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Review

The coronavirus 3CL protease: Unveiling its complex host interactions and central role in viral pathogenesis

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ABSTRACT

The 3CL protease, a highly conserved enzyme in the coronavirus, plays a crucial role in the viral life cycle by facilitating viral replication through precise cleavage of polyproteins. Beyond its proteolytic function, the 3CL protease also engages in intricate interactions with host cell proteins involved in critical cellular processes such as transcription, translation, and nuclear-cytoplasmic transport, effectively hijacking cellular machinery to promote viral replication. Additionally, it disrupts innate immune signaling pathways, suppresses interferon activity and cleaves antiviral proteins. Furthermore, it modulates host cell death pathways including pyroptosis and apoptosis, interferes with autophagy and inhibits stress granule formation to maintain viral infection and exacerbate viral pathogenesis. This review highlights the molecular mechanisms by which the 3CL protease orchestrates virus-host interactions, emphasizing its central role in coronavirus pathogenesis and highlighting potential therapeutic targets for future interventions.

INTRODUCTION

Coronaviruses (CoVs), members of the subfamily *Orthocoronavirinae* within the family *Coronaviridae* of the order *Nidovirales*, are enveloped viruses with a single-stranded positive-sense RNA genome (Woo et al., 2005). The subfamily *Orthocoronavirinae* encompasses four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. While seasonal human coronaviruses (HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1) typically cause mild to moderate respiratory illnesses (Kesheh et al., 2022), highly pathogenic *Betacoronaviruses* such as SARS-CoV (severe acute respiratory syndrome coronavirus), MERS-CoV (Middle East respiratory syndrome coronavirus), and SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) can lead to severe respiratory diseases, pneumonia, multi-organ failure, and even death (Chen et al., 2020).

The coronavirus genomes (26–32 kilobases) features a 5′-cap and a 3′-poly-A tail (Fig. 1A). The initial open reading frame (ORF1a/1b), accounting for approximately two-thirds of the genome length, encodes two polyproteins (pp1a and pp1ab) through a ribosomal frameshifting mechanism between ORF1a and ORF1b. Subsequently, pp1a and pp1ab are cleaved into multiple non - structural proteins (NSPs, NSP1–16) by the 3C- like protease (3-chymotrypsin-like protease, 3CL, encoded by NSP5) and papain-like protease (PLP, encoded by NSP3) (Yang et al., 2003; Liu and Wang, 2020) (Fig. 1B). The maturation of 3CL protease depends on autocleavage at two sites located at the ends of the protein (referred to as NSP4–5 and NSP5–6) (Duan et al., 2023). In SARS-CoV-2, the 3CL protease cleaves 11 sites within pp1a and pp1ab to generate from NSP4 to NSP16, which are essential for viral replication, transcription, and immune evasion (Chen et al., 2021; Hu et al., 2022) (Fig. 1B). The coronavirus 3CL protease, a cysteine protease composed of approximately 300 amino acids (Bacha et al., 2004), has a catalytic center formed by His41 and Cys145 (Webber et al., 1998; Chen et al., 2008).

The coronavirus 3CL protease demonstrates both conserved and variable features in its cleavage sites on viral polyproteins (Table 1 and Fig. 2). According to the Schechter-Berger nomenclature, amino acids surrounding the cleavage site are numbered from the N- to the C-terminus as P4 – P3 – P2 – P1↓P1′ - P2′ - P3′, with the cleavage site between P1 and P1′ (Anand et al., 2002; Hsu et al., 2005). The coronavirus 3CL proteases exhibit strong sequence specificity at the P1, P2, and P1′ positions, while showing more flexibility in their requirements for other Pn residues (Xiong et al., 2021). Notably, a glutamine (Gln, Q) residue is

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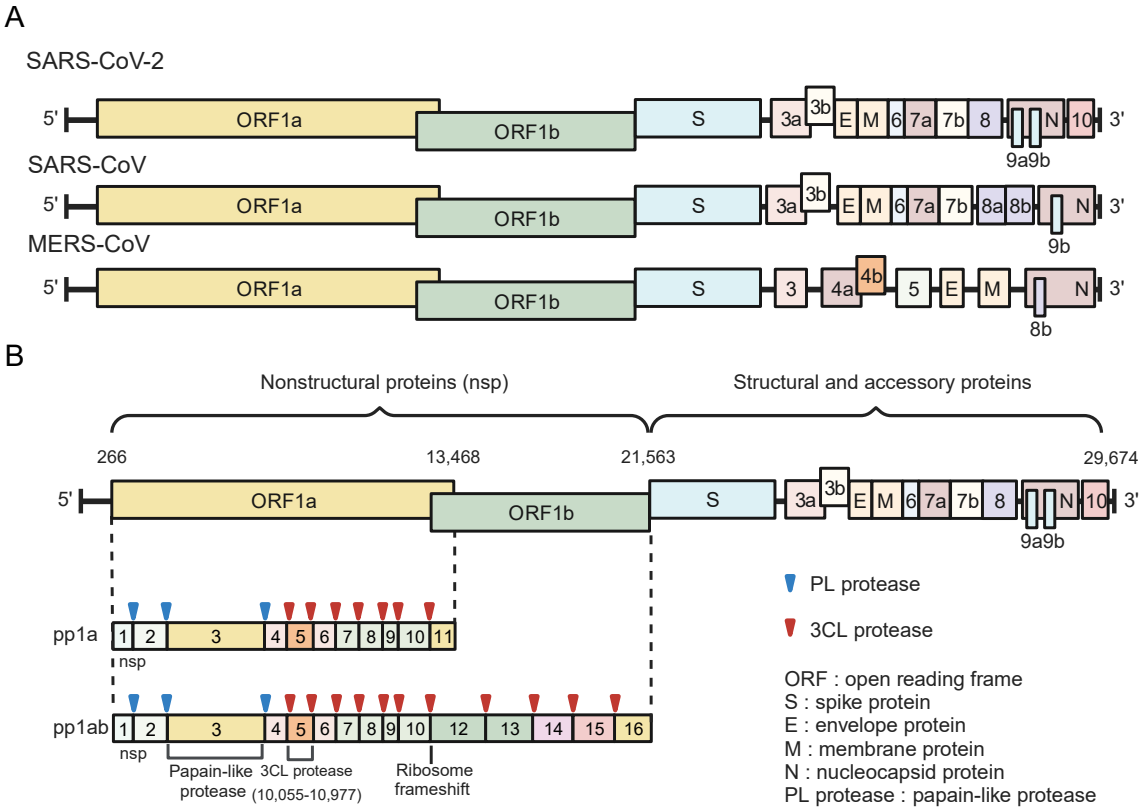

Fig. 1. Genome organization of coronaviruses. **A** The illustration of the genome organization of three representative coronaviruses: SARS-CoV, SARS-CoV-2, and MERS-CoV. **B** Polyprotein 1a and polyprotein 1 ab (pp1a and pp1ab) are translated by ribosome frameshift of open reading frame 1a/1b (ORF1a/1b). Subsequently, pp1a and pp1ab are cleaved by virus encoded papain like protease (PL protease) and 3C like protease (3CL protease) to form non-structural proteins (NSPs). The illustration takes SARS-CoV-2 as an example, with the cleavage sites of PL protease and 3CL protease marked by blue and red inverted triangles, respectively.

