Multi-level inhibition of coronavirus replication by chemical ER stress

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Coronaviruses (CoVs) are important human pathogens for which no specific treatment is available. Here, we provide evidence that pharmacological reprogramming of ER stress pathways can be exploited to suppress CoV replication. The ER stress inducer thapsigargin efficiently inhibits coronavirus (HCoV-229E, MERS-CoV, SARS-CoV-2) replication in different cell types including primary differentiated human bronchial epithelial cells, (partially) reverses the virus-induced translational shut-down, improves viability of infected cells and counteracts the CoV-mediated downregulation of IRE1α and the ER chaperone BiP. Proteome-wide analyses revealed specific pathways, protein networks and components that likely mediate the thapsigargin-induced antiviral state, including essential (HERPUD1) or novel (UBA6 and ZNF622) factors of ER quality control, and ER-associated protein degradation complexes. Additionally, thapsigargin blocks the CoV-induced selective autophagic flux involving p62/SQSTM1. The data show that thapsigargin hits several central mechanisms required for CoV replication, suggesting that this compound (or derivatives thereof) may be developed into broad-spectrum anti-CoV drugs.
Coronaviruses are enveloped plus-strand RNA viruses with a broad host range, including humans. The four seasonal human CoVs (HCoV-229E, -NL63, -HKU1, -OC43) generally cause a spectrum of (mild) symptoms that are mainly restricted to the upper respiratory tract. In contrast, the three highly pathogenic CoVs that emerged from animal reservoirs over the past two decades are frequently associated with significant disease burden and mortality in humans. The latter include the severe acute respiratory syndrome CoV (SARS-CoV), SARS-CoV-2, SARS-CoV-2, and Middle-East respiratory syndrome CoV (MERS-CoV).

The current SARS-CoV-2 pandemic highlights the urgent need to identify new antiviral strategies, including drugs that target the host side. CoVs impose multiple functional but also structural changes to a wide range of cellular pathways and there is increasing evidence that some of these pathways may be exploited therapeutically.

In common with other cellular stress conditions, including infections by diverse pathogens, CoVs are known to activate the NF-κB, JNK, and p38 MAPK pathways and to reprogram host cell transcriptional programs. In addition, they induce the formation of replicative organelles (ROs), an intracellular network of convoluted membranes (CMs), and double-membrane vesicles (DMV) that harbor the viral replication/transcription complexes (RTC) and shield these complexes from recognition by cellular defense mechanisms. The origins of the membranes used for RO are still under debate but can be linked, at least partly, to ER- and autophagy-related processes. The combination of these and other events leads to cell damage and cell death upon virus budding and release within a few days. The virus-induced cellular changes are associated with an activation of the unfolded protein response (UPR), which is evident from a profound transcriptomic endoplasmic reticulum (ER) stress signature, as reported for cells infected with HCoV-229E.

The ER is critically involved in surveying the quality and fidelity of membrane and secreted protein synthesis, as well as the folding, assembly, transport, and degradation of these proteins. The accumulation of unfolded or misfolded proteins in the ER lumen leads to ER stress and UPR activation, thereby slowing down protein synthesis and increasing the folding capacity of the ER. As a result, cellular protein homeostasis can be restored and the cell survives. If this compensatory mechanism fails, ER stress pathways can also switch their functions, inducing oxidative stress and resulting in cell death.

The system relies on sensors residing in the ER membrane which include the protein kinase R (PKR)-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and cyclic AMP-dependent transcription factor 6α (ATF6α). PERK and IRE1α are Ser/Thr kinases whose conserved N termini are oriented towards the ER lumen. In non-stressed cells, the highly abundant major ER kinases whose conserved N termini are oriented towards the ER include the severe acute respiratory syndrome CoV (SARS-CoV), SARS-CoV-2, SARS-CoV-2, and Middle-East respiratory syndrome CoV (MERS-CoV).

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Active PERK phosphorylates the eukaryotic translation initiation factor 2 (eIF2) subunit α to shut down translation and also activates ATF4, the master transcription factor orchestrating ER stress-induced genes. Phosphorylated IRE1α activates its own RNase domain to generate spliced (s)XBP1 protein, a multifunctional transcriptional regulator responsible for adaptive responses or cell death. The specific function(s) of yet another ER stress-activated transcription factor, ATF3, is less well understood. Generally, the various branches of the UPR act in concert, allowing a multitude of potential outcomes, ranging from the compensation of ER stress and restoration of proteostasis to cell death.

The activation of ER stress by microbial and viral infections has been widely observed. However, with few exceptions, it remains to be studied how this response is shaped in an infectious agent-specific manner and whether or not these responses are beneficial or detrimental to the host. Moreover, there is a lack of knowledge on CoV-mediated (de)regulation of ER stress components at the protein level. The latter is important because CoVs, in common with many RNA viruses, are known to cause a global shutdown of host protein synthesis.

Here, we report that CoV infection activates ER stress signaling and induces UPR components at the mRNA level while suppressing them at the protein level. Strikingly, the well-known chemical activator of the UPR, thapsigargin, exerts a profound antiviral effect in the lower nanomolar range on three different CoVs in four different cell types, including human primary bronchial epithelial cells. A detailed proteomics analysis revealed multiple thapsigargin-regulated pathways and a network of proteins involved in ER quality control (ERQC) or ER-associated degradation (ERAD) that are suppressed by CoV but (re)activated by chemically stressed infected cells. Additionally, we discovered that thapsigargin blocks the autophagic flux in CoV-infected cells. Taken together, these data provide important new insight into central factors involved in CoV replication and open new avenues for targeted CoV antivirals.

**Results**

To investigate how CoVs modulate ER stress components at the mRNA compared to the protein level, we determined the expression levels of 166 components of the ER stress pathway KEGG hsa04141 “protein processing in ER” in human HuH7 liver cells, a commonly used cellular model for CoV replication, in response to infections with HCoV-229E and MERS-CoV, respectively. For untreated HuH7 cells, we obtained mRNA (by RNA-seq) and protein (by LC-MS/MS) expression data for 119 components which revealed a positive correlation between mRNA and protein abundances. However, in cell lysates obtained at 24 h post infection (p.i.), this effect was largely lost. Pearson correlation matrix confirmed a progressive loss of correlation between mRNA levels and protein levels for this pathway over a time course from 3 to 24 h p.i. (Fig. 1b). Thus, out of 39 (for HCoV-229E) or 56 (for MERS-CoV) ER stress factors that were found to be regulated at the mRNA level, only a few remained regulated at the protein level at late time points (Fig. 1c, shown as a projection on KEGG hsa04141 in Supplementary Fig. 1).

To determine the functional consequences of this opposing regulation at the mRNA and protein levels in CoV-infected cells, we focused on HCoV-229E and assessed key regulatory features of the ER stress pathway as shown in Fig. 2a. As a reference, we included samples from cells exposed to thapsigargin, a compound that has been widely used to study prototypically activated ER stress mechanistically. This setup included experiments, in which thapsigargin and virus were added simultaneously to the cell culture medium (followed by a further incubation for 24 h) or thapsigargin was added to the cells at 8 h p.i. for 16 h (Fig. 2b). The presence of thapsigargin in the growth medium resulted in a major drop in viral titers by more than 150-fold (from 9.18 × 10^6 to 5.7 × 10^4 pfu/ml), which was paralleled by reduced amounts of viral RNA isolated from thapsigargin-treated, HCoV-229E-infected cells at 24 h p.i. (Fig. 2c). Immunofluorescence analysis of HCoV-229E-infected cells treated with thapsigargin...
confirmed the impaired formation of functional viral RTCs as shown by the reduced levels of both double-stranded RNA (an intermediate of viral RNA replication) and nonstructural protein (nsp) 8 (an essential part of the viral RTC) (Fig. 2d).

