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REVIEW

Recent Progress on Functional Genomics Research of Enterovirus 71

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Abstract
Enterovirus 71 (EV71) is one of the main pathogens that causes hand-foot-and-mouth disease (HFMD). HFMD caused by EV71 infection is mostly self-limited; however, some infections can cause severe neurological diseases, such as aseptic meningitis, brain stem encephalitis, and even death. There are still no effective clinical drugs used for the prevention and treatment of HFMD. Studying EV71 protein function is essential for elucidating the EV71 replication process and developing anti-EV71 drugs and vaccines. In this review, we summarized the recent progress in the studies of EV71 non-coding regions (5′ UTR and 3′ UTR) and all structural and nonstructural proteins, especially the key motifs involving in viral infection, replication, and immune regulation. This review will promote our understanding of EV71 virus replication and pathogenesis, and will facilitate the development of novel drugs or vaccines to treat EV71.

Keywords Enterovirus 71 (EV71) · Functional genomics · Structural protein · Nonstructural protein · Viral protein function

Introduction

Enterovirus 71 (EV71), a single-stranded RNA virus with a genome length of approximately 7.5 kb, belongs to the genus *Enterovirus* within the family *Picornaviridae* (Wang *et al.* 2012a, b). Since it was first discovered in 1969, EV71 has caused numerous outbreaks and epidemics worldwide, particularly in Asian and Pacific regions, such as China, Korea, Singapore, Japan, and Vietnam (WHO 2018). EV71 usually infects infants and young children under the age of 5 years, and hand-foot-and-mouth disease caused by EV71 infection is usually self-limited. However, some infections can cause aseptic meningitis, brain stem encephalitis, and other nervous system diseases (Weng *et al.* 2010; Teoh *et al.* 2016). At present, there are no effective drugs to prevent and treat EV71 infection, and ribavirin, interferon (IFN), and other drugs are used only for symptomatic treatment (Yi *et al.* 2011; Wang *et al.* 2017a). Studying the structure and function of the EV71 viral genome is essential for understanding the EV71 replication process and developing anti-EV71 drugs and vaccines.

In this review, we discuss the important functions of the EV71 genome, based on recent functional genomics research.

Structural Features and Replication Process of the EV71 Genome

The EV71 genome contains only one open reading frame (ORF), which encodes 2193 amino acids and is flanked by 5′ and 3′ untranslated regions (UTRs). As shown in Fig. 1, the low-molecular-weight protein VPg covalently binds to the 5′ UTR, and a variable length poly-A tail is located at the terminus of the 3′ UTR. During the EV71 replication process, the polyprotein is subdivided into P1, P2, and P3 regions. P1 encodes four structural proteins (VP1–VP4), whereas P2 and P3 encode seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Cardosa *et al.* 2003; McMinn 2002). These 11 proteins are closely related to EV71 infection, inflammatory responses, and host immune responses.

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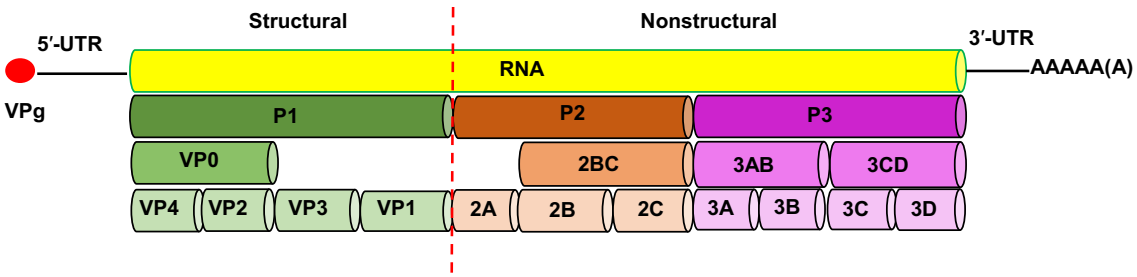

Fig. 1 Structure of the enterovirus 71 (EV71) genome.

As a nonenveloped virus, the host cell entry mechanism of EV71 remains largely unknown. Recent data have indicated that the interaction between viral particles and receptors causes the spatial configuration to change and results in loss of VP4; eventually, the virus particles enter the cell through the endocytotic pathway. Then, the viral shell is removed, the viral genomic RNA is released into the cytoplasm, and the translation of the viral polymeric protein begins with the viral genomic RNA as mRNA (Hu *et al.* 2003; Solomon *et al.* 2010). The replication of EV71 is similar to that of other positive-stranded RNA viruses. First, polymeric precursor protein is synthesized by viral RNA as mRNA template. Then, polymer precursor protein is cut into four structural proteins and seven nonstructural proteins. The nonstructural protein 3D, an RNA-dependent RNA polymerase (RdRp), synthesizes negative-chain RNA using RNA as a template. A large number of positive-strand RNAs are further synthesized using negative-strand RNA as a template. With the accumulation of a large number of viral positive-strand RNAs in the cytoplasm, the subgeneration virus particles begin to be assembled (Bedard and Semler 2004).

