Coronaviruses exploit a host cysteine-aspartic protease for efficient replication

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Coronaviruses exploit a host cysteine-aspartic protease for efficient replication

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Summary

Highly pathogenic coronaviruses including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-1 vary in their transmissibility and pathogenicity. However, infection by all three viruses result in substantial apoptosis in cell culture and in patient samples, suggesting a potential link between apoptosis and the pathogenesis of coronaviruses. To date, the underlying mechanism of how apoptosis modulates coronavirus pathogenesis is unknown.

Here we show that a cysteine-aspartic protease of the apoptosis cascade, caspase-6, serves as an essential host factor for efficient coronavirus replication. We demonstrate that caspase-6 cleaves coronavirus nucleocapsid (N) proteins, generating N fragments that serve as interferon (IFN) antagonists, thus facilitating virus replication. Inhibition of caspase-6 substantially attenuates the lung pathology and body weight loss of SARS-CoV-2-infected golden Syrian hamsters and improves the survival of mouse-adapted MERS-CoV (MERS-CoVMA)-infected human DPP4 knock-in (hDPP4 KI) mice. Overall, our study reveals how coronaviruses exploit a component of the host apoptosis cascade to facilitate their replication.

These results further suggest caspase-6 as a potential target of intervention for the treatment of highly pathogenic coronavirus infections including COVID-19 and MERS.
Seven coronaviruses are known to infect humans. Among them, three highly pathogenic coronaviruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)\textsuperscript{1,2}, Middle East respiratory syndrome coronavirus (MERS-CoV)\textsuperscript{3,4}, and SARS-CoV-1\textsuperscript{5}, have emerged over the last two decades and have dramatically impacted the global public health of the human populations\textsuperscript{12}. These highly pathogenic coronaviruses vary in their transmissibility and pathogenicity with underlying mechanism remains largely unexplained\textsuperscript{13}. A common feature of these coronaviruses is their propensity to induce apoptosis in the infected target cells. SARS-CoV-1 infection induced apoptosis in cell culture and infected patients\textsuperscript{9,10}. MERS-CoV triggered severe apoptosis in lung epithelial cells\textsuperscript{6}, primary T cells\textsuperscript{7}, and infected animals\textsuperscript{14}. More recently, SARS-CoV-2-induced apoptosis was similarly documented in infected human tracheobronchial epithelial cells\textsuperscript{8}, lungs of infected hamsters\textsuperscript{15}, and lung specimens of Coronavirus Disease 2019 (COVID-19) patients\textsuperscript{11}. These findings suggest a role of apoptosis in the pathogenesis of coronaviruses. In line with these observations, we recently demonstrated that inhibition of apoptosis with a pan-caspase inhibitor significantly attenuated MERS-CoV replication\textsuperscript{14}, suggesting a previously unrecognized connection between the apoptosis cascade and coronavirus replication.

In this study, we further investigated how apoptosis modulates coronavirus replication. Our results identify caspase-6, which is a cysteine-aspartic protease serving as an executor caspase of the apoptosis cascade, as a novel host factor that facilitates coronavirus replication. \textit{In vitro} inhibition of caspase-6 reduces the replication of all evaluated human pathogenic coronaviruses including MERS-CoV, SARS-CoV-2, SARS-CoV-1, human coronavirus (HCoV)-229E, and HCoV-OC43. In human DPP4 knock-in (hDPP4 KI) mice, caspase-6 inhibition attenuates the replication of mouse-adapted MERS-CoV (MERS-CoV\textsubscript{MA}) and significantly improves the survival of infected mice. Similarly, caspase-6 inhibition reduces SARS-CoV-2 replication in lungs of golden Syrian hamsters and markedly
ameliorates lung pathology, leading to significantly improved body weight in the infected hamsters. Mechanistically, we demonstrate that caspase-6 cleaves coronavirus N proteins and generates N fragments, which subsequently serve as interferon (IFN) antagonists that promote efficient virus replication. Importantly, caspase-6-mediated N cleavage and its associated IFN antagonism are conserved among all evaluated human-pathogenic coronaviruses. Overall, our study reveals a novel mechanism on how human-pathogenic coronaviruses exploit a component of the apoptosis cascade for their efficient replication. Caspase-6 inhibition can be further explored as an intervention strategy for coronavirus infections including MERS and COVID-19.

Results

Inhibition of caspase-6 limits coronavirus replication

We recently demonstrated that MERS-CoV infection triggered substantial apoptosis while inhibition of apoptosis with the pan-caspase inhibitor, z-VAD-fmk, significantly limited MERS-CoV replication (Fig. 1a,b)\textsuperscript{14}. Interestingly, z-VAD-fmk similarly limited the replication of other human pathogenic coronaviruses including SARS-CoV-2, SARS-CoV-1, HCoV-229E, and HCoV-OC43 (Fig. 1b), suggesting that the dependency on apoptosis or caspase activity for efficient virus replication is a conserved mechanism for coronaviruses. Caspases are cysteine-aspartic proteases that regulate the host apoptosis cascade\textsuperscript{16}. To investigate which caspase is most responsible for modulating coronavirus replication, we used MERS-CoV as a model virus and evaluated virus replication in the presence of specific inhibitors against individual caspases. Our results revealed that caspase-6 inhibition most dramatically limited MERS-CoV replication (Fig. 1c,d). The inhibitory effect on MERS-CoV replication by caspase-6 inhibition was conserved across different cell types with the exception of VeroE6 cells (Extended Data Fig. 1). Importantly, inhibition of caspase-6 with
its specific inhibitor, z-VEID-fmk, attenuated the replication of all evaluated coronaviruses including that of SARS-CoV-2 and SARS-CoV-1, but did not impact the replication of influenza virus (H1N1) or enterovirus (enterovirus A71) (Fig. 1e). According to RT-qPCR and TCID$_{50}$ assays, the IC$_{50}$ of z-VEID-fmk against coronaviruses ranged from 3.3 µM for SARS-CoV-2 to 21.1 µM for MERS-CoV in the cell lysate samples and 1.2 µM for SARS-CoV-2 to 30.6 µM for HCoV-OC43 in the supernatant samples, respectively (Fig. 1f).

