [105], allerdings lieferten sie keinen formalen Beweis für diese Hypothese. Außerdem blieb die Frage offen, ob die ACE2-Spaltung eine Voraussetzung für die TMPRSS2-abhängige Aktivierung von SARS-S ist. Zur Beantwortung dieser Fragen wurde zunächst mit Hilfe von rekombinant hergestellten Proteinen (HAT und Spike) und Massenspektrometrie die Spaltstelle untersucht. Die massenspektrometrische Analyse ergab eine Spaltung von ACE2 durch HAT an Aminosäureposition R621, allerdings hatte eine Aminosäure-Austauschmutation an dieser Stelle keinen Verlust der TMPRSS2/HAT-vermittelten Spaltung zur Folge. Daher wurde vermutet, dass nicht ein einzelnes Spaltmotiv als Erkennungssequenz durch die Proteasen abgelesen wird, sondern dass vielmehr die übergeordnete dreidimensionale Struktur für die Protease von Bedeutung ist, so das mehrere Spaltstellen möglich sind. Daraufhin wurden weitere Mutanten hergestellt um die Spaltstelle zu identifizieren. Dazu wurde alle Cluster an basischen Aminosäuren zwischen R621 und der ACE2-Transmembrandomäne in Alanine mutiert. Die Analyse der proteolytischen Prozessierung sämtlicher Mutanten durch TMPRSS2/HAT ergab, dass die Lysine und Arginine zwischen Aminosäuren 697 und 716 (Mutante C4) für die Prozessierung durch TMPRSS2 und HAT wichtig sind. Wurde in der Mutante C4 zusätzlich die zuvor in Massenspektronomie identifizierte Position R621 mutiert, war kaum Spaltprodukt

detektierbar. Diese Ergebnisse sprechen für eine Spaltkaskade in deren Rahmen ACE2 sowohl an R621 als auch zwischen Aminosäuren 697 und 716 gespalten wird.

Die Mutante C4 wurde robust exprimiert und vermittelte den SARS-S getriebenen Zelleintritt ähnlich effizient wie Wildtyp (wt) ACE2. Daher wurde die Mutante C4 genutzt, um die Rolle der ACE2-Spaltung im SARS-S-vermittelten Zelleintritt zu klären. Die Koexpression von TMPRSS2/HAT und wt ACE2 verstärkte, wie bereits erwähnt, den S Protein-getriebenen Eintritt [105]. Im Gegensatz dazu konnte keine Steigerung des SARS-S-vermittelten Zelleintritts nach Koexpression von Protease und Mutante C4 nachgewiesen werden. Der TMPRSS2-abhängige, Cathepsin L-unabhängige Zelleintritt wurde durch die Mutation von Cluster 4 nicht gestört. Das bedeutet, dass die aktivierende SARS-S-Spaltung durch TMPRSS2 auch dann möglich ist, wenn ACE2 nicht zuvor proteolytisch prozessiert wurde. Die beiden Prozesse sind also unabhängig voneinander. Auch die Affinität von SARS-S an ACE2 ist unabhängig von der ACE2-Spaltung (nicht gezeigt); so bindet lösliches SARS-S nahezu genauso gut an durch TMPRSS2 prozessiertes ACE2, wie an unprozessiertes. Es wurde postuliert, dass die Bindung von SARS-S an ACE2 eine Konformationsänderung im S Protein auslöst, die die Prozessierung der SARS-S Spaltstelle durch aktivierende Proteasen fördert [83, 84]. Da die ACE2-Spaltung durch TMPRSS2/HAT keinen Einfluss auf die Aktivierung und Affinität von SARS-S hatte, scheint diese Spaltung die Konformationsänderung in SARS-S, die die Empfänglichkeit gegenüber proteolytischer Prozessierung erhöht, nicht auszulösen.

Die vorliegende Arbeit lieferte den formalen Beweis dafür, dass Spaltung von ACE2 durch TMPRSS2/HAT für die infektionsverstärkende Wirkung dieser Proteasen verantwortlich ist. Diese Beobachtung warf die Frage auf, wie ACE2-Proteolyse den Eintritt von SARS-CoV in Zielzellen verstärkt. Analysen mit rekombinantem SARS-S zeigten, dass die Internalisierung in ACE2/TMPRSS2 doppelt-positive Zellen effizienter ist als die Aufnahme in Zellen, die nur den Rezeptor exprimieren. Diese Verstärkung der SARS-S-Aufnahme konnte in Anwesenheit der ACE2 Mutante C4 und TMPRSS2 nicht beobachtet werden (nicht gezeigt). Insgesamt weisen diese Ergebnisse darauf hin, dass die TMPRSS2-abhängige ACE2-Spaltung für die Partikelaufnahme in Rezeptor-positive Zellen verantwortlich ist. Der molekulare Mechanismus hinter der verstärkten Aufnahme ist zurzeit noch unklar, aber es ist denkbar, dass die Spaltung von ACE2 zu einer rapiden Internalisierung der ACE2-Ektodomäne führt, wie sie auch nach Prozessierung des ACE2-Homologs ACE beobachtet wurde [140]. Vielleicht nutzt das SARS-CoV diesen Prozess aus, um mittels Endozytose in die Zelle zu gelangen.

