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Cleavage of Stau2 by 3C protease promotes EV-A71 replication

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Abstract

Background Enterovirus A71 (EV-A71), as a neurotropic virus, mainly affects infants and young children under the age of 5. EV-A71 infection causes hand-foot-mouth disease and herpetic angina, and even life-threatening neurological complications. However, the molecular mechanism by which EV-A71 induces nervous system damage remains elusive. The viral protease 3C plays an important role during EV-A71 infection and is also a key intersection of virus-host interactions. Previously, we used yeast two-hybrid to screen out the host protein Double-stranded RNA-binding protein Staufen homolog 2 (Stau2), an important member involved in neuronal mRNA transport, potentially interacts with 3C.

Methods We used coimmunoprecipitation (Co-IP) and immunofluorescence assay (IFA) to confirm that EV-A71 3C interacts with Stau2. By constructing the mutant of Stau2, we found the specific site where the 3C protease cleaves Stau2. Detection of VP1 protein using Western blotting characterized EV-A71 viral replication, and overexpression or knockdown of Stau2 exhibited effects on EV-A71 replication. The effect of different cleavage products on EV-A71 replication was demonstrated by constructing Stau2 truncates.

Results In this study, we found that EV-A71 3C interacts with Stau2. Stau2 is cleaved by 3C at the Q507-G508 site. Overexpression of Stau2 promotes EV-A71 VP1 protein expression, whereas depletion of Stau2 by small interfering RNA inhibits EV-A71 replication. Stau2 is essential for EV-A71 replication, and the product of Stau2 cleavage by 3C, 508–570 aa, has activity that promotes EV-A71 replication. In addition, we found that mouse Stau2 is also cleaved by EV-A71 3C at the same site.

Conclusions Our research provides an example for EV-A71-host interaction, enriching key targets of host factors that contribute to viral replication.

Keywords EV-A71, 3C protease, Stau2, Cleavage

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Introduction

Enterovirus A71 (EV-A71), a member of the *Picornaviridae* family, is one of the main pathogens that cause hand, foot and mouth disease (HFMD). Some EV-A71 strains cause severe neuronal system damage, especially in children under five [1]. EV-A71 was first isolated from sputum specimens of infants in California, USA in 1969, and has become a continuing threat to global public health [2]. At present, the specific pathogenic mechanism of EV-A71 is still unclear, and there is no effective treatment for the disease caused by EV-A71 [3].

EV-A71 is a single strand RNA virus with a positive strand and the coding is divided into three regions: P1, P2 and P3. The P1 region encodes four structural proteins, including VP1, VP2, VP3 and VP4, which form the capsid of the virus. The P2 and P3 regions encode seven non-structural proteins, which are involved in the replication of the viral genome, the packaging and release of viral particles [4]. EV-A71 3C protein is a 183 amino acid cysteine protease responsible for the cleavage of most structural and non-structural proteins of EV-A71 [5]. At present, 3C has been proved to be involved in multiple pathological processes of EV-A71 infection [6] and is required for the production of mature viral proteins [7]. Furthermore, 3C protein is involved in the cleavage of various host cytokines, such as histone H3 [8], TATA-binding protein (TBP) [9], transcriptional activator p53 [10]and telomere-binding protein PinX1 [11]. 3C induces the activation of Casepase9 through the host cell mitochondria-mediated apoptotic pathway, which in turn induces apoptosis [12]. In the process of virus resistance to host innate immunity, 3C also plays an important role. 3C blocks the interferon (IFN) antiviral immune response by binding to retinoid acid-inducible gene I (RIG-I) receptors to inhibit IFN regulatory factor 3 (IRF3) activation and IFN-β release, and it degrades IFN regulatory factor 9 to block IFN antiviral immune responses [13]. Since 3C plays a vital role in EV-A71 replication and anti-host immune, also has no homology with mammalian proteases, it has become one of the important targets for the development of anti-EV-A71 drugs [14].

Staufen (Stau) protein belongs to the RNA binding protein (RBP) family and was first discovered in Drosophila [15]. In mammal, there are two different genes encoding two different Stau proteins, Stau1 and Stau2 [16]. Stau1 is ubiquitously expressed in various tissue cells, while Stau2 is mainly expressed in nerve cells [17, 18]. There are at least four subtypes of Stau2 protein with different molecular weights, including $Stau2^{62}$, $Stau2^{59}$, $Stau2^{56}$ and $Stau2^{52}$. Each subtype contains 3 ~ 5 double-stranded RNA binding domains (dsRBD) and one suspected tubulin binding domain (TBD), suggesting that Stau2 may connect the cytoskeleton and RNA complex [19]. The mRNA in mammalian neuronal cells is transported to the dendrites of neurons through ribonucleoproteins (RNPs) or RNA particles [16]. Most of the early studies of Stau2 focused on its role as a component of RNPs to participate in mRNA transport, translation and degradation [20]. Studies have shown that Stau2 can affect virus replication. Atoshi Banerjee et al. found that the Stau2 interacts with the Rev protein of human immunodeficiency virus type 1 (HIV-1) as a host factor [21]. And studies have shown that Stau specifically interacts with Gag protein and genomic RNA of HIV-1 to affect the replication of HIV-1 [22].

In this study, we first identified Stau2 as a novel 3C-interacting protein that promotes EV-A71 replication. Further experiments showed that EV-A71 3C cleaves Stau2 at Q507-G508 through its protease activity. In addition, we found that cleavage of Stau2 by EV-A71 3C contributes to the promotion of EV-A71 replication. These findings extend the mechanisms of virus-host interactions and provide ideas for the development of novel antiviral therapies.

Materials and methods

Plasmids and transfection

After extracting total RNA from HeLa, Stau2 cDNA was obtained by reverse transcription-PCR. The Stau2 cDNA was inserted into pCMV-Tag-2B (Flag tag) (Stratagene). Stau2 was constructed into different eukaryotic expression vectors based on pCMV-Tag2B-Stau2 using multiple primers by PCR. The plasmids were transfected into HeLa, HEK293T, SH-SY5Y or RD cells with polyethyleneimine (PEI) (Polysciences) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The primers used in this study are listed in Table 1.

Cell lines and viruses

Adherent (HEK293T, HeLa, SH-SY5Y and RD) cell lines were cultured in Dulbecco's modified Eagle Culture medium (DMEM) (high glucose; Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (HyClone) and 100 U/ml penicillin-streptomycin (GIBCO/BRL), and in 37℃, 5% CO_2 humidified atmosphere.

