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Review Article

# Update on enteroviral protease 2A: Structure, function, and host factor interaction

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ABSTRACT

Enteroviruses (EVs) are human pathogens commonly observed in children aged 0–5 years and adults. EV infections usually cause the common cold and hand-foot-and-mouth disease; however, more severe infections can result in multiorgan complications, such as polio, aseptic meningitis, and myocarditis. The molecular mechanisms by which enteroviruses cause these diseases are still poorly understood, but accumulating evidence points to two enterovirus proteases, $2A^{pro}$ and $3C^{pro}$, as the key players in pathogenesis. The $2A^{pro}$ performs post-translational proteolytic processing of viral polyproteins and cleaves several host factors to evade antiviral immune responses and promote viral replication. It was also discovered that coxsackievirus-induced cardiomyopathy was caused by $2A^{pro}$-mediated cleavage of dystrophin in cardiomyocytes, indicating that cellular protein proteolysis may play a key role in enterovirus-associated diseases. Therefore, studies of $2A^{pro}$ could reveal additional substrates that may be associated with specific diseases. Here, we discuss the genetic and structural properties of $2A^{pro}$ and review how the protease antagonizes innate immune responses to promote viral replication, as well as novel substrates and mechanisms for $2A^{pro}$. We also summarize the current approaches for identifying the substrates of $2A^{pro}$ to discover novel mechanisms relating to certain diseases.


## 1. Introduction

Human enteroviruses (EV), members of the genus *Enterovirus* of the family *Picornaviridae*, are a group of viruses that primarily cause the common cold and hand-foot-and-mouth disease. Owing to multiorgan tissue tropism, EV infection can lead to multiple organ dysfunction, including a variety of disorders, such as poliomyelitis, flaccid paralysis, encephalitis, meningitis, viral myocarditis, neurogenic pulmonary edema, and type I diabetes [1–3]. According to genetic variances in the whole genome sequence, EV is currently categorized into four groups with more than 100 serotypes, and novel enteroviruses have been continuously identified in recent years. The genome of EV is approximately 7.5 kb in length. Except for the untranslated portions at its polar ends, it can be separated into three parts (P1-P3) and translated into 11 proteins [4,5]. Among these, the 2A protease ($2A^{pro}$) of the P2 region and the 3C protease ($3C^{pro}$) of the P3 region are multifunctional cysteine proteases that process viral polyprotein to structural (VP4-VP1) and nonstructural (2A-3D) proteins [4].

Additionally, $2A^{pro}$ and $3C^{pro}$ have been found to launch their specific proteolytic activity in critical pathways, including viral replication, immune escape, inflammation, and pyroptosis. $2A^{pro}$ is closely associated with EV pathogenesis. For instance, $2A^{pro}$ cleaves dystrophin in cardiomyocytes, leading to myocarditis [6]. As a result, fundamental studies on protease conformation, sequence analysis, and pathogenic mechanisms have been conducted to uncover more potential substrates and novel mechanisms of $2A^{pro}$. Given the crucial role of $2A^{pro}$ in viral infections, we aimed to provide a more thorough understanding of the structure and functional characteristics of $2A^{pro}$.

## 2. Sequence feature of enteroviral protease 2A

$2A^{pro}$, encoded by the P2 region of the virus genome, belongs to the chymotrypsin-related endopeptidase protease family [7] and is engaged *in cis* cleavage between the capsid protein and the nonstructural protein during the early precursor proteolysis of EV (Fig. 1A). The genome segment encoding for $2A^{pro}$ is 441–450 bp and encodes 147–150 amino acids across all serotypes, with an average amino acid similarity of 50%–75% among EV subgroups [8]. Most EV-A members

