TMPRSS11A activates the influenza A virus hemagglutinin and the MERS coronavirus spike protein and is insensitive against blockade by HAI-1

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The influenza virus hemagglutinin (HA) facilitates viral entry into target cells. Cleavage of HA by host cell proteases is essential for viral infectivity and the responsible enzymes are potential targets for antiviral intervention. The type II transmembrane serine protease (TTSP) TMPRSS2 has been identified as an HA activator in cell culture and in the infected host. However, it is less clear whether TMPRSS2-related enzymes can also activate HA for spread in target cells. Moreover, the activity of cellular serine protease inhibitors against HA-activating TTSPs is poorly understood. Here, we show that TMPRSS11A, another member of the TTSP family, cleaves and activates the influenza A virus (FLUAV) HA and the Middle East respiratory syndrome coronavirus spike protein (MERS-S). Moreover, we demonstrate that TMPRSS11A is expressed in murine tracheal epithelium, which is a target of FLUAV infection, and in human trachea, suggesting that the protease could support FLUAV spread in patients. Finally, we show that HA activation by the TMPRSS11A-related enzymes HAT and DESC1 but not TMPRSS11A itself is blocked by the cellular serine protease

inhibitor hepatocyte growth factor activator inhibitor type-1 (HAI-1). Our results suggest that TMPRSS11A could promote FLUAV spread in target cells and that HA-activating TTSPs exhibit differential sensitivity to blockade by cellular serine protease inhibitors.

Influenza viruses, members of the family *Orthomyxoviridae*, are enveloped viruses with a negative sense, segmented RNA genome. Influenza viruses readily adapt to immune pressure and thus constantly circulate in the human population, causing annual influenza epidemics (1,2). Moreover, reassortment of genomic segments between different influenza A viruses can result in the emergence of antigenically new viruses, which may spread pandemically (1,2). The constantly changing nature of influenza viruses also compromises the efficacy of currently available antivirals, since the viruses can rapidly acquire resistance-conferring mutations (3). This may not be the case if host cell factors were targeted, which are required for viral spread but dispensable for cellular survival, and the identification of such factors is in the focus of current research efforts (4).

The viral hemagglutinin protein (HA) is incorporated into the viral envelope and facilitates influenza virus entry into target cells (5,6). For this, the surface unit of HA, termed HA1, binds to sialic acids on cell surface receptors and stimulates viral uptake into host cell endosomes. Thereafter, the transmembrane unit HA2 fuses the viral and the endosomal membrane, allowing the delivery of the viral genomic information into the cellular cytoplasm (5,6). HA is synthesized as an inactive precursor protein, HA0, and cleavage of HA0 by host cell proteases primes HA for membrane fusion, which is triggered by endosomal low pH (7,8). The priming of HA, which, for historic reasons, will subsequently be referred to as HA activation, is essential for viral infectivity.

As a consequence, the host cell proteases responsible for HA activation are potential targets for antiviral intervention.

Evidence is constantly accumulating that the type II transmembrane serine protease TMPRSS2 plays an important role in HA activation. Thus, TMPRSS2 activates HA upon directed (9,10) and upon endogenous expression in cell culture (11,12) and the protease is expressed in viral target cells in the human respiratory tract (13). Moreover, polymorphisms in the TMPRSS2 promoter were found to be associated with the severity of influenza in humans (14). Finally, analysis of TMPRSS2 knock-out mice revealed that TMPRSS2 is essential for spread and pathogenesis of several influenza A virus (FLUAV) subtypes (15-17), although certain H3N2 viruses seem to be able to use proteases other than TMPRSS2 for HA activation (18,19). HAT, DESC1, TMPRSS4 and other TTSP members can activate HA upon directed expression in cell culture (9,10,20-23) and TMPRSS4 was shown to contribute to H3N2 FLUAV spread in mice (18). However, some TTSPs remain to be examined for their ability to activate HA and to promote viral spread. Moreover, it is incompletely understood how natural inhibitors of TTSPs impact FLUAV infection, although a recent study reported that interferon (IFN)-induced upregulation of the serine protease inhibitor PAI-1 blocks viral activation by TMPRSS2 and viral spread in mice (24). Moreover, hepatocyte growth factor activator inhibitor 2 (HAI-2) was found to block H1N1 FLUAV infection in cell culture and a mouse model (25) but whether this activity was due to inhibition of TTSPs was not examined.

Here, we investigated whether TMPRSS11A, a member of the HAT/DESC subfamily of TTSPs, can activate HA and whether activation is blocked by an endogenous serine protease inhibitor, hepatocyte growth factor activator inhibitor type-1 (HAI-1, also termed serine peptidase

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inhibitor, Kunitz type 1 (SPINT1)). We show that TMPRSS11A cleaves and activates FLUAV HA and MERS coronavirus spike protein (MERS-S) and that endogenous TMPRSS11A is expressed in trachea, a target of FLUAV infection. Finally, we demonstrate that HA activation by TMPRSS2 but not TMPRSS11A is repressed by HAI-1, indicating that HA-activating TTSP are differentially sensitive to inhibition by cellular serine protease inhibitors.

Results

TMPRSS11A activates influenza A virus hemagglutinin

Although previous studies investigated the ability of diverse TTSPs to activate HA (9,20-23), TMPRSS11A, a member of the HAT/DESC subfamily of TTSPs, has so far not been examined. In order to close this gap, we cloned the open reading frame of human TMPRSS11A and first analyzed protein expression in transfected 293T cells. Other members of the HAT/DESC subfamily, which were previously shown to be able (HAT, DESC1; (9,23)) or unable (TMPRSS11B; (23)) to cleave and activate HA, were included as controls. In order to determine protease expression, the enzymes were equipped with an N-terminal c-Myc antigenic tag and protease levels in transfected 293T cells were analyzed by immunoblot, employing anti-C-Myc antibody. The immunoblot revealed that DESC1, TMPRSS11A and TMPRSS11B were readily and comparably expressed, while the signal detected for HAT was somewhat weaker (Fig. 1A). For all proteases a prominent band with the molecular weight roughly expected for the zymogen form (46-48 kDa amino acid chain, up to three N-glycans) was observed (Fig. 1A). In addition, modest signals potentially corresponding to N-terminal auto-activation products were detected for HAT and DESC1 (21-22 kDa amino acid chain, up to two N-glycans for all

proteases tested) but relative signal intensities varied between experiments (see also Fig. 6) and no such signals were consistently detected for TMPRSS11B. In contrast, strong signals for N-terminal fragments were observed in TMPRSS11A expressing cells (Fig. 1A). Finally, intracellular FACS (Fig. 1B) confirmed that all proteases examined were expressed in transfected 293T cells and could thus be subjected to further analysis.

