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RASSF4 promotes EV71 replication to accelerate the inhibition of the phosphorylation of AKT

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ABSTRACT

Enterovirus 71 (EV71) is a neurotropic virus that causes hand, foot and mouth disease (HFMD), occasionally leading to death. As a member of the RAS association domain family (RASSFs), RASSF4 plays important roles in cell death, tumor development and signal transduction. However, little is known about the relationship between RASSF4 and EV71. Our study reveals for the first time that RASSF4 promotes EV71 replication and then accelerates AKT phosphorylation inhibition in EV71-infected 293T cells, suggesting that RASSF4 may be a potential new target for designing therapeutic measures to prevent and control EV71 infection.

1. Introduction

Enterovirus 71 (EV71) is one of the major pathogens causing hand, foot and mouth disease (HFMD). Since EV71 was first isolated in California in 1969 [1], thousands of HFMD outbreaks caused by EV71 infection have been reported in Asia [2]. In China, HFMD has been classified as a certifiable class C infectious disease since 2008 [3]. EV71, belonging to the Enterovirus genus of the Picornaviridae family [4], has a positive-sense, single-stranded RNA genome containing only one open reading frame that encodes a large polyprotein. The polyprotein can be cleaved into 4 capsid proteins (VP1–VP4) and 7 nonstructural proteins (2A–2C and 3A–3D) [2], the latter of which play important roles in RNA replication, viral genome translation, polyprotein processing and the regulation of host-protein expression [5]. Some host proteins also participate in regulating the life cycle of EV71. For example, the host protein binding protein 2 (FBP2) can decrease viral protein synthesis in EV71-infected cells [6], whereas another host protein, scavenger receptor class B 2 (SCARB2), serves as a receptor for EV71 and can increase viral attachment to target cells and viral uncoating [7]. Therefore, understanding the relationships between host proteins and EV71 will provide insight into EV71 pathogenesis [8].

As members of the RAS effector family, host proteins RAS association domain family (RASSFs), regulate various normal biological processes, such as cell cycle arrest, senescence, cell adhesion and motility [9]. Ten members (RASSF1-10) have been identified to date. RASSF4 is broadly expressed in normal tissues, such as the heart, brain, placenta, lung, liver, bone, muscle and pancreas [10]. Kristin et al. demonstrated that RASSF4 is silenced by promoter methylation in tumor cells [10]. In addition, Crose et al. reported that RASSF4, considered to be an oncoprotein, can regulate the Hippo and MAPK pathways to mediate tumorigenesis [11]. Interestingly, the methylation of RASSF1A was found to activate the AKT pathway in all tested EBV-infected tissues [12]. Additionally, RASSF6 might play a role in adjusting and controlling the extent of the inflammatory reaction to respiratory syncytial virus by suppressing the NF-κB pathway [13]. However, it remains unknown whether RASSF4 regulates certain signaling pathways to assist in EV71 infection.

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A previous report showed that EV71 infection can activate the AKT pathway in both Vero and MRC-5 cells to promote viral infection [14]. However, there is no literature reporting the relationship between RASSF4 and the AKT pathway upon EV71 infection. In this study, we investigated the effect of RASSF4 on the AKT phosphorylation induced by EV71 infection and found that RASSF4 can accelerate AKT phosphorylation inhibition in EV71-infected 293T cells. Moreover, RASSF4 can increase virus replication and promote apoptosis in EV71-infected cells. These findings are important contribution to the understanding of the pathogenesis of EV71.

2. Materials and methods

2.1. Ethicsstatement

This study obtained ethics approval from the ethics committee at the School of Medicine, Wuhan University, in accordance with the guidelines for the protection of human subjects. Written informed consent was obtained from the parents of all children involved in our study.