Next, we used MERS-CoV and SARS-CoV-2 as model coronaviruses and evaluated the impact of caspase-6 inhibition on coronavirus replication in infected human ex vivo lung tissues, human intestinal organoids, and animals. In human lung tissues, caspase-6 inhibition with z-VEID-fmk significantly reduced MERS-CoV nucleocapsid (N) protein and gene expression (Fig. 2a,b). Similarly, z-VEID-fmk inhibited MERS-CoV replication in human intestinal organoids and inhibited the production of infectious virus particles by approximately 80% (p<0.0001) at 24 hours post infection (hpi) (Fig. 2c-f). Caspase-6 is largely conserved among mammals and the z-VEID-fmk binding pocket is conserved among humans, mice, and hamsters, allowing us to evaluate the effect of caspase-6 inhibition with z-VEID-fmk in these animal models (Extended Data Fig. 2). To evaluate the impact of caspase-6 inhibition on MERS-CoV replication in vivo, we infected human DPP4 knock-in (hDPP4 KI) mice with mouse adapted MERS-CoV (MERS-CoV$_{MA}$) and treated the mice with z-VEID-fmk or DMSO (Fig. 2g). Our results demonstrated that z-VEID-fmk effectively reduced MERS-CoV$_{MA}$ replication in the lungs of the infected mice at both day 2 and day 4 post infection (Fig. 2h-j), and significantly attenuated the expression of pro-inflammatory cytokines and chemokines (Fig. 2k and Extended Data Fig. 3). Importantly, the z-VEID-fmk treatment largely inhibited body weight loss and significantly improved the survival of the infected hDPP4 KI mice from 33.3% to 80% (3/9 vs 8/10; p=0.0388) (Fig. 2l,m).
We next asked whether z-VEID-fmk could similarly inhibit SARS-CoV-2 replication in vivo. To this end, we infected golden hamsters with SARS-CoV-2 through the intranasal route and treated the hamsters with z-VEID-fmk or DMSO (Fig. 3a). Our results demonstrated that caspase-6 inhibition with z-VEID-fmk significantly reduced SARS-CoV-2 replication in the hamster lungs (Fig. 3b,c) including small airways (Fig. 3d) and alveoli (Fig. 3e), and ameliorated the expression of virus-induced pro-inflammatory cytokines and chemokines (Fig. 3f). The attenuated virus replication and expression of pro-inflammatory markers resulted in significant improvements of the body weight of the infected hamsters (Fig. 3g). In addition, we harvested lung tissues from the hamsters at day 4 post infection to evaluate histological changes. Infected lungs from hamsters in the mock treatment group showed severe bronchiolar epithelial cell death and desquamation, extensive alveolar space mononuclear cell infiltration, protein rich fluid exudation, alveolar haemorrhage, and severe destruction of alveolar structure. Pulmonary blood vessel wall inflammation and endothelium infiltration were frequently observed (Fig. 3h, middle panels). These histopathological changes were consistent with what we previously reported in SARS-CoV-2-infected golden hamsters. In contrast, in the lungs of z-VEID-fmk-treated hamsters, all categories of tissue damage, including bronchiolitis, alveolitis and vasculitis, were significantly ameliorated. In these animals, we observed a mild degree of bronchiolar epithelium desquamation, focal alveolar septal congestion, localized infiltration or hemorrhage, and mild perivascular infiltration. At the same time, the z-VEID-fmk treatment dramatically inhibited alveolar space immune cells infiltration (Fig. 3h, bottom panels). In order to quantitatively evaluate the severity of lung damage, we performed semi-quantitative histopathological examination of the bronchioles, alveoli, and blood vessels using our previously described methods. According to the histopathological scores, the z-VEID-fmk treatment significantly ameliorated lung damage in the infected hamsters (Fig. 3i). Taken together, our results
demonstrate that caspase-6 inhibition attenuates coronavirus replication in cell culture, human lung tissue, organoid, and animal settings.

Caspase-6 cleaves nucleocapsid (N) protein and modulates coronavirus replication at a post-entry step

Next, we seek to understand the role of caspase-6 in coronavirus replication. Using MERS-CoV as a model, we first assessed the effect of z-VEID-fmk in a time-of-addition assay, which showed that z-VEID-fmk added during MERS-CoV inoculation did not reduce virus replication (Fig. 4a). Consistent with this finding, MERS-CoV entry in caspase-6-stable knockdown A549 and BEAS2B cells was not compromised (Fig. 4b and Extended Data Fig. 4a), confirming the notion that caspase-6 did not play a role in MERS-CoV entry. Next, we evaluated virus gene expression in caspase-6 stable knockdown cells harvested at 24 hpi. Among these samples, MERS-CoV replication was significantly reduced in the presence of caspase-6 knockdown (Fig. 4c). The role of caspase-6 on MERS-CoV replication was further investigated in human primary monocyte-derived macrophages (MDMs). In these cells, transient depletion of caspase-6 with siRNA markedly reduced MERS-CoV replication in both cell lysates and supernatant samples (Fig. 4d and Extended Data Fig. 4b). In addition to the gene depletion studies, we examined MERS-CoV replication in caspase-6-overexpressed cells. Our results demonstrated that caspase-6 but not caspase-3 overexpression efficiently promoted MERS-CoV replication (Fig. 4e,f). Thus, these results suggest that caspase-6 is required for efficient MERS-CoV replication at a post entry step.

Together with caspase-3 and caspase-7, caspase-6 is one of the three executor caspases that execute apoptosis by proteolytic cleavage of host substrates\textsuperscript{19}. Caspase-6 is cleavage activated when apoptosis is induced but can also undergo autoactivation (Extended Data Fig. 5)\textsuperscript{20}. As a cysteine-aspartic protease, we speculate that caspase-6 may modulate...
coronavirus replication by acting on a viral component. To this end, we co-expressed caspase-6 with various MERS-CoV components and evaluated viral protein cleavage by caspase-6 when apoptosis was induced. In these assays, we used staurosporine (STS) to trigger apoptosis to mimic the apoptotic environment in MERS-CoV-infected cells. Intriguingly, our results demonstrated that caspase-6 mediated the cleavage of the viral N protein but not other viral components (Fig. 4g and Extended Data Fig. 6). Cleavage of N was completely abolished in the presence of the specific caspase-6 inhibitor, z-VEID-fmk, suggesting that the cleavage is caspase-6 specific (Fig. 4g). Importantly, N cleavage was readily detected in cells infected by MERS-CoV (Fig. 4h), and was similarly inhibited by caspase-6 inhibition in the infected cells (Fig. 4i). In addition, N was only cleaved by capsase-6, but not by the key executor caspase, caspase-3 (Fig. 4j). Together, these results demonstrate that caspase-6 specifically cleaves MERS-CoV N and modulates virus replication at a post entry step.

Caspase-6-mediated N cleavage modulates interferon response and is conserved across human-pathogenic coronaviruses

Next, we seek to further investigate how caspase-6-mediated N cleavage modulates coronavirus replication. We demonstrated earlier that caspase-6 inhibition attenuated MERS-CoV replication in all evaluated cell types with the exception of VeroE6 (Extended Data Fig. 1), which is deficient in interferon (IFN) signaling due to a homozygous deletion in the type-I IFN gene cluster. These results hinted that caspase-6-mediated N cleavage might modulate coronavirus replication through regulating IFN signaling. To test this hypothesis, we evaluated the role of caspase-6 and MERS-CoV N on regulating IFN response with IFN-β-reporter assays. Interestingly, our results demonstrated that MERS-CoV N co-expressed with caspase-6 suppressed IFN-β-reporter activation in a dose-dependent manner (Fig. 5a).
parallel, co-expression of caspase-3 and MERS-CoV N or caspase-6 and MERS-CoV envelope (E) protein did not significantly impact IFN-β-reporter activation (Fig 5b). In addition to IFN-β-reporter activity, co-expression of caspase-6 and MERS-CoV N similarly reduced the expression of IFN-β and representative interferon-stimulated genes (ISGs) including IFIT1, IFIT2, IFIT3, IFITM3, TRIM22, and OAS1 (Fig. 5c and Extended Data Fig. 7). Recent studies have reported MERS-CoV ORF4a, ORF4b, and membrane (M) protein as potent IFN antagonists. Our data showed that caspase-6 did not modulate the IFN antagonism of these known IFN antagonists (Fig. 5d). Importantly, our further investigations demonstrated that caspase-6 similarly mediated N cleavage of other human pathogenic coronaviruses, including that of SARS-CoV-2 and SARS-CoV-1 (Fig. 5e), which is in agreement with our earlier findings that caspase-6 inhibition attenuated the replication of these coronaviruses (Fig. 1e,f). We next expressed the N genes of these coronaviruses together with caspase-6, which revealed that the co-expression of coronavirus N with caspase-6 antagonized IFN-β-reporter activation and reduced the expression of representative ISGs including IFIT3 and OAS1 (Fig. 5f,g). Collectively, these results suggest that caspase-6-mediated N cleavage modulates coronavirus replication by regulating IFN signaling.