In order to analyze HA cleavage, the proteases under study were coexpressed with FLUAV HA of the H1 (Fig. 2A, left panel) or H3 (Fig. 2A, right panel) subtype and HA cleavage was examined by immunoblot, employing anti-FLUAV polyclonal antibodies. Trypsin treatment of cells served as positive control. Coexpression of HAT and DESC1 resulted in cleavage of both HA proteins tested (Fig. 2A), as evidenced by the conversion of the precursor form of HA, termed HA0, into HA1 and HA2 (not detected), in keeping with published reports (9,23). Similarly, coexpression of TMPRSS11A resulted in cleavage of H1 and H3 HA while no cleavage was observed in cells coexpressing HA and TMPRSS11B (Fig. 2A), as expected (23). These results identify TMPRSS11A as an HA-cleaving protease. In order to determine whether cleavage results in activation, we analyzed FLUAV infection of 293T cells transiently expressing the proteases investigated here. In keeping with the cleavage analysis, coexpression of HAT, DESC1 and TMPRSS11A or treatment of cells with trypsin increased spread of A/PR/8/34 (H1N1) or A/Panama/2007/1999 (H3N2) FLUAV (Fig. 2B). In contrast, FLUAV infection of cells expressing TMPRSS11B was within the background range (Fig. 2B), as expected (23). Collectively, these results show that TMPRSS11A can cleave and activate FLUAV HA of different subtypes.

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TMPRSS11A activates the MERS-coronavirus spike protein for virus-cell fusion

Several TTSP members previously shown to activate HA were also demonstrated to activate the spike (S) protein of coronaviruses (23,26,27). Therefore, we addressed whether TMPRSS11A can cleave and activate the S protein of Middle East respiratory syndrome coronavirus (MERS-CoV). The MERS-CoV S protein (MERS-S) is cleaved by furin in virus producing cells (28,29) and can be activated by furin (29) and endosomal cysteine proteases, cathepsin B and cathepsin L (30,31), (CatB/CatL) during entry into target cells. Therefore, activation of MERS-S by TTSPs can be studied by asking whether inhibition of viral entry due to blockade of CatB/CatL activity can be rescued by directed expression of TTSPs (30). Indeed, coexpression of HAT and DESC1 resulted in cleavage of MERS-S (Fig. 3A), increased MERS-S entry (Fig. 3B) and rescued MERS-S-driven entry from inhibition by the CatB/CatL inhibitor MDL 28170 (Fig. 3B), as expected (23,32). Similar activities were detected for TMPRSS11A but not TMPRSS11B (Fig. 3), indicating that TMPRSS11A could be exploited by both FLUAV and coronaviruses for activation of viral surface proteins.

TMPRSS11A is expressed in trachea

We next sought to investigate whether endogenous TMPRSS11A could promote FLUAV spread. For this, we employed quantitative RT-PCR to assess expression of TMPRSS11A mRNA in diverse cell lines derived from liver (Huh-7, HepG2), kidney (293T), cervix (HeLa) and lung (A549, Calu-3, BEAS-2B, NCI-H292, NCI-H727, NCI-H1299). None of the cell lines tested expressed robust levels of endogenous TMPRSS11A mRNA, although a very low signal was detected in NCI-H292 and NCI-H727 cells (Fig. 4A). Thus, the cell lines at

hand did not afford the opportunity to test whether endogenous TMPRSS11A can activate FLUAV HA. Instead, we asked whether TMPRSS11A is expressed at appreciable levels in the human respiratory tract, the major target of FLUAV infection, which would suggest that the protease might be able to support viral spread in infected patients. In addition, we assessed TMPRSS11A expression in non-respiratory tissues since evidence for occasional spread of FLUAV outside the respiratory tract has been reported. For comparison, we determined expression of the HA activating proteases TMPRSS2 and TMPRSS4. Analysis of a commercially available cDNA panel (supplemental table S1) revealed no appreciable expression of TMPRSS11A mRNA in brain, heart, liver, kidney, pancreas, placenta, skeletal muscle and lung, although TMPRSS2 and/or TMPRSS4 expression was detected in some of these organs (Fig. 4B). In contrast, TMPRSS11A mRNA was readily detectable in trachea and tracheal tissues also contained TMPRSS2 and TMPRSS4 transcripts (Fig. 4B). Thus, TMPRSS11A jointly with functionally redundant proteolytic enzymes could activate FLUAV for spread in tracheal epithelium.

Endogenous TMPRSS11A is expressed in murine tracheal epithelium, which is a target for FLUAV infection

In order to further explore the potential contribution of TMPRSS11A to FLUAV activation in the host, we investigated protease expression in the trachea of FLUAV infected mice. For this, C57BL/6J mice were intranasally inoculated with A/PR/8/34 and expression of TMPRSS11A and the viral NP protein was analyzed in serial trachea sections using immunohistochemistry. Single NP-positive cells were detected in tracheal epithelium (Fig. 5A-C), as expected, and the vast majority of tracheal epithelial cells expressed TMPRSS11A (Fig. 5D-F),

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providing further evidence that this protease could contribute to viral spread in the infected host.

HAI-1 blocks autocatalytic activation and hemagglutinin cleavage by HAT and DESC1 but not TMPRSS11A

The activity of TTSPs can be regulated by cellular protease inhibitors but the impact of these inhibitors on activation of FLUAV HA is poorly understood. Therefore, we asked whether the hepatocyte growth factor activator inhibitor type-1 (HAI-1, a membrane-associated Kunitz type 1 serine protease inhibitor), which was previously shown to inhibit activity of the TTSP members, matriptase (33) and prostasin (34), also blocks HA activation by HAT/DESC proteases. For this, the effect of directed HAI-1 expression in 293T cells on HA cleavage and activation was examined. Coexpression of HAI-1 interfered with HA cleavage by HAT and DESC-1 but not TMPRSS11A (Fig. 6A). In keeping with these results, HAI-1 expression inhibited FLUAV spread in cells transfected with HAT and DESC1 but not TMPRSS11A (Fig. 6B) while treatment with camostat mesylate, a serine protease inhibitor previously found to be active against TMPRSS2 (30,31), blocked viral spread promoted by all three proteases (Fig. 6C). Finally, we sought to obtain insights into why HAI-1 selectively inhibited HA-activation by HAT and DESC1 but not TMPRSS11A. Analysis of protease expression in the presence of rising amounts of HAI-1 revealed that autocatalytic activation of HAT and DESC1 was inhibited by HAI-1 in a dose-dependent fashion, as indicated by the reduced production of the N-terminal 25 kDa (HAT) and 27 kDa (DESC1) fragments (Fig. 7). In contrast, autocatalytic activation of TMPRSS11A was not efficiently blocked by coexpression of HAI-1 (Fig. 7), indicating that HAI-1 can interfere with HA activation by some TTSPs but not others.

