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REVIEW

SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

Picornavirus 3C – a protease ensuring virus replication and subverting host responses

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ABSTRACT

The protease 3C is encoded by all known picornaviruses, and the structural features related to its protease and RNA-binding activities are conserved; these contribute to the cleavage of viral polyproteins and the assembly of the viral RNA replication complex during virus replication. Furthermore, 3C performs functions in the host cell through its interaction with host proteins. For instance, 3C has been shown to selectively 'hijack' host factors involved in gene expression, promoting picornavirus replication, and to inactivate key factors in innate immunity signaling pathways, inhibiting the production of interferon and inflammatory cytokines. Importantly, 3C maintains virus infection by subtly subverting host cell death and modifying critical molecules in host organelles. This Review focuses on the molecular mechanisms through which 3C mediates physiological processes involved in virus–host interaction, thus highlighting the picornavirus-mediated pathogenesis caused by 3C.

KEY WORDS: 3C protease, Picornavirus, Virus replication, Innate immunity, Pathogenesis

Introduction

Picornaviruses are one of the major virus groups that infect multiple organs in humans and animals, including the gastrointestinal tract, upper respiratory tract, central nervous system, heart, liver and skin (Tapparel et al., 2013). The family *Picornaviridae* comprises 147 species that are divided into 63 genera (as of March 2020), such as *Aphthovirus*, *Cardiovirus*, *Enterovirus* and *Hepatovirus* (Zell, 2018).

Both the virion and genome structures of picornaviruses are highly conserved. Externally, the virus has an icosahedral symmetrical spherical structure with an approximate diameter of 30 nm, which is assembled into a non-enveloped virion (Rossmann et al., 1985; Hogle et al., 1985). Internally, the viral genome consists of a single-stranded positive RNA that ranges from 6.7 kb to 10.1 kb in length, including a 5′ untranslated region (5′ UTR), an open reading frame (ORF), and a 3′ untranslated region (3′ UTR) containing a polyadenylated [poly(A)] tail (Wimmer et al., 1993) (Fig. 1). A viral genome-linked protein (VPg, also known as 3B) that participates in the production of viral RNA synthesis primers is covalently linked to the 5′ end of the positive-strand RNA. An internal ribosome entry site (IRES) within the 5′ UTR is used to recruit ribosomes and other host factors and directly initiate virus translation. The ORF initially encodes a single polyprotein. During co- and post-translation processes, the single polyprotein is cleaved into capsid proteins and nonstructural proteins, including 1A to 1D (VP4, VP2, VP3 and VP1), 2A, 2B, 2C, 3A to 3D, and some intermediates (e.g. 3ABCD, 3ABC, 3BCD and 3CD) (Fig. 1). Viruses of some genera (e.g. *Aphthovirus* and *Cardiovirus*) have a leader (L) protein attached to the N-terminus of the polyprotein (Jiang et al., 2014) (Fig. 1). This Review mainly focuses on the protease 3C.

In 1968, proteolysis was shown to be a prerequisite for the formation of picornavirus capsid proteins (VP0, VP1 and VP3) and for the assembly of virions (Jacobson and Baltimore, 1968; Holland and Kiehn, 1968). This activity was assigned to 3C (Palmenberg et al., 1979), which contains a trypsin catalytic triad typical of trypsin-like family of serine proteases, but the catalytic nucleophile is a cysteine (Cys) residue and not a serine (Ser) (Bazan and Fletterick, 1988). Subsequently, 3C was shown to be an RNA-binding protein (Andino et al., 1993). Based on these particular activities, 3C serves as a common virulence factor of picornaviruses that not only acts on the virus, but also on host proteins to ensure virus viability (Sun et al., 2016). Recently, the detailed roles of 3C during picornavirus infection have been uncovered in several viruses, including enterovirus (EV), coxsackievirus (CV) and poliovirus (PV) (see Table S1), foot and mouth disease virus (FMDV) (see Table S2), encephalomyocarditis virus (EMCV) (see Table S3), senecavirus (SVV) (see Table S4); hepatitis A virus (HAV) (see Table S5) and human rhinovirus (HRV) (see Table S6). This Review aims to summarize the general structural features and functions of picornaviral protease 3C. We will compare and contrast the strategies used by different picornavirus 3C proteases to modulate virus–host interactions.

Structural features of picornaviral 3C

Following the first report of the HAV 3C structure (Matthews et al., 1994), crystal structures of 3C proteases from HRV (Bergmann et al., 1997), PV (Mosimann et al., 1997), FMDV (Birtley et al., 2005), CVB (Lee et al., 2009; Ohlenschläger et al., 2004) and EV71 (Cui et al., 2011) were reported in succession. 3C has a classical trypsin-like fold structure (Bazan and Fletterick, 1988) consisting of six β-sheets, which are folded into two β-barrel domains that are packed perpendicularly to each other. The surface groove between two β-barrel domains constitutes the substrate-binding capsule, with a centrally-located cysteine-histidine (His)-aspartic acid (Asp) or glutamine (Glu) catalytic triad in the active site. The third residue varies depending on the species; for instance, in HRV (Matthews et al., 1994), PV (Mosimann et al., 1997), CVB (Lee et al., 2009; Ohlenschläger et al., 2004) and EV71 (Cui et al., 2011), it is glutamine, but in HAV and FMDV, it is aspartic acid (Bergmann et al., 1997; Birtley et al., 2005). The strict substrate-specificity of 3C is determined by the groove, as the β-ribbon located above it controls the shape and depth of the active site. In the absence of substrate, the β-barrel adapts a flexible conformation to increase the chances of substrate recognition. Once the substrates are bound, the β-ribbons and substrates tightly associate, which increases the efficiency of catalysis (Sweeney et al., 2007; Cui et al., 2011).

Apart from the structural feature forming the active site, there are conserved regions in 3C that have RNA-binding activity, such as the

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Journal of Cell Science (2021) 134, jcs253237. doi:10.1242/jcs.253237

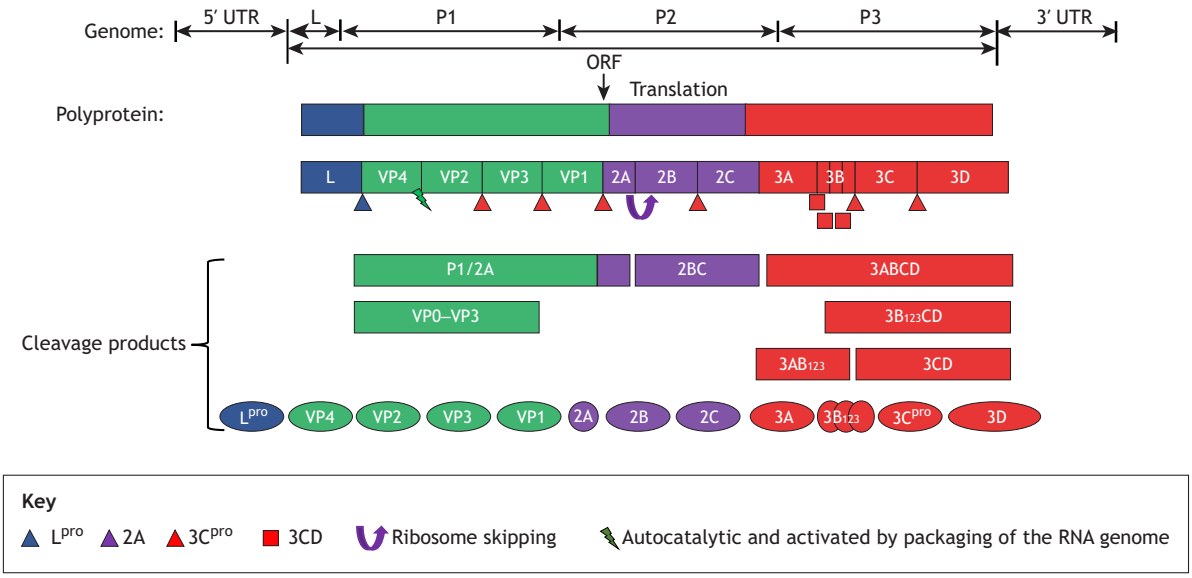

Fig. 1. Schematic illustration of *Aphthovirus* genome organization and polyprotein processing. The picornavirus genome includes a 5′ untranslated region (5′ UTR), an open reading frame (ORF) and a 3′ untranslated region (3′ UTR). In *Aphthovirus*, the ORF is divided into leader protein (L) (also in *Cardiovirus*), precursor 1 (P1), P2 and P3. The ORF initially encodes a single polyprotein. L, 2A 3C, 3CD, ribosome skipping and autocatalysis and activation by packaging of the RNA genome mediates the processing of the polyprotein, which is cleaved into capsid proteins and nonstructural proteins, including L, 1A to 1D (VP4, VP2, VP3 and VP1), 2A, 2B, 2C, and 3A to 3D (ovals), as well as some intermediate proteins (rectangles). Processing of polyproteins of different genera of the picornavirus family mainly differs in the L-P1, P1/2A-2B and VP1-2A sites (Sun et al., 2016).

KFRDI motif and the VGK motif (Bergmann et al., 1997; Ohlenschläger et al., 2004; Nayak et al., 2006). The KFRDI motif is located opposite to the protease active sites, while the VGK motif lies in the loop. Both regions have been verified to bind to the secondary RNA structures of viral genomes, indicating that 3C might preferentially bind to a conformation motif on the viral RNA rather than a sequence.

Moreover, a study has revealed that a highly dynamic α helix on PV 3C binds to phosphatidylinositol phosphate (PIP) on the cell membrane; PIP binding is competitive with RNA binding as there is an overlap between the PIP- and RNA-binding sites (Shengjuler et al., 2017). Thus, picornaviruses could utilize phosphoinositide lipids to regulate viral RNA replication (Hsu et al., 2010).

Further explorations of the structural features of 3C and their functional relevance might help to better explain the roles of 3C in the viral replication cycle.

Roles of 3C in picornavirus replication

3C has essential roles in the cleavage of the viral polyprotein and in the promotion of the RNA replication complex assembly during viral replication (Fig. 1; Box 1). Moreover, 3C also cleaves host cell proteins, which is indispensable for picornavirus replication (Fig. 2).