Function of the Noncoding Region

The 5′ UTR

The 5′ UTR of EV71 usually folds into a specific spatial structure, including a cloverleaf structure and an internal ribosome entry site (IRES). The IRES contains six major stem loop structures associated with viral RNA replication, which regulate the initiation of viral protein translation by binding to host cell protein factors (Lin *et al.* 2008, 2009a; Yeh *et al.* 2011). IRES directs the initiation of translation in a cap-independent manner and can be used as a target for antiviral drugs. A single nucleotide change from cytosine to uridine at base 158 in the second stem ring of 5′ UTR reduces viral translation and EV71 virulence in mice (Yeh *et al.* 2011). Moreover, Chang *et al.* found that a single nucleotide T to C mutation at nucleotide 494 of the 5′-UTR affects EV71 virulence (Chang *et al.* 2018). During EV71 infection, the process of viral RNA translation relies mainly on translation initiation factors and IRES-specific trans-acting factors (ITAFs). These ITAFs interact with various IRES elements to regulate their activities by affecting ribosome recruitment or modifying the structure of the IRES itself. As shown in Fig. 2, several host proteins have been shown to be involved in modulating EV71 IRES function as ITAFs, including heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), hnRNP K, far upstream element binding protein 1 (FBP1), FBP2, and poly real (rC)-binding protein 1 (PCBP1) (Huang *et al.* 2011; Lin *et al.* 2008, 2009b, c; Luo *et al.* 2014). During EV71 infection, hnRNPA1, hnRNP K, FBP1, FBP2, and PCBP1 are enriched in the cytoplasm, where EV71 replication occurs, whereas hnRNPA1, hnRNP K, FBP1, FBP2, and PCPB1 are localized in the nucleus in mock-infected cells. The cytoplasmic relocalization of hnRNP A1, hnRNP K, FBP1, and PCBP1 in EV71-infected cells leads to enhancement of EV71 IRES-mediated translation and directly regulates the viral protein translation process, thus promoting the replication of EV71; hnRNP A1 interacts with stem-loops II and VI, hnRNP K interacts with stem-loops I–II and IV, FBP1 interacts with the linker region downstream of the EV71 IRES (637–745 nt), and PCBP1 interacts with stem-loops I and IV (Huang *et al.* 2011; Lin *et al.* 2008, 2009c; Luo *et al.* 2014). In contrast to hnRNP A1, hnRNP K, FBP1, and PCBP1, FBP2 inhibits IRES activity by directly binding to nucleotides 1–167 (stem-loops I and II), 91–228 (stem-loops II and III), and 566–745 (stem-loop VI and the spacer region) regions in the EV71 5′ UTR, thereby inhibiting EV71 replication (Lin *et al.* 2009b). During EV71 infection, AU-rich element binding factor 1 (AUF1) accumulates in the cytoplasm where viral replication occurs, binds the IRES of EV71, and negatively regulates IRES-dependent translation (Lin *et al.* 2014). In contrast to AUF1, the mRNA stability factor HuR and the RISC subunit Argonaute 2, as two ITAFs that bind stem-loop II, can promote EV71 IRES activity and viral replication (Fig. 2) (Lin *et al.* 2015). EV71 promotes the expression of silent mating type information regulation 2 homolog 1 (SIRT1), stimulates SIRT1 sumoylation and deacetylase activity, and enhances

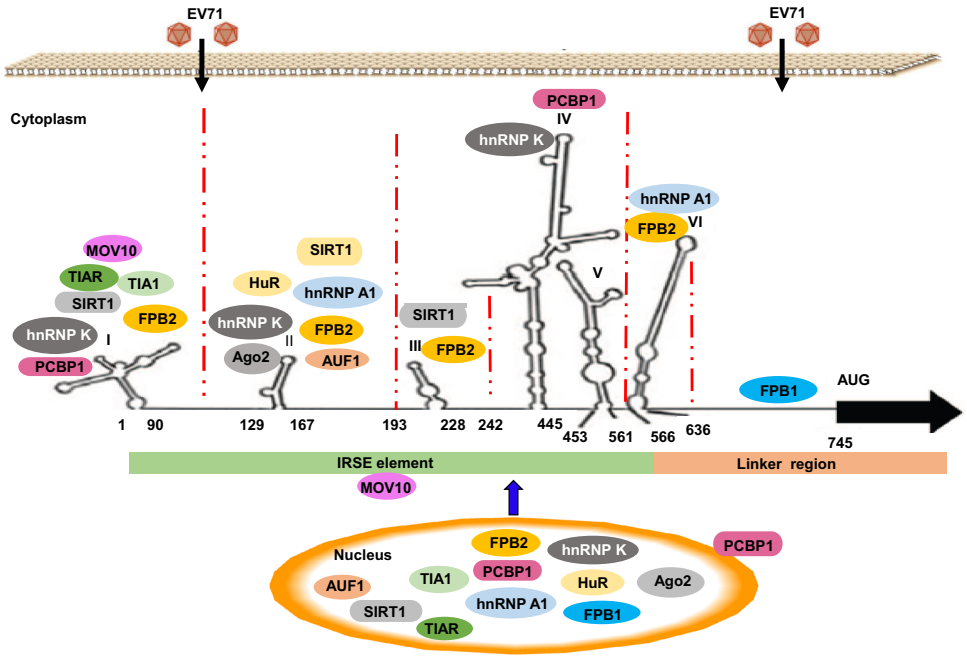

Fig. 2 Host proteins bind to the 5′ UTR and regulate EV71 replication.

SIRT1 translocation from the nucleus to the cytoplasm. After being enriched in the cytoplasm, SIRT1 interacts with the cloverleaf structure of the 5′ UTR to inhibit viral RNA transcription and binds to the IRES to attenuate viral RNA translation (Fig. 2) (Han *et al.* 2016). As shown in Fig. 2, T cell-restricted intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR) are translocated from the nucleus to the cytoplasm after EV71 infection. TIA-1 and TIAR can both bind to stem-loop I of the 5′ UTR and improve the stability of viral genomic RNA, facilitating EV71 replication by enhancing synthesis of the viral genome in host cells (Wang *et al.* 2015c). Moloney leukemia virus 10 (MOV10), a highly conserved cellular protein belonging to the SF1 helicase family, positively regulates EV71 replication by binding to the EV71 cloverleaf-like structure and IRES with its C-terminus (Wang *et al.* 2016). Based on the highly conserved 5′ UTR and its important role in EV71 replication, the 5′ UTR can be targeted to effectively inhibit EV71 replication through RNAi strategies (Deng *et al.* 2012).