Discussion

Activation of viral glycoproteins by host cell proteases is essential for the infectivity of many viruses and important insights into the proteolytic systems responsible for this process have been obtained in the last decades. It has long been believed that HA proteins with monobasic cleavage sites (found in low pathogenic avian and human FLUAV) are activated by secreted proteases (35). However, recent studies point towards an important role of the type II transmembrane serine protease TMPRSS2 (16,17,36). Here, we show that the TMPRSS2-related enzyme TMPRSS11A is expressed in trachea and can also activate HA. Moreover, we demonstrate that the cellular protease inhibitor HAI-1 can block HA-activation by TMPRSS2 but not TMPRSS11A. These findings support the notion that proteases other than TMPRSS2 could promote FLUAV spread, at least in certain target cells, and that cellular protease inhibitors might selectively target certain HA-activating enzymes.

In the aftermath of the discovery of TMPRSS2 as an HA-activating protease in cell culture (9,10) and in the infected host (15-17), several other members of the TTSP family were shown to activate HA, at least upon directed expression. These proteases are members of the TMPRSS/hepsin and HAT/DESC subfamilies and include HAT (9), DESC1 (23), MSPL (23), matriptase (20-22) and TMPRSS4 (10). The present study identified TMPRSS11A as an HA-activating protease, using DESC1 and HAT as positive and TMPRSS11B as negative controls. TTSPs are synthesized as zymogens and can be activated upon autoproteolytic cleavage at a site located between protease domain and stem region (37). Bands with a molecular weight expected for zymogen forms and N-terminal products of autocatalytic processing were indeed observed for most proteases, although with variable intensity (HAT, DESC1), and roughly comparable banding

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patterns were previously documented by us (23) and others (38,39). In general, more than one N-terminal protease fragment was observed upon expression of HAT, DESC1 and TMPRSS11A, which might reflect multiple autoprocessing events and/or processing of different protease glycoforms. Finally, it is noteworthy that N-terminal fragments of TMPRSS11B expected for autocatalytic activation were not consistently detected and it can at present not be excluded that this protease was not active in 293T cells.

The multitude of proteases able to activate HA in transfected cells raises the question which of these enzymes might contribute to viral spread in the host. A prerequisite for an important role in vivo is the expression in cells of the respiratory tract. Our results show that TMPRSS11A meets this prerequisite: Robust expression of TMPRSS11A mRNA was detected in human trachea, which is in keeping with a previous study (40). Moreover, TMPRSS11A protein was detected in the tracheal epithelium of mice, which was shown to be infected by FLUAV. Finally, it is noteworthy that TMPRSS11A activated both H1 and H3 HA, since other TTSPs, including matriptase, were shown to activate HA in a subtype-dependent manner (20-22). Collectively, our results demonstrate that TMPRSS11A activates diverse HA proteins and is expressed in trachea, a target of FLUAV infection, suggesting that the enzyme could contribute to FLUAV spread in the host and thus deserves further investigation. However, it should also be stated that other HA activating proteases, TMPRSS2 and TMPRSS4, were expressed in trachea. Thus, redundant proteases are available to FLUAV for spread in trachea and these enzymes might need to be simultaneously inhibited in order to efficiently suppress viral spread. Notably, TMPRSS11A (40) and TMPRSS2 (41) are dispensable for normal development and health of mice, suggesting that parallel blockade of these

enzymes may be possible without major unwanted side effects.

TMPRSS2 and other TTSPs able to activate FLUAV can also activate coronavirus S proteins and allow entry into cells in which the activity of the endosomal S protein-activating proteases CatB/L is blocked (23,27,30). Our finding that TMPRSS11A displays the same activity may therefore not be surprising, also taking into account that recombinant TMPRSS11A was previously shown to cleave the SARS-CoV S protein (42). It is still noteworthy that TMPRSS11A cleaved MERS-S with higher efficiency as compared to HAT and DESC1, at least upon coexpression in transfected cells, and that S protein-driven entry into TTSP-expressing cells was generally more efficient than CatB/L-dependent entry into control cells. Thus, S protein activation by TMPRSS11A and other TTSPs at or close to the cell surface might be more efficient than activation by CatB/L in host cell endosomes.

Although HA-activating proteases are in the focus of present research, comparatively little is known about the impact of their endogenous inhibitors on HA-activation. A recent study constitutes a notable exception. Dittmann and colleagues showed that expression of the cellular protease inhibitor PAI-1 (encoded by SERPINE1) is upregulated upon IFN-treatment, inhibits activation of FLUAV HA by TMPRSS2 and reduces FLUAV-associated death in experimentally infected mice (24). Although upregulation of PAI-1 expression and the ensuing antiviral effects were modest, these results suggest that endogenous protease inhibitors could impact FLUAV spread and pathogenesis. However, we observed that not all HA-activating proteases might be equally susceptible to blockade by cellular protease inhibitors. Thus, TMPRSS11A autoactivation and HA-activation were insensitive to HAI-1, a serine protease inhibitor expressed to high levels in stomach, pancreas, uterus and liver and to low

levels in the bronchial epithelium (43). In contrast, HAT and DESC1 were blocked by HAI-1, in keeping with published findings (39). These observations suggest that upon expression of certain protease inhibitors and ensuing blockade of TMPRSS2, FLUAV might be able to employ alternative HA-activating proteases for spread, which are resistant to inhibition. The mechanism underlying the differential inhibitor sensitivity remains to be clarified but one could speculate that it depends on the cellular localization of protease activation. Thus, HAT and DESC1 may activate HA mostly at the cell surface (44), where also HAI-1 is localized (43), while HA-activation by TMPRSS11A might occur in the constitutive secretory pathway and might thus be insensitive to HAI-1.

Collectively, our results identify a new HA-activator, TMPRSS11A, and demonstrate that HA-activating TTSP are differentially susceptible to cellular protease inhibitors. Thus, FLUAV cell tropism might not only be determined by TTSP expression but also by expression of protease inhibitors and by TTSP sensitivity to these inhibitors.

Experimental procedures

Cells and viruses

293T, HeLa and Huh-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, PAN Biotech), MDCK, HepG2 and Calu-3 cells were cultivated in minimum essential medium (MEM, Gibco), A549 and BEAS-2B cells were incubated in DMEM/F-12 medium (Gibco) and NCI-H292, NCI-H727 and NCI-H1299 cells were incubated in Roswell Park Memorial Institute 1640 medium (RPMI, Gibco). All media were supplemented with 10% fetal bovine serum (Biochrome) and 100 U/ml penicillin and streptomycin (PAN Biotech). The influenza A viruses A/PR/8/34 (H1N1) and A/Panama/2007/99 (H3N2) were reconstituted from previously described 8-plasmid systems

(45,46). Both viruses were propagated in the chorio-allantoic cavity of 10-day-old embryonated hen eggs (Valo Biomedia GmbH, Germany) for 48 h at 37°C, as described previously (47,48). Thereafter, the eggs were chilled overnight at 4°C and the allantoic fluid was harvested.