The EV-A71 infectious clone was digested with SalI for 6 h, and the linearized DNA obtained was purified and transcribed into RNA in vitro. After purification using the lithium chloride (LiCl) method, the RNA was transfected into RD cells using Lipofectamine 2000 (Invitrogen). After the RD cells died, the supernatant medium was collected and reinfected with fresh RD cells, and the culture was expanded for 2–3 rounds. A 50% Tissue Culture Infective Dose ($TCID_{50}$) assay was used to measure the titers of viruses. When performing viral infection experiments, in DMEM without FBS, EV-A71 with the indicated multiplicity of infection (MOI) was

Table 1 Primers used in this study

Primer name	Sequence (5′→3′)	Target plasmid
Stau2-F	GCGAGATATCATGGCAAACCCA AAAGAG	pCMV-Tag2B-Stau2 (Flag tag)
Stau2-R	GTTAGTCGACCTAGACGGCCGA GTTTGATT	pCMV-Tag2B-Stau2 (Flag tag)
Stau2-508-F	ATTTAGCAAGGATTCAAGCCTTT CAGGCAG	Stau2 mutant G508A
Stau2-508-R	CTGCCTGAAAGGCTTGAATCCT TGCTAAAT	Stau2 mutant G508A
Stau2-523-F	TTTTCTGAACAAGCACTGGATC CAATCG	Stau2 mutant G523A
Stau2-523-R	CGATTGGATCCAGTGCTTGTTCA GAAAATTG	Stau2 mutant G523A
Stau2-F	CGAGCTCATGGCAAACCCAAAA GAGAAAAC	pEGFP-C3-Stau2
Stau2-R	TCACGTCGACCTAGACGGCCGA GTTTGATT	pEGFP-C3-Stau2
Stau2-F	GCGATCTAGAGATGGCAAACCC AAAAGAG	pCMV-3HA-Stau2
Stau2-R	GTTAGATATCCTAGACGGCCGAG TTTGATT	pCMV-3HA-Stau2
Stau2-F	ATTAGCGGCCGCATGGGCCGCA TCTTTTAC	HA-pQCXIP-Stau2
Stau2-R	GCCTACGCGTCTAGACGGCCGA GTTTGATTT	HA-pQCXIP-Stau2
Stau2-1–507-F	ATTAGCGGCCGCATGGGCCGCA TCTTTTAC	HA-pQCXIP-Stau2-1–507
Stau2-1–507-R	GCCTACGCGTCTATTGAATCCT TGCT	HA-pQCXIP-Stau2-1–507
Stau2-508–570-F	ATTAGCGGCCGCATGGGCCGCA TCTTTTAC	HA-pQCXIP-Stau2-508–570
Stau2-508–570-R	TCACGTCGACCTAGACGGCCGA GTTTGATT	HA-pQCXIP-Stau2-508–570
Stau2shRNA1-F	gatccGCGAAATATGCCTGT-CAGTTTCAAGAGAACTGACAG-GCATATTTCGCTTTTTTg	pSIREN-RetroQ-shStau2#1
Stau2shRNA1-R	aattcAAAAAAGCGAAATATGCCT-GTCAGTTCTCTTGAAACTGA-CAGGCATATTTCGCg	pSIREN-RetroQ-shStau2#1
Stau2shRNA2-F	gatccGTTTATTAGGTCCT-GTTCCTTTCAAGAGAAGGAA-CAGGACCTAATAAATTTTTTg	pSIREN-RetroQ-shStau2#2
Stau2shRNA2-R	aattcAAAAAATTTATTAGGTCCT-GTTCCTTCTCTTGAAAGGAA-CAGGACCTAATAAACg	pSIREN-RetroQ-shStau2#2

incubated with the cells for 1.5 h, and then the medium was changed to contain 2% FBS.

Generation of Stau2 knockdown cell lines

Double-stranded oligonucleotides corresponding to the target sequences were synthesized, annealed, and cloned into pSIREN-RetroQ (Clontech). The pairs of targeting oligonucleotides were as follows: 5'-GATCCGCGAAATA TGCCTGTCAGTTTCAAGAGAACTGACAGGCATAT TTCGCTTTTTTG-3' and 5'-AATTCAAAAAAGCGAA ATATGCCTGTCAGTTCTCTTGAAACTGACAGGCA TATTTCGCG-3', 5'-GATCCGTTTATTAGGTCCTGTT CCTTTCAAGAGAAGGAACAGGACCTAATAATTTT TTG-3' and 5'-AATTCAAAAAATTTATTAGGTCCTG TTCCTTCTCTTGAAAGGAACAGGACCTAATAAAC G-3'. The retrovirus particles were prepared by co-transfecting HEK293T cells with pVSV-G, pMLV-Gag-Pol and pQCXIP-Stau2. After 48 h, the supernatant was collected and centrifuged at 3000 rpm for 10 min to remove cell debris. SH-SY5Y cells were cultured in a 12-well plate at a density of 0.2×10^6/well, containing 500 μl complete medium (1:1000 with polybrene added) and 500 μl lentivirus stock solution, centrifuged at 1500 rpm for 30 min, and then cultured in a 37℃ incubator. Cells were subcultured in selection medium containing 2 μg/ml puromycin (Sigma) until the control cells were completely dead. Knockdown efficiency was confirmed by Western blotting using specific antibodies.

Immunofluorescence assay (IFA)

HeLa cells were cultured on glass coverslips in 12-well cell culture plates. After washing with 1×phosphate buffered saline (PBS), the cells were treated with 4% paraformaldehyde for 10 min at room temperature. The cells were then treated with 4% paraformaldehyde containing 0.2% Triton X-100 for 10 min. After incubation in a blocking buffer containing 15% BSA for 2 h, cells were incubated with anti-Myc antibody for 90 min. After washing with 0.1% Triton X-100 PBS four times for 5 min, the cells were added with fluorescein isothiocyanate (FITC)-conjugated secondary antibody and incubated for 45 min. The nuclei were stained with 0.2 μg/ml DAPI for 10 min. Then the cover glass coverslips were fixed with 90% glycerol in PBS containing 1 mg/ml p-phenylenediamine and observed with an Olympus IX71 fluorescence microscope.

