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Tribbles pseudokinase 3 promotes enterovirus A71 infection via dual mechanisms

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ABSTRACT

Enterovirus A71 (EV-A71) is the main pathogen causing hand, foot and mouth disease (HFMD) in children and occasionally associated with neurological diseases such as aseptic meningitis, brainstem encephalitis (BE) and acute flaccid paralysis. We report here that cellular pseudokinase tribbles 3 (TRIB3) facilitates the infection of EV-A71 via dual mechanisms. In one hand, TRIB3 maintains the metabolic stability of scavenger receptor class B member 2 (SCARB2), the *bona fide* receptor of EV-A71, to enhance the infectious entry and spreading of the virus. On the other hand, TRIB3 facilitates the replication of EV-A71 RNA in a SCARB2-independent manner. The critical role of TRIB3 in EV-A71 infection and pathogenesis was further demonstrated *in vivo* in mice. In comparison to wild-type C57BL/6 mice, EV-A71 infection in TRIB3 knockdown mice (*Trib3*$^{+/-}$) resulted in significantly lower viral loads in muscular tissues and reduced lethality and severity of clinical scores and tissue pathology. In addition, TRIB3 also promoted the replication of coxsackievirus B3 (CVB3) and coxsackievirus A16 (CVA16) *in vitro*. In conclusion, our results suggest that TRIB3 is one of key host cellular proteins required for the infection and pathogenesis of EV-A71 and some other human enteroviruses and may thus be a potential therapeutic target for combating the infection of those viruses.

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Introduction

Enterovirus A71 (EV-A71), a single-stranded positive sense RNA virus belonging to the Enterovirus genus of the *Picornaviridae* family, is a major aetiological agent of hand, foot and mouth disease (HFMD) in children mostly under 5 years old [1-3]. EV-A71 is spread through contact with virus-containing body fluids, respiratory droplets, and faeces. EV-A71 infections usually cause mild clinical symptoms, and are occasionally associated with neurological diseases such as aseptic meningitis, brainstem encephalitis (BE), acute respiratory failure, acute flaccid paralysis and fatal pulmonary oedema (PE) [4-5]. However, clinical data have shown that severe EV-A71 infection can lead to death [6]. EV-A71 was first isolated from patients with central nervous system (CNS) diseases in California in 1969 [7]. Since then, periodic outbreaks have been reported worldwide, especially in the Asia Pacific Region. Currently, EV-A71 has become a medically important neurotropic enterovirus in the post-polio era. However, there is no approved antiviral drug to treat EV-A71 infection.

EV-A71 enters host cells through three stages: attachment, endocytosis and uncoating. Scavenger receptor class B member 2 (SCARB2) is a major receptor for EV-A71, which can support all three stages of EV-A71 entry [8]. In addition, other molecules such as P-selectin glycoprotein ligand-1 (PSGL-1), sialylated glycans, annexin II (Anx2) and heparan sulphate (HS) proteoglycan etc. serve as "attachment receptors" since they support viral attachment to the cell surface but cannot trigger uncoating [9]. Viral genomic RNA is released into the cytoplasm upon capsid uncoating [10]. Viral genomic RNA first serves as a mRNA to translate the polyprotein, which is subsequently processed by viral proteases to produce 4 structural proteins (VP1~VP4) and seven non-structural

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proteins (2A∼2C, 3A∼3D) [1,2]. VP1∼VP4 are involved in the assembly of the viral protein coats, while 2A∼2C and 3A∼3D participate in the replication of the viral genome in the endoplasmic reticulum (ER)-membrane derived replication organelles. The progeny viral genomic RNAs are then packaged by VP1∼VP4 to form viral particles and released from infected cells [11,12].

As for all the other viruses, EV-A71 relies on many different cellular proteins to support its replication in susceptible cells. For instance, fibronectin, a high-molecular-weight extracellular glycoprotein, interacts with EV-A71 capsid protein VP1 to facilitate virus entry into host cells [13]. Several cellular nuclear proteins, including heterogeneous nuclear ribonucleoproteinA1 (hnRNP A1), heterogeneous nuclear ribonucleoprotein K (hnRNP K), far upstream element binding protein 1 (FBP1) and far upstream element binding protein 2 (FBP2), are translocated into the cytoplasm upon EV-A71 infection and promote EV-A71 internal ribosome entry site (IRES)-regulated viral protein translation through direct binding to IRES [14-17]. ER-resident acetyltransferase N-acetyltransferase 8 (NAT8) facilitates EV-A71 replication by increasing the stability of EV-A71 2B, 3AB, and 3C proteins [18]. Studies have shown that heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70) isoforms HSPA8 and HSPA9 promote the assembly of progeny EV-A71 particles, while HSPA1 and HSPA8 participate in the release of virus particles [19,20]. It is, therefore, apparent that EV-A71 exploits host cellular proteins to support almost every step of its life cycle.

Tribbles (TRIB) proteins are pseudokinases first discovered in Drosophila [21-24]. Due to the lack of at least one conserved catalytic residue in its kinase domain, TRIB proteins showed very low or no kinase activity [21-24]. TRIB proteins have three functional regions, including central kinase-like domain (KD), N-terminal and C-terminal protein-binding domains, among which the KD domain is relatively conserved [25,26]. The expression of TRIB3 (also known as TRB3, NIPK, SKIP3, and SINK), a member of the TRIB family, is regulated by ER stress, oxidative stress and nutrient deficiencies [27-29]. TRIB3 regulates cell growth, metabolism and cell stress under physiological conditions, and it is also involved in the development of a variety of diseases, such as diabetes, cancer, cardiovascular diseases and neurological diseases [30-35]. In addition, TRIB3 acts as a scaffolding protein to regulate the stability and activity of client proteins to reach its impact on diverse cellular processes. TRIB3 promotes acute promyelocytic leukaemia (APL) progression through stabilization of the oncoprotein PML-RARα and inhibition of p53-mediated senescence [36]. By interacting with MDM2, TRIB3 obstructs the ubiquitination degradation of SLUG, causing SLUG accumulation and thus promoting pulmonary fibrosis [37]. Through physical interaction with STAT3, TRIB3 enhances STAT3-mediated transcriptional activity and increases VEGF-A expression to promote tumour angiogenesis [38]. Taken together, TRIB3 is considered to be a key "stress regulatory switch," a biomarker and potential therapeutic target for many diseases.

At present, the role of TRIB3 in viral replication was rarely reported. Previous studies had found that hepatitis C virus (HCV), bovine viral diarrhoea virus (BVDV), Japanese encephalitis virus (JEV) and Zika virus infections all increase the expression of TRIB3 [39-41]. While TRIB3 restricts the entry of HCV into hepatocytes, interaction of HCV NS3 with TRIB3 disrupts TRIB3-Akt interaction and promotes the persistent infection of HCV in hepatocytes [39]. However, the effects of TRIB3 on the replication of BVDV, JEV and Zika virus remain to be determined [40,41]. Recently, Carvalho et al. found that the decreased expression of TRIB3 in the aged lung may be associated with more severe cases of the COVID-19, and they predicted that TRIB3 may interact with the nucleocapsid protein and the RNA-dependent RNA polymerase of SARS-CoV-2 to inhibit the virus replication [42, 43].

In this study, the expression of TRIB3 was found to be induced in EV-A71-infected cells and mice, and TRIB3 may thus play an important role in EV-A71 infection. Further genetic and biochemical analyses clearly demonstrated that TRIB3 promotes EV-A71 infection and pathogenesis by facilitating the infectious entry and genomic RNA replication of the virus in cultured cells and *in vivo* in mice.

Materials and methods

Cells and viruses

Human colon cancer (HCT-8) cells, human embryonic malignant rhabdomyoma (RD) cells and human embryonic kidney (293 T) cells were purchased from the Cell Culture Center of Chinese Academy of Sciences, and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, USA) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin, Invitrogen) at 37°C in a 5% CO_2 incubator. TRIB3-KO cells [44] were kind gift from Prof Zhuowei Hu, Institute of Materia Medica, Chinese Academy of Medical Science and Peking Union Medical School, Beijing, China. African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection and cultured in Modified Eagle's Medium (Invitrogen) supplemented with 10% inactivated FBS (Gibco) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) at 37°C in a 5% CO_2 incubator. Normal human colon epithelium (FHC) cells, purchased from Shanghai Jining Shiye Co., Ltd. and

cultured in RPMI-1640 supplemented with 10% inactivated FBS (Gibco) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) at 37°C in a 5% CO_2 incubator. HCCLM3 cells were purchased from the China Center for Type Culture Collection (CCTCC), and were cultured in DMEM supplemented with 10% FBS (Gibco) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin, Invitrogen) at 37° C in a 5% CO_2 incubator. The construction of SCARB2-KO HCCLM3 cells is as described in our previous study [45].

EV-A71 strain H (VR-1432) and strain BrCr (VR-1775) were purchased from the ATCC. EV-A71 strain SZ-98 and CVA16 strain (shzh05-1/GD/CHN/2005) were kindly provided by Jianwei Wang, Institute of Pathogen Biology, Chinese Academy of Medical Science and Peking Union Medical School. EV-A71 strain JS-52 were kindly provided by Xiangzhong Ye, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. CVB3 (strain Nancy) was obtained from ATCC. The mouse-adapted EV-A71 strain (EV-A71-H-MA) used in *in vivo* experiments was obtained by the adaptive transmission of mice in our laboratory. All the viruses were propagated in Vero cells.

Mice

C57BL/6 mice, obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., were housed under specific pathogen-free conditions in individual ventilated cage. *Trib3* heterozygous C57BL/6 mice (C57BL/6-*Trib3*^{tm1Cyagen}, referred as *Trib3*$^{+/-}$), generated by Cyagen Biosciences Inc (Guangzhou, China), were housed under specific pathogen-free conditions in ventilated cage. The mice were raised and cared according to the guidelines of the Animal Care and Welfare Committee in the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College for the Ethics of Animal Care and Treatment.