3C drives polyprotein maturation and RNA replication complex assembly

After the entry of a picornavirus, the viral genome is released in the cytoplasm, where it attaches to a cellular ribosome. A polyprotein, encoded by the ORF is initially translated. However, the release of mature structural and nonstructural viral proteins requires co- and post-translation steps that are performed by viral proteases (L, 2A, 3C and 3CD), ribosome skipping and autocatalysis (Fig. 1). Although different genera of *Picornaviridae* have developed various strategies for this, 3C participates in most of the polyprotein cleavage processes (Sun et al., 2016) (Fig. 1).

During the processing of the polyprotein, precursors are key factors for the formation of the RNA replication complex (Spear et al., 2015) (Fig. 2; Box 1). They interact with the virus RNA replication elements to cyclize the virus RNA and trigger the assembly of the viral RNA replication complex (Fig. 2, Box 1). This

Box 1. Roles of 3C and 3CD in picornavirus RNA replication

The synthesis of new picornavirus RNA requires RNA-replication elements, including the 5′ UTR, cis-acting replication element (cre) and 3′ UTR. Several studies have revealed the initiation model of PV RNA synthesis (Andino et al., 1993; Parsley et al., 1997; Herold and Andino, 2001; Paul and Wimmer, 2015) (Fig. 2). 3CD binds to the stem-loop d of cloverleaf on the 5′ UTR (Ohlenschläger et al., 2004), which increases the binding of poly(rC)-binding protein 2 (PCBP2) to the stem-loop b (Parsley et al., 1997; Gamarnik and Andino, 2000). The 3CD–cloverleaf complex then recruits 3AB (Harris et al., 1994; Xiang et al., 1995). 3AB or VPg stimulates the cleavage of 3CD to release free 3D with polymerase activity (Molla et al., 1994), leading to the uridylylation of the peptide primer VPg. During this process, 3CD directly interacts with cre, converting cre to the template for VPg uridylylation (Yang et al., 2004). In addition, 3AB-3CD binds to the 3′ UTR (Harris et al., 1994), and PABP cleaved by 3CD binds to the poly(A) tail (Roehl et al., 1997), thus linking both ends of the viral genome to form a circular RNA replication complex (Herold and Andino, 2001), initiating synthesis of a new RNA strand.

Similarly, HRV-14 3C binds to the stem-loop d of the cloverleaf (Leong et al., 1993; Walker et al., 1995), and 3C or 3CD interacts with cre directly during uridylylation of VPg (Shen et al., 2007; Yang et al., 2004). HAV 3C, 3CD, and 3ABC are able to interact with the 5′ UTR and 3′ UTR (Kusov and Gauss-Müller, 1997; Kusov et al., 1997). The binding capacity of 3ABC has been shown to be 50-fold stronger than that of 3AB and 3C, but the RNA-binding ability of 3CD is weaker than that of 3C. FMDV 3C can substitute for 3CD to stimulate VPg uridylylation but with lower efficiency. $3B_3C$ and $3B_{123}C$ can also function as substrates for uridylylation in the absence of 3C and 3CD (Nayak et al., 2006). In Aichi virus, both 3ABC and 3CD interact with the 5′ UTR, but the interaction of 3ABC with the 5′ UTR, rather than that of 3CD, is involved in new RNA strand synthesis (Nagashima et al., 2008).

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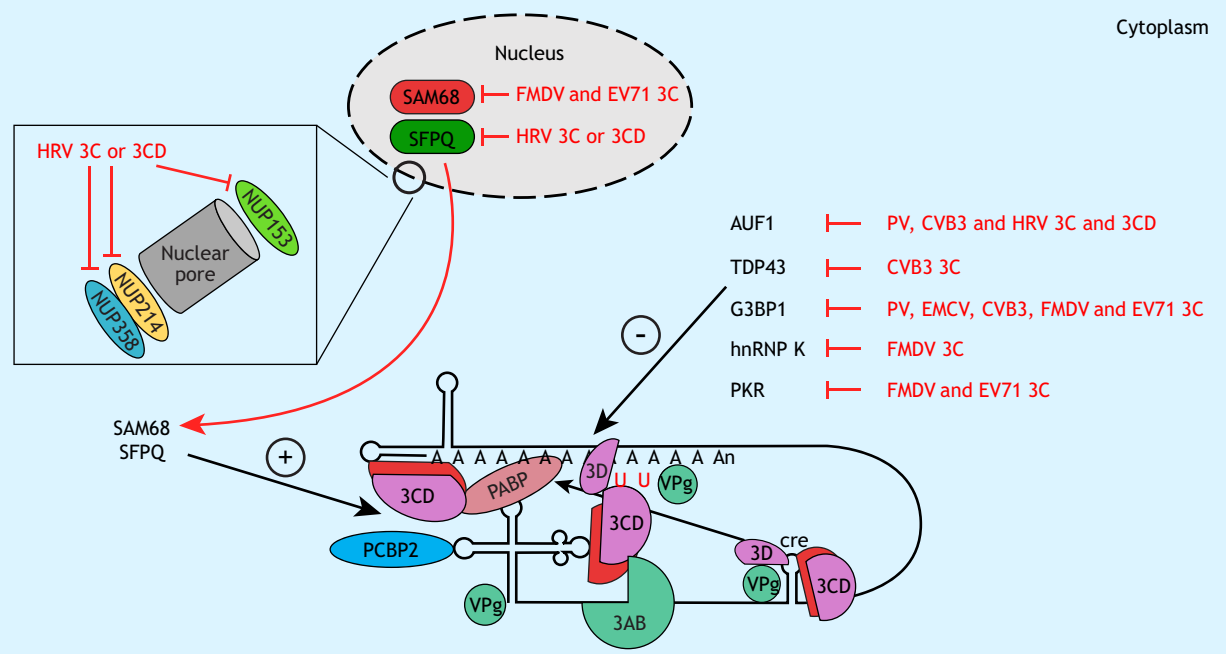

Fig. 2. 3C hijacks host factors to promote picornavirus RNA replication. 3C and host factors are essential for the picornavirus RNA replication (see Box 1). During picornavirus infection, 3C or 3CD degrades some nucleoporins (NUP153, NUP214 and NUP358) to disrupt nuclear transport (red inhibition bars), which may help picornavirus to take over the host-cell gene expression machinery. 3C or 3CD also cleaves SAM68 and SFPQ in the nucleus, which facilitates the export of SAM68 and SFPQ to the cytoplasm (red arrows), where the truncated fractions can interact with the picornavirus genome and promote viral RNA replication. In addition, 3C or 3CD cleaves or degrades several host factors that restrict infection in the cytoplasm to impair their antiviral functions (red inhibition bars).

is an energy-efficient mechanism that allows the virus to store enormous biological information while regulating viral replication. Further clarification of the interaction between 3C and its precursors, including its conformational regulation, will provide important insights into the replication of picornaviruses.

3C hijacks host factors to promote picornavirus replication

Picornavirus translation and RNA synthesis is achieved with the assistance of cellular proteins (Fig. 2), some of which are targeted by 3C as discussed below.

3C blocks the host transcription and translation machineries

It was been initially shown that infection of FMDV induces a new polypeptide called Pi in BHK cells (Grigera and Tisminetzky, 1984). Further *in vitro* cleavage assays have demonstrated that Pi is generated by 3C-mediated cleavage of histone H3 at its N-terminus and remains associated with chromatin (Grigera and Tisminetzky, 1984; Falk et al., 1990) (Table S2). Modification of the N-terminus of histones typically induces the decondensation of chromosomes and provides binding sites for transcription factors, thus promoting transcription (Kouzarides, 2007). Therefore, the cleavage of H3 into Pi might shut down host cell transcription. Subsequently, PV 3C has also been shown to cleave transcription factors (Table S1), including transcription factor IIIC (TFIIIC) (Clark et al., 1991; Shen et al., 1996), cAMP response element-binding protein-1 (CREB1) (Yalamanchili et al., 1997a), octamer binding protein-1 (OCT1; also known as POU2F1) (Yalamanchili et al., 1997b), p53 (TP53) (Weidman et al., 2001), TATA-binding protein-associated factor 110 (TAF110; also known as TAF1C) (Banerjee et al., 2005), and TATA box-binding protein (TBP) (Clark et al., 1993; Kundu et al., 2005), leading to inhibition of transcription initiation by RNA polymerases. In addition, cleavage stimulation factor subunit 2 (CSTF-64; also known as CSTF2), which is responsible for 3′-end pre-mRNA processing and polyadenylation, is also cleaved by EV71 3C (Weng et al., 2009) (Table S1).

Eukaryotic translation includes cap- and IRES-dependent translation (Jackson et al., 2010). Because picornaviruses also initiate IRES-dependent translation to produce viral proteins, which requires the cellular initiation factors, they have evolved a series of strategies that interfere with host translation. FMDV 3C cleaves the eukaryotic translation initiation factors (eIFs) eIF4A1 and eIF4G1 (Belsham et al., 2000; Li et al., 2001) (Table S2), while 3C of PV, CVB and HRV induce the cleavage of eIF5B (de Breyne et al., 2008). Interestingly, these cleavage events are also associated with the shutoff of viral translation. This may lead to an increase in the packaging of viral RNA into new virus particles instead of production of virus proteins in late infection.