Table 1
Cleavage sequences (P4 – P3 – P2 – P1↓P1′ - P2′ - P3′) on viral polyprotein of 3CL protease.

Position	SARS-CoV-2	SARS-CoV	MERS	PEDV
NSP4↓5	AVLQ↓SGF	AVLQ↓SGF	GVLQ↓SGL	STLQ↓AGL
NSP5↓6	VTFQ↓SAV	VTFQ↓GKF	VVMQ↓SGV	VNLQ↓GGY
NSP6↓7	ATVQ↓SKM	ATVQ↓SKM	AAMQ↓SKL	SSVQ↓SKL
NSP7↓8	ATLQ↓AIA	ATLQ↓AIA	SVLQ↓ATL	SMLQ↓SVA
NSP8↓9	VKLQ↓NNE	VKLQ↓NNE	VKLQ↓NNE	VKLQ↓NNE
NSP9↓10	VRLQ↓AGN	VRLQ↓AGN	VRLQ↓AGS	VRLQ↓AGK
SP10↓12	PMLQ↓SAD	PLMQ↓SAD	ALPQ↓SKD	SIMQ↓STD
NSP12↓13	TVLQ↓AVG	TVLQ↓AVG	TTLQ↓AVG	AVLQ↓SAG
NSP13↓14	ATLQ↓AEN	ATLQ↓AEN	YKLQ↓SQI	SDLQ↓ANE
NSP14↓15	TRLQ↓SLE	TRLQ↓SLE	TKVQ↓GLE	NNLQ↓GLE
NSP15↓16	PKLQ↓SSQ	PKLQ↓ASQ	PRLQ↓ASA	PQLQ↓ASE

Notes: ↓ means the cleavage site between P1 and P1′. SARS-CoV-2, SARS-CoV, MERS-CoV, PEDV stand for Severe Acute Respiratory Syndrome Coronavirus 2, Severe Acute Respiratory Syndrome Coronavirus, Middle East Respiratory Syndrome Coronavirus, and Porcine Epidemic Diarrhea Coronavirus respectively.

typically present at the P1 position, although non-canonical residues such as methionine (Met) or histidine (His) have been identified in certain 3CL protease substrates of SARS-CoV-2, HCoV-NL63, and HCoV-HKU1 (Koudelka et al., 2021; Pablos et al., 2021; Xiong, M. et al., 2021). The P2 position is predominantly occupied by hydrophobic residues, while the P1′ position shows a preference for small-sized amino acids (Xiong, M. et al., 2021) (Fig. 2).

Recent advances in computational and experimental approaches have significantly enhanced our understanding of 3CL protease-host interactions. Multiple studies employing machine learning algorithms and high-throughput proteomic techniques have systematically identified host substrates of the 3CL protease, providing comprehensive insights into its functional repertoire during viral infection (Kiemer et al., 2004; Zhu et al., 2017b; Gordon, D.E. et al., 2020; Miczi et al., 2020; Koudelka et al., 2021; Pablos et al., 2021; Chen, H. et al., 2022; Luo et al., 2023). This review aims to summarize current knowledge regarding the coronavirus 3CL protease, elucidates its regulatory mechanisms in virus-host interactions, and discusses emerging perspectives on its role in viral replication and pathogenesis.

REGULATION OF HOST CELLULAR PROCESSES BY 3CL PROTEASE

Cleavage and functional reprogramming of the host transcription machinery

The 3CL protease of SARS-CoV-2 has been shown to cleave multiple transcription associated factors, including RNA polymerase II associated protein 1 (RPAP1), heterogeneous ribonucleoprotein U (hnRNP U), cleavage stimulation factor subunit 2 (CSTF2), and polypyrimidine tract binding protein 1 (PTBP1), by N-terminomic analysis using TAILS approach (Pablos et al., 2021) (Fig. 3). Notably, the 3CL protease of SARS-CoV-2 cleaves PTBP1 at Q153, resulting in its relocalization from the nucleus to cytoplasm (Pablos et al., 2021). This cleavage-induced redistribution is hypothesized to disrupt PTBP1's transcriptional regulatory functions, potentially providing an advantage for viral replication. However, the exact contribution of these cleavage events, particularly of PTBP1, to SARS-CoV-2 replication and pathogenesis requires further investigation.

Additionally, the 3CL protease cleaves tonicity-responsive enhancer-binding protein (TonEBP), a transcription factor regulating the Rel family (Park et al., 2024). This cleavage not only impairs TonEBP's

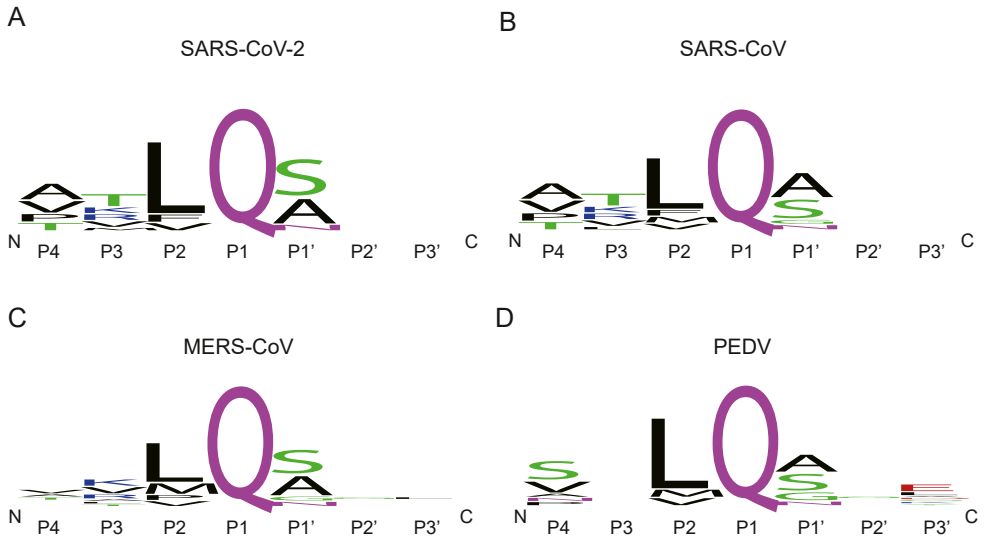

Fig. 2. Sequence logos indicating the conservation and variability features in the cleavage site of substrates for coronaviruses 3CL protease. Cleavage sequence logos (P4 – P3 – P2 – P1–P1′ - P2′ - P3′) on viral polyprotein of SARS-CoV-2 (**A**), SARS-CoV (**B**), MERS-CoV (**C**) and PEDV (**D**) 3CL protease, with the cleavage site between P1 and P1′. The sequence logos generated by Weblogo (Crooks et al., 2004).