Plasmid preparation

The plasmids encoding HAT, DESC1, TMPRSS11B, HA of subtype H1 (A/South Carolina/1/1918) and H3 (A/Hong Kong/1/68), MERS-CoV spike protein, hepatocyte growth activator inhibitor 1 HA1-1 and DPP4 were described previously (23,30,49). The sequence encoding human TMPRSS11A was amplified by nested PCR with reverse-transcribed human trachea mRNA (Agilent Technologies) as template with the following outer primers 5'AATTTGTTTCCTTATTTCTTTGAGCCGTAGACTTCT3' (forward) and 5'ATTGCCATAAGCCTTATAAGCTATTTGGCTGTAGC3' (reverse), and inner primers 5'GCGAATTCACCATGATGTATCGGACAGTGGGATTTGGCACCCG3' (forward) and 5'CGCTCGAGTTAGATGCCTGTTTTTGAAGCAATCCAGTTTCGGTAATAAG3' (reverse). The protease sequence was inserted into plasmid pCAGGS by using the EcoRI/XhoI restriction sites, and verified by automated sequencing. The expression plasmid for TMPRSS11A with an N-terminal c-Myc-tag was generated by PCR using the above mentioned plasmid as a template and employing a previously described strategy (23). The resulting plasmids encode human TMPRSS11A isoform 2 identical to the amino acid sequence deposited in GenBank entry AAI11797.1.

Protease expression and blockade of autocatalytic activation by HAI-1

For analysis of protease expression, 293T cells seeded into 6-well plates at a density of 2.8 x10^5 cells/well were transiently transfected

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with plasmids encoding proteases equipped with an N-terminal c-Myc tag, or empty plasmid as a control. For analysis of the inhibition of auto-proteolytic activation of TTSPs by HAI-1, 293T cells were transfected with plasmids encoding proteases and increasing amount of plasmid encoding HAI-1. After overnight incubation, the medium was replaced with fresh DMEM, and at 48 h post transfection the cells were washed with phosphate-buffered saline (PBS), resuspended in 100 µl of 2x sodium dodecyl sulfate (SDS) loading buffer per well and then heated at 95°C for 30 min. All samples were separated by SDS-PAGE, and blotted onto a nitrocellulose membrane (GE Healthcare). The protease expression was detected using a mouse anti-c-Myc antibody (Biomol) as the primary antibody and a horseradish peroxidase (HRP)-coupled antibody (Dianova) as the secondary antibody. The HAI-1 expression was detected with rabbit anti-HAI-1 (Affymetrix eBioscience) as primary antibody and a horseradish peroxidase (HRP)-coupled antibody (Dianova) as secondary antibody. Expression of β-actin, detected with anti-β-actin antibody (Sigma Aldrich), served as a loading control. A self-made ECL Western blotting solution was used to detect the bound antibodies, and the image acquisition was performed with a ChemoCam Imager (Intas). For analysis of protease expression by flow cytometry, 293T cells were transiently transfected with TTSP-encoding plasmids as described above and then detached, washed with PBS, incubated with ice-cold ethanol for 10 min, and stained with a mouse anti-c-Myc antibody (Biomol) diluted in 0.1% saponin. Mouse IgG1 (R&D Systems) was used as an isotype-matched control. After 30 min of incubation with primary antibodies at 4°C, cells were washed twice with PBS and incubated for 30 min at 4°C with DyLight 647-coupled anti-mouse secondary antibodies (Dianova) diluted in 0.1% saponin. After two final washing steps, cells were fixed with 2% paraformaldehyde and staining was analyzed with an LSR II flow cytometer (BD Biosciences).

Cleavage of FLUAV HA and MERS-S by TMPRSS11A

For the detection of FLUAV HA and MERS-S cleavage by TTSPs, 293T cells were seeded in 6-well plates at a density of 2.8 x10^5 cells/well, cultured for 24 h and then calcium phosphate cotransfected with plasmids encoding FLUAV HA of the H1 or H3 subtype or MERS-S, equipped with a C-terminal V5 tag, and plasmids encoding the indicated proteases. At 16 h post transfection, the medium was changed and at 48 h post transfection, the cells were harvested in PBS and treated with PBS or 250 µg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin (Sigma Aldrich) for 10 min at 37°C and processed for Western blot analysis as described above. Expression of MERS-S was detected by staining with a mouse monoclonal antibody reactive against the C-terminal V5 tag (Invitrogen), followed by incubation with an HRP-coupled anti-mouse secondary antibody (Dianova). The FLUAV HA cleavage was detected by staining with a goat anti-FLUAV polyclonal antibody (Millipore) raised against the H1 subtype or with a rabbit anti-H3 HA serum (Immune Technology) and HRP-coupled anti-goat or anti-rabbit antibodies (Dianova), respectively. As a loading control, the expression of β-actin was detected with anti-β-actin antibodies (Sigma Aldrich). To analyze inhibition of FLUAV HA cleavage by HAI-1, 293T cells were cotransfected with plasmids encoding FLUAV H1 hemagglutinin, the indicated proteases and HAI-1 or empty vector, as described above. At 8 h post transfection, the medium was replaced with fresh DMEM, and at 48 h post transfection the cells were harvested and processed for Western blot analysis as described above.

FLUAV infection experiments

For infection experiments, 293T cells were seeded in 12-well plates at a density of 1.4×10^5 cells/well. After 24 h, the cells were transfected with expression plasmids encoding the indicated proteases or empty plasmid using the calcium phosphate transfection method. The transfection medium was replaced by fresh medium after an overnight incubation. At 24 h post transfection, the culture medium was removed and the cells incubated with infection medium (DMEM supplemented with 0.2% BSA) containing A/PR/8/34 (H1N1) at an MOI of 0.01 or 0.1 or A/Panama/2007/99 (H3N2) at an MOI of 0.1. After 1 h of incubation at 37°C, the infection medium was removed, the cells were gently washed with PBS and fresh infection medium was added. Culture supernatants were collected at 48 h post infection. The amount of infectious units within the culture supernatants was determined by focus formation assay, as described previously (50). In brief, serial 5-fold dilutions of samples were prepared in infection medium (DMEM with 1% P/S and 0.1% BSA) and added to MDCK cells. After 1 h of incubation, the medium was replaced by infection medium containing Avicel overlay and 2.5 µg/ml N-acetylated trypsin (Sigma), and the cells were incubated for 24 h. Subsequently, the cells were fixed with 4% formalin in PBS, and incubated for 1 h with anti-FLUAV (Milipore), followed by 1 h incubation with anti-goat-HRP antibodies (Dianova), and 10 min incubation with True Blue substrate (KPL). Foci were counted and viral titers were calculated as focus forming units (FFU) per ml of culture supernatant.

TMPRSS11A-mediated activation of MERS-S for virus-cell fusion

MERS-S activation was analyzed employing retroviral pseudotyping as previously described (30,51). Briefly, expression plasmids encoding MERS-S, MLV gag-pol and a firefly-luciferase harboring MLV vector were transiently cotransfected into 293T cells. At 16 h post transfection, the culture medium was replaced by fresh medium and at 48 h post transfection the supernatant was harvested, passed through 0.45-µm filters, aliquoted, and stored at −80°C. For transduction experiments, 293T cells were cotransfected with expression plasmids for DPP4 and the indicated TTSPs or an empty plasmid. The culture medium was replaced with fresh medium at 8 h post transfection, and the cells were seeded into 96-well plates at 24 h post transfection. At 48 h post transfection, cells were preincubated with dimethyl sulfoxide (DMSO) or 10 µM cathepsin B/L inhibitor MDL 28170 (Calbiochem) for 1 h and then incubated with equal volumes of MERS-S-bearing vector for 8 h. Thereafter, the medium was changed and the luciferase activities in cell lysates were determined at 72 h posttransduction employing a commercially available kit (Beetle Juice, PJK).