The 3′ UTR

The 3′ UTR of EV71 contains a variable poly(A) tail, which is very important for EV71 replication. The poly(A) tail in eukaryotic cells can confer mRNA stability, promote the translational efficiency of mRNA, and transport mRNA from the nucleus to the cytoplasm (Weng *et al.* 2009). However, the specific function of the 3′ UTR and poly(A) tail for EV71 is not fully understood, and studies of PV have shown that the recombinant 3′ UTR of PV is active, although its replication rate is slower than that of the wild-type sequence (Fernandez-Miragall *et al.* 2009; Herold and Andino 2001; Silvestri *et al.* 2006; Todd *et al.* 1997). Sim and others have shown that the chemical synthesis of siRNA targeting the 3′ UTR can reduce the replication of EV71. Transfection of rhabdomyosarcoma cells with siRNA targeting the 3′ UTR region significantly decreases viral RNA, viral proteins, and plaque formation; therefore, RNA interference may also be used as a method for clinical antiviral therapy (Sim *et al.* 2005). A recent study showed that *miR-23b* is significantly downregulated in EV71-infected cells and that upregulation of *miR-23b* inhibits the replication of EV71 by targeting the EV71 3′ UTR conserved sequence through seven consecutive nucleic acids (Wen *et al.* 2013). Feng *et al*. found that *miR-127-5p* expression was upregulated during EV71 infection and *miR-127-5p* can inhibit EV71 infection through downregulating the expression of SCARB2 via targeting two potential sites in 3′ UTR region (Feng *et al.* 2017).

Protein-Coding Region

VP1 Protein

The VP1 protein of EV71 contains 297 amino acids and shows complete genetic diversity corresponding to the viral serotype, which can be used as the basis of EV71 serotype classification (McMinn 2002). Human scavenger receptor class B member 2 (SCARB2) serves as a cellular receptor for EV71 entry, and its cellular entry is through a clathrin-

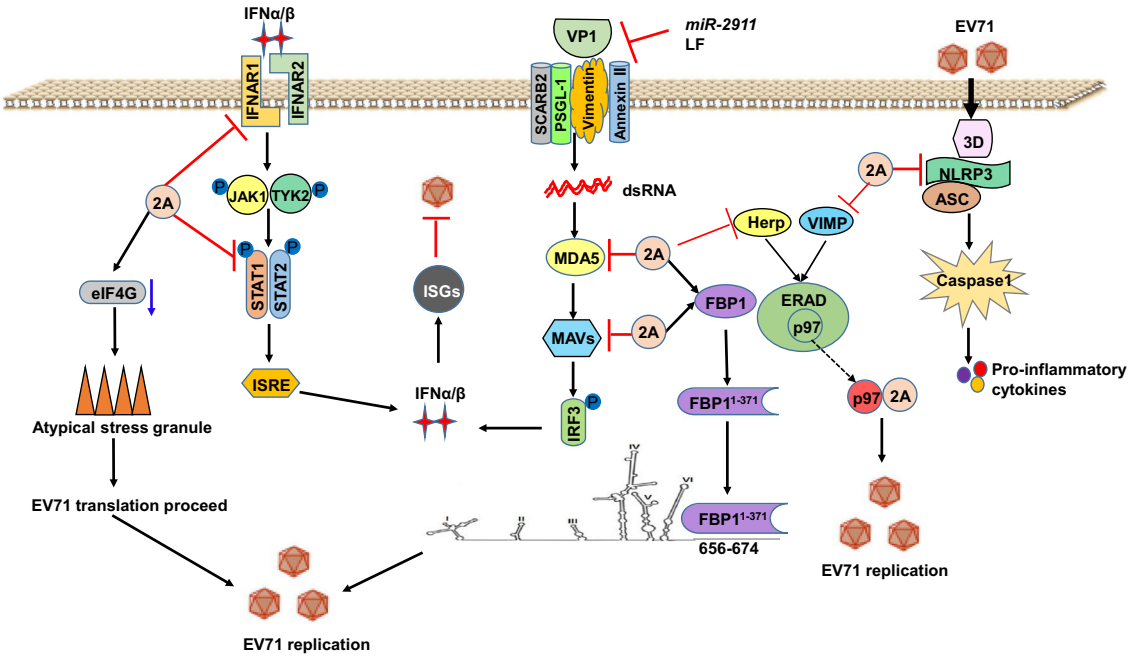

Fig. 3 The protein functions of VP1 and 2A.

mediated and pH-dependent endocytic pathway (Fig. 3). EV71 binds to SCARB2 via a canyon in VP1 around residue Gln-172 (Chen *et al.* 2012). In addition, another study found that the three VP1 mutations K98E, E145A, and L169F enhanced the combination of VP1 with the mSCARB2 protein on murine cells and permitted the virus to infect murine cells (Fujii *et al.* 2018; Kobayashi *et al.* 2018; Victorio *et al.* 2016). P-selectin glycoprotein ligand-1 (PSGL-1), another receptor of EV71, enhances EV71 entry through binding to VP1, and the binding depends on sulfated tyrosine residues and the interaction between negatively charged sulfate groups and positively charged basic residues in the viral capsid (Fig. 3) (Nishimura *et al.* 2013). PSGL-1 cannot bind to VP1 when VP1-145G/Q is replaced with E. VP1-145 is in close proximity to conserved lysine residues at VP1-242 and VP1-244. Moreover, VP1-145 controls the orientation of the lysine side-chain of VP1-244 as follows: with VP1-145Q, the lysine side chain faces outward, and VP1 combines with PSGL-1 to regulate virus entry; however, with VP1-145E, the lysine side chain is turned toward the virus surface, and VP1 cannot bind to PSGL-1 (Nishimura *et al.* 2013). The VP1-145 amino acids vary in different strains, which may be the reason why not all EV71 strains use PSGL-1 as a receptor. In addition, a VP1 mutation (K244E) was found to be necessary for mouse-adapted EV71 virulence in adult mice using reverse genetics (Caine *et al.* 2016). EV71 uses heparan sulfate as an attachment receptor, and both VP1-98 and VP1-145 cannot modulate heparin binding (Tan *et al.* 2017; Tseligka *et al.* 2018). Galectin-1, a soluble beta-galactoside binding lectin, may be associated with EV71 VP1 via carbohydrate residues and is subsequently released and bound to another cell surface along with the virus. When galectin-1 is knocked down, EV71 exhibits low infectivity in cells and less pathogenicity in mice, and galectin-1-free EV71 virus is sensitive to high temperature and loses its viability after long-term storage (Lee *et al.* 2015). Cell surface vimentin is an attachment receptor for EV71, and vimentin expressed on the cell surface binds to VP1 via its N-terminal to promote EV71 infection (Fig. 3) (Du *et al.* 2014). During EV71 infection, EV71 VP1 protein activates calmodulin-dependent protein kinase II, which phosphorylates the N-terminal domain of vimentin on serine 82. Vimentin phosphorylation and rearrangement may enhance EV71 replication by playing structural roles in the formation of the replication factories (Cong *et al.* 2013). In addition, human annexin II protein can bind to EV71 VP1 via VP1 amino acids 40–100, a region different from the known receptor binding domain, thereby enhancing EV71 replication (Fig. 3) (Yang *et al.* 2011). *miR-2911*, a honeysuckle-encoded atypical microRNA, can directly inhibit EV71 replication by targeting the *VP1* gene (Fig. 3) (Li *et al.* 2018a). The mutation K215A in the VP1 GH loop results in a significant increase in thermal stability, indicating that conditional thermostable mutants can be generated by altering the charge characteristics of VP1 (Yuan *et al.* 2015). In addition, studies have found that cattle and human lactoferrin (LF) can effectively inhibit EV71 infection through binding to VP1 and that LF inhibits EV71-induced interleukin (IL)-6 production and protects