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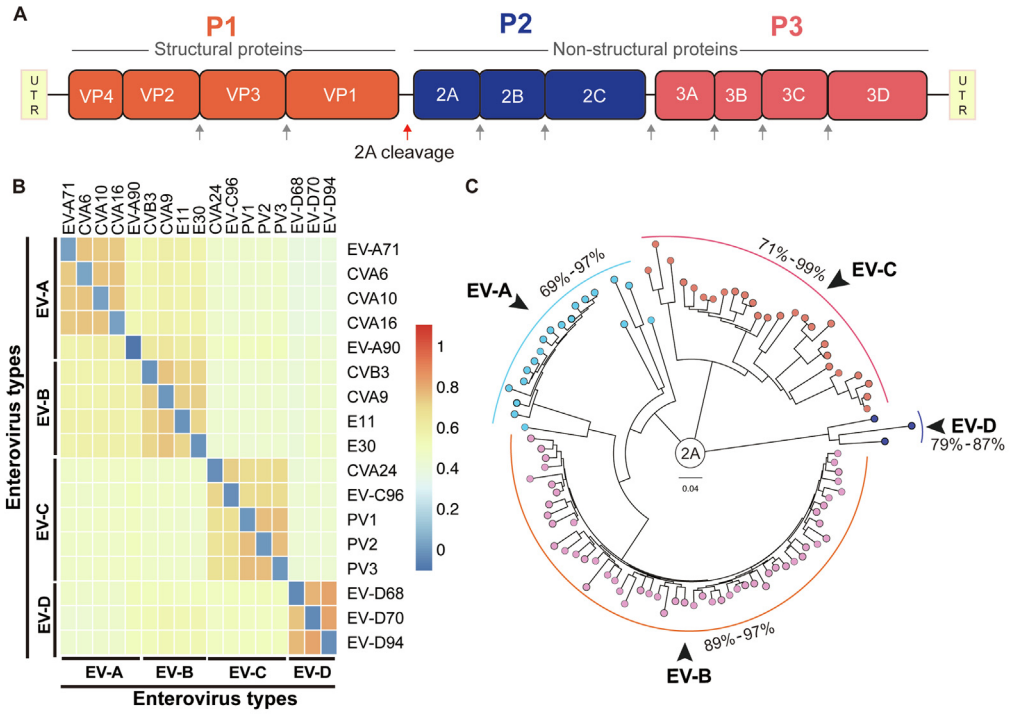


**Fig. 1.** Sequence features of 2A protease ($2A^{pro}$). A) The genome of enterovirus and cleavage site of $2A^{pro}$, red arrow represents 2A cleavage, and gray arrows represent $3C^{pro}$ cleavage. B) Sequence similarity comparison of important serotypes constructed based on the amino acid sequence of $2A^{pro}$. C) Phylogenetic tree of $2A^{pro}$ of EV-A to EV-D constructed based on the amino acid sequence of $2A^{pro}$. Abbreviations: EV, enterovirus; EV-A to D, enterovirus group A to D; CVA, coxsackievirus A; CVB, coxsackievirus B; E11/30, echovirus 11/30; PV, poliovirus.

share the highest amino acid similarities (93%–96%) with $2A^{pro}$ of enterovirus A71 (EV-A71), followed by coxsackievirus serotype A (CVA), coxsackievirus serotype B (CVB), and echovirus at approximately 75%, and poliovirus (60%) and EV-D (48%) with lower similarity, whereas that of rhinoviruses was lower with a 35%–55% resemblance (Fig. 1B). Interestingly, based on amino acid sequence comparison, EV-A serotypes 76, 89, 90, and 121 clustered separately in EV-A with approximately 70% similarity compared to both EV-A and EV-B, which could be the result of recombination with simian EVs [9] (Fig. 1C).

After extensive research, the junction of VP1 (C-terminus) and P2 (N-terminus of $2A^{pro}$) on enteroviral precursor proteins has been identified as the cleavage site of $2A^{pro}$. The amino acid residues P4, P2, P1, P1′, and P2′ are critical determinants of the sequence specificity of EV proteases [7]. Although previous studies have shown that $2A^{pro}$ shows a tendency to cleave the LTTYG motif for poliovirus [10], the particular proteolysis motif of $2A^{pro}$ in EV has not been established or comprehensively studied. Previous studies show that P1′ is the crucial determinant, exclusively glycine, whereas P2 is predominantly threonine and asparagine. Although P1 can tolerate a variety of residues, it favors threonine, tyrosine, and phenylalanine [11]. P2′ tends toward proline, alanine, and phenylalanine, whereas leucine and threonine are most frequently found in P4.

## 3. Structure of enteroviral protease 2A

The crystal structures of $2A^{pro}$ in EV-A and EV-B have been elucidated in recent years, promoting the understanding of its specificity, spatial conformation, catalytic core, proteolytic function, and antiviral inhibitors [12,13]. The tertiary structure of $2A^{pro}$ has been described as a four-stranded β-sheet (bI2, cI, eI2, and fI) in the N-terminal domain and a six-stranded antiparallel β-barrel in the C-terminal domain (aII, bII, cII, dII, eII, and fII), connected by a long interdomain loop [14] (Fig. 2A). In addition, the catalytic triad (His21, Asp39, and Cys110) and rigid zinc ion-binding sites (Cys56, Cys58, Cys116, and His118) that contribute to the stability of the overall folding [15], were identified and found to be highly conserved in EV (Fig. 2B and C). EV-D68 of EV-D is slightly different from EV-A to EV-C because it contains only 147 amino acids with a catalytic triad consisting of His18, Asp36, and Cys107.