Quantification of TTSP mRNA transcripts in cell lines and tissues

For the assessment of TTSP mRNA expression in human cell lines (293T, A549, BEAS-2B, Calu-3, HeLa, HepG2, Huh-7, NCI-H292, NCI-H727, NCI-H1299), total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Next, 1 µg of RNA was treated with DNase I (NEB) according to the manufacturer's protocol and reverse transcribed into cDNA employing the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) following the instructions for random hexamers. In addition, commercially available RNAs cDNAs originating from various human tissues were purchased from Amsbio, BioChain, Clontech and TaKaRa (detailed sample information are summarized in the Supplementary Information section). RNA samples were first reverse transcribed into cDNA as described above.

TTSP transcript levels were analyzed by quantitative PCR (qPCR). For the different cell lines and tissues for which RNA was the starting material, 1µl of a total of 20µl cDNA was used per reaction, while for the commercially purchased cDNAs, 2 ng cDNA were subjected to qPCR analysis, as recommended by the manufacturers. All samples were analyzed in three separate reactions for mRNA transcript levels of ß-actin (housekeeping gene control), TMPRSS2, TMPRSS4 and TMPRSS11A (genes of interest) using the QuantiTect SYBR Green PCR Kit on a Rotor-Gene Q platform (both Qiagen). Primer sequences are available upon request. Further, plasmid standards ranging from 1 to 1,000,000 copies/reaction for each target were analyzed in parallel to define the limit of detection (LOD) and generate standard curves for each target to calculate the absolute copy numbers from the cycle threshold (Ct) values. Of note, copy numbers for samples that did not yield signals above the LOD were set as 0. Reactions in which water instead of sample/plasmid standard was used served as negative controls. For data normalization, the calculated copy numbers for TMPRSS2, TMPRSS4 and TMPRSS11A in each sample were divided by the respective copy number for β-actin, resulting in the relative transcript levels per β-actin transcript.

Virus, mouse strain, infection

The original stock of PR8M was obtained from Stefan Ludwig, University of Münster (PR8M, A/Puerto Rico/8/34 H1N1, Münster variant). Virus stock was amplified by infection of 10 days-old embryonated hen eggs for 48 h at 37°C. The C57BL/6J mice were purchased from Janvier, France. All mice were maintained under specific pathogen free conditions and according to the German animal welfare law. Eight weeks old females were anesthetized by intra-peritoneal injection with a mixture of Ketamine/Xylazine (100mg/ml Ketamine and 20mg/ml Xylazine)

in sterile sodium chloride solution. The doses were adjusted to the individual body weight using 200 µl per 20 g body weight. Mice were then intranasally infected with 20 µl of virus solution in sterile phosphate-buffered saline, containing 2×10^3 focus forming units (FFU).

Immunohistochemistry

Tracheae were stored in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 µm. For detection of FLUAV antigen, tissues were stained with a primary antibody against the FLUAV nucleoprotein, as described previously (52). TMPRSS11A-positive cells were identified by immunohistochemistry with anti-TMPRSS1A antibody (chicken polyclonal reactive against the TMPRSS11A C-terminus, Abcam). Binding of the primary antibody was detected using a peroxidase labeled goat-anti-chicken IgY H&L-HRP (Abcam). Peroxidase activity was revealed using 3-amino-9-ethylcafrbazole (AEC) (Sigma). Hematoxylin was used for counterstaining.

Ethics statement

All experiments in mice were approved by an external committee according to the national guidelines of the animal welfare law in Germany (BGBl. I S. 1206, 1313 and BGBl. I S. 1934). Moreover, the protocol used in these experiments has been reviewed by an ethics committee and approved by the 'Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany' (Permit Number: 3392 42502-04-13/1234).

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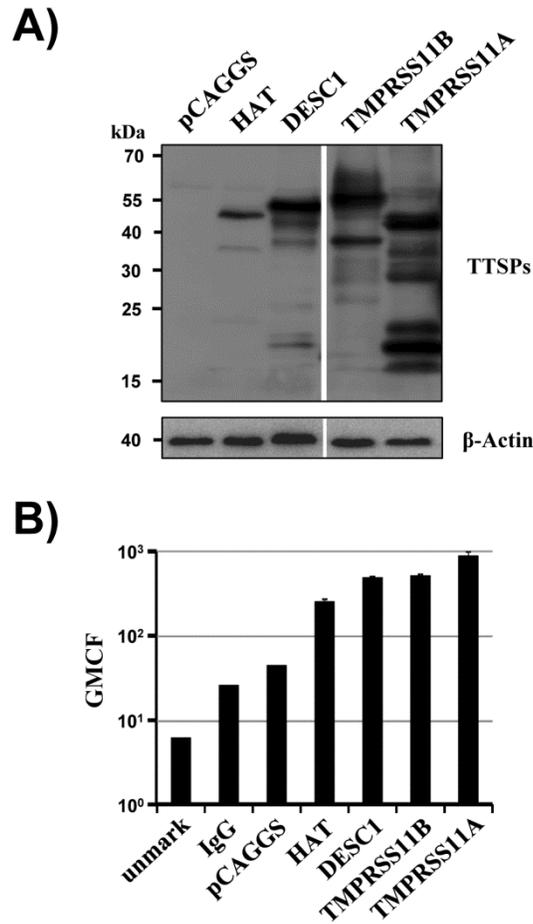
Figure 1. Expression of TMPRSS11A in transfected 293T cells. Plasmids encoding the indicated proteases equipped with an N-terminal c-Myc tag were transiently transfected into 293T cells. Empty plasmid (pCAGGS) served as a negative control. Protease expression in cell lysates was detected via Western blotting (*A*) and intracellular FACS analysis (*B*) employing anti-c-Myc antibody. Staining of empty plasmid-transfected cells with isotype-matched antibody was used as negative control for FACS analysis. The results of single blots from which irrelevant lanes were cut out are shown in panel A and were confirmed in two separate experiments. The geometric mean channel fluorescence (GMCF) measured in a representative experiment performed with triplicate samples is shown in panel B. Error bars indicate standard deviations (SD). Similar results were obtained in a separate experiment.