ACE2 wird ebenfalls von der Metalloprotease ADAM17/TACE gespalten, woraufhin die Ektodomäne in das Zytoplasma entlassen wird [113, 118, 141]. Es konnte gezeigt werden, dass das

ADAM17 abhängige Ektodomänen *shedding* auch von SARS-S stimuliert wird und dass diese Prozessierung die SARS-CoV-Aufnahme in Zellen fördert [111, 112]. Der Schluss liegt also nahe, dass auch die Prozessierung von ACE2 durch ADAM17, ähnlich wie die durch TMPRSS2, zur gesteigerten Aufnahme von Viruspartikeln in die Zelle führt. Dieser Hypothese wurde in der vorliegenden Arbeit untersucht. Zunächst wurde überprüft, ob TMPRSS2/HAT und ADAM17 ACE2 an der gleichen Stelle spalten, was für eine ähnlichen Mechanismus der Regulation des SARS-CoV Zelleintritts sprechen würde. Die Analyse der Spaltprodukte offenbarte allerdings einige Unterschiede. Die ACE2-Ektodomäne wurde nach ADAM17-Spaltung in den Zellkultur-Überstand abgegeben, wohingegen nach TMPRSS2-Spaltung kein ACE2 im Überstand nachgewiesen werden konnte; vielmehr inhibierte TMPRSS2 das ADAM17-vermittelte konstitutive *shedding* von ACE2. Diese Inhibierung war abhängig von der proteolytischen Aktivität von TMPRSS2, denn eine katalytisch inaktive TMPRSS2-Mutante verhinderte das *shedding* nicht. Die Inhibition des ACE2-*sheddings* durch TMPRSS2 könnte auf die Bindung von TMPRSS2 an ACE2 und damit das Besetzen der Spaltstelle zurückzuführen sein. Alternativ könnte die Expression von TMPRSS2 mit der ADAM17-Biogenese interferieren.

Die Analyse der durch TMPRSS2 und ADAM17 generierten, zellassoziierten ACE2-Spaltprodukte zeigte weitere interessante Unterschiede: Ein C-terminales ACE2-Spaltprodukt konnte nach TMPRSS2-Expression effizient nachgewiesen werden. Wesentlich aufwändiger war die Detektion des C-terminalen Spaltprodukts, das nach ADAM17-Spaltung entstand. Erst nach Optimierung des Nachweisverfahrens konnte ein extrem unstabiles Fragment nachgewiesen werden (nicht gezeigt). Die molekulare Masse dieses Fragments war größer als die seines Pendants, das durch TMPRSS2 generiert wurde [111, 113, 142]. Es wird angenommen, dass das nach ADAM17-Verdau resultierende Fragment direkt nach Spaltung weiter prozessiert wird [143, 144]. Die Mutante C4, die durch TMPRSS2 nicht mehr gespalten werden kann, wurde durch ADAM17 prozessiert und das Spaltfragment konnte effizient nachgewiesen werden. Es ist daher denkbar, dass die in Mutante C4 eingefügten Aminosäureaustausche eine weitere Prozessierung des C-terminalen Fragments inhibieren.

Die Beobachtungen, dass TMPRSS2 und ADAM17 unterschiedliche ACE2-Fragmente generieren und dass Mutante C4 durch ADAM17 aber nicht TMPRSS2 prozessiert werden konnte, zeigen, dass diese Proteasen ACE2 an unterschiedlichen Stellen spalten. In der Tat zeigte eine Mutationsanalyse, dass die Lysine und Arginine zwischen 652 und 659 (ACE2 Mutante C2) für die Spaltung durch ADAM17 aber nicht TMPRSS2 wichtig sind. Die extrazelluläre Domäne dieser Mutante wird weder konstitutiv noch nach Stimulation des Ektodomänen *sheddings* von Zellen in den Zellkulturüberstand abgegeben. Frühere Studien lieferten Hinweise auf eine ACE2-Spaltung

durch ADAM17 zwischen Aminosäure 706 und 707 [145], 708 und 709 [146] oder zwischen 716-741 [113]. Gegenwärtig kann nicht ausgeschlossen werden, dass die in der vorliegenden Studie identifizierte Region zwischen Aminosäuren 652 und 659 nicht direkt an der ACE2-Spaltung durch ADAM17 beteiligt ist, sondern dass die eingeführten Mutationen die ACE2-Struktur so beeinflussen, dass die eigentliche Spaltstelle nicht mehr erkannt werden kann. Dieses Szenario steht im Einklang mit Arbeiten, die zeigen, dass die Struktur der membranproximalen Region wichtiger für die Spaltung, als ihre Aminosäuresequenz ist [118, 147, 148].

Die nicht mehr *sheddbare* ACE2 Cluster 2-Mutante ist in ihrer Fähigkeit, SARS-S als Rezeptor zu dienen, stark eingeschränkt. Ob das an der reduzierten Expression oder der enormen Wichtigkeit der Prozessierung von ACE2 für die virale Aufnahme liegt, muss noch geklärt werden. Da die Cluster 2-Mutante noch durch TMPRSS2 prozessiert werden kann, könnte nun in nachfolgenden Experimenten untersucht werden, ob TMPRRS2 diesen Defekt wieder wettmachen kann.

