

Research Article

ARRDC3 promotes lysosome-mediated YAP degradation to inhibit enterovirus replication

Xia Huang^{a,1}, Siyuan Wang^{b,1}, Yan Huang^{a,1}, Yue Wang^a, Guangchao Zang^a, Yan Liang^a, Juntong Liu^a, Xinyue Han^a, Jingjing Liao^a, Tingting Chen^{a,*}, Nan Lu^{a,*}, Guangyuan Zhang^{a,*}^a Pathogen Biology and Immunology Laboratory, Lab Teaching & Management Center, School of Basic Medical Sciences, Chongqing Medical University, Chongqing, 400016, China^b Department of Thyroid, Breast and Vascular Surgery, Xi Jing Hospital, Air Force Medical University, Xi'an, 710032, China

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ABSTRACT

Enterovirus D68 (EV-D68) and enterovirus A71 (EV-A71) are two major types of enteroviruses that pose emerging challenges to public health and have the potential to cause outbreaks, yet their pathogenic mechanisms remain largely unexplored. Arrestin domain containing 3 (ARRDC3) is a vital regulator of glucose metabolism, cancer development, and inflammation. Whether ARRDC3 contributes to innate antiviral immunity is undefined. Here, we found that enterovirus infection induces ARRDC3 expression at both the mRNA and protein levels, thereby inhibiting enterovirus replication. Moreover, we demonstrate that the expression of Yes-associated protein (YAP), a key effector of the Hippo pathway, is severely downregulated by ARRDC3 via lysosomal pathway. YAP facilitates enterovirus replication by suppressing the interferon pathway during the later stage of enterovirus infection, independent of its transcriptional activity. Finally, the ARRDC3-YAP pathway exhibits a broad-spectrum antiviral effect in various viral infections, including those caused by human parainfluenza virus type 3 (HPIV3) and vesicular stomatitis virus (VSV). Collectively, our results identify the critical role of ARRDC3 and its negative regulatory effect on YAP in the innate antiviral response, suggesting a novel therapeutic strategy against virus infection.

INTRODUCTION

Enteroviruses belong to the *Picornaviridae* family and contain positive-sense, single-stranded RNA genome of about 7400 nucleotides (Lukashev et al., 2018). Based on the molecular and biological characteristics, human enterovirus (EV) are classified into four species: EV-A, -B, -C, and -D. In recent years, human enterovirus D68 (EV-D68) and enterovirus A71 (EV-A71) are the two predominate enterovirus types circulating in numerous regions (Poelman et al., 2015; Solomon et al., 2010; Wang et al., 2024). EV-D68 is associated with severe respiratory tract illness and acute flaccid myelitis (AFM), whereas EV-A71 is a leading cause of hand, foot, and mouth disease (HFMD) (Fall et al., 2022; Ooi et al., 2010; Kreuter et al., 2011). Because no antiviral agents are currently available for EV-D68 and EV-A71 infection, it is urgent to

elucidate their pathogenic mechanisms to develop effective preventive and therapeutic strategies.

Arrestin domain containing 3 (ARRDC3), a member of the α -arrestins family, is a newly-identified multi-functional adaptor protein that modulates receptor trafficking and cellular signaling. It regulates G protein signaling by controlling the ubiquitination and endocytic trafficking of G protein-coupled receptor (GPCR), including the β 2-adrenergic receptor (β 2AR) and protease-activated receptors 1 (PAR1) (Nabhan et al., 2010; Does et al., 2015). Recent studies revealed a surprising functional diversity for ARRDC3 in various disease conditions, such as obesity, cancer, and inflammation. For example, ARRDC3 inhibits energy expenditure to promote obesity through suppressing β -adrenergic signaling (Patwari et al., 2011). Furthermore, ARRDC3 has been reported to repress breast cancer by regulating GPCR degradation

* Corresponding authors.

E-mail addresses: cherrychen@cqmu.edu.cn (T. Chen), ficus@cqmu.edu.cn (N. Lu), sanqinyouthzhang@126.com (G. Zhang).¹ Xia Huang, Siyuan Wang, and Yan Huang contributed equally to this work.

and trafficking (Arakaki et al., 2018; Draheim et al., 2010). In addition, ARRDC3 interacts with and negatively regulates PAR1, thereby promoting gastric inflammation during *Helicobacter pylori* infection (Liu et al., 2020). Knocking down ARRDC3 in HeLa cells reduces their susceptibility to human papillomavirus 16 (HPV16) infection (Takeuchi et al., 2019). However, less is understood about whether ARRDC3 plays a direct role in other viral infections.