mice against lethal EV71 challenge (Fig. 3) (Weng *et al.* 2005; Wu *et al.* 2010a).

VP1 is the main virus-neutralizing determinant, directly determining the antigenicity of the virus, and contains the main antigen-binding site (Huang *et al.* 2008). The N-terminal of VP1 capsid protein possesses an important antigen region, which is highly immunogenic. The peptides (amino acids 66–77 or 208–222) of the C-terminal of VP1 capsid protein may stimulate the production of neutralizing antibodies. In addition, three regions on the VP1 protein (amino acids 66–77, 145–159, and 247–261) are capable of inducing human EV-71-specific $CD4^+$ T-cell proliferation (Foo *et al.* 2008). The full-length VP1 is capable of self-association and forms a dimerization structure to improve the pathogenicity of the virus and the ability to adapt to the external environment. VP1 (amino acids 66–132) contains the major dimerization domain, and VP1 (amino acids 132–297) contributes largely to increasing the strength of the interaction (Lal *et al.* 2006).

VP1, a surface antigen, is a suitable candidate for EV71 vaccines. Two peptides, SP55 (amino acids 163–177) and SP70 (amino acids 208–222) are capable of eliciting neutralizing antibodies against EV71. Immunization of mice with either SP55 or SP70 triggers an EV71-specific IgG response as high as that obtained with the whole virion as immunogen; thus, SP70 represents a promising candidate for an effective synthetic peptide-based vaccine against EV71 (Foo *et al.* 2007). Oral immunization with recombinant VP1 protein (rVP1) induces VP1-specific IgA antibodies, serum-specific IgG, and neutralization antibodies in mice and may be a promising subunit vaccine candidate for preventing EV71 infection (Zhang *et al.* 2014). Immunization of hamsters with an EV71 VP1 fragment (NPt-VP11-100) protein can induce good immune responses, but the high level of antibodies fails to neutralize EV71 viruses or protect vaccinated hamsters in viral challenge studies (Ch'ng *et al.* 2012).

VP2–VP4 Proteins

The EV71 viral particle capsid composition complex and three structural proteins VP1, VP2, and VP3, which are exposed on the surface of the shell with no homology of nucleotide sequences among them, show certain similarities of the protein topology structure. The VP4 package is embedded in the inside of the virus shell, is closely connected with the virus core, and exhibits an extended spatial conformation feature, which is a bridge connecting the inside and outside (Chen et al. 2010; Rowlands *et al.* 2010). When the virus binds to the receptors, the spatial configuration will change, and the VP4 is lost. Eventually, the viral shell is removed, the viral genomic RNA is released into the cytoplasm, and the translation of the viral polymeric protein begins with the viral genomic RNA as mRNA. The N-terminal myristoylation signal (MGXXXS) of VP4 plays an important role in EV71 replication, and different myristic acid analogs elicit differential effects on EV71 replication *in vitro*, suggesting that removal of the myristate moiety in the viral structural protein precursor can be an effective antiviral target for further research (Tan *et al.* 2016). EV71 virus capsid proteins VP2 and VP3, which are important parts of the shell protein, are associated with the antigenicity of the virus. Thus, VP2 and VP3 may be potential candidates with structures similar to that of VP1, and VP2 (amino acids 142–146) contains a single, linear, non-neutralizing epitope, which is located in the E–F loop of the VP2 protein (Chen *et al.* 2010; Kiener *et al.* 2012). VP2 149 M mutation enhances viral binding and RNA accumulation of EV71, which promotes EV71 infectivity *in vitro* and mouse lethality *in vivo* (Huang *et al.* 2012). T cells play an important role in the host immune response against EV71 infection. Compared with the other three capsid proteins, VP2 shows a more extensive distribution and immunogenicity in T cells (Tan *et al.* 2013). The VP2-28 epitope containing residues 136–150 of VP2 was identified as another neutralizing epitope. Xu *et al.* (2015) constructed a bivalent chimeric virus-like particle (VLP) presenting the VP1 (amino acids 208–222) and VP2 (amino acids 141–155) epitopes of EV71 that could induce higher IgG titers and neutralization titers and protect neonatal mice against lethal EV71 and CA16 infections. Moreover, they found that anti-VP2 (amino acids 141–155), but not anti-VP1 (amino acids 208–222), could crossreact with normal EV71 and CA16 virions (Xu *et al.* 2015). Kiener *et al.* (2014) found that the knob of EV71 VP3 encompassing residues 55–69 of VP3 is a new conformational epitope of EV71 involved in EV71 virus neutralization. The importance of this novel neutralization epitope lies in the optimization of putative EV71 vaccines because the VP3 knob can be combined with VP1 to form a bivalent subunit vaccine (Kiener *et al.* 2014).