3C mediates the conversion of the viral RNA template

Picornaviral RNA serves as a template for both translation and RNA replication. When viral RNA is being translated, it cannot be used as the template for RNA replication; the usage of the viral RNA template must be shifted from translation to replication. It has been reported that 3C proteases of PV (Joachims et al., 1999; Kuyumcu-Martinez et al., 2002, 2004), HAV (Zhang et al., 2007a), EMCV (Kobayashi et al., 2012) and duck hepatitis A virus (DHAV) (Sun et al., 2017) all cleave poly(A)-binding protein (PABP, herein referring to PABP1; also known as PABPC1), crucial regulators of translation for both host cells and picornavirus. In HAV (Zhang et al., 2007a), EMCV (Kobayashi et al., 2012) and DHAV (Sun et al., 2017), 3C-mediated cleavage separates the N-terminal RNA-binding domain (NTD) of PABP from its C-terminal protein-interaction domain (CTD). The NTD of PABP inhibits the translation of HAV RNA, but facilitates its replication (Zhang et al., 2007a). Moreover, viral RNA synthesis and production of infectious virus particles are both reduced in EMCV-infected cells expressing a cleavage-resistant PABP variant

(Kobayashi et al., 2012). The above results suggest that 3C-mediated cleavage of PABP has only a subtle effect on its function, in that it allows the RNA template to be replicated, but not translated, which may be a strategy to promote viral replication to ensure that adequate virus RNA is packaged into mature virus particles. Recently, SVV 3C has been reported to also cleave PABP (Xue et al., 2020), but in this case, it is unclear whether the cleavage mediates a similar shift of viral RNA template to viral RNA replication. Similarly, PV 3C or 3CD cleaves PCBP2, which binds to viral RNA; this results in the loss of the KH3 domain of PCBP2, which is involved in translation, thus constituting the switch from the translation of viral RNA to its replication (Chase et al., 2014; Perera et al., 2007). This cleavage of PCBP2 by 3C also takes place in HAV-infected cells (Zhang et al., 2007b). In addition, HAV 3C similarly cleaves the polypyrimidine tract-binding protein (PTB; including PTBP1 and PTBP2) to downregulate viral translation and increase viral genome replication (Kanda et al., 2010), as has also been shown for PV 3C (Back et al., 2002). Therefore, 3C can be considered as one of the switches that induces viral RNA replication through cleavage of translation-associated proteins bound to viral RNA. It has been shown that the cleavage efficiency of 3C for ribosome-associated PABP is higher than that for other PABPs (Kuyumcu-Martinez et al., 2002). This suggests that 3C prefers to cleave the PABP fraction that acts on the translation process; however, it is unknown how 3C recognizes these translation-associated proteins. Does the increasing concentration of viral proteins or the ribosome-associated protein cause the cleavage? It remains unclear how picornaviruses modulate the cleavage of these proteins via 3C without completely inhibiting viral translation.

3C redistributes nuclear proteins to the cytoplasm

Picornaviruses produce RNA in the cytoplasm, but cellular RNA synthesis occurs in the nucleus. This raises the question of how nuclear host proteins required for virus replication are shuttled to the cytoplasm. There is increasing evidence that 3C triggers this shuttling process. For instance, transient transfection of HRV 3C or 3CD leads to the eventual degradation of nucleoporins (Nups), such as NUP153, NUP214 (KIAA0023) and NUP358 (also known as RANBP2), thereby increasing the permeability of the nuclear pore complex (Ghildyal et al., 2009). Another study found that FMDV 3C induces the cleavage of Src-associated in mitosis 68 kDa protein (SAM68; also known as KHDRBS1), thus resulting in its accumulation in the cytoplasm, where it interacts with IRES of the FMDV genome and increases viral IRES-driven translation (Lawrence et al., 2012). Moreover, co-immunoprecipitation, structural modeling, and subcellular fractionation have shown that FMDV 3C and 3CD interact with SAM68 (Rai et al., 2015), and EV71 3C also cleaves SAM68 (Zhang et al., 2014). Therefore, SAM68 appears to be a specific host factor hijacked by 3C to ensure viral translation and replication. Similarly, HRV 3C or 3CD cleaves the splicing factor SFPQ (also known as PSF). The resulting C-terminal fragment of SFPQ, which retains RNA-binding activity, translocates to the cytoplasm, where it binds to the viral RNA to promote viral RNA stability and replication but not translation (Flather et al., 2018); however, the specific mechanism and roles of SFPQ remains to be elucidated.

Taken together, these findings clearly demonstrate that 3C-mediated cleavage of nuclear host proteins is another mechanism by which this protease promotes picornavirus replication.

Roles of 3C in subverting host responses

During picornavirus infection, host cells adopt multiple antiviral strategies, including inducing stress granules, programmed cell death, innate immunity responses and autophagy among others. In order to ensure their survival, picornaviruses thus have to target the cellular proteins involved in counteracting these antiviral responses, and as discussed below, 3C plays a pivotal role in this process.

3C targets host factors that restrict infection

Cells have developed several mechanisms to impede virus invasion. During PV, CVB3 and HRV infection, AU-rich binding factor 1 (AUF1; also known as HNRNPD) interacts with different sites on the viral genome to inhibit viral replication by mediating the degradation of viral RNA (Cathcart et al., 2013; Wong et al., 2013). However, 2A mediates the relocalization of AUF1 to the cytoplasm, where it is subsequently cleaved by 3C or 3CD, thereby reducing its effect on inhibition of viral replication (Wong et al., 2013; Cathcart et al., 2013; Rozovics et al., 2012). TARDNA/RNA-binding domain protein 43 (TDP-43; also known as TARDBP) is a host RNA-binding protein involved in the regulation of RNA metabolism, and its knockdown increases virus titers (Fung et al., 2015). CVB 3C has been shown to cleave TDP-43 and inhibit its functions in the alternative splicing of RNA, suggesting that 3C-mediated cleavage of TDP-43 allows picornaviruses to overcome the antiviral effect of TDP-43 (Fung et al., 2015). Furthermore, stress granules (SGs) are induced in the early stages of viral infection and they inhibit viral replication (Montero and Trujillo-Alonso, 2011). Double-stranded RNA-activated protein kinase (PKR; also known as EIF2AK2) recognizes viral RNA in the cytoplasm, thereby inducing the phosphorylation of eIF2α to block host translation and trap viral RNA, stalling viral protein synthesis, and leading to the formation of SGs (Montero and Trujillo-Alonso, 2011). EV71 3C directly cleaves PKR at glutamine (Gln) 188 (Q188); this leads to the induction of PKR phosphorylation, but not its dimerization or eIF2α phosphorylation, resulting in an N-terminal fragment that instead promotes viral protein expression and replication (Chang et al., 2017). In addition, FMDV 3C mediates the degradation of PKR through the lysosomal pathway, thus benefiting virus replication; this process does not rely on its protease activity (Li et al., 2017a), but the underlying mechanisms remain unclear. Furthermore, Ras GTPase-activating protein-binding protein 1 (G3BP1; also known as G3BP) is another critical component of SGs that is involved in blocking viral replication and translation (Yang et al., 2020), and 3C of PV (White et al., 2007), CVB3 (Fung et al., 2013), EMCV (Ng et al., 2013), FMDV (Ye et al., 2018) and EV71 (Zhang et al., 2018) all cleave G3BP1 to improve viral survival. However, in cells infected by SVV or transfected with SVV 3C, the cleavage of G3BP1 was not detected (Wen et al., 2020). This study further showed that SVV 3C cleaves eIF4G1, impairing the interaction between eIF4G1 and G3BP1, and thus disrupting SG formation, albeit without significant promotion of virus replication (Wen et al., 2020). Furthermore, FMDV 3C has been found to mediated cleavage, at Q364, of heterogeneous nuclear ribonucleoprotein K (hnRNP K; also known as HNRPK), a newly described IRES-transacting factor that competes with PTB to inhibit FMDV IRES-mediated translation (Liu et al., 2020). Interestingly, the inhibitory effect on the viral translation of hnRNP $K_{1–364}$ is attenuated compared to that of the full-length hnRNP K, and the hnRNP $K_{364–465}$ fragment promotes FMDV replication (Liu et al., 2020); therefore, two advantageous effects on picornavirus replication are obtained with a single 3C cleavage event. Based on these findings, it is evident that 3C has a crucial role in blocking host responses aimed at resisting the establishment of virus infection (Fig. 2).

3C exerts control over and subverts cell death pathways

Cell death is an inevitable consequence of virus proliferation, and is necessary for the release of virus particles to the extracellular matrix

Journal of Cell Science (2021) 134, jcs253237. doi:10.1242/jcs.253237

and for expansion of the infection range of the virus. However, as viruses need to replicate in living cells, cell death also limits virus transmission; hence, viruses, including picornaviruses, have evolved several mechanisms to regulate cell death and to precisely control both pro-apoptotic and anti-apoptotic processes (Croft et al., 2017). Apart from apoptosis, cells also execute other forms of cell death, including necroptosis and pyroptosis (Miyake et al., 2019), and 3C controls and subverts all these cell death pathways using multiple strategies (Fig. 3).

The first evidence for 3C regulating cell death came when it was shown that PV 3C initiated the degradation of cellular DNA and production of apoptotic bodies in the late infection (Barco et al., 2000), which could be inhibited by the caspase 1 inhibitor vad-fmk (Weidman et al., 2001), indicating that 3C initiates caspase cascades in apoptosis during late infection of PV. Moreover, the production of apoptotic bodies during EV71 infection has also been linked to 3C (Li et al., 2002), and several studies have revealed the underlying mechanisms (Fig. 3). 3C associates with and activates caspase-8 and caspase-9, thus activating caspase-3, which induces apoptosis (Song et al., 2018). Accordingly, a protease-deficient mutant form of 3C remains associated with caspase-8 and caspase-9, but is unable to activate caspase-8, caspase-9 and caspase-3 (Song et al., 2018). However, whether caspase activation by 3C indeed involves a cleavage event needs to be confirmed. EV71 3C also cleaves heterogeneous ribonucleoprotein A1 (hnRNP A1), leading to the dissociation of hnRNP A1 from the IRES region of the apoptotic protease activating factor 1 (*APAF1*) mRNA. This results in *APAF1* translation and subsequent apoptosis triggered by activated caspase-3 (Li et al., 2019) (Fig. 3); however, it is unclear whether the RNA-binding ability of 3C promotes its cleavage of HnRNP A1. The telomere-binding protein PIN2/TERF1-interacting telomerase 1 (PINX1; also known as LPTL) has been shown to reduce the sensitivity of cells to apoptosis inducers and protect DNA from damage (Tian et al., 2014). EV 71 3C directly cleaves PINX1 at Q50 (Li et al., 2017b). Here, overexpression of PINX1 was sufficient to reduce apoptosis, while the overexpression of both 3C and PINX1 increased the incidence of apoptosis, suggesting that EV71 3C indeed induces apoptosis through the cleavage of PINX1 (Li et al., 2017b) (Fig. 3). Additionally, 3C-induced apoptosis has also been discovered during infection of CVB3 (Chau et al., 2007), SVV (Fernandes et al., 2019; Liu et al., 2019b) and HAV (Shubin et al., 2015) (Fig. 3). CVB3 3C induces caspase-8-mediated activation of caspase-3 and cleavage of BH3-interacting domain death agonist (BID), thus triggering the caspase-9 dependent apoptosis pathway (Chau et al., 2007) (Fig. 3). Similarly, 3C is indispensable for SVV-induced apoptosis (Fernandes et al., 2019; Liu et al., 2019b); here, SVV 3C induces the cleavage of poly ADP-ribose polymerase 1 (PARP1; PPOL) and p65 subunit of nuclear transcription factor-κB (NF-κB) (also known as RELA) through the activation of caspase-3, but it does not interact with PARP1 and NF-κB p65 directly (Liu et al., 2019b). In contrast, HAV 3C induces cell death independently of the caspase pathway (Shubin et al., 2015), but the underlying