To further explain how caspase-6-mediated N cleavage regulates IFN response, we analyzed the potential caspase-6 cleavage sites on MERS-CoV N based on known caspase-6 substrate specificity and the size of the cleavage fragments, and generated the corresponding N mutants that potentially interfered with caspase-6 cleavage (Fig. 6a). Western blot-based cleavage assays demonstrated that caspase-6-mediated cleavage was abolished for the T239KKΑ242 mutant, suggesting that caspase-6 cleaves MERS-CoV N at the T239KKD242 motif (Fig. 6b). Interestingly, the T239KKD242 motif is located within the intrinsically disordered region (IDR) of MERS-CoV N that bridges the N-terminal domain and C-terminal domain of N, which are structurally conserved among coronaviruses. Similar putative
caspase-6 cleavage motifs are also present in the IDR of N of other human-pathogenic coronaviruses (Extended Data Table. 1). In IFN-β-reporter assays, caspase-6 and N-mediated IFN antagonism was attenuated when T^{239}KKA^{242} was expressed in place of the wildtype N (Fig. 6e). These findings suggested that caspase-6-mediated MERS-CoV N cleavage is essential for its IFN antagonism. Next, we generated the N(1-241) and N(242-413) fragments that mimicked the N cleavage products (Fig. 6a). In Western blots, we showed that the two fragments were no longer cleaved by caspase-6 (Fig. 6d). Consistent with this result, in IFN-β-reporter assays, the two N cleavage products individually limited IFN-β-reporter activity but were no longer modulated by caspase-6 (Fig. 6e). To dissect how the N fragments modulate IFN signaling, we explored their capacity of interacting with different components of the IFN signaling pathway with co-immunoprecipitation assays. Our results demonstrated that both N(1-241) and N(242-413) fragments interacted with IRF3 but not other components of the IFN signaling pathway (Fig. 6f and Extended Data Fig. 8). In poly(I:C)-treated 293T cells, IRF3 translocated to the cell nuclei regardless of MERS-CoV N expression. In stark contrast, both N(1-241) and N(242-413) fragments co-localized with IRF3 and abolished its nucleus translocation (Fig. 6g). Taken together, our study identifies caspase-6-mediated N cleavage as a novel mechanism that serves to dampen the host IFN response for efficient coronavirus replication. Inhibition of caspase-6 markedly attenuates coronavirus replication and ameliorates coronavirus-induced lung pathology in vivo, suggesting that caspase-6 inhibition should be further explored as an option for the treatment of highly pathogenic coronaviruses (Extended Data Fig. 9).

Discussion

In this study, we reveal caspase-6 as an essential host factor for efficient coronavirus replication. Caspase-6 inhibition limits the replication of all evaluated human-pathogenic
coronaviruses including MERS-CoV, SARS-CoV-2, SARS-CoV-1, HCoV-229E, and HCoV-OC43. In addition, caspase-6 inhibition significantly lowers the replication of highly pathogenic coronaviruses including MERS-CoV and SARS-CoV-2 in vivo, which improves the survival of MERS-CoV<sub>MA</sub>-infected hDPP4 KI mice and attenuates the body weight loss and lung pathology of SARS-CoV-2-infected golden Syrian hamsters. Mechanistically, caspase-6 mediates the cleavage of coronavirus N protein. The N cleavage products serve as IFN antagonists and interfere with activation of IFN signaling, which reduces the expression of ISGs, leading to efficient coronavirus replication. Overall, our study reveals a novel mechanism for efficient coronavirus replication. Upon coronavirus infection, the host initiates apoptosis to eliminate infected cells and terminate virus propagation. At the same time, coronaviruses exploit a component of the activated apoptosis cascade to facilitate virus replication in order to maximize virus production before the cells are obliterated due to apoptosis induction. This is an elegant example of virus-host interaction that exemplifies the long-standing arms race between humans and coronaviruses.

The replication of coronaviruses including MERS-CoV and SARS-CoV-2 is known to depend on a host serine protease, transmembrane protease serine 2 (TMPRSS2), which cleavage activates the spike (S) protein of coronaviruses for efficient entry and replication<sup>28,29</sup>. In contrast, the role of host cysteine-aspartic protease on coronavirus replication has not been explored. Previous studies have suggested that influenza viruses require a host cysteine-aspartic protease, caspase-3, for efficient translocation of viral ribonucleoprotein (RNP) complexes across the nuclear membrane, which is essential for efficient virus replication<sup>30,31</sup>. In the current study, we show that caspase-6 but not caspase-3 facilitates coronavirus replication. Caspase-6 is most known for its role as an executor caspase and its catalytic role in neurodegeneration in Huntington's and Alzheimer's disease<sup>32</sup>. Here, we demonstrate that caspase-6 mediates efficient coronavirus replication by cleaving
coronavirus N proteins, which in turn serve as IFN antagonists that block IFN activation. Importantly, inhibiting caspase-6 attenuates virus replication and disease severity in highly pathogenic coronavirus-infected mice and hamsters. These findings suggest that in addition to the serine protease TMPRSS2, inhibiting the cysteine-aspartic protease caspase-6 should also be further explored as a therapeutic option against the infection by highly pathogenic coronaviruses including SARS-CoV-2.

A large body of studies identified viral components of different viruses on their potential role as IFN antagonists by individually expressing the viral components. Our study reveals host proteases as previously unappreciated factors that can modulate viral antagonism. Taking this new knowledge into consideration, our current understanding on viral components that antagonize IFN signaling may be incomplete since viral components may be processed by host proteases, thus modulating their capacity in interacting with the host IFN signaling pathways.

In the current study, we demonstrated that caspase-6 mediated the cleavage of N protein of all evaluated human pathogenic coronaviruses including MERS-CoV, SARS-CoV-2, SARS-CoV-1, HCoV-229E, HCoV-OC43, and HCoV-NL63. In addition to these human pathogenic coronaviruses, the N protein of transmissible gastroenteritis coronavirus (TGEV) and porcine epidemic diarrhea virus (PEDV), which are alphacoronaviruses that infect pigs, can also be cleaved by caspase-6. To this end, it will be interesting to further investigate if the caspase-6-mediated N cleavage mechanism and its associated IFN antagonism can be further generalized beyond human-pathogenic coronaviruses to other members of the coronavirus family.

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Author contributions


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Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Competing Interests

