

## Research Article

## Ubiquitin-specific protease 24 promotes EV71 infection by restricting K63-linked polyubiquitination of TBK1

Lichao Zang<sup>a,1</sup>, Jin Gu<sup>d,1</sup>, Xinyu Yang<sup>a</sup>, Yukang Yuan<sup>b,c</sup>, Hui Guo<sup>a</sup>, Wei Zhou<sup>a</sup>, Jinhong Ma<sup>a</sup>, Yan Chen<sup>a</sup>, Yumin Wu<sup>a</sup>, Hui Zheng<sup>b,c,\*</sup>, Weifeng Shi<sup>a,\*</sup><sup>a</sup> Department of Laboratory Medicine, The Third Affiliated Hospital of Soochow University, Changzhou, 213003, China<sup>b</sup> International Institute of Infection and Immunity, Institutes of Biology and Medical Sciences, Soochow University, Suzhou, 215123, China<sup>c</sup> Jiangsu Key Laboratory of Infection and Immunity, Soochow University, Suzhou, 215123, China<sup>d</sup> Hubei Hospital of Integrated Chinese and Western Medicine, Wuhan, 430015, China

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## ABSTRACT

TANK-binding kinase 1 (TBK1) is an essential protein kinase for activation of interferon regulatory factor 3 (IRF3) and induction of the type I interferons (IFN-I). Although the biochemical regulation of TBK1 activation has been studied, little is known about how enterovirus 71 (EV71) employs the deubiquitinases (DUBs) to regulate TBK1 activation for viral immune evasion. Here, we found that EV71 infection upregulated the expression of ubiquitin-specific protease 24 (USP24). Further studies revealed that USP24 physically interacted with TBK1, and can reduce K63-linked polyubiquitination of TBK1. Knockdown of USP24 upregulated TBK1 K63-linked polyubiquitination, promoted the phosphorylation and nuclear translocation of IRF3, and in turn improved IFN-I production during EV71 infection. As a consequence, USP24 knockdown dramatically inhibited EV71 infection. This study revealed USP24 as a novel regulator of TBK1 activation, which promotes the understanding of immune evasion mechanisms of EV71 and could provide a potential strategy for treatment of EV71 infection.

## 1. Introduction

The innate immune response constitutes the first line of host defense against pathogen invasion, particularly in the early stage of viral infections. Multiple pattern recognition receptors (PRRs) of the innate immune system recognize pathogen-associated pattern molecules (PAMPs), when a pathogen invades host cells. PRRs include retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and cytoplasmic DNA recognition receptors (Kawai and Akira, 2006). The activation of these receptor proteins can stimulate the relevant natural immunity to resist the infection of pathogenic microorganisms.

Enterovirus 71 (EV71) is one of the main pathogens of hand foot and mouth disease (HFMD), belonging to the *Enterovirus* genus of the *Picornavirus* family. EV71 was first isolated in 1969 from the feces of a California infant with central nervous system disease. It has been reported that humans are the only natural host of EV71, which invades cells pri-

marily through scavenger receptor B2 (SCARB2), p-selectin glycoprotein ligand-1 (PSGL-1), sialo glycan, heparin sulfate, and membrane II (Anx2) (Kobayashi and Koike, 2020). When EV71 binds to its receptor, the spatial configuration of its virus particles immediately changes. Subsequently, the virus removes its protein shell, releases viral nucleic acid into the cell, and begins to translate viral peptides using multiple host factors (Shih et al., 2011).

EV71 has evolved perfectly good immune escape strategies. It was found that the 2A protease of EV71 not only cleaved Melanoma differentiation-associated gene 5 (MDA5) and Mitochondrial antiviral signaling protein (MAVS) in the RLR signaling pathway but also down-regulated type I interferon receptor (IFNAR1) to inhibit IFN-I antiviral immune response. Whereas, the 3C protease of EV71 can inhibit the interaction between RIG-I and MAVS, and block antiviral immune signal transduction (Lei et al., 2010; Jing et al., 2012; B. Wang et al., 2013; Qian et al., 2014). The protease 2B of EV71 induces apoptosis through activation of the pro-apoptotic protease BAX (Cong et al., 2016). In addition,

\* Corresponding authors.

E-mail addresses: [huizheng@suda.edu.cn](mailto:huizheng@suda.edu.cn) (H. Zheng), [swf67113@163.com](mailto:swf67113@163.com) (W. Shi).<sup>1</sup> Lichao Zang and Jin Gu contributed equally to this work.

2A protease and 3C protease cleave NOD-like receptor protein 3 (NLRP3) protein to inhibit the inflammation signal transduction pathway (Wang et al., 2015). The complex and powerful immune escape mechanisms of EV71 prevent the antiviral immune response of host cells. Therefore, it is important to find new targets to enhance the antiviral immune activity.

Ubiquitination is a covalent posttranslational modification in eukaryotic cells, during which ubiquitin forms different ubiquitin chains on target proteins by linking different lysine residues. Target protein molecules can be either mono-ubiquitin modified or poly-ubiquitin modified (Calistri et al., 2014). Different forms of lysine connections between ubiquitin molecules determine the types of polyubiquitin chains on target proteins, which regulate varieties of biological processes in cells, including endocytosis of macromolecules, protein transport, DNA damage repair, and cellular immune function. As an example, the K63-linked polyubiquitination modification plays an important role in the transmission of innate immune signals (Zeng et al., 2010; Hou et al., 2011). Ubiquitination modification is a highly reversible process, in which ubiquitin chains can be removed by deubiquitinases (DUBs). There are more than one hundred known DUBs, which are divided into six groups based on their structural properties. Ubiquitin-specific protease (USP) as the biggest family of DUBs plays a key role in the regulation of antiviral immune signaling pathways. For example, USP and USP21 specifically remove the K63 ubiquitin chain on RIG-I, thereby inhibiting IRF3 activation induced by vesicular stomatitis virus (VSV) (Cui et al., 2014; Fan et al., 2014). USP4 specifically cleaves the K48 ubiquitin chain on RIG-I, which promotes RIG-I mediated IFN-I antiviral immune response (L. Wang et al., 2013).