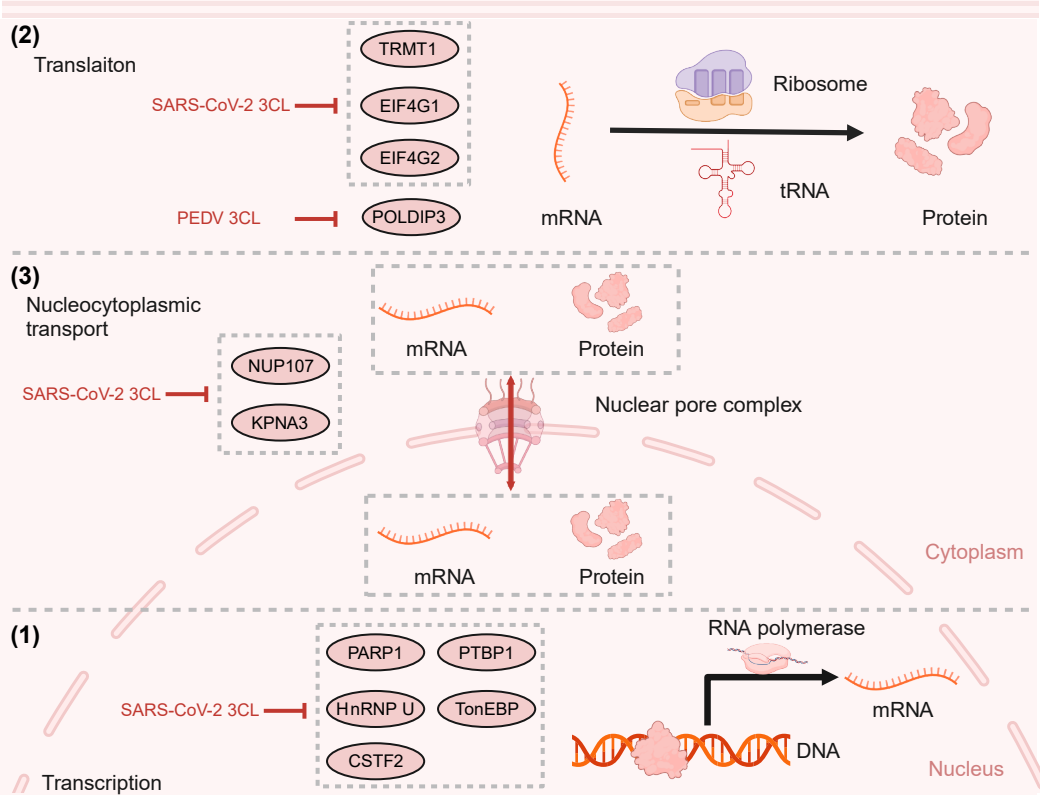

Fig. 3. 3CL protease of coronavirus hijacks transcription, translation and nuclear-cytoplasmic transport to promote viral replication. During coronavirus infection, 3CL protease targets multiple host factors involved in transcription, such as RPAP1, hnRNP U, CSTF2, PTBP1 and TonEBP and disrupt their normal functions (red inhibition bars in part 1). The 3CL protease also cleaves factors related to translation, including TRMT1, POLDIP3, EIF4G1 and EIF4G2. This cleavage may suppress host protein synthesis and promote the cellular pathogenesis of viral infection (red inhibition bars in part 2). Additionally, the 3CL protease disrupts the nuclear-cytoplasmic transport process by cleaving key proteins such as NUP107 and KPNA3, thereby facilitating viral replication (red inhibition bars in part 3).

ability to stimulate TonE-driven transcription but also generates fragments that enhance viral replication and suppress interferon expression (Park et al., 2024) (Fig. 3). These findings reveal a sophisticated mechanism by which the 3CL protease manipulates host transcription to favor viral propagation.

Hijacking and redirection of host translation system

The 3CL protease significantly impacts host translation machinery through multiple mechanisms (Fig. 3). Eukaryotic translation occurs through cap- and IRES- dependent mechanisms. EIF4G1 and EIF4G2, essential cap-dependent translation initiation factors, are cleaved by the 3CL protease of SARS-CoV-2 (Pablos et al., 2021). Once upon the cleavage of EIF4G1 and EIF4G2, host protein translation is likely impeded, which may represent a strategic maneuver by the virus to redirect cellular resources towards its own replication.

Additionally, TRMT1 (tRNA methyltransferase 1), responsible for modifying tRNAs in the nucleus, cytoplasm, and mitochondria, is also cleaved at Q530 by the 3CL protease of SARS-CoV-2 (Gordon, C.J. et al., 2020; Lu and Zhou, 2023; Xiong et al., 2023; D'oliviera et al., 2025). This cleavage removes the zinc-finger domain of TRMT1 and abolishing both its tRNA modification activity and binding affinity (Lu and Zhou, 2023; D'oliviera et al., 2025), which leads to decreased TRMT1-catalyzed tRNA modifications and potentially inhibits host protein synthesis. Intriguingly, TRMT1-deficient human cells exhibit reduced intracellular viral RNA levels (Lu and Zhou, 2023), suggesting complex interplay between TRMT1 function and viral replication.

Furthermore, DNA polymerase delta interacting protein 3 (POLDIP3), which enhances translation efficiency through S6K1 recruitment (Ma et al., 2008), is cleaved at the Q176 by the 3CL protease of multiple coronaviruses including porcine deltacoronavirus (PDCoV), porcine epidemic diarrhea virus (PEDV), and SARS-CoV-2 (Wu et al., 2023). This cleavage inhibits the antiviral effects of POLDIP3 and promotes viral infection (Wu et al., 2023). Key questions remain regarding whether POLDIP3 inhibits coronavirus replication through translation-dependent mechanisms and how viral translation persists despite general host translation suppression.

Disruption of nuclear-cytoplasmic transport

The 3CL protease also disrupts nuclear-cytoplasmic transport, a critical cellular homeostasis mechanism (Fig. 3). Nuclear pore complex protein 107 (NUP107), a vital component of the nuclear pore complex (NPC), is specifically cleaved at Q32 by the 3CL protease of SARS-CoV-2 (Meyer et al., 2021; Pablos et al., 2021), likely impairing nuclear pore complex (NPC) function and causing nuclear-cytoplasmic transport abnormalities. The 3CL protease of SARS-CoV-2 also cleaves karyopherin subunit alpha 3 (KPNA3), a key nuclear import factor (Pablos et al., 2021).

By selectively cleaving these nuclear-cytoplasmic transport machinery components, the 3CL protease may disrupts normal protein localization patterns, potentially facilitating viral replication and immune evasion. However, the precise mechanistic contributions of NUP107 and KPNA3 cleavage to viral replication require further elucidation.