Crystal structures enable an enhanced understanding of the function of each structural domain, thus laying the foundation for antiviral drug design and functional studies. Studies on the $2A^{pro}$ of CVA16 uncovered conformational conversion — “open and close” states triggered by two “switcher” residues, Glu88 and Tyr89, located within the bll2-cII loop [12]. Along with these important sites, the C-terminal region of $2A^{pro}$ is critical for boosting viral replication. The hydrophobic motif “LLWL” and the acidic motif “DEE” at the C-terminus of $2A^{pro}$, which are highly conserved in EV-A (Motif 1 in Fig. 2D), play a significant role in viral RNA replication [16]. Meanwhile, deleting the “LLWL” pattern abolished the proteolytic activity of $2A^{pro}$, demonstrating that the motif is required for the active proteinase conformation and viral replication. Another study examined the negatively charged motif (EAMEQ-NH (2)) at the C terminus of poliovirus $2A^{pro}$ is required for viral RNA replication rather than proteolytic processing (Motif 2 in Fig. 2D). Finally, EV infection typically results in the activation of the interferon (IFN) signaling pathway and increases interferon-stimulated genes (ISGs); however, $2A^{pro}$ in EV-A71 infection leads to the complete opposite, with decreased ISGs and significant IFN resistance [17]. Furthermore, $2A^{pro}$ of EV-A71 is more effective than CVA16 in cleaving the VP1-2A substrate, reflecting faster polyprotein processing that potentially contributes to a shorter replication cycle than CVA16 [12].