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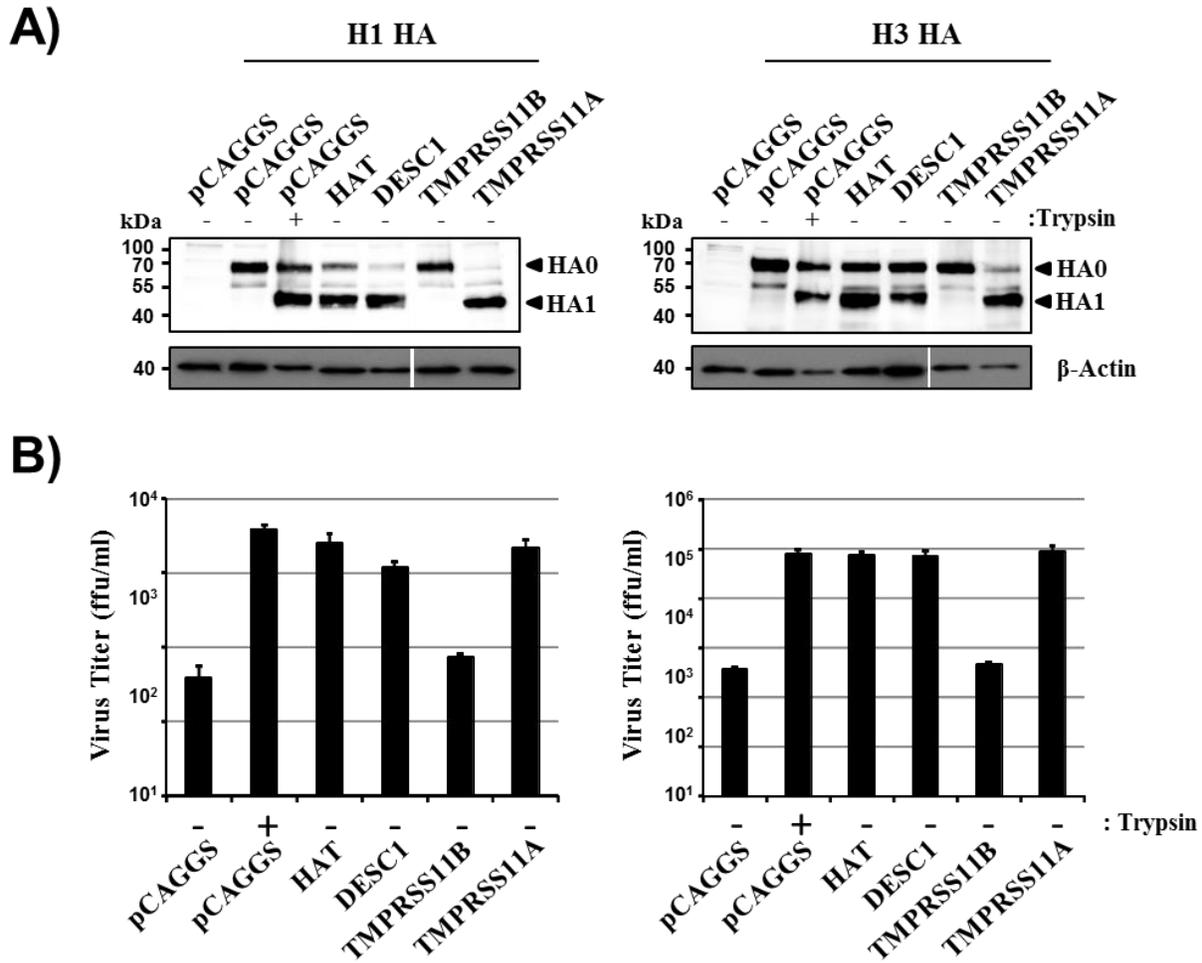
Figure 2. TMPRSS11A cleaves and activates the influenza A virus hemagglutinin. A, Expression plasmids encoding the influenza HA (H1 left panel and H3 right panel) and the indicated proteases or empty plasmid (pCAGGS) were transiently cotransfected into 293T cells. The cells were then treated with PBS or trypsin and HA cleavage was detected by Western blot analysis of cell lysates using a monoclonal antibody specific for HA. Bands corresponding to the uncleaved HA precursor protein HA0 and the cleavage product HA1 are indicated. The results of single blots from which irrelevant lanes were cut out are shown. Cuts are marked by black lines. Similar results were obtained in three independent experiments. B, The indicated proteases were transiently expressed in 293T cells and the cells were infected with influenza A virus A/PR/8/34 H1N1, left panel) at an MOI of 0.01 or A/Panama/2007/1999 (H3N2, right panel) at an MOI of 0.1 and either treated with trypsin or PBS. At 48 h post infection the release of infectious particles into the culture supernatants was quantified by focus formation assay. Error bars indicate SD. Similar results were obtained in three independent experiments. HA, hemagglutinin.

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A)
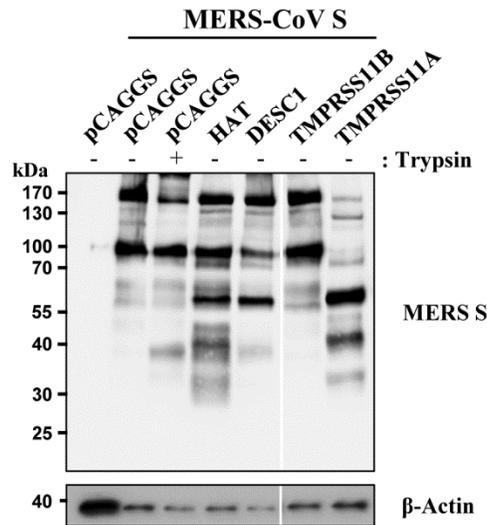
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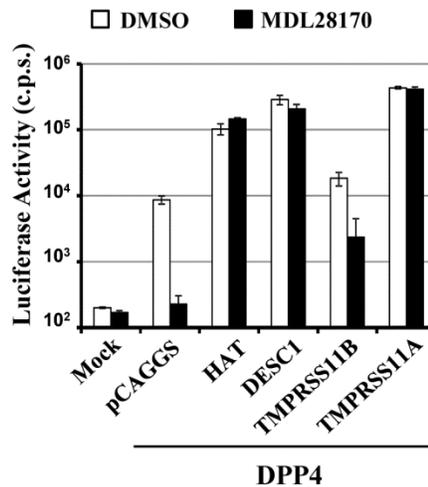
Figure 3. TMPRSS11A cleaves and activates the MERS-CoV spike protein. *A*, Expression plasmids encoding MERS-S equipped with a C-terminal V5 antigenic tag and the indicated proteases or empty plasmid (pCAGGS) were transiently cotransfected into 293T cells. The S protein cleavage was detected by Western blot analysis of cell lysates using a monoclonal antibody specific for the V5-tag. The results of a single blot are shown, from which irrelevant lanes were removed. Similar results were obtained in two separate experiments. *B*, Retroviral particles bearing MERS-S were used for transduction of 293T cells, which were transfected to express the viral receptor, DPP4, and the indicated proteases and which were preincubated with 10 µM of the cathepsin B/L inhibitor MDL-28170 or DMSO. Cells transfected with empty plasmid (pCAGGS) served as negative control. Luciferase activities in the cell lysates were determined at 72 h post transduction. Error bars indicate SD. Similar results were obtained in a separate experiment. c.p.s., counts per second. MERS-S, MERS-coronavirus spike protein.