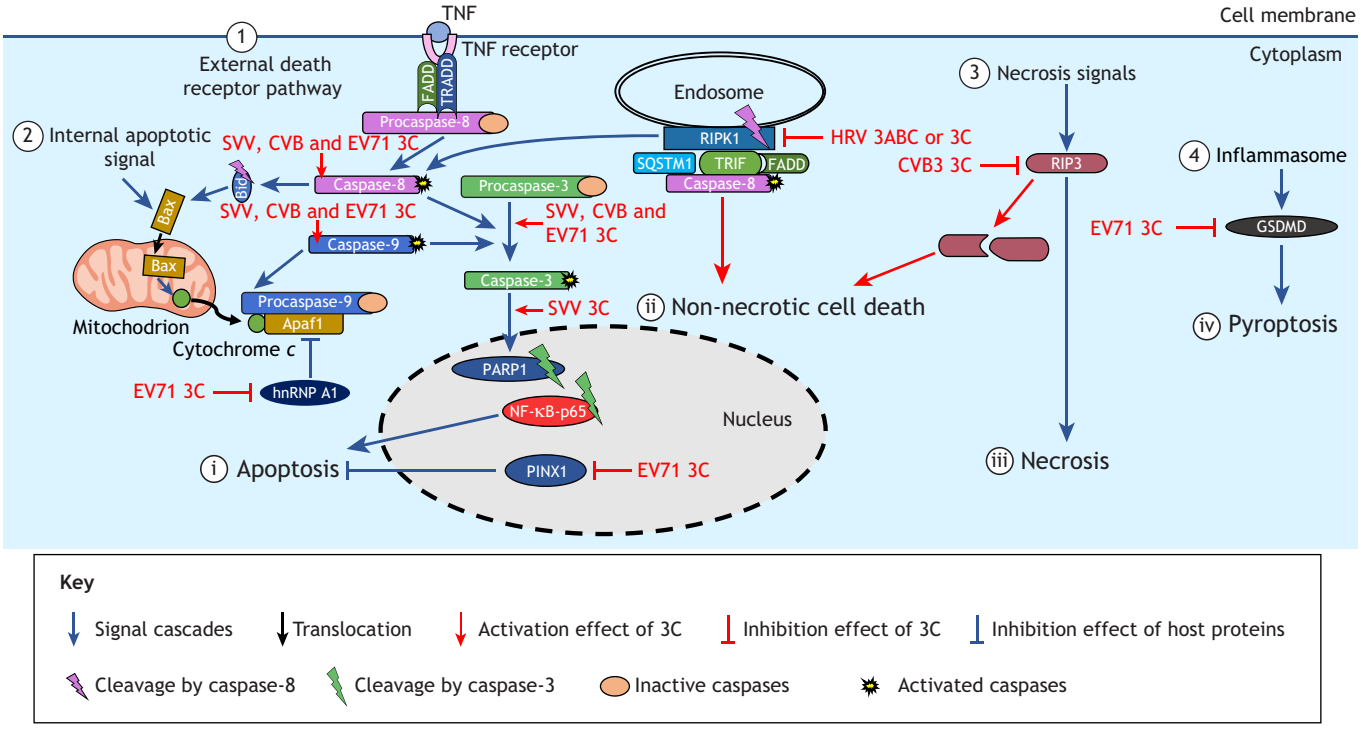

Fig. 3. Roles of 3C in modulating host cell-death pathways. Illustrated here are different cell-death pathways, including apoptosis (i), non-necrotic cell death (ii), necrosis (iii) and pyroptosis (iv). Apoptosis has two pathways – an external pathway that is typically triggered by activation of external death receptor (such as TNF receptor) and the assembly of a death complex (external death receptors–FADD–TRADD–caspase-8) (1), and an internal pathway, which is triggered by translocation of Bax to the mitochondrion and the subsequent release of cytochrome *c*, which binds to Apaf1, thus activating caspase-9 (2). Moreover, activated caspase-8 cleaves Bid, which also leads to translocation of Bax to the mitochondrion and subsequent caspase-9 activation, connecting the two apoptotic pathways. Both caspases can cleave and activate caspase-3, which cleaves PARP transcription factors, triggering the production of apoptotic bodies. 3C activates caspases, which induce apoptosis. EV71 3C targets the anti-apoptotic proteins PINX1 and hnRNP A1 to induce apoptosis. In addition, 3C can also inhibit cell death; for instance, HRV 3ABC or 3C cleaves RIPK1 at a different site to caspase-8, disrupting the cell death complex, suppressing apoptosis, and inducing non-necrotic cell death. CVB3 3C cleaves RIP3 into two fragments; this inhibits necrosis (3), but induces non-necrotic cell death. EV71 3C cleaves the pore-forming GSDMD and abrogates pyroptosis (4).

mechanism by which HAV 3C causes this cell death needs to be further studied.

Despite the fact that 3C can activate caspase-3 and cleave host factors to promote apoptosis, it has also been reported to have a vital role in avoiding cell death. For instance, in the late stages of infection, CVB3 3C cleaves receptor interaction protein 3 (RIP3; also known as RIPK3) into two fragments, RIP3NT and RIP3CT, which are incapable of inducing necrosis; however, RIP3CT induces the non-necrotic form of cell death (Harris et al., 2015). During HRVA16 infection, 3C cleaves receptor-interacting serine/threonine-protein kinase 1 (RIPK1; also known as RIP1) at a different site (Q430) compared to that targeted by caspase-8 (D324), thereby generating a new RIPK1 fragment (Croft et al., 2018). This suggests that 3C mediates further fragmentation of the pro-apoptotic caspase-8-generating cleavage product, which allows picornaviruses to inhibit apoptosis. Interestingly, another study demonstrated that HRVA16 3C and 3ABC cleave RIPK1 at specified sites that disrupt death signaling-complexes containing RIPK1, Toll-IL-1R domain-containing adaptor-inducing interferon-β (IFNβ) factor (TRIF; also known as TICAM1), Fas-associated-protein with death domain (FADD; also known as GIG3) and sequestosome 1 (SQSTM1; also known as OSIL), which trigger apoptosis and necroptosis (Lötzerich et al., 2018) (Fig. 3). A recent study provided significant new insight by demonstrating that RIPK1 is a caspase-8 substrate and its cleavage limits apoptosis and necroptosis (Newton et al., 2019); this points to a link between 3C, caspase-8 and RIPK1-induced cell death, which could help explain the strategies adopted by picornaviruses to modulate cell death to maintain persistent infection rate and cytolysis.

Additionally, pyroptosis is a recently described form of programmed cell death that is mediated by the pore-forming protein gasdermin D (GSDMD; GSDMDC1) (Shi et al., 2017) (Fig. 3). EV71 3C cleaves GSDMD at Q193, and the resulting GSDMD fragments can no longer induce pyroptosis (Lei et al., 2017). Overall, 3C is involved in the timely inhibition or induction of apoptosis, as well as in the appropriate regulation of the modes of cell death, which are considered advantageous for virus replication. In light of these findings, it has been recently reported that caspase-8 acts as a switch to control apoptosis, necroptosis and pyroptosis; fully functional caspase-8 induces apoptosis and necroptosis, whereas a mutant that lacks caspase activity induces necrosis and pyroptosis (Fritsch et al., 2019). These findings present an interesting premise to further explore the roles of 3C in mediating different cell death pathways that contribute to picornavirus pathogenesis.

3C inhibits innate immunity pathways

Upon picornavirus infection, viral RNA is recognized by the RNA sensors, retinoic acid-inducible gene I (RIG-I; also known as DDX58), melanoma differentiation-associated gene 5 (MDA5; also known as IFIH1), laboratory of genetics and physiology 2 (LGP2; also known as DHX58), and Toll-like receptor 3 (TLR3) (Takeuchi and Akira, 2009) (Fig. 4). They recruit the downstream adaptors, including mitochondrial antiviral signaling (MAVS), TRIF, tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3), TRAF6, and TRAF family member-associated NF-κB activator (TANK), inducing formation of the TANK-binding kinase 1 (TBK1)–IκB kinase ε (IKKε)–NF-κB essential modulator (NEMO; also known as IKBKG) complex directly or that of the IKKα–IKKβ–NEMO complex through activation of the transforming growth factor β (TGF-β)-activated kinase 1 (TAK1; also known as MAP3K7) complexes. These signal cascades lead to phosphorylation, dimerization, and translocation of interferon regulatory factors (IRFs) and NF-κB into the nucleus, where they promote the expression of interferons (IFNs), interferon stimulated genes (ISGs) and proinflammatory cytokines (Takeuchi and Akira, 2009) (Fig. 4). Moreover, IFNs binds to the IFN-α/β receptors (IFNARs), activating the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway to amplify IFN production (Ivashkiv and Donlin, 2014), thereby inhibiting virus proliferation. However, picornaviruses have evolved multiple strategies to evade the innate immunity response, with 3C playing a key role in the process (Fig. 4), as discussed below.