Intriguingly, ARRDC3 has been identified as an influential upstream regulator of Yes-associated protein (YAP) in various cancer cell types (Xiao et al., 2018; Shen et al., 2018). YAP was initially discovered in *Drosophila* as a developmental transcriptional co-activator that mediates the biological functions of the Hippo pathway (Moroishi et al., 2015). Subsequent investigations have revealed intricate interplay of YAP in cell proliferation, apoptosis, and stem cell differentiation, which is closely associated with the onset and progression of cancer (Yu et al., 2015; Mo et al., 2014; Tang et al., 2022). It is interesting that, in addition to its well-known oncogenic role, YAP also appears to be critical for regulating viral infection. Recent studies reported that YAP can strongly reduce the innate antiviral immunity by dampening the cytosolic RNA and DNA sensing mechanisms. Specifically, YAP suppresses TLR3 expression and inhibits the production of pro-inflammatory and antiviral cytokines (Zhang et al., 2022). YAP also abolishes virus-induced TBK1 activation by preventing Lys63-linked ubiquitylation and its binding with adaptor and substrates (Zhang et al., 2017). Besides, YAP impedes the dimerization of IRF3 and its subsequent nucleus translocation, leading to the blockade of IFN- β signaling (Wang et al., 2017). However, other researches have shown that YAP can also suppress viral replication. For example, YAP interacts with Marburg virus (MARV) VP40 to hinder virus-like particles (VLPs) budding (Han et al., 2020). Besides, stimulator of interferon genes (STING)-mediated nuclear transport of viral genome was impaired by YAP, thus inhibiting human cytomegalovirus (HCMV) replication (Lee et al., 2022). Nevertheless, the effect of YAP on enterovirus infection and the precise mechanisms governing YAP activity have yet to be fully elucidated.

In this report, we demonstrate that ARRDC3 is significantly upregulated in enterovirus-infected cells, driving the lysosomal degradation of YAP, which is subsequently proved to facilitate enterovirus replication. The pro-viral effect of YAP is associated with its inhibition of the IFN- β signaling and is independent of its transcriptional activity. Moreover, we find that the ARRDC3-YAP pathway exerts potential broad-spectrum antiviral activity against diverse viral infections. Taken together, our findings not only illuminate the function of ARRDC3 on enterovirus replication but also propose a novel regulatory mechanism for YAP in the context of viral infection.

RESULTS

EV-D68 and EV-A71 infection upregulate ARRDC3 expression, which in turn inhibits viral replication

Our previous transcriptome sequencing of RD cells infected with EV-D68 revealed significant alterations in the expression levels of cellular α -arrestins family genes (Si et al., 2021). Specifically, ARRDC2, ARRDC3, ARRDC4, and TXNIP were upregulated, whereas ARRDC1 was downregulated after EV-D68 infection (Fig. 1A). We then analyzed the changes in α -arrestins family genes following EV-A71 infection using Gene Expression Omnibus (GEO) data sets and observed a similar expression pattern (Fig. 1B). Given that the biological functions of ARRDC3 remain largely unexplored, we then investigated whether it participates in the host response to enterovirus replication. The RT-qPCR and Western blotting analyses showed a gradual increase in both the mRNA and protein levels of ARRDC3 in RD cells infected with EV-D68 in a multiplicity of infection (MOI)-dependent manner (Fig. 1C and D). Similarly, ARRDC3 expression was also elevated in a dose-dependent manner following EV-A71 infection (Fig. 1E and F).

To investigate the effect of ARRDC3 on viral replication, we constructed stable RD cell line overexpressing ARRDC3 (RD-ARRDC3) and infected these cells with either EV-D68 or EV-A71. In RD-ARRDC3 cells, EV-D68 VP1 expression was markedly reduced (Fig. 1G), and viral titers were also lower compared to those in the control RD-GFP cells (Fig. 1H). Similar results were observed during EV-A71 infection (Fig. 1I and J). Conversely, siRNA-mediated knockdown of ARRDC3 resulted in a substantial increase in viral VP1 protein and viral titers for EV-D68 and EV-A71 (Fig. 1K–N). Collectively, these findings suggest that ARRDC3 inhibits enterovirus replication.