USP24 belongs to the USP family of DUBs. USP24 has been reported to play crucial roles in the regulation of the occurrence and development of tumors, neurodegenerative diseases, autophagy and DNA damage repair (Li et al., 2010; Zhang and Gong, 2016; Thayer et al., 2020; Bedekovics et al., 2021). However, whether and how USP24 regulates viral infection remains unknown. In this study, we found that EV71 upregulated the expression of USP24 in human rhabdomyosarcoma cells (RD). Furthermore, we revealed the mechanism by which USP24 inhibits the production of IFN-I to achieve immune escape during EV71 infection.

## 2. Materials & methods

### 2.1. Cell culture

HEK293T cells and the RD cells (National Collection of Authenticated Cell Cultures) were maintained in Dulbecco modified Eagle medium (DMEM) (HyClone, UT, USA) supplemented with 100 U/mL penicillin–streptomycin antibiotics (Invitrogen) and 10% fetal bovine serum (Gibco, OK, USA) at 37 °C under a 5% CO<sub>2</sub> atmosphere. Mouse peritoneal macrophages were harvested from mice 4 days after injection of thioglycolate (BD) and were cultured in DMEM with 10% fetal bovine serum medium (FBS).

### 2.2. Virus infection

The EV71 strain (BrCr strain, ATCC VR784, GenBank accession number: U22521) came from China Centre for Type Culture collection. Vesicular stomatitis virus (VSV) was a gift from Dr. Chen Wang (Shanghai Institutes for Biological Sciences, Chinese Academy of Science). Influenza A virus (H1N1, PR/8/34) was a gift from Dr. Jianfeng Dai (Institutes of Biology and Medical Sciences, Soochow University). Herpes simplex virus was a gift from Dr. Chunfu Zheng (Fujian Medical University). Cells were seeded in 6-well plates and cultured to a density of 70%–80%. The medium was discarded and the cells were washed twice with sterile 1 × PBS. Then the cells were infected with the viruses (MOI = 1.0) in serum-free DMEM for 1.5 h. After that, the viruses were removed and the cells were cultured in fresh medium containing 10% fetal bovine serum.

### 2.3. Plasmids and antibodies

The pcDNA3.1-Flag-RIG-I, pcDNA3.1-Flag-MAVS, pcDNA3.1-Flag-TBK1, pcDNA3.1-Flag-TRAF3, pcDNA3.1-Flag-TRAF6, pcDNA3.1-Flag-IRF3, Wild-type (WT) HA-tagged ubiquitin and its mutated plasmids (R48K: only lysine (Lys, K)-48 retained; R63K: only lysine-63 retained) were gifts from Dr. Lingqiang Zhang (State Key Laboratory of Proteomics, Beijing). PcDNA3.1-Flag-TBK1-K154R was generated by the Quik-Change site-directed mutagenesis kit (Stratagene, CA, USA). All plasmids were confirmed by DNA sequencing. The transient transfection was carried out by Lipofectamine 2000 (Invitrogen, CA, USA) in accordance with standard protocols.

Antibodies against IRF3 (11904s), phosphorylated IRF3 (4947s), TBK1 (3504s) and phosphorylated TBK1 (5504s) were purchased from the Cell Signaling Technology (CST, MA, USA). Antibodies for USP24 (13126-1-AP), β-Actin (66009-1-Ig), hemagglutinin (HA)-horseradish peroxidase (HRP) (51064-2-AP), Flag (66008-3-Ig), GAPDH (60004-1-Ig), Tubulin (11224-1-AP), HRP conjugated goat anti-rabbit IgG secondary antibodies (SA00001-2) and HRP conjugated goat anti-mouse IgG secondary antibodies (SA00001-1) were obtained from the Protein TECH Group (Chicago, USA) and MBL (Japan). Antibodies against EV71/VP1 (ab169442) were from Abcam (UK) and anti-Flag (M2)-horseradish peroxidase (A8592) was purchased from the Sigma (USA). Antibodies against IRF3 (sc-33641) and anti-rabbit-IgG (sc-52336) were from Santa Cruz (USA). The sequence for silencing USP24 (Gene ID:23358) was synthesized by HanBio (Shanghai, China) and listed as follows: shRNA 1: 5'-CTCGTATGTAACGTATTTG-3'; shRNA 2: 5'-GCGCTATGTGATCATCATA-3'; shRNA 3: 5'-CACTTACCGGGAGTATTTA-3'.

### 2.4. Stable cell lines with USP24 knockdown (shUSP24)

The shUSP24 vectors and the control vectors (shNC) were transfected into HEK293T cells to make lentiviral particles. RD cells were seeded in 6-well plates and then treated with 1 mL DMEM with 1 μL (10 mg/mL) polybrene, 5 μg shNC or 5 μg shUSP24 lentiviral particles. Twelve hours after infection, the medium was replaced by fresh medium and then cells were cultured for another 72 h. Cells were treated the optimal antibiotic screening concentration until the control cells died.

### 2.5. EV71 plaque assay

RD cells were infected with EV71 (MOI = 1.0). After 24 h, the supernatant was collected in EP tubes. RD cells in a 96-well plate were infected with an equal dilution of viral supernatant for 1.5 h. Next, the viral supernatant was discarded, and cells were washed with 1 × PBS, and maintained in DMEM with 10% fetal bovine serum (FBS). The cell morphology was observed and recorded every day. The TCID<sub>50</sub> was calculated using the Spearman-Kärber algorithm.

### 2.6. RNA quantitation

The total RNA was extracted by the Trizol method and reversely transcribed using a Prime Script RT Reagent kit (TaKaRa, Japan). Quantitative real-time PCR (RT-qPCR) was performed using an ABI 7500 PCR system (Applied Biosystems, USA). The GAPDH gene was used to make a control. Fluorescent dye for amplification was used for SYBR Green Premix Ex Taq II PCR mix (TaKaRa, Japan). The reaction conditions were: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min, and extension at 72 °C for 30 s, 40 cycles. The CT value at the end of the reaction was used to analyze the expression levels of the target genes. The calculation method of the target gene is as follows: three replicate wells are set for each sample, and the relative expression level of the target gene is calculated by the 2<sup>-ΔΔCt</sup> method (ΔΔCt = (Ct target gene – Ct reference gene) experiment – (Ct

target gene – Ct internal reference gene) control). RT-qPCR primers are listed in [Supplementary Table S1](#).