JFW Chan has received travel grants from Pfizer Corporation Hong Kong and Astellas Pharma Hong Kong Corporation Limited, and was an invited speaker for Gilead Sciences Hong Kong Limited and Luminex Corporation. The other authors declared no conflict of interests. The funding sources had no role in study design, data collection, analysis or interpretation or writing of the report.
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Figure 1. Caspase-6 inhibition limits coronavirus replication. a MERS-CoV-infected Huh7 cells were treated with 100µM z-VAD-fmk or DMSO. Cells were fixed at 24 hpi and immunolabeled with an in-house guinea pig immune serum against MERS-CoV N. b Replication of human-pathogenic coronaviruses treated with 100µM z-VAD-fmk or DMSO. Samples were harvested at 24 hpi and viral gene expression was quantified with RT-qPCR (n=3). c-d Flow cytometry of MERS-CoV-infected BEAS2B cells treated with 75µM specific caspase inhibitors. Cells were fixed at 24 hpi and labelled with the MERS-CoV N immune
serum and an active caspase-3 antibody (n=3). e Virus replication of MERS-CoV (BEAS2B), SARS-CoV-2 (Calu3), SARS-CoV-1 (Huh7), HCoV-229E (Huh7), HCoV-OC43 (BSC-1), H1N1 (A549), and EV-71 (RD) with or without 100µM z-VEID-fmk. Virus gene copy was quantified with RT-qPCR. f The half-maximal inhibitory concentrations (IC$_{50}$) of z-VEID-fmk on the replication of MERS-CoV (HFL), SARS-CoV-2 (Calu3), SARS-CoV-1 (Huh7), HCoV-229E (Huh7) and HCoV-OC43 (BSC-1) in cell lysate and supernatant samples were determined with RT-qPCR and TCID$_{50}$ assays, respectively (n=4 for HCoV-OC43 and EV-71, n=3 for other viruses). Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (d) or two way-ANOVA (b and e). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant. Bars in (a) represented 20µm.
Figure 2. Caspase-6 inhibition attenuates MERS-CoV replication in human lung tissues, human intestinal organoids, and improves the survival of hDPP4 KI mice. a-b Ex vivo human lung tissues were infected with MERS-CoV and treated with z-VEID-fmk. The tissues and supernatants were harvested at 24 hpi for immunostaining with an in-house guinea pig
immune serum against MERS-CoV N (a) and RT-qPCR (n=8) (b). c-f Human intestinal
organoids were infected with MERS-CoV and treated with z-VEID-fmk. Organoids were fixed
at 24 hpi for immunostaining for N protein expression (c). The percentage of infected cells per
organoids was calculated from counting the number of infected cells and uninfected cells per
organoid (n=5) (d). N gene expression at the indicated time points was quantified with RT-
qPCR (n=3) (e). Infectious titer was determined with plaque assays (n=6) (f). g hDPP4 KI mice
were intranasally inoculated with 2.5x10³PFU MERS-CoV followed by intraperitoneal
administration of 12.5mg/kg/day z-VEID-fmk or DMSO for 6 days or until sample harvest. h A
subset of mice were harvested at day 2 and day 4 post infection. Mouse lungs were
immunolabelled to detect MERS-CoV N expression. i Viral gene expression in mouse lungs
were quantified with RT-qPCR (n=3). j Infectious titer was determined with plaque assays
(n=3). k Expression of pro-inflammatory cytokines and chemokines were quantified with RT-
qPCR. l-m Body weight and survival of the infected mice were monitored for 14 days. Data
represented mean and standard deviations from the indicated number of biological repeats.
Statistical significance between groups was determined with one way-ANOVA (k), two way-
ANOVA (e), Student’s t-test (b, d, f, i, j, and l), or Log-rank (Mantel-Cox) test (m). *
represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p <
0.0001. ns = not significant. Bars in (a, c, and h) represented 50µm, 20µm, and 100µm,
respectively.
Figure 3. Caspase-6 inhibition ameliorates lung pathology and improves the body weight of SARS-CoV-2-infected golden Syrian hamsters.  

**a** Golden Syrian hamsters were intranasally inoculated with 3x10^3 PFU SARS-CoV-2 followed by intraperitoneal administration of 12.5mg/kg/day z-VEID-fmk or DMSO for 4 days.  

**b-c** Hamsters were sacrificed at day 4 post infection, viral gene copy and infectious titer of hamster lungs were quantified with RT-qPCR and TCID\(_{50}\) assays, respectively (n=6).  

**d-e** Viral N protein expression in the small airways and alveoli of infected hamster lungs with or without z-VEID-fmk treatment was revealed with immunofluorescence staining with the in-house rabbit immune serum against SARS-CoV-2 N.  

**f** Expression of pro-inflammatory cytokines and chemokines was quantified with RT-qPCR.
Body weight change of SARS-CoV-2-infected hamsters with z-VEID-fmk or mock treatment was documented from day 0 to day 4 post infection. Representative images of haematoxylin and eosin (H&E) stained hamster lungs. (Top panels) mock-infected hamster lung sections showed normal histology, boxed areas were magnified showing (i) intact bronchiolar epithelium lining, (ii) thin alveolar wall and clear air sac, and (iii) a normal structure of pulmonary blood vessel section. (Middle panels) In SARS-CoV-2-infected hamsters, lung tissues showed diffuse inflammatory infiltration and exudation with disappearing air-exchange structures. Boxed areas were magnified to demonstrate the characteristic histopathological changes (i) peribronchiolar infiltration and bronchiolar epithelium desquamation, (ii) alveolar infiltration and haemorrhage with alveolar space filled with infiltrated immune cell and protein-rich exudate, (iii) pulmonary blood vessel showed immune cells infiltration in the vessel wall, endothelium, and perivascular connective tissue. (Bottom panels) The hamster lung pathology was markedly improved with z-VEID-fmk treatment. Magnified images demonstrated (i) milder degree of immune cell infiltration in bronchiolar epithelium and peribronchiolar tissue, (ii) thickened alveolar wall with red blood cells but alveolar space showed no immune cell infiltration nor exudation, (iii) pulmonary vessel wall showed a few immune cells attached to the endothelium.

Quantitative scores for the lung histopathological changes of SARS-CoV-2-infected hamsters with or without z-VEID-fmk treatment. Three categories of characteristic histopathological changes including bronchiolitis, alveolitis and vasculitis were examined and scored. (n=6 and two-three lung lobes were examined from each hamster). Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (f) or Student’s t-test (b, c, g, and i). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant. Bars in (d and e) represented 100µm.
Figure 4. Caspase-6 modulates MERS-CoV replication at a post entry step and cleaves MERS-CoV N protein. a MERS-CoV-infected BEAS2B cells were incubated with z-VEID-fmk in a time of addition assay. Virus gene copy in the supernatant was determined with RT-qPCR at 24hpi (n=4). b-c Caspase-6 stable knockdown A549 and BEAS2B cells were infected with MERS-CoV at 0.1MOI. (b) Cell lysates were harvested at 1 hpi to quantify virus entry with RT-qPCR (n=4). (c) Virus replication at 24 hpi were quantified with RT-qPCR (n=4). d MDMs were treated with caspase-6 or nontargeting siRNA and infected with MERS-CoV. Virus gene
copy was quantified at 24 hpi (n=4). e-f Caspase-6- or caspase-3-overexpressed 293T cells were infected with MERS-CoV at 1MOI. Virus replication was quantified at 1 and 24 hpi with RT-qPCR (n=4). g MERS-CoV N and caspase-6 were co-expressed in 293T cells with or without 100µM z-VEID-fmk. Cells were harvested for Western blot at 24 hours post transfection. 1µM staurosporine (STS) was added as an apoptosis trigger at 6 hours before sample harvest. h N cleavage in cell lysates from MERS-CoV-infected Huh7 and BEAS2B cells was compared to the N cleavage in cell lysates from MERS-CoV N and caspase-6 co-transfected 293T cells. i N cleavage in MERS-CoV-infected cells with or without z-VEID-fmk. j Comparison of MERS-CoV N cleavage in caspase-6-overexpressed and caspase-3-overexpressed cells. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (a), two-way ANOVA (b and f), or Student’s t-test (c and d). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant.
Figure 5. Caspase-6-mediated coronavirus N cleavage interferes with IFN signalling.