Multiple inhibition of host innate immunity by 3CL protease

Upon coronavirus infection, viral RNA is detected by innate immune sensors like retinoic acid-inducible gene I (RIG-I/DDX58) (Fig. 4). RIG-I recognizes viral RNA and initiates antiviral response by recruiting adapter proteins such as mitochondrial antiviral signaling protein (MAVS) and TNF receptor-associated factors (TRAFs) (Chow et al., 2018; Rehwinkel and Gack, 2020). These adapters activate signaling cascades through the formation of the TANK-binding kinase 1 (TBK1)-IκB kinase ε (IKKε)-NF-κB essential modulator (NEMO) complex or the IKKα-IKKβ-NEMO complex. These cascades ultimately activate transcription factors, including interferon regulatory factors (IRF3 and IRF7) and nuclear factor kappa-B (NF-κB), which translocate to the nucleus to induce expression of interferons (IFNs), interferon-stimulated genes (ISGs), and proinflammatory cytokines.

Type I IFNs (IFN-α and IFN-β) mediate crucial antiviral responses by binding to IFN-α/β receptors (IFNARs) on cell surfaces (Fig. 4). This interaction activates the JAK-STAT pathway, leading to phosphorylation of STAT1 and STAT2 by JAK kinases, their heterodimerization, and nuclear translocation (Stark and Darnell, 2012). In the nucleus, these complexes enhance expression of IFNs and ISGs, which encode antiviral proteins with immunomodulatory and antiviral functions. However, coronaviruses have evolved multiple strategies to subvert innate immune response by 3CL protease (Fig. 4).

Disruption of RIG-I mediated antiviral signaling

The SARS-CoV-2 3CL protease profoundly disrupts RIG-I-mediated immune signaling through multiple mechanisms. The 3CL protease cleaves RIG-I at the Q10 residue in its N-terminus and inhibits phosphorylation of both IRF3 and TBK1 (Liu et al., 2021). Additionally, it reduces RIG-I ubiquitination and weakens its interaction with TRIM25 during infection (Wu et al., 2020). The 3CL protease also disrupts the interaction between RIG-I and MAVS (Zheng et al., 2022), thereby preventing formation of antiviral stress granules that normally restrict viral replication (Zheng et al., 2022). These combined strategies may effectively suppress RIG-I-mediated antiviral signaling.

Cleavage and modification of the adaptor proteins

The 3CL protease targets multiple adaptor proteins to evade immune response. While the SARS-CoV-2 3CL protease promotes MAVS ubiquitination and proteasomal degradation (Liu et al., 2021), MERS-CoV 3CL protease directly cleaves MAVS to inhibit IFN-β and NF-κB responses (Van Huizen et al., 2024). Interestingly, the SARS-CoV-2 protease can also enhance the SUMOylation of MAVS, increasing its stability and activating NF-κB signaling (Li et al., 2021). These conflicting findings highlight the complex interactions between coronaviruses 3CL proteases and MAVS.

The PDCoV 3CL protease cleaves multiple adaptor proteins, such as TRAF2, TRAF3 and AZI2 (Zhou, J. et al., 2024). Notably, while both porcine and human TRAF3 are susceptible to cleavage, only porcine TRAF2 is cleaved due to sequence differences in human TRAF2 (Zhou, J. et al., 2024). These findings suggest potential species-specific adaptations in viral immune evasion strategies.

Disruption of NF-κB and interferon pathway

The 3CL protease targets key components of NF-κB and interferon pathways, including NEMO, IRFs, and STATs. Multiple coronaviruses 3CL proteases cleave NEMO at distinct sites to inhibit downstream IFN production: PEDV and PDCoV at Q231 (Wang et al., 2016; Zhu et al., 2017a), feline infectious peritonitis virus (FIPV) at Q132, Q205, and Q231 (Chen et al., 2019), and SARS-CoV/SARS-CoV-2 at E152, Q205, and Q231 (Chen, J. et al., 2022). Notably, SARS-CoV-2 exhibits particularly strong NEMO cleavage activity and IFN suppression compared with SARS-CoV (Chen, J. et al., 2022). The cleavage of NEMO by the 3CL proteases of multiple coronaviruses may be a common strategy for immune evasion.

Although SARS-CoV-2 3CL protease doesn't cleave IRF3 directly, it traps phosphorylated IRF3 in the cytoplasm, preventing nuclear translocation and activation of IRF3 and IFN production (Fung et al., 2021). The 3CL protease of MERS-CoV employs a similar strategy to inhibit IFN-β and cytokines production (Zhang et al., 2024). Notably, the inhibition of IFN-β production depends on the protease activity of MERS-CoV 3CL protease (Zhang et al., 2024). Although coronavirus 3CL