3C modulates RNA sensors

RIG-I, MDA5 and LGP2 can detect viral RNA through their C-terminal domains (CTDs), and the caspase activation and recruitment domains (CARDs) interact with the CARDs of the downstream adaptor MAVS to transduce signals (Takeuchi and Akira, 2009) (Fig. 4). Cleavage assays *in vitro* have shown that EMCV 3C cleaves RIG-I (Papon et al., 2009); however, *in vivo* experiments did not detect the cleaved fraction, which might have been degraded by cellular caspases (Papon et al., 2009). Similarly, HRV and SVV 3C degrade RIG-I in a caspase-dependent manner (Pang et al., 2017; Wen et al., 2019). Interestingly, SVV 3C has been identified as a viral deubiquitylase, counteracting the ubiquitylation of RIG-I and thus reducing IFN-β and ISG expression (Xue et al., 2018b). This study is the first evidence for picornavirus 3C acting as deubiquitylase, pointing to a novel mechanism through which 3C can target different components of a pathway; however, it remains to be determined whether any other proteins are targeted by SVV 3C to reduce the level of RIG-I ubiquitylation. EV71 3C interacts with CARDs of RIG-I, thereby blocking the recruitment of MAVS independent of its protease activity (Lei et al., 2010). During CV-A16, CV-A6, and EV-D68 infection, 3C interacts with MDA5 to inhibit IFN production (Rui et al., 2017). In addition, overexpression of FMDV 3C decreases the expression of exogenous LGP2 to increase FMDV replication (Zhu et al., 2017). Collectively, 3C can block interferon downstream signaling cascades to impair the host defense against viruses.

3C cleaves the adaptors

Apart from RNA sensors, 3C also degrades or inactivates the adaptors in the IFN response pathway, disrupting the signaling transduction process (Fig. 4). For instance, the protease activity of 3C is required for HAV 3ABC to cleave MAVS to ablate IFN responses (Yang et al., 2007). Further evidence has shown that HAV 3CD disrupts TLR3 signaling by cleaving TRIF (Qu et al., 2011), and EV71 and EV68 3C directly induce TRIF cleavage at multiple sites (Lei et al., 2011; Xiang et al., 2014). During CVB3 infection, 3C cleaves TRIF and MAVS to ablate interferon downstream signaling (Mukherjee et al., 2011), and HRV 3C also cleaves MAVS (Pang et al., 2017). EMCV 3C cleaves TANK at Q197 and Q291, which disrupts the tetrameric TANK–TBK1–IKKε–IRF3 complex, thus reducing IRF3 phosphorylation and subsequent IFN production (Huang et al., 2017). SVV 3C inhibits IFN production by cleaving the adaptor proteins MAVS, TRIF and TANK at specific sites (Qian et al., 2017). Moreover, SVV 3C inhibits the ubiquitylation of TBK1 and TRAF3 through its deubiquitylase activity, similar to the effect on RIG-I described above (Xue et al., 2018b). HAV 3C also cleaves NEMO at Q304 to inhibit both the IFN and TLR3 signaling pathways, blocking the production of IFNs (Wang et al., 2014), while FMDV 3C cleaves NEMO at Q383, impairing the innate immune response (Wang et al., 2012) (Fig. 4).

3C cleaves IRFs

IRFs are another group of key transcription factors that stimulate IFN expression (Jefferies, 2019). Picornavirus 3C has been shown

Journal of Cell Science (2021) 134, jcs253237. doi:10.1242/jcs.253237

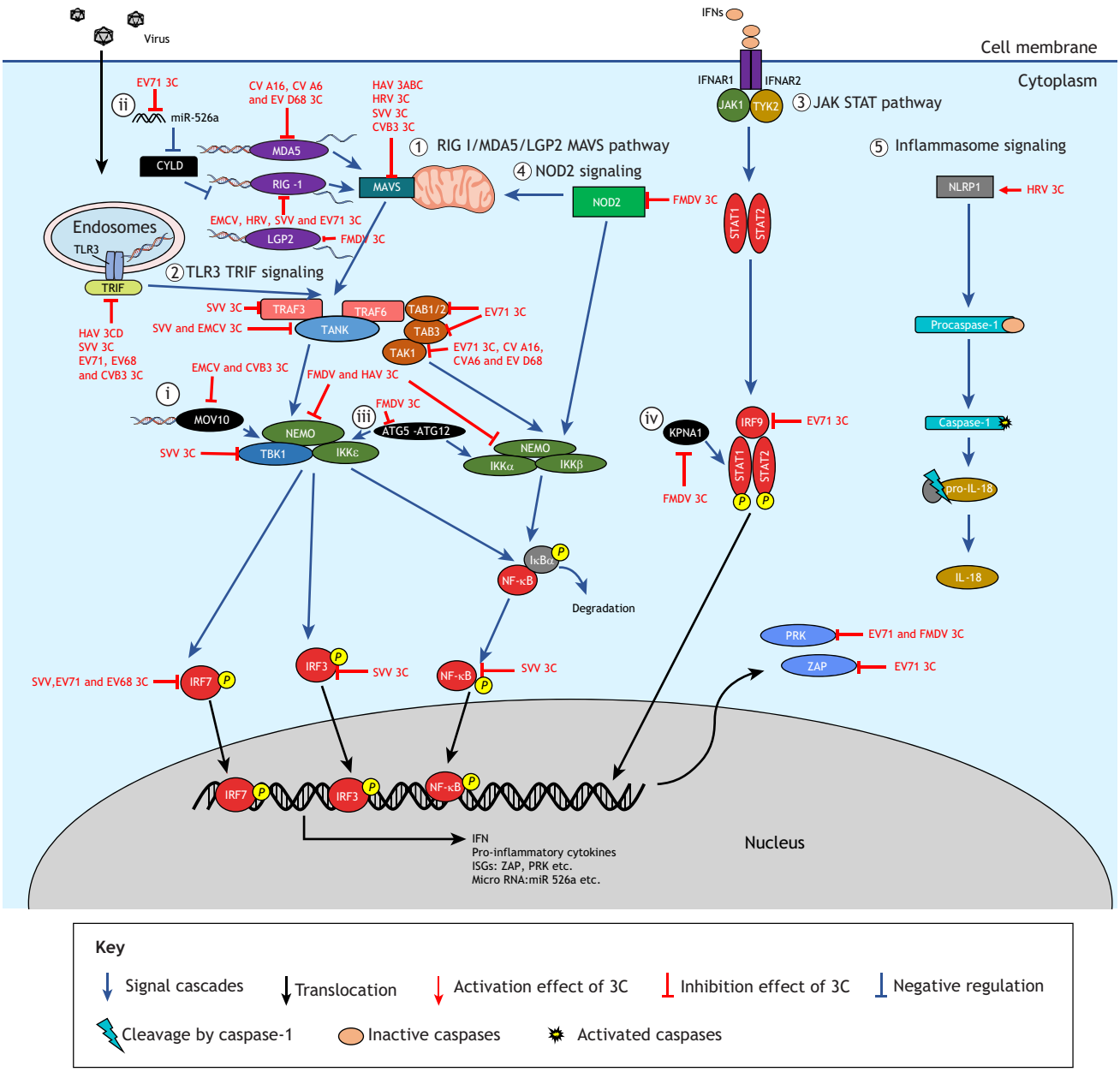

Fig. 4. Roles of 3C in subverting host innate immunity. Upon picornavirus infection, viral RNA triggers two interferon response pathways, the RIG-I/MDA5/LGP2-MAVS pathway (1) and the TLR3–TRIF pathway (2). Both pathways involve the recruitment of downstream adaptors, including TRAF3, TRAF6, and TANK, to induce the TBK1–IKKε–NEMO complex directly or induce the IKKα–IKKβ–NEMO complex through activation of TAK1 complexes (TAB1–TAB2–TAB3–TAK1). These signal cascades lead to the phosphorylation of IRFs and NF-κB, and their dimerization and translocation into the nucleus, thus promoting the expression of IFNs, ISGs, and proinflammatory cytokines. Binding of the secreted IFNs to their receptors IFNAR1 and IFNAR2 triggers the JAK-STAT pathway (3), which culminates in IFN production. NOD2 signaling (4) induces the activation of MAVS and the IKKα–IKKβ–NEMO complex. In addition, MOV10 acts as an RNA sensor to induce formation of the TBK1–IKKε–NEMO complex to activate IRF3 (i), miR-526 and CYLD modulate RIG-I (ii), ATG5-ATG12 promotes activation of IRF3 and NF-κB (iii), and KPNA1 helps the nuclear translocation of STAT1–STAT2 (iv). 3C targets numerous factors involved in these signaling cascades in order to inhibit IFN signaling. As an exception, HRV 3C cleaves NLRP1 (5); this triggers NLRP1-dependent inflammasome activation, induces caspase-1 activation and subsequent IL-18 secretion. Overactivation of IL-18 is associated with HRV-related inflammatory diseases.

to cleave or degrade them, thus directly influencing the phosphorylation, dimerization and translocation of IRFs to the nucleus (Hung et al., 2011; Lei et al., 2013; Xiang et al., 2016; Xue et al., 2018a) (Fig. 4).

3C affects NF-κB activation

NF-κB is another important transcription factor for the host defense against virus infection and it is activated by the kinase TAK1 (Vidal et al., 2001) (Fig. 4). EV71 3C interacts with TAK1 and TAK binding protein 2 (TAB2) and cleaves components of the TAK1 complex (TAK1–TAB1–TAB2–TAB3) at specific sites (Lei et al., 2014) (Table S1); this leads to the inactivation of the downstream IKK complex and NF-κB. In addition, the 3C of CV-A16, CV-A6 and EV-D68 also cleave TAK1 to suppress the NF-κB response (Rui et al., 2017) (Table S1). SVV 3C mediates the cleavage of p65 subunit of NF-κB through the caspase pathway (Wen et al., 2019;

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Fernandes et al., 2019). Notably, EMCV 3C activates NF-κB by cleaving TANK to impair the inhibition of TRAF6-mediated NF-κB signaling (Huang et al., 2015), indicating that 3C can modulate the NF-κB pathway through different means.