Coimmunoprecipitation (Co-IP)

HEK293T cells were collected after 48 h of transfection, and resuspended in a radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl buffer [pH 7.4] containing 1% NP-40, 150 mM NaCl, and 0.25% sodium deoxycholate) containing protease inhibitor cocktail (Roche, Indianapolis, IN). The cell suspension was lysed by sonication on ice and centrifuged at 12,000 rpm to collect the supernatants. 50 μl of the supernatants were taken out as the input sample, while the remaining lysates were slowly mixed with 3 μl of mouse anti-HA antibody on a 4℃ chromatographic cabinet rotating mixer for 3 h. After adding pre-treated Protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), the cell lysates were slowly mixed on a 4℃ chromatography cabinet rotating mixer for 3 h. The immunocomplex captured on the protein A-agarose was added to 40 μl 2 × Loading Buffer as the IP sample. The IP sample and Input sample were

fractionated by 10% SDS-PAGE and subjected to Western blotting.

Western blotting

Cells were collected after 48 h of transfection, and the cell lysates or immunoprecipitation materials were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). The PVDF membranes were placed in a 5% nonfat milk blocking solution prepared with PBST (0.05% Tween 20 in PBS) for 45 min or in a refrigerator overnight at 4℃. The membranes were incubated with specific primary antibodies for 90 min at room temperature, and then incubated with suitable secondary antibodies for 45 min. The signal from the immunoreactive proteins were detected using Luminata™ Western HRP substrate (Millipore) and X-ray film.

Results

EV-A71 3C interacts with Stau2

In our previous research, using 3C as the bait and human universal cDNA library, a yeast two-hybrid screen was performed to identify potential proteins that interact with EV-A71 3C [23]. Sixty positive clones were obtained. One positive clone contained an in-frame 222 bp partial cDNA encoding amino acids 184 to 257 of the human Stau2 protein. The yeast two-hybrid screen has a high false-positive rate, so we confirmed the interaction between 3C and Stau2 by coimmunoprecipitation in mammalian cells. To prevent Stau2 from being cleaved by 3C protease and thus undetectable, we used 3C-C147S, a 3C mutant with impaired protease activity, instead of 3C wild-type for immunoprecipitation experiments [23]. GFP-3C-C147S and HA-Stau2 were co-transfected into HEK293T cells, followed by coimmunoprecipitation with anti-HA. The results showed that 3C-C147S interacted with Stau2 (Fig. 1A). Immunofluorescence analysis also showed that 3C and Stau2 could co-localize in the cytoplasm (Fig. 1B). Altogether, these results consistently revealed the interaction between 3C and Stau2.

EV-A71 3C cleaves Stau2, and 3C protease activity is required for the cleavage

EV-A71 3C is an important protease and has been shown to cleave a variety of host proteins [24–27]. We performed cleavage assays to examine whether 3C cleaves Stau2. When 3C and Stau2 were co-expressed, full-length Stau2 and a smaller band of approximately 55 kDa were detected (Fig. 2A), suggesting the detection of cleavage band. HEK293T cells were transfected with increasing amounts of Myc-3C, along with Flag-Stau2. At 48 h after transfection, Stau2 was detected by Western blotting. As shown in Fig. 2B lanes 2 to 4, 3C reduced the full length of Stau2 and increased the cleavage band in a dose-dependent manner. EV-A71 3C also possesses RNA binding activity in addition to its protease activity [28]. 3C-R84Q, a mutant that has been shown to abolish only RNA-binding activity but not protease activity, still cleaved Stau2 (Fig. 2B, lane 6), while the 3C-C147S with impaired protease activity failed to cleave Stau2 (Fig. 2B, lane 5). These results suggest that protease activity, but not RNA-binding activity, is necessary for 3C to cleavage Stau2. Next, we further explored whether the cleavage was related to the RNA-binding activity of Stau2. GFP-Stau2-ΔdsRBD-3, a Stau2 mutant that has been shown to abolish RNA-binding activity [17, 29], remains cleaved by 3C (Fig. 2C, lane 2). Taken together, these results demonstrated that EV-A71 3C cleaved Stau2 and that the protease activity of 3C, but not the RNA binding activity, is essential for cleavage.

EV-A71 3C cleaves Stau2 at Q507-G508

EV-A71 3C specifically recognizes and cleaves polypeptides with Gln-Gly (Q-G) or Gln-Ser (Q-S) linkages [30]. To identify the 3C cleavage site(s) on Stau2, we performed mutational analysis of Stau2. There are three potential cleavage sites on Stau2: Q304-G305, Q507-G508 and Q522-G523 (Fig. 3A). Since Stau2 was cleaved by 3C to generate a specific fragment of approximately 55 kDa, we deduced that Q507-G508 or Q522-G523 were potential cleavage sites for 3C. The Stau2 G508A and G523A mutants were co-expressed with 3C or 3C-C147S in HEK293T cells, respectively. The result of Western blotting showed that the wild-type Stau2 and G523A were cleaved by 3C but not 3C-C147S, while G508A was resistant to cleavage, suggesting that Q507-G508 is the cleavage site on Stau2 (Fig. 3B). However, only the larger N-terminal cleavage band of Stau2 was detected, the smaller C-terminal cleavage band was not detected. To detect the smaller cleavage band at the C-terminus, we constructed the double-tagged eukaryotic expression plasmid GFP-Stau2-GST. HEK293T cells were co-transfected with GFP-Stau2-GST and 3C or 3C-C147S. At 48 h after transfection, Stau2 was detected by Western blotting. As shown in Fig. 3C, 3C cleaved GFP-Stau2-GST and produced two cleavage bands of 82 kDa and 33 kDa. These results suggested that EV-A71 3C cleaves Stau2 at Q507-G508.

Stau2 promotes EV-A71 replication

The EV-A71 3C protease is a key point of interaction between the virus and the host, and a variety of host proteins that interact with it affect EV-A71 replication. To explore whether Stau2 influences EV-A71 replication, Stau2 was overexpressed in RD and HeLa cells followed by EV-A71 infection. The cells were infected with EV-A71 at the indicated multiplicity of infection (MOI).