A strong suppression of viral replication was also demonstrated by the reduced protein levels observed for the nucleocapsid (N) protein (a major coronavirus structural protein) as well as nsp 8 and 12, both representing essential components of the viral replication complex36 (Fig. 2e, f). In all cases, the antiviral effect of thapsigargin remained readily detectable when the compound was added at 8 h pi, suggesting that it does not prevent viral entry but rather suppresses intracellular pathways required for efficient viral RNA replication and virus formation and release, or, activates unknown antiviral effector systems (Fig. 2c, e, f).

**Fig. 1 CoVs uncouple mRNA and protein levels of ER stress components in infected cells.** a–c HuH7 cells were left untreated or were infected with HCoV-229E or MERS-CoV (MOI = 1) for 3, 6, 12, or 24 h. Transcriptomic and proteomic data were derived from samples obtained at the indicated time points post infection (p.i.). Subsequently, mRNA and protein expression values for the KEGG pathway “protein processing in endoplasmic reticulum” from two biologically independent experiments were extracted and used for further analysis. a Scatter plots show mean normalized protein/mRNA expression values for the number (n) of expressed components, fitted linear regression lines, 95% confidence intervals, and coefficients of determination for non-infected HuH7 cells and HuH7 cells infected with virus for 24 h. P values were calculated from an F test to test the null hypothesis that the overall slope is zero. b Correlation matrix of Pearson’s r across all conditions. All corresponding p values are provided in the Source data. c The heatmaps show mean ratio values of differentially expressed mRNAs or proteins based on significant differences (fold change ≥ 2, adjusted p ≤ 0.01) calculated from the replicates by moderated t-tests. See also Supplementary Fig. 1 for pathway mappings of mRNA and protein data and Source data for complete data sets.
Next, we investigated ER stress signaling under these conditions. Both virus and thapsigargin were confirmed to activate the PERK branch of ER stress (Fig. 2e, f), as shown by the retarded mobility of PERK in SDS gels (indicating multisite phosphorylation) and by phosphorylation of the PERK substrate eIF2α at Ser51 (Fig. 2e, f). HCoV-229E infection led to a weak but significant decrease of PERK (mean 71 ± 15%) and eIF2α (mean 67 ± 13%) levels compared to the controls. Infection also caused an approximately twofold (mean 42 ± 22%) reduction of BiP expression (Fig. 2e, f). In contrast, long-term thapsigargin treatment (for 16 h or 24 h) caused a 3–4-fold increase of BiP expression, also in HCoV-229E-infected cells, thus reversing the suppression by viral infection (Fig. 2e, f). Similarly, thapsigargin treatment for 16 h or 24 h caused a 1.5–2-fold increase in IRE1α expression (but not phosphorylation), again also in infected cells (Fig. 2e, f). In this set of experiments, ATF3 proved to be the only protein that was induced by the virus alone (Fig. 2e, f), while the expression levels of ATF4 remained largely unchanged (Fig. 2e, f).

To reveal the role of PERK in these effects, we treated cells with the protein kinase inhibitor GSK2656157. This compound suppressed PERK autophosphorylation, PERK activity (on eIF2α), and CoV replication without having a major impact on cell viability (Supplementary Fig. 2a–c). However, inhibition of viral replication by GSK2656157 was less effective than thapsigargin and, in combined treatments, it did not abolish the thapsigargin-mediated suppression of N protein expression and virus replication, placing the thapsigargin-mediated viral suppression downstream of PERK (Supplementary Fig. 2d, e).

These data show that both CoV infection and chemicals like thapsigargin activate ER stress through the same proximal PERK pathway, although they affect downstream cellular outcomes differentially. The restoration of BiP and IRE1α levels by long-term thapsigargin treatment further suggests that the CoV-induced block of inducible host factors is reversible and can be reprogrammed by a (presumably protective) thapsigargin-mediated response. Our comparative analyses of viral replication and host...
response, along with the effects of PERK inhibition, lead us to conclude that chemically and virus-induced forms of ER stress, although proceeding through the same core PERK pathway, do not simply potentiate each other but rather (somewhat counter-intuitively) counteract each other.

To explore a potential pharmacological exploitation of this effect, we assessed the (half-maximal) effective and cytotoxic concentrations (EC₅₀, CC₅₀) of the combined thapsigargin treatment and virus infection, because both conditions are known to promote cell death. HCoV-229E replication was suppressed with an EC₅₀ of 1 nM (Fig. 3a), as judged by virus titration of cell culture supernatants obtained from these cells. At 24 h p.i., the cell viability of HCoV-229E-infected HuH7 cells was only marginally reduced (mean 90.02 ± 12.32%) (Fig. 3b, upper graph). After 24 h of incubation, thapsigargin decreased cell viability in a dose-dependent manner with a CC₅₀ of 5.9 µM in line with previous reports (Fig. 3b, middle graph, Fig. 3c). The combination of thapsigargin treatment and HCoV-229E infection did not cause additional cytotoxicity as shown by a nearly identical CC₅₀ of 4.6 µM (Fig. 3b, lower graph, Fig. 3c). At 1 µM thapsigargin, i.e., a concentration shown to completely abolish viral protein translation and production of infectious virus progeny (see above), the cell viability of cells infected with HCoV-229E and treated with thapsigargin was 76.6 ± 7.9% (Fig. 3b, c).

Furthermore, the antiviral effects of thapsigargin remained detectable for three days after a single dose, with a profound reduction of viral titers and RNA levels by several orders of magnitude (Fig. 3d). After three days, 50% of the cells infected with HCoV-229E and treated with thapsigargin were still viable, compared to only 20% of cells that survived the infection in the absence of thapsigargin (Fig. 3d). The data suggest that thapsigargin exerts long-lasting antiviral effects at concentrations well below its cytotoxic concentrations.

To further characterize the metabolic state of the cells under the conditions used in these experiments, we investigated protein de novo synthesis. Newly produced proteins were quantified by in vivo puromycinylation tagging of nascent protein chains followed by immunoblotting using anti-puromycin antibodies. HCoV-229E infection was found to shut down cellular protein biosynthesis by 90.3 ± 5.4%, while treatment with thapsigargin for
1 h led to a translational shut-down by 94.3 ± 4.3% (Fig. 3e). However, in infected cells, the simultaneous or delayed addition of thapsigargin restored (or rescued) protein biosynthesis to approximately 50% of the level observed in untreated cells (Fig. 3e). These data demonstrate that, although both viral infection and thapsigargin treatment (individually) induce ER stress and cause a translational shut-down, their combination shows no additive harmful effects on the cells. On the contrary, their combination appears to have opposing effects that result in a partial restoration of the cellular metabolic capacity while retaining a profound antiviral effect.