2A Protein

The EV71 2A protease exhibits cysteine protease activity and contains 150 amino acid residues; it is an enzyme that cleaves at its own N-terminus at the junction between VP1 and 2A of the polyprotein. A polyprotein, translated from the single ORF of EV71, is processed into mature proteins by 2A (Hellen *et al.* 1992; Hsu *et al.* 2007; Ventoso and Carrasco 2003). Eukaryotic translation initiation factor 4G (eIF4G) induces the synthesis of the host cell proteins by promoting the interaction between mRNA and the 40S ribosomal subunit. $2A^{pro}$ inhibits host cap-dependent protein synthesis by cleaving the elongation factor eIF4G and promoting EV71 replication (Fig. 3) (Morley *et al.* 1997).

2A can block typical stress granule formation and induce atypical stress granule formation by cleaving eIF4GI to sequester cellular mRNA but release viral mRNA, thereby facilitating viral translation (Yang *et al.* 2018). In addition, the 2A protease of EV71 shows great transcription activity in yeast, which is independent of its protease activity. EV71 2A protease retains its transcriptional activity after truncation of 40 amino acids at the N-terminus but loses this activity after truncation of 60 amino acids at the N-terminus or deletion of 20 amino acids at the C-terminus. The acidic structure domain at the C terminal is necessary for its transcriptional activity, and deletion of amino acids 146–149 (EAME) in this acidic domain causes the loss of transcriptional activity (Yang *et al.* 2010).

As shown in Fig. 3, 2A is capable of cleaving FBP1 at the Gly-371 residue of FBP1, which generates a functional cleavage product, $FBP1^{1-371}$. $FBP1^{1-371}$ can bind to the 5′ UTR linker region, which is different from full-length FBP1. Moreover, FBP1 and $FBP1^{1-371}$ act additively to promote IRES-mediated translation and enhance EV71 replication (Hung *et al.* 2016). 2A significantly inhibits cellular endoplasmic reticulum-associated degradation (ERAD) by inhibiting the transcription of the *de novo* synthesis of key molecules Herp and VIMP. p97, a host factor that is distributed and co-exists with the viral protein and EV71 replication-related molecules, is hijacked from cellular ERAD by EV71 to promote viral replication (Wang *et al.* 2017b).

Type I IFNs (IFN-Is) are key players in the innate antiviral response against viral infections. However, IFN therapy does not significantly affect EV71 infection, and induction of downstream IFN-stimulated genes is inhibited by EV71. The 2A protease can reduce IFN-I receptor protein 1 levels and suppress interferon regulatory factor 3 (IRF3) signaling by cleaving mitochondrial antiviral protein and retinoid acid-inducible gene I (RIG-I)-like receptor MDA5, thus inhibiting IFN signaling pathways and causing the virus to escape the immune response (Fig. 3) (Kuo *et al.* 2013; Lu *et al.* 2012; Wang *et al.* 2013). In addition, 2A attenuates IFN-γ signaling using another mechanism by reducing the serine phosphorylation of signal transducer and activator of transcription 1 (STAT1) following the inactivation of extracellular signal-regulated kinase without affecting STAT1 expression (Fig. 3) (Wang *et al.* 2015b). 2A protein can also induce apoptotic cell death (Kuo *et al.* 2002a). EV71 infection induces the production of inflammasomes, whereas inflammasomes inhibit the replication of EV71. 2A protein can block the production of inflammasomes by eliminating NLRP3 protein at the G493-L494 or Q225-G226 junction (Fig. 3) (Wang *et al.* 2015a).

2B Protein

The EV71 2B protein, a small hydrophobic ion channel protein with 99 amino acid residues, may mediate a chloride-dependent rather than calcium-dependent current in oocytes; furthermore, DIDS, an inhibitor of this current, can significantly inhibit the replication of EV71 (Xie *et al.* 2011). Currently, little is known about the function of EV71 2B proteins. We speculate that they may have the same function as other enteroviruses. The expression of protein 2B from polioviruses shows relatively high similarity, with two transmembrane domain (TM1 and TM2) structures that are essential for the function of viroporins, such as damaging organelle membrane integrity, improving the permeability function, promoting viral RNA replication, and releasing viral particles from the host cell (Agirre *et al.* 2002; De Jong et al. 2003; Johnson and Sarnow 1991; Madan *et al.* 2007). The C-terminal region of 2B (amino acids 63–80) is reported to be responsible for the location of 2B in the mitochondria, and 2B induces cell apoptosis by interacting directly with and recruiting the proapoptotic protein Bax and inducing Bax conformational activation (Fig. 4). A hydrophilic region of 14 amino acids in the N-terminal region of 2B is important for Bax interaction and subsequent activation. Moreover, overexpression of the anti-apoptotic protein Bcl-XL abrogates 2B-induced release of cytochrome *c* and caspase activation (Cong *et al.* 2016). Although little is known about the function of 2B protein, this protein may be a potential target for anti-EV71 drug development.

2C Protein

The 2C protein of EV71 is one of the most highly conserved nonstructural proteins, containing 329 amino acid residues. 2C harbors an adenosine triphosphatase (ATPase) domain, a zinc finger structure, and an alpha helix at the end of the C-terminal region (Guan *et al.* 2017). The $2C^{ATPase}$, an RNA helicase that 3′-to-5′ unwinds RNA helices in an ATP-dependent manner and an RNA chaperone independently of ATP, facilitates EV71 RNA synthesis *in vitro*. $2C^{ATPase}$-mediated RNA remodeling plays a critical role in the EV71 life cycle (Xia *et al.* 2015). The N terminus of the 2C protein, which exhibits both RNA- and membrane-binding activity, interacts with reticulon 3 (RNT3) via its highly conserved reticulon homology domain and then combines with double-stranded RNA (dsRNA) viruses to form viral replication complex and participate in viral replication (Fig. 4). Reduced production of RNT3 by RNA interference markedly reduces the synthesis of EV71-encoded viral proteins and replicative dsRNA, reducing plaque formation and apoptosis (Tang