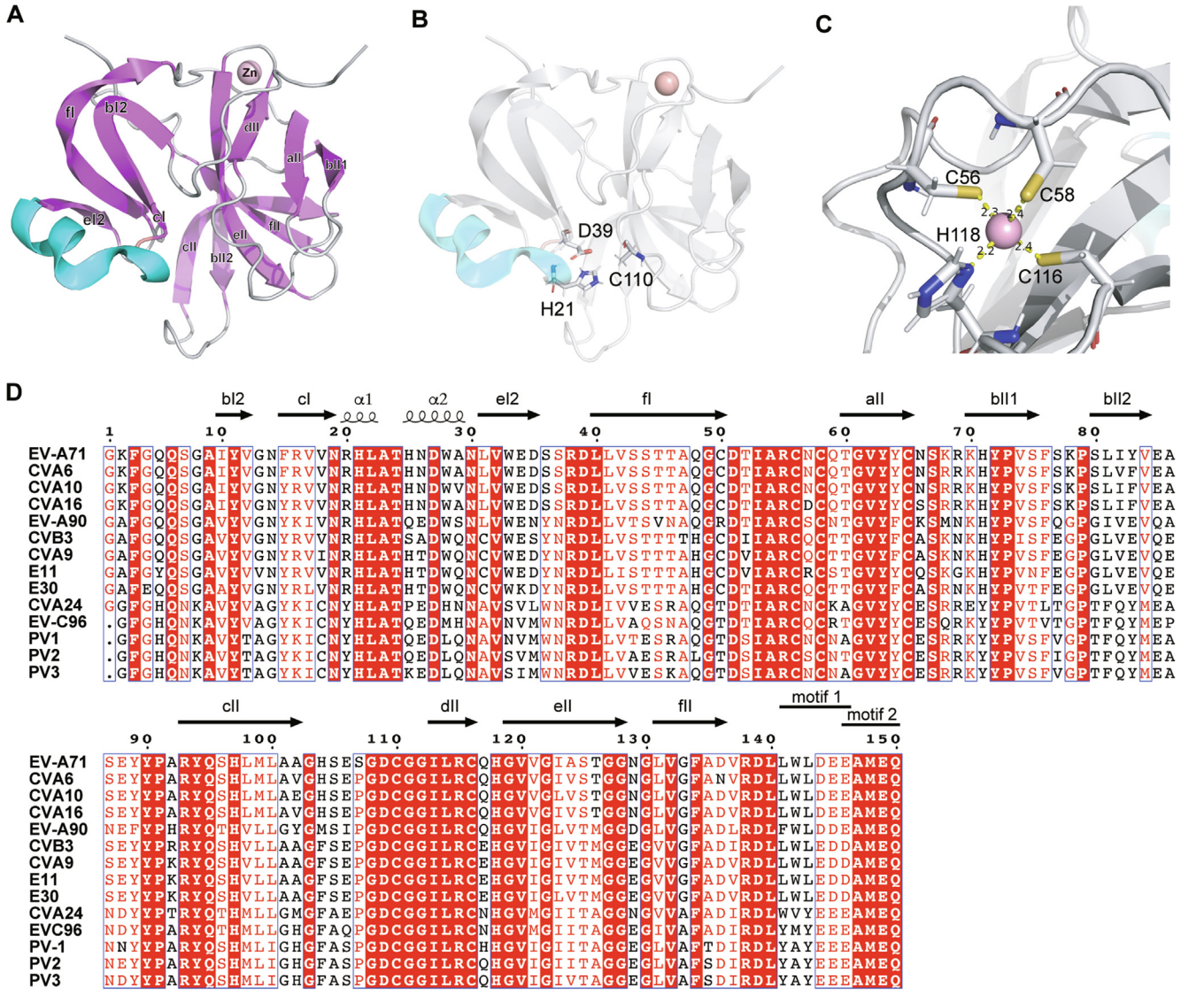


**Fig. 2.** Crystal structure and sequence comparison of 2A protease ($2A^{pro}$) in EV. A) Crystal structure of EV-A71 (PDB number: 3w95). B) The catalytic triad of $2A^{pro}$. C) Zinc ion-binding site of $2A^{pro}$. D) Sequence alignment, secondary structure, and essential motifs of $2A^{pro}$ in representative enterovirus serotypes. Abbreviations: EV, enterovirus; EV-A to D, enterovirus group A to D; CVA, coxsackievirus A; CVB, coxsackievirus B; E11/30, echovirus 11/30; PV, poliovirus.

## 4. The function of enteroviral protease 2A

### *4.1. The role of enteroviral protease 2A in apoptosis*

When EV genomic RNA is translated into precursor protein, $2A^{pro}$, as one of the proteins with hydrolysis function, cuts off the junction between nonstructural protein and structural protein and cuts 3CD protein into 3C and 3D proteins [18], while 3C protease cuts the remaining 8/11 proteins. Although the mechanism behind VP0 cleavage remains unknown, several studies suggest that VP0 cleavage is an autocatalytic process that involves RNA, and His195 of VP2 is crucial for efficient cleavage [19,20]. In addition to cleaving precursor proteins, $2A^{pro}$ and $3C^{pro}$ hijack cellular proteins to promote viral propagation (Table 1). For instance, $2A^{pro}$ has been found to cleave eukaryotic initiation factor 4 gamma 1 (eIF4G), a cap-dependent translation initiation factor. This cleavage shuts down the normal synthesis process of host proteins while promoting virus replication because EV replication is initiated by an internal ribosome entry site (IRES) rather than a 7-methylguanosine cap [21,22]. Another translation initiation factor, death-associated protein 5 (DAP5) specific to IRES-containing mRNAs, has been cleaved by $2A^{pro}$. Fragments of DAP5 retain the ability to initiate the IRES-driven translation of apoptosis-associated p53 and promote EV replication and offspring release. The $2A^{pro}$-mediated cleavage of eIF4G and DAP5 enhances host apoptosis and viral replication [23].

In addition to directly hindering host protein synthesis, $2A^{pro}$ can indirectly affect the normal replication of host proteins. Studies have found that $2A^{pro}$ targets and cleaves various nuclear pore complexes, such as nucleoporins 62 [24], 98 [25], and 153 [26], and that cleavage affects the transport process of host proteins, thereby interfering with the cargo shuttle between the nucleus and cytoplasm and the sending of antiviral signals. Another apoptosis-related gene, TXNIP, is elevated after EV-A71 infection. Its expression is increased by $2A^{pro}$, which also leads to enhanced cellular apoptosis [27]. All this evidence suggests that $2A^{pro}$ promotes viral replication and induces cell apoptosis by cleaving several apoptosis-related proteins.

**Table 1**
The functions and associated host factors of $2A^{pro.}$

| Function | Host factors* | Reference |
|---|---|---|
| Promote virus replication | SETD3 | [28] |
| Apoptosis | eIF4G | [22] |
| | DAP5 | [23] |
| | TXNIP | [27] |
| Inhibit nuclear transport | nucleoporins 62, 98, 153 | [24] |
| Antagonize antiviral response | MDA5 | [31] |
| | MAVS, TRAF3 | [33] |
| | STAT1 | [40] |
| | IFNAR1 | [17] |
| | YTHDF | [37] |
| Inflammation | NLRP3 | [43] |
| | CARD8 | [46] |
| Etiology | Dystrophin | [6] |

* STED3, methyltransferase set domain containing 3; eIF4G, eukaryotic initiation factor 4 gamma 1; DAP5, death-associated protein 5; TXNIP, thioredoxin-interacting protein; MDA5, mitochondrial antiviral protein 5; MAVS, mitochondrial antiviral signaling; TRAF3, tumor necrosis factor receptor-associated factor 3; STAT1, signal transducer and activator of transcription 1; IFNAR1, IFN-α/β receptor 1; YTHDF, YTH N6-methyladenosine RNA binding proteins; NLRP3, nucleotide-binding oligomerization domain, leucine- rich repeat and pyrin domain containing 3; CARD8, caspase recruitment domain-containing protein 8.