Mouse infection experiments

Mouse infection experiments were performed under animal biosafety level 2 conditions. All the animal procedures were conducted according to the protocols approved by the Animal Care and Welfare Committee in the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College. For *in vivo* assessment of mortality and morbidity under the lethal EV-A71 challenge, 12-day C57BL/6 wide-type (WT) mice and *Trib3*$^{-/+}$ mice were infected with 10 LD_{50} (20,000 $TCID_{50}$) of EV-A71-mouse adapted strain (EV-A71-H-MA), mice survival was observed for 11 days, and photos were taken 5 days post infection (dpi). For the sub-lethal EV-A71 challenge, 12-day C57BL/6 WT mice and *Trib3*$^{-/+}$ mice were infected with 1 LD_{50} (2000 $TCID_{50}$) of EV-A71-H-MA, mice survival was observed for 12 days. For the non-lethal EV-A71 challenge, 12-day C57BL/6 WT mice and *Trib3*$^{-/+}$ mice were infected with 0.1 LD_{50} (200 $TCID_{50}$) of EV-A71-H-MA, mice were weighed daily and observed for morbidity daily for 13 days. The clinical manifestation scores were graded with the previously described standards [46]. The severity of clinical symptoms was scored as: 0, healthy; 1, hair scattered; 2, limb-shaking weakness; 3, hind limb paralysis; and 4, moribund or dead.

For *in vivo* assessment of viral propagation and histopathology in the sub-lethal EV-A71 infected mice, the protocol for EV-A71 infection was the same as described above. Three mice enrolled in each group were dissected at 3 or 5 dpi. Muscle tissues prepared from the mice were used for viral protein expression and viral titer assays. Tissues prepared from mice were fixed in 10% neutral buffered formalin for applied in pathological for haematoxylin and eosin (H&E) assay and immunohistochemical (IHC) analyses with antibody against EV-A71 VP1 (GeneTex) [47].

Plasmids

TRIB3-Myc (HG10731-CM), SCARB2-Flag (HG11063-CF), SCARB2-Myc (HG11063-CM) and NEDD8-Myc (HG15620-CM) were purchased from Sino Biological Inc (Beijing, China). Myc-Ub-K63R and Myc-Ub-K48R were purchased from miaolingbio Inc (Wuhan, China). TRIB3-HA, control-HA, TRIB3-FL-GFP, TRIB3-C-GFP, TRIB3-KD-GFP, TRIB3-KDC-GFP, TRIB3-DKDC-GFP, Ub-Flag, Ub-K63-Flag, Ub-K48-Flag were kindly provided by Zhuowei Hu, Institute of Materia Medica, Chinese Academy of Medical Science and Peking Union Medical School, Beijing, China [44, 48]. VP1-HA, 2A-V5, 2B-HA, 2C-HA, 3A-HA, 3C-HA were purchased from Inovogen Tech. Co. (Chongqing, China) [49]. pGFP-VP2-4, pGFP-3B and pGFP-3D were kindly provided by Zhaohua Zhong, Department of Microbiology, Harbin Medical University [49].

Production of EV-A71 subgenomic replicon RNA

According to the sequence of EV-A71 (FY strain, GenBank accession number EU703812), we generated a subgenomic replicon by replacing the structural genes with *a* luciferase reporter gene. The cDNA encoding a luciferase reporter gene of EV-A71 subgenomic replicon was synthesized and constructed into pUC57 vectors through total gene synthesis. A luciferase reporter gene was inserted between the 5′-UTR and 2A, and an EV-A71 2A protease recognition site (-AITTL-) was inserted between luciferase and 2A. A T7 promoter was placed at the 5′-end for *in vitro*

transcription. A mMESSAGE mMACHINE™ T7 transcription kit (Invitrogen) was used for *in vitro* transcription. EV-A71 subgenomic replicon RNA was prepared through *in vitro* transcription with a Not I-linearized plasmid harbouring EV-A71 subgenomic replicon as templates [49].

EV-A71 binding assay

The cells were rested at 4°C and infected with EV-A71 (H, MOI = 1.0) on ice for 30 min. The cells were rinsed with PBS and the total RNA was extracted using the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The RNA level of EV-A71 was detected with qRT-PCR assay.

Tandem mass tag™-LC-MS/MS assay

300 µL of 8 M urea (Amresco, Solon, OH, USA) was added to the sample and the protease inhibitor (Roche, Basel, Switzerland) was added at 10% of the lysate. After centrifuging at 14,100 ×g for 20 min, the supernatant was collected. Tandem Mass Tag™-LC-MS/MS analysis was performed by Beijing Cnkingbio Biotechnology Corporation (Beijing, China).

Western blot analysis

The cells were lysed in the M-PER mammalian protein extraction reagent (Thermo, Waltham, MA, USA) containing halt protease inhibitor single-use cocktail (Thermo). The protein concentration was determined by the BCA reagents (Thermo). About 15 µg proteins were denatured and applied to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis products were transferred to a polyvinyl idenefluoride (PVDF) membrane. The membranes were then incubated at room temperature with specific primary antibody. After a standard washing, membranes were incubated with horse radish peroxidase (HRP)-labelled secondary antibody. The assay developed using a chemiluminescent substrate. The primary antibodies used in this study included antibodies against β-actin, GFP-tag, Flag-tag, HA-tag, and Myc-tag from Cell Signalling Technology (Beverly, MA, USA), SCARB2 from Abcam (Cambridge, MA, USA), TRIB3 from OriGene (Wuxi, China) and EV-A71 VP1 from GeneTex (California, USA). The goat anti-rabbit and anti-mouse HRP-labelled antibodies were obtained from Cell Signalling Technology.

In-cell Western

Cells cultured in 96-well black plate were infected with EV-A71 for 24 h, then cells were fixed by 4% paraformaldehyde for 15 min, permeabilized in 0.5% Triton X-100 for 15 min, and blocked with Odessey Blocking Buffer for 1 h at room temperature. Cells were immunolabeled for 2 h at room temperature with GAPDH antibody (1:1,000 dilution, Proteintech) and EV-A71-VP1 antibody (1:1,000 dilution, GeneTex), 1 h with IRDye 800CW Goat anti-Mouse (1:1,000 dilution, LI-COR, USA) and IRDye 680RD Goat anti-Rabbit secondary antibodies (1:1,000 dilution, LI-COR). After washing with PBST, images were taken with Odessey CLx (LI-COR USA) [49].

Immunofluorescence assay

Cells were seeded on glass coverslips (Thermo). After the preprocessing, the culture medium was removed and cells were fixed in 4% buffered paraformaldehyde for 15 min at room temperature. The cells were permeabilized in 0.5% Triton X-100 at room temperature for 15 min and blocked in PBS containing 1% BSA for 60 min at room temperature. Cells were then incubated with an anti-SCARB2 antibody (Abcam, 1: 500) or an anti-VP1 antibody (GeneTex, 1: 500) for 2 h at room temperature. After washing three times with PBS, the samples were reacted with FITC conjugate goat anti-rabbit secondary antibody (TransGen Biotech, Beijing, China) at a dilution of 1: 500 for 1 h at room temperature. After washing with PBS, add DAPI (Beyotime Institute of Biotechnology, Shanghai, China) for 10 min and Images were acquired using a confocal microscope (Olympus Microsystems, CA, USA) or a fluorescence microscope (Olympus, IX71).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) quantification

The total RNA was extracted using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. All RNA was analyzed with TransScript II Green One-Step qRT-PCR SuperMix (TransGen Biotech) using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). The specific primers were used for one-step qRT-PCR assay as shown in Supplementary Table 1.

Immunoprecipitation

Co-immunoprecipitation experiment was performed as described previously [49]. Briefly, cells were collected and lysed for 30 min on ice. Soluble lysates were incubated with indicated antibodies 4°C overnight, followed by incubation of Protein A/G Agarose (Roche) at 4 °C for 3 h. Complexes were separated from the beads and then boiled for 10 min. The precipitated proteins were subjected to SDS-PAGE and blotted with specific antibodies.

Flow cytometry

For Flow cytometry, 1×10^6 cell suspensions from cells were incubated with primary anti-LIMPII (ab176317, Abcam, 1:100) for 1 h at room temperature, followed by staining with secondary antibodies labelled with FITC. Then, cells were washed by MACs buffer, and data were acquired using a PARTEC CyFlow (Munich, Germany) and analyzed with FCS EXPRESS software.

Statistical analysis

Statistical analyses were performed using SPSS (Statistical Product and Service Solutions) 13.0 or GraphPad Prism 7.0 software. All data are expressed as the mean ± SD. Statistical significance was assessed using Student's *t*-test or two-way ANOVA with Holm-Sidak multiple comparisons test. A Log-Rank (Mantel–Cox) test was used for survival studies with GraphPad Prism 7.0 software. The score of clinical symptoms was analyzed by Ridit assay with SPSS 13.0. $P < 0.05$ was considered significant.

Results

EV-A71 infection upregulates TRIB3 expression

Our group's previous study found that TRIB3 impeded the degradation of PML-RARα and promoted the development of acute promyelocytic leukaemia [36]. We also found that TRIB3 promotes MYC-associated lymphoma development through suppression of UBE3B-mediated MYC degradation [50]. Considering that TRIB3 has been widely reported in cancer research and few studies related to viruses, we believe that it will be important to explore the role of TRIB3 in viral infection. During the course of EV-A71 infection, the mRNA level of *TRIB3* in EV-A71 infected cultures increased at the early phase of infection (1, 2 and 4 h) in HCT-8 cells, RD cells and FHC cells (Figure 1(a)). A time-course analysis showed that the protein level of TRIB3 was also induced in a time- and infective dose-dependent manner (Figure 1(b) and (c)). These results suggest that EV-A71 infection rapidly stimulates TRIB3 expression in host cells. Therefore, we further investigated the role and mechanism of TRIB3 in EV-A71 infection.