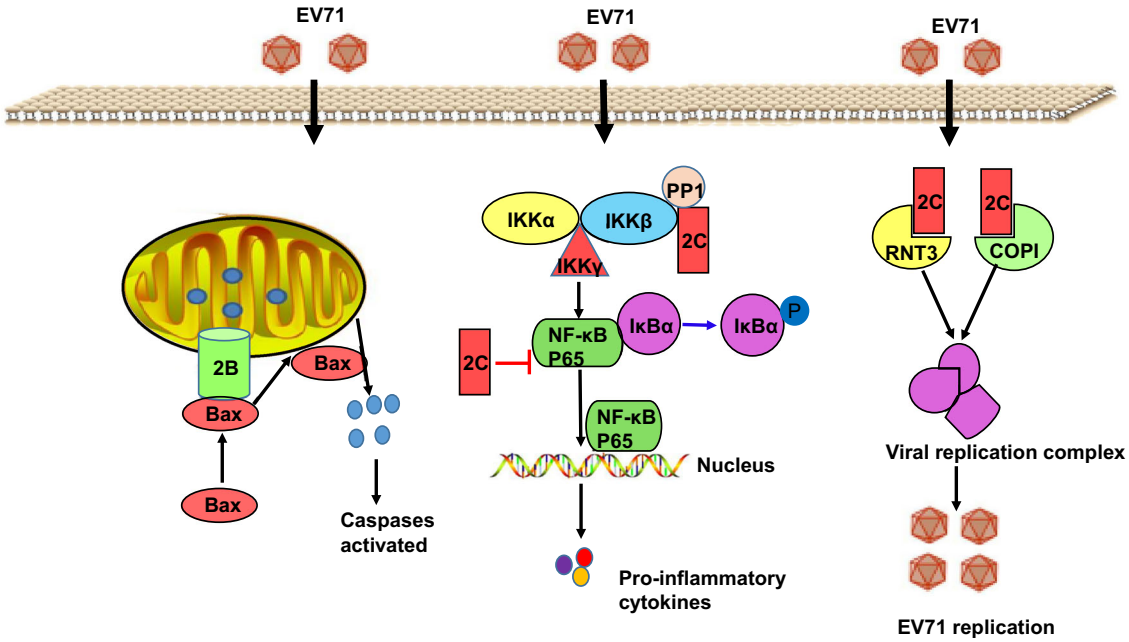

Fig. 4 The protein functions of 2B and 2C.

et al. 2007). 2C proteins can control the activity of nucleoside triphosphate and participate in the synthesis of negative-strand RNA and the capsid formation of PV subgeneration viral particles (Wu *et al.* 2010a). By interaction with the IPT domain (amino acids 194–290) of p65, 2C can reduce the formation of the heterodimer p65/p50 and then inhibit nuclear factor (NF)-κB activation (Fig. 4) (Du *et al.* 2015). The N-terminal of 2C (amino acids 1–125) interacts with all isoforms of the protein phosphatase 1 (PP1) catalytic subunit through PP1-docking motifs, which is efficient for EV71 2C-mediated inhibition of IKKβ phosphorylation and NF-κB activation (Fig. 4). Moreover, 2C forms a complex with PP1 and IKKβ to dephosphorylate IKKβ activation (Li *et al.* 2016; Zheng *et al.* 2011). Coat protein complex I (COPI) may be directed to the viral replication complex through viral 2C protein to enhance EV71 infection, whereas the inhibition of COPI activity can weaken the replication of EV71 (Fig. 4) (Wang *et al.* 2012a).

3A Protein

The EV71 3A protein, containing 86 amino acid residues, is a membrane binding protein that regulates intracellular transport of host cells. During RNA replication, the 3A protein promotes the combination of capsule membrane and replication complex and viral RNA synthesis through its hydrophobic zone (Fujita *et al.* 2007; Giachetti *et al.* 1992). The 3A protein expressed in mammalian cells can lead to cleavage of the endoplasmic reticulum and the transport dysfunction of the Golgi complex protein. It may be closely related to the inhibition of host antiviral immune function and can inhibit the expression of cytokines, such as IL-1β, IL-6, and IL-8; furthermore, 3A can also reduce the expression of major histocompatibility complex I and tumor necrosis factor (TNF) receptor in the infected cells (Choe and Dodd 2005). EV71 3AB displays RNA chaperone activity. Moreover, 3B and the last seven amino acids at the C-terminal of 3A (termed 3B + 7) possess RNA chaperone activity, and five amino acids, i.e., Lys-80, Phe-82, Phe-85, Tyr-89, and Arg-103, are critical and probably the active sites of 3AB for its RNA chaperone activity (Tang *et al.* 2014). The Golgi resident protein acyl-coenzyme A binding domain-containing 3 (ACBD3) promotes EV71 replication by interacting with the 3A protein (Fig. 5) (Lei *et al.* 2017). EV71 infection induces the production of phosphatidylinositol-4-phosphate, and ACBD3 is required for the recruitment of phosphatidylinositol-4-kinase IIIβ (PI4 KB) to the viral RNA replication site for EV71 replication (Fig. 5). 3A stimulates the interaction of PI4 KB and ACBD3, and three proteins are all located at the viral RNA replication site to form a complex containing 3D protein to facilitate EV71 replication. However, I44A or H54Y mutation in the 3A protein can block its interaction with PI4 KB and ACBD3 (Xiao *et al.* 2017). Qiu *et al.* (2017) found that 3A protein, as a viral suppressor of RNAi (VSR), effectively inhibits the production of virally derived siRNAs and antiviral RNAi immunity *in vitro* and *in vivo*.