We next assessed if these effects were cell type or virus-specific. In line with the results described above, the antiviral effects of thapsigargin, the reconstitution of the BiP and IRE1 levels, and the lack of additional cytotoxicity in infected cells could be confirmed for diploid MRC-5 embryonic lung fibroblasts infected with HCoV-229E (Fig. 4a–d), as well as for HuH7 cells infected with MERS-CoV and Vero E6 African green monkey kidney epithelial cells infected with SARS-CoV-2 (Fig. 4e–k and Supplementary Fig. 3a, b). MERS-CoV and SARS-CoV-2 replication were suppressed by thapsigargin with an EC$_{50}$ of 4.8 and 260 nM, respectively (Fig. 4i, j), while the CC$_{50}$ for thapsigargin in Vero E6 cells was 18.25 µM (based on MTT assay) or 20.27 µM (based on ATPlitez assay) (Fig. 4k), resulting in selectivity indices (SI, CC$_{50}$/EC$_{50}$) of 1229 for MERS-CoV and 70 (MTT) to 78 (ATPlitez) for SARS-CoV-2, respectively. In contrast to thapsigargin, the inhibition of PERK by GSK2656157 required higher concentrations in the low micromolar range to attain a significant drop of MERS-CoV replication, once again supporting the exceptional efficacy of thapsigargin (Supplementary Fig. 2f).

Next, we tested potential antiviral activities of thapsigargin against other RNA viruses and used (as a reference) remdesivir, an adenosine analog that inhibits coronavirus RNA-dependent RNA polymerases. We found that thapsigargin suppressed influenza A virus (IAV) but not poliovirus replication (Supplementary Fig. 4a, b). As shown in Supplementary Fig. 5a–c, remdesivir reduced the replication of HCoV-229E (EC$_{50}$ < 10 nM), MERS-CoV (EC$_{50}$ = 5.3 nM), and SARS-CoV-2 (EC$_{50}$ = 2.38 µM) in HuH7 and Vero E6 cells at non-toxic concentrations (Supplementary Fig. 5d, e), in line with previously published studies. The data show that thapsigargin has potent antiviral activity against another family of enveloped RNA viruses and is at least as effective as remdesivir against HCoV-229E and MERS-CoV, while thapsigargin is approximately 10-times more effective than remdesivir against SARS-CoV-2.

To corroborate these observations in a physiologically more relevant system, we established cell cultures of differentiated normal human bronchial epithelial (NHBE) cells. After the initial expansion, the cells were exposed to air-liquid interfaces allowing their differentiation into various airway cell types, which was validated by fluorescence microscopy using antibodies detecting tight junctions and marker proteins specific for the goblet, ciliated and basal cells, respectively (Fig. 5a, b). As shown in Fig. 5c, thapsigargin inhibited the replication of all three coronaviruses included in this experiment (HCoV-229E, MERS-CoV, SARS-CoV-2) in a dose-dependent manner in NHBE cells obtained from different donors. Cell viability following thapsigargin treatment (as judged by measuring the integrity of the epithelial monolayer using TEER) ranged between 70 and 80% after 72 h (Supplementary Fig. 5f). Similar to the cell lines used before, SARS-CoV-2 was found to be slightly less sensitive to thapsigargin treatment. Importantly, in the presence of 1 µM thapsigargin, no infectious virus progeny of any of the three CoVs was detectable at later time points p.i. (Fig. 5c).

To characterize the underlying molecular mechanisms responsible for the observed antiviral effects of thapsigargin, we focused on the two highly pathogenic coronaviruses, MERS-CoV and SARS-CoV-2, for which, to our knowledge, no side-by-side comparison of proteomic changes has been reported at the time of our study. The large-scale proteomic study included (i) untreated cells and cells that were (ii) infected with MERS-CoV, (iii) infected with SARS-CoV-2, (iv) treated with thapsigargin, or, (v and vi) infected with one of these viruses in the presence of thapsigargin. We used label-free quantification to determine the expression levels of >5000 protein IDs from total cell extracts.

In a systematic approach, we identified differentially expressed proteins (DEPs) based on pairwise comparisons of proteins obtained from untreated cells, virus-infected cells, or thapsigargin-treated cells using a p value of −log$_{10}$ (p) ≥ 1.3 as cut-off. As visualized by Volcano plot representations, MERS-CoV infection suppressed 412 (at 12 h p.i.) and 1171 proteins (at 24 h p.i.), respectively, and increased the levels of 150 proteins (at 12 h p.i.) and 508 proteins (at 24 h p.i.), respectively (Fig. 6a, b), while SARS-CoV-2 suppressed the expression of 250 proteins at 12 h p.i. and 159 proteins at 24 h p.i. and increased the expression of 224 proteins at 12 h p.i. and 63 proteins at 24 h p.i. (Fig. 6c, d). Thapsigargin treatment alone suppressed induced large numbers of proteins in HuH7 cells (918 down, 893 up at 12 h; 1711 down, 958 up at 24 h) and in Vero E6 cells (225 down, 191 up at 12 h; 249 down, 162 up at 24 h) (Fig. 6a–d). As expected, this analysis also identified viral proteins as the most strongly regulated DEPs. A comparison of virus-infected cells with virus-infected cells treated with thapsigargin revealed a complete suppression of all viral proteins and a large number of proteins with increased expression in thapsigargin-treated cells infected with MERS-CoV (843, 12 h p.i.; 1208, 24 h p.i.; red groups of proteins) or SARS-CoV-2 (299, 12 h; 362, 24 h; red groups of proteins) (Fig. 6a–d, right graphs). Also, similar numbers of proteins were identified with higher expression in virus-infected cells compared to virus-infected cells treated with thapsigargin (Fig. 6a–d, right graphs; blue groups of proteins). Together, these data lead us to conclude that thapsigargin causes a profound shift in protein expression in infected cells that likely contributes to the antiviral effects of this compound.

We then devised a bioinformatics strategy to identify patterns of co-regulated or unique pathways and link deregulated protein sets identified in these data to specific (known) biological functions. As shown schematically in Fig. 6e, we sorted the DEPs from each of the four groups shown in Fig. 6a–d into four multiple gene ID lists, annotated the gene IDs to biological pathways, and generated hierarchically clustered heatmaps of the top 100 differentially enriched pathway categories for the 12 h p.i. and 24 h p.i. time points of MERS-CoV and SARS-CoV-2-infected cells, respectively, versus thapsigargin-treated cells by over-representation analysis (ORA) using Metascape software. In this analysis, the groups of up- or downregulated proteins were kept separate to preserve information on whether specific DEPs belonging to particular overrepresented pathway terms were regulated in the same or opposite direction. Inspection of the four top 100 clustered heatmaps shows many similarities but also differences in pathways and their enrichment p values in response to virus infection or thapsigargin, with the combined data revealing the complexity of cellular responses to CoV infections or chemical stressors (Supplementary Figs. 6, 7). By condensing this information to the top 5 pathways for up- or downregulated DEPs, we found that many of the most highly enriched categories are related to RNA, DNA, metabolic functions and localization (Supplementary Fig. 8a). We then combined the 400 pathway categories and searched this list for identical or unique GO terms in response to MERS-CoV, SARS-CoV-2, or thapsigargin. By filtering 229 pathways (out of 400) with enrichment p values of log$_{10}$ (p) ≤ −3, we found 36 pathway categories shared by both viruses and by thapsigargin, which are mostly related to RNA,
folding, stress, and localization (Fig. 6f and Supplementary Fig. 8b). Fifty-two pathway categories unique to thapsigargin almost exclusively represented metabolic and biosynthetic pathways as shown for the top 20 overrepresented pathways containing up- or downregulated DEPs, suggesting that thapsigargin on its own, unlike CoV infection, initiates a broad metabolic response (Fig. 6f and Supplementary Fig. 8b).