## 2.7. Immunoblotting and immunoprecipitation

Cells were harvested using NP-40 lysis buffer. The lysates were incubated with Flag beads for 3 h at 4 °C or IRF3 antibody overnight, and then with protein A/G plus agarose beads (Santa Cruz, USA) at 4 °C for 3 h. All beads were collected via centrifugation for 1 min at 12,000 ×g. The supernatant was discarded, and the pellets were washed 4–5 times. Then 3× loading buffer was added to the samples. Western blot was performed to analyze the immunoprecipitants. 1%–2% of the input lysates of whole cells served as a control.

## 2.8. Immunofluorescence and confocal microscopy assay

Ten hours after EV71 infection of RD cells, the effect of RD cell lesions under different treatment conditions was observed under an inverted microscope. Images are magnified at 200×. The nuclear translocation of IRF3 was determined by the immunofluorescence technique. Ten hours after EV71 infection of RD cells, cells were washed with 1× PBS buffer, followed by the addition of 4% paraformaldehyde and fixed on ice. Cells were then permeabilized by adding 0.2% Triton X-100. After washing with 1× PBS buffer, 5% BSA was added to the cells. Cells were incubated with IRF3 antibody (1:50, Santa Cruz, sc-33641) diluted in 1% Triton X-100 at 4 °C overnight, and were incubated with a fluorescent antibody (Alexa Fluor, A21053) for 1 h at room temperature. Meanwhile, the cells in the IgG control group were incubated with rabbit IgG antibody (1:50, Santa Cruz, sc-52336) and a fluorescent antibody in the same manner. Nuclei were stained with DAPI. Fluorescent images were taken using a Nikon A1 confocal microscope.

## 2.9. CCK8 assay

RD cells were first transfected with different constructs. After centrifuging at 800 ×g for 3 min at 25 °C, the number of cells was calculated using a counting plate. RD cells were seeded at a proportion of  $5 \times 10^3$ /well in 96-well plates. The media was changed to DMEM with 2% FBS. One group was infected with EV71 (MOI = 1.0) at different time points, and another group was infected by EV71 at different MOI. Meanwhile, the control group (containing only cells) and blank holes (containing only culture medium) were set up. After 24 h, CCK-8 solution (10 μL) was immediately added to each well. The plate was put in an incubator at 37 °C, and incubated for 1 h. Absorbance at 450 nm was measured with a microplate reader. The results were calculated by the following formula: cell survival = [(Absorbance of test group – Absorbance of blank)/(Absorbance of control group – Absorbance of blank)] × 100%.

## 2.10. Statistical analysis

Data were expressed as means ± standard deviation (SD). The significance of differences was evaluated using one-way ANOVA or *t*-test implemented in SPSS statistics 17.0 software; *P* < 0.05 was used as a threshold for determination of inferential statistical significance.

## 3. Results

### 3.1. EV71 infection significantly promotes the expression of the deubiquitinase USP24

We analyzed 88 common human deubiquitinase genes present in EV71-infected RD cells by PCR microarray, aiming at identifying the potential deubiquitinases regulated by EV71 infection. The data showed that the expression of host deubiquitinases, including *Usp54*, *Usp43*, and *Usp4*, were downregulated during EV71 infection, while the expression

of *Usp24*, *Otud7b*, and *Usp19* were differently up-regulated ([Fig. 1A](#)). Previous studies have suggested the involvement of USP24 in tumorigenesis and Alzheimer's disease (AD) ([Li et al., 2010](#); [Bedekovics et al., 2021](#)). Whether USP24 is associated with the viral infection and innate immunity remains unknown. Therefore, we focused on USP24 to explore its regulatory role in the innate immune response against EV71 infection. We further confirmed that the expression levels of USP24 were up regulated during EV71 infection by RT-qPCR ([Fig. 1B](#)) and Western blot ([Fig. 1C](#)). Interestingly, we noticed that USP24 expression levels were not significantly affected by two RNA viruses, vesicular stomatitis virus (VSV) and influenza A virus (H1N1), as well as a DNA virus herpes simplex virus (HSV) in RD cells and mouse peritoneal macrophages cells ([Fig. 1D](#)). Taken together, these findings revealed that EV71 infection can promote USP24 expression.