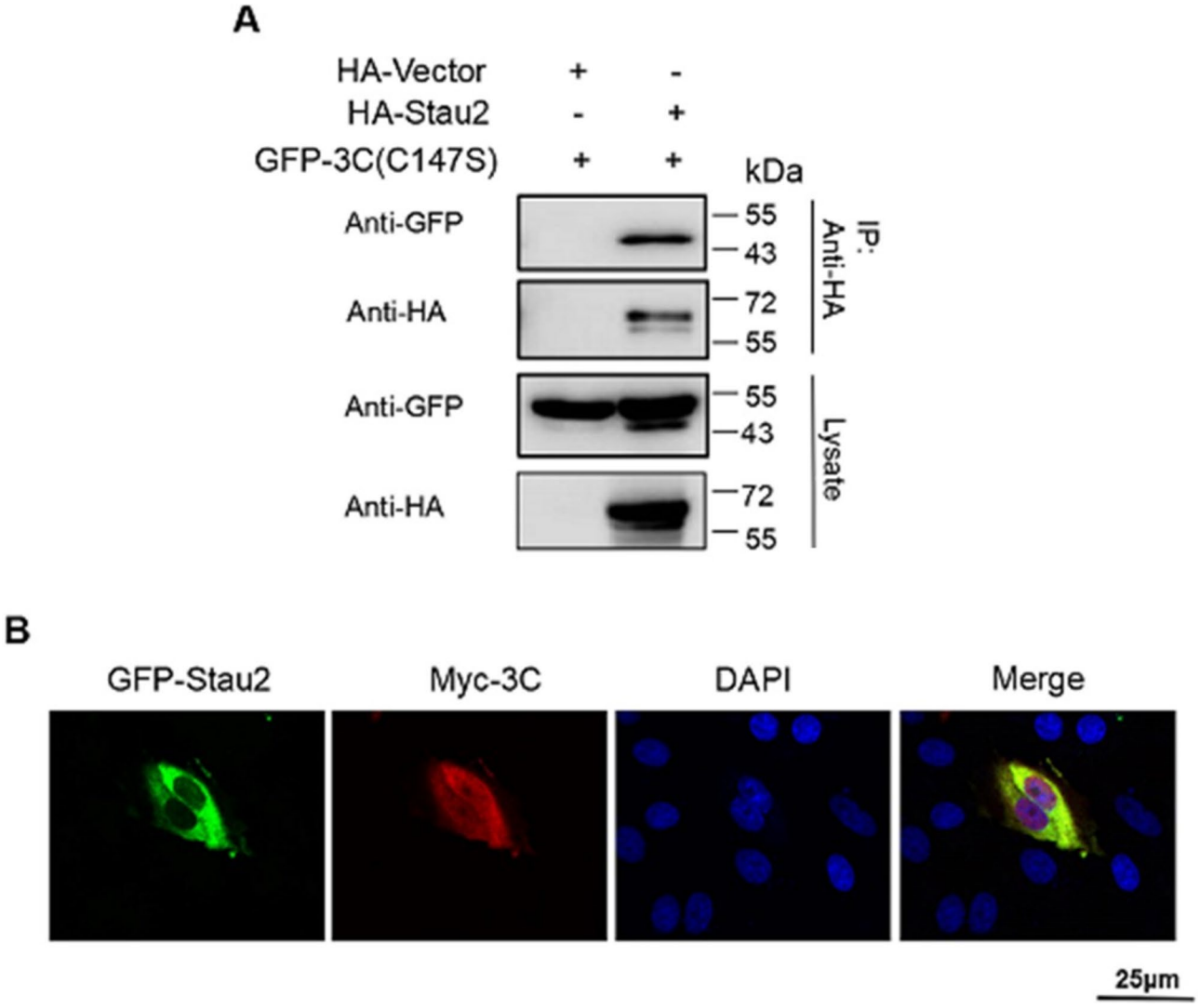

Fig. 1 EV-A71 3C interacts with Stau2. (**A**) HEK293T cells were co-transfected with HA-vector or HA-Stau2 and GFP-3C-C147S. Cells were lysed for CO-IP 48 h after transfection, and the proteins were detected by Western blotting. (**B**) HeLa cells were co-transfected with Myc-3C and GFP-Stau2 for 48 h. Cells were subjected to immunofluorescence staining, and the cell nuclei were stained with DAPI. 400× magnification was used

At 24 h post-infection, cell lysates were subjected to Western blotting. The EV-A71 VP1 protein expression level was increased in the Stau2-overexpressing groups compared with the negative control in both HeLa and RD cells (Fig. 4A and B). Stau2 is reported to be highly expressed in neural cells [18]. So we further explored the effect of Stau2 on EV-A71 virus replication in human neuroblastoma SH-SY5Y cells. Lentiviral packaging was used to construct shRNA knockdown cell lines targeting Stau2. The shRNA control, shRNA Stau2#1 and shRNA Stau2#2 SH-SY5Y cells were infected with EV-A71 at the indicated MOI for 24 h. As shown in Fig. 4C, shRNA #2, which knocked down Stau2 better, inhibited EV-A71 replication more than #1. Our results suggested that Stau2 promotes EV-A71 replication.

Cleavage of Stau2 by 3C contributes to promoting EV-A71 replication

To explore the influence of Stau2 cleavage on EV-A71 replication, the effects of wild-type Stau2 and the mutant Stau2-G508A, which cannot be cleaved by 3C, on EV-A71 replication were compared. HA-Stau2, cleavage products HA-Stau2-1–507, 508–570, and mutant HA-Stau2-G508A were transfected into RD cells or HeLa cells. After 24 h, cells were infected with EV-A71 at the indicated MOI. Cells were collected 24 h after infection, and the expression of VP1 protein was detected by Western blotting. The results showed that in both RD cells (Fig. 5A) and HeLa cells (Fig. 5B), wild-type Stau2 promoted EV-A71 replication, while mutant Stau2-G508A did not. This suggested that the cleavage of Stau2 by EV-A71 3C facilitates viral replication. Among the