TRIB3 significantly enhances EV-A71 replication

To investigate the functional involvement of TRIB3 in EV-A71 infection, HCT-8 cells were transfected with a plasmid expressing TRIB3-HA or a control vector (control-HA). Twenty-four hours later, the transfected cells were infected with EV-A71 (MOI = 0.01). As shown in Figure 2, overexpression of TRIB3-HA significantly increased the levels of intracellular EV-A71 VP1 (Figure 2(a)–(c)) and viral RNA (Figure 2(d)) as well as virus yield (Figure 2(e)). Similar proviral effects of TRIB3 overexpression on EV-A71 were also observed in RD, Vero and FHC cell lines (Figure S1(A)) or on three other different strains of EV-A71 in HCT-8 cells (Figure S1(B)). Interestingly, in addition to EV-A71, overexpression of TRIB3 also promoted the replication of CVB3 and CVA16, two distantly related human enteroviruses (Figure S1(C)). Finally, knockout of TRIB3 in HCT-8 cells significantly reduced the levels of intracellular EV-A71 VP1 protein (Figure 2(f)–(h)) and RNA (Figure 2(i)) as well as virus yield (Figure 2(j)). As anticipated, supplementation of TRIB3 expression in TRIB3-KO HTC-8 cells by transfection of a plasmid expressing TRIB3 efficiently restored the levels of EV-A71 replication (Figure 2(k)). In summary, those results clearly demonstrated that TRIB3 is a cellular proviral factor that facilitate the replication of EV-A71 and other distantly related human enteroviruses.

TRIB3 does not interact with viral proteins

According to previous reports, many host proteins interact with viral proteins to regulate the stability or function of viral proteins and thus affect viral replication [51,52]. To investigate whether TRIB3 interacts with EV-A71 proteins, 293 T cells were co-transfected with a plasmid expressing TRIB3-Myc and a plasmid expressing HA-tagged VP1, 2A, 2B, 2C, 3A, 3C or GFP-tagged VP2-4, 3B, 3D protein or V5-tagged 2A protein. Interaction of TRIB3 with the different viral proteins was determined by immunoprecipitation and Western blot assays. As shown in Figure 3(a), immunoprecipitation with antibody against Myc epitope tag and probing the precipitated viral proteins with antibodies against HA tag, V5 tag or GFP tag indicated that TRIB3 protein did not interact with HA-tagged VP1, 2A, 2B, 2C, 3A, 3C or GFP-tagged VP2-4, 3B, 3D protein or V5-tagged 2A protein. To investigate whether EV-A71 proteins interact with TRIB3 in the context of EV-A71 infection, 293 T cells transfected with TRIB3-Myc plasmid were infected by EV-A71 and harvested at 24 h post infection. Immunoprecipitation from the cell lysates with anti-Myc antibody and probed with antibodies against viral VP0, VP2, 2B, 2C, 3A or 3C proteins. As shown in Figure 3(b), consistent with our previous findings [48], the positive control NEDD8 protein interacts with the EV-A71 VP2 protein. However, there is no interaction between TRIB3 and any of EV-A71 proteins in the context of EV-A71 infection was identified (Figure 3(b)).

TRIB3 enhances SCARB2 expression and EV-A71 attachment to cells

Because TRIB3 does not interact with all the EV-A71 proteins, it is conceivable that TRIB3 may facilitate

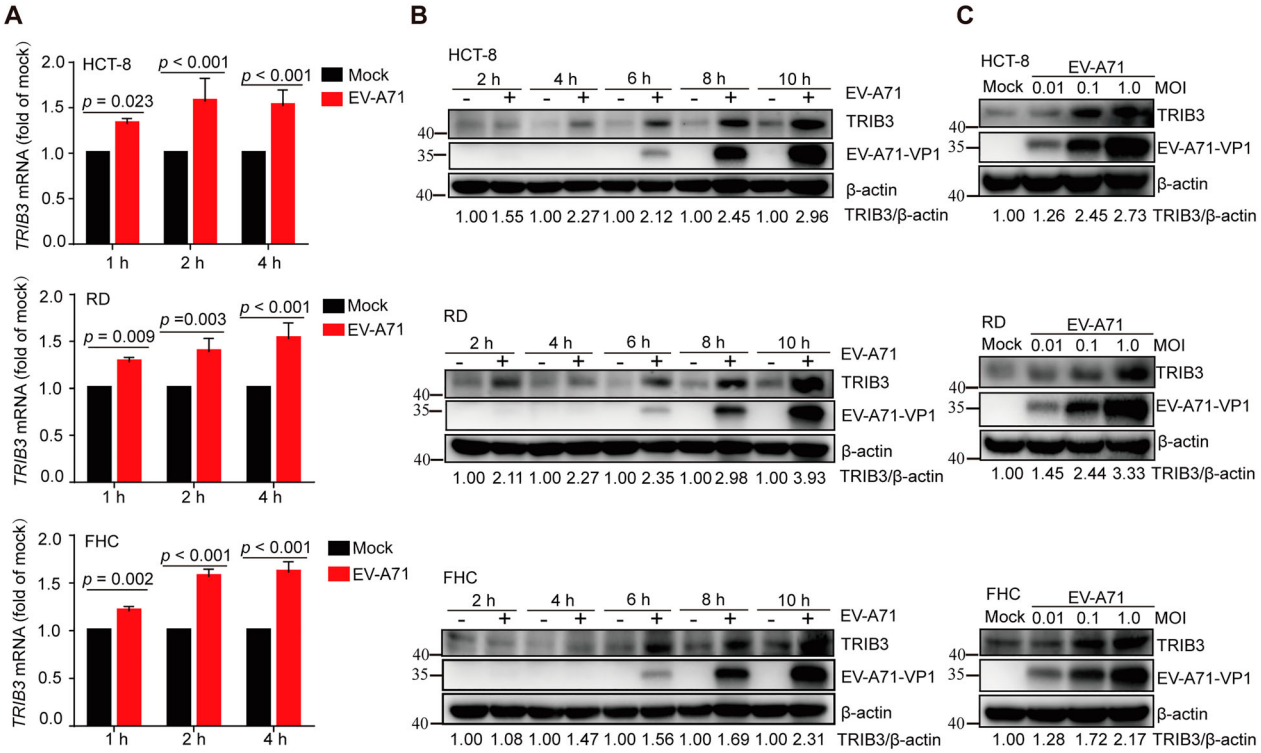

Figure 1. EV-A71 infection upregulated TRIB3 expression. (a) HCT-8 cells, RD cells and FHC cells were infected with EV-A71 (MOI = 1.0) and harvested at the indicated time post infection. The mRNA level of *TRIB3* was analyzed by qRT-PCR ($n = 3$). $P < 0.05$, two-way ANOVA with Holm-Sidak multiple comparisons test. (b) EV-A71 infection increased TRIB3 expression *in vitro* in a time dependent manner. HCT-8 cells, RD cells and FHC cells were infected with EV-A71 (MOI = 1.0) and harvested at the indicated time post infection. Cell extracts were applied for WB assay. (c) EV-A71 infection increased TRIB3 expression *in vitro* in an inoculum dose dependent manner. HCT-8 cells, RD cells and FHC cells were infected with EV-A71 at the indicated MOI (MOI = 0.01, 0.1, 1.0) and harvested at 10 h post infection. Cell extracts were applied for WB assay.

viral replication via interacting with or altering the expression of other host proviral or restriction factors. Accordingly, a Tandem Mass Tag™-LC-MS/MS analysis was performed to compare the proteomes of parental HTC-8 cells and TRIB3-KO cells. Interestingly, the level of SCARB2 (also known as lysosomal integral membrane protein II or CD36b like-2), the *bona fide* receptor for EV-A71, was lower in the TRIB3-KO cells as compared to that in parental WT HTC-8 cells (Figure 4(a)) [8,53]. This result was further validated by Western blot assays (Figure 4(b)). However, it is interesting that knockout of TRIB3 in HTC-8 cells did not alter the level of SCARB2 mRNA, as revealed by qRT-PCR analysis (Figure 4(c)). In agreement with those findings, it was further demonstrated that that overexpression of TRIB3-HA in HTC-8 cells significantly increased the level of SACRB2 protein (Figure 4(d)), but did not alter the level of SCARB2 mRNA (Figure 4(e)). Moreover, flow cytometry and immunofluorescent staining assays were performed to compare SCARB2 expression on the cell surface at single cell level. The results showed that overexpression of TRIB3 increased the level of SCARB2 (Figure 4(f) and (h)), whereas knockout of TRIB3 decreased the level of SCARB2 (Figure 4(g) and (i)).

Since SCARB2 is the receptor for EV-A71 and TRIB3 up-regulates the level of SCARB2, it is possible that TRIB3 facilitates EV-A71 infection by promoting the receptor binding and entry into host cells. In support of this hypothesis, it was demonstrated that overexpression of TRIB3 increased the binding of EV-A71 to HTC-8 cells (Figure 4(j)), knockout of TRIB3 significantly reduced the binding of EV-A71 to the cells (Figure 4(k)). Those results indicate that TRIB3 promotes EV-A71 infection is, at least in part, by maintaining the level of SCARB2 at cell surface to support efficient virus entry into host cells.