Es gibt weitere offene Fragen die Rolle ADAM17s während des SARS-S getriebenen Zelleintritts betreffend. So zum Beispiel führt die Modulierung der ADAM17 Aktivität nicht in jedem experimentellen Aufbau zu einer Veränderung der Infektion. Haga und Kollegen konnten zeigen, dass die Inhibition von ADAM17 mit einer reduzierten SARS-CoV Infektion einhergeht [111, 112], wohingegen Glowacka und Kollegen sowie Daten aus der vorliegenden Arbeit diese Beobachtungen nicht bestätigten [149]. Das liegt vermutlich daran, dass die experimentellen Bedingungen dieser Studie nicht dazu geeignet waren, die Aktivität von ADAM17 hinreichend zu beeinflussen. Denn die Regulation von ADAM17 verläuft in einem engen zeitlichen Rahmen, sehr schnell und auch sehr vielschichtig ab [150].

Zusammengefasst lässt sich sagen, dass die Spaltung von ACE2 durch TMPRSS2, HAT und potentiell auch anderen TTSPs die Aufnahme des Virus-Rezeptor Komplexes verstärkt. Dieser Verstärkung liegt weder die proteolytische Aktivierung von SARS-S noch die verstärkte Bindung an prozessiertes ACE2, sondern eine verstärkte SARS-S Partikel-Aufnahme zu Grunde.

Diese Ergebnisse müssten in einem nächsten Schritt durch den Einsatz authentischer SARS- CoV überprüft werden. Außerdem sollten die Ergebnisse auf andere Coronaviren ausgeweitet werden, die Ektopeptidasen als Rezeptoren nutzen. Vielleicht liegt dem ein genereller Mechanismus der Rezeptorspaltung und der damit provozierten Internalisierung zu Grunde. Wenn dem so ist, könnten ausgewählte Protease Inhibitoren als „Breitband“ Therapie gegen die meisten Coronaviren eingesetzt werden.

5 Literaturverzeichnis

1. Rohde, G., et al., *[Detection of respiratory viruses--how, when, where and why?].* Pneumologie, 2009. **63**(1): p. 14-22.
2. Daubin, C., et al., *Epidemiology and clinical outcome of virus-positive respiratory samples in ventilated patients: a prospective cohort study.* Crit Care, 2006. **10**(5): p. R142.
3. Stock, I., *[Human rhinovirus diseases--epidemiology, treatment and prevention].* Med Monatsschr Pharm, 2014. **37**(2): p. 44-53.
4. Kramarev, S.A. and A.P. Moshchich, *[The treatment of influenza and acute respiratory viral infections].* Lik Sprava, 2013(2): p. 99-106.
5. Bawage, S.S., et al., *Recent advances in diagnosis, prevention, and treatment of human respiratory syncytial virus.* Adv Virol, 2013. **2013**: p. 595768.
6. Rynans, S., T. Dzieciatkowski, and G. Mlynarczyk, *[Adenovirus infection in immunocompromised patients].* Postepy Hig Med Dosw (Online), 2013. **67**: p. 964-72.
7. Jartti, T., et al., *New respiratory viral infections.* Curr Opin Pulm Med, 2012. **18**(3): p. 271-8.
8. Taubenberger, J.K. and D.M. Morens, *The pathology of influenza virus infections.* Annu Rev Pathol, 2008. **3**: p. 499-522.
9. Taubenberger, J.K. and J.C. Kash, *Insights on influenza pathogenesis from the grave.* Virus Res, 2011. **162**(1-2): p. 2-7.
10. Taubenberger, J.K., et al., *Initial genetic characterization of the 1918 "Spanish" influenza virus.* Science, 1997. **275**(5307): p. 1793-6.
11. Stadler, K., et al., *SARS--beginning to understand a new virus.* Nat Rev Microbiol, 2003. **1**(3): p. 209-18.
12. Raj, V.S., et al., *MERS: emergence of a novel human coronavirus.* Curr Opin Virol, 2014. **5C**: p. 58-62.
13. Ware, L.B. and M.A. Matthay, *The acute respiratory distress syndrome.* N Engl J Med, 2000. **342**(18): p. 1334-49.
14. Luyt, C.E., et al., *Virus-induced acute respiratory distress syndrome: epidemiology, management and outcome.* Presse Med, 2011. **40**(12 Pt 2): p. e561-8.
15. He, X., et al., *Angiotensin-converting enzyme inhibitor captopril prevents oleic acid-induced severe acute lung injury in rats.* Shock, 2007. **28**(1): p. 106-11.
16. He, X.L., Z. Liu, and S.Y. Xia, *Vascular endothelial injuries and changes of blood coagulation and fibrinolysis indexes in patients with acute respiratory distress syndrome.* Chin Med Sci J, 2004. **19**(4): p. 252-6.
17. Wenzel, C., et al., *Endothelial cell activation and blood coagulation in critically ill patients with lung injury.* Wien Klin Wochenschr, 2002. **114**(19-20): p. 853-8.
18. Hendrickson, C.M. and M.A. Matthay, *Viral pathogens and acute lung injury: investigations inspired by the SARS epidemic and the 2009 H1N1 influenza pandemic.* Semin Respir Crit Care Med, 2013. **34**(4): p. 475-86.
19. Imai, Y., et al., *Angiotensin-converting enzyme 2 protects from severe acute lung failure.* Nature, 2005. **436**(7047): p. 112-6.
20. Hagiwara, S., et al., *Antagonist of the type-1 ANG II receptor prevents against LPS-induced septic shock in rats.* Intensive Care Med, 2009. **35**(8): p. 1471-8.