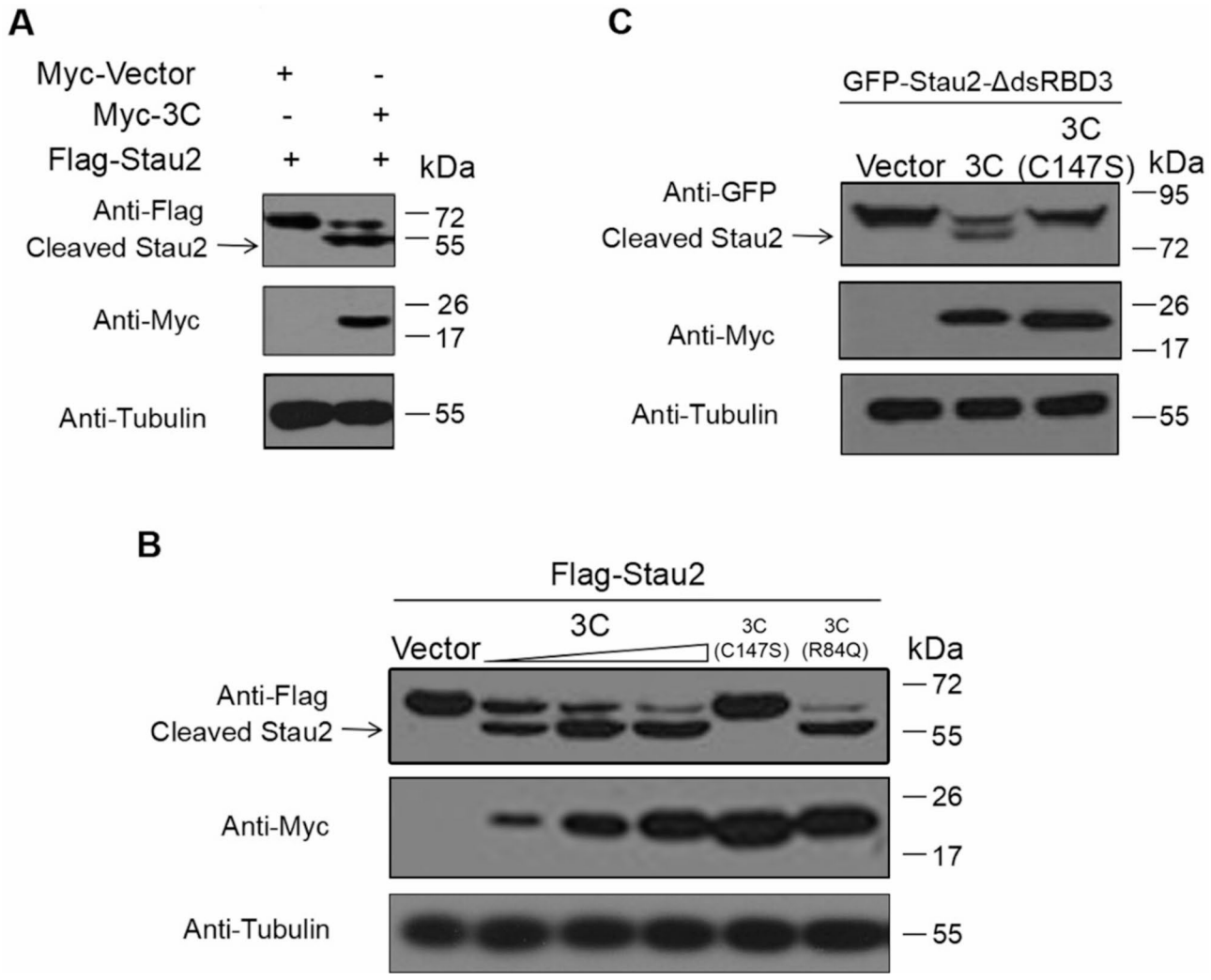

Fig. 2 3C cleaves Stau2 through protease activity but not RNA binding activity. (**A**) HEK293T cells were co-transfected with Myc-vector or Myc-3C and Flag-Stau2. After 48 h, cell lysates were harvested and subjected to Western blotting. (**B**) The increasing amounts of Myc-3C (0, 0.1 μg, 0.3 μg, and 0.6 μg for lanes 1 to 4, respectively), different 3C variants including 3C-C147S (lane 5) or 3C-R84Q (lane 6) and Flag-Stau2 were co-transfected into HEK293T. After 48 h, Stau2 was detected to be cleaved by Western blotting. (**C**) Co-transfection analysis of Myc-3C or Myc-3C-C147S and GFP-Stau2-ΔdsRBD-3. After 48 h, cell lysates were tested by Western blotting

cleavage products, C-terminal product Stau2-508–570 upregulated EV-A71 replication, while N-terminal product Stau2-1–507 did not. This result indicated that 508–570 aa are the key domain of Stau2 promoting EV-A71 replication. Overall, the cleavage of Stau2 by EV-A71 3C contributes to promoting EV-A71 replication, and the functional domain is present at 508–570 aa of Stau2.

EV-A71 3C cleaves mouse Stau2 at Q507-G508

The above results have shown that human Stau2 (hStau2) is one of the substrates of the EV-A71 3C protease. Based on sequence alignment analysis, the nucleic acid sequence similarity between human and mouse Stau2 is over 90%. We speculated that mouse Stau2 (mStau2) was also a substrate for 3C protease. The mStau2 gene was obtained by PCR amplification from the mouse brain cDNA library and used to construct the eukaryotic expression plasmid pCMV-Tag2B-mStau2 (N-Flag). Flag-mStau2 was co-transfected into HEK293T cells together with a gradient increase of Myc-3C, mutant 3C-C147S or 3C-R84Q. The Western blotting results showed that 3C and 3C-R84Q cleaved mStau2 to produce a cleavage band, whereas 3C-C147S, which was deficient in enzymatic activity, did not cleave (Fig. 6A), suggesting that mStau2, the same as hStau2, is a cleavage substrate for EV-A71 3C. We speculated that the cleavage site of mStau2 was the same as that of hStau2. Flag-mStau2 or mutant Flag-mStau2-G508A was co-transfected with Myc-3C or 3C-C147S in HEK293T cells. The Western blotting results showed that mStau2-G508A did not produce the cleavage band under the action of 3C (Fig. 6B), that is, Q507-G508 is also the site where mStau2 is