ARRDC3 mediates YAP degradation via lysosomal pathway during enterovirus infection

To investigate the mechanism by which ARRDC3 inhibits enterovirus infection, we used GeneMANIA to predict potential ARRDC3-interacting proteins (Fig. 2A). Among these candidates, YAP has been reported to play a crucial role in viral infection, leading us to hypothesize that ARRDC3 might regulate YAP to exert its antiviral effect. We first examined the correlation between ARRDC3 and YAP expression during enterovirus infection. RD cells were infected with EV-D68 or EV-A71 and the expression levels of ARRDC3 and YAP were analyzed. Notably, as the infection time increased, ARRDC3 mRNA levels showed a gradient increase, whereas YAP mRNA levels remained largely unchanged (Fig. 2B and C). However, at the protein level, ARRDC3 levels rose significantly over time, while YAP levels markedly decreased (Fig. 2D and E).

To explore whether ARRDC3 could negatively regulate YAP at the post-translational level, we examined the protein levels of YAP in RD-ARRDC3 cell. Compared with RD-GFP cells, YAP protein levels significantly diminished in RD-ARRDC3 cells (Fig. 2F). Conversely, knockdown of endogenous ARRDC3 resulted in a marked increase in YAP protein levels compared to the siNC group (Fig. 2G), indicating that ARRDC3 promotes YAP degradation.

To elucidate the mechanism by which ARRDC3 downregulates YAP, we assessed the protein-protein interaction between ARRDC3 and YAP through co-immunoprecipitation experiments. Results showed that ARRDC3 notably co-precipitated with YAP, indicating that ARRDC3 forms a complex with YAP (Fig. 2H). Next, we investigated the pathway through which ARRDC3 degrades YAP during viral infection by treating RD-ARRDC3 cells with either the lysosome inhibitor NH₄Cl or the proteasome inhibitor MG-132. Only NH₄Cl inhibited YAP degradation, demonstrating that ARRDC3 promotes the lysosomal degradation of YAP (Fig. 2I). Furthermore, we performed immunofluorescence co-staining for ARRDC3 and YAP to evaluate their colocalization with the mCherry-fused lysosome-associated membrane protein 1 (mCherry-LAMP1), a resident lysosomal membrane protein. Results showed evident co-localization between ARRDC3, YAP and LAMP1 (Fig. 2J). Consistently, in EV-D68-infected RD cells, NH₄Cl treatment inhibited YAP degradation and increased the EV-D68 VP1 expression, whereas MG-132 or DMSO had no similar effect (Fig. 2K). Moreover, YAP co-localized with LAMP1 after EV-D68 infection (Fig. 2L). These findings suggest that ARRDC3 interacts with YAP and promotes its lysosomal degradation following EV-D68 infection.

YAP facilitates enterovirus replication

Given that ARRDC3 promotes YAP degradation during viral infection, we next tested the effect of YAP on enterovirus replication. When endogenous YAP was knocked down by siRNA, EV-D68 VP1 expression and viral titers were strongly decreased in both RD cells (Fig. 3A) and 293T cells (Fig. 3B). Similarly, the knockdown of YAP inhibited EV-A71 replication in RD and 293T cells (Fig. 3C and D). Conversely, ectopic expression of YAP significantly augmented VP1 expression and viral titers (Fig. 3E). To ensure a robust transfection effect, we performed

this overexpression experiment in 293T cells, where a more pronounced pro-viral effect of YAP was observed (Fig. 3F). Similar outcomes were obtained in RD and 293T cells infected with EV-A71 (Fig. 3G and H). Together, these data show that YAP promotes enterovirus replication.

YAP suppresses the interferon pathway at later stage of enterovirus infection

Previous studies have suggested that YAP sequesters IRF3 in the cytoplasm, thereby inhibiting the transcriptional activity of IRF3 and