TRIB3 represses ubiquitylation and degradation of SCARB2

Because modulation of TRIB3 expression did not alter the level of SCARB2 mRNA, but significantly change the level of total and cell surface SCARB2 protein, it is conceivable that TRIB3 may regulate the translation of SCARB2 mRNA and /or metabolism stability SCARB2 protein. It is well known that TRIB3 can regulate the stability and activity of client proteins to reach its impact on diverse cellular processes [54]. Accordingly, we first examined the effect of TRIB3 on the stability of SCARB2. The results presented in Figure 5(a) showed that overexpression of TRIB3 significantly increased the stability of SCARB2. Since ubiquitination plays an important role in protein

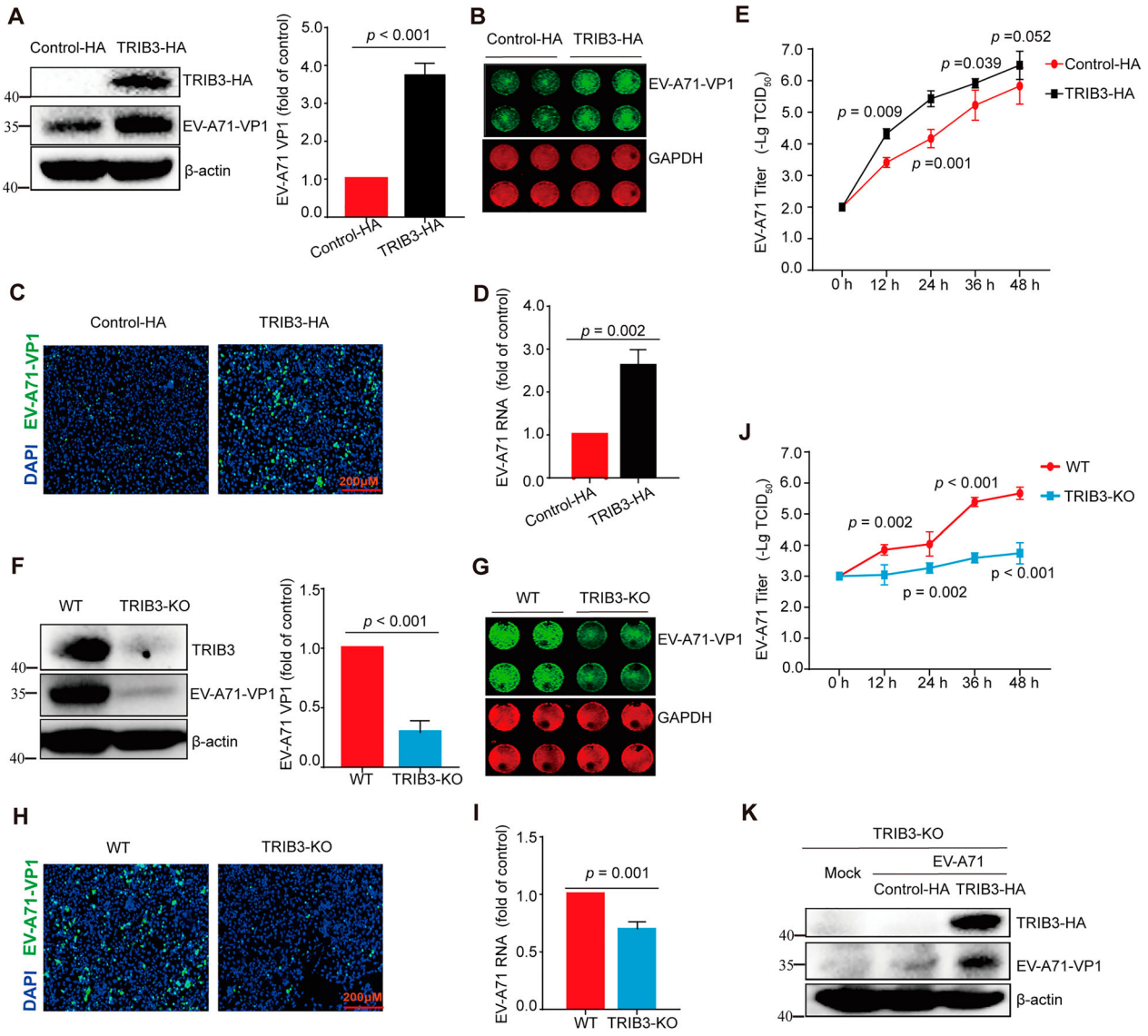

Figure 2. TRIB3 enhances EV-A71 replication. (a to e) TRIB3 overexpression promoted EV-A71 infection. HCT-8 cells were transfected with control-HA or TRIB3-HA plasmids. At 24 h after transfection, HCT-8 cells were mock-infected or infected with EV-A71 (MOI = 0.01) for 24 h. The cells were harvested for WB assay with indicated antibodies (a, $n = 3$), In-cell WB assay with indicated antibodies (b), IF (c), qRT-PCR assay (d, $n = 3$) or virus titer assay (e, $n = 3$) 24 h post infection. (f to j) TRIB3 depletion reduced the replication of EV-A71. Parental or TRIB3-KO HCT-8 cells were infected with EV-A71 (MOI = 0.1) for 24 h. The cells were harvested for WB assay with indicated antibodies (f, $n = 3$), In-cell WB assay with indicated antibodies (g), IF (h), qRT-PCR assay (i, $n = 3$) or titer assay (j, $n = 3$) 24 h post infection. (k) TRIB3-KO cells were transfected with control-HA or TRIB3-HA plasmids. At 24 h after transfection, TRIB3-KO cells were mock-infected or infected with EV-A71 (MOI = 0.1) for 24 h. The cells were harvested for WB assay with indicated antibodies. $P < 0.05$, two-way ANOVA with Holm-Sidak multiple comparisons test (e, j) or Student's *t*-test (a, d, f, i).

degradation, we tested the effect of TRIB3 on ubiquitination of SCARB2. SCARB2-Myc was overexpressed in 293 T cells for 24 h, along with expression of Ub-Flag or TRIB3-HA. As shown in Figure 5(b), co-immunoprecipitation analysis showed that SCARB2 was modified by ubiquitin and TRIB3 overexpression represses SCARB2 ubiquitylation. Ubiquitin has seven lysine residues, of which the two most fully characterized forms of polyubiquitination occur on lysine 48 (K48) or lysine 63 (K63) [55]. We found that TRIB3 inhibited SCARB2 ubiquitination by K48 rather than K63 linkages in co-immunoprecipitation assay (Figure 5(c) and (d)). The C terminal kinase domain (KDC) of TRIB3 was reported to exert the function of full length TRIB3 protein in cancer cells [36, 44]. We also determined that the KDC region of TRIB3 was responsible for its promoting EV-A71 infection (Figure 5(e) and (f)). Indeed, co-immunoprecipitation showed that the absence of KDC in TRIB3 did not inhibit ubiquitination and degradation of SCARB2, and thus did not increase the replication of EV-A71 (Figure 5(e), (f) and (g)). In addition, the expression of KDC domain alone can compensate for the influence of KDC deletion on EV-A71 replication (Figure 5(h)). Intriguingly, co-immunoprecipitation results showed that there was no interaction between exogenously expressed SCARB2-Flag and TRIB3-HA (Figure 5 (i)). To further verify this result, we detected the

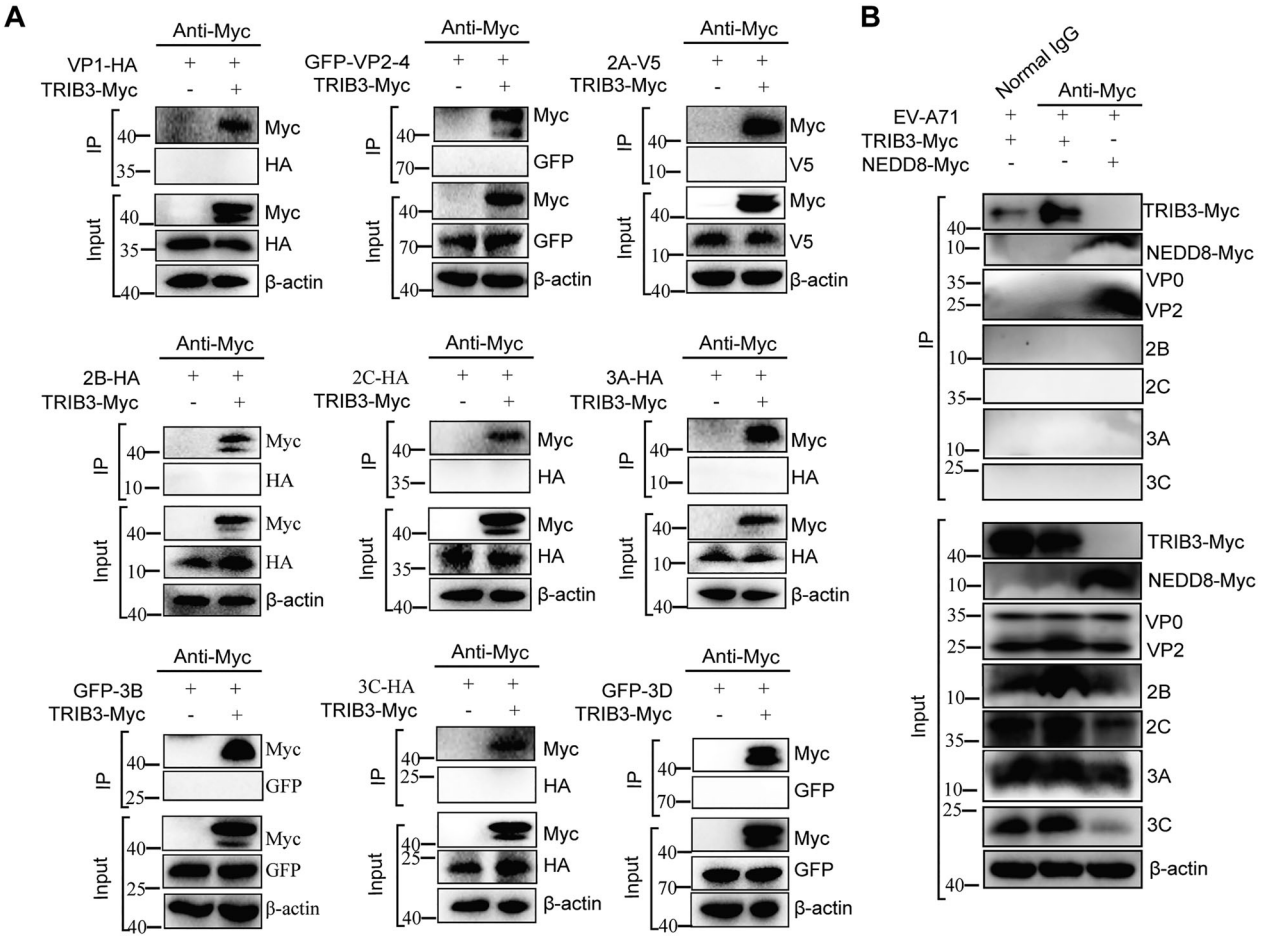

Figure 3. TRIB3 does not interact with viral proteins. (a) 293 T cells were co-transfected with a plasmid expressing Myc-tagged TRIB3 and plasmid expressing HA-tagged VP1, 2A, 2B, 2C, 3A, 3C or GFP-tagged VP2-4, 3B, 3D protein or V5-tagged 2A. Immunoprecipitation from cell lysates were performed and probed with the indicated antibodies. (b) 293 T cells were transfected with plasmid expressing TRIB3-Myc or NEDD8-Myc, the cells were infected with EV-A71 at 24 h post transfection and harvested at 24 h post infection. Immunoprecipitation from cell lysates were performed and probed with the indicated antibodies.