a-b 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of MERS-CoV N or E and caspase-6 or caspase-3, with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual-luciferase reporter assays (n=4). c 293T cells were transfected with the same set of plasmids. Gene expression of IFN-β, IFIT3, and OAS1 was quantified with RT-qPCR (n=6 for IFN-β and IFIT1, n=4 for OAS1). d 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and MERS-CoV N, ORF4a, ORF4b, or M, with or without poly(I:C). Cells were incubated for 24 hours.
before harvesting for dual-luciferase reporter assays (n=3). e N protein of the indicated coronaviruses were co-expressed with caspase-6. N cleavage was detected with Western blots. f 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and coronavirus N, with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual-luciferase reporter assays (n=3). g 293T cells were transfected with the same set of plasmids. Gene expression of IFIT3 and OAS1 was quantified with RT-qPCR (n=4). Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (a-f and h-i). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant.
Figure 6. Caspase-6-mediated MERS-CoV N cleavage generates N fragments that block IRF3 translocation to the cell nucleus. a Schematic of N mutants. b Caspase-6-mediated cleavage of N mutants was evaluated with Western blots. c 293T cells were transfected with an
IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and MERS-CoV N or N mutants, with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual-luciferase reporter assays (n=3). Caspase-6-mediated cleavage of N, N(1-241), and N(242-413) was evaluated with Western blots. 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and MERS-CoV N, N(1-241), or N(242-413), with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual-luciferase reporter assays (n=4). Interaction between IRF3 and N, N(1-241), or N(242-413) was evaluated with co-immunoprecipitation assays using IRF3 as the bait protein. 293T cells were transfected with expression constructs of IRF3, N, N(1-241), or N(242-413), and poly(I:C). Cells were fixed at 24 hours post transfection. Localization of N was detected with an in-house guinea pig anti-N immune serum and IRF3 was detected with a rabbit anti-HA antibody. Cell nuclei were identified with the DAPI stain. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (c and e). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant. Bars in (g) represented 10µm.
Extended Data Figure 1. Caspase-6 inhibition reduces MERS-CoV replication in different cell types. MDM, Caco2, Calu3, A549, and VeroE6 cells were infected with MERS-CoV at 1MOI and were treated with z-VEID-fmk, z-VAD-fmk, or DMSO. Cell lysate and supernatant samples were harvested at 24hpi. Virus gene copy was quantified with RT-qPCR (n=3). Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA. * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant.
Extended Data Figure 2. The z-VEID-fmk binding pocket is conserved in human, mouse, and golden Syrian hamster caspase-6. a Multiple sequence alignment of human, mouse and golden Syrian hamster caspase-6 full-length sequences. The indices were labelled according to human caspase-6. VEID binding sites on caspase-6 were indicated with orange triangles. b VEID binding mode represented in 3D structure. Binding sites on caspase-6 and VEID were shown in green and magenta sticks, respectively. The binding site residues were labelled in red.
Extended Data Figure 3. Caspase-6 inhibition reduces the expression of pro-inflammatory cytokines and chemokines in the lungs of hDPP4 KI mice. hDPP4 KI mice were intranasally inoculated with $2.5 \times 10^3$ PFU MERS-CoV followed by intraperitoneal administration of 12.5mg/kg/day z-VEID-fmk or DMSO for 6 days or until sample harvest. Mouse lungs were harvested at day 2 and day 4 post infection. Expression of pro-inflammatory cytokines and chemokines were quantified with RT-qPCR. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA. * represented $p < 0.05$, ** represented $p < 0.01$, *** represented $p < 0.001$. ns = not significant.
Extended Data Figure 4. Caspase-6 knockdown efficiency in cell lines and MDMs.

a Caspase-6 RNA (n=3) and protein expression from caspase-6 stable knockdown A549 and BEAS2B cells. b Caspase-6 protein expression from caspase-6 siRNA transfected MDMs. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with two way-ANOVA. **** represented p < 0.0001.
Extended Data Figure 5. Caspase-6 is activated by apoptosis and can undergo autoactivation. a Caspase-6 is activated by apoptosis triggered by MERS-CoV infection or staurosporine (STS) stimulation. Huh7 cells were infected with MERS-CoV at 1MOI for 12 hours. In parallel, Huh7 cells were stimulated with STS at 1µM for 6 hours. Caspase-6 activity in the cell lysate was determined with the caspase-Glo-6 assay kit (n=4). b Autoactivation and STS-mediated activation of caspase-6 were demonstrated with Western blots. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with two way-ANOVA. ** represented p < 0.01 and *** represented p < 0.001.
Extended Data Figure 6. Caspase-6 cleaves MERS-CoV N but not other MERS-CoV proteins. Caspase-6 and expression constructs for MERS-CoV proteins were transfected in 293T cells. Caspase-6-mediated cleavage was detected with Western blots. Asterisks at Nsp2 and Nsp14 indicated the predicted protein sizes according to the number of amino acid residues. Caspase-6-specific cleavage product was only observed when N was co-expressed with caspase-6 (arrow).
Extended Data Figure 7. Caspase-6-mediated N cleavage reduces ISG expression. 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of MERS-CoV N or E and caspase-6 or caspase-3, with or without poly(I:C). Gene expression of IFIT1, IFIT2, IFITM3, and TRIM22 was quantified with RT-qPCR (n=6 for IFIT1 and IFIT2, n=4 for IFITM3 and TRIM22). Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA. * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant.
Extended Data Figure 8. Caspase-6-cleaved N fragments do not bind MAVS, IKKε, TBK1, MDA5, or RIG-I. Interaction between MAVS, IKKε, TBK1, MDA5, and RIG-I with N, N(1-241), or N(242-413) was evaluated with co-immunoprecipitation assays.
Extended Data Figure 9. Current model of how caspase-6 facilitates coronavirus replication using MERS-CoV as an example. a Upon coronavirus infection, the host initiates apoptosis to eliminate infected cells, aiming to terminate virus propagation. The triggered apoptosis cascade leads to activation of executor caspases (caspase-3, -6, -7). b MERS-CoV exploits caspase-6 to cleave its N protein, generating N fragments that bind to IRF3, attenuating the activation of IFN signalling, thus benefits virus replication. c In the presence of caspase-6 inhibition, N is not cleaved and IFN signalling is more intact, resulting in restricted virus replication.
Extended Data Figure 10. Gating Strategy for the flow cytometry experiment in Figure 1.

The BEAS2B cell population was gated with SSC-A vs FSC-A. Most cells with the exception of the cell debris at the lower left corner were gated. A mock-infected sample treated with the
apoptosis inhibitor, z-VAD-fmk, was used as the gating control for active caspase-3-positive cells. A mock-infected sample treated with DMSO was used as the gating control for MERS-CoV-N-positive cells.

**Materials and methods**

**Cell lines**

A549, BSC-1, Caco2, Huh7, VeroE6, and 293T cells were maintained in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 µg/ml streptomycin. BEAS2B and Calu3 cells were maintained in DMEM/F12 supplemented with 10% heat-inactivated FBS, 100 unit/ml penicillin and 100 µg/ml streptomycin. HFL (primary human embryonic lung fibroblast) were maintained in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated FBS, 100 unit/ml penicillin and 100 µg/ml streptomycin. Human primary monocytes were obtained from human peripheral blood mononuclear cells (PBMCs) taken from healthy donors, collected from Hong Kong Red Cross Blood Transfusion Service according to a protocol approved by the Institutional Review Board of the University of Hong Kong. Primary human monocyte-derived macrophages (MDMs) were differentiated from monocytes in Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 10% heat-inactivated FBS, 100 unit/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1% sodium pyruvate, 1% non-essential amino acids, and 10 ng/ml recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems) as we previously described.

**Viruses**

The MERS-CoV (EMC/2012) strain of MERS-CoV was provided by Dr. Ron Fouchier (Erasmus Medical Center). The mouse-adapted MERS-CoV (MERS-CoV<sub>MA</sub>) was a gift from
Dr. Paul McCray (University of Iowa, IA, USA). SARS-CoV-2 HKU-001a was isolated from a nasopharyngeal aspirate specimen taken from a laboratory-confirmed COVID-19 patient in Hong Kong. SARS-CoV-1 GZ50, HCoV-229E, HCoV-OC43, enterovirus A71, and influenza A virus strain A/Hong Kong/415742/2009(H1N1)pdm09 were archived clinical isolates at Department of Microbiology, HKU. All infectious experiments involving MERS-CoV, SARS-CoV-2 and SARS-CoV-1 followed the approved standard operating procedures of the Biosafety Level 3 facility at the Department of Microbiology, HKU.

Chemical modulators

The pan-caspase inhibitor, z-VAD-fmk, was obtained from Invivogen. The caspase-1-to-caspase-10 inhibitor sampler kit was purchased from R&D Systems. The caspase-6 inhibitors, z-VEID-fmk, used for in vitro and in vivo experiments, were obtained from R&D Systems and APExBIO, respectively. The apoptosis enhancer, staurosporine, was obtained from Sigma.