2.2. Cells and antibodies

Human embryonic kidney (293T) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C with 5% CO_2. At 80–90% confluence, the cells were trypsinized with 0.25% trypsin (Solarbio) and supplemented with complete medium. Antibodies against RASSF4, GAPDH, and AKT as well as polyclonal antibodies recognizing phosphorylated AKT were purchased from Sigma–Aldrich; antibodies against PARP and caspase-3 were obtained from Cell Signaling. The anti-EV71 antibody was obtained from the serum of an HFMD patient.

2.3. RASSF4 overexpression and shRNA gene silencing

To construct a plasmid for RASSF4 overexpression, a fragment of RASSF4 was cloned into the *EcoR* I and *Sal* I sites of the PBABE vector using the targeting sequences RASSF4-S (5′-GGGGAAATTCATGAAGGAAGACTGTCTGCCGAG-3′) and RASSF4-A (5′-TTTGTCGAACTTACTT-GGCCTCCACCAGCTG-3′). For RASSF4 depletion, the target sequence was cloned into the *Bgl* II and *Hind* III sites of the pSUPER vector using the corresponding sequences RASSF4-shRNA-S (5′-GATCCCCGGCCACTTCTACAATCATATTCAAGAGATATGATTGTAGAAGTGGC-CTTTTTA-3′) and RASSF4-shRNA-A (5′-GGGCCGGTGAAGATGTTAGTATAAGTTCTCTATACTAA-CATCTTCACCGGAAAAATTCGA-3′).

2.4. Virus propagation and ultraviolet (UV) radiation-induced inactivation of EV71

EV71-BrCr was a gift from Professor Songya Lu (College of Life Sciences, Wuhan University, China). Cells were grown to 70%–80% confluence in complete medium, and virus infections were carried out as follows. 293T cells were infected with EV71 at the indicated multiplicity of infection (MOI) or were mock-infected using the same growth medium without virus for 1 h. The virus was removed with PBS, and then cells were cultured with fresh medium supplemented with 2% FBS. The virus-infected cells were harvested at the indicated time points post-infection, and the virus was inactivated by exposure using a portable UV lamp with a 225-nm wavelength at a distance of 2 cm for 1 h. Inactivation was confirmed by titration of the virus before and after UV exposure and by the absence of cytopathic effects after infection of 293T cells with UV-inactivated EV71.

2.5. Western blotting

Cells were lysed in buffer containing 150 mM NaCl, 25 mM Tris (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, and a proteinase inhibitor cocktail (Roche). The samples were centrifuged at 12,000 rpm for 15 min to harvest the supernatant, which was electrophoresed using 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (BioRad). The membranes were blocked with 5% dried skim milk and then incubated with the appropriate primary antibody overnight at 4 °C. The membranes were washed with PBST and incubated with a 1:5000 dilution of anti-goat or anti-mouse horseradish-peroxidase-conjugated antibody for 1 h. Following incubation, the membranes were washed extensively with PBST. Immunoreactive bands detected using ECL reagents (GE Healthcare) were developed with Super RX film.

2.6. Real-time quantitative reverse transcription PCR (qRT-PCR)

293T cells were seeded in a 12-well tissue-culture plate and transfected with the desired plasmids. After 48 h, the culture medium was then replaced with medium containing EV71 at the indicated MOI. After 1 h, the virus was removed by washing, and the cells were cultured with fresh medium supplemented with 2% FBS. Total cellular RNA was isolated with the TRIZOL reagent using standard protocols. Primers for the qRT-PCR assay included β-actin-S (5′-CACGATGGAGGGGCCGGACTCATC-3′), β-actin-A (5′-TAAA-GACCTCTATGCCAACACAGT-3′), EV71-VP1-S (5′-GCAGCCCAAAA-GAACTTCAC-3′) and EV71-VP1-A (5′-ATTTCAGATTTCAGCAGCTTGG AGTGC-3′). The assay was performed using the QuantiTect SYBR Green RT-PCR kit (QIAGEN) following the manufacturer's protocol.