relationship between endogenous TRIB3 and SCARB2. The results showed that there was also no interaction between endogenous TRIB3 and SCARB2 (Figure 5(j)), suggesting that TRIB3 may indirectly affect ubiquitination of SCARB2 by regulating other host proteins such as E3 ligase. Overall, these results suggest that TRIB3 increases SCARB2 stability by inhibiting its ubiquitylation and proteasomal degradation.

TRIB3 also facilitates EV-A71 replication through a non-SCARB2-mediated mechanism

Although the above results suggest that maintenance of SCARB2 metabolic stability by TRIB3 is important for EV-A71 infection of susceptible cells, the biological function of EV-A71 infection-induced up-regulation of TRIB3 expression remains to be determined. Because the increase of TRIB3 protein occurred at 2, 4 and 6 h post EV-A71 infection (Figure 1(b)), we postulated that the elevated TRIB3 in EV-A71 infected cells may regulate the viral genome replication and/or virion production. To investigate this hypothesis, the effect of TRIB3 on EV-A71 replication was evaluated with a one-step replication curve assay. Specifically, the ectopically expressed TRIB3-HA was not detectable at 3 h, but accumulated at a high level at 10 h post transfection. In agreement with the kinetics of TRIB3-HA expression in the transfected HCT-8 cells, the level of SCARB2 was not changed at 3 h, but significantly increased at 10 h post transfection (Figure 6(a)). Accordingly, the HTC-8 cells were infected with EV-A71 after 3 h post transfection with control vector or plasmid expressing TRIB3-HA. The level of EV-A71 VP1 at 7 h post infection was significantly higher in the cultures with TRIB3-HA overexpression (Figure 6(b)). The results thus suggest that the observed enhancement of EV-A71 replication by TRIB3 under this experimental condition is not due to the enhancement of SCARB2-mediated infectious entry, but most likely the promotion of viral genome replication via a TRIB3-dependent, but SCARB2-independent mechanism. To further validate this notion, we constructed a subgenomic replicon of EV-A71 with a luciferase reporter, which

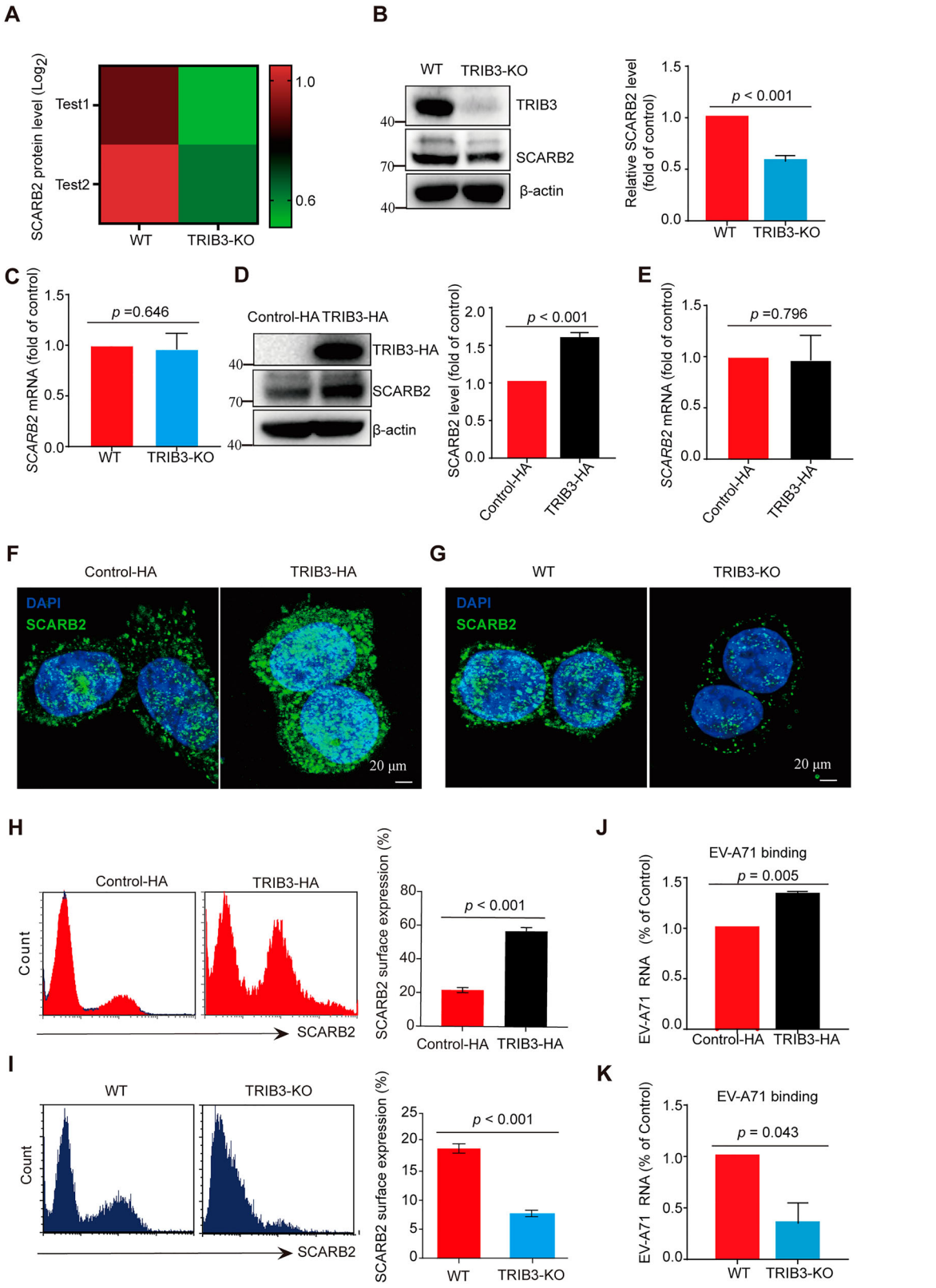

Figure 4. TRIB3 enhances SCARB2 expression and EV-A71 attachment to cells. (a) TRIB3 depletion decreased the protein level of *SCARB2*. Tandem Mass Tag™-LC-MS/MS analysis evaluated the protein level of SCARB2 in WT cells and TRIB3-KO cells ($n = 2$). (b, c, g, i) TRIB3 depletion decreased the protein level of SCARB2. SCARB2 expression were detected with WB (b, $n = 3$), qRT-PCR assay (c, $n = 4$), Immunofluorescence (g) and flow cytometry (i, $n = 3$) in WT cells and TRIB3-KO cells. (d, e, f, h) TRIB3 overexpression increased the protein level of SCARB2. HCT-8 cells were treated with Control-HA or TRIB3-HA plasmids. At 24 h after transfection, cells were harvested for WB assay with indicated antibodies (d, $n = 3$), qRT-PCR assay (e, $n = 4$), Immunofluorescence (f) and flow cytometry (h, $n = 3$). Percentage of SCARB2 positive cells was calculated with FCS express software. (j, k) TRIB3 overexpression promoted EV-A71 binding and TRIB3 knockout decreased EV-A71 binding. HCT-8 cells were transfected with indicated plasmids. At 24 h after transfection, HCT-8 cells were rested at 4°C for 1 h and infected with EV-A71 (MOI = 1.0) on ice for 30 min. The cells were rinsed with PBS and harvested. Cell-associated EV-A71 RNA were measured by a qRT-PCR assay (j. $n = 3$). WT cells and TRIB3-KO cells were rested at 4°C for 1 h and then infected with EV-A71 (MOI = 1.0) on ice for 30 min. The cells were rinsed with PBS and harvested. Cell-associated EV-A71 RNA were measured by were a qRT-PCR assay (k, $n = 3$). $P < 0.05$, Student's *t*-test (b, c, d, e, h, i, j, k).

lacks the structural genes necessary for the assembly of progeny viruses, but is able to support efficient viral genome replication (Figure 6(c)). The luciferase activity quantitatively reflected the replication levels of the replicon RNA. The EV-A71 subgenomic replicon RNA transcribed *in vitro* by a mMESSAGE mMACHINE™ T7 transcription kit. 293 T cells were firstly transfected with control-HA or TRIB3-HA plasmid. After 24 h of transfection, cells were then transfected with replicon RNA and luciferase reporter activities were determined at the different times. The results showed that overexpression of TRIB3 significantly increased the luciferase activity of replicon-transfected cells and thus replicon RNA replication (Figure 6(c)). To rule out the role of SCARB2 in TRIB3 promotion of EV-A71 replicon replication, SCARB2-KO HCCLM3 cells (Figure 6(d)) were transfected with control-HA or TRIB3-HA plasmid and followed by replicon RNA transfection at 24 h later. Similar extents of TRIB3-enhanced replication of EV-A71 replicon RNA were observed (Figure 6(e)). More importantly, we found that overexpression of TRIB3-HA still significantly increased the levels of intracellular EV-A71 VP1 (Figure 6(f)) and viral RNA (Figure 6(g)) in SCARB2-KO HCCLM3 cells. Taking together, those results strongly support the hypothesis that in addition to promoting EV-A71 entry into host cells by maintaining the stability of SCARB2, TRIB3 also facilitate the genome replication of EV-A71 in a SCARB2-independent manner.