This raised the question of whether the thapsigargin effects were retained in infected cells or, alternatively, drug-sensitive pathway patterns were reprogrammed (or masked) by the virus infection. To address this point, we pooled all pathways enriched under virus + thapsigargin conditions and compared them to virus infection or thapsigargin treatment alone. 59% (147 out of 249) pathway terms were shared by these three conditions, reflecting multiple stress-related catabolic, RNA regulatory, and vesicle or autophagy processes (Fig. 6g, h). 20 pathway terms were unique to the virus + thapsigargin situation. They primarily mapped to specific splicing, signaling (TORC, RHOA, ARF3) and transport/localization pathways (Fig. 6g, h). The 37 categories shared by virus + thapsigargin and thapsigargin conditions but not detectable in cells infected with virus (only) recapitulate the thapsigargin-regulated metabolic pathways (metabolism of...
Fig. 4 Thapsigargin inhibits the replication of high- and low-pathogenic human coronaviruses in multiple cell types. a–d Human embryonic MRC-5 lung fibroblasts were infected with HCoV-229E according to the scheme shown in Fig. 2b. a Viral titers (upper graph, five biologically independent experiments) and expression of viral S gene-encoding RNAs (lower graph, four biologically independent experiments). b, c Expression of viral and host cell proteins. b Shows one representative immunoblot of total cell extracts and c shows quantification from four or more biologically independent experiments. d Cell viability was analyzed and quantified as described in the legend of Fig. 3 (four biologically independent experiments). e–j Similarly, HuH7 cells or Vero E6 African green monkey kidney epithelial cells were infected with MERS-CoV (MOI = 0.5) or SARS-CoV-2 (MOI = 0.5) for 12 h or 24 h in the presence/absence of 0.4 µM or 1 µM thapsigargin. e, f Show viral titers and g, h display representative images of the corresponding expression of MERS-CoV/SARS-CoV-2 nucleocapsid (N) and host cell proteins, respectively (three biologically independent experiments). i Dose-dependent suppression of MERS-CoV-2 replication by thapsigargin in HuH7 cells infected with an MOI of 0.5 (upper graph, two or more biologically independent experiments) and the estimated EC_{50} concentration calculated from the mean values (lower graph). j Dose-dependent suppression of SARS-CoV-2 replication by thapsigargin in Vero E6 cells infected with an MOI of 0.5 (upper graph, three or more biologically independent experiments) and the estimated EC_{50} concentration from the mean values (lower graph). k The CCR_{5} of thapsigargin in Vero E6 cells was calculated by MTT or APTitre assays as described in the legend of Fig. 3b. c, d Data are from three or more biologically independent experiments. All bar graphs show means ± s.d.; asterisks indicate p values (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001) obtained by two-tailed unpaired t-tests (a, c, e, f, j) or ordinary one-way ANOVA (i). See Supplementary Figs. 3–5 for quantifications from replicates for MERS-CoV/SARS-CoV-2 immunoblot experiments and for further inhibitor data.

monosaccharide, cysteine, glutathione, methionine, fructose, mannose, pyruvate, TCA cycle, ERAD pathway) (Fig. 6g, h). For some of these pathways (e.g., ERAD, monokarboxylic acid metabolism), some DEPs were induced while others were repressed, indicating remodeling of pathway functions at the protein level (Fig. 6g, h). The 36 pathway terms that were absent from thapsigargin group of terms (groups 15, 9, 12 of the Venn diagram shown in Fig. 6g) represent a distinct set of terms, most of which being related to nucleotide and DNA-related processes, such as DNA repair, DNA unwinding, chromatin silencing (Fig. 6g, h). In summary, the functional analysis of DEPs at the level of differentially enriched pathway categories shows that the antiviral effects of thapsigargin strongly correlate with the activation/suppression of a range of metabolic programs.

The enriched pathway terms provided important overarching information on shared and unique biological processes but not necessarily encompassed identical sets of DEPs as exemplified by the twelve pathways shown in Fig. 6i. We, therefore, refined our analysis to the individual component level to identify proteins with similar regulation between both viruses across both cell types. The proteomes of HuH7 and Vero E6 cells overlap by 57% (Fig. 6g, h). In this group, only 43 identical proteins were found to be deregulated by both MERS-CoV and SARS-CoV-2 (Fig. 7b, left Venn diagrams). However, under thapsigargin + virus conditions, 120 proteins were upregulated and 63 proteins were downregulated (Fig. 7b, right Venn diagrams). Using the top 50 DEPs as an example, it becomes apparent that the majority of proteins are regulated into the same direction by thapsigargin alone; demonstrating that thapsigargin largely overrides any virus-induced modulation of host processes (Fig. 7c).

In the absence of thapsigargin, the virus infection generally has little or opposite effects on the levels of the 120 proteins, as exemplified by the suppression observed for BiP (HSPA5) or HERPUD1 (Fig. 7c, highlighted in green). The 120 induced factors map to pathways involving Golgi vesicle transport, ER stress, fiber organization, and apoptosis (Fig. 7d). Across their pathway annotations, 71 out of the 120 proteins were reported to strongly interact, thus probably being involved in protein:protein networks that coordinate activities of the enriched pathways (Fig. 7c, left graph). Likewise, the 63 repressed proteins map to specific (though different) pathways, such as monokarboxylic acid metabolism or viral life cycle (Fig. 7d). 26 components can be allocated to a few small protein interaction networks (Fig. 7e, right graph).

In our proteomic/bioinformatics analysis, HERPUD1 and p62/SQSTM1 were revealed to be among the most prominent thapsigargin-regulated factors in MERS-CoV- and SARS-CoV-2-infected cells (Fig. 7c, highlighted in green, orange). Both proteins are key regulators of two major and highly interconnected intracellular degradation pathways, ERAD and autophagy, leading us to focus our further analyses of the antiviral effects of thapsigargin on these processes.

The protein HERPUD1 has an essential scaffolding function for the organization of components of the core ERAD complex. ERQC and ERAD pathways are critically involved in the qualitative and quantitative control of misfolded or excessively abundant proteins in the ER. If protein folding in the ER fails, the proteins are retro-translocated through a HERPUD1-dependent ER membrane complex to the cytosol for proteasomal degradation. By searching our proteomics data for further ERAD factors, we were able to retrieve a total of 33 (for MERS-CoV) and 20 (for SARS-CoV-2) proteins of the canonical ERQC and ERAD pathways for which a differential expression was observed in virus-infected cells treated with thapsigargin (Fig. 7f). Mapping of these data on the KEGG hsa04141 pathway suggests that thapsigargin enhances or restores these mechanisms at key nodes of ERQC and ERAD in coronavirus-infected cells (Supplementary Fig. 9).