### 3.2. USP24 promotes EV71 infection

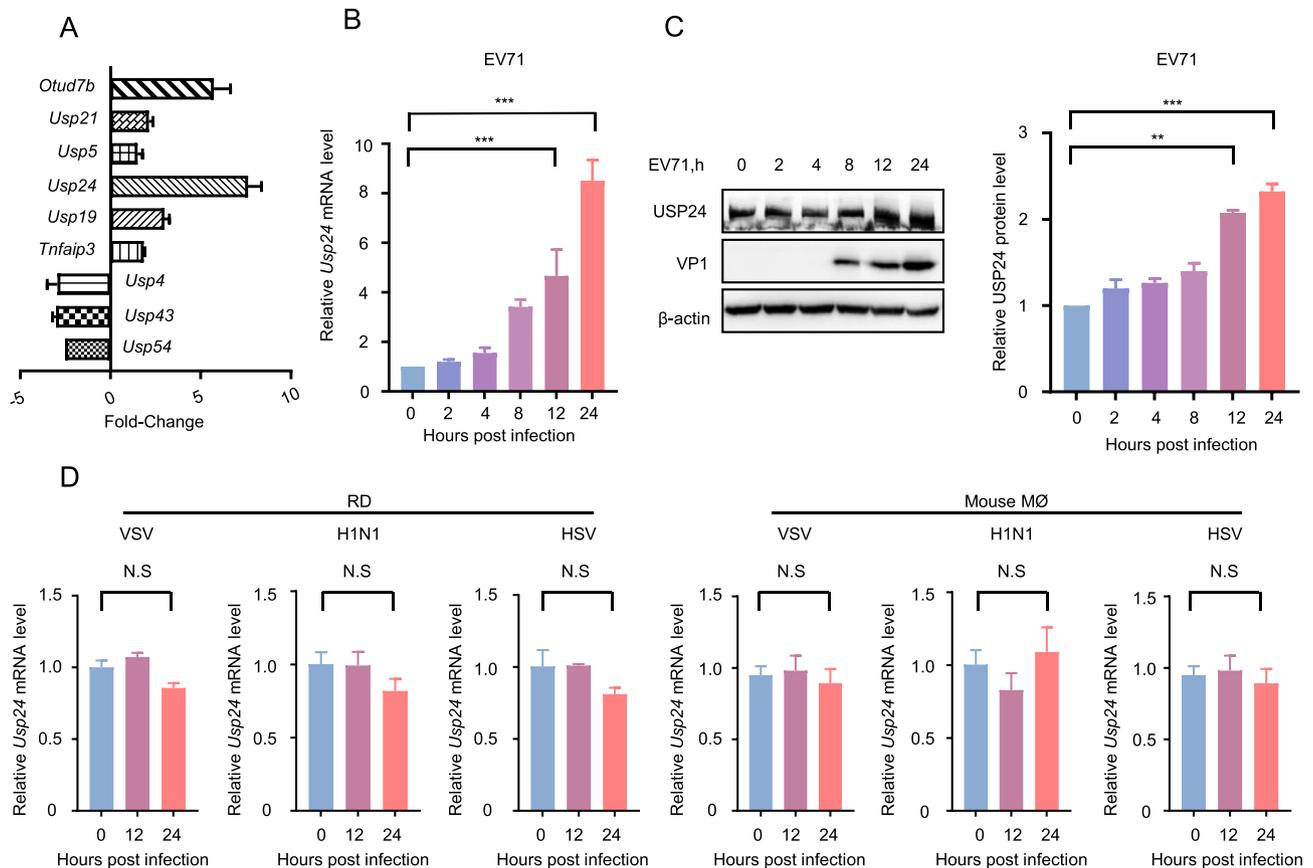
To explore the regulatory role of USP24 in EV71 replication, we designed three shRNAs to knockdown USP24 expression. Given that the shRNA (#2) showed the highest efficiency for USP24 knockdown ([Fig. 2A & B](#)), we next utilized the shUSP24 (#2) to observe the effects of USP24 on EV71 infection and antiviral immune signaling. We found that during EV71 infection, knockdown of USP24 down regulated the expression of EV71-encoded VP1 proteins ([Fig. 2C](#)) in a time-dependent manner ([Fig. 2D](#)). Consistently, EV71 viral RNA levels ([Fig. 2E](#)) and viral titers ([Fig. 2F](#)) were also down regulated by USP24 knockdown. Furthermore, we found that knockdown of endogenous USP24 significantly suppressed the cytopathogenic effect of EV71 infection ([Fig. 2G](#)). Therefore, we speculated that knockdown of USP24 could assist host cells to resist EV71 infection to some extent. Further experiments confirmed that USP24 knockdown alleviated the disease progression caused by EV71 infection in RD cells and therefore sustained cell survival ([Fig. 2H & I](#)). Together, our findings suggested that USP24 is a positive regulator of EV71 infection.

### 3.3. USP24 negatively regulates EV71-induced IFN-I production

We next sought to determine the mechanism by which USP24 promotes EV71 infection. IFN-I is one of the most important signaling molecules in innate immunity, so we hypothesized that USP24 may affect IFN-I responses during EV71 infection. We found that USP24 knockdown promoted the mRNA expression of IFNβ induced by EV71 infection ([Fig. 3A](#)). Consistently, the secreted IFNβ also increased in EV71-infected cells when USP24 was knocked down ([Fig. 3B](#)). Given that increased levels of IFN-I could stimulate higher expression of IFN-stimulated genes (ISGs), we next determined whether USP24 could influence ISGs expression in EV71 infection. In line with our above findings, knockdown of endogenous USP24 significantly increased the mRNA levels of the representative ISGs, including *Mx1* and *Viperin*, in cells with EV71 infection ([Fig. 3C](#)). These findings suggested that USP24 negatively regulates IFN-I production and subsequent ISGs expression, which could help the understanding of how USP24 promotes EV71 infection.

### 3.4. USP24 negatively regulates IRF3 activation and nuclear translocation

These above results have raised our interest in how USP24 affects IFN-I production. IRF3 is a member of the interferon regulatory family that controls multiple interferon induction pathways. Pathogenic microorganisms can induce IRF3 phosphorylation (p-IRF3), which forms a dimer of IRF3 and then translocates into the nucleus to induce IFN-I production. We found that knockdown of USP24 upregulated the phosphorylation levels of IRF3 induced by EV71 infection, but did not affect the protein levels of IRF3 ([Fig. 4A](#)). This suggested that USP24 affects IFN-I production by regulating IRF3 phosphorylation and activation rather than directly regulating total IRF3 protein levels. Furthermore, we observed the nuclear translocation of IRF3 in cells by



**Fig. 1.** EV71 infection significantly promotes the deubiquitinase USP24 expression. **A** Differential gene expressions of human DUBs in RD cells infected with EV71 were analyzed by PCR microarray after 8 hpi. **B** RT-qPCR analysis of the USP24 mRNA levels in RD cells infected by EV71 (MOI = 1.0) at different time points. **C** Western blot analysis of the expression of USP24 and EV71/VP1 in RD cells infected by EV71 (MOI = 1.0) at different time points. The independent experiments were performed in triplicate. **D** RT-qPCR analysis of the USP24 mRNA levels in RD cells and mouse peritoneal macrophages infected by VSV, H1N1 and HSV (MOI = 1.0) at different time points. Data (**B**, **D**) were shown relative to GAPDH expression and are presented as the mean  $\pm$  SD from three independent experiments. N.S, not significant ( $P > 0.05$ ),  $**P < 0.01$  and  $***P < 0.001$ .

immunofluorescence technique. The results showed that USP24 knockdown upregulated nuclear IRF3 levels during EV71 infection (Fig. 4B). Thus, these findings suggested that USP24 regulates IRF3 activation and nuclear translocation during EV71 infection.