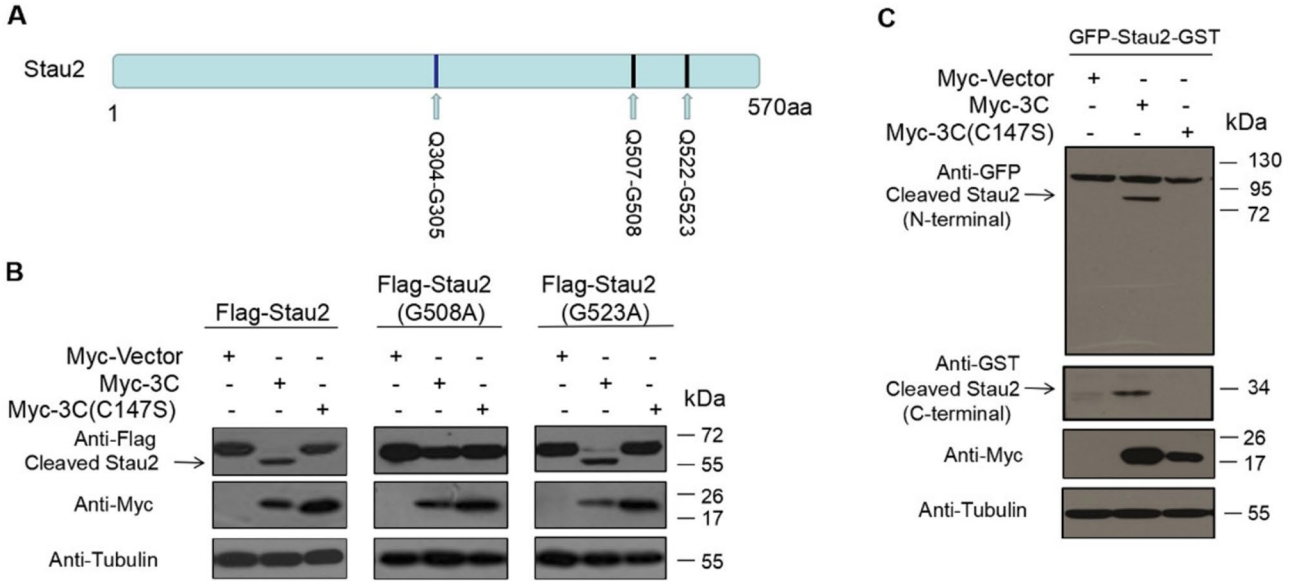

Fig. 3 Q507-G508 is the site where Stau2 is cleaved by 3C. (**A**) Schematic illustration of the potential cleavage sites by 3C on Stau2. (**B**) Flag-Stau2 wild-type or variants and Myc-3C or Myc-3C-C147S were co-transfected in HEK293T cells, and the Stau2 cleavage products were detected by Western blotting 48 h later. (**C**) HEK293T cells were co-transfected with Myc-vector or Myc-3C or Myc-3C-C147S and GFP-Stau2-GST. After 48 h, cell lysates were harvested and subjected to Western blotting

cleaved by 3C. These results indicated that the cleavage of Stau2 by EV-A71 3C protease is conservative in human and mouse, providing a theoretical basis for possible future experiments in animal models.

Discussion

As one of the seven nonstructural proteins encoded by the EV-A71 viral genome, 3C plays an important role in viral genome replication, viral particle maturation and virus-host interactions. 3C cleaves a variety of host proteins to disrupt normal host physiological activities to promote viral replication [24, 26]. In this study, we identified Stau2 as a novel target of EV-A71 3C and clarified the detailed site of Stau2 cleavage mediated by 3C protease. Both overexpression and knockdown results indicated that Stau2 promotes EV-A71 replication and that cleavage of Stau2 by 3C has a synergistic effect on promoting EV-A71 replication. The functional domain of Stau2 that promotes EV-A71 replication is localized at 508–570 aa. In addition, our data support that EV-A71 3C cleavage sites for both human Stau2 and mouse Stau2 are at Q507-G508.

We have been exploring the interaction of the EV-A71 3C protease with the host protein. In previous studies, we screened out the host proteins PinX1 and TRAF3IP3 interacting with 3C using yeast two-hybrid, identified the cleavage sites in detail, and explained the significance of cleavage for the host proteins affecting EV-A71 replication [23, 31]. Stau2 in this study was similar to PinX1 and TRAF3IP3. Co-IP and IFA experiments confirmed the yeast two-hybrid result that Stau2 interacts with EV-A71 3C. By detecting the cleavage bands and constructing mutants, Stau2 was identified as a substrate for 3C and the only cleavage site was at Q507-G508. There are three Q-G sites in the full-length Stau2 sequence, and only Q507-G508 (^{504}ARIQ/G^{508}) matches the characteristic sequence AxxQ/G cleaved by 3C (x represents any amino acid and the cleavage site is indicated by a slash) [32, 33]. Stau2 is another typical host protein we identified that interacts with the EV-A71 3C protease and is cleaved by 3C. However, in contrast to the inhibitory effects of PinX1 and TRAF3IP3, Stau2 promotes EV-A71 replication, and the cleavage of Stau2 by 3C is more conducive to this promotion. This reflects the versatility, complexity and flexibility of viral proteases in interacting with the host and regulating host protein function. But the ultimate responsibility of viral proteases is to tend to contribute to viral replication.

Stau2 is a double-stranded RNA-binding protein (dsRBP) with multiple double-stranded RNA-binding domains (dsRBD) [29, 34]. As a core component of RNPs, Stau2 is involved in the transport, translation and degradation of mRNA. Stau2 has been reported to promote HIV-1 proliferation by positively regulating RNA export activity of viral protein Rev. Although both are RBPs, human Stau2 and HIV-1 Rev interact in an RNA-independent manner [21, 35]. Similarly, chicken Stau2 interacts with nonstructural protein 1 and promotes replication of H5N1 avian influenza virus by enhancing the nuclear export of NS1 mRNA [36]. On the other hand, Stau2 interacts with HIV-1 Gag and is incorporated into viral particles, and encapsulated Stau2 enhances viral