### *4.2. The role of enteroviral protease 2A in promoting viral replication*

Furthermore, recent studies have demonstrated that $2A^{pro}$ can directly bind host proteins and promote viral replication. $2A^{pro}$ interacts with the methyltransferase set domain containing 3 (SETD3), which is essential for a variety of EVs [28]. Unexpectedly, this interaction is independent of either the methyltransferase activity of SETD3 or the protease activity of $2A^{pro}$. In contrast, SETD3 binds to $2A^{pro}$ directly via distinct domains without recruiting additional host factors, and the two then form a stable complex that promotes viral RNA replication in the cytoplasm [29]. Structural studies have shown that mutations in the interaction interface of SETD3 can severely affect the combination of the two and reduce the proviral effect of SETD3. In summary, $2A^{pro}$, directly and indirectly, promotes viral replication in the host by cleaving host proteins, and a less well-known mechanism of $2A^{pro}$ is that, like 2C and 3A proteins, it can directly bind to crucial host factors and drive viral reproduction without cleavage.

### *4.3. The role of enteroviral protease 2A in antiviral response*

The natural/innate immune response is the first line of defense against viral infections. After much research, it was determined that numerous signaling pathways, including mitochondrial antiviral signaling (MAVS) protein-mediated antiviral signaling, antiviral signals mediated by toll-like receptors (TLR), type I IFN signaling pathways, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways, and inflammasome formation, are involved in the process of EV infection. All these routes can potentially increase cytokine production, leading to inflammation and disorders in multiple organs. Viruses such as influenza and Zika have developed different evolutionary strategies to antagonize host antiviral defenses. As evolved strategies of EV, $2A^{pro}$, and $3C^{pro}$ antagonize natural antiviral immunity by cleaving numerous host factors through crucial pathways (Fig. 3).

#### *4.3.1. MDA5-mediated antiviral response*

Type I IFN is one of the three types of IFN and is the main activation pathway upon EV infection. Type I IFN signaling pathway can be divided into two processes. The upstream process first increases the synthesis of interferon regulatory factor (IRF) 3/7 and other interferon regulatory factors, stimulating interferon receptors downstream and activating the Janus kinase(JAK)-signal transducer and activator of transcription (STAT) signaling pathway, leading to ISG production [30].

Upstream, identification of pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs) constitutes the first line of defense, which primarily detects viral RNA during infection and activates the downstream adapter protein. Retinoic acid-inducible gene I (RIG-I)-like receptors and TLR3 and TLR7 are the significant PRRs that identify EV replication intermediates (dsRNA) are predominantly detected by the RIG-I-like receptor mitochondrial antiviral protein 5 (MDA5) [31], which stimulates adapter proteins such as MAVS and cardif-mediated downstream signaling and partially activates IRF and TANK-binding kinase 1 (TBK1) and IκB kinase (IKK). These activated kinases activate IRF3 and IRF7 and induce type I IFN expression. MDA5 and MAVS are preferentially targeted by $2A^{pro}$ of EV-A71 in the upstream IFN signaling cascade, resulting in an effective blockage of IFN transcription, evasion of the host antiviral innate immune system and promotion of viral replication [32–34].

#### *4.3.2. Tlrs-mediated antiviral response*

TLRs, including intracellular TLR3, 7, 8, and 9, are another critical class of PRRs that mainly recognize viral RNA and activate adapter proteins myeloid differentiation primary response 88 (Myd88) and toll-interleukin receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF), respectively. It has been shown that $2A^{pro}$ inhibits the TLR3- and TLR4-triggered TRIF-dependent pathway, primarily involved in the IFN signaling pathway. One of the targets for $2A^{pro}$ of EV-D68 is tumor necrosis factor receptor-associated factor 3 (TRAF3), coupled to the adaptor TRIF and necessary for marshaling the protein kinase TBK1 into TIR signaling complexes. The cleavage of TRAF3 suggests that modification of the IFN-β pathway may be a viral mechanism underlying EV-D68 infection [35] (Fig. 3). Furthermore, the $2A^{pro}$ of EV-A71 has been implicated in the downregulation of TLR3 [36]. The evidence above suggests that $2A^{pro}$ could, directly and indirectly, suppress the TLRs-mediated type I IFN pathway.