21. Liu, X., et al., *Downregulation of angiotensin-converting enzyme 2 by the neuraminidase protein of influenza A (H1N1) virus.* Virus Res, 2014. **185**: p. 64-71.

22. Kuba, K., et al., *A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury.* Nat Med, 2005. **11**(8): p. 875-9.

23. Snijder, E.J., M.C. Horzinek, and W.J. Spaan, *The coronaviruslike superfamily.* Adv Exp Med Biol, 1993. **342**: p. 235-44.

24. van der Hoek, L., et al., *Croup is associated with the novel coronavirus NL63.* PLoS Med, 2005. **2**(8): p. e240.

25. van der Hoek, L., et al., *Human coronavirus NL63 infection is associated with croup.* Adv Exp Med Biol, 2006. **581**: p. 485-91.

26. Bradburne, A.F., M.L. Bynoe, and D.A. Tyrrell, *Effects of a "new" human respiratory virus in volunteers.* Br Med J, 1967. **3**(5568): p. 767-9.

27. van Elden, L.J., et al., *Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction.* J Infect Dis, 2004. **189**(4): p. 652-7.

28. Woo, P.C., et al., *Phylogenetic and recombination analysis of coronavirus HKU1, a novel coronavirus from patients with pneumonia.* Arch Virol, 2005. **150**(11): p. 2299-311.

29. Lau, S.K., et al., *Coronavirus HKU1 and other coronavirus infections in Hong Kong.* J Clin Microbiol, 2006. **44**(6): p. 2063-71.

30. Hendley, J.O., H.B. Fishburne, and J.M. Gwaltney, Jr., *Coronavirus infections in working adults. Eight-year study with 229 E and OC 43.* Am Rev Respir Dis, 1972. **105**(5): p. 805-11.

31. van der Hoek, L., *Human coronaviruses: what do they cause?* Antivir Ther, 2007. **12**(4 Pt B): p. 651-8.

32. Zaki, A.M., et al., *Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia.* N Engl J Med, 2012. **367**(19): p. 1814-20.

33. Ksiazek, T.G., et al., *A novel coronavirus associated with severe acute respiratory syndrome.* N Engl J Med, 2003. **348**(20): p. 1953-66.

34. Drosten, C., et al., *Identification of a novel coronavirus in patients with severe acute respiratory syndrome.* N Engl J Med, 2003. **348**(20): p. 1967-76.

35. Gonzalez, J.M., et al., *A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae.* Arch Virol, 2003. **148**(11): p. 2207-35.

36. Gorbalenya, A.E., et al., *Nidovirales: evolving the largest RNA virus genome.* Virus Res, 2006. **117**(1): p. 17-37.

37. de Groot, R.J., et al., *Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group.* J Virol, 2013. **87**(14): p. 7790-2.

38. Mahy, B.W., *Molecular biology of the coronaviruses.* Nature, 1983. **305**(5934): p. 474-5.

39. Sturman, L.S. and K.V. Holmes, *The molecular biology of coronaviruses.* Adv Virus Res, 1983. **28**: p. 35-112.

40. Lai, M.M. and D. Cavanagh, *The molecular biology of coronaviruses.* Adv Virus Res, 1997. **48**: p. 1-100.

41. Masters, P.S., *The molecular biology of coronaviruses.* Adv Virus Res, 2006. **66**: p. 193-292.

42. Holmes, K.V. and L. Enjuanes, *Virology. The SARS coronavirus: a postgenomic era.* Science, 2003. **300**(5624): p. 1377-8.

43. Smith, E.C., et al., *Coronaviruses lacking exoribonuclease activity are susceptible to lethal mutagenesis: evidence for proofreading and potential therapeutics.* PLoS Pathog, 2013. **9**(8): p. e1003565.

44. Thiel, V., et al., *Mechanisms and enzymes involved in SARS coronavirus genome expression.* J Gen Virol, 2003. **84**(Pt 9): p. 2305-15.

45. Brierley, I., P. Digard, and S.C. Inglis, *Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot.* Cell, 1989. **57**(4): p. 537-47.

46. Ziebuhr, J., *Molecular biology of severe acute respiratory syndrome coronavirus.* Curr Opin Microbiol, 2004. **7**(4): p. 412-9.

47. Hofmann, H. and S. Pohlmann, *Cellular entry of the SARS coronavirus.* Trends Microbiol, 2004. **12**(10): p. 466-72.

48. Hofmann, H., et al., *Susceptibility to SARS coronavirus S protein-driven infection correlates with expression of angiotensin converting enzyme 2 and infection can be blocked by soluble receptor.* Biochem Biophys Res Commun, 2004. **319**(4): p. 1216-21.

49. Yeung, K.S., G.A. Yamanaka, and N.A. Meanwell, *Severe acute respiratory syndrome coronavirus entry into host cells: Opportunities for therapeutic intervention.* Med Res Rev, 2006. **26**(4): p. 414-33.

50. Du, L., et al., *The spike protein of SARS-CoV--a target for vaccine and therapeutic development.* Nat Rev Microbiol, 2009. **7**(3): p. 226-36.