Trib3 knockdown significantly reduces the lethality and severity of EV-A71 infection in mice

To investigate whether TRIB3 facilitates EV-A71 replication *in vivo*, we conducted a series of experiments in *Trib3* heterozygous C57BL/6 mice (referred as $Trib3^{+/-}$) as description in schematic presentation of animal experiment design (Figure 7(a)). These mice were viable and fertile and born at the expected Mendelian ratio without apparent abnormalities in adult mice. C57BL/6 wild-type (WT) mice and $Trib3^{+/-}$ mice at 12 days of age were mock-infected or infected with the indicated amount of EV-A71 (10 LD_{50}, 1 LD_{50}, 0.1 LD_{50}). As shown in Figure 7(b), Trib3 knockdown significantly attenuated EV-A71 induced hind limb paralysis on day 5 after lethal dose of EV-A71 infection (10 LD_{50}). Compared with age-matched WT mice, Trib3 knockdown delayed the death of mice caused by the lethal dose of EV-A71 infection (Figure 7(c)). The WT C57BL/6 mice succumbed to EV-A71 infection with mean survival time (MST) of 6.4 ± 1.5 days, and all mice in both WT and $Trib3^{+/-}$ group died within 10 dpi (Table 1). Trib3 knockdown provided partial but statistically significant protection from mortality with MST of 9.4 ± 1.0 days (Table 1). Next, we detected the protective effect of Trib3 knockdown on the death of mice against sub-lethal dose EV-A71 challenge (1 LD_{50}). Compared with WT mice, Trib3 knockdown significantly reduced the lethality from sub-lethal EV-A71 challenge (Figure 7(d)). Moreover, compared with age-matched WT mice, Trib3 knockdown also alleviated infected mice weight loss (Figure 7(e)) and lower clinical manifestation scores when mice were challenged with non-lethal dose EV-A71 (0.1 LD_{50}) (Figure 7(f)). These results indicate that Trib3 knockdown significantly reduce the lethality and severity of EV-A71 infection in mice.

Trib3 knockdown significantly attenuates EV-A71 replication in mice

Considering that TRIB3 promotes EV-A71 replication in cultured cells, it is reasonable to consider that the amelioration of EV-A71 infection manifestations in $Trib3^{+/-}$ mice is due to the reduced replication of EV-A71. In our previous study, we found that EV-A71 titers in hind-limb muscle tissues were much higher than other tissues [56]. We, therefore, examined the expression of viral and cellular proteins, viral titers and histopathology of hind-limb muscles of mice at 3 or 5 days post sub-lethal dose of EV-A71 infection (1 LD_{50}). In agreement with that observed in EV-A71 infected cells *in vitro*, EV-A71 infection increased the protein level of TRIB3 in muscles, and Trib3 knockdown resulted in decreased SCARB2 expression and reduced levels of VP1 protein (Figure 8(a)). In addition, Trib3 knockdown decreased viral titers in the infected muscles by approximately 1 log (Figure 8(b)). In parallel with the decrease of viral protein expression, Trib3 knockdown also improved muscle pathology, as shown in the H&E staining of muscle tissue sections (Figure 8(c)). IHC studies demonstrated that the EV-A71 VP1 protein expression in virus-infected muscles was significantly reduced in $Trib3^{+/-}$ mice at 3 and 5 dpi, compared with the infected WT mice (Figure 8(d)). Those results thus indicate that Trib3 knockdown significantly attenuated EV-A71 replication and pathogenesis *in vivo*.

Discussion

As an obligate intracellular pathogen, EV-A71 hijacks host factors to support its replication. Using qRT-PCR analysis of gene transcription changes in EV-A71-infected cells, we found that the cellular pseudokinase TRIB3 mRNA was significantly increased after EV-A71 infection in HCT-8 cells, RD cells and FHC cells. The increased accumulation of TRIB3 protein was also observed in EV-A71 infected cells *in vitro* as well as *in vivo* in mice (Figures 1(b), (c) and 8(a)).

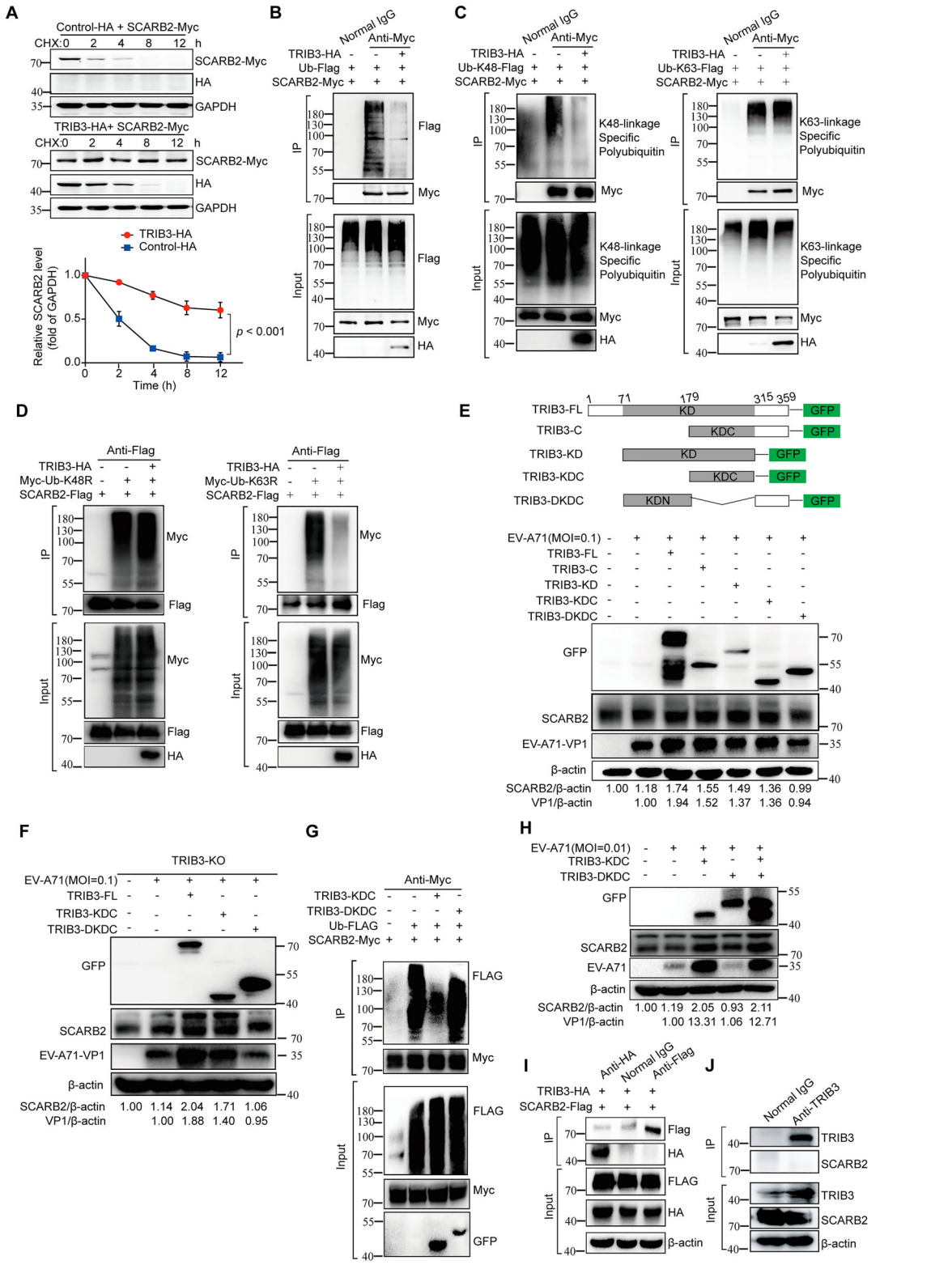

Figure 5. TRIB3 represses ubiquitylation and degradation of SCARB2. (a) 293 T cells were co-transfected with a plasmid expressing SCARB2-Myc and TRIB3-HA or a vector plasmid. At 24 h post transfection, cells were incubated with cycloheximide (CHX) (10 μg/ml) for indicated times. Proteins were detected by WB with the indicated antibodies. $P < 0.05$, two-way ANOVA with Holm-Sidak multiple comparisons test. (b) The effect of TRIB3 overexpression on SCARB2 ubiquitylation *in vitro*. 293 T cells were transfected with indicated plasmids and cell extracts were IP with anti-Myc Ab. The ubiquitylated SCARB2 was detected with WB assay. (c, d) 293 T cells were transfected with indicated plasmids and cell extracts were IP with anti-Myc Ab or anti-Flag Ab. The ubiquitylated SCARB2 was detected with WB assay. (e) The KDC region of TRIB3 was responsible for its promoting EV-A71 infection. (Upper panel) Schematic diagram of TRIB3 deletion mutants. (Lower panel) Cell extracts from 293 T cells transfected with the indicated plasmids and infected with EV-A71 were resolved by SDS-PAGE, proteins were detected by WB with the indicated antibodies. (f) Cell extracts from TRIB3-KO cells transfected with the indicated plasmids and infected with EV-A71 were resolved by SDS-PAGE, proteins were detected by WB with the indicated antibodies. (g) Effect of KDC deletion in TRIB3 on SCARB2 ubiquitination in 293 T cells. (h) Cell extracts from 293 T cells transfected with the indicated plasmids and infected with EV-A71 were detected by WB with the indicated antibodies. (i) The relationship between TRIB3 and SCARB2. 293 T cells were transfected with indicated plasmids and cell extracts were IP with anti-HA or anti-Flag Ab. (j) The relationship between endogenous TRIB3 and SCARB2. HCT-8 cells extracts were IP with anti-TRIB3.