We also intersected the 120 + 63 proteins jointly regulated by thapsigargin in MERS-CoV- and SARS-CoV-2-infected cells with data from a recent genome-wide sgRNA screen that reported new ERAD factors required for protein degradation. This analysis identified 30 additional thapsigargin-regulated factors that may further support antiviral ERAD, including the E1 ubiquitin ligase UBA6 and the zinc finger protein ZNF622 (also called ZPR9), which were recently described either as negative regulators of autophagy or of some DNA virus infections.

The protein p62/SQSTM1 is a multifunctional signaling protein and cargo receptor that targets clients for destruction by selective autophagy. This raised the question of whether the elevated p62/SQSTM1 levels observed in thapsigargin-treated cells affected autophagy pathways during CoV infection. We, therefore, assessed, under these conditions, viral replication and the autophagic flux by determining the levels of p62/SQSTM1 and the non-lipidated/lipidated forms of the ATG8 ortholog LC3B, a protein that is central to autophagosome formation, in the presence/absence of lysosomal inhibition. Pre-treatment of HuH7 cells with the lysosomal V-ATPase inhibitor bafilomycin A1 for 4 h suppressed HCoV-229E infection to a similar extent as thapsigargin (by about 100-fold) (Fig. 8a, lanes 3 and 4), while the addition of bafilomycin A1 at 8 h p.i. reduced viral replication only 10-fold (Fig. 8a, lanes 5 and 6). Under these conditions, bafilomycin A1 alone showed no negative effects on cell viability (Supplementary Fig. 10). Bafilomycin A1 alone did not suppress MERS-CoV replication but inhibited SARS-CoV-2 (Fig. 8b).
These (variable) antiviral effects of bafilomycin A₁ are in line with other alkylating agents that suppress lysosomal pathways\textsuperscript{54}.

Bafilomycin A₁ and thapsigargin strongly increased the appearance of p62/SQSTM1-positive foci representing autophagosomes in untreated but also in infected cells, suggesting that HCoV-229E infection but also thapsigargin treatment stimulate early events in autophagosome biogenesis (Fig. 8c).

With respect to viral protein synthesis, pretreatment of cells with bafilomycin A₁ partially reduced HCoV-229E N protein levels but had no effect on viral N protein accumulation when
Fig. 5 Thapsigargin suppresses CoV replication in differentiated primary human bronchial epithelial cells. a Scheme showing the expansion in a liquid —liquid interphase (LLI) followed by the differentiation at an air-liquid interphase (ALI) of normal human bronchial epithelial cells (NHBE). b Three-dimensional immunofluorescence analysis (z-stacks) of representative NHBE cells stained with antibodies specific for the indicated differentiation markers. Shown is one representative out of two biologically independent experiments. c NHBE cells were left untreated or infected with the indicated CoV (MOI = 3) and treated for up to three days with thapsigargin (0.1 or 1 μM). Supernatants were collected at five time points p.i. and virus titers determined by plaque assay. Data represent three biologically independent experiments using NHBE cells derived from two or three independent donors. Shown are means ± s.d. of technical duplicates. For HCoV-229E and MERS-CoV, cells from donor 1 were plated and differentiated a second time to generate an additional independent experiment (labeled donor 1.2). See Supplementary Fig. 5f for cell viability experiments.

Discussion

In this study, we report a potent inhibitory effect of the chemical thapsigargin on the replication of three human CoVs in four different cell types. Following up on observations that CoV globally suppresses UPR/ER stress factors, we find that thapsigargin counteracts the CoV-induced downregulation of BiP, HERPUD1 (and CTH) and increases IRE1α levels. In this context, thapsigargin also plays a role in overcoming the coronavirus-induced block of global protein biosynthesis. Proteome-wide data revealed a thapsigargin-mediated reprogramming of metabolic pathways and helped to identify a network of specific thapsigargin-regulated factors, including candidates from the ERQC/ERAD pathways that, most likely, are involved in the destruction of viral proteins. The positive effects of prolonged thapsigargin treatment on the expression of cellular BiP and HERPUD1 are well documented37–60. Thus, one key finding of our study is that the thapsigargin-mediated induction of ER factors overrides suppressive effects of CoVs on ER functions, as illustrated here for BiP, IRE1α and HERPUD1, but also at the global proteomic scale.

BiP is one of the most abundant cellular proteins (also in our mass spectrometry data) and plays an essential role in development and disease61,62. In yeast, the inducible expression of the BiP homolog Karp2 was shown to be essential for disposing of toxic proteins and reducing cellular stress63. Hence, a reduction of BiP levels (as seen during CoV infection) and the contrary effect of thapsigargin-mediated upregulation are likely to have opposing consequences for the host cell following viral infection. Similarly, IRE1α is suggested to mediate protective and adaptive responses suitable to alleviate ER stress, e.g., by balancing lipid bilayer stress, an aberrant perturbation of ER membrane structures, which may be expected to occur upon DMV formation in CoV-infected cells18,31,64. Accordingly, high levels of BiP, HERPUD1, and IRE1α may increase in general the resilience of cells when infected by diverse pathogens. In line with this, our data show that, in cells infected with representative coronaviruses, a protective ER/UPR response is initially elicited at the mRNA level (Fig. 1c and ref. 17). However, the global suppression at the protein level (or the lack of induction) indicates that CoVs have evolved strategies at the posttranscriptional or translational level to escape the protective antiviral activities of BiP, IRE1α, and HERPUD1.

Together with PERK, all three proteins are key regulators of ERQC/ERAD pathways and there is ample evidence to suggest that their expression, regulation, and activities are intimately linked31,22. A recent study reported that PERK activation induces the RPAP2 phosphatase, inactivates IRE1α kinase activity, and aborts IRE1α-mediated adaptive functions in response to the chemical stressor Brefeldin A65. In another report, high BiP levels exerted negative control of IRE1α by directly binding the kinase or by promoting IRE1α degradation66. Here, we show a different scenario, in which high BiP and IRE1α protein levels coincide with an antiviral state, as well as with improved metabolic functions, suggesting unique modes of cross-regulation of PERK, BiP, and IRE1α in CoV-infected cells exposed to chemical stress.

The (up)regulation of HERPUD1 and several ERAD factors by thapsigargin provides an additional layer of control contributing to the rapid suppression of CoV proteins. While ERAD is generally needed to dispose of unwanted proteins in the ER57,68, a process called “ERAD tuning” that has been suggested to dampen or balance ERAD activity by...
segregating ERAD components into specific ER-derived vesicles called EDEMsomes. During CoV infection, ERAD tuning may prevent the destruction of CoV proteins. Our data are compatible with a model in which a modulation of ERAD components by small molecules may antagonize “ERAD tuning” promoted by CoVs, thereby preserving normal ERAD topology, as well as high ERAD activity.