51. Zhong, N.S., et al., *Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003.* Lancet, 2003. **362**(9393): p. 1353-8.

52. Ge, X.Y., et al., *Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor.* Nature, 2013. **503**(7477): p. 535-8.

53. Lau, S.K., et al., *Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 14040-5.

54. Li, W., et al., *Bats are natural reservoirs of SARS-like coronaviruses.* Science, 2005. **310**(5748): p. 676-9.

55. Drexler, J.F., et al., *Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences.* J Virol, 2010. **84**(21): p. 11336-49.

56. Tong, S., et al., *Detection of novel SARS-like and other coronaviruses in bats from Kenya.* Emerg Infect Dis, 2009. **15**(3): p. 482-5.

57. Ren, W., et al., *Difference in receptor usage between severe acute respiratory syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin.* J Virol, 2008. **82**(4): p. 1899-907.

58. Song, H.D., et al., *Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human.* Proc Natl Acad Sci U S A, 2005. **102**(7): p. 2430-5.

59. Chan, P.K. and M.C. Chan, *Tracing the SARS-coronavirus.* J Thorac Dis, 2013. **5**(Suppl 2): p. S118-21.

60. Sheahan, T., et al., *Pathways of cross-species transmission of synthetically reconstructed zoonotic severe acute respiratory syndrome coronavirus.* J Virol, 2008. **82**(17): p. 8721-32.

61. Lim, W., K.C. Ng, and D.N. Tsang, *Laboratory containment of SARS virus.* Ann Acad Med Singapore, 2006. **35**(5): p. 354-60.

62. Wang, M., et al., *SARS-CoV infection in a restaurant from palm civet.* Emerg Infect Dis, 2005. **11**(12): p. 1860-5.

63. Li, W., et al., *Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus.* Nature, 2003. **426**(6965): p. 450-4.

64. Rota, P.A., et al., *Characterization of a novel coronavirus associated with severe acute respiratory syndrome.* Science, 2003. **300**(5624): p. 1394-9.

65. Belouzard, S., et al., *Mechanisms of coronavirus cell entry mediated by the viral spike protein.* Viruses, 2012. **4**(6): p. 1011-33.

66. Heald-Sargent, T. and T. Gallagher, *Ready, set, fuse! The coronavirus spike protein and acquisition of fusion competence.* Viruses, 2012. **4**(4): p. 557-80.

67. Wong, S.K., et al., *A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2.* J Biol Chem, 2004. **279**(5): p. 3197-201.

68. Xiao, X., et al., *The SARS-CoV S glycoprotein: expression and functional characterization.* Biochem Biophys Res Commun, 2003. **312**(4): p. 1159-64.

69. Li, F., et al., *Structure of SARS coronavirus spike receptor-binding domain complexed with receptor.* Science, 2005. **309**(5742): p. 1864-8.

70. Weissenhorn, W., et al., *Structural basis for membrane fusion by enveloped viruses.* Mol Membr Biol, 1999. **16**(1): p. 3-9.

71. Sainz, B., Jr., et al., *Identification and characterization of the putative fusion peptide of the severe acute respiratory syndrome-associated coronavirus spike protein.* J Virol, 2005. **79**(11): p. 7195-206.

72. Madu, I.G., et al., *Characterization of a highly conserved domain within the severe acute respiratory syndrome coronavirus spike protein S2 domain with characteristics of a viral fusion peptide.* J Virol, 2009. **83**(15): p. 7411-21.

73. Chen, J., et al., *Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation.* Cell, 1998. **95**(3): p. 409-17.

74. Bosch, B.J., W. Bartelink, and P.J. Rottier, *Cathepsin L functionally cleaves the severe acute respiratory syndrome coronavirus class I fusion protein upstream of rather than adjacent to the fusion peptide.* J Virol, 2008. **82**(17): p. 8887-90.

75. Xu, Y., et al., *Crystal structure of severe acute respiratory syndrome coronavirus spike protein fusion core.* J Biol Chem, 2004. **279**(47): p. 49414-9.

76. Kielian, M. and F.A. Rey, *Virus membrane-fusion proteins: more than one way to make a hairpin.* Nat Rev Microbiol, 2006. **4**(1): p. 67-76.

77. Donoghue, M., et al., *A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9.* Circ Res, 2000. **87**(5): p. E1-9.

78. Danilczyk, U., et al., *Essential role for collectrin in renal amino acid transport.* Nature, 2006. **444**(7122): p. 1088-91.

79. Guang, C., et al., *Three key proteases--angiotensin-I-converting enzyme (ACE), ACE2 and renin--within and beyond the renin-angiotensin system.* Arch Cardiovasc Dis, 2012. **105**(6-7): p. 373-85.

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

81. Crackower, M.A., et al., *Angiotensin-converting enzyme 2 is an essential regulator of heart function.* Nature, 2002. **417**(6891): p. 822-8.

82. Barrett, A.J., *Classification of peptidases.* Methods Enzymol, 1994. **244**: p. 1-15.

83. Matsuyama, S., et al., *Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection.* Proc Natl Acad Sci U S A, 2005. **102**(35): p. 12543-7.

84. Simmons, G., et al., *Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry.* Proc Natl Acad Sci U S A, 2005. **102**(33): p. 11876-81.

85. Bertram, S., et al., *Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease.* J Virol, 2011. **85**(24): p. 13363-72.