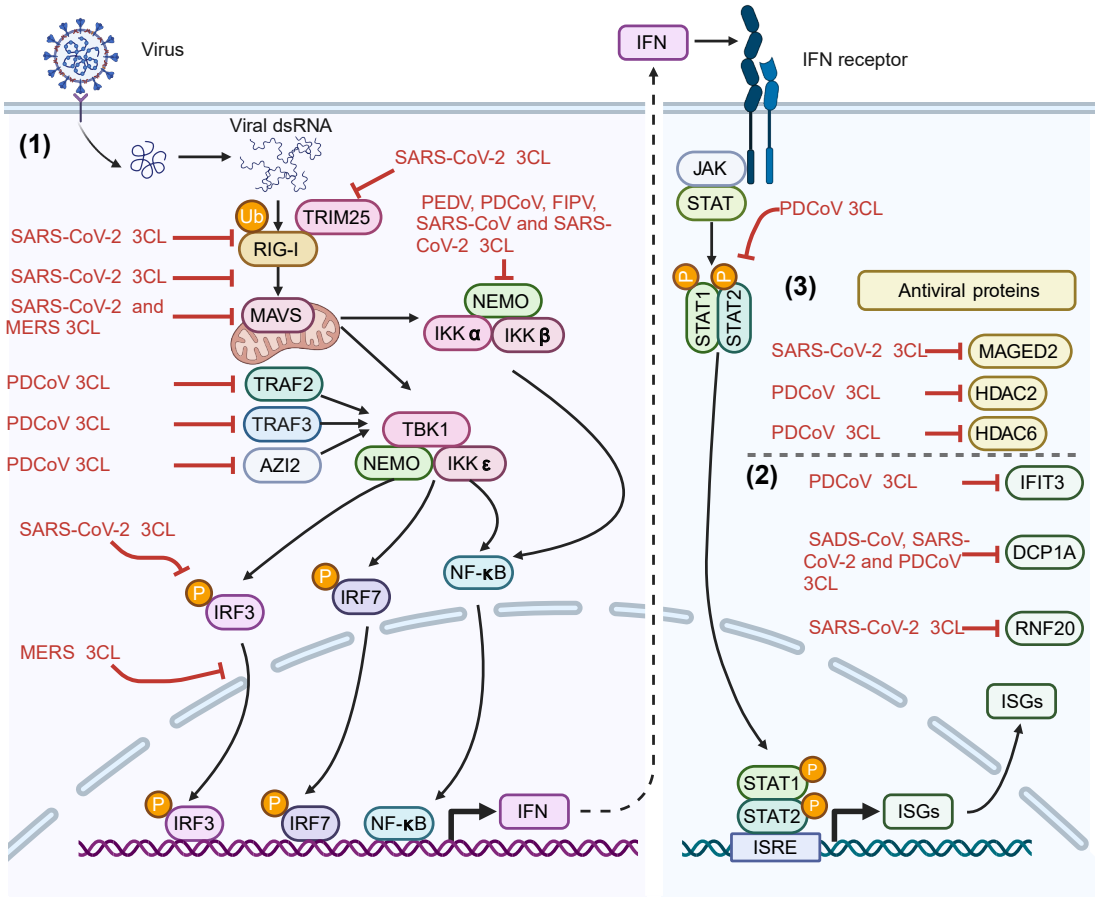

Fig. 4. Roles of 3CL protease in subverting host innate immunity. Upon infection with coronaviruses, the viral RNA is sensed by innate immune RNA sensors such as RIG-I, which recruits downstream adapters like MAVS and TRAFs, triggering the formation of complexes like TBK1-IKKε-NEMO or IKKα-IKKβ-NEMO. This leads to the activation of transcription factors like IRFs and NF-κB, which get phosphorylated, dimerize, and move into the nucleus to prompt the expression of IFNs, ISGs, and proinflammatory cytokines. Subsequently, type I IFNs, binding to IFNARs on cell surfaces, activate the JAK-STAT pathway involving STAT1 and STAT2. The activated STAT1-STAT2 heterodimer relocates to the nucleus, amplifying IFN and ISG production. However, the 3CL protease cleaves or hijack viral RNA sensor RIG-I to disrupt downstream signaling for interferon induction. The 3CL protease also hijacks key factors of IFN pathway, such as MAVS, TRAF2/3, AZI2, IRF3 and NEMO to interfere with innate immune signaling cascades by impeding NF-κB and IFN pathways. Additionally, the 3CL protease cleaves STAT2 to impair ISG induction. Moreover, the 3CL protease targets both ISGs (IFIT3, DCP1A, RNF20) and antiviral proteins (MAGED2, HDAC6, and HDAC2) to inhibit their antiviral activity.

proteases may trap IRF3 in the cytoplasm as a common strategy to inhibit the production of interferon, the detailed mechanisms between different coronavirus 3CL proteases and IRF3 remains to be further investigated. The PDCoV protease cleaves porcine STAT2 at Q685 and Q758, impairing STAT2 function and ISG induction (Zhu et al., 2017b). However, whether the cleavage of STAT2 by coronaviruses 3CL protease is conserved in other species such as human remains to be further investigated.

Inactivation of interferon-stimulated genes

Coronavirus 3CL proteases target multiple ISGs to evade host defenses. IFIT3 (interferon induced protein with tetratricopeptide repeats 3), an interferon-induced protein, is upregulated in individuals exposed to SARS-CoV-2 but not infected or asymptomatic (De Castro et al., 2024). PDCoV protease cleaves IFIT3 (interferon induced protein with tetratricopeptide repeats 3) at Q406, counteracting type I interferon signaling (Huang et al., 2024). This strategy appears conserved across human coronaviruses, indicating that the cleavage of IFIT3 is a common strategy for coronaviruses to counter the host's innate immune response (Huang et al., 2024).

In addition, DCP1A (Decapping mRNA 1A), an IFN-α induced ISG (He et al., 2006), is cleaved at the conserved Q343 site by the 3CL proteases of PDCoV, SARS-CoV-2 and other coronaviruses, which inhibit the antiviral activity of DCP1A (Zhu et al., 2020; Song et al., 2023). Swine acute diarrhea syndrome coronavirus (SADS-CoV) 3CL protease also cleaves DCP1A to decrease IFN and inflammatory cytokine production (Huang et al., 2023).

Moreover, RNF20 (ring finger protein 20) is cleaved by the 3CL protease of SARS-CoV-2 at the conserved Q521 residue across species. This cleavage disrupts the normal function of RNF20 in mediating the degradation of SREBP1 and promotes viral replication (Zhang et al., 2021). Future studies should prioritize systematic mapping of viral protease and ISG interaction networks, revealing previously unrecognized mechanisms by which viral protease subvert host immune defenses.

Cleavage of antiviral proteins

Beyond ISGs, the 3CL protease also cleaves antiviral factors. MAGED2, which inhibits SARS-CoV-2 by binding viral nucleocapsid protein in an RNA-dependent manner, is cleaved at Q263 by multiple coronavirus proteases (Ju et al., 2023). The PDCoV 3CL protease cleaves two members of the HDAC (histone deacetylase) family: HDAC6 and HDAC2. HDAC6 plays a key role in RIG-I mediated viral RNA sensing and viral protein degradation (Choi et al., 2016; Li, Z. et al., 2023). The PDCoV 3CL protease cleaves HDAC6 at Q519, impairing its deacetylase activity, protein degradation ability, and interferon activation capacity (Li, Z. et al., 2024). Similarly, the PDCoV 3CL protease cleaves HDAC2 at

Q261, a highly conserved site in mammals, to inhibit antiviral activities of intact HDAC2 (Li, Z. et al., 2022). Further investigation of viral protease and antiviral proteins interactions is expected to significantly broaden our understanding of viral immune evasion mechanisms.

REGULATION OF HOST CELL DEATH BY 3CL PROTEASE

Bidirectional regulation of pyroptosis

Pyroptosis, a type of programmed cell death, is characterized by cell swelling, membrane rupture, and the release of cellular contents, serving as a crucial component of the innate immune response to eliminate infected or damaged cells (Bergsbaken et al., 2009; Schroder and Tschopp, 2010; Ding et al., 2016; Evavold et al., 2018). Pyroptosis is closely associated with inflammasome activation (Fig. 5A), which senses pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Upon recognition of cytosolic LPS or other stimuli, NLR (NOD-like receptor) family proteins recruit the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) to form the inflammasome, ultimately activaing caspase proteases including caspase-1. Caspase-1 mediates pyroptosis through cleavage of gasdermin D (GSDMD), generating membrane pores that compromise cellular integrity, leading to cell swelling and rupture (Aglietti et al., 2016; Ding et al., 2016; Van Opdenbosch and Lamkanfi, 2019; Broz et al., 2020). Concurrently, caspase-1 processes pro-inflammatory cytokines IL-1β and IL-18 into their mature forms, amplifying inflammatory responses.

The 3CL protease exhibits complex, dual roles in pyroptosis regulation during coronavirus infection. First, it can promote excessive inflammation through multiple mechanisms, contributing to viral pathogenesis. The 3CL protease of SARS-CoV-2 cleaves the Q333 site of NLRP1 to initiates NLRP1 inflammasome assembly and induces cell death via caspase-3 and GSDME (Planès et al., 2022). Similarly, CARD8 (caspase recruitment domain family member 8), an analogue of NLRP1 (Taabazuing et al., 2020), is cleaved by the 3CL protease of SARS-CoV-2 and other coronaviruses (Tsu et al., 2023). The activation of CARD8 inflammasome and release of IL-1β are likely related to the inflammatory response triggered by coronavirus infection (Tsu et al., 2023). These findings align with that inflammasome activation contributes to excessive inflammatory responses in COVID-19 (Freeman and Swartz, 2020). Additionally, NLRP12 is also cleaved by the 3CL protease of SARS-CoV-2, contributing to the heightened production of cytokines and inflammatory responses observed in COVID-19 patients (Moustaqil et al., 2021). Since NLRP12 negatively regulates NLRP3 inflammasome activation (Coombs et al., 2024), cleavage of NLRP12 may further exacerbate inflammatory responses mediated by NLRP3.