2.7. 50% tissue culture infective dose (TCID50)

The virus was diluted from 10^{-1} to 10^{-8} and added to 293T cells in 96-well plates; the cells were cultured at 37 °C for 5 days and observed daily. The TCID50 values were measured by counting the cytopathic effect (CPE). Further calculations were conducted using the Reed-Muench formula.

2.8. Flow cytometry

293T cells were infected with EV71 after transfection. The cells were collected, washed with PBS and incubated with FITC-labeled annexin V and stained with PI (Baosai Biotech) at room temperature for 15 min and then analyzed by flow cytometry (BD CantoII). Early apoptotic cells only bind to Annexin V, but late apoptotic cells become stained with both annexin V-FITC and PI. A quantitative analysis was conducted by determining the percentage of stained cells among the total cells.

3. Results

3.1. RASSF4 accelerates inhibition of the AKT phosphorylation induced by EV71 infection

Wong et al. reported that EV71 infection activates various host cellular signaling pathways, including the AKT pathway, which benefits viral replication [15]. However, the activation of AKT by a phosphoinositide-dependent kinase is different in various cell types and exhibits cellular specificity [14]. Wen-Rou Wong et al. demonstrated that AKT is activated during the early stage of EV71 infection in both Vero and MRC-5 cells but does not occur in RD

cells [14]. To evaluate the effect of the association between RASSF4 and EV71 in 293T cells on the AKT signaling pathway, we first verified whether EV71 has an impact on AKT phosphorylation in 293T cells. To this end, 293T cells were infected with EV71 (MOI = 1.5) and harvested at different time points, and AKT activity was determined using western blotting. As shown in Fig. 1A, the amount of phosphorylated AKT in EV71-infected 293T cells peaked at 12 h post-infection (pi.) and then significantly decreased at 14 h, 24 h, and 32 h pi. At 14 h pi, a typical cytopathic effect (CPE) was observed in the cells infected with EV71, characterized by cell detachment and rounding. Therefore, we chose 14 h pi as the late infection time point for the ensuing experiments. These results suggested that EV71 could inhibit AKT phosphorylation in the late stages of infection. To determine whether AKT activity in EV71-infected cells is influenced by RASSF4, 293T cells were transfected with PBABE-RASSF4-puro, pSUPER-RASSF4-shRNA and their empty-vectors. The transfected cells were then infected with EV71 at an MOI of 1.5 for 1 h, and AKT activity was detected by western blotting at different time points. As shown in Fig. 1B, we found that the PBABE-puro plasmid-transfected 293T cells treated with EV71 had a notable reduction in AKT phosphorylation at 14 h pi ($p < 0.01$, Fig. 1B) compared to the mock-treated cells (transfected with PBABE-puro and mock-infected). However, we found a statistically significant reduction in AKT phosphorylation in 293T cells overexpressing RASSF4 and EV71-infected at 12 h pi ($p < 0.001$, Fig. 1B) compared to mock-treated cells (transfected with PBABE-RASSF4-puro and mock-infected). Interestingly, at 12 h pi, the phosphorylation of AKT was significantly decreased in EV71-infected 293T cells overexpressing RASSF4 compared to EV71-infected 293T cells transfected with the PBABE-puro plasmid ($p < 0.001$, Fig. 1B). This result suggests that RASSF4 can make the inhibition of AKT phosphorylation induced by EV71 ahead of time. As shown in Fig. 1C, pSUPER-shRNA plasmid-transfected 293T cells infected with EV71 exhibited reduced amounts of phosphorylated AKT at 14 h pi compared to mock-infected cells ($p < 0.001$, Fig. 1C). However, EV71-infected cells underexpressing RASSF4 showed a significant decrease in AKT phosphorylation at 32 h pi ($p < 0.001$, Fig. 1C) compared to mock-treated cells (transfected with pSUPER-RASSF4-