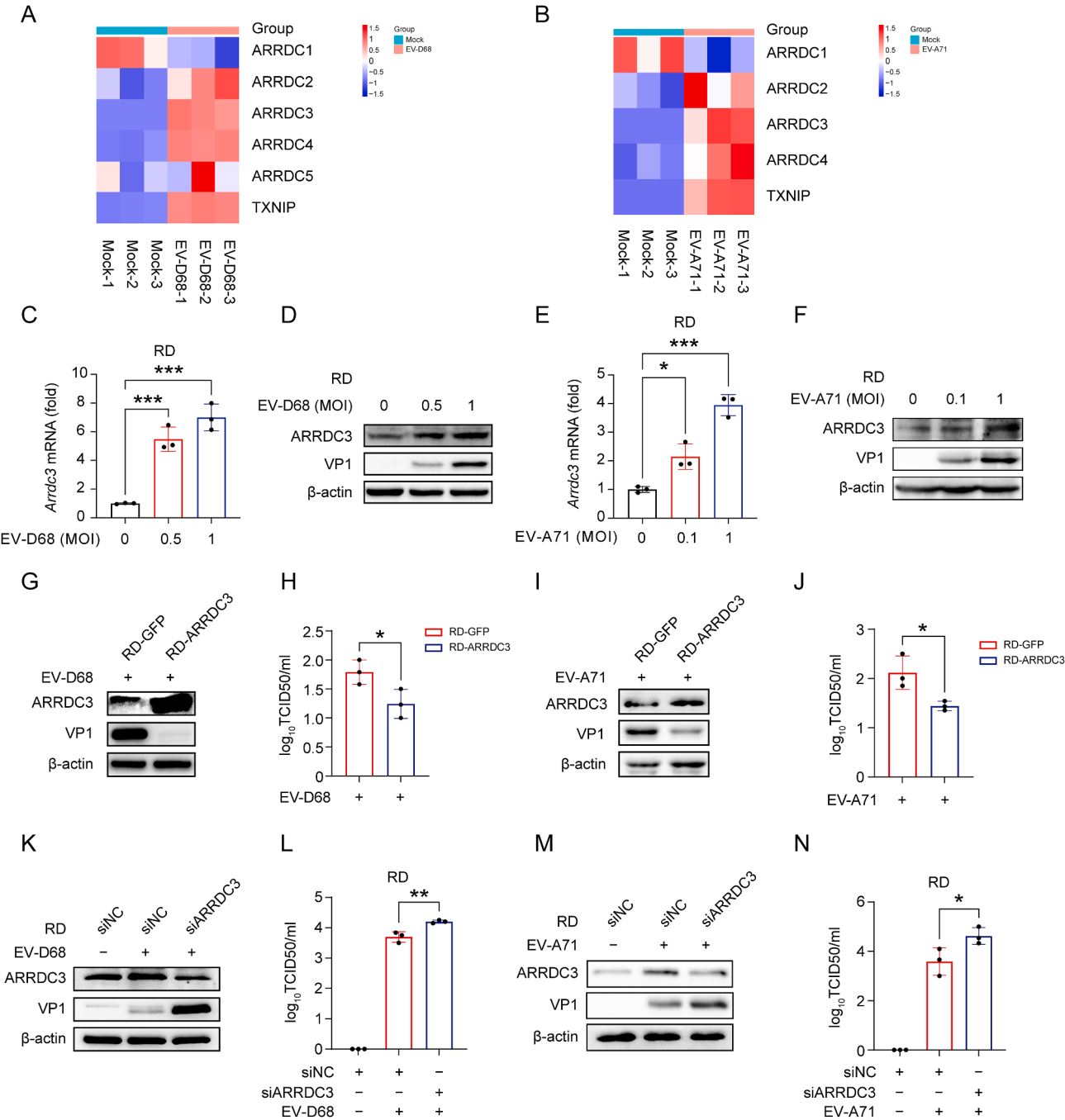
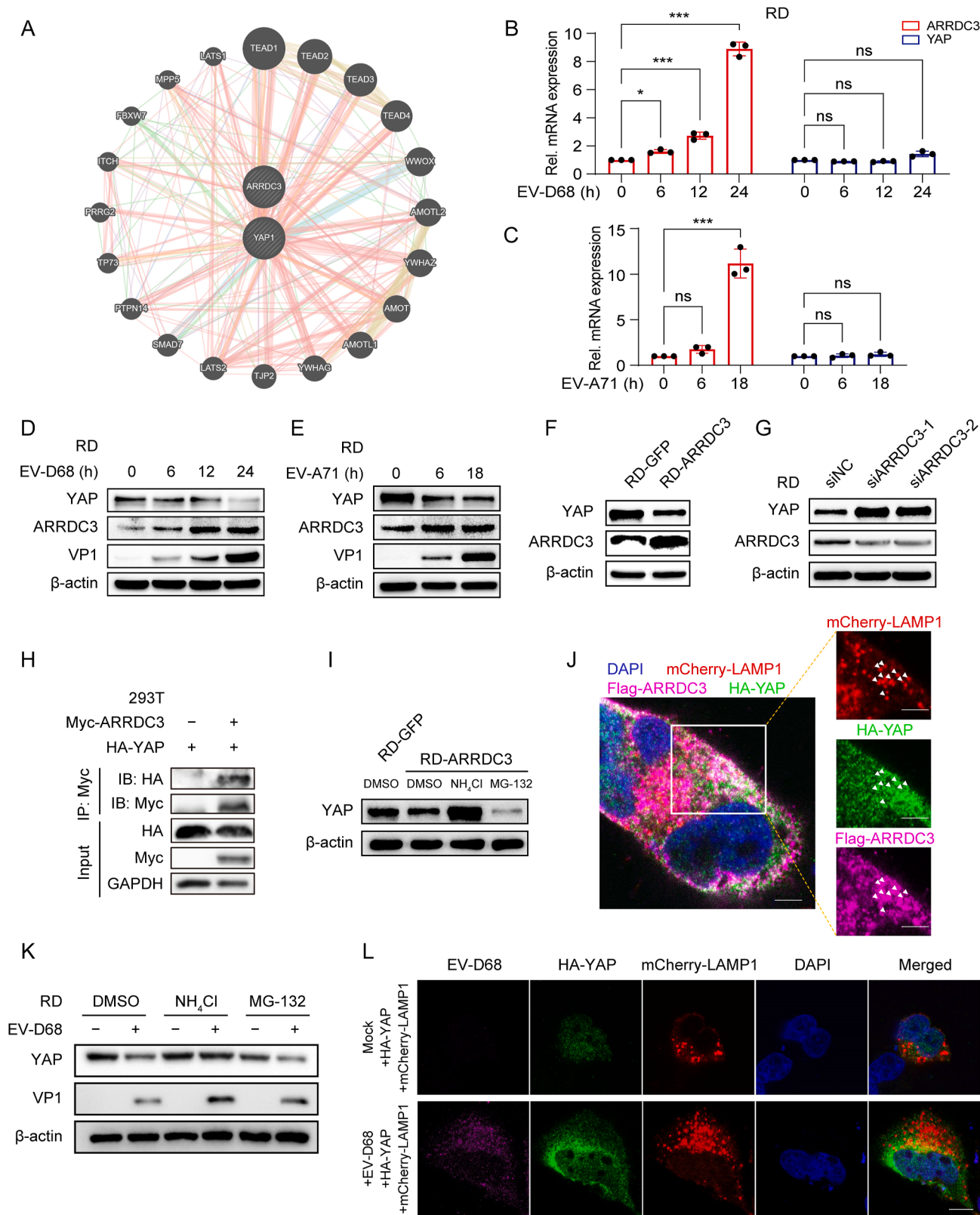