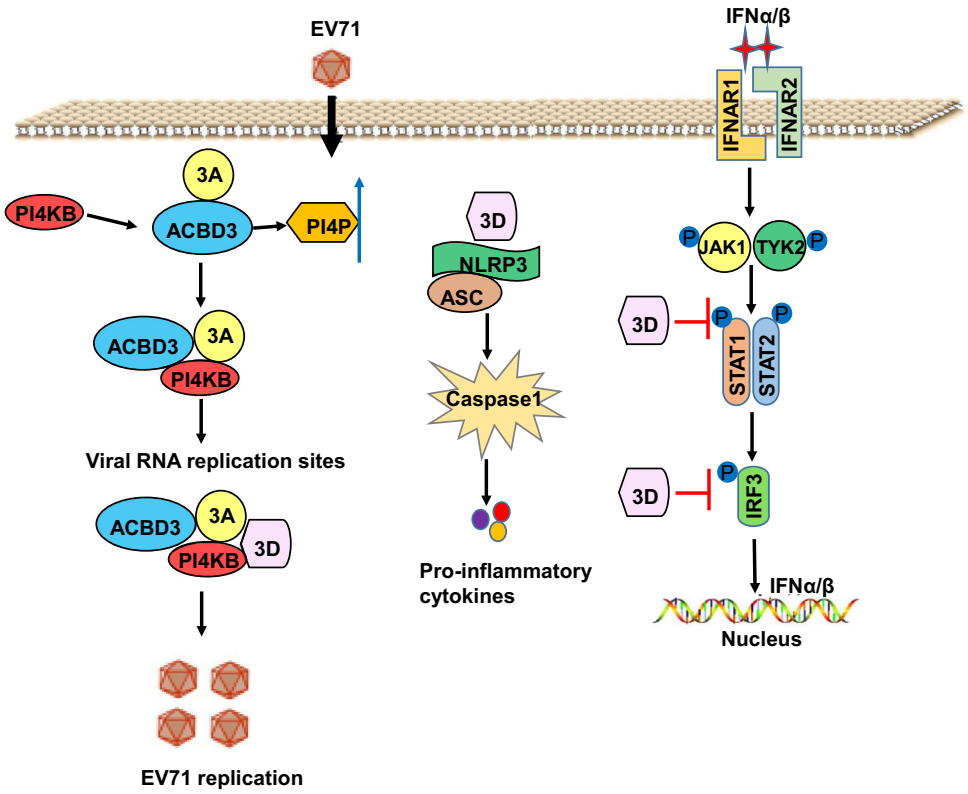

Fig. 5 The protein functions of 3A and 3D.

3B Protein

The 3B protein, also known as a VPg protein, is a small protein that contains 22 amino acid residues. The VPg protein forms phosphodiester bonds with pUpU at the 5′ UTR of the EV71 genome through the hydroxyl groups of the Try residues and then uses vpg-pUpU as the primer to participate in the synthesis of the negative chain and plus strand RNA (Herrero *et al.* 2003; Liu *et al.* 2007; McMinn 2002). VPg protein can interact with the polymerase 3D, and VPg is catalyzed by 3D polymerase to uridine acidification, which is a primer for viral RNA synthesis (Paul *et al.* 2003).

3C Protein

The 3C protein of EV71 contains 183 amino acids and shows serine protease and cysteine protease activities. During the replication of EV71, 3C proteins catalyze the lysis of virus precursor proteins and form mature structural proteins and nonstructural proteins (Cui *et al.* 2011). The catalytic activity site of 3C protease comprises His40, Glu71, and Cys147 (Qiu 2008). Studies have shown that 3C protease has many functions, such as promoting viral replication and enhancing apoptosis in host cells (Lee *et al.* 2008; Li *et al.* 2002). 3C protease can enter the nucleus through its precursor 3 CD′ and 3 CD, and its nuclear localization signal is located in the 126–129 sequence "KKKRD". The 3C protease enters the nucleus in its precursor form, shears itself to form the 3C protease, and shears transcription factors in the nucleus, such as TATA-box binding protein, the transcription factor p53, histone H3, transcription factor C, and the pre-mRNA cleavage stimulation factor (CstF-64). 3C inhibits host gene expression by regulating host pre-mRNA processing and polyadenylation, resulting in a decline in transcription in the host cell (Lin *et al.* 2009a, b, c; Weng *et al.* 2009; Sharma *et al.* 2004). 3C protease exhibits RNA binding activity, among which the "KFRDI" (amino acids 82–86) and "VGK" (amino acids 154–156) sequences are the binding sequences for RNA (Shih *et al.* 2004). Changes in the sequence of the RNA binding region of 3C can affect the activity of 3C protease, whereas mutations in the catalytic position will not change the RNA binding capacity of 3C protein (Shih *et al.* 2004). Apoptosis may be an important host defense mechanism, whereby virus-infected cells are eliminated, thus preventing the generation and spread of viral progeny during viral infection. A study showed that poly (ADP-ribose) polymerase, a DNA repair enzyme, is cleaved by 3C protease, which then activates caspase and induces apoptosis, and is closely related to central nervous system diseases (Fig. 6) (Kuo *et al.* 2002b).

EV71 3C protease plays an important role in viral replication, and sequence analysis showed that no

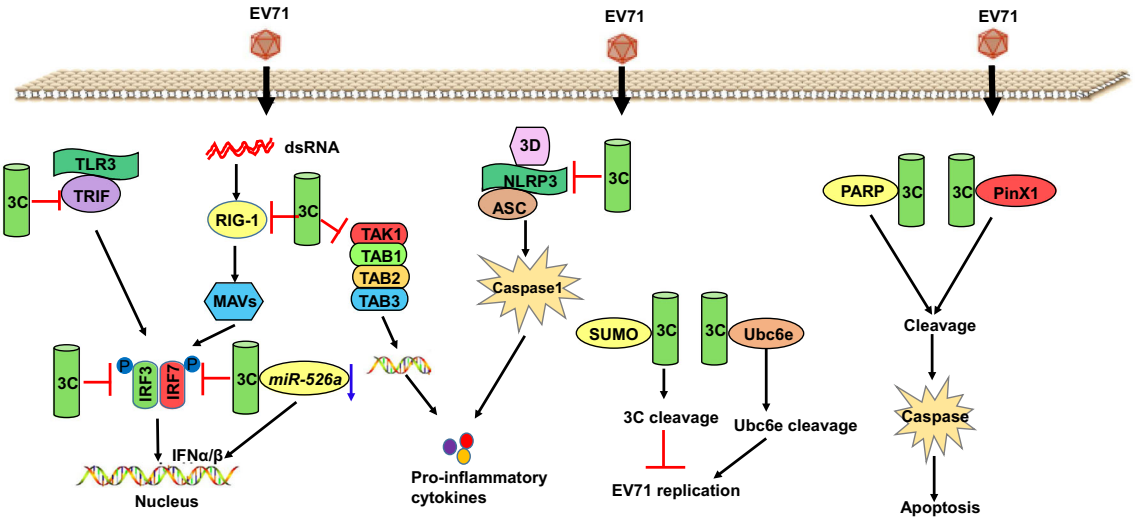

Fig. 6 The protein functions of 3C.