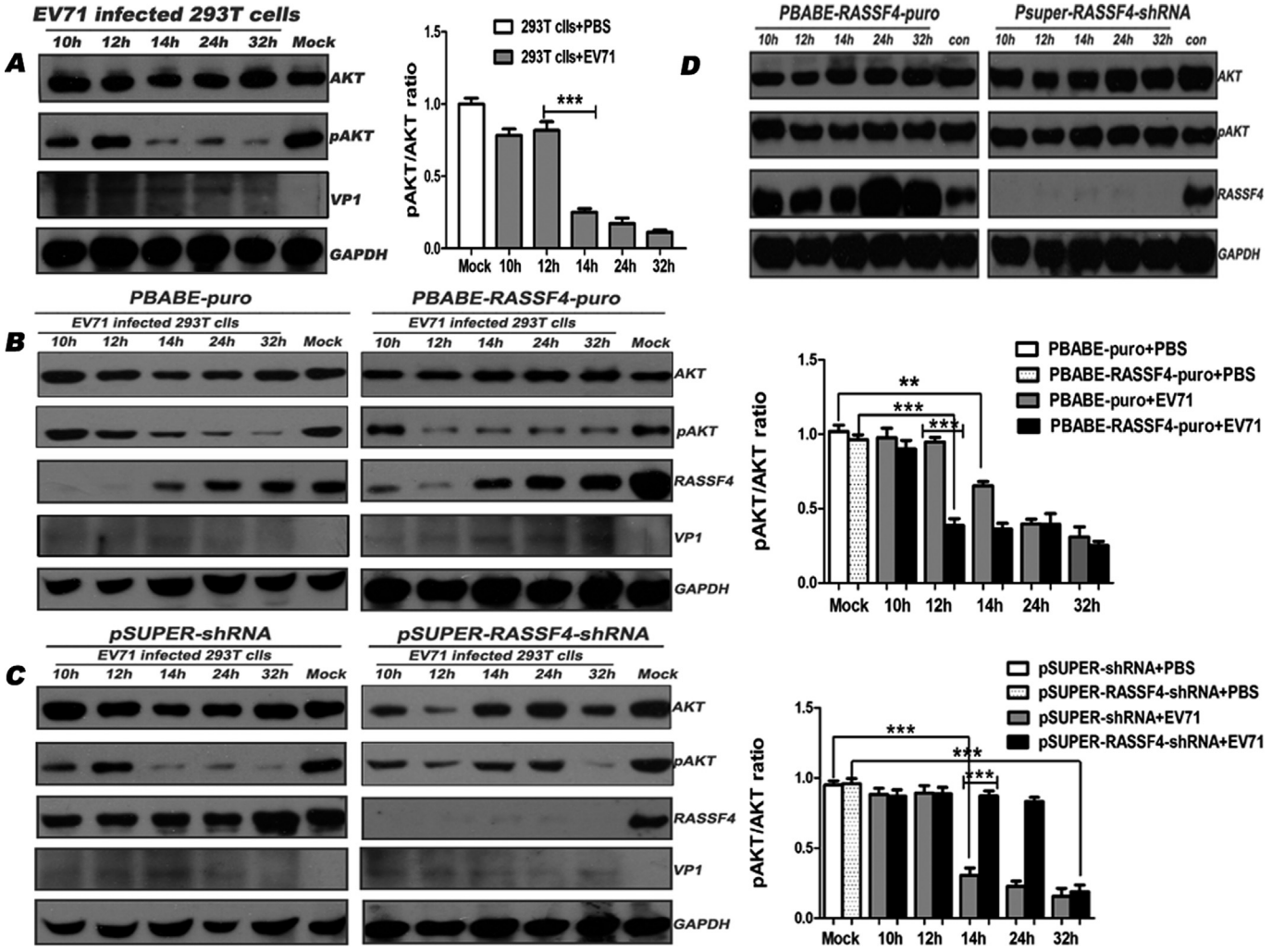

Fig. 1. The effect of RASSF4 on the AKT phosphorylation induced by EV71. (A) 293T cells were infected with EV71-BrCr at an MOI of 1.5 for 1 h, and cell lysates were collected at the indicated times. Western blotting assays were used to assess the levels of phosphorylated AKT (pAKT) and total AKT (AKT). The levels of pAKT and AKT were quantified using Quantity One software. 293T cells were transfected with PBABE-RASSF4-puro or control plasmid (B) and pSUPER-RASSF4-shRNA or mock vector (C) for 48 h. The cells were then treated with EV71-BrCr at an MOI of 1.5, cell lysates were harvested, and western blotting was performed to determine AKT activation. The ratios of pAKT to AKT are presented graphically on the right. (D) 293T cells were transfected with the PBABE-RASSF4-puro or pSUPER-RASSF4-shRNA plasmid to generate overexpressing or underexpressing RASSF4 cells, respectively. At 48 h post-transfection, whole-cell lysate was harvested and subjected to western blot analyses. All the data presented were analyzed using unpaired the two-tailed Student's *t*-test and a two-way ANOVA. **, $p < 0.01$; ***, $p < 0.001$.

shRNA and mock-infected). Interestingly, the amount of phosphorylated AKT in EV71-infected 293T cells transfected with plasmid pSUPER-RASSF4-shRNA was greater than that in cells transfected with the control plasmid pSUPER-shRNA at 14 h pi ($p < 0.001$, Fig. 1C). These results suggested that RASSF4 underexpression delayed the phosphorylation of AKT in the EV71-infected 293T cells. To rule out the influence of RASSF4 on the AKT pathway, we detected the impact of RASSF4 on AKT phosphorylation without EV71 infection at different time points. In 293T cells, overexpression or underexpression of RASSF4 did not affect the expression of AKT or AKT phosphorylation at different time points (Fig. 1D). Taken together, these results (Fig. 1) show that RASSF4 can accelerate the inhibition of AKT phosphorylation induced by EV71 infection.

3.2. RASSF4 promotes EV71 replication and the production of viral progeny after viral entry into 293T cells