Antibodies

MERS-CoV N, MERS-CoV Spike, SARS-CoV-1 N and SARS-CoV-2 N were detected with specific in-house immune serum. Primary antibodies including rabbit anti-caspase-3, rabbit anti-caspase-6, rabbit anti-HA, mouse anti-Flag, mouse anti-His, and mouse anti-β-actin were from Abcam. Secondary antibodies including goat anti-mouse horseradish peroxidase (HRP), goat anti-rabbit HRP and goat anti-guinea pig HRP from Thermo Fisher Scientific were used for Western blots. Alexa Fluor 488 goat anti-guinea pig, Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 568 goat anti-rabbit from Thermo Fisher Scientific were used for immunohistochemistry staining.

Ex vivo human lung tissues
Ex vivo human lung tissues were processed and infected with MERS-CoV we previously described\textsuperscript{41,42}. Human lung tissues for ex vivo studies were retrieved from patients underwent surgical operations at the Queen Mary Hospital, Hong Kong. All donors gave written consent as approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Normal nonmalignant lung tissue fragments in excess for clinical diagnosis were used. The freshly obtained lung tissues were processed into small rectangular pieces and were rinsed with the primary tissue culture medium, which contained the advanced DMEM/F12 medium supplemented with 2mM HEPES (Gibco), 1x GlutaMAX (Gibco), 100\text{unit/ml} penicillin, 100\text{µg/ml} streptomycin, 20\text{µg/ml} vancomysin, 20\text{µg/ml} ciprofloxacin, 50\text{µg/ml} amikacin, and 50\text{µg/ml} nystatin. The specimens were infected with MERS-CoV at a titer of $1 \times 10^8$ PFU/ml. After 2 h, the inoculum was removed and the specimens were washed thoroughly with the primary tissue culture medium. The infected tissues were then incubated with primary tissue culture medium supplemented with 100\text{µM} caspase-6 inhibitor z-VEID-fmk dissolved in DMSO or DMSO only. Tissues were harvested at 24 hpi with either 10% neutral-buffered formalin for immunofluorescence staining or with RL buffer for RT-qPCR analysis.

**Human intestinal organoids**

Human Intestinal organoids were established using biopsied human intestinal tissues from patients who underwent surgical operations at the Queen Mary Hospital, Hong Kong\textsuperscript{43}. All donors had written consent as approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Human intestinal organoids were maintained in expansion medium and induced differentiation by incubating with differentiation media for 5 days as we previously described\textsuperscript{43}. Differentiated intestinal organoids were sheared mechanically and inoculated with MERS-CoV at 1MOI for 2 h. After the inoculum was removed, the intestinal organoids were rinsed with PBS and embedded in Matrigel and
maintained in differentiation medium containing 100µM z-VEID-fmk. At the indicated time points after inoculation, intestinal organoids were harvested for the quantification of intracellular viral load and immunofluorescence staining, whereas the cell-free Matrigel and culture medium were combined for viral titration of extracellular virions using standard plaque assays.

**Human DPP4 mouse model**

The hDPP4 knockin (KI) mice were kindly provided by Dr. Paul McCray (University of Iowa, IA, USA). The use of animals has complied with all relevant ethical regulations and was approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong. On the day of infection, hDPP4 KI mice were intranasally (i.n.) inoculated with 2.5x10^3 PFU mouse-adapted MERS-CoV (MERS-CoV<sub>MA</sub>) pre-diluted in 20µl DMEM, followed by intraperitoneal (i.p.) injection with 12.5mg/kg/day z-VEID-fmk or DMSO diluted in 200µl 0.3% methylcellulose/0.1% tween-80/PBS for 6 days or until sample harvest.

The health status and body weight of the mice were monitored for 14 days on a daily basis or until the animal is sacrificed or euthanized because of reaching the humane endpoint of the experiment. Mice were sacrificed at the designated time points and lung tissue from mice of both treatment and control groups were harvested for immunofluorescence staining, RT-qPCR, and plaque assay analysis.

**Golden Syrian hamster model**

Infection of golden Syrian hamsters was performed as we described previously<sup>15</sup>. Golden Syrian hamsters aged 6-8 weeks old were obtained from the Chinese University of Hong Kong Laboratory Animal Service Centre through the HKU Centre for Comparative Medicine Research (CCMR). The use of animals has complied with all relevant ethical regulations and was approved by the Committee on the Use of Live Animals in Teaching and Research of
The University of Hong Kong. On the day of infection, each hamster was intranasally inoculated with $3 \times 10^3$ PFU SARS-CoV-2 pre-diluted in 50µl DMEM under intraperitoneal ketamine (100mg/kg) and xylazine (10mg/kg) anesthesia. Infected hamsters were treated with 12.5mg/kg/day z-VEID-fmk or DMSO diluted in 600µl 0.3% methylcellulose/0.1% tween-80/PBS for 4 days. The health status and body weight of the hamsters were monitored on a daily basis or until the animal is sacrificed or euthanized because of reaching the humane endpoint of the experiment. Hamsters were sacrificed at day 4 post infection and lung tissues were harvested for immunofluorescence staining, histopathology examination, RT-qPCR, and TCID$_{50}$ assay analysis$^{44,45}$.

RNA extraction and quantitative RT-PCR

Cells were lysed in RL buffer and extracted with the MiniBEST Universal RNA Extraction Kit (TaKaRa). Viral RNA in the supernatant was extracted with the MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa). Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) were performed with Transcriptor First Strand cDNA Synthesis Kit and LightCycler 480 master mix from Roche. All primer and probe sequences were provided in Extended Data Table 2.

Plaque assays and TCID$_{50}$ assays

Infectious titers of MERS-CoV and SARS-CoV-2 were determined with standard plaque assays$^{35}$. In brief, VeroE6 cells were seeded in 24-well plates 1 day before the experiment. The harvested supernatant samples were serially diluted and inoculated to the cells for 2 h at 37°C. After inoculation, the cells were washed with PBS 3 times, and covered with 2% agarose/PBS mixed with $2 \times$ DMEM/2%FBS at 1:1 ratio. The cells were fixed after incubation at 37°C for 72 h. Fixed samples were stained with 0.5% crystal violet in 25% ethanol/distilled water for 10 min.
for plaque visualization. In some experiments, infectious titers of coronaviruses were determined with standard TCID\textsubscript{50} assays. In brief, VeroE6 cells were seeded in 96-well plates 1 day before the experiment. The harvested supernatant samples were serially diluted and inoculated to the cells for 2 h at 37°C. After inoculation, the cells were washed with PBS 3 times and incubation at 37°C. After 72 hpi, virus titer was calculated using the Muench and Reed method.

siRNA and shRNA knockdown

On-Targetplus caspase-6 siRNA was obtained from Dharmacon. Transfection of siRNA on MDMs was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) as we previously described\textsuperscript{46}. In brief, the cells were transfected with 50nM caspase-6 siRNA for two consecutive days. At 24 hours after the second siRNA transfection, the cells were harvested in RIPA buffer for Western blot analysis. In parallel, siRNA-transfected cells were challenged with MERS-CoV at 1MOI for 1 h at 37°C. Following the inoculation, the cells were washed with PBS and incubated for 24 h. The virus copy number at 24 hpi was determined with RT-qPCR. pLKO.1 lentiviral caspase-6 shRNA plasmid was obtained from Dharmacon. Transfection of caspase-6 shRNA plasmid, psPAX2 packaging plasmid and pMD2.G envelope plasmid on 293T cells was performed using Lipofectamine 3000 (Thermo Fisher Scientific) following manufacturer’s manual. In brief, 293T cells in 10cm dishes were transfected with 6 µg caspase-6 shRNA plasmid, 4.5µg packaging plasmid, and 1.5µg envelope plasmid in FBS-supplemented DMEM medium, followed by aspirating supernatant at 6 h post transfection and replacing with FBS-free medium. On the next day, the supernatant containing caspase-6 shRNA lentivirus particles was harvested and was used to transduce A549 and BEAS2B cells. A549 and BEAS2B caspase-6 stable knockdowns cells were selected by 0.5µg/mL and 0.7µg/mL puromycin, respectively. The selected cells were challenged with MERS-CoV at 0.1MOI for 1 h at 37°C. The virus copy number at 1 and 24 hpi was determined with RT-qPCR.
Caspase-6 activity assay

Huh7 cells were infected with MERS-CoV at 1MOI for 12 h. In parallel, Huh7 cells were stimulated with STS at 1µM for 6 h. Caspase-6 activity in the cell lysate was determined with the caspase-Glo-6 assay kit (Promega). The luminescence signal of caspase-6 activity was measured following manufacturer’s manual with a multilabel plate reader Victor X3 (Perkin-Elmer).