### 3.5. USP24 can interact with TBK1

The RIG-I/MAVS signaling mediates IFN-I antiviral immune response against RNA virus invasion. Thus, we next determined how USP24 affects the RIG-I/MAVS pathway during EV71 infection. To this end, HEK293T cells were transfected with the key components of the RIG-I/MAVS pathway, including RIG-I, MAVS, TRAF6/3, TBK1 and IRF3, and then IP experiments were performed to pull down the potential proteins that interact with USP24 (Fig. 5A–F). We found that USP24 could interact with TBK1, but not RIG-I, MAVS, TRAF6/3, or IRF3 (Fig. 5A–F). Furthermore, we confirmed that endogenous TBK1 could interact with endogenous USP24 in HEK293T cells (Fig. 5G). Collectively, these findings suggested that USP24 could target TBK1 to regulate IRF3 activation and IFN-I production.

### 3.6. USP24 reduces K63-linked polyubiquitination of TBK1

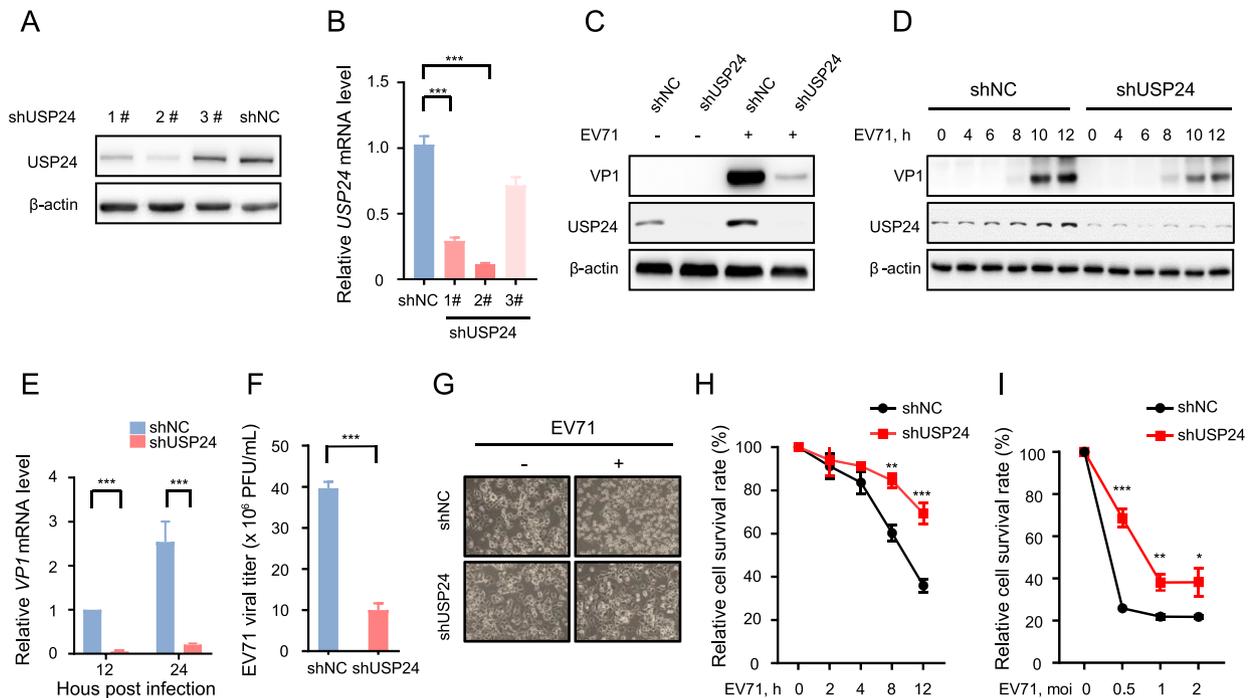
Our above studies have demonstrated that USP24 interacts with TBK1. Thus, we further investigated the effect of USP24 on TBK1. Given that USP24 is a deubiquitinase, we observed how USP24 regulates protein levels and ubiquitination modifications of TBK1. We found that knockdown of USP24 did not affect the expression levels of endogenous

TBK1 proteins (Fig. 6A). Furthermore, we observed that knockdown of USP24 increased total polyubiquitination levels of TBK1 (Fig. 6B). To analyze which types of ubiquitination of TBK1 are regulated by USP24, we transfected RD cells with WT-Ub, or R48K-Ub, or R63K-Ub constructs. The results showed that the K63-linked but not K48-linked polyubiquitination of TBK1 was dramatically upregulated by USP24 knockdown (Fig. 6C).

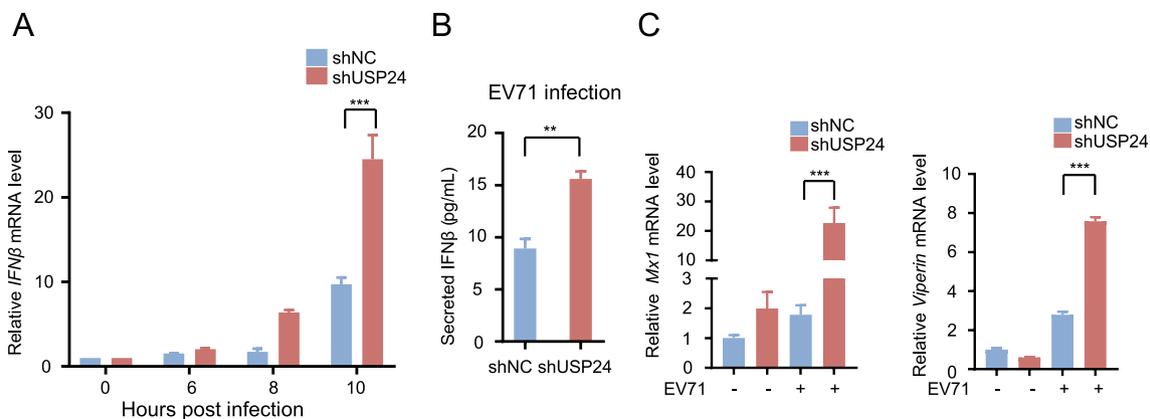
It has been reported that Lys69, Lys154 and Lys372 of TBK1 are acceptor sites for K63-linked polyubiquitination, although other lysine residues could be involved in the regulation of TBK1 K63-linked polyubiquitination (Wang et al., 2012). Among them, Lys154 exhibited the strongest effect on the regulation of both TBK1 phosphorylation and IRF3 phosphorylation (Wang et al., 2012). Thus, we transfected RD cells with Flag-TBK1 (WT) and Flag-TBK1-K154R. The results showed that USP24 knockdown dramatically promoted the K63-linked polyubiquitination of wild type TBK1 but not TBK1-K154R (Fig. 6D), suggesting that USP24 regulates TBK1 K63-linked polyubiquitination dependently on the Lys 154 residue. Taken all together, these findings demonstrated that USP24 can target TBK1 and regulate K63-linked polyubiquitination of TBK1, which affect IRF3 activation and IFN-I production during EV71 infection.