Because we speculated that RASSF4 might enhance EV71 replication to accelerate the inhibition of AKT phosphorylation, qRT-PCR was performed to detect the mRNA level of the VP1 gene of EV71. 293T cells were transfected with the RASSF4 overexpression plasmid or the control plasmid; at 48 h post-transfection, the cells were infected with EV71 at an MOI of 10 for 1 h and then harvested at 12 h and 24 h pi. The results of qRT-PCR showed that the amount of VP1 mRNA in 293T cells overexpressing RASSF4 was significantly increased at 12 h ($p < 0.001$, Fig. 2A) and 24 h pi ($p < 0.05$, Fig. 2A) compared with the control group (Fig. 2A). Moreover, we found that RASSF4 underexpression remarkably decreased EV71 VP1 replication at both 12 h ($p < 0.05$, Fig. 2B) and 24 h pi ($p < 0.01$, Fig. 2B). These results suggest that RASSF4 might increase EV71 replication in 293T cells to accelerate the inhibition of the AKT pathway. This was further confirmed by using TCID50 to measure the production of viral particles. These results showed that RASSF4 overexpression led to a significant increase in virus titers at 12 h ($p < 0.01$, Fig. 2C) and 24 h pi ($p < 0.01$, Fig. 2C) compared to the control group. In contrast, RASSF4 underexpression remarkably decreased virus titers at both 12 h ($p < 0.01$, Fig. 2D) and 24 h pi ($p < 0.05$, Fig. 2D). Together, these results led us to conclude that RASSF4 plays important roles in AKT phosphorylation by increasing EV71 VP1 gene expression and replication in 293T cells.

Because RASSF4 affects EV71 gene expression and replication at 12 h and 24 h pi (Fig. 2A–D), we hypothesized that RASSF4 might affect the stages of EV71 after entry into host cells. To confirm this hypothesis, the VP1 gene of UV-inactivated EV71 was detected by qRT-PCR in 293T cells transfected with the PBABE-RASSF4-puro and pSUPER-RASSF4-shRNA plasmids. Neither RASSF4 overexpression nor underexpression had an effect on the mRNA level of the VP1 gene in 293T cells (Fig. 2E and F). Therefore, RASSF4 has no effect on EV71 attachment and internalization into host cells, suggesting that RASSF4 might promote EV71 replication after entry into host cells. However, the detailed mechanism remains unclear and needs to be clarified in future studies.