High HERPUD1 levels were also reported to impair autophagy and, conversely, its depletion increased autophagic flux upon glucose stress, suggesting that HERPUD1 may contribute to the...
antiviral effects of thapsigargin by a dual-mode, through ERAD and as a repressor of autophagy. The contribution of (macro) autophagy to CoV replication is still controversial with evidence for both pro- and antiviral roles, depending on the virus strains and model systems used (reviewed in refs. 76,77). Here, we provide evidence to suggest that human CoVs induce (and require) p62/SQSTM1-mediated selective autophagy early in their replication cycle because N protein translation and replication are repressed to some extent by inhibitors of lysosomal acidification. Recently, p62/SQSTM1 was shown to act as an adaptor to mediate a selective form of autophagy, called ER-phagy, that normally serves to remove larger parts of damaged ER or bulky protein aggregates that cannot readily be disposed of by ERAD74–76. Based on our findings, we speculate that CoV may leverage ER-phagy, in addition to “ERAD tuning”, to generate membrane sources for their specific CM/DMV environment. Compared to lysosomal inhibition, the thapsigargin effects on blocking CoV-mediated autophagic flux were much more sustained and durable. This observation is in line with the work from Ganley et al. who showed that thapsigargin blocks the fusion of autophagosomes with lysosomes, a late step in the autophagy pathway, by an unknown mechanism27. Genetic experiments in Drosophila revealed a role of the SERCA Ca^{2+} pump in membrane fusion events of lysosomes28. Because thapsigargin inhibits SERCA27, it, therefore, remains a possibility that thapsigargin is particularly efficient in blocking autophagy and CoV-replication because it prevents the Ca^{2+} gradients required for multiple vesicle fusion events known to occur during the CoV replication cycle.

Clearly, the precise mechanistic basis for these effects remains to be identified in additional studies. One approach will be to identify direct interactions between CoV proteins and ER stress or autophagy pathway components. A number of affinity purification- or proximity labeling-based mass spectrometry data sets are now available for host cell proteins interacting with individually expressed SARS-CoV-2 proteins (reviewed in ref. 80). These data reveal a large number of host interactors from a broad range of pathways, several of which including regulators of ER stress or autophagy28. However, it remains to be seen which of the reported protein:protein interactions can be confirmed in CoV-infected cells with their unique subcellular compartmentalization.

In line with the interaction studies, our proteomic and functional data show that thapsigargin affects multiple pathways beyond the core ER stress response. Overall, the available evidence indicates that it will not be trivial to identify the essential targets that mediate thapsigargin’s multimodal antiviral effects.

Our data provide a rich resource for further drug target analysis, also in conjunction with the few deep protein sequencing studies available for SARS-CoV-2 (but not MERS-CoV)82–85. Thus, our study fills an important knowledge gap by providing a direct side-by-side comparison of pharmacologically targeted cells infected with two highly pathogenic human coronaviruses.

In the absence of specific and effective therapeutic strategies to combat coronaviruses, and in view of the current SARS-CoV-2 pandemic, we hope that our observations will stimulate a broader investigation of this potential therapeutic avenue. This will also include more detailed studies on the antiviral effects and specificity of thapsigargin for other RNA viruses, such as influenza A virus. Given that thapsigargin concentrations in the lower nanomolar range were shown to abolish CoV replication in cultured cells (including primary bronchial epithelial cells) for up to three days after a single application, this work identifies thapsigargin as an interesting drug candidate. The Ca^{2+} mobilizing and cytotoxic features of plant-derived thapsigargin have been studied for 40 years86,87. Several analogs have already been designed and efficient and scalable purification or synthesis is now available for application in humans88–90. Recently, a protease-cleavable produg of thapsigargin, mipsagarin, has been evaluated in phase I and II clinical trials for prostate cancer91,92. It is not uncommon to adapt anti-proliferative cytostatic drugs (e.g., azathioprine, cyclophosphamide, methotrexate) for the treatment of autoimmune and inflammatory disorders by applying lower doses than those needed for treating cancer93. Similarly, low doses of thapsigargin combined with short-term systemic or topical application in the airways might reduce viral load early on or in critically ill patients with a favorable therapeutic index with respect to antiviral versus cytotoxic effects. CoVs also activate inflammatory, NF-kB-dependent cytokine and chemokines at the mRNA level97, some of which (CXCL2, CCL20) escaping translational shut-down and being secreted in a cell-type-specific manner (Supplementary Fig. 11). Some of these cytokines may contribute to the cytokine storm observed in some COVID-19 patients98. While thapsigargin had no effect on IL-8, IL-6, CXCL2, and CCL20 in cell culture (Supplementary Fig. 11), a single bolus of the compound was shown to efficiently reduce the translation of pro-inflammatory cytokines in preclinical models of sepsis99. Thus, an additional benefit of thapsigargin treatment may arise from dampening overshooting tissue inflammation in COVID-19 patients. In summary, the study provides several lines of evidence that thapsigargin hits a unique combination of central mechanisms required for CoV replication, which may be exploited to develop novel therapeutic strategies. This compound or derivatives thereof with improved specificity, pharmacokinetics, and safety profiles may also turn out to be suitable for prophylactic and therapeutic applications in the clinic.
Fig. 7 Thapsigargin regulates a specific network of proteins involved in transport, ERQC/ERAD, and ER stress in MERS-CoV or SARS-CoV-2-infected cells. a Overlap of orthologous proteins expressed in HuH7 and Vero cells. b Overlap of virus- and thapsigargin-regulated proteins common to HuH7 and Vero cells showing 120 proteins with higher and 63 proteins with lower expression in thapsigargin-treated infected cells compared to virus infection alone (ratio > 0, p value of −log10 (p) ≥ 1.3). c Heatmaps showing individual mean ratio values of normalized protein intensities for the top 50 up- or downregulated proteins in virus-infected and thapsigargin-treated cells. Ratio values of infected or thapsigargin-treated conditions compared to untreated cells are shown for comparison and are sorted according to the virus plus thapsigargin conditions. Green colors highlight HERPUD1 and BiP (HSPA5), while orange colors highlight p62/SQSTM1. d Top pathways mapping to gene IDs with increased (120 proteins, red) or decreased (63 proteins, blue) expression levels in thapsigargin-treated and infected cells compared to virus infection alone as revealed by overrepresentation analysis using Metascape software. e Protein:protein interactions (PPI) amongst the 120 up- and 63 downregulated thapsigargin-sensitive proteins based on experimental evidence, co-occurrence, co-expression, and confidence scores from the STRING database. According to experimental evidence and combined STRING score criteria, 71 and 26 coregulated proteins are engaged in defined PPI networks; the remaining proteins are not known to interact. f Heatmap showing thapsigargin-reprogrammed proteins of KEGG hsa04141 (mean ratio ≥ 1.5 fold). See also Supplementary Fig. 9 for projection of thapsigargin-mediated protein changes on the KEGG pathway map. g Venn diagram showing the intersection of thapsigargin-/virus-regulated proteins with all novel ERAD components (FDR of 1%) identified by ref. 50. The regulation of 30 overlapping components is shown as a heatmap displaying mean ratio values in thapsigargin-treated or infected cells. Red colors highlight UBA6 and ZNF622 as discussed in the text. (b, c, f, g) Mean ratio and p values were determined as described in the legend of Fig. 6a–d. Abbreviations: M MERS-CoV; S SARS-CoV-2; T thapsigargin.
for mitigating the consequences of potential future CoV epidemics more effectively.