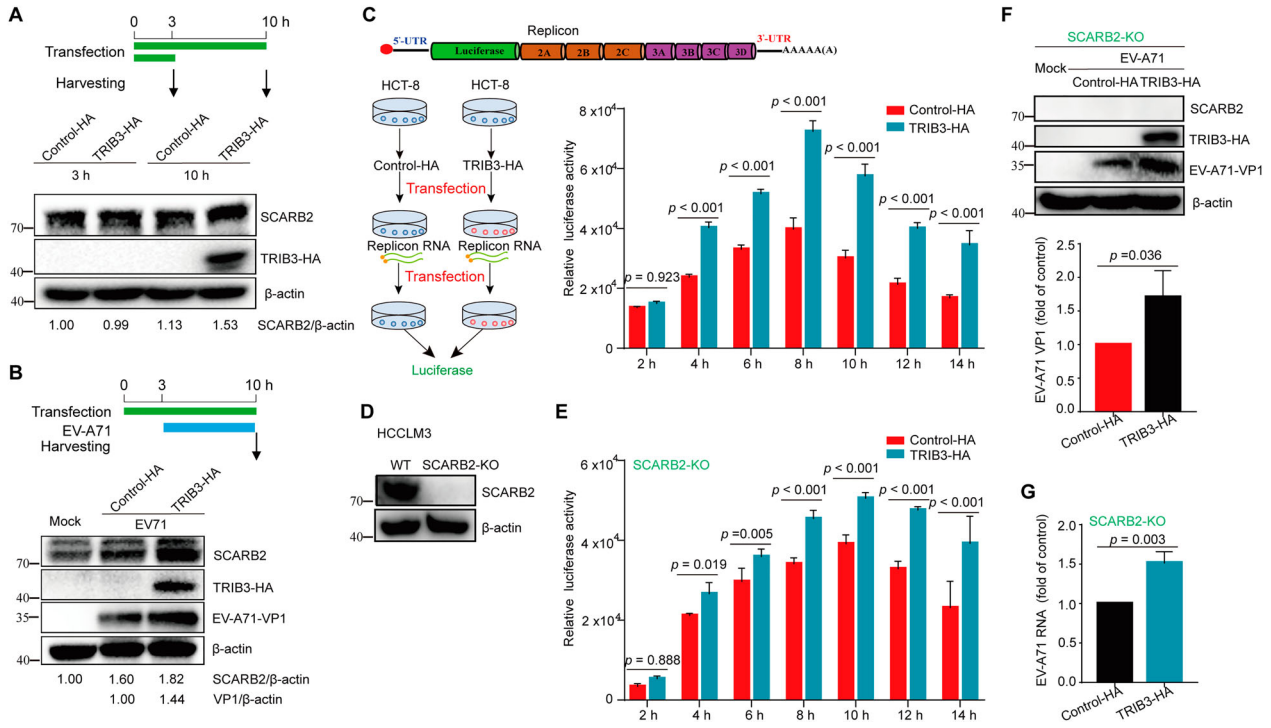

Figure 6. TRIB3 facilitates EV-A71 replication in a SCARB2-independent manner. (a) HCT-8 cells were transfected with control-HA or TRIB3-HA plasmids. At 3 h or 10 h post transfection, HCT-8 cells were harvested for WB assay with indicated antibodies. (b) HCT-8 cells were transfected with control-HA or TRIB3-HA plasmids. At 3 h post transfection, HCT-8 cells were mock-infected or infected with EV-A71 (MOI = 1.0) for 7 h. The cells were harvested for WB assay with indicated antibodies. (c) 293 T cells were transfected with control-HA or TRIB3-HA plasmids. At 24 h post transfection, the cells were transfected again with EV-A71 subgenomic replicon RNA and luciferase reporter activities were determined at different time ($n = 6$). (d) HCCLM3 cells and SCARB2-KO HCCLM3 cells were harvested for WB assay with indicated antibodies. (e) SCARB2-KO HCCLM3 cells were transfected with control-HA or TRIB3-HA plasmids. At 24 h post transfection, the cells were transfected again with EV-A71 subgenomic replicon RNA and luciferase reporter activities were determined at different time ($n = 6$). (f–g) SCARB2-KO HCCLM3 cells were transfected with control-HA or TRIB3-HA plasmids. At 24 h after transfection, cells were mock-infected or infected with EV-A71 (MOI = 1) for 24 h. The cells were harvested for WB assay with indicated antibodies (f, $n = 3$) and qRT-PCR assay (g, $n = 3$). $P < 0.05$, two-way ANOVA with Holm-Sidak multiple comparisons test (c, e) or Student's *t*-test (f, g).

Interestingly, the upregulation of TRIB3 mRNA and protein had also been observed in cells infected by hepatitis C virus (HCV), bovine viral diarrhea virus (BVDV), Japanese encephalitis virus (JEV) and Zika virus [39-41]. This suggests that upregulation of TRIB3 expression may be a stress response of host cells to different viral infections. Of course, more research is needed to find out what kind of stress host cells use to regulate TRIB3 expression during viral infection.

While TRIB3 inhibits the infectious entry of HCV into hepatocytes, the effect of TRIB3 on the replication of BVDV, JEV and Zika virus remains to be determined [39-41]. In this study, we demonstrated that overexpression of TRIB3 promoted EV-A71 replication, while knockout of TRIB3 inhibited EV-A71 replication (Figure 2). Consistent with the findings in infected cells, Trib3 knockdown significantly reduced the level of VP1 protein and viral titers in hind-limb muscle tissues of mice infected with sublethal dose of EV-A71 (Figure 8). Furthermore, we found that the proviral effect of TRIB3 on EV-A71 was not cell line- specific and viral strain-specific (Figure S1 (A) and (B)). Together, these results suggest that unlike its negative role in HCV infection, TRIB3 plays a positive role in regulating EV-A71 infection.

Previous study has shown that TRIB3 regulates the metabolic stability and activity of client proteins to execute its biological functions on diverse cellular processes [54]. In HCV-infected cells, HCV non-structural protein 3 (NS3) interacts with TRIB3 to disrupt the TRIB3-Akt signalling pathway to promote persistent HCV infection [39]. However, our results shown that TRIB3 promotion of EV-A71 infection most likely did not work through interaction with any of the viral proteins (Figure 3). Instead, Tandem Mass Tag™-LC-MS/MS analysis revealed that TRIB3 knockout significantly reduced the level of EV-A71 receptor SCARB2. This finding was further validated in EV-A71 infected cells by Western blot, flow cytometry and confocal microscopic analysis (Figure 4). TRIB3 was considered to connect metabolic stressors to tumour progression by affecting the ubiquitylation and degradation of multiple cellular proteins, such as SQSTM1 and PML-RARα [36]. Similarly, in this study, we found that TRIB3 inhibited SCARB2 K48-

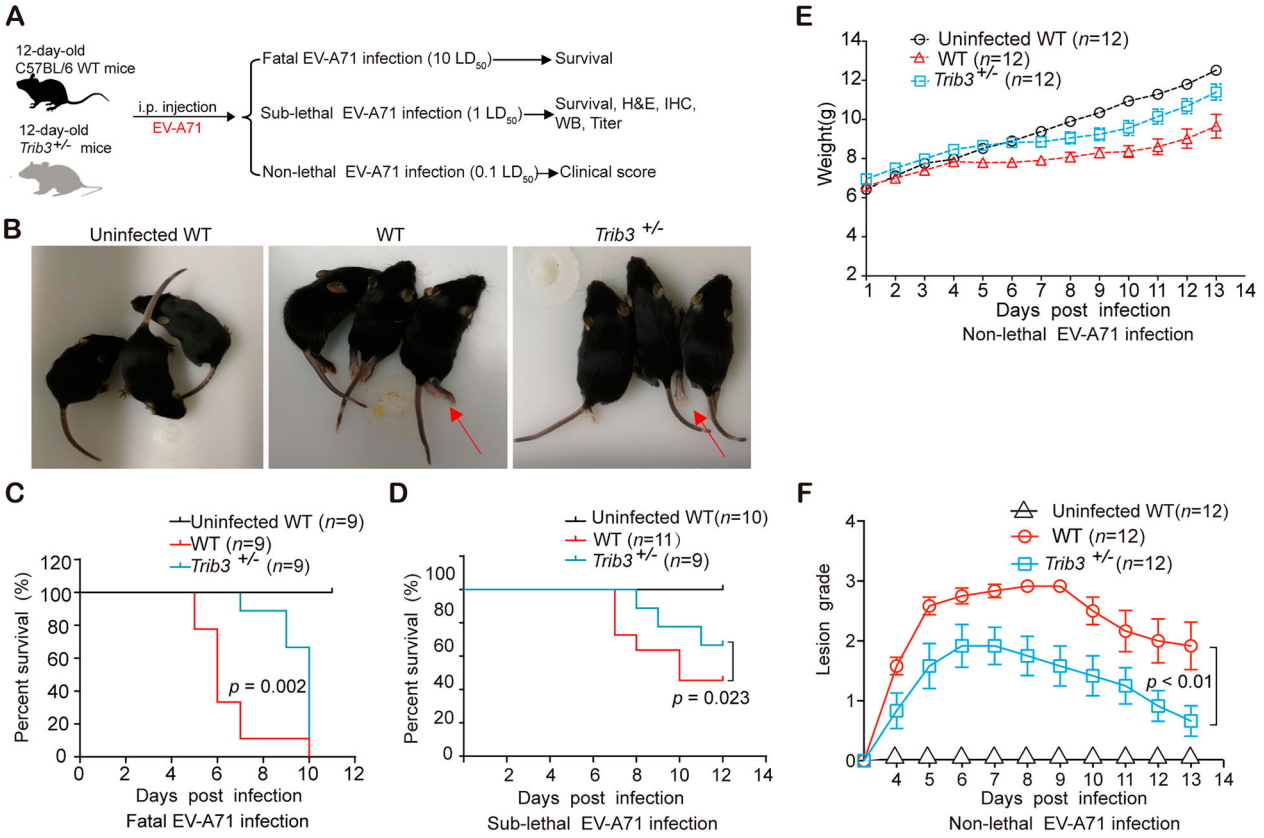

Figure 7. Trib3 knockdown significantly reduce the lethality and severity of EV-A71 infection in mice. (a) Schematic presentation of animal experiment design. (b, c) Trib3 knockdown could delay the death of mice upon lethal EV-A71 challenge and photos were taken at 5 dpi. (d) 12-day C57BL/6 WT mice and $Trib3^{-/+}$ mice were infected with 1 LD_{50} of EV-A71, mice survival was observed every day until day 12. (e, f) 12-day C57BL/6 WT mice and $Trib3^{-/+}$ mice were infected with 0.1 LD_{50} of EV-A71, mice were weighed daily (e) and observed for clinical scores for 13 days (f). $P < 0.05$, A Log-Rank (Mantel-Cox) test (c, d) or Ridit assay (f).