#### *4.3.3. Interferon signaling pathway*

In the downstream processes of the IFN signaling pathway, IFN-α/β receptor 1 (IFNAR1) is activated through IFN binding, mediating the phosphorylation of STAT1, STAT2, JAK1, and tyrosine kinase 2 (TYK2). Phosphorylated STAT1 and STAT2 form heterodimers and bind with IRF9 to form the transcription factor ISGF3, ultimately initiating ISG activation. As a countermeasure, $2A^{pro}$ of EV was found to cleave IFNAR1 directly and block IFN-mediated phosphorylation in the JAK-STAT pathway [17]. In addition to directly cleaving pathway members, other studies have found that the $m6^A$ reader YTH $N^6$-methyladenosine RNA binding proteins (YTHDF) can be cleaved by $2A^{pro}$ and antagonize JAK-STAT signaling to impede RNA translation in vivo [37]. YTHDF proteins are reported to be critical regulators for stress granule (SG) formation [38], whereas $2A^{pro}$ of EV-A71, CVB3, CVA21, and EV-D68 could strongly suppress the formation of SGs [39], indicating that the cleavage of YTHDF may contribute to inhibiting the formation of SGs in infected cells.

In addition to the type I IFN response, $2A^{pro}$ also blocked IFN-γ-induced IRF1 transactivation following reduced phosphorylation of STAT1 in the type III IFN response [40]. Another study found that EV infection in the intestinal tract mainly causes a type III IFN response, and $2A^{pro}$ and $3C^{pro}$ can reduce the expression of IFN-λR, thereby inhibiting the production of type III IFN in intestinal epithelial cells [41]. Finally, it should be noted that most studies only use representative strains of the particular virus, raising the question of whether they truly reflect the entire serotype, given that certain divergences in $2A^{pro}$ may affect its cleavage activity.