**Methods**

**Cells and viruses.** HuH7 human hepatoma cells (Japanese Collection of Research Bioresources (JCRB) cell bank) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, including 3.7 g/l NaHCO3; PAN Biotech Cat No P04-03550) complemented with 10% filtered bovine serum (FBS Good Forte; PAN Biotech, Cat No. P40-47500), 2 mM L-glutamine (Gibco, 21935-028), 100 U/ml penicillin and 100 μg/ml streptomycin. MRC-5 human embryonic lung fibroblasts (ATCC, CCL-171) were maintained in DMEM containing 1.5 g/l (w/v) NaHCO3 and complemented with 10% fetal calf serum (FCS; PAN Biotech Cat No. 1502-P110704), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, 1% minimum essential medium non-essential amino acids (100x MEM NEAA; Gibco Cat No 11140-035) and 1 mM sodium pyruvate (100 mM; Gibco 11360-039). Vero E6 African green monkey kidney epithelial cells (ATCC CRL-1586), A549 cells (ATCC CCL-185), and MDCK-II (ATCC CRL-2936) cells were grown...
in DMEM, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. HuH7 and MRC-5 cells were confirmed to be free of mycoplasma using the Venor* Gm Classic kit (Minvira Biolabs).

Cryopreserved normal human bronchial epithelial (NHBE) cells were obtained from Lonza. All donors (donor 1: TAN 24717, Lot No. 00031262; donor 2: TAN 36385, Batch: 18TL269120; donor 3: TAN 28986, Lot No. 00048590) were non-smoking and lacking respiratory pathology. Undifferentiated cells were seeded on transwell plates (Corning Costar, CLS3470-48EA) coated with collagen IV (Invitrogen) and grown in a mixture of DMEM (Invitrogen) and BEGM (Lonza, CC-3170) supplemented with retinoic acid (75 nM). Fresh medium was added regularly after 2 days. After reaching confluence, the cells were cultured under air liquid conditions for four additional weeks until full differentiation into pseudostratified human airway epithelia was observed. Medium from the basolateral compartment was renewed every 2–3 days and the apical surface was washed every week with PBS (Invitrogen).

Genome sequences of coronavirus strains used in this study are as follows: HCoV-229E (NCBI accession number JX69059, NCBI reference sequence NC_019833), MERS-CoV and SARS-CoV-2 were kindly provided by Christian Drosten. For infection with other RNA viruses, influenza A virus (A/Thailand/1/2004/HSN1), IAV; NCBIBiobaseD68872) and human poliovirus type 1 (strain Mahoney, NCBITaxid12081) were used.

Virus infections and assessments of antiviral activity. To analyze the antiviral activity of thapsigargin, HuH7 cells (for HCoV-229E, MERS-CoV), MRC-5 cells (for HCoV-229E and SARS-CoV-2), and NHBE cells (for SARS-CoV-2 and poliovirus), and 293T cells (for IAV), respectively, were infected at the indicated multiplicities of infection (MOI) and incubated at 33 °C (for HCoV-229E and SARS-CoV-2) or 37 °C (for MERS-CoV, IAV, poliovirus) in the presence or absence of thapsigargin, or with the appropriate volume of solvent control (DMSO) as indicated. At 24 h.p.i., supernatants were collected and stored at −80 °C. Virus titers in the supernatants were determined by plaque assay.

Cell lysis, in vivo puromycinylation, and immunoblotting. For whole-cell extracts, samples derived from experiments performed with HCoV-229E were lysed in Triton cell lysis buffer (10 mM Tris, pH 7.0, 50 mM NaF, 50 mM NaCl, 1% Triton X-100, 2 mM Na3VO4, 50 mM NaF, 20 mM β-glycerophosphate and freshly added 0.5 mM PMSF, 2.5 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µM microcin). After 10–15 min on ice, lysates were cleared by centrifugation at 15,000 × g for 15 min at 4 °C. Protein concentrations of supernatants were determined by Bradford assay and samples were stored at −80 °C.

To label nascent polypeptides in intact cells10, HuH7 cells were seeded in 6 cm cell culture dishes (3 × 106 cells) and treated as described in the figure legends. Thirty minutes prior to harvest, the medium was supplemented with 3 µM puromycin (InvitroGen, #ant-pr-1). Then, cells were lysed as described above. After immunoblotting (see below), membranes were stained with Coomassie brilliant blue and then hybridized with an anti puromycin antibody (Kerafast, #EQ0001) to detect puromycylated polypeptides.
Fig. 9 Thapsigargin induces key regulators of UPR, ERQC, ERAD, and autophagy in HCoV-229E, MERS-CoV- and SARS-CoV-2-infected cells.

a, b Validation of thapsigargin-induced proteins in CoV-infected HuH7 or Vero E6 cells by immunoblotting of whole-cell extracts from cells treated as indicated. BiP and IRE1α levels are shown for comparison. a Depicts representative images and b shows quantification of four biologically independent experiments, except for HERPUD1 (HCoV-229E samples) or LC3B, LC3B-I, and LC3B-II levels from MERS-CoV-infected cells which were quantified from three experiments. Bar graphs show means ± s.d.; asterisks indicate p values (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001) obtained by two-tailed ratio-paired or Mann–Whitney t-tests. Note that LC3B-I and LC3B-II images represent different exposures of the same blot membrane (see source files).

c Summary of the main findings of our study.
Total cell lysates of MERS-CoV- and SARS-CoV-2-infected cells used for immunoblotting or mass spectrometry were prepared as follows. Cells were scraped (500 x g for 5 min at 4 °C) and pelleted in ice-cold PBS and stored in liquid N2 (or lysed and processed immediately). After thawing, cell pellets (corresponding to ~300,000 cells seeded in 60 mm dishes at the start of the experiment) were resuspended in 90 µl of ice-cold Ca2+/Mg2+-free PBS and transferred to fresh tubes. After the addition of 10 µl of 10% SDS, samples were heated at 100 °C for 10 min and centrifuged at 100,000 x g for 1 min at room temperature. Supernatants were transferred to a fresh tube and heated again at 100 °C for 10 min and centrifuged at 60,000 x g for 1 min at room temperature. Protein concentrations were determined by the detergent compatible Bradford assay (Biorad, D600-250). A 150-fold dilution of aliquots (diluted in water) was added to a 96-well plate containing 95 µl of 5× Bradford assay buffer (BioRad, D600-250) and incubated at 37 °C for 30 min. After this reaction mixture was used to amplify cDNAs using Taqman assays on demand (Applied Biosystems/Thermo Fisher Scientific). Moloney murine leukemia virus reverse transcriptase (RevertAid Reverse Transcriptase, Thermo Fisher Scientific, #M2616) was used for multiple testing by the Benjamini and Hochberg procedure104. From the entire analysis of the samples was performed using a timsTOF Pro mass spectrometer (Bruker Daltonics). A nanoElute HPLC system (Bruker Daltonics), equipped with a Finnigan ion trap mass spectrometer, was used for proteome analysis protocol using Microcon YM-30 filter devices by the addition of sequencing grade modified trypsin (Promega, #G5382). In brief, 1.2 x 10⁶ HuH7 or 1 x 10⁵ MRC-5 cells were seeded in 96-well plates for 24 h and thereafter treated with DMSO, thapsigargin, remdesivir, GSK2656157, bafilomycin A1, virus alone or virus plus chemical for 16, 24, or 28 h as indicated in the figure legends. Then, the medium was replaced by 100 µl complete cell culture medium including 4 µl or 20 µl CellTiter 96 AQueous one solution reagent according to the manufacturer’s recommendations. Cells were further incubated for 1 h at 37 °C. Then, absorbance values were measured at 490 nm. Control diluting only medium and reagent were used to correct for background absorbance. Relative values for cell viability were calculated in relation to the mean of all untreated controls (set to 100%).