## 4. Discussion

The production of type I interferon caused by viral infection is a crucial mechanism for the antiviral innate immunity and later-stage adaptive immunity (Tian et al., 2019; Zhu et al., 2019). This study found that USP24 can target TBK1 protein and affect its K63-linked



**Fig. 2.** USP24 promotes EV71 infection. **A, B** Western blot (**A**) and RT-qPCR (**B**) analysis of USP24 levels in RD cells transfected with control shRNA (shNC) or USP24-specific shRNAs (shUSP24, #1, #2 and #3) for 72 h. **C, D** Western blot analysis of EV71 protein levels in RD cells transfected with shNC or shUSP24 (#2) and then infected by EV71 (MOI = 1.0) for 24 h (**C**) or different times (**D**). **E** RT-qPCR analysis of EV71-VP1 RNA levels in RD cells transfected with shNC or shUSP24 (#2) and then infected by EV71 (MOI = 1.0) for indicated times. **F** Viral titers of EV71 in RD cells with stable knockdown of USP24 were analyzed. **G** Phase contrast microscopy of RD cells with stable knockdown of USP24 infected with EV71 (MOI = 1.0) at 10 h post infection. Magnification = 20×. **H** CCK8 analysis of RD cell survival after infection with EV71 (MOI = 1.0) for the indicated times. The cell survival rates at 0 h time points were normalized as 100%. **I** CCK8 analysis of RD cells survival after infection with different amount of EV71. The cell survival rates when MOI = 0 was normalized as 100%. Data (**B, E, F, H, I**) were shown as the mean ± SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . The independent experiments (**A, D**) were performed in triplicate.

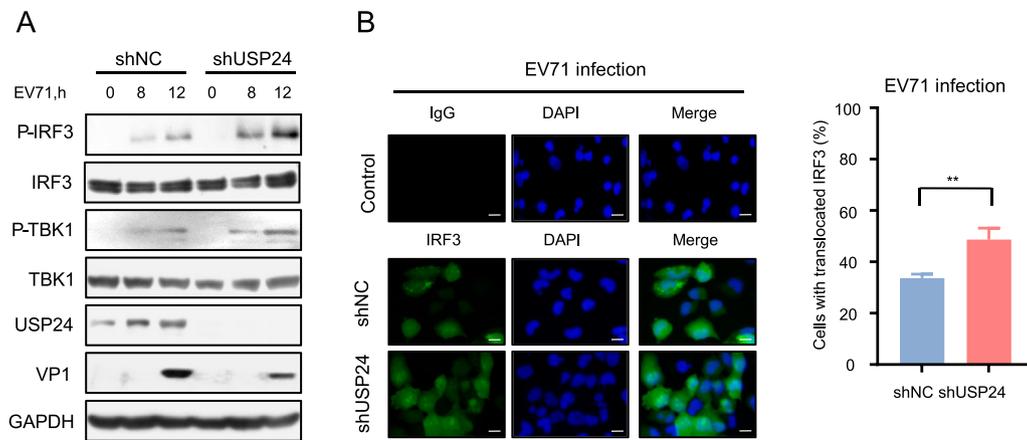


**Fig. 3.** USP24 negatively regulates EV71-induced IFN-I production. **A** RT-qPCR analysis of IFN $\beta$  mRNA levels in RD cells with stable knockdown of USP24 that infected with EV71 (MOI = 1.0) at the indicated time. **B** ELISA analysis of the IFN $\beta$  protein levels in RD cells with stable knockdown of USP24 that infected with EV71 (MOI = 1.0) for 24 h. **C** The mRNA levels of the Mx1 and Viperin in RD cells with stable knockdown of USP24 that infected with EV71 (MOI = 1.0) for 24 h were analyzed by RT-qPCR. Data were shown relative to GAPDH expression and are presented as the mean ± SD from three independent experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

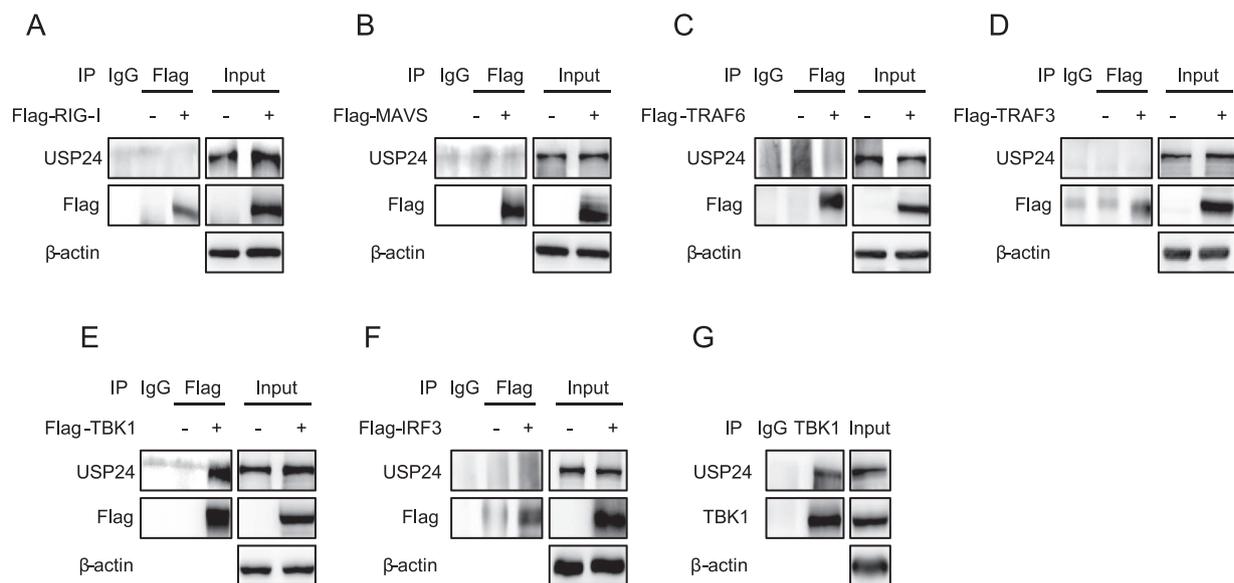
polyubiquitination in cells with EV71 infection. Importantly, USP24 was upregulated by EV71 infection, which suggested a novel regulatory mechanism of the RIG-I/MAVS signaling pathway. As a consequence, USP24-mediated regulation of TBK1 ubiquitination and IRF3 activation weakens the host's antiviral innate immunity and ultimately benefits the immune escape of EV71. In addition, it is possible that USP24 could regulate infection of other viruses, even if these viruses cannot promote USP24 expression. In fact, one can speculate that USP24 could affect both host proteins and virus-encoded proteins, which eventually either