3C targets other proteins associated with the innate immunity response

Alternative mechanisms through which 3C targets other host proteins to regulate the IFN signaling pathway have also been described (Fig. 4). In FMDV-infected cells, 3C is involved in the degradation of nucleotide-binding oligomerization domain 2 (NOD2), thereby inhibiting the IFN-β and NF-κB signaling pathways (Liu et al., 2019a). In addition, FMDV 3C degrades karyopherin 1 (KPNA1) through a proteasome- and caspase-independent pathway (Du et al., 2014); this impairs the nuclear translocation of signal transducers and activators of transcription 1 and 2 (STAT1–STAT2), thereby inhibiting IFN signaling (Du et al., 2014). Although the underlying mechanism remains unclear, it might be related to the lysosomal pathway, such as in the case of PKR degradation (Li et al., 2017a). In contrast, KPNA1 degradation is mediated by the caspase-3 pathway during EV71 infection and not by 3C (Wang et al., 2017a). FMDV 3C also degrades the ATG5–ATG12 complex, which is involved in autophagosome formation and promoting IκBα degradation, which activates the p65 subunit of NF-κB and IRF3 (Fan et al., 2017). Similarly, it has been shown that EMCV and CVB 3C degrade the RNA helicase MOV10, which mediates IRF3 activation through a pathway that is independent of RIG-I–MAVS (Cuevas et al., 2016), suggesting that 3C might target this RNA sensor to help picornavirus escape the host innate immunity response. Furthermore, EV71 3C downregulates microRNA-526a (miR-526a), which suppresses the expression of the deubiquitylase cylindromatosis (CYLD) and its subsequent ubiquitylation of RIG-1 to inhibit the IFN response (Xu et al., 2014). EV71 3C also directly cleaves the ISG zinc-finger antiviral protein (ZAP; also known as ZC3HAV1) at Q369, which reduces the inhibitory effect of ZAP on EV71 replication (Xie et al., 2018). In addition, FMDV and EV71 3C also target PKR, another ISG, which impairs its antiviral activity of mediating the formation of SGs as discussed above (Li et al., 2017a; Chang et al., 2017).

In summary, a number of factors that act in different innate immunity signaling pathways are targeted by 3C during picornavirus infection. In addition, HRV 3C has been shown to cleave NOD-like receptor protein 1 (NLRP1) at Q130 (Robinson et al., 2020); this liberates the activating C-terminus of NLRP1, thus triggering inflammasome assembly and interleukin-18 (IL-18) production in primary human airway epithelial cells. This study provides new insights into how picornaviruses cause inflammatory diseases. We anticipate that additional factors and mechanisms will be discovered to contribute to our understanding of the pathogenic mechanisms of picornaviruses.

The effects of 3C on cellular organelles

During virus infection, the functions of organelles are typically altered and 3C is also involved in this aspect (Fig. 5). For instance, PV 3C or 3CD cleaves microtubule-associated protein 4 (MAP-4), leading to the collapse of microtubules (Joachims et al., 1995) and their dissociation from the microtubule-organizing center (MTOC)

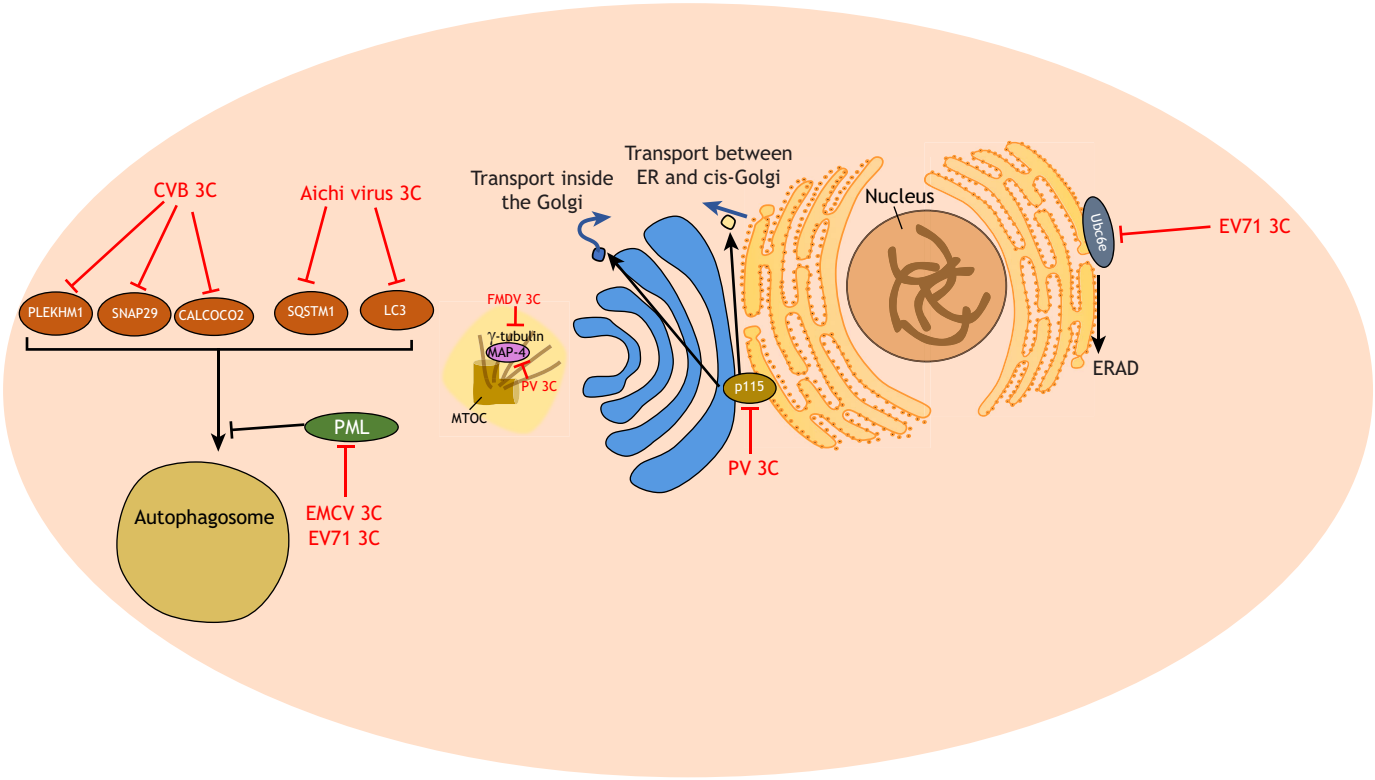

Fig. 5. Effects of 3C on cellular organelle. 3C modulates the physiological activity of cellular organelles (red inhibition bars). 3C targets both γ-tubulin and MAP-4 to disrupt the organization of microtubules. p115, which modulates transport inside the Golgi and between the ER and cis-Golgi, is also cleaved by 3C. Both are strategies of picornaviruses to hijack cellular transport and secretory pathways. PLEKHM1, SNAP29, CALCOCO2, SQSTM1 and LC3 are key factors involved in autophagy formation, and are cleaved by 3C to disrupt autophagy, thus supporting picornavirus survival. PML exerts an antiviral effect by inhibiting autophagy, and EV71 3C degrades PML to enhance infection. Ubc6e, a key molecule for ERAD, is cleaved by 3C. This ERAD and promotes ER membrane rearrangements, which might benefit virus replication.

(Joachims et al., 1995). FMDV 3C overexpression in Vero cells resulted in a loss of tubulin organization, disruption of the MTOC, fragmentation of the Golgi and blockage of intra-Golgi transport, which are all mediated through its protease activity (Zhou et al., 2013). Subsequently, it has been shown that PV 3C cleaves p115 (also known as USO1) to increase PV replication (Jagdeo et al., 2018). Interestingly, the endoplasmic reticulum (ER)–Golgi vesicle tethering protein p115 is responsible for the regulation of transport inside the Golgi and between the ER and cis-Golgi (Allan et al., 2000). These findings indicate that 3C modulates intracellular membrane trafficking and the secretory pathway during picornavirus infection.

Autophagy and endoplasmic reticulum-associated degradation (ERAD) regulate physiological processes in the cell by degrading protein aggregates, misfolded proteins and damaged organelles, as well as pathogens (Munz, 2017; Mehrbod et al., 2019; Glingston et al., 2019). Recently, 3C of CVB3 (Mohamud et al., 2019), EV71 (Mohamud et al., 2018) and Aichi virus (Kung et al., 2020) have been shown to subvert autophagy. CVB3 3C specifically cleaves two critical autophagosome-fusion-related proteins, synaptosomal-associated protein, 29 kDa (SNAP29) and adaptor protein pleckstrin homology domain-containing family M member 1 (PLEKHM1), resulting in a decrease in autophagy flux during CVB3 infection (Mohamud et al., 2018). Interestingly, the autophagy receptor calcium-binding and coiled-coil domain-containing protein 2 (CALCOCO2) is also cleaved by 3C, but the truncated C-terminal fragment still mediates the degradation of MAVS (as is the case for the full-length receptor), thus indirectly promoting viral replication (Mohamud et al., 2019). In case of Aichi virus, 3C degrades two autophagy markers, microtubule-associated protein light chain 3 (LC3) and sequestosome-1 (SQSTM1) (Kung et al., 2020). In EMCV-infected cells, 3C, the proteasome and SUMO modification have all been shown to be involved in degradation of promyelocytic leukemia (PML) (El McHichi et al., 2010). As a stress sensor, PML maintains mitochondrial complex II activity to inhibit reactive oxygen species (ROS) production, thus limiting autophagy (Guo et al., 2014). However, ROS generation promotes viral replication during EV71 infection (Cheng et al., 2014), which could account for the inhibitory effect of PML on EV71 replication (Chen et al., 2018). Accordingly, EV71 3C degrades PML to counter this antiviral response and support viral replication (Chen et al., 2018). EV71 3C also cleaves the ubiquitin-conjugating enzyme Ubc6e (also known as UBE2J1) to inhibit ERAD, which might promote ER membrane rearrangements to benefit viral replication (Wang et al., 2017b). Taken together, 3C disrupts the physiological functions of membrane-associated organelles (Fig. 5), which serves to positively modulate virus infection by promoting virus replication and motility that are associated with its pathogenesis.