Immunofluorescence and histology

Immunofluorescence staining was performed as we previously described with slight modifications\(^4^7\). Briefly, infected human and animal lung tissues were fixed overnight in 10% formalin. The fixed samples were then embedded in paraffin with a TP1020 Leica semi-enclosed benchtop tissue processor and sectioned at 5µm. Tissue sections were fished and dried to fix on Thermo Fisher Scientific Superfrost Plus slides at 37°C overnight. Antigen retrieval was performed by heating the slides in antigen unmasking solution (Vector Laboratories) for 90 seconds. MERS-CoV and SARS-CoV-2 were detected with an in-house guinea pig anti-MERS-CoV-N immune serum and an in-house rabbit anti-SARS-CoV-2-N immune serum, respectively. Cell nuclei were labeled with the DAPI nucleic acid stain (Thermo Fisher Scientific). Alexa Fluor secondary antibodies were obtained from Thermo Fisher Scientific. Mounting was performed with the Diamond Prolong Antifade Mountant from Thermo Fisher Scientific. Images were captured with an Olympus BX53 fluorescence microscope (Olympus Life Science, Tokyo, Japan) or a Carl Zeiss LSM 780 confocal microscope in the faculty core facility of HKU. For H&E staining, hamster lung tissue sections were stained with Gill’s hematoxylin and eosin Y (Thermo Fisher Scientific). H&E stained hamster lung tissue sections were blinded for the identities of experimental settings and examined by a trained
histopathologist. Lung pathology was graded on a scale of 0 (normal) to 4 (most severe) according to a grading system we previously described\textsuperscript{18}.

\section*{Western blot}

Cells were lysed by RIPA buffer (Thermo Fisher Scientific) with protease inhibitor (Roche, Basel, Switzerland). Proteins were separated with SDS-PAGE and transferred to PVDF membranes (Thermo Fisher Scientific). Specific primary antibodies were incubated with the blocked membranes at 4°C overnight, followed by horseradish peroxidase (HRP) conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature. The signal was developed by Immobilon Crescendo Western HRP Substrate (Merck Millipore, MA, USA) and detected using automatic x-ray film processor (Advanta) or an Alliance Q9 Advanced imager (Uvitec, Cambridge, UK).

\section*{Flow cytometry}

BEAS2B cells were infected with MERS-CoV at 1MOI. At 24 hpi, the cells were detached with 10mM EDTA in PBS, fixed in 4% paraformaldehyde, followed by immunolabeling with an in-house guinea pig anti-MERS-CoV-N immune serum and a rabbit anti-active caspase-3 antibody (BD). Flow cytometry was performed using a BD FACSCanto II flow cytometer (BD) and data was analyzed using FlowJo X 10.0.7 (BD) as we previously described\textsuperscript{48}. The gating strategy was demonstrated in Extended Data Figure 10.

\section*{IFN-\(\beta\)-luciferase reporter assays}

IFN-\(\beta\)-luciferase reporter assays were performed as we previously described\textsuperscript{23,24}. In brief, 500ng IFN-\(\beta\)-luciferase reporter plasmid, 10ng transfection efficiency control plasmid (pNL1.1.TK, Promega), 1\(\mu\)g coronavirus N plasmids, 3\(\mu\)g caspase-6 expression plasmid, together with or
without 5µg Poly(I:C) were co-transfected into 293T cells for 24 h. On the next day, the cells were harvested for luciferase measurement with the dual-luciferase reporter assay system kit (Promega) according to the manufacturer’s protocol using a multilabel plate reader Victor X3 (Perkin-Elmer).

**Alignment of human, mouse, and hamster caspase-6**

Caspase-6 protein sequences of homo sapiens (Uniprot ID: P55212), mus musculus (Uniprot ID: O08738) and mesocricetus auratus (Uniprot ID: A0A1U7QNN7) were downloaded from UniProt. Multiple sequence alignment was performed with MUSCLE. The crystal structure of caspase-6 and VEID complex was retrieved from the Protein Data Bank (PDB code: 3OD5). Caspase-6 residues within 4Å of VEID were defined as the binding sites and visualized with Pymol.

**Prediction of potential caspase-6 cleavage sites**

The protein sequence of MERS-CoV N (NC_019843) was used for caspase-6 cleavage site analysis. Potential caspase-6 cleavage motifs on MERS-CoV N was determined based on published substrate specificity of caspase-6. The amino acid pattern "[TVILENYF]..D" was search against the N sequence, where "." represented any amino acid.

**Statistical analysis**

Data on figures represented means and standard deviations. Statistical comparison between different groups was performed by one-way ANOVA, two-way ANOVA, Student’s t-test, or Log-rank (Mantel-Cox) test using GraphPad Prism 6. Differences were considered statistically significant when $p < 0.05$. 
Figures

Figure 1

Caspase-6 inhibition limits coronavirus replication. a MERS-CoV-infected Huh7 cells were treated with 100μM z-VAD-fmk or DMSO. Cells were fixed at 24 hpi and immunolabeled with an in-house guinea pig immune serum against MERS-CoV N. b Replication of human-pathogenic coronaviruses treated with 100μM z-VAD-fmk or DMSO. Samples were harvested at 24 hpi and viral gene expression was quantified with RT-qPCR (n=3). c-d Flow cytometry of MERS-CoV-infected BEAS2B cells treated with 75μM specific
caspase inhibitors. Cells were fixed at 24 hpi and labelled with the MERS-CoV N immune serum and an active caspase-3 antibody (n=3). e Virus replication of MERS-CoV (BEAS2B), SARS-CoV-2 (Calu3), SARS-CoV-1 (Huh7), HCoV-229E (Huh7), HCoV-OC43 (BSC-1), H1N1 (A549), and EV-71 (RD) with or without 100μM z-VEID-fmk. Virus gene copy was quantified with RT-qPCR. f The half-maximal inhibitory concentrations (IC50s) of z-VEID-fmk on the replication of MERS-CoV (HFL), SARS-CoV-2 (Calu3), SARS-CoV-1 (Huh7), HCoV-229E (Huh7) and HCoV-OC43 (BSC-1) in cell lysate and supernatant samples were determined with RT-qPCR and TCID50 assays, respectively (n=4 for HCoV-OC43 and EV-71, n=3 for other viruses). Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (d) or two way-ANOVA (b and e). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant. Bars in (a) represented 20μm.
Caspase-6 inhibition attenuates MERS-CoV replication in human lung tissues, human intestinal organoids, and improves the survival of hDPP4 KI mice. a-b Ex vivo human lung tissues were infected with MERS-CoV and treated with z-VEID-fmk. The tissues and supernatants were harvested at 24 hpi for immunostaining with an in-house guinea pig immune serum against MERS-CoV N (a) and RT-qPCR (n=8) (b). c-f Human intestinal organoids were infected with MERS-CoV and treated with z-VEID-fmk. Organoids
were fixed at 24 hpi for immunostaining for N protein expression (c). The percentage of infected cells per organoids was calculated from counting the number of infected cells and uninfected cells per organoid (n=5) (d). N gene expression at the indicated time points was quantified with RT qPCR (n=3) (e). Infectious titer was determined with plaque assays (n=6) (f). g hDPP4 KI mice were intranasally inoculated with 2.5x10^3PFU MERS-CoVMA followed by intraperitoneal administration of 12.5mg/kg/day z-VEID-fmk or DMSO for 6 days or until sample harvest. h A subset of mice were harvested at day 2 and day 4 post infection. Mouse lungs were immunolabelled to detect MERS-CoV N expression. i Viral gene expression in mouse lungs were quantified with RT-qPCR (n=3). j Infectious titer was determined with plaque assays (n=3). k Expression of pro-inflammatory cytokines and chemokines were quantified with RT qPCR. l-m Body weight and survival of the infected mice were monitored for 14 days. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (k), two way- ANOVA (e), Student’s t-test (b, d, f, i, j, and l), or Log-rank (Mantel-Cox) test (m). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant. Bars in (a, c, and h) represented 50μm, 20μm, and 100μm, respectively.
Figure 3