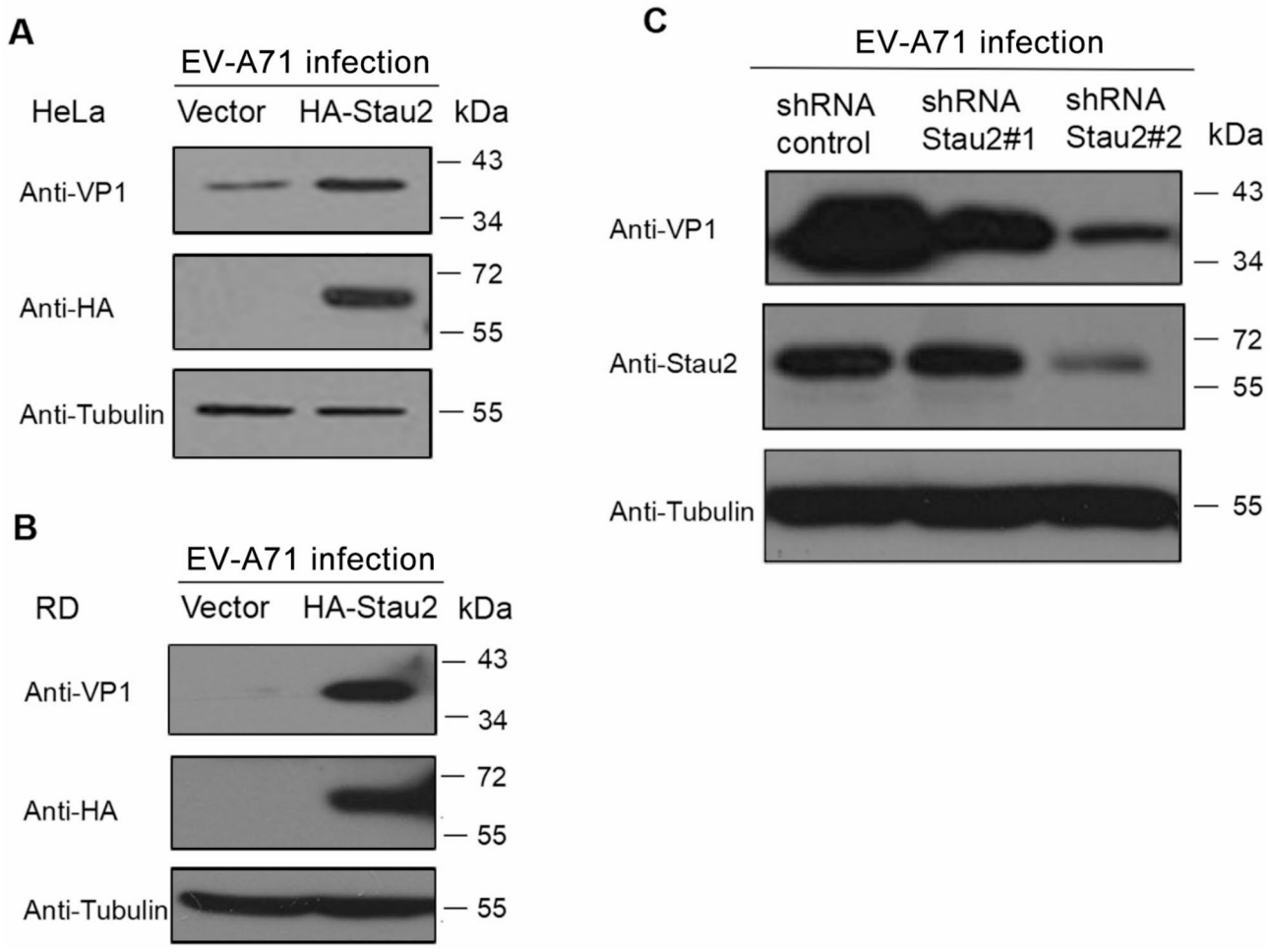

Fig. 4 Stau2 promotes EV-A71 replication. (**A** and **B**) The HA-vector or HA-Stau2 was transfected into HeLa cells (**A**) or RD cells (**B**). 24 h after transfection, cells were infected with 0.2 MOI EV-A71 (**A**) or 0.01 MOI EV-A71 (**B**) for 24 h. In harvested cells, the expression of EV-A71 VP1 was detected by Western blotting. (**C**) SH-SY5Y-shRNA control, SH-SY5Y-shRNA Stau2#1 and SH-SY5Y-shRNA Stau2#2 cells were infected with 0.01 MOI EV-A71 for 24 h. The expression of EV-A71 VP1 and endogenous Stau2 were detected by Western blotting

infectivity [37]. The interaction of Stau2 with Nsp2 protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also been reported [38]. Our study shows that Stau2 interacts with EV-A71 3C independently of RNA and that cleavage by 3C contributes to Stau2's role in promoting EV-A71 replication. The functional domains retained on 3C-cleaved Stau2 that promote EV-A71 replication are 508–570, not 1–507, which contains all dsRBDs. This suggests that Stau2 promotes EV-A71, as opposed to the previously reported RNA binding activity, possibly by interacting with viral proteins such as 3C, and may act at multiple stages of viral replication. The interaction of Stau2 with EV-A71 viral proteins remains to be investigated.

Stau protein is a key regulator of neuronal development and function [20]. Stau2 is enriched in the brain and is involved in RNA transport, mRNA stability and translation, and is also associated with dendritic spine extension and synapse number [20, 39]. Although HFMD is a self-limiting disease, it may induce severe neurological complications. There is increasing evidence that EV-A71 can directly infect neurons in the central nervous system [40]. However, the molecular mechanisms of EV-A71-associated neuroinflammation and pathogenesis are not fully clear. To investigate whether the interaction between EV-A71 3C and Stau2 plays a role in the process of EV-A71 infection triggering neurological disease, we will next introduce an animal experimental model. Our results suggest that the cleavage of Stau2 by EV-A71 3C protease is conservative in human and mouse. This will allow to validate Stau2 function in mouse models in the future.

In summary, the limitation of this study is that it has not yet delved into the detailed molecular mechanisms of how 3C cleavage plays a role in the promotion of EV-A71 replication by Stau2. In what function does Stau2 promote EV-A71 replication? How is 3C involved? Where are the specific sites of interaction between Stau2 and