Conclusions and perspectives

3C is a conserved protease among the picornaviruses, and it plays important roles in promoting picornavirus replication and subverting host responses. These functions mostly depend on its proteolytic and RNA-binding activities. 3C has been demonstrated to interact with viral RNA secondary structures, but there is no evidence yet that 3C can bind to the IRES of host cells. With regard to its proteolytic function, 3C clearly targets a wealth of cellular substrates (EV, CV and PV, see Table S1; FMDV, see Table S2; EMCV, see Table S3; SVV, see Table S4; HAV, see Table S5; HRV, see Table S6), but the 3C proteins of the various picornaviruses differ in certain aspects, including their cleavage sites, types of substrates, and the pathways they affect. These differences may be attributed to two factors: (1) proteolytic substrates are shared by other proteases (i.e. L, 2A) in some picornaviruses, and (2) the differences among the respective 3C amino acid sequences contribute to the difference in cleavage sites of the different 3C proteins. Deciphering the molecular basis of these similarities and differences remains a challenging issue. As for the abundant substrates of 3C, each of them performs multiple functions in host cell, and the cleavage events may result in multiple consequences that benefit the viruses in different lifecycle stages. Terminal amine isotopic labeling of substrates has been used to identify multiple candidate substrates of PV and CVB3 3C *in vitro* (Jagdeo et al., 2018), and further advances in high-throughput technologies might facilitate the identification and verification of novel cellular substrates for 3C and provide a better understanding of how 3C modulates and usurps host processes, while also helping to uncover the mechanisms underlying pathogenesis. Importantly, there are currently only limited therapies for the treatment of picornavirus infection. 3C is a valuable therapeutic target owing to its multiple functions in the virus lifecycle and in subverting host cell response, but the clinical development of 3C inhibitors still could be improved (Ma et al., 2019; Kassem et al., 2019; Banerjee et al., 2019). Further efforts to elucidate the structures and the molecular functions of 3C protease might help to achieve this goal.

Acknowledgements

We thank the other members of the Haixue Zheng's lab for their constructive comments.

Competing interests

The authors declare no competing or financial interests.

Funding

Our work in this area is supported by the National Natural Science Foundation of China (grant no. 31672585); and the Fundamental Research Funds of Chinese Academy of Agricultural Sciences (grant nos 1610312016013 and 1610312017003).

Supplementary information

Supplementary information available online at
https://jcs.biologists.org/lookup/doi/10.1242/jcs.253237.supplemental

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Table S1. Known functions of Enteriviruses 3C on host responses

Classification	Virus	Cells	Host proteins	Full name of the host proteins	*In Vivo* or *In Vitro*	Cleage Sites (Evidences)	References
Shut off host transcription and translation	PV	HeLa	TFIIIC	transcription factor IIIC	*In Vivo* and *In Vitro*	Q732–G733 (Site-directed mutagenesis)	(Clark et al., 1991, Shen et al., 1996)
		HeLa	CREB-1	cAMP-response element binding protein 1	*In Vivo* and *In Vitro*	Q172–G173 (Site-directed mutagenesis)	(Yalamanchili et al., 1997a)
		HeLa	Oct-1	Octamer-binding transcription factor-1	*In Vivo* and *In Vitro*	Q330–G331 (predicted)	(Yalamanchili et al., 1997b)
		HeLa	p53	tumor suppressor protein p53	*In Vivo* and *In Vitro*	Degrades p53 with a cellular activity	(Weidman et al., 2001)
		HeLa	TAF110	TATA-box binding protein associated factor 110	*In Vitro*	Q265-G266, Q805-G806 (Site-directed mutagenesis)	(Banerjee et al., 2005)
		HeLa	TBP	TATA-box binding protein	*In Vivo* and *In Vitro*	Q104-S105 (Site-directed mutagenesis)	(Clark et al., 1993, Kundu et al., 2005)
	EV71	RD SF268	CstF-64	Cleavage stimulation factor subunit 2, 64 kDa	*In Vivo* and *In Vitro*	Q251-A252 (Site-directed mutagenesis)	(Weng et al., 2009)
	PV,CVB	293T Hela	eIF5B	eukaryotic Initiation Factor 5B	*In Vivo* and *In Vitro*	Q478-G479 (sequencing)	(de Breyne et al., 2008)
Mediate the conversion of the viral RNA template	PV	HeLa	PABP	poly(A)-binding protein	*In Vivo* and *In Vitro*	Q537 (Site-directed mutagenesis)	(Kuyumcu-Martinez et al., 2004)
	PV	HeLa	PCBP2	poly (rC) binding protein 2	*In Vivo* and *In Vitro*	Q253-S254, Q306-G307 (Site-directed mutagenesis)	(Perera et al., 2007, Chase et al., 2014)
	PV	HeLa 293T	PTB	polypyrimidine tract-binding protein	*In Vivo* and *In Vitro*	Q148-A149, Q152-A153, Q321-A322 (Site-directed mutagenesis)	(Back et al., 2002)

Redistribute nuclear proteins to the cytoplasm	EV71	HeLa	Sam68	SRC associated in mitosis of 68 kD	*In Vivo*	Not shown	(Zhang et al., 2014)
Target host factors that restrict infection	CVB3, PV	HeLa	AUF1	AU-rich binding factor 1	*In Vivo* and *In Vitro*	Q-G sites in the Q-rich domain (Site-directed mutagenesis)	(Rozovics et al., 2012, Wong et al., 2013)
	CVB3	Hela	TDP-43	TARDNA/RNA-binding domain protein 43	*In Vivo* and *In Vitro*	Q327-A328 (Site-directed mutagenesis)	(Fung et al., 2015)
	EV71	RD 293T	PKR	Double-stranded RNA-activated protein kinase	*In Vivo*	Q188-S189 (Site-directed mutagenesis)	(Chang et al., 2017)
	EV71 CVB3 PV	Hela 293T MCF7 Vero	G3BP1	Ras GTPase-activating protein-binding protein 1	*In Vivo* *In Vivo* and *In Vitro* *In Vivo*	Q326 Q325 Q326 (Site-directed mutagenesis)	(Zhang et al., 2018) (Fung et al., 2013) (White et al., 2007)
Exert control over and subvert cell-death pathways	EV71	RD HepG2	caspase-8 caspase-9	caspase-8 caspase-9	*In Vivo*	Not shown (3C binds to and activates caspase-8 and caspase-9)	(Song et al., 2018)
	CVB3	Hela	caspase-8 caspase-9	caspase-8 caspase-9	*In Vivo*	Not shown (3C activates caspase-8 and caspase-9)	(Chau et al., 2007)
	CVB3	Caco-2 HT29 Hela 293T	RIP3	receptor interaction protein 3	*In Vivo*	Q430 (Site-directed mutagenesis)	(Harris et al., 2015)
	EV71	SF268 RD Vero	HnRNP A1	heterogeneous ribonucleoprotein A1	*In Vivo* and *In Vitro*	Not shown	(Li et al., 2019)
	EV71	Hela	PinX1	PIN2/TERF1-interacting telomerase 1	*In Vivo* and *In Vitro*	Q50-G51 (Site-directed mutagenesis)	(Li et al., 2017)

	EV71	RD 293T	GSDMD	gasdermin D	*In Vivo*	Q193 (Site-directed mutagenesis)	(Lei et al., 2017)
Inhibit the innate immunity pathway	EV71	RD 293T	RIG-I	retinoic acid-inducible gene I	*In Vivo*	Not shown (3C interacts with RIG-I, but not cleaves or degrades it.)	(Lei et al., 2010)
	CV-A16, CV-A6, EV-D68	RD 293T	MDA5	melanoma differentiation-associated gene 5	*In Vivo*	Not shown (3C binds MDA5 to inhibit MDA5-MAVS complex)	(Rui et al., 2017)
	EV71	RD 293T Hela	TRIF	TIR domain-containing adaptor inducing IFN-b	*In Vivo*	Q312-S313 (Site-directed mutagenesis)	(Lei et al., 2011)
	EV68	293T Hela				Q312 and Q653 (Site-directed mutagenesis)	(Xiang et al., 2014)
	CVB3	293 HeLa U2OS			*In Vivo* and *In Vitro*	Q190, Q653, Q659, Q671, Q702 (Site-directed mutagenesis)	(Mukherjee et al., 2011)
	CVB3	293 HeLa U2OS	MAVS	mitochondrial antiviral signaling	*In Vivo* and *In Vitro*	Q148 (Site-directed mutagenesis)	(Mukherjee et al., 2011)
	EV71	RD Vero	IRF9	interferon regulatory factor 9	*In Vivo* and *In Vitro*	Q202, Q237, Q381, E168, Q337 (Predicted)	(Hung et al., 2011)
	EV71	293T HeLa RD	IRF7	interferon regulatory factor 7	*In Vivo* and *In Vitro*	Q189 (Site-directed mutagenesis)	(Lei et al., 2013)
	EV68	293T HeLa THP1	IRF7	interferon regulatory factor 7	*In Vivo*	Q189 and Q167 (Site-directed mutagenesis)	(Xiang et al., 2016)

		PBMCs					
	EV71	RD 293T HeLa	TAB2 TAK1 TAB1 TAB3	TAK binding protein 2 TGF-β-activated kinase 1 TAK binding protein 1 TAK binding protein 3	*In Vivo*	Q113-S114 (Site-directed mutagenesis) Q360-S361 (Site-directed mutagenesis) Q414-G415 and Q451-S452 (Site-directed mutagenesis) Q173-G174 and Q343-G344 (Site-directed mutagenesis)	(Lei et al., 2014)
	CV-A16 CV-A6 EV-D68	293T RD	TAK1	TGF-β-activated kinase 1	*In Vivo*	Not shown	(Rui et al., 2017)
	CVB3	293 293T HT1080 HEF	MOV10	Moloney leukemia virus 10	*In Vivo*	Q129 and Q869 (Site-directed mutagenesis)	(Cuevas et al., 2016)
	EV71	293T Vero RD MDCK THP-1	miR-526a	microRNA miR-526a	*In Vivo*	Not shown (3C downregulates miR-526a through cleaving IRF7),	(Xu et al., 2014)
	EV71	293T HeLa RD	ZAP	zinc-finger antiviral protein	*In Vivo*	Q369 (Site-directed mutagenesis)	(Xie et al., 2018)
Destroy cellular organelles	PV	Hela	MAP-4	Microtuble-assocoated protein 4	*In Vivo* and *In Vitro*	Not shown	(Joachims et al., 1995)
	PV	BHK-38 BHK-21	γ-tubulin	γ-tubulin	*In Vivo* and *In Vitro*	Not shown (3C induces γ-tubulin degradation)	(Armer et al., 2008)
	PV	Hela	p115	the endoplasmic reticulum (ER)-Golgi	*In Vivo* and *In Vitro*	Q832 (Site-directed mutagenesis)	(Jagdeo et al., 2018)

				vesicle-tethering protein			
	CVB3	Hela	SNAP29 PLEKHM1	synaptosomal-associated protein, 29 kDa pleckstrin homology domain-containing family M member 1	*In Vivo* and *In Vitro*	Q161 (Site-directed mutagenesis) Q668 (Site-directed mutagenesis)	(Mohamud et al., 2018)
	CVB3	Hela	CALCOCO2	calcium-binding and coiled-coil domain-containing protein 2	*In Vivo* and *In Vitro*	Q139 (Site-directed mutagenesis)	(Mohamud et al., 2019)
	EV71	RD Hela 293T	PML	promyelocytic leukemia	*In Vivo*	Nor shown (3C degrades PML)	(Chen et al., 2018)
	EV71	RD 293T BSRT7	Ubc6e	E2 ubiquitin-conjugating enzyme for ERAD	*In Vivo* and *In Vitro*	Q219-G220, Q260-S261, and Q273-G274 (Site-directed mutagenesis)	(Wang et al., 2017)