Caspase-6 inhibition ameliorates lung pathology and improves the body weight of SARS-CoV-2-infected golden Syrian hamsters. a Golden Syrian hamsters were intranasally inoculated with 3x10^3 PFU SARS-CoV-2 followed by intraperitoneal administration of 12.5 mg/kg/day z-VEID-fmk or DMSO for 4 days. b–c Hamsters were sacrificed at day 4 post infection, viral gene copy and infectious titer of hamster lungs were quantified with RT-qPCR and TCID50 assays, respectively (n=6). d–e Viral N protein expression in the small airways and alveoli of infected hamster lungs with or without z-VEID-fmk treatment was revealed.
with immunofluorescence staining with the in-house rabbit immune serum against SARS-CoV-2 N. f
Expression of pro-inflammatory cytokines and chemokines was quantified with RT-qPCR (n=6). g Body
weight change of SARS-CoV-2-infected hamsters with z-VEID-fmk or mock treatment was documented
from day 0 to day 4 post infection. h Representative images of haematoxylin and eosin (H&E) stained
hamster lungs. (Top panels) mock-infected hamster lung sections showed normal histology, boxed areas
were magnified showing (i) intact bronchiolar epithelium lining, (ii) thin alveolar wall and clear air sac,
and (iii) a normal structure of pulmonary blood vessel section. (Middle panels) In SARS-CoV-2-infected
hamsters, lung tissues showed diffuse inflammatory infiltration and exudation with disappearing air-
exchange structures. Boxed areas were magnified to demonstrate the characteristic histopathological
changes (i) peribronchiolar infiltration and bronchiolar epithelium desquamation, (ii) alveolar infiltration
and haemorrhage with alveolar space filled with infiltrated immune cell and protein rich exudate, (iii)
pulmonary blood vessel showed immune cells infiltration in the vessel wall, endothelium, and
perivascular connective tissue. (Bottom panels) The hamster lung pathology was markedly improved
with z-VEID-fmk treatment. Magnified images demonstrated (i) milder degree of immune cell infiltration in
bronchiolar epithelium and peribronchiolar tissue, (ii) thickened alveolar wall with red blood cells but
alveolar space showed no immune cell infiltration nor exudation, (iii) pulmonary vessel wall showed a
few immune cells attached to the endothelium. i Quantitative scores for the lung histopathological
changes of SARS-CoV-2- infected hamsters with or without z-VEID-fmk treatment. Three categories of
characteristic histopathological changes including bronchiolitis, alveolitis and vasculitis were examined
and scored. (n=6 and two-three lung lobes were examined from each hamster). Data represented mean
and standard deviations from the indicated number of biological repeats. Statistical significance between
groups was determined with one way-ANOVA (f) or Student’s t-test (b, c, g, and i). * represented p < 0.05,
** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant. Bars in
d and e) represented 100μm.
Figure 4

Caspase-6 modulates MERS-CoV replication at a post entry step and cleaves MERS-CoV N protein. a MERS-CoV-infected BEAS2B cells were incubated with z-VEID fmk in a time of addition assay. Virus gene copy in the supernatant was determined with RT qPCR at 24hpi (n=4). b-c Caspase-6 stable knockdown A549 and BEAS2B cells were infected with MERS-CoV at 0.1MOI. (b) Cell lysates were harvested at 1 hpi to quantify virus entry with RT-qPCR (n=4). (c) Virus replication at 24 hpi were quantified with RT-qPCR.
d MDMs were treated with caspase-6 or nontargeting siRNA and infected with MERS-CoV. Virus gene copy was quantified at 24 hpi (n=4). e-f Caspase-6- or caspase-3-overexpressed 293T cells were infected with MERS-CoV at 1MOI. Virus replication was quantified at 1 and 24 hpi with RT qPCR (n=4). g MERS-CoV N and caspase-6 were co-expressed in 293T cells with or without 100μM z-VEID-fmk. Cells were harvested for Western blot at 24 hours post transfection. 1μM staurosporine (STS) was added as an apoptosis trigger at 6 hours before sample harvest. h N cleavage in cell lysates from MERS-CoV-infected Huh7 and BEAS2B cells was compared to the N cleavage in cell lysates from MERS-CoV N and caspase-6 co-transfected 293T cells. i N cleavage in MERS-CoV-infected cells with or without z-VEID-fmk. j Comparison of MERS CoV N cleavage in caspase-6-overexpressed and caspase-3-overexpressed cells. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (a), two-way ANOVA (b and f), or Student's t-test (c and d). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant.
Caspase-6-mediated coronavirus N cleavage interferes with IFN signalling. a–b 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of MERS-CoV N or E and caspase-6 or caspase-3, with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual-luciferase reporter assays (n=4). c 293T cells were transfected with the same set of plasmids. Gene expression of IFN-β, IFIT3, and OAS1 was quantified with RT-qPCR (n=6 for IFN-β and IFIT1, n=4 for...
OAS1). d 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and MERS CoV N, ORF4a, ORF4b, or M, with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual-luciferase reporter assays (n=3). e N protein of the indicated coronaviruses were co-expressed with caspase-6. N cleavage was detected with Western blots. f 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and coronavirus N, with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual-luciferase reporter assays (n=3). g 293T cells were transfected with the same set of plasmids. Gene expression of IFIT3 and OAS1 was quantified with RT-qPCR (n=4). Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (a-f and h-i). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001, ns = not significant.
Figure 6

Caspase-6-mediated MERS-CoV N cleavage generates N fragments that block IRF3 translocation to the cell nucleus. a Schematic of N mutants. b Caspase-6-mediated cleavage of N mutants was evaluated with Western blots. c 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and MERS-CoV N or N mutants, with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual luciferase reporter assays (n=3). d Caspase-6-mediated cleavage of N, N(1-241), and N(242-413) was evaluated with Western blots. e 293T cells were transfected with an IFN-β-Luc reporter plasmid, expression constructs of caspase-6 and MERS-CoV N, N(1-241), or N(242-413), with or without poly(I:C). Cells were incubated for 24 hours before harvesting for dual luciferase reporter assays (n=4). f Interaction between IRF3 and N, N(1-241), or N(242-413) was evaluated with co-immunoprecipitation assays using IRF3 as the bait protein. g 293T cells were transfected with expression constructs of IRF3, N, N(1-241), or N(242-413), and poly(I:C). Cells were fixed at 24 hours post transfection. Localization of N was detected with an in-house guinea pig anti-N immune serum and IRF3 was detected with a rabbit anti-HA antibody. Cell nuclei were identified with the DAPI stain. Data represented mean and standard deviations from the indicated number of biological repeats. Statistical significance between groups was determined with one way-ANOVA (c and e). * represented p < 0.05, ** represented p < 0.01, *** represented p < 0.001, **** represented p < 0.0001. ns = not significant. Bars in (g) represented 10μm.

Supplementary Files

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