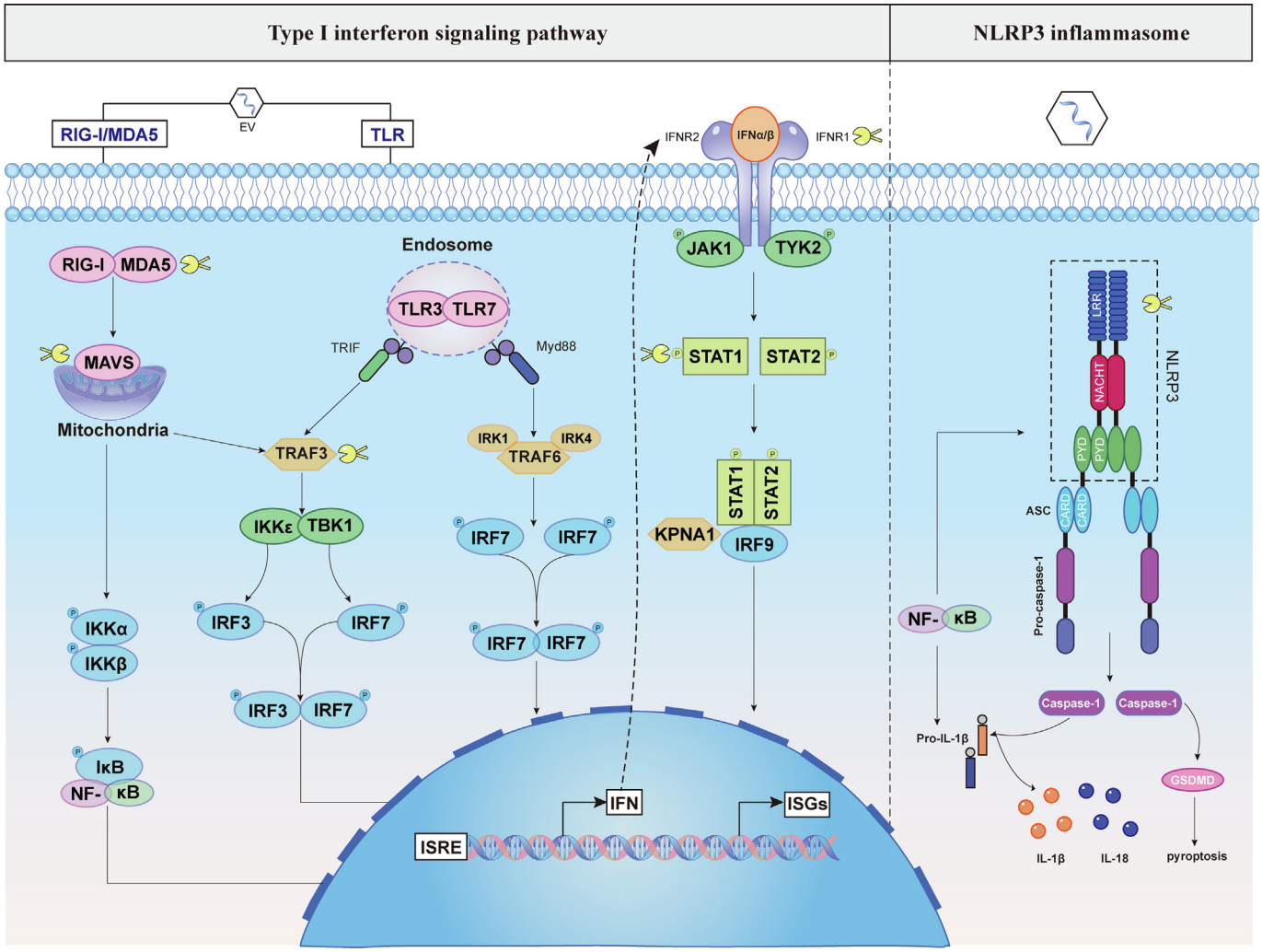


**Fig. 3.** Immune-related signaling pathways during enterovirus infection. Enterovirus protease $2A^{pro}$ cleaves mitochondrial antiviral protein 5 (MDA5) and mitochondrial antiviral signaling (MAVS) protein in the MDA5-mediated pathway and tumor necrosis factor receptor-associated factor 3 (TRAF3) in the toll-like receptor (TLR)-mediated pathway. $2A^{pro}$ also degrades IFN-α/β receptor 1 (IFNAR1) in the interferon (IFN) signaling pathway and nucleotide-binding domain, leucine-rich–containing family, pyrin domain-containing-3 (NLRP3) in the NLRP3 inflammasome. Abbreviations: RIG-I, retinoic acid-inducible gene I-like receptors; IKK, IκB kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TRIF, toll-interleukin receptor (TIR)-domain-containing adapter-inducing interferon-β; Myd88, myeloid differentiation primary response 88; TRAF6, tumor necrosis factor receptor-associated factor 6; TBK1, TANK-binding kinase 1; IRF, interferon regulatory factor; ISRE, interferon-sensitive response element; ISGs, interferon-stimulated genes; IRK, interleukin receptor-associated kinase; IFNR, interferon receptor; JAK, janus kinase; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription; KPNA1, karyopherin subunit alpha 1; LRR, leucine-rich repeat; NLRP3, nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3; PYD, pyrin domain; CARD, caspase recruitment domain-containing protein; ASC, apoptosis-associated speck-like protein containing a CARD; GSDMD, Gasdermin D; IL, interleukin.

*4.3.4. Enteroviral protease function in inflammasome*

The inflammasome family, including nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 1 (NLRP1), NLRP3, NLR family caspase recruitment domain-containing protein (CARD) domain-containing protein 4 (NLRC4), CARD8 inflammasomes, etcetera., is a crucial part of innate immunity because it recognizes damage-associated molecular patterns and PAMPs [42]. Studies have demonstrated that the systemic inflammation brought on by EV-A71 is partially mediated by the NLRP3 inflammasome [43]. Oligomerized NLRP3 attaches to the adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and subsequently recruits CARD to form the inflammasome complex in the normal process of the NLRP3 inflammasome, which eventually promotes the maturation of the pro-inflammatory cytokine interleukin-18 (IL-18) and interleukin-1β (IL-1β). Conversely, EV-A71 inhibits inflammasome activation by cleaving leucine-rich repeat (LRR) region of NLRP3 via $2A^{pro}$ and $3C^{pro}$ (Fig. 3). In contrast, the NLRP3 inflammasome may also induce pyroptosis by cleaving pyroptosis-median Gasdermin D (GSDMD) through activating caspase-1, thereby interfering with virus replication and eliminating infected immune cells. As a countermeasure, $3C^{pro}$ suppresses pyroptosis by cleaving GSDMD, eventually resulting in more severe pathogenesis and death [44].

Other inflammasome members CARD8 and NLRP1 are external viral protease sensors [45]. Studies have found that the cleavage of CARD8 by HIV-1 protease generated the release of the cleaved C-terminus and N-terminus, which are required for inflammasome assembly and activation [46]. Interestingly, in contrast to their involvement in suppressing the formation of the NLRP3 inflammasome, $2A^{pro}$ and $3C^{pro}$ activate the CARD8 inflammasome through fragment cleavage during CVB3 infection in endothelial cells, which is the first line of defense against blood-borne pathogens. Furthermore, the reduction of CARD8 in cardiomyocytes can significantly reduce

CVB3 infection [47,48]. In conclusion, $2A^{pro}$ may have complicated effects on the inflammasome, acting as both an activator and an inhibitor.