Fig. 1. EV-D68 and EV-A71 infection upregulate ARRDC3 expression, which in turn inhibits viral replication. **A, B** Heatmap shows α -arrestins family genes expression upon EV-D68 infection or EV-A71 infection. EV-A71 data from GEO: GSE15323. Different colors show gene expression level differences, with increased level colored red and decreased level colored blue. **C, D** RD cells were infected with EV-D68 at MOI of 1 for 18 h. Relative mRNA expression levels of ARRDC3 in RD cells were determined by RT-qPCR (**C**). Western blot analysis of ARRDC3, EV-D68 VP1 and β -actin levels in RD cells (**D**). **E, F** RD cells were infected with EV-A71 at MOI of 1 for 14 h and subjected to RT-qPCR (**E**) and Western blot analysis (**F**). **G–J** RD-GFP cells or RD-ARRDC3 cells were infected with EV-D68 or EV-A71 for 18 h or 14 h. The expression of ARRDC3, viral VP1 and β -actin were detected (**G, I**). Viral titers in the cell supernatant were qualified by TCID₅₀ assay (**H, J**). **K–N** ARRDC3 expression was silenced in RD cells using nonspecific siRNA (siNC) or ARRDC3-specific small interfering RNA (siARRDC3) (50 nM) for 48 h, followed by infection with EV-D68 or EV-A71 for 18 h or 14 h. ARRDC3, viral VP1 and β -actin protein levels were assessed by Western blotting (**K, M**) and viral titers were quantified by TCID₅₀ (**L, N**). Data are representative of results from at least three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