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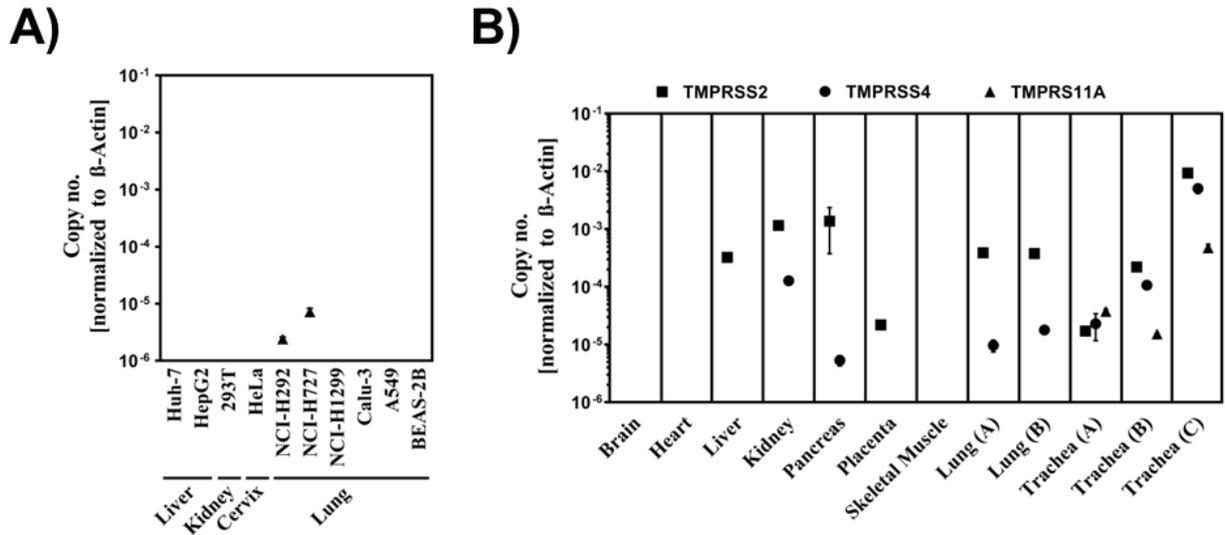
Figure 4. TMPRSS11A transcript levels in human cell lines, organs and tissues. *A*, Total cellular RNA was extracted from the indicated cell lines, reverse transcribed into cDNA and the cDNA analyzed for TMPRSS11A transcripts by quantitative PCR (qPCR). The mean copy numbers measured for triplicate samples are shown, errors bars indicate standard deviation. Reactions containing water instead of cDNA served as controls. *B*, Commercially available cDNAs and cDNA produced from commercially available RNA originating from the indicated human organs and tissues were analyzed for TMPRSS2 (boxes), TMPRSS4 (circles) and TMPRSS11A (triangles) transcripts by qPCR. Both panels show the relative copy numbers (normalized against ß-actin) measured for triplicate samples, errors bars indicate SD. Lung_A and lung_B represent lung cDNAs obtained from two different sources. Similarly, trachea A, B and C represent trachea cDNA/RNA obtained from three different sources. Mean copy numbers measured for triplicate samples with SD are shown. Dashed lines indicate the limit of detection (i.e. the lowest dilution of the plasmid standards that gave a positive signal).