86. Simmons, G., et al., *Different host cell proteases activate the SARS-coronavirus spike-protein for cell-cell and virus-cell fusion.* Virology, 2011. **413**(2): p. 265-74.

87. Glowacka, I., et al., *Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response.* J Virol, 2011. **85**(9): p. 4122-34.

88. Kominami, E., et al., *Biosyntheses and processing of lysosomal cysteine proteinases in rat macrophages.* FEBS Lett, 1988. **231**(1): p. 225-8.

89. Nishimura, Y., T. Kawabata, and K. Kato, *Identification of latent procathepsins B and L in microsomal lumen: characterization of enzymatic activation and proteolytic processing in vitro.* Arch Biochem Biophys, 1988. **261**(1): p. 64-71.

90. Huang, I.C., et al., *SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells.* J Biol Chem, 2006. **281**(6): p. 3198-203.

91. Beniac, D.R., et al., *Conformational reorganization of the SARS coronavirus spike following receptor binding: implications for membrane fusion.* PLoS One, 2007. **2**(10): p. e1082.

92. Biniossek, M.L., et al., *Proteomic identification of protease cleavage sites characterizes prime and non-prime specificity of cysteine cathepsins B, L, and S.* J Proteome Res, 2011. **10**(12): p. 5363-73.

93. Bosch, B.J., W. Bartelink, and P.J. Rottier, *Cathepsin L functionally cleaves the severe acute respiratory syndrome coronavirus class I fusion protein upstream of rather than adjacent to the fusion peptide.* J.Virol., 2008. **82**(17): p. 8887-8890.

94. Belouzard, S., V.C. Chu, and G.R. Whittaker, *Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites.* Proc Natl Acad Sci U S A, 2009. **106**(14): p. 5871-6.

95. Belouzard, S., I. Madu, and G.R. Whittaker, *Elastase-mediated activation of the severe acute respiratory syndrome coronavirus spike protein at discrete sites within the S2 domain.* J Biol Chem, 2010. **285**(30): p. 22758-63.

96. Hofmann, H., et al., *Highly conserved regions within the spike proteins of human coronaviruses 229E and NL63 determine recognition of their respective cellular receptors.* J Virol, 2006. **80**(17): p. 8639-52.

97. Qiu, Z., et al., *Endosomal proteolysis by cathepsins is necessary for murine coronavirus mouse hepatitis virus type 2 spike-mediated entry.* J Virol, 2006. **80**(12): p. 5768-76.

98. Kawase, M., et al., *Protease-mediated entry via the endosome of human coronavirus 229E.* J Virol, 2009. **83**(2): p. 712-21.

99. Gierer, S., et al., *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, 2013. **87**(10): p. 5502-11.

100. Bertram, S., et al., *TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium.* J Virol, 2013. **87**(11): p. 6150-60.

101. Antalis, T.M., T.H. Bugge, and Q. Wu, *Membrane-anchored serine proteases in health and disease.* Prog Mol Biol Transl Sci, 2011. **99**: p. 1-50.

102. Simmons, G., et al., *Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research.* Antiviral Res, 2013. **100**(3): p. 605-14.

103. Netzel-Arnett, S., et al., *Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer.* Cancer Metastasis Rev, 2003. **22**(2-3): p. 237-58.

104. Matsuyama, S., et al., *Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2.* J Virol, 2010. **84**(24): p. 12658-64.

105. Shulla, A., et al., *A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry.* J Virol, 2011. **85**(2): p. 873-82.

106. Kam, Y.W., et al., *Cleavage of the SARS coronavirus spike glycoprotein by airway proteases enhances virus entry into human bronchial epithelial cells in vitro.* PLoS.One., 2009. **4**(11): p. e7870.

107. Böttcher-Friebertshauser, E., et al., *Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors.* J.Virol., 2010. **84**(11): p. 5605-5614.

108. Yasuoka, S., et al., *Purification, characterization, and localization of a novel trypsin-like protease found in the human airway.* Am J Respir Cell Mol Biol, 1997. **16**(3): p. 300-8.

109. Kim, T.S., et al., *Phenotypic analysis of mice lacking the Tmprss2-encoded protease.* Mol Cell Biol, 2006. **26**(3): p. 965-75.

110. Potts, W., et al., *Cathepsin L-deficient mice exhibit abnormal skin and bone development and show increased resistance to osteoporosis following ovariectomy.* Int J Exp Pathol, 2004. **85**(2): p. 85-96.

111. Haga, S., et al., *Modulation of TNF-alpha-converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF-alpha production and facilitates viral entry.* Proc Natl Acad Sci U S A, 2008. **105**(22): p. 7809-14.

112. Haga, S., et al., *TACE antagonists blocking ACE2 shedding caused by the spike protein of SARS-CoV are candidate antiviral compounds.* Antiviral Res, 2010. **85**(3): p. 551-5.

113. Jia, H.P., et al., *Ectodomain shedding of angiotensin converting enzyme 2 in human airway epithelia.* Am J Physiol Lung Cell Mol Physiol, 2009. **297**(1): p. L84-96.

114. Burrell, L.M., et al., *ACE2, a new regulator of the renin-angiotensin system.* Trends Endocrinol Metab, 2004. **15**(4): p. 166-9.