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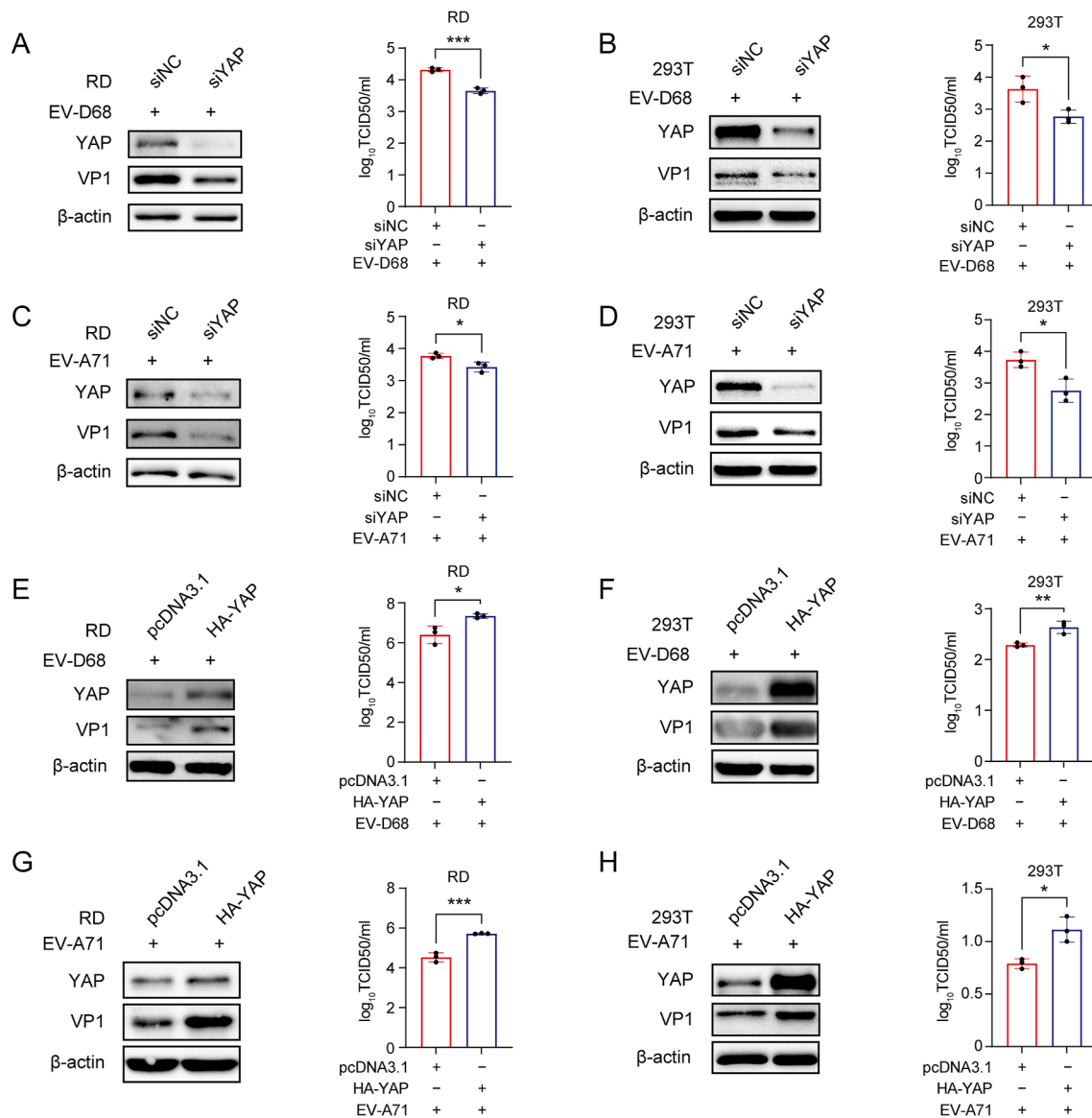


Fig. 3. YAP facilitates enterovirus replication. **A, B** RD or 293T cells were transfected with siNC or siYAP (30 nM) for 48 h, followed by infection with EV-D68 at MOI of 1 for 18 h. YAP and viral VP1 protein levels were assessed by Western blotting (left) and viral titers were quantified by TCID₅₀ (right). **C, D** Similar experiment described in (**A, B**) was conducted, but the cells were infected with EV-A71 at MOI of 1 for 14 h. **E, F** RD or 293T cells were transfected with YAP (4 μg). After 48 h, cells were infected with EV-D68 for 18 h. YAP and viral VP1 protein levels (left) and viral titers (right) were then analyzed. **G, H** The procedures were repeated as in (**E, F**) upon EV-A71 infection. Data are representative of results from at least three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

subsequent interferon production (Wang et al., 2017). To assess whether YAP also influences type I interferon signaling during EV-D68 infection, we measured IFN-α and IFN-β mRNA levels. Although EV-D68 infection did not induce IFN-α mRNA expression

(data not shown), IFN-β mRNA levels were significantly elevated at later time points (12 and 24 h post-infection) compared to the early stage (9 h post-infection). Furthermore, ectopic expression of YAP significantly suppressed the elevation in IFN-β mRNA levels