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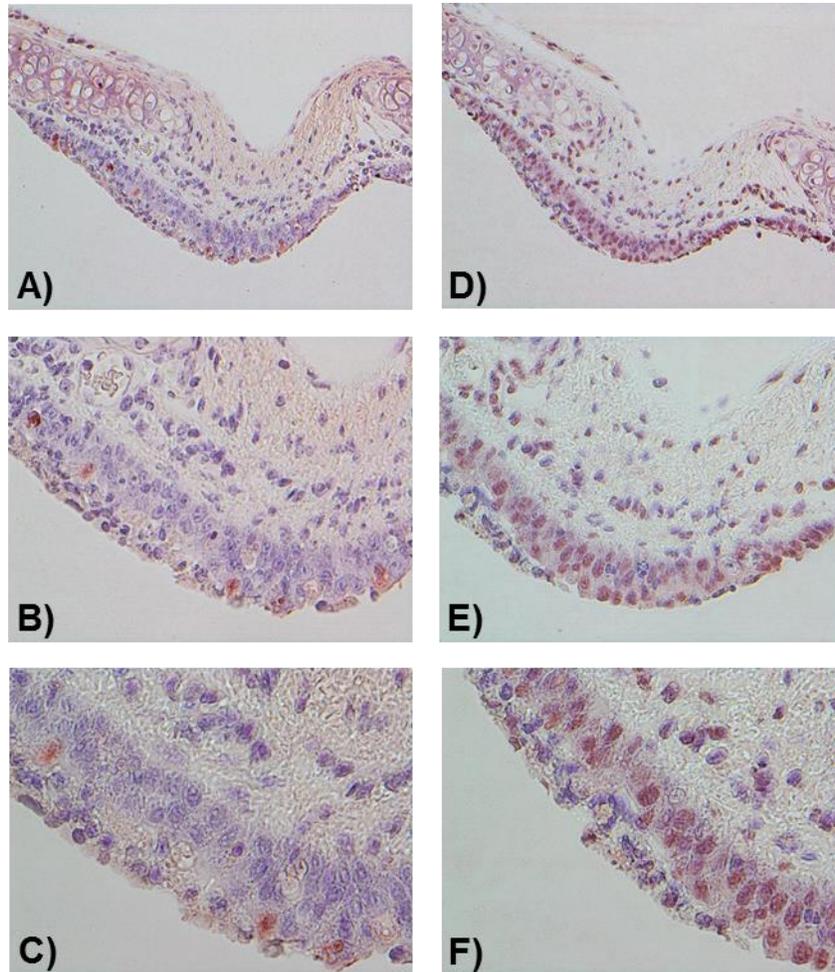
Figure 5. FLUAV infected and TMPRSS11A-positive cells are located in epithelial cells of mouse trachea. Serial trachea sections were stained with anti-NP (*A-C,* brown signal) and anti-TMPRSS11A (*D-F,* brown signal) antibodies at three days after intranasal infection of C57BL/6J mice with 2 x 10^3 FFU of A/PR/8/34 (Münster variant, PR8M). Hematoxylin (violet) was used for counterstaining. Staining was analyzed by microscopy. Original magnification x 20 (*A*, *D*), x 40 (*B*, *E*), and x 60 (*C*, *F*).
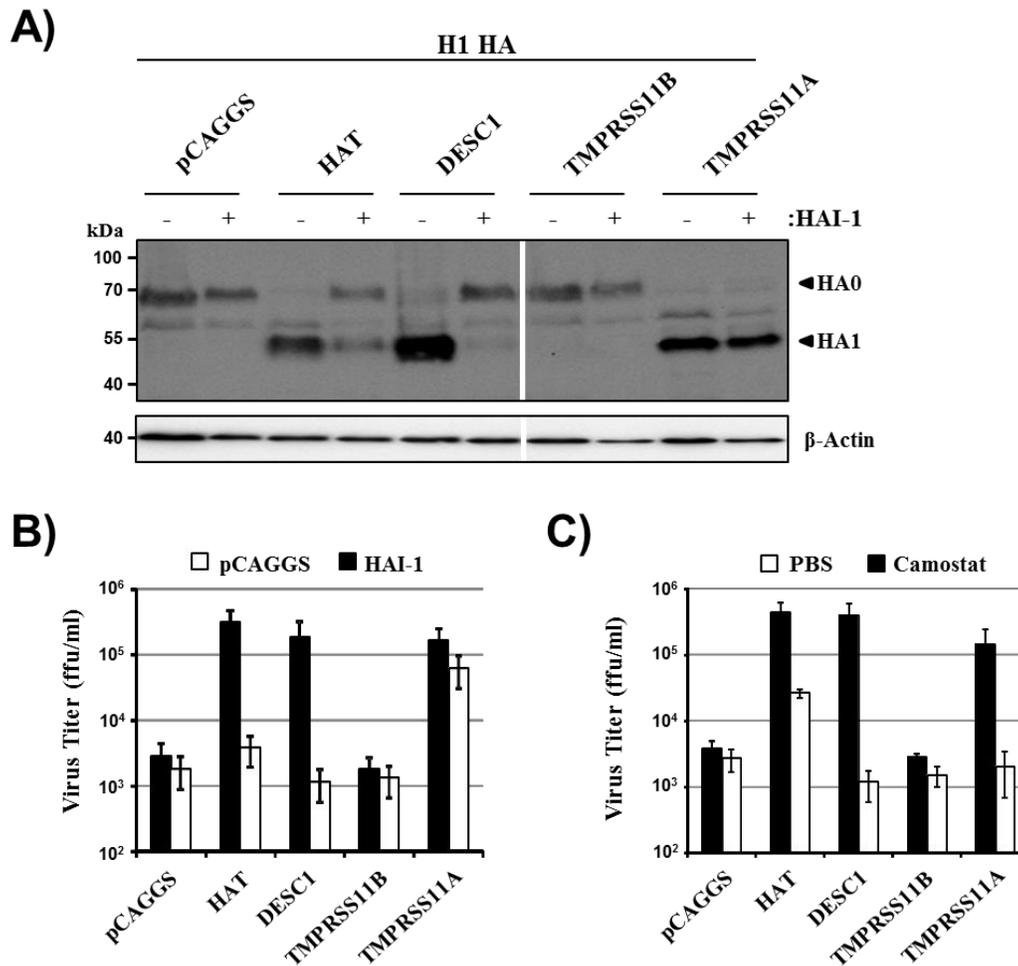
Figure 6. Hemagglutinin activation by TMPRSS11A is resistant HAI-1. *A*, Expression plasmids encoding HA, the indicated proteases or empty plasmid (pCAGGS) and hepatocyte growth factor activator inhibitor type 1 (HAI-1) were transiently cotransfected into 293T cells. The cleavage of HA was determined by Western blot analysis of cell lysates using a polyclonal antibody against FLUAV. Bands corresponding to the uncleaved HA precursor protein HA0 and the cleavage product HA1 are indicated. The results of a single blot from which irrelevant lanes were removed are presented. Similar results were obtained in three separate experiments. *B*, Plasmids encoding the indicated proteases and HAI-1 or empty plasmid as control were transiently cotransfected into 293T cells and the cells were infected with influenza A virus A/PR/8/34 (H1N1) at an MOI of 0.1. At 48 h post infection, viral spread was quantified as the release of infectious particles into the culture supernatants, as measured by a focus formation assay. The results of a single experiment carried out with triplicate samples are shown. Error bars indicate SD. Similar results were obtained in three separate experiments. *C*, 293T cells were transiently cotransfected with plasmids encoding the indicated proteases, infected with A/PR/8/34 (H1N1) at an MOI of 0.1 and treated with 100 μM of camostat mesylate. The viral titers in culture supernatant were quantified by focus formation assay at 48h post infection. The results of a single experiment carried out with triplicate samples are shown. Error bars indicate SD. Similar results were obtained in a separate experiment.

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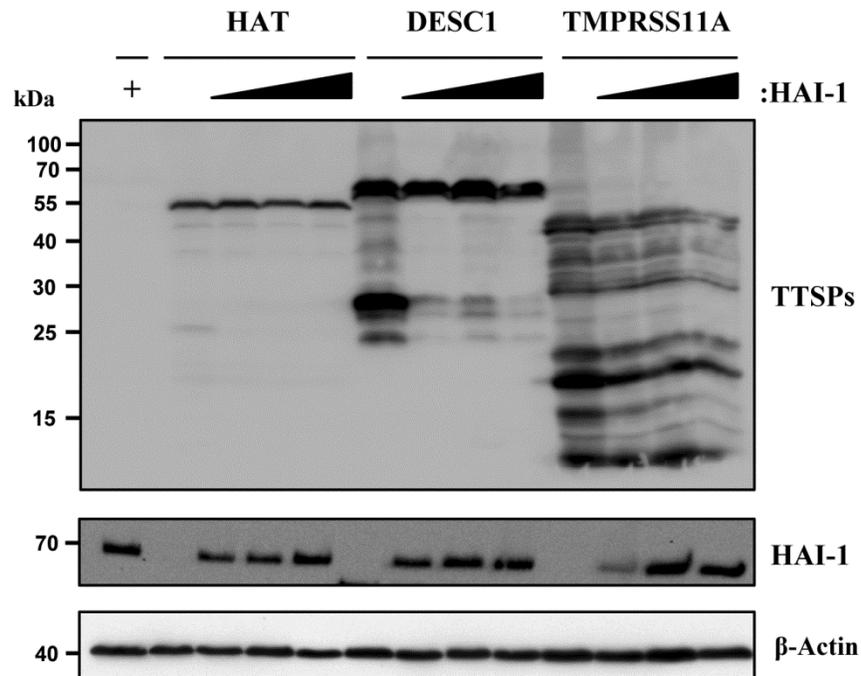
Figure 7. HAI-1 blocks HAT and DESC1 autoactivation and hemagglutinin cleavage but is inactive against TMPRSS11A. 293T cells were transiently cotransfected with plasmids encoding the indicated proteases jointly with increasing amounts of HAI-1 encoding plasmid or empty plasmid. At 48 h post transfection expression of TTSPs was analyzed by Western blot, using a monoclonal antibody specific for the c-Myc-tag. The results were confirmed in two separte experiments with different plasmid preparations.

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TMPRSS11A activates the influenza A virus hemagglutinin and the MERS coronavirus spike protein and is insensitive against blockade by HAI-1

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