115. Ohtsu, H., et al., *ADAM17 mediates epidermal growth factor receptor transactivation and vascular smooth muscle cell hypertrophy induced by angiotensin II.* Arterioscler Thromb Vasc Biol, 2006. **26**(9): p. e133-7.

116. Lambert, D.W., et al., *Calmodulin interacts with angiotensin-converting enzyme-2 (ACE2) and inhibits shedding of its ectodomain.* FEBS Lett, 2008. **582**(2): p. 385-90.

117. Doedens, J.R. and R.A. Black, *Stimulation-induced down-regulation of tumor necrosis factor-alpha converting enzyme.* J Biol Chem, 2000. **275**(19): p. 14598-607.

118. Lambert, D.W., et al., *Tumor necrosis factor-alpha convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2).* J Biol Chem, 2005. **280**(34): p. 30113-9.

119. Glowacka, I., et al., *Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63.* J.Virol., 2010. **84**(2): p. 1198-1205.

120. Haga, S., et al., *Modulation of TNF-alpha-converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF-alpha production and facilitates viral entry.* Proc.Natl.Acad.Sci.U.S.A, 2008. **105**(22): p. 7809-7814.

121. Rockx, B., et al., *Early upregulation of acute respiratory distress syndrome-associated cytokines promotes lethal disease in an aged-mouse model of severe acute respiratory syndrome coronavirus infection.* J Virol, 2009. **83**(14): p. 7062-74.

122. Oudit, G.Y., et al., *SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS.* Eur J Clin Invest, 2009. **39**(7): p. 618-25.

123. Bertram, S., et al., *TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells.* J Virol, 2010. **84**(19): p. 10016-25.

124. Bottcher, E., et al., *Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium.* J Virol, 2006. **80**(19): p. 9896-8.

125. To, K.F., et al., *Tissue and cellular tropism of the coronavirus associated with severe acute respiratory syndrome: an in-situ hybridization study of fatal cases.* J Pathol, 2004. **202**(2): p. 157-63.

126. Ye, J., et al., *Molecular pathology in the lungs of severe acute respiratory syndrome patients.* Am J Pathol, 2007. **170**(2): p. 538-45.

127. Ding, Y., et al., *Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways.* J Pathol, 2004. **203**(2): p. 622-30.

128. Mossel, E.C., et al., *SARS-CoV replicates in primary human alveolar type II cell cultures but not in type I-like cells.* Virology, 2008. **372**(1): p. 127-35.

129. To, K.F. and A.W. Lo, *Exploring the pathogenesis of severe acute respiratory syndrome (SARS): the tissue distribution of the coronavirus (SARS-CoV) and its putative receptor, angiotensin-converting enzyme 2 (ACE2).* J Pathol, 2004. **203**(3): p. 740-3.

130. Gu, J., et al., *Multiple organ infection and the pathogenesis of SARS.* J Exp Med, 2005. **202**(3): p. 415-24.

131. Hatesuer, B., et al., *Tmprss2 is essential for influenza H1N1 virus pathogenesis in mice.* PLoS Pathog, 2013. **9**(12): p. e1003774.

132. Sakai, K., et al., *The host protease TMPRSS2 plays a major role in in vivo replication of emerging H7N9 and seasonal influenza viruses.* J Virol, 2014. **88**(10): p. 5608-16.

133. Tarnow, C., et al., *TMPRSS2 is a host factor that is essential for pneumotropism and pathogenicity of H7N9 influenza A virus in mice.* J Virol, 2014. **88**(9): p. 4744-51.

134. Raj, V.S., et al., *Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC.* Nature, 2013. **495**(7440): p. 251-4.

135. Hofmann, H., et al., *Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry.* Proc Natl Acad Sci U S A, 2005. **102**(22): p. 7988-93.

136. Clarke, N.E., et al., *Angiotensin converting enzyme (ACE) and ACE2 bind integrins and ACE2 regulates integrin signalling.* PLoS One, 2012. **7**(4): p. e34747.

137. Thatcher, S.E., et al., *Angiotensin-converting enzyme 2 deficiency in whole body or bone marrow-derived cells increases atherosclerosis in low-density lipoprotein receptor-/- mice.* Arterioscler Thromb Vasc Biol, 2011. **31**(4): p. 758-65.

138. Bauvois, B., *Transmembrane proteases in cell growth and invasion: new contributors to angiogenesis?* Oncogene, 2004. **23**(2): p. 317-29.

139. Clarke, N.E. and A.J. Turner, *Angiotensin-converting enzyme 2: the first decade.* Int J Hypertens, 2012. **2012**: p. 307315.

140. Lucero, H.A., et al., *Cell signaling, internalization, and nuclear localization of the angiotensin converting enzyme in smooth muscle and endothelial cells.* J Biol Chem, 2010. **285**(8): p. 5555-68.

141. Iwata, M., J.E. Silva Enciso, and B.H. Greenberg, *Selective and specific regulation of ectodomain shedding of angiotensin-converting enzyme 2 by tumor necrosis factor alpha-converting enzyme.* Am J Physiol Cell Physiol, 2009. **297**(5): p. C1318-29.

142. Le Gall, S.M., et al., *ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site.* J Cell Sci, 2010. **123**(Pt 22): p. 3913-22.