Conversely, the 3CL protease can suppress pyroptosis through GSDMD cleavage. In SARS-CoV-2, 3CL protease cleaves human GSDMD at Q193 to inhibit pyroptosis, but induces GSDME dependent pyroptosis by NLRP1-driven caspase-3 activation (Planès et al., 2022). The 3CL

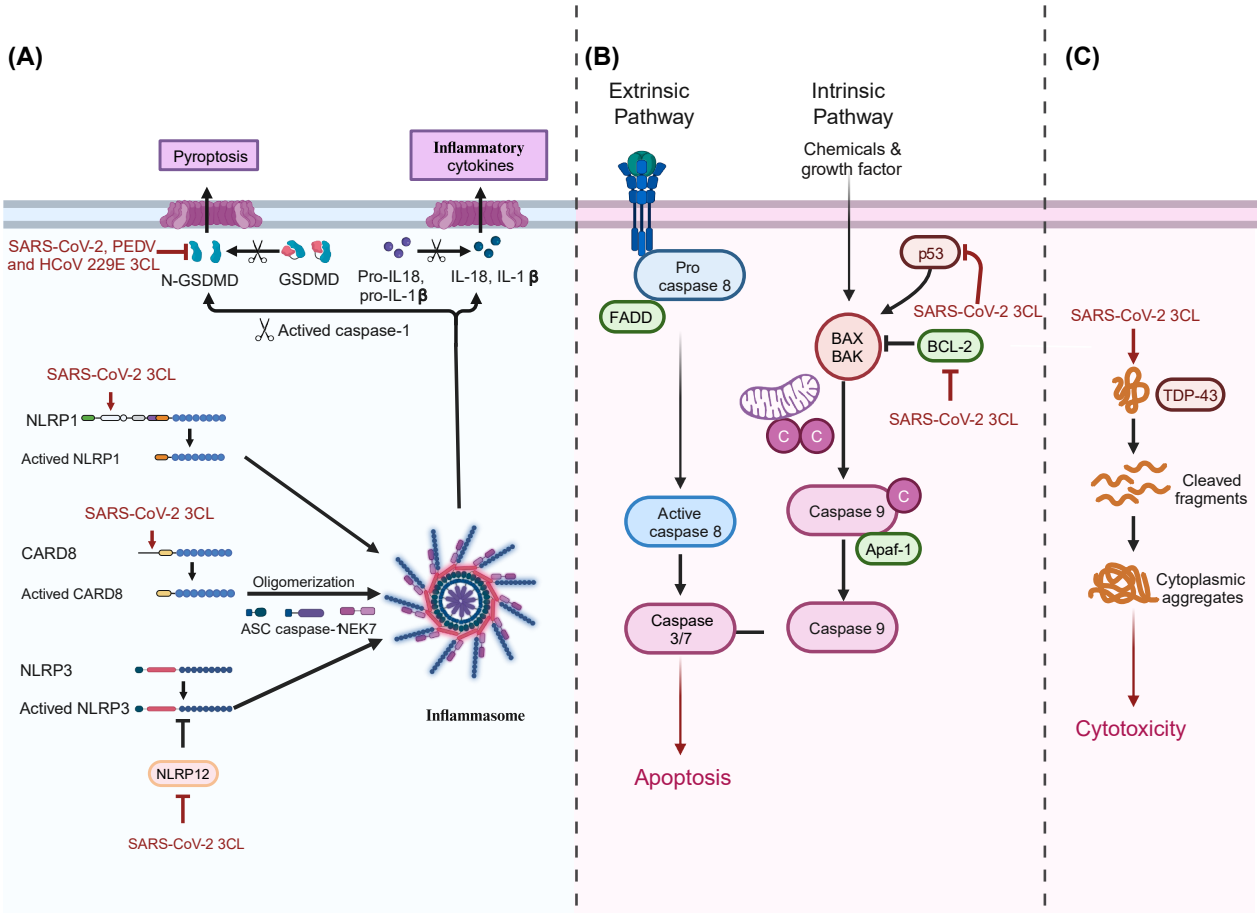

Fig. 5. Roles of 3CL protease in modulating host cell-death pathways. In the context of host cell death, the 3CL protease plays a pivotal regulatory role in both pyroptosis and apoptosis. **A** In the case of pyroptosis, the 3CL protease directly cleaves NLRP12, potentially increasing cytokine production and inflammatory responses. Additionally, the 3CL protease cleaves NLRP1 and CARD8, triggering inflammasome activation and leading to pyroptosis. Paradoxically, the 3CL protease also cleaves GSDMD (at Q193 and Q29), thereby inhibiting pyroptosis, which may represent a common viral evasion strategy. **B** Regarding apoptosis, the SUD2-3CL complex downregulates Bcl2, which enhances apoptosis in respiratory cells. Furthermore, 3CL protease also represses p53 transcriptional activity. **C** Additionally, the cleavage of TDP-43 by 3CL protease leads to neuroinflammation.

protease from several coronavirus species, including SARS-CoV-2, MERS-CoV, PDCoV, and PEDV, can cleave both porcine and human GSDMD proteins at the Q193 to inhibit pyroptosis and promote viral replication (Shi et al., 2022). While another study reported that the GSDMD is cleaved at both Q193 and Q29 by the 3CL protease of SARS-CoV-2 (Shen et al., 2024). Our recently study shows that 3CL protease of SARS-CoV-2 cleave GSDMD at Q29 and Q193 to inhibit GSDMD mediated pyroptosis and promote viral replication (Zhang et al., 2025). In addition, the 3CL protease of Human coronavirus 229E (HCoV-229E) cleaves GSDMD at Q29 and Q193, preventing GSDMD mediated pyroptosis (Martiáñez-Vendrell et al., 2024). Notably, lytic cell death still occurs due to caspase-3 mediated cleavage and activation of GSDME, which may contribute to the sustained release of infectious virus particles (Martiáñez-Vendrell et al., 2024). Further research is required to comprehensively characterize the different types of cell death and their roles in virus replication. Thus, the inhibition of GSDMD mediated pyroptosis by the 3CL protease represents a common strategy employed by viruses to evade the host immune response.

Manipulation of apoptosis pathway

Apoptosis, another form of programmed cell death, can be triggered by cell damage or infection and is categorized into intrinsic and extrinsic pathways (Yuan et al., 2023) (Fig. 5B). In the extrinsic pathway, extracellular ligands such as Fas ligand (FasL) bind to and activate cell surface death receptors (e.g., Fas), forming the death-inducing signal transduction complex (DISC). This complex includes Fas, Fas-associated protein with a death domain (FADD), and procaspase-8, leading to the activation of caspase-8 and ultimately inducing apoptosis. In the intrinsic pathway, signals of DNA damage, cellular stress, or intracellular signals activate proteins from the pro-apoptotic B-cell lymphoma-2 (Bcl-2) family, specifically Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer (Bak), which causes the release of cytochrome *c* (Cytc) from mitochondria into the cytoplasm. In the cytoplasm, free Cytc, along with procaspase-9 and apoptotic protease activating factor-1 (Apaf-1), assembles into the apoptosome, which activates caspase-9 and leads to intrinsic apoptosis.