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Table S2. Known functions of FMDV 3C on host responses

Classification	Cells	Host proteins	Full name of the host proteins	*In Vivo or In Vitro*	Cleavage sites	References
Shut off host transcription and translation	BHK-21 IB-RS2 Hela	H3	Histone 3	*In Vivo* and *In Vitro*	L20-A21 (predicted)	(Grigera and Tisminetzky, 1984, Falk et al., 1990)
	BHK	eIF4AI eIF4GI	eukaryotic Initiation Factor 4AI eukaryotic Initiation Factor 4GI	*In Vivo* *In Vivo* and *In Vitro*	E143-V144 (predicted) E712-P713 (predicted)	(Belsham et al., 2000, Li et al., 2001)
Redistribute nuclear proteins to the cytoplasm	BHK-21 LFBK	sam68	SRC associated in mitosis of 68 kD	*In Vivo* and *In Vitro*	Not shown(Induces the cleavage of the NLS)	(Lawrence et al., 2012)
Target host factors that restrict infection	PK-15 293T	PKR	Double-stranded RNA-activated protein kinase	*In Vivo*	Not shown (PKR are degraded through the lysosomal pathway independent of protease activity)	(Li et al., 2017)
	IBRS-2 293T	G3BP1	Ras GTPase-activating protein-binding protein 1	*In Vivo*	E284(Site-directed mutagenesis)	(Ye et al., 2018)
	BHK-21 IBRS-2 293T pBK	hnRNP K	heterogeneous nuclear ribonucleoprotein K	*In Vivo* and *In Vitro*	Q364-G365 (Site-directed mutagenesis)	(Liu et al., 2020)
	BHK-21 PK-15	LGP2	laboratory of genetics and physiology 2	In Vivo	Not shown	(Zhu et al., 2017)

	293T					
Inhibit the innate immunity pathway	PK-15 IBRS-2	NEMO	NF-κB essential modulator	*In Vivo*	Q383 (Site-directed mutagenesis)	(Wang et al., 2012)
	Hela BHK-21 PK-15	KPNA1	Karyopherin 1	*In Vivo*	Not shown (3C degrades KPNA1 through proteasome- and caspase-independent pathway)	(Du et al., 2014)
	BHK-21 PK-15	ATG5-ATG12	ATG5-ATG12	*In Vivo*	Not shown (3C degrades ATG5-ATG12)	(Fan et al., 2017)
	PK-15	NOD2	nucleotide-binding oligomerization domain 2	*In Vivo*	Not shown (3C induces NOD2 degradation)	(Robinson et al., 2020)
Destroy cellular organelles	Vero	γ-tubulin	γ-tubulin	*In Vivo*	Not shown (3C induce γ-tubulin degradation)	(Zhou et al., 2013)

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Table S3. Known functions of EMCV 3C on host responses.

Classification	Cells	Host proteins	Full name of the host proteins	*In Vivo* or *In Vitro*	Cleavage sites	References
Shut off host translation	293	PABP	poly(A)-binding protein	*In Vivo* and *In Vitro*	Q437-G438 (N-terminal sequencing)	(Kobayashi et al., 2012)
Target host factors that restrict infection	HeLa	G3BP1	Ras GTPase-activating protein-binding protein 1	*In Vivo*	Q325 (Site-directed mutagenesis)	(Ng et al., 2013)
Inhibit the innate immunity pathway	Hela MEF	RIG-I	retinoic acid-inducible gene	*In Vivo* and *In Vitro*	Not shown	(Papon et al., 2009)
	293T BHK-21 HeLa MDBK	TANK	TRAF family member-associated NF-κB activator	*In Vivo*	Q197 and Q291 (Site-directed mutagenesis)	(Huang et al., 2017)
	293 293T HT1080 HEF	MOV10	Moloney leukemia virus 10	*In Vivo*	Not shown (3C induce MOV10 degradation)	(Cuevas et al., 2016)

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Table S4. Known functions of SVV 3C on host responses

Classification	Cells	Host proteins	Full name of the host proteins	*In Vivo* or *In Vitro*	Cleavage sites	References
Shut off host translation	293T PK-15	PABP	poly(A)-binding protein	*In Vivo* and *In Vitro*	Q437-G438(Site-directed mutagenesis)	(Xue et al., 2020)
Impair stress granule formation	293T SK6	eIF4GI	eukaryotic Initiation Factor 5B	*In Vivo*	Not shown	(Wen et al., 2020)
Exert control over and subvert cell-death pathways	BHK-21 293T	caspase-3 caspase-8 caspase-9	caspase-3 caspase-8 caspase-9	*In Vivo*	Not shown (3C activates caspase-8 and caspase-9)	(Liu et al., 2019)
Inhibit innate immunity pathways	293T SW620 SK6	RIG-I	retinoic acid-inducible gene I	*In Vivo*	Not shown (RIG-I is degraded through the caspase signaling pathway or is ubiquitinated by 3C)	(Wen et al., 2019, Xue et al., 2018b)
	BHK-21 293T	MAVS	mitochondrial antiviral signaling	*In Vivo*	Q148(Site-directed mutagenesis)	(Qian et al., 2017)
		TRIF	TIR domain-containing adaptor inducing IFN-b		Q159(Site-directed mutagenesis)	
		TANK	TRAF family member-associated NF-κB activator		E272 and Q291(Site-directed mutagenesis)	
	293T	TBK1	TANK-binding kinase 1	*In Vivo*	Not shown (3C deubiquitinates TBK1 and TRAF3)	(Xue et al., 2018b)
		TRAF3	TNF receptor-associated factor 3			

	293T PK-15	IRF3 IRF7	interferon regulatory factor 3 interferon regulatory factor	*In Vivo*	Not shown (3C degrades IRF3 and IRF7)	(Xue et al., 2018a)
	STu H1299	NF-κB-p65	nuclear transcription factor-κB-p65	*In Vivo*	L444 by caspase (Site-directed mutagenesis))	(Fernandes et al., 2019)

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Table S5. Known functions of HAV 3C on host responses

Classification	Cells	Host proteins	Full name of the host proteins	*In Vivo* or *In Vitro*	Cleavage sites	References
Mediate the conversion of the viral RNA template	Huh-7	PABP	poly(A)-binding protein	*In Vivo* and *In Vitro*	near or at Q415-T416(predicted) Q367-G368 (Site-directed mutagenesis)	(Zhang et al., 2007a)
	DEF					(Sun et al., 2017)
	Hela	PCBP2	poly (rC) binding protein 2	*In Vivo* and *In Vitro*	Q306-G307(predicted)	(Zhang et al., 2007b)
	Huh-7	PTB	polypyrimidine tract-binding protein	*In Vivo* and *In Vitro*	Not shown	(Kanda et al., 2010)
Inhibits the innate immunity pathway	293T Huh7	MAVS	mitochondrial antiviral signaling	*In Vivo*	Q428 (Site-directed mutagenesis)	(Yang et al., 2007)
	293T Huh7 Bla-C	TRIF	TIR domain-containing adaptor inducing IFN-b	*In Vivo* and *In Vitro*	Q554-H555(Site-directed mutagenesis) Q190-G191(Site-directed mutagenesis)	(Qu et al., 2011)
	293T	NEMO	NF-κB essential modulator	*In Vivo*	Q304(Site-directed mutagenesis)	(Wang et al., 2014)

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Table S6. Known functions of HRV 3C on host response

Classification	Cells	Host proteins	Full name of the host proteins	*In Vivo* or *In Vitro*	Cleavage sites	References
Shut off host translation	293T Hela	eIF5B	eukaryotic Initiation Factor 5B	*In Vivo* and *In Vitro*	Q478-G479 (sequencing)	(de Breyne et al., 2008)
Mediate the conversion of the viral RNA template	HeLa	PCBP2	poly (rC) binding protein 2	*In Vivo* and *In Vitro*	Q253-S254 (Site-directed mutagenesis)	(Chase et al., 2014)
Redistribute nuclear proteins to the cytoplasm	COS-7	Nup 153, Nup 214, Nup 358	nucleoporins	*In Vivo*	Not shown (nucleoporins are degraded)	(Ghildyal et al., 2009)
	HeLa	SFPQ	splicing factor proline and glutamine-rich	*In Vivo* and *In Vitro*	Q257-G258 (predicted)	(Flather et al., 2018)
Target host factors that restrict infection	HeLa	AUF1	AU-rich binding factor 1	*In Vivo* and *In Vitro*	Q-G sites in the Q-rich domain (Site-directed mutagenesis)	(Rozovics et al., 2012)
Exert control over and subvert cell-death pathways	A549 HeLa	RIPK1	Receptor-interacting protein kinase-1	*In Vivo* and *In Vitro*	Q430 (predicted) Q401, Q464, Q573 (predicted)	(Croft et al., 2018) (Lotzerich et al., 2018)
Inhibit the innate immunity pathway	293T Hela	RIG-I	retinoic acid-inducible gene I	*In Vivo*	Not shown	(Pang et al., 2017)
		MAVS	mitochondrial antiviral signaling		Q148-G149 (Site-directed mutagenesis)	
	293T Hela normal bronchial epithelial cells	NLRP1	NOD-like receptor protein 1	*In Vivo* and *In Vitro*	Q130-G131 (Site-directed mutagenesis)	(Robinson et al., 2020)

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