promotes or inhibits infection of different viruses by different mechanisms. It will be interesting to study the roles of USP24 in different types of viral infections in the future.

Although the critical roles of MDA5 (Ren et al., 2020; Zhu et al., 2021), RIG-I (Wang et al., 2012), and TLR-3 in mediating host innate immune responses (Chathuranga et al., 2021) against EV71 infection have been reported, the virus actually has developed multiple complex mechanisms to evade or antagonize the host innate immune response. TBK1 mediates the activation of IRF3 and IRF7, inducing IFN- $\alpha$  or



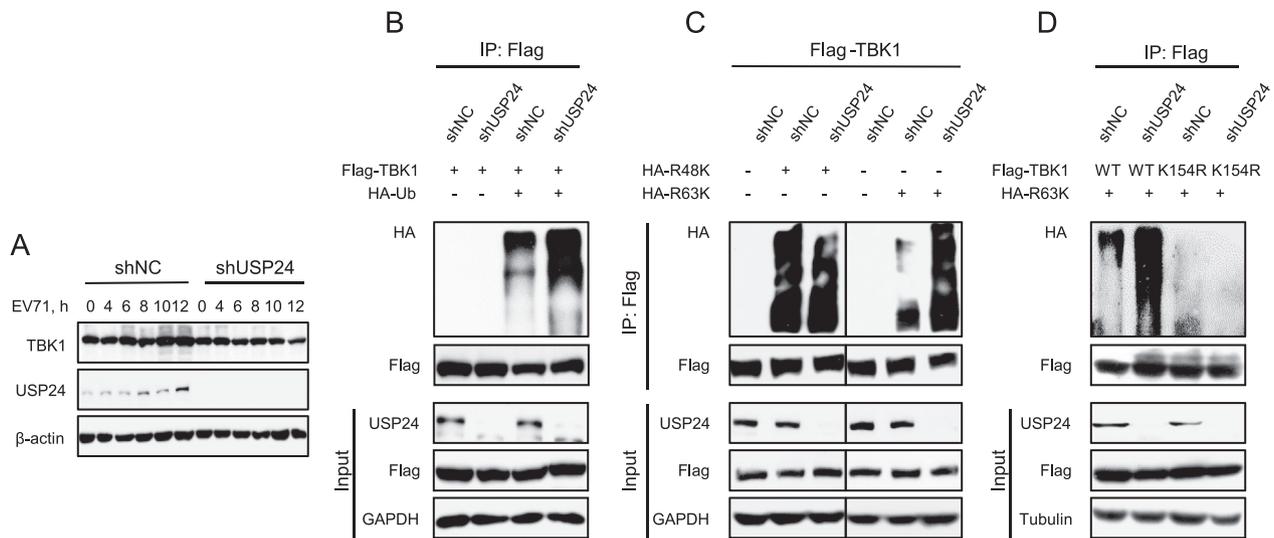
**Fig. 4.** USP24 negatively regulates IRF3 activation and nuclear translocation. **A** Western blot analysis of p-IRF3 (pSer396-IRF3), total IRF3, p-TBK1 (pSer172-TBK1), total TBK1 levels in RD cells with stable knockdown of USP24 that were infected with EV71 (MOI = 1.0) at the indicated time. **B** Immunofluorescence analysis of nuclear IRF3 in RD cells with stable knockdown of USP24 that infected with EV71 (MOI = 1.0) at 10 h. The percentage of cells in which IRF3 was translocated into the nucleus was calculated: a total of 300 cells from different fields were counted. Results are shown as means  $\pm$  S.D. from three independent experiments. Scale bar, 20  $\mu$ m. \*\* $P$  < 0.01.



**Fig. 5.** USP24 can interact with TBK1. **A–F** Co-immunoprecipitation analysis of the interaction between USP24 and the key components of RIG-I/MAVS signaling. HEK293T cells were transfected with Flag-RIG-I or Flag-MAVS or Flag-TRAF6 or Flag-TRAF3 or Flag-TBK1 or Flag-IRF3, and immunoprecipitated with anti-Flag agarose beads. The eluted immunocomplex was then subjected to SDS-PAGE analysis with anti-USP24 antibody. **G** Co-immunoprecipitation analysis the interaction between endogenous USP24 and TBK1 in HEK293T cells. The independent experiments were performed in triplicate.

IFN- $\beta$ ) in innate antiviral immune response (He et al., 2017; Brown et al., 2021). As a key protein kinase for IFN-I production, TBK1 is involved in the host's homeostasis maintenance (Basit et al., 2020; Khoshnood et al., 2021). However, its activity may be associated with phosphorylation and ubiquitination modifications (Deng et al., 2020; Guo et al., 2020; Khoshnood et al., 2021). To date, there is no relevant report between EV71 infection and the activity of TBK1 protein. Here, we found that USP24 can downregulate TBK1 ubiquitination and influence IRF3 activation during EV71 infection. Thus, this study revealed the regulation of the USP24-TBK1 axis on EV71 infection.