Fig. 2. ARRD3 mediates YAP degradation via lysosomal pathway during enterovirus infection. **A** Predicted network of proteins that interact with ARRD3 by GeneMANIA. **B–E** RD cells were infected with EV-D68 at MOI of 1 for 0, 6, 12, 24 h or EV-A71 at MOI of 1 for 0, 6, 18 h. The mRNA levels of ARRD3 and YAP in RD cells were measured by RT-qPCR (**B, C**). ARRD3, YAP, viral VP1 and β-actin protein levels were detected by Western blotting (**D, E**). **F** RD-GFP cells and RD-ARRD3 cells were collected and analyzed for the protein levels of ARRD3, YAP and β-actin. **G** RD cells were transfected with siNC or siARRD3 (50 nM) for 48 h and subjected to Western blot analysis. **H** HA-YAP (15 μg) were co-transfected with Myc-ARRD3 (15 μg) into 293T cells. At 48 h posttransfection, the cells were harvested and subjected to co-immunoprecipitation assay. Immunoprecipitation (IP) of the whole cell lysates was done with anti-Myc antibody. **I** RD-ARRD3 cells were treated with the lysosome inhibitor NH₄Cl (10 mM) or proteasome inhibitor MG-132 (10 μM) for 12 h, with DMSO serving as control. YAP and β-actin protein levels were then assessed. **J** RD cells were transfected with plasmids expressing Flag-ARRD3 (5 μg), HA-YAP (5 μg) and mCherry-LAMP1 (5 μg) for 24 h. Cells were stained with anti-Flag and anti-HA antibody. The nuclei were stained by DAPI. White arrows indicate representative co-localization of ARRD3, YAP and LAMP1. Scale bar: 5 μm. **K** RD cells were infected with EV-D68 for 18 h and treated with DMSO, NH₄Cl or MG-132 for 6 h before harvest. Cell lysates were analyzed by Western blotting. **L** RD cells were transfected with plasmids expressing HA-YAP (5 μg) and mCherry-LAMP1 (5 μg) for 6 h and then infected with EV-D68 for 18 h. Cells were stained with anti-dsRNA and anti-HA antibodies. The nuclei were stained by DAPI. Scale bar: 10 μm. Data are representative of results from at least three independent experiments. **P* < 0.05; ****P* < 0.001; ns, not significant.

(Fig. 4A), suggesting that YAP acts as a negative regulator of IFN-β signaling.

Since YAP primarily acts as a transcription factor and exerts its function in the nucleus, we next examined whether its pro-viral effect depends on its transcriptional activity. First, we investigated whether YAP4, a truncated isoform of YAP lacking nuclear localization signals, retains the capacity to promote EV-D68 replication. Immunofluorescence analysis confirmed that, in comparison to the wild-type YAP, exogenously expressed YAP4 failed to translocate to the nucleus in RD cells as anticipated (Fig. 4B). Overexpression of YAP4 in 293T cells led to a gradual increase in both EV-D68 VP1 protein levels and viral titers, and the pro-viral effect of YAP4 was not attenuated compared to the wild-type YAP (Fig. 4C and D). Furthermore, treating RD cells with the YAP transcriptional inhibitor verteporfin, which disrupts the interaction between YAP and TEAD (Liu-Chittenden et al., 2012), did not decrease the expression level of viral VP1 compared with the DMSO-treated group (Fig. 4E). These findings suggest that the pro-viral effect of YAP is independent on its transcriptional activity. Together, the above results

suggest that YAP inhibits IFN-β expression at later stage of enterovirus infection through a mechanism unrelated to its transcriptional regulation.

The ARRDC3-YAP pathway emerges as a broad host response to viral infection

The findings described above indicate that the upregulation of ARRDC3 leads to YAP protein degradation, thereby dampening the pro-viral effect of YAP in the innate immune pathway. To explore whether the ARRDC3-YAP pathway exerts broader antiviral activity, we extended our analysis to other viral infections.

GEO data reveal that beyond enterovirus (such as EV-D68 and EV-A71), ARRDC3 expression is also elevated during infection with diverse viruses, including Sendai virus (SeV), coxsackievirus A6 (CV-A6), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and Middle East respiratory syndrome coronavirus (MERS-CoV) (Fig. 5A–D). Subsequently, we validated these observations in human parainfluenza