3.3. RASSF4 promotes EV71-induced apoptosis

We here demonstrate that RASSF4 could accelerate the inhibition of the AKT phosphorylation induced by EV71 infection by promoting EV71 replication and the production of viral progeny. Previous studies have shown that activated AKT can inhibit the apoptosis of target cells [16,17] and that apoptosis promotes the release of EV71 virus [18]. To test whether RASSF4 promotes EV71-induced apoptosis, 293T cells were transfected with our experimental plasmids and then infected with EV71 at an MOI of 10; cell lysates were collected for flow cytometry and western blot analysis. As shown in Fig. 3A, 62.4% of the RASSF4-overexpressing cells infected with EV71 were in various stages of apoptosis. In contrast, the number of dying cells decreased to 9.5% with RASSF4 underexpression. These results demonstrate that RASSF4 increased the number of apoptotic cells induced by EV71 infection. Poly (ADP-Ribose) polymerase (PARP) and caspase-3 are considered to be hallmarks of apoptosis [19]. As shown in Fig. 3B, the cleavage of PARP and caspase-3 and the expression of VP1 were apparently increased in RASSF4-overexpressing cells, whereas cleavage and VP1 products were decreased in RASSF4-underexpessing cells. Thus, RASSF4 increases EV71-induced apoptosis, which may be beneficial for the release of viral particles.

4. Discussion

In this paper, we identified the roles of RASSF4 in EV71 replication. Evidence is provided to demonstrate that RASSF4, a tumor suppressor, can promote EV71 replication to accelerate the inhibition of AKT phosphorylation during EV71 infection. Thus, RASSF4 may be a potential target for anti-EV71 therapy.

It is important to understand the relationships among EV71 and host cell signaling pathways to reveal mechanisms of viral infection. Previous studies have focused on the activation of AKT during viral infection. With regard to respiratory syncytial virus (RSV) [20] and CVB3 [21], EV71 infection can activate the AKT pathway to promote short-term survival in the early stages of infection [22]. The activation of AKT can regulate apoptosis in favor of viral survival, replication, and dissemination and also maintain a constant infection in host cells [23]. However, few reports focus on the opposite impact of EV71 on the AKT pathway. In the present study, we confirmed that AKT is activated during the process of EV71 infection (data not shown), consistent with previous reports [14]. Notably, we found a different effect of EV71 on the AKT pathway, with data showing that EV71 can inhibit AKT phosphorylation in the later stages of infection. Similar to influenza virus [24], EV71 can activate AKT in the early phases of infection, with EV71 suppressing AKT phosphorylation in the later stages. We presume that the activation of AKT results in the avoidance of premature death, which facilitates viral replication in living cells; the inhibition of AKT can induce cell apoptosis, which may be beneficial to the survival and transmission of viral particles. As cell apoptosis promotes the release of EV71 virus in a lysogenic way [18], this also explains why EV71 inhibits AKT phosphorylation in the later stages of infection.

RASSF4 is a member of the RASSFs gene family and has been identified as a tumor suppressor gene [11]. Studies have found that RASSF4 can mediate oncogenesis by regulating the Hippo and MAPK pathways [11], and RASSF4 expression inhibits the growth of human tumor cells [11]. As it has been demonstrated that EV71 can induce apoptosis via the AKT pathway [25], we investigated the influence of RASSF4 and EV71 on AKT signaling. Our research reveals for the first time relationships between RASSF4 and EV71 in this pathway. In this study, we found that RASSF4 might assist EV71 in inhibiting AKT phosphorylation (Fig. 1). In addition, many host proteins, such as vascular endothelial growth factor A (VEGF-A), can promote vaccinia virus (VV) replication, resulting in the phosphorylation of AKT [26]. We suspect that promotion of the viral titer by RASSF4 may be involved in the effects of AKT phosphorylation. Our results suggested that RASSF4 promotes EV71 replication but has no effect on the entry phase of EV71 into target cells (Fig. 2).