143. Lichtenthaler, S.F., C. Haass, and H. Steiner, *Regulated intramembrane proteolysis--lessons from amyloid precursor protein processing.* J Neurochem, 2011. **117**(5): p. 779-96.

144. Cao, X. and T.C. Sudhof, *A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60.* Science, 2001. **293**(5527): p. 115-20.

145. Xiao, F., et al., *Characterization of angiotensin-converting enzyme 2 ectodomain shedding from mouse proximal tubular cells.* PLoS One, 2014. **9**(1): p. e85958.

146. Lai, Z.W., et al., *Angiotensin-converting enzyme 2 ectodomain shedding cleavage-site identification: determinants and constraints.* Biochemistry, 2011. **50**(23): p. 5182-94.

147. Ehlers, M.R., et al., *Proteolytic release of membrane-bound angiotensin-converting enzyme: role of the juxtamembrane stalk sequence.* Biochemistry, 1996. **35**(29): p. 9549-59.

148. Sadhukhan, R., et al., *Unaltered cleavage and secretion of angiotensin-converting enzyme in tumor necrosis factor-alpha-converting enzyme-deficient mice.* J Biol Chem, 1999. **274**(15): p. 10511-6.

149. Glowacka, I., et al., *Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63.* J Virol, 2010. **84**(2): p. 1198-205.

150. Vincent, B. and F. Checler, *alpha-Secretase in Alzheimer's disease and beyond: mechanistic, regulation and function in the shedding of membrane proteins.* Curr Alzheimer Res, 2012. **9**(2): p. 140-56.

6 Anhang

Abkürzungen

%	Prozent
aa	*amino acids*
Abb.	Abbildung
ACE	*angiotensin- converting enzyme*
ACE2	*angiotensin- converting enzyme 2*
ADAM17	*A Disintegrin And Metalloproteinase*
ANG	Angiotensin
AT1R	Angiotensin II-Rezeptors Typ 1
AT2R	Angiotensin II-Rezeptors Typ 2
Bp	Basenpaare
Bzw.	beziehungsweise
ca.	circa
CDC	*Centers of Disease Control and Prevention*
et al.	*et alteri* (und andere)
FP	Fusionspeptid
HA	Hämagglutinin
HAT	*Human airway trypsin-like*
hCoV	humanes Coronavirus
HEF	Hämagglutinin- Esterase- Fusions- Protein
HIV	humanes Immundefizienzvirus
HPAIV	*high pathogenic avian influenza virus*
hr	*heptad repeats*
K	Lysin
kb	Kilobasen

kDa	Kilodalton
LPAIV	*Low pathogenic avian influenza virus*
M- Protein	Matrix- Protein
MERS- CoV	*middle east respiratory syndrome*- Coronavirus
mRNA	*messenger* Ribonukleinsäure
N- Protein	Nukleokapsid- Protein
NA	Neuraminidase
nm	Nanometer
NP- Protein	Nukleoprotein
NSP	Nichtstrukturprotein
ORF	*open reading frame*
PA	*polymerase acidic protein*
PB	*polymerase basic protein*
pH	*potentia hydrogenii*
PMA	Phorbol- Myristat- Acetat
R	Arginin
RAS	Renin- Angiotensin- System
RBD	Rezeptor- Bindedomäne
RNA	Ribonukleinsäure
S- Protein	Spike- Protein
SARS- CoV	*acute respiratory syndrome*- Coronavirus
Tab.	Tabelle
TACE	*tumor necrosis factor-α-converting enzyme*
TM	Transmembrandomäne
TMPRSS2	Transmembran Protease, Serin 2
TTSP	Type II Transmembran Serin Protease
WHO	*World Health Organization*
Wt	Wildtyp
z.B.	zum Beispiel

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Gierer, S.; Müller, M.A.; Heurich, A.; Ritz, D.; Springstein, B.; Karsten, C. B.; Schendzielorz, A.; Gnirß, K.; Drosten, C. and Pöhlmann, S.: **Inhibition of proprotein convertases abrogates processing of the MERS-coronavirus spike protein in infected cells but does not reduce viral infectivity**, submitted JID/ 2014

Heurich A, Hofmann-Winkler H, Gierer S, Liepold T, Jahn O, Pöhlmann S.: **TMPRSS2 and ADAM17 cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the SARS-coronavirus spike-protein**, JVI/2014

Simmons G, Zmora P, Gierer S, Heurich A, Pöhlmann S.: **Proteolytic activation of the SARS-coronavirus spike protein: Cutting enzymes at the cutting edge of antiviral research**, AVR/2013

Bertram S, Dijkman R, Habjan M, Heurich A, Gierer S, Glowacka I, Welsch K, Winkler M, Schneider H, Hofmann-Winkler H, Thiel V, Pöhlmann S.: **TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium**, JVI/ 2013

Gierer S, Bertram S, Kaup F, Wrensch F, Heurich A, Krämer-Kühl A, Welsch K, Winkler M, Meyer B, Drosten C, Dittmer U, von Hahn T, Simmons G, Hofmann H, Pöhlmann S.: **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**, JVI/ 2013

Bertram S, Heurich A, Lavender H, Gierer S, Danisch S, Perin P, Lucas JM, Nelson PS, Pöhlmann S, Soilleux EJ.: **Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts**, PLoS One/ 2012

„Wo Worte selten sind, haben sie Gewicht“

William Shakespeare

Danke.