For MTT and ATPlite assay (Perkin Elmer), Vero E6 cells seeded at near confluency were incubated with a serial dilution of thapsigargin in a 96-well format. After 24 h, either 200 µl MTT mix (MDEM supplemented with 10% FCS containing 250 µg/ml tetrazolium bromide, Sigma) or 100 µl ATPlite assay buffer was added to each well. For ATPlite assay, cells were incubated for 10 min and luminescence was measured using Spark 10 M (Tecan). For MTT assay, cells were incubated for 90–120 min at 37 °C and fixed using 3.7% PFA in PBS. The tetrazolium crystals were dissolved by adding 200 µl/well isopropanol and the absorbance at 490 nm was measured using an ELISA reader (BioTek). To determine CC₅₀ values, the MTT/ATPlite values were calculated in relation to the untreated control (which was set to 100%). CC₅₀ values were then calculated by non-linear regression using GraphPad Prism 5.0 (GraphPad Software). For analyzing cytotoxicity in NIH/3T3 cells after 72 h treatment, the transpethelial electrical resistance (TEER) was measured using Epithelial Volt/Ohm Meter 3 (EVOM3, WPI). The obtained TEER values were compared to those obtained for untreated cells.

ELISA. Sandwich ELISAs from R&D Systems (DuoSet ELISA for human IL-8 (DY208), IL-6 (DY206), CXCL2 (DY276-05), CCL20 (DY360) were used to detect secreted human cytokine/chemokine protein concentrations in cell culture supernatants of HuH7 or MRC-5 cells treated as described in the figure legends. The cell culture supernatants were harvested, centrifuged at 15,000 × g at 4 °C for 15 s, and stored at −80 °C. 100 µl of the supernatants were either undiluted or were diluted in cell culture medium as follows (HuH7: IL-8 (1:10), CXCL2 (1:3), CCL20 (1:8), MRC-5: IL-5 (1:10), IL-6 (1:20), CXCL2 (1:1.5) and MELISA was performed according to the manufacturer’s instructions using serial dilutions of recombinant proteins as standards. All measurements were within the linear range of the standard curve. In some experiments, an IL-1α (10 ng/ml) stimulation for 16 h was used as a positive control.

RNA-seq and bioinformatics. For the data shown in Fig. 1, total RNA was isolated from uninfected and infected cells obtained at 3, 6, 12, 24 h.p.i. (or mock infection) using the detergent compatible dye QIAGEN RNeasy mini kit (Qiagen) according to the manufacturer instructions using serial dilutions of recombinant proteins as standards. All measurements were within the linear range of the standard curve. In some experiments, an IL-1α (10 ng/ml) stimulation for 16 h was used as a positive control.

Mass spectrometry and bioinformatics. Protein extracts were lysed in SDS lysis buffer as described above. Prior to digestion, the SDS-containing solution was exchanged to 8 M urea applying the following to complete the analysis. For proteome analysis protocol using Microcon YM-30 filter devices (Millipore, Cat. MRFCOR030)102. Cysteines were alkylated with iodoacetamide and 8 M urea buffer for multiple testing by the Benjamini and Hochberg procedure104. From the entire data set, only normalized read count values and ratio values for 166 gene IDs assigned to KEGG Isao41144 were extracted and further analyzed.

Cell viability assays. MTS assay of HCoV-229E experiments were performed according to the manufacturer instructions using serial dilutions of recombinant proteins as standards. All measurements were within the linear range of the standard curve. In some experiments, an IL-1α (10 ng/ml) stimulation for 16 h was used as a positive control.

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Sampling load was performed at a constant pressure of 800 bar. Separation of the tryptic peptides was achieved at 50 °C column temperature with the following gradient (solvent A) and acetonitrile (solvent B) at a flow rate of 400 nL/min: Linear increase from 2% B to 17% B within 60 min, followed by a linear gradient to 25% B within 30 min and linear increase to 37% solvent B in additional 10 min. Finally, B was increased to 95% within 10 min and held for an additional 10 min. The benchtop HPLC system was equipped with a C18 column and the data was acquired using DataAnalysis (version 4.3c, Bruker Daltonics). 

The mass spectrometry proteomics data in this study shown in Fig. 6a–d have been deposited to the ProteomeXchange Consortium [11] via the PRIDE partner repository [12] with the dataset identifier PXD021222 (https://doi.org/10.6019/PXD021222). The remaining data generated in this study are provided in the Supplementary Information/Source Data files. Source data are provided with this paper.

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Statistics, quantification, and reproducibility. Quantification of data and statistical parameters (means, t-tests, standard deviations, confidence intervals, Pearson correlations, linear regressions, non-linear fittings for EC50 and IC50 values) were calculated using SigmaPlot 11, DESeq2 (version 1.22.1), GraphPad Prism 5.0 or 8.4.3, Perseus (versions 1.6.10.50 (MERS-CoV) or 1.6.14 (SARS-CoV-2)), ImageLab (versions 5.2.1 or 6.0.1), or Microsoft Excel 2016. All of the data of cell viability assay results (Figs. 1b, d, 4d and Supplementary Figs. 2c, 5d, e, 10) show technical replicates according to the numbers of independent experiments indicated in the legends.

All statistical tests for pathway enrichment analyses (Figs. 6c–d, 7d and Supplementary Figs. 6–8) were calculated online by Metascape software (https://metascape.org) using the Entrez sources, KEGG Pathway, GO Functional Processes, Reactome Gene Sets, Canonical Pathways, CORUM, TRUST, DisGeNET, PagenBase, Transcription Factor Targets, WikiPathways, PANTHER Pathway, and COVID and all genes in the genome as the enrichment background. P-values were based on the cumulative hypergeometric distribution and q-values were calculated using the Benjamini–Hochberg method for multiple testing. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 were selected. Kappa scores were used as the similarity metric for hierarchical clustering on the enriched terms, and sub-trees with a similarity of > 0.3 were considered a cluster. The most statistically significant terms within a cluster were chosen to represent the cluster.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The mass spectrometry proteomics data in this study shown in Fig. 6a–d have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021222 (https://doi.org/10.6019/PXD021222). The remaining data generated in this study are provided in the Supplementary Information/Source Data files. Source data are provided with this paper.
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Author contributions

M.S.S., C.M., C.M.-B., H.W., B.V.A., N.K. and J.M-S. performed experiments, analyzed, and visualized data. U.L. performed mass spectrometry measurements and together with A.W., M.K. and I.B. analyzed proteomics data sets. N.H. analyzed RNA-seq data. T.H. sequenced SARS-CoV-2. M.K. conceived and supervised the study and analyzed and visualized data. M.K. wrote the initial paper draft. J.Z. supervised and analyzed MERS-CoV and SARS-CoV-2 experiments. J.Z., S.B., S.H., and M.L.S. (and all other authors) helped to finalize the paper. All authors approved the submitted version of the paper.

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Competing interests

The authors declare no competing interests.

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