The 3CL protease also regulates apoptosis. It interacts with the SUD2 (SARS unique domain) domains of NSP3 to enhance binding affinity for the G-quadruplex (G4) structure in the Bcl2 promoter, downregulating Bcl2 expression (Li, Y. et al., 2023). The 3CL protease also represses the transcriptional activity of p53, an activator of apoptosis, in a manner dependent on its protease function (Kumar et al., 2022; Wang et al., 2023). Notably, p53 overexpression reduces SARS-CoV-2 production, indicating its potential antiviral function (Kumar et al., 2022). However, a deeper understanding of the mechanisms underlying the interaction between the 3CL protease and p53 requires further research.

Induction of cytotoxicity

Beyond pyroptosis and apoptosis, the 3CL protease contributes to cytotoxicity through cleavage of TDP-43 (Fig. 5C) (Yang et al., 2023), a primary component of insoluble aggregates implicated in neurodegenerative disorders (Tziortzouda et al., 2021). This cleavage not only alters the solubility of TDP-43, promoting its aggregation into harmful deposits within neurons, but also generates a cytotoxic fragment that significantly increases cellular toxicity (Yang et al., 2023). Thus, the cleavage of TDP-43 by the 3CL protease potentially explains COVID-19-associated neuroinflammation. Whether 3CL protease regulates additional cell death modalities (e.g., necrosis, ferroptosis) remains an important area for future research.

THE REGULATION OF AUTOPHAGY AND STRESS GRANULES BY 3CL PROTEASE

Disruption of autophagy

Autophagy, an evolutionarily conserved and vital degradative process, significantly contributes to the immune system's ability to clear invading pathogens (Levine and Kroemer, 2008; Levine et al., 2011) (Fig. 6A). There are three major pathways of autophagy: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (Mizushima et al., 2011). Among them, macroautophagy is the most extensively studied. In macroautophagy, cytoplasmic proteins and damaged organelles are encapsulated within a double-membrane vesicle called the autophagosome. This autophagosome then matures and fuses with lysosomes, enabling the degradation of its contents and the recycling of nutrients (Lamb et al., 2013; Shibutani and Yoshimori, 2014).

Selective autophagy, a specialized form of autophagy, specifically recognize and target intracellular microorganisms including viruses for degradation (Levine, 2005). The 3CL protease of SARS-CoV-2 targets key proteins involved in selective autophagy (Fig. 6A). SQSTM1 (sequestosome 1, p62) is a multifunctional autophagy receptor that links ubiquitinated cargo to the autophagosome membrane, facilitating the degradation of damaged proteins and organelles (Lamark et al., 2017). The 3CL protease of SARS-CoV-2 directly cleaves SQSTM1 at Q354, disrupting ability of SQSTM1 to mediate selective autophagy and preventing SQSTM1 from binding to ubiquitinated SARS-CoV-2 M protein (Zhang et al., 2022). As a result, the cleaved fragments of SQSTM1 are unable to facilitate the autophagic degradation of the M protein. Similarly, PEDV 3CL protease cleaves NBR1 (NBR1 autophagy cargo receptor) at Q353 to inhibit the autophagic degradation of viral envelope (E) protein, thereby promote viral proliferation (Li et al., 2025). These findings suggest that targeting selective autophagy receptors may represent a conserved strategy among coronaviruses. Although the impact of the 3CL protease mediated cleavage of SQSTM1 and NBR1 on coronavirus replication has been confirmed, future studies should investigate whether additional autophagy receptors are targeted by 3CL proteases across coronavirus species.

Interestingly, the SARS-CoV-2 3CL protease also cleaves Galectin-8 at Q158 (Pablos et al., 2021). This cleavage prevents Galectin-8 from binding to the glycosylated spike S1 protein of SARS-CoV-2, disrupts its interaction with NDP52, and interferes with the autophagic degradation process (Pablos et al., 2021). These coordinated actions demonstrate how coronaviruses subvert autophagy to promote viral survival and replication.

Suppression of stress granule formation

Stress granules (SGs) are cytoplasmic aggregates that assemble in response to various cellular stressors, such as heat shock, oxidative stress, viral infection, or nutrient deprivation (Fig. 6B). They act as dynamic centers for the sequestration of mRNAs and associated proteins. In viral infection, the RNA and protein of the virus can activate protein kinase R (PKR) and its downstream translation initiation factor 2α (eIF2α) in the host cell. This activation leads to the disassembly of polysomes and the shutdown of translation. Subsequently, SGs nucleating proteins and mRNAs associated with stalled ribosome complexes in the cytoplasm aggregate to form SGs. Ras GTPase-activating protein-binding protein 1 (G3BP1) is a key player in SGs formation, acting as a main scaffold to facilitate the SGs assembly. SGs formation is generally believed to inhibit viral replication by affecting host translation and the G3BP1-mediated antiviral innate response (Nikolic et al., 2016; Yang et al., 2019).

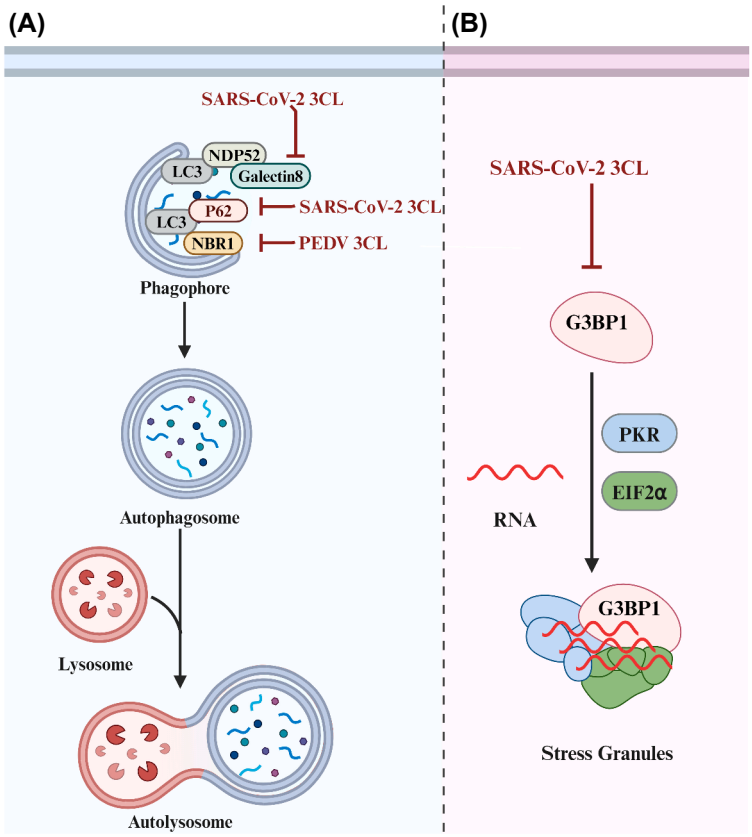

Fig. 6. Effects of 3CL protease on autophagy and stress granules. **A** The 3CL protease cleaves p62 at Q354, disrupting its function in selective autophagy and preventing the degradation of viral M protein. Similarly, the 3CL protease of PEDV cleave NBR1 at Q353 to inhibit the autophagic degradation of viral E protein, thereby promote viral proliferation. Moreover, Galectin-8, which is involved in autophagy regulation, is also cleaved by 3CL protease, impairing its glycan-binding ability and interaction with NDP52, thus thwarting autophagic degradation. **B** The formation of stress granules is a significant host defense strategy. However, the 3CL protease of SARS-CoV-2 specifically inhibits the formation of stress granules through its interaction with G3BP1, independent of its enzymatic activity.