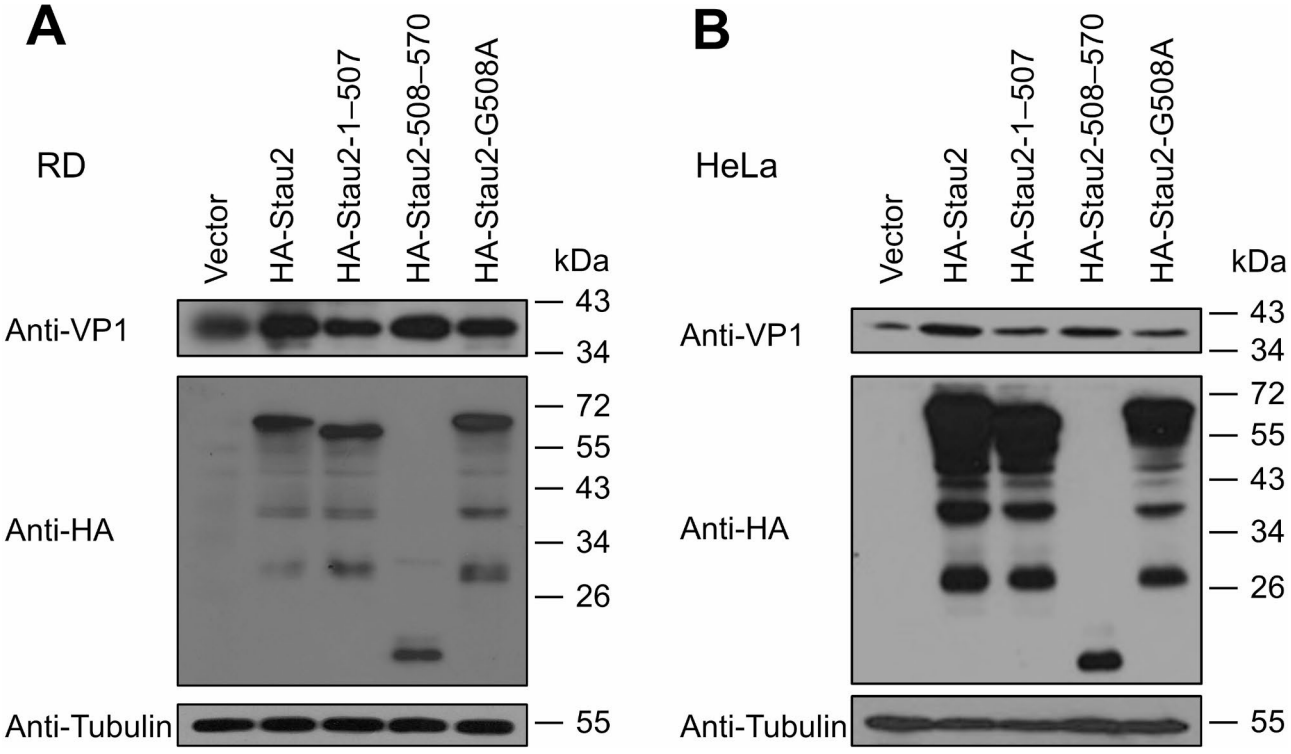

Fig. 5 The C-terminal cleavage product of Stau2 promotes EV-A71 replication. (**A** and **B**) RD cells (**A**) and HeLa cells (**B**) were transfected with HA-vector, HA-Stau2, HA-Stau2-1–507, HA-Stau2-508–570 or HA-Stau2-G508A. After 24 h of transfection, cells were infected with EV-A71 virus at 0.01 MOI (**A**) and 0.2 MOI (**B**), respectively, and harvested for Western blotting 24 h after infection

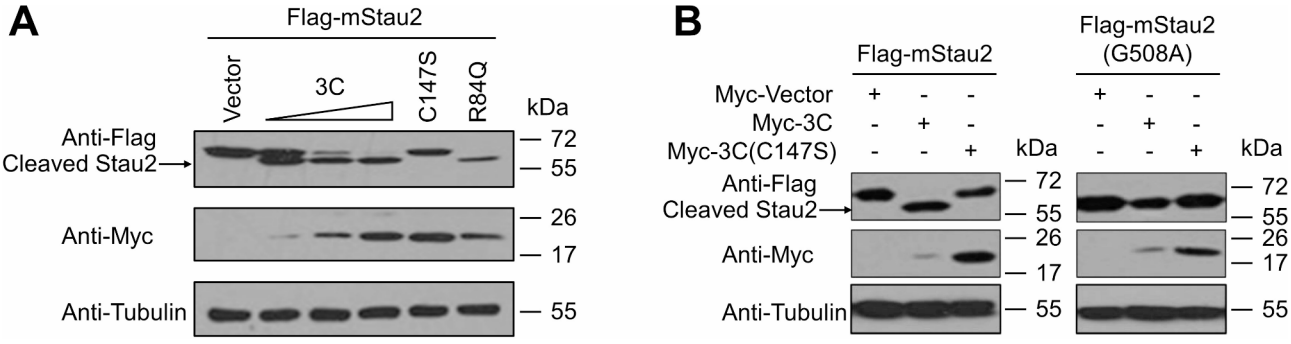

Fig. 6 EV-A71 3C cleaves mouse Stau2 at the Q507-G508. (**A**) Flag-mStau2 and Myc-vector, the increasing amounts of Myc-3C (0.1 μg, 0.3 μg and 0.6 μg for lanes 2 to 4, respectively), Myc-3C-C147S or Myc-3C-R84Q were co-transfected into HEK293T cells. After 48 h, mStau2 cleavage was detected by Western blotting. (**B**) Flag-mStau2 or Flag-mStau2-G508A and Myc-3C or Myc-3C-C147S were co-transfected into HEK293T cells. Western blotting was performed after 48 h

3C? How does Stau2 behave in nerve cells? These outstanding issues are the direction of our next research. Answering these questions will be of greatly benefit in the search for novel drug targets as well as in the use of mouse models to study pathogenic mechanisms and antiviral therapies.

Conclusions

In this study, Stau2 was identified for the first time as a cleavage substrate for EV-A71 3C and the specific cleavage site were determined. We found that Stau2 promotes EV-A71 replication and that 3C cleavage to Stau2 contributes to the promotion. In addition, the presence of the same 3C cleavage site in mouse Stau2 provides a basis for further studies. In conclusion, our study provides a novel example of host-virus protease interactions and offers new ideas for drug development.

Abbreviations

Co-IP	Coimmunoprecipitation
dsRBD	Double-stranded RNA binding domains
EV-A71	Enterovirus A71
HFMD	Hand, foot and mouth disease

hStau2	Human Stau2
IFA	Immunofluorescence assay
MOI	Multiplicity of infection
mStau2	Mouse Stau2
RBP	RNA binding protein
Stau2	Staufen homolog 2

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Author contributions
HL, JS, ZD, and YY performed the experiments. HL and JS analyzed the data and wrote the manuscript. JT and WQ provided general guidance and financial support.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors consent to the publication of the manuscript.

Competing interests
The authors declare no competing interests.

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