## 5. Mutational analysis on $2A^{pro}$

To better understand the conformational changes in the hydrolysis process of $2A^{pro}$ and the relationship between sequence mutation and the development of diseases, researchers conducted experiments to identify the key sites that alter the hydrolysis efficiency of $2A^{pro}$. The unnatural mutation in the catalytic triad C110A (C107A for EVD68) site is essential in that the "suicide" mutation will directly inactivate $2A^{pro}$ and render it incapable of performing proteolysis, which usually serves as a common control site for inactivating $2A^{pro}$ in experiments. Mutations in $2A^{pro}$, for instance, Y64H, M68R, and D85E may increase viral virulence and replication and increase the severity of clinical symptoms [49]. The $2A^{pro}$ mutant with G122E in the eII barrel exhibited lower cleavage efficiency for eIF4G, significantly attenuating its SGs-inducing ability [50]. Another study suggested that the Q29K mutation in the $2A^{pro}$ of CVB3 can facilitate the cleavage of cellular eIF4G under polyamine depletion conditions [51]. The CVB3 mutation at S35G, which is located in the eI2 β-barrel for $2A^{pro}$, potentially develops resistance to the polyamine analog diethylnorspermidine (DENSpm), leading to increased proteolytic activity in the presence of DENSpm [52]. Although $2A^{pro}$ is relatively conserved, the impact of scattered variations implies that the mechanism behind its structural changes requires further investigation.

## 6. Methodology in finding potential substrate

Enteroviral proteases are closely associated with enteroviral pathogenesis. For instance, $2A^{pro}$ cleaves dystrophin in cardiomyocytes, leading to myocarditis. Therefore, identifying the potential cleavage substrates of $2A^{pro}$ could provide insight into the novel pathogenic mechanisms of EV infection. Researchers have recently employed various means, such as co-immunoprecipitation, motif search, protein interaction networks, and proteomics, to uncover potential substrates of $2A^{pro}$. Among these, proteomics with mass spectrometry detection optimizes high-throughput detection methods in fragment identification and enables precise prediction on substrate cleavage sites using the N-terminal labeling technique [53]. Based on the Terminal Amine Isotopic Labeling of Substrates N-terminal labeling technique, phosphoribosylformylglycinamidine synthetase, heterogeneous nuclear ribonucleoprotein (hnRNP)K, and hnRNPM were discovered as novel substrates of $2A^{pro}$ [54]. Another study adopted the subtiligase labeling method and discovered that multiple known and new cellular targets were cleaved by $2A^{pro}$ [11].

Based on the known proteolysis motif (LTTYG) for poliovirus $2A^{pro}$, other researchers searched the protein library and found that the $m6^A$ reader YTHDF protein can be cleaved by $2A^{pro}$ and antagonize JAK-STAT signaling to impede RNA translation in vivo. Furthermore, the protein interaction network is a derivation method for predicting cleavage substrates of $2A^{pro}$, which validates the interaction between the 2A protein and the substrates; numerous techniques, such as western blotting, co-immunoprecipitation, overexpression, and siRNA are usually used to verify the results. Given the complexity and size of the $2A^{pro}$ recognized cleavage substrates, much more research is required to understand the interaction between the protease and host factory completely.

## 7. Conclusion

EV infection can result in several neurological disorders; therefore, neurovirulence is a prominent area for EV research, and it has been a topic of concern as to whether $2A^{pro}$ contributes to or expedites the onset of neurological illness [55]. Several studies have indicated that inflammatory responses caused by cytokines and interferons play critical roles in the severity of the disease; therefore, $2A^{pro}$ may contribute to the development of the disease process through its impact on the IFN pathway and inflammation. Furthermore, even though some research has been done on how EV affects host natural immune responses, many unstudied substrates and pathways are yet to be uncovered. As a result, investigating the substrates and function of $2A^{pro}$ will continue to be a key area of study, which could provide evidence for cutting-edge clinical treatment with the EV-A71 vaccine with high efficiency has been developed [56]. However, it cannot shield children from other EV infections. Several studies also showed that antibodies against viral proteases were observed in human serum samples. For example, SARS-CoV-2 cysteine-like protease antibodies can be detected in the serum and saliva of COVID-19-seropositive individuals [57]. Therefore, antibodies against viral proteases may be a feasible option for antiviral treatment. The other alternative and potentially more effective technique for preventing viral infection is the broad-spectrum antivirals that target highly conserved proteins, such as 2A and 3C protease, which leads to the development of broad-spectrum antiviral medications. Rupintrivir, an antiviral inhibitor designed based on $3C^{pro}$, has significantly inhibited group B EVs such as CVB2, CVB5, and echovirus [58]. However, no known potent and specific 2Apro inhibitor exists, making it a relatively unexplored therapeutic target. Given the crucial role of $2A^{pro}$ in viral infections, broad-spectrum antiviral medicines for EVs will likewise prioritize $2A^{pro}$ [59,60].

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## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Author contributions

**Ying Liu:** Conceptualization, Writing – review & editing. **Jichen Li:** Writing – review & editing. **Yong Zhang:** Conceptualization, Writing – review & editing, Supervision.

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