Interestingly, we found that the amount of viral RNA at 12 h pi was greater than the amount of viral RNA at 24 h pi after RASSF4 stimulation (Fig. 2A). The reason for this may be that (i) EV71

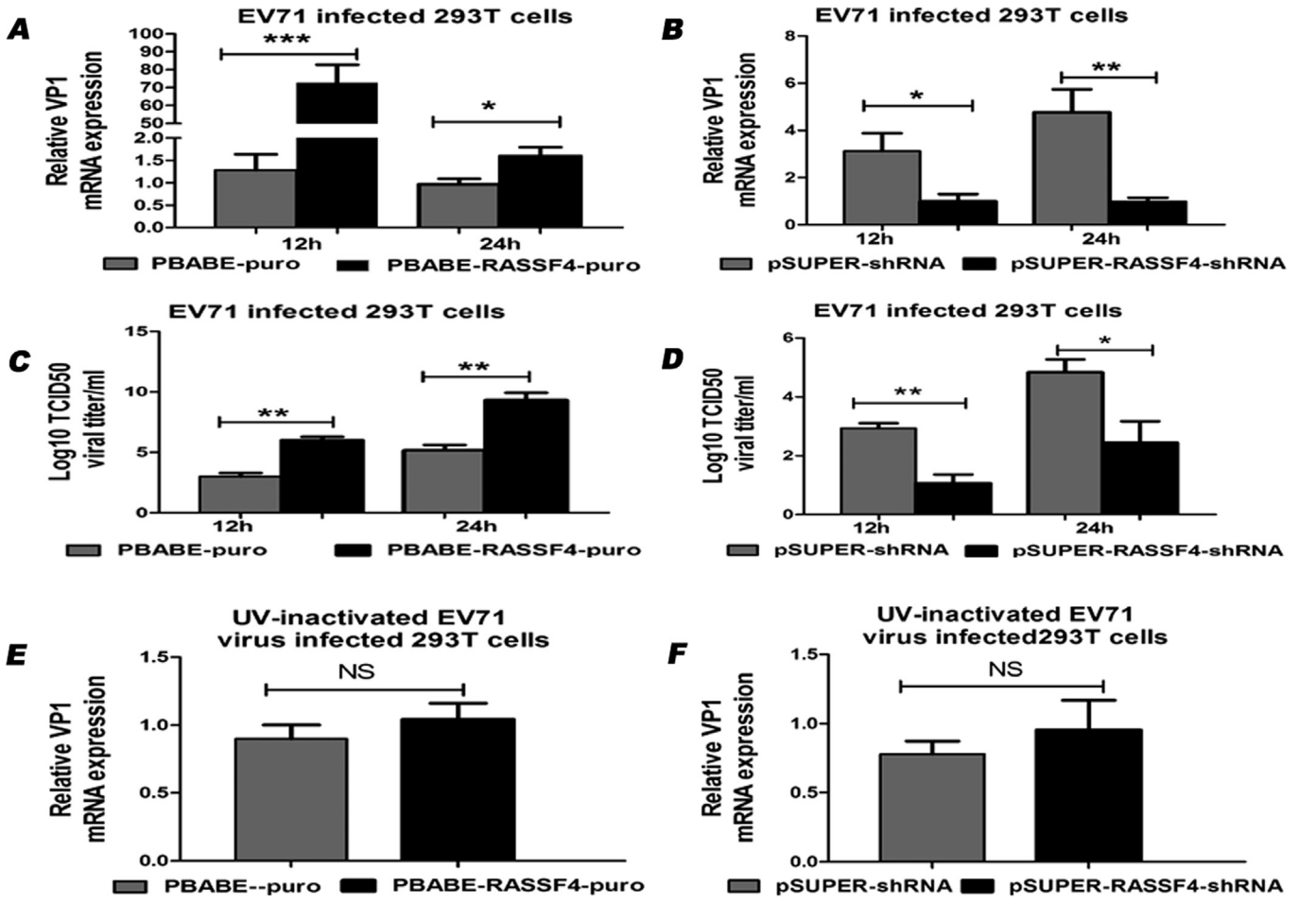

Fig. 2. Analysis of the role of RASSF4 in the EV71 replication cycle. 293T cells were transfected with plasmids expressing RASSF4 or control plasmids and RASSF4 shRNA or mock vectors for 48 h before infection with wild-type (A–D) or UV-inactivated EV71 (E, F) at an MOI of 10. QRT-PCR (A, B, E, F) was used to determine the viral DNA copy number at 12 and 24 h pi, and infectious virion production was measured at 12 and 24 h pi using a TCID50 assay (C, D). The statistical analysis was conducted using a two-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

experiences rapid replication and quickly accumulates intracellular viral RNA at the early stages of infection, similar to poliovirus infection, or (ii) as an increasing number of infected cells become unhealthy and die, the intracellular viral RNA enters a plateau phase, and viral replication decreases [27].