USP24 was first recognized due to its association with a rare human nervous system disease Parkinson's disease (PD), which results in a tremble, amyotrophic, bradykinesia, and other symptoms (Oliveira et al., 2005). At present, most studies on USP24 focused on the cancer or nerves field (Bedeckovics et al., 2021; He et al., 2021; Wang et al., 2021). In this study, we revealed a new biological function of USP24, that is, USP24 can restrict the innate antiviral immune response and downregulate interferon production. We showed that USP24 inhibited IFN-I production by targeting TBK1 and reduced the expression of ISGs, thereby promoting EV71 virus infection. However, the lack of reliable animal models is an



**Fig. 6.** USP24 reduces K63-linked polyubiquitination of TBK1. **A** Western blot analysis of TBK1 protein levels in RD cells with stable knockdown of USP24 that infected with EV71 (MOI = 1.0) at the indicated time. **B** Immunoprecipitation analysis of total ubiquitination of TBK1 in HEK293T cells transfected with Flag-TBK1, HA-Ub and shUSP24. **C** Immunoprecipitation analysis of the ubiquitination types of TBK1 in HEK293T cells transfected with Flag-TBK1, HA-Ub (R48K) or HA-Ub (R63K), together with or without shUSP24 as indicated. **D** Immunoprecipitation analysis of the K63 ubiquitination of TBK1 in HEK293T cells transfected with Flag-TBK1, Flag-TBK1-K154R, together with shUSP24 as indicated. The independent experiments were performed in triplicate.

issue when studying EV71-induced disease manifestations in humans (Wang and Yu, 2014). Current studies have shown that SCARB2 receptors in mice are different significantly from humans, and mouse models of EV71 infection do not generalize all aspects of human disease. Existing mouse models of infection have been constructed by adapting mice to increase the virulence of EV71 clinical isolates or by constructing adult immunodeficient mice (Chua et al., 2008; Khong et al., 2012). However, mouse-adapted viruses are still unable to infect mice beyond 14 days of age (Liu, 2005). Another animal model is hSCARB2 transgenic mice, however, EV71-infected mice do not exhibit the full range of human infection symptoms and disease mechanisms similar to those of human infection. Therefore, we consider that USP24-deficient mice could be used to confirm the *in vivo* function of USP24 during EV71 infection when a mouse model with a similar mechanism of infection to that of humans emerges in the future.

The genetic relationship between hand, foot, and mouth disease caused by EV71 and patients has not been linked, but several studies have found a correlation between the genetic diversity of patients and susceptibility to EV71 and disease severity. Chen found that patients with congenital TLR3 or MDA5 defects increased susceptibility to EV71 (Jie et al., 2021); Li found that genetic polymorphisms in the endothelial nitric oxide synthase gene correlated with the degree of EV71 infection (Li et al., 2013). Most of the USP24 gene mutation or deletion studies have been associated with PD or tumor development (Li et al., 2010; Bedekovics et al., 2021). However, there is no conclusive evidence on the association of children with USP24 defects or mutations with EV71 infection. In our study, we found that during EV71 infection, USP24 was associated with the production of IFN-I, which is protective against EV71 infection; therefore, we hypothesized that children with USP24 deficiency or mutation may be resistant to EV71 infection. However, this hypothesis needs to be supported by further clinical cases and large-scale epidemiological screening studies.

Currently, it is still unclear how EV71 inhibits IFN-I production by up-regulating USP24 protein for the purpose of immune escape. It has been suggested that SP1 is one of the transcription factors that regulates

USP24 (Wang et al., 2014). Thus, it will be interesting to further observe whether and how EV71 infection could regulate SP1 in the future. In addition, TLR9-MyD88 signal activation can downregulate the expression of USP24 (Chouri et al., 2021), while EV71 was reported to inhibit MyD88 expression by inducing miR-21 expression (Blasius and Beutler, 2010; Feng et al., 2017). Therefore, it is also possible that EV71 could affect USP24 levels through miR-21-MyD88-TLR signaling. In terms of EV71-encoded proteins, it has been reported that EV71 inhibits IFN signaling pathway mainly by 2A and 3C protein (Rasti et al., 2018). 2A protein, a non-structural protein of EV71, can target IFNAR1, MAVS and MDA5 protein levels to inhibit IFN signaling pathway (Lu et al., 2012; Wang et al., 2016). The EV71 3C protein can also interact with RIG-I protein to block the IFN signaling pathway and exert its protease activity to degrade IRF7, TRIF and IRF9 protein, ultimately inhibiting the production of IFN (Lei et al., 2010, 2011, 2013; Hung et al., 2011). Thus, there is a possibility that EV71 2A and 3C proteases may be involved in EV71-induced USP24 expression, which deserves further investigation in the future. However, it is possible that other proteins of EV71 upregulate USP24 expression.

## 5. Conclusions

In this paper, we reveal that EV71 infection promotes host USP24 gene expression and therefore inhibits IFN-I antiviral immune response. Mechanistically, USP24 can target TBK1 and reduce TBK1 K63-linked polyubiquitination modifications dependently on the Lys154 residue of TBK1, resulting in decreased IRF3 activation and nuclear translocation. Consequently, USP24 inhibits IFN-I production and promotes EV71 infection. These findings could facilitate the development of novel strategies to inhibit EV71 infection.

## Data availability

All the data generated during the current study are included in the manuscript.

## Ethics statement

This study does not contain any studies with human or animal objects by any of the authors.

## Author contributions

Weifeng Shi: conceptualization, review & editing, visualization. Hui Zheng: conceptualization. Lichao Zang: writing original draft preparation, data curation, investigation. Jin Gu: data curation, formal analysis, project administration, investigation. Xinyu Yan: data curation, investigation. Yukang Yuan: writing – review & editing. Hui Guo: software. Wei Zhou: formal analysis. Jinhong Ma: formal analysis. Yan Chen: data curation. Yumin Wu: funding acquisition, formal analysis.

## Conflict of interest

All authors have no competing interests to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.11.001>.

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