While the precise mechanism is not yet fully elucidated, SARS-CoV-2 3CL protease interacts with G3BP1 independently of its enzymatic activity to inhibit SGs formation (Zheng et al., 2022). In the mouse infection model of SARS-CoV-2, conditional knockout of G3BP1 resulted in higher viral load in the lungs, indicating that G3BP1 may inhibit the pathogenicity of SARS-CoV-2 (Liu et al., 2022). These research findings illustrate the intricate relationship between SARS-CoV-2 and SGs. However, the conservation of this mechanism across other coronaviruses warrants further investigation.

HOST DEFENSE MECHANISMS AGAINST CORONAVIRUS 3CL PROTEASE

The coronavirus 3CL protease strategically manipulates critical host cellular processes and subverts antiviral immune responses through multifaceted mechanisms. In response, the host has evolved sophisticated countermeasures targeting this essential viral enzyme. FBXO22, a F-box protein family memeber, binds directly to 3CL protease and mediates K48-linked polyubiquitination at lysine residues 5 and 90, marking the protease for proteasomal degradation (Zhou, Y. et al., 2024). Experimental knockdown of FBXO22 significantly reduces 3CL protease ubiquitination while enhancing its stability, and its expression levels show an inverse correlation with SARS-CoV-2 infectivity. Similarly, ZBTB25, a zinc finger and BTB domain-containing protein, directly binds to 3CL protease and ubiquitinates the 3CL protease at the Lys-100 (Lear et al., 2023). Genetic studies demonstrate that ZBTB25 ablation enhances human coronavirus OC43 infectivity, while its overexpression restricts viral replication, establishing its role as a bona fide restriction factor (Lear et al., 2023). Notably, Parkin, a mitochondrial E3 ubiquitin ligase, also regulates SARS-CoV-2 3CL protease stability (Zhou, L. et al., 2024). Mechanistically, Parkin induces 3CL protease ubiquitination and degradation, thereby suppressing viral replication (Zhou, L. et al., 2024). This represents a novel intersection between mitochondrial homeostasis and antiviral immunity. These defense mechanisms exemplify the host's multilayered strategy to countermeasures viral proteases. Future investigations should explore additional post-translational modifications that may regulate 3CL protease activity, potentially revealing new therapeutic avenues.

CONCLUSIONS AND PERSPECTIVES

In summary, the coronavirus 3CL protease functions as a conserved and pivotal virulence factor in the viral life cycle, playing dual roles in facilitating viral replication and subverting host antiviral responses. These functions mainly relying on its unique cysteine protease activity and specific interactions with host proteins. The 3CL protease has been demonstrated to cleave a broad spectrum of host proteins involved in multiple essential cellular processes, including transcription, translation, and nuclear-cytoplasmic transport. Cumulatively, these proteolytic events reshape the host cellular environment, creating a proviral milieu that exacerbates viral pathogenesis. Furthermore, through its capacity to disrupt multiple signaling pathways—particularly by

targeting key innate immune components such as RIG-I, MAVS, and NEMO—the protease effectively suppresses interferon production, NF-κB signaling, and stress granule formation, thereby crippling antiviral defenses. Beyond immune evasion, the 3CL protease also plays a critical role in regulating host cell death, autophagy, and stress granule formation. It exerts bidirectional regulation over pyroptosis, manipulates the apoptosis pathway, and induces cytotoxicity effects, all of which may contribute to the pathogenicity of the virus.

Comparative analyses reveal both conserved and divergent roles of 3CL proteases across coronavirus species. Although SARS-CoV-2 and other coronaviruses share common substrates such as NEMO, DCP1A, and GSDMD, significant differences exist in cleavage site specificity and efficiency. Notably, the SARS-CoV-2 3CL protease demonstrates superior NEMO cleavage efficiency compared to SARS-CoV. Species-specific adaptations are exemplified by PDCoV 3CL protease's unique ability to cleave porcine TRAF2 rather than human homologs. These functional divergences likely arise from structural variations in 3CL proteases and substrate sequence heterogeneity, potentially explaining differences in host tropism and clinical outcomes. Additionally, while SARS-CoV-2 3CL protease cleaves novel substrates including NLRP1, CARD8, and TDP-43, whether other coronaviruses share these cleavage activities—and with comparable efficiency—remains a critical unanswered question. Resolving these disparities may elucidate mechanisms underlying interspecies pathogenicity differences.

Current therapeutic strategies targeting the 3CL protease through direct-acting antivirals (DAAs) include Paxlovid (nirmatrelvir/ritonavir), simnotrelvir/ritonavir (SIM0417), leritrelvir (RAY1216), and mindeudesivir (VV116) (Cao et al., 2023). Clinical data demonstrate that nirmatrelvir/ritonavir significantly reduces hospitalization and mortality risks (Lewnard et al., 2023), though its utility is limited by emerging resistance mutations (e.g., L50F, E166V) (Zhou et al., 2022; Jochmans et al., 2023). While simnotrelvir/ritonavir, leritrelvir, and mindeudesivir show promising safety and efficacy profiles (Cao et al., 2023), their clinical validation requires further large-scale studies (Zheng et al., 2024). Future development should prioritize pan-coronavirus inhibitors targeting conserved regions (e.g., substrate-binding pockets), combination therapies to mitigate resistance, and computational approaches like AlphaFold-guided structural optimization for rapid drug design.

Despite significant advances, key knowledge gaps persist. The molecular mechanisms governing substrate specificity across coronavirus species remain poorly characterized, as do the structural determinants of cleavage efficiency variations. Furthermore, the pathophysiological consequences of cleaving newly identified substrates (e.g., NLRP1, CARD8) and the evolutionary dynamics of protease-host interactions warrant systematic investigation. Addressing these questions will require integrating high-throughput proteomics with advanced structural biology techniques to comprehensively map 3CL protease-host interaction networks. Such efforts will not only deepen our understanding of coronavirus biology but also accelerate the development of novel antiviral strategies. Sustained research on the 3CL protease is therefore essential for combating both current and emerging coronavirus threats.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. Prof. Xinwen Chen is an editorial board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article.

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