In addition, viral protein VP1 in 293T cells (transfected with the control plasmids PBABE-puro and pSUPER-shRNA), reaching a peak at 14 h pi and then rapidly decreasing at 24 h pi (Fig. 1B, C). These findings suggested that the cells could no longer sustain further viral replication [28]. However, overexpression of RASSF4 enhanced

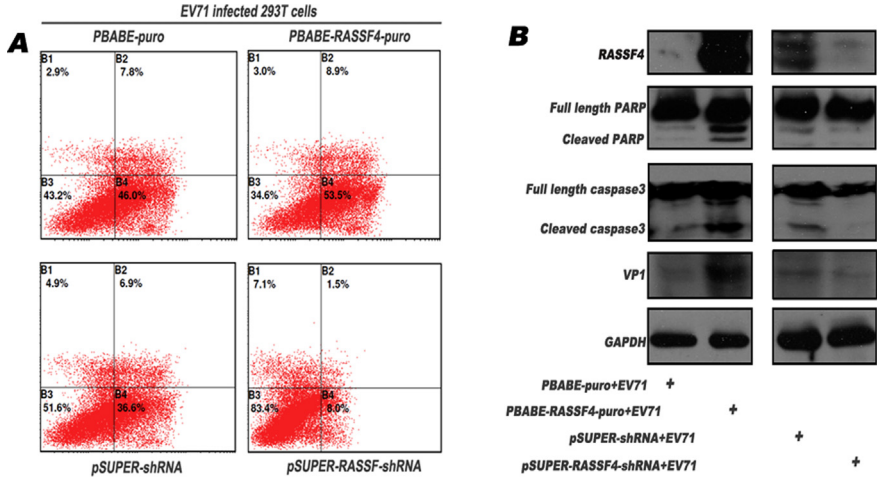

Fig. 3. RASSF4 increases apoptosis induced by EV71. 293T cells were transfected with PBABE-puro, PBABE-RASSF4-puro, pSUPER-shRNA or pSUPER-RASSF4-shRNA for 48 h, and the cells were then infected with EV71-BrCr at an MOI of 10. (A) After 24 h of infection, the cells were collected and stained with annexin V-FITC and PI and analyzed by flow cytometry. (B) After 24 h of infection, 293T cells were harvested for lysis and analyzed by western blotting.

EV71 replication. Therefore, EV71 VP1 continued to increase at 24 h pi in 293T cells overexpressing RASSF4 (Figs. 1 and 3B). It is possible that an increased number of EV71 particles might accelerate the inhibition of AKT phosphorylation. RASSF4 promotion of EV71-induced apoptosis is also a defensive mechanism by which the host can prevent the generation and spread of viral progeny during viral infection.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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