homologous sequence of EV71 3C is present in mammals; therefore, the 3C protein may be a potential target for antiviral drugs (Kuo *et al.* 2008). 3C can bind to the SUMO E2 conjugating enzyme Ubc9 after binding of the K52 amino acid and SUMO E2 ligase and be SUMO modified at residue K52 for degradation, correlating with a decrease in EV71 in virus replication and cell apoptosis (Fig. 6) (Chen *et al.* 2011). Overexpression of the telomere binding protein PinX1 can inhibit the apoptosis induced by EV71 infection, whereas the 3C protein interacts with PinX1 to degrade PinX1, which can promote host cell apoptosis (Fig. 6) (Li *et al.* 2017). Ubc6e, an E2 ubiquitin-conjugating enzyme, plays a key role in EV71-dependent ERAD disruption, and EV71 3C cleaves Ubc6e at Q219G, Q260S, and Q273G, which can inhibit ERAD to promote EV71 replication (Fig. 6) (Wang *et al.* 2017b). EV71 induces the production of inflammatory cytokines, and 3C interacts with transforming growth factor-β activated kinase 1 (TAK1) and the TAK1 binding protein 1 (TAB 1), which inhibits NF-κB activation. Furthermore, 3C mediates the cleavage of TAK1/TAB 1/TAB 2/TAB 3 complexes to interfere with inflammatory responses (Fig. 6) (Lei *et al.* 2014).

EV71 inhibits antiviral immunity by inhibiting RIG-I to downregulate IFN-β, IFN-stimulated gene 54 (ISG54), ISG56, and TNF in virus-infected cells. The 3C protein can inhibit IFN-β activation by virus and RIG-I but does not inhibit MDA5. 3C is associated with RIG-I via the caspase recruitment domain, which prevents the recruitment of an adaptor IPS-1 and subsequent nuclear translocation of IRF3 (Fig. 6) (Lei *et al.* 2010). In addition, *miR-526a* positively regulates virus-triggered IFN-I production, thus suppressing viral replication. 3C can inhibit interferon production by downregulating *miR-526a* or inhibiting interferon regulation factor 7 to block the RIG-I signaling pathway (Lei *et al.* 2013; Xu *et al.* 2014). Toll-like receptor (TLR)-related pathways play an important role in antiviral immune responses. TLR3 in the endosome recognizes viral dsRNA and recruits a TIR domain-containing adaptor inducing IFN-β (TRIF) to transmit signals to IRF3 and NF-κB. 3C is capable of cleaving TRIF and impairing type I IFN production in response to TLR3 activation (Fig. 6) (Lei *et al.* 2011). EV71 infection induces the production of inflammasomes, and the NLRP3 inflammasome plays a protective role against EV71 infection. 3C protein can block the production of the inflammasome by eliminating NLRP3 protein (Fig. 6) (Wang *et al.* 2015a).

3D Protein

The EV71 3D protein, containing 462 amino acid residues, is an RdRp, which mainly completes the extension of the RNA chain during viral replication. The 3D polymerase is indispensable for the initiation of viral replication and plays an important role in changing viral virulence. When 3D is cleaved from 3CD, the nucleic acid location signal of the 3D protein transmits 3CD to the host cell nucleus and shuts down the transcription of the host cell (Wang *et al.* 2010; Wu *et al.* 2010b). The EV71 3D protein is a polymerase dependent on Mn^{2+}, which is completely inactive in the presence of Mg^{2+}. Studies of EV71 transcription activity *in vitro* have shown that 3D can use dinucleotide and 10-nucleotide RNA as a primer and initiates transcription using genomic RNA as a template (Jiang *et al.* 2011). In addition, the EV71 3D protein attenuates STAT1 tyrosine phosphorylation independent of Janus kinase 2 inactivation, without interfering with IFN-γ receptor expression. Then, 3D blocks IRF1 activation and

antagonizes the antiviral activity of IFN-γ (Fig. 5) (Agirre *et al.* 2002). EV71 induces the production of IL-1β through activation of the NLRP3 inflammasome. 3D interacts with NLRP3 to facilitate the assembly of the inflammasome complex by forming a 3D-NLRP3-ASC ring-like structure and then stimulates activation of the NLRP3 inflammasome and the cleavage of pro-caspsase-1, which causes the release of IL-1β (Fig. 5) (Wang *et al.* 2017c).

Summary

In summary, different viral proteins of EV71 exert various functions to guarantee the replication of the virus itself. Although some progress has been made in research on the function of the EV71 genome, further studies are still needed. For example, the function of the 3′ UTR is relatively unclear. Notably, however, EV71 infection induces the host immune response, resulting in the expression of many host factors that inhibit EV71 replication through different mechanisms. For example, the promyelocytic leukemia protein contributes to cellular antiviral effects by inhibiting autophagy (Chen *et al.* 2018), and A3G competitively binds to the 5′ UTR to inhibit the 5′ UTR activity of EV71 and the synthesis of EV71 viral proteins and RNA (Li *et al.* 2018b). In the future, researchers will need to focus not only on further structural and functional studies of EV71 proteins but also on how viral virulence factors interact with the human immune system, which will have a profound impact on the development of vaccines and drugs. Such studies will improve our comprehensive understanding of EV71 genome structure and function. This will also help us elucidate the pathogenesis of EV71, which will provide insights into the design of therapeutic strategies against EV71 infection.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement The authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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