linked polyubiquitination and maintained the stability of SCARB2, thereby increasing the SCARB2 protein level at cell surface to facilitate EV-A71 infection (Figure 5). Interestingly, we did not detect the interaction between TRIB3 and SCARB2 (Figure 5(i) and (j)), indicating that TRIB3 may indirectly affect ubiquitination of SCARB2 by regulating other host proteins such as E3 ligase. Subsequent adsorption and binding experiments confirmed that TRIB3 did enhance EV-A71 infection by increasing SCARB2 protein level to promote EV-A71 binding of susceptible cells (Figure 4(j) and (k)). Consistent with the *in vitro* results, SCARB2 protein level was significantly reduced in muscle tissue of $Trib3^{+/-}$ mice compared with that in WT mice (Figure 8(a)), suggesting that the downregulation of SCARB2 caused by Trib3 knockdown was one of the reasons for the reduced replication of EV-A71 *in vivo*. In summary, our *in vitro* and *in vivo* results suggest that TRIB3 can indeed regulate EV-A71 infection in a SCARB2-dependent manner.

Table 1. Impact of Trib3 knockdown on mortality rates and mean survival time (MST) of mice infected with lethal EV-A71.

Groups	Mortality rate	MST (days)	Life extension rate (%)
Uninfected WT	0	11.0 ± 0.0	—
WT	100%	6.4 ± 1.5**	—
$Trib3^{+/-}$	100%	9.4 ± 1.0*##	46.9

Notes: $n = 9$; MST value represents mean ± SD; Life extension rate = (MST ($Trib3^{+/-}$)-MST (WT))/MST(WT); *$P < 0.05$ *vs* Uninfected WT; #$P < 0.05$ *vs* WT.

Although our results confirm that TRIB3 can modulate EV-A71 binding of susceptible cells by regulating SCARB2 expression, EV-A7 induced TRIB3 in infected cells may modulate EV-A71 replication at distinct steps of viral life cycle. By studying the effects of TRIB3 on one-step growth curve of EV-A71 and EV-A71 replicon, we found that TRIB3 also facilitates EV-A71 RNA genome replication (Figure 6). The dual proviral mechanisms of TRIB3 on the SCARB2-mediated entry and viral RNA replication may explain our finding that TRIB3 more potently promoted the replication of EV-A71 and CVA16 that use SCARB2 as cellular receptor, but less efficiently promoted the replication of CVB3 whose receptor is not SCARB2 (Figure S1(C)). More importantly, we found that overexpression of TRIB3-HA still significantly promoted EV-A71 replication in SCARB2-KO cells (Figure 6(f) and (g)). These results suggest that TRIB3 may play a broad-spectrum role in promoting enterovirus infection through several different mechanisms. Among them, the mechanism of TRIB3 promoting enterovirus infection through SCARB2-independent way is worthy of further research, and the new findings

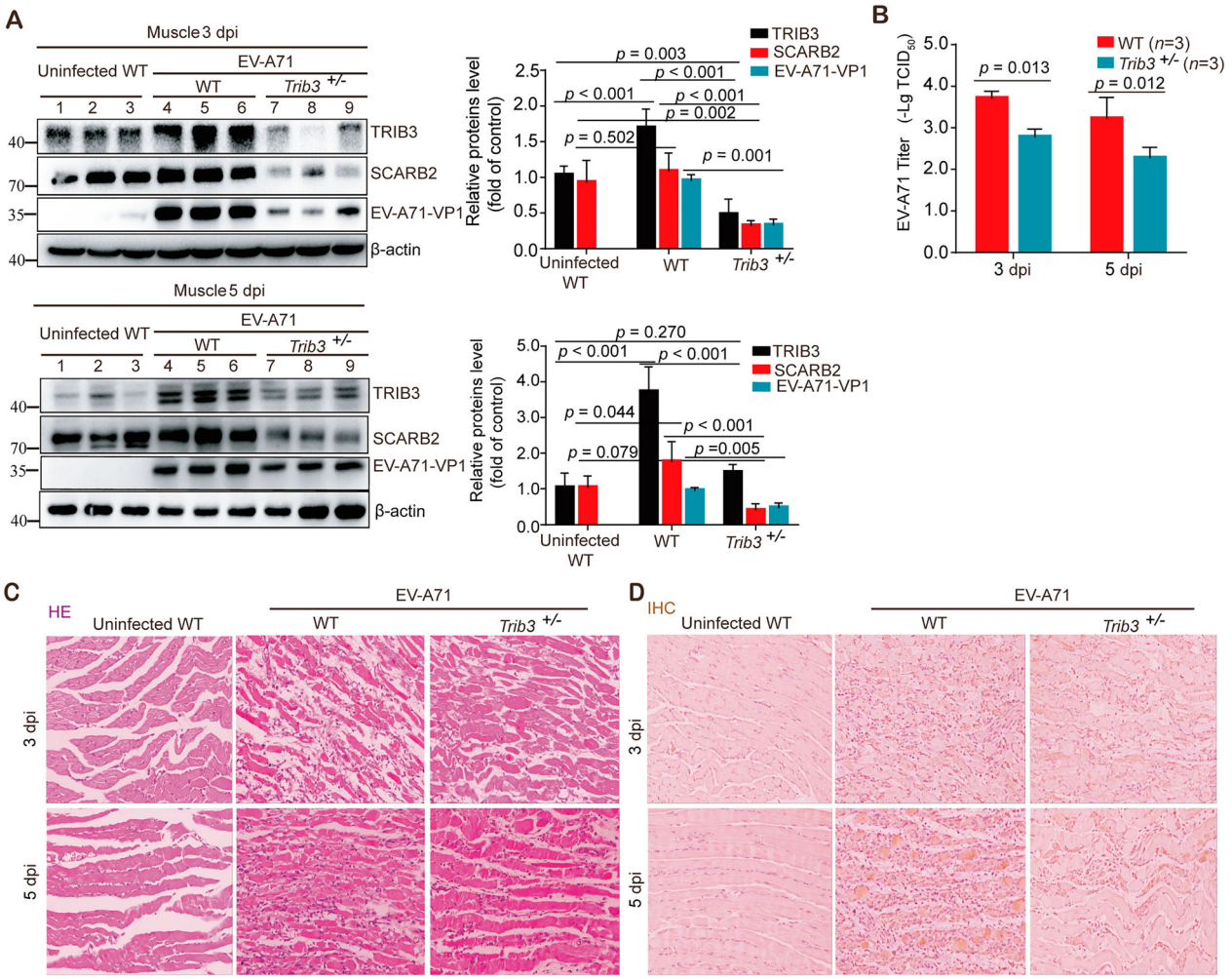

Figure 8. Trib3 knockdown significantly attenuated EV-A71 replication in mice. (a to d) 12-day C57BL/6 WT mice and *Trib3*$^{-/+}$ mice were infected with 1 LD_{50} of EV-A71. Three mice enrolled in each group were dissected at 3 or 5 dpi. Muscle tissues proteins were detected by WB assay with indicated antibodies (a) and viral titer assays (b). Paraffin-embedded sections of muscle tissues were prepared from mice at 3 or 5 dpi and examined with H&E stain (c). The muscle tissue sections prepared from mice at 3 or 5 dpi were stained with EV-A71 VP1 antibody for IHC analyses (d). $P < 0.05$, two-way ANOVA with Holm-Sidak multiple comparisons test (a, b).

may provide a meaningful reference for the development of broad-spectrum anti-enterovirus drugs in the future. As a cellular stress sensor, TRIB3 is responsible for regulating a variety of physiological and pathological functions [27-35]. For example, the up-regulated expression of TRIB3 promotes lipid accumulation and apoptosis of macrophages, and TRIB3 suppresses proinflammatory cytokine production in macrophages in response to oxidized low density lipoprotein (OX-LDL) stimulation [57,58]. TRIB3 can act as a negative regulator to inhibit the production of proinflammatory cytokines and chemokines, thus regulating inflammatory response [59]. Considering that many biological processes regulated by TRIB3 are related to viral infection, it provides a reference direction for the subsequent research on the mechanism of the regulation of viral infection by SCARB2-independent mode of TRIB3.

In summary, we show for the first time that host protein TRIB3, which is up-regulated during EV-A71 infection, promotes EV-A71 infection via enhancing the replication of the EV-A71 genome. TRIB3 can also inhibit the ubiquitination and degradation of EV-A71 receptor protein SCARB2 and up-regulate the level of SCARB2, thereby promoting EV-A71 infection. Our findings reported herein thus deepen the understanding of the molecular mechanism of EV-A71 infection and pathogenesis and reveal TRIB3 as a potential target for treatment of EV-A71 and other enteroviruses infections.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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