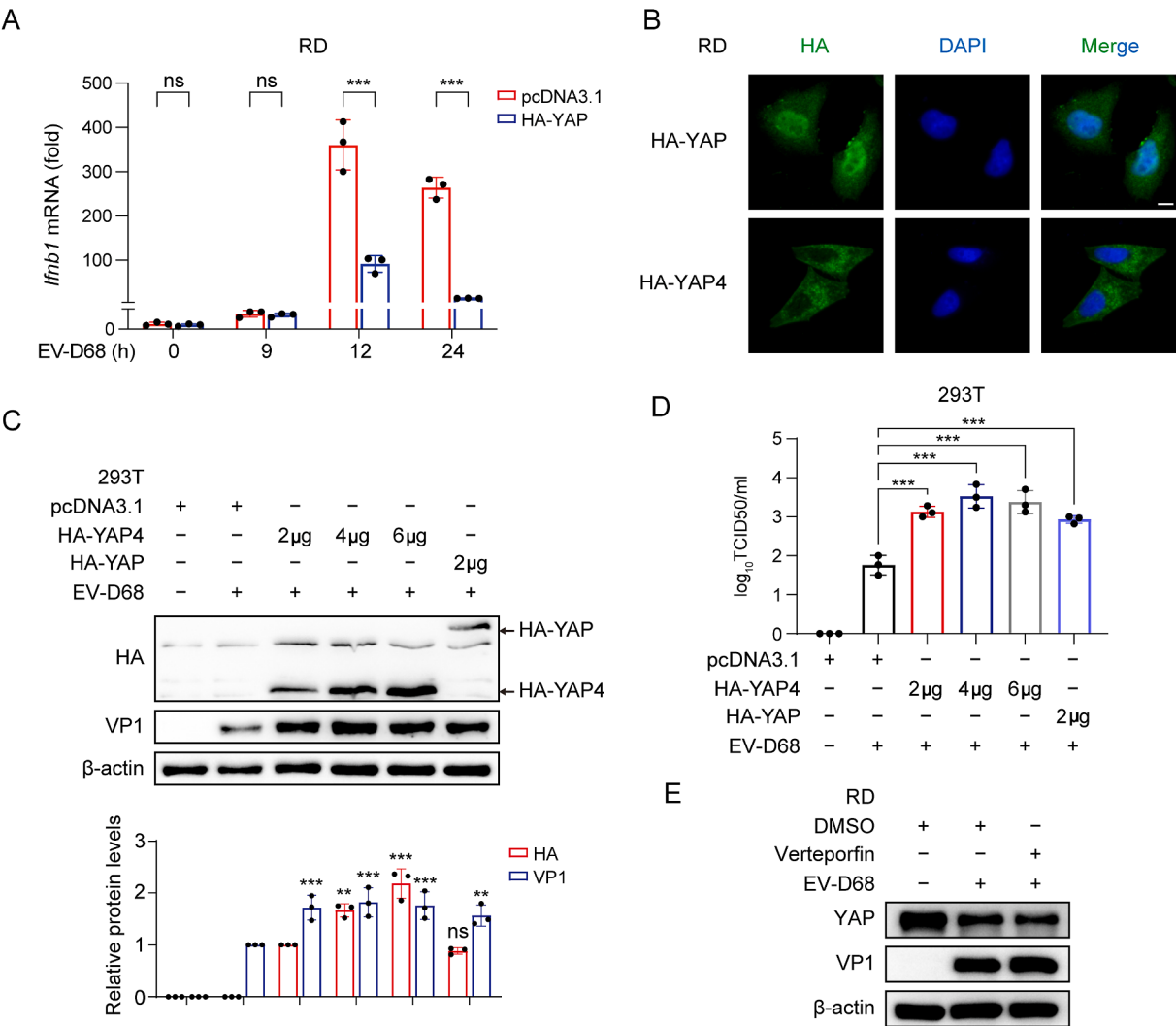


Fig. 4. YAP suppresses the interferon pathway at later stage of enterovirus infection. **A** RD cells were transfected with HA-YAP (4 μg) for 48 h and then infected with EV-D68 at MOI of 1 for 0, 9, 12, 24 h. Relative expression levels of *Ifnb1* in cells were determined by RT-qPCR. **B** RD cells were transfected with plasmids expressing HA-YAP or HA-YAP4 (4 μg). After 48 h, cells were subjected to immunofluorescence staining. Scale bar: 10 μm. **C, D** 293T cells were transfected with HA-YAP (2 μg) or HA-YAP4 (2, 4 or 6 μg) for 48 h and then infected with EV-D68 for 18 h. HA and EV-D68 VP1 protein levels were assessed (C). Lane-loading differences were normalized by levels of β-actin. The amount of HA/β-actin was normalized to the value obtained in the 2 μg HA-YAP4 group, and the amount of VP1/β-actin was normalized to the value obtained in the pcDNA3.1+EV-D68 group. Viral titers in the cell supernatant were qualified by TCID₅₀ assay (D). **E** RD cells were treated with verteporfin (1 μM) for 24 h, and then infected with EV-D68 for 18 h. Cell lysates were subjected to Western blot analysis. Data are representative of results from at least three independent experiments. ***P* < 0.01; ****P* < 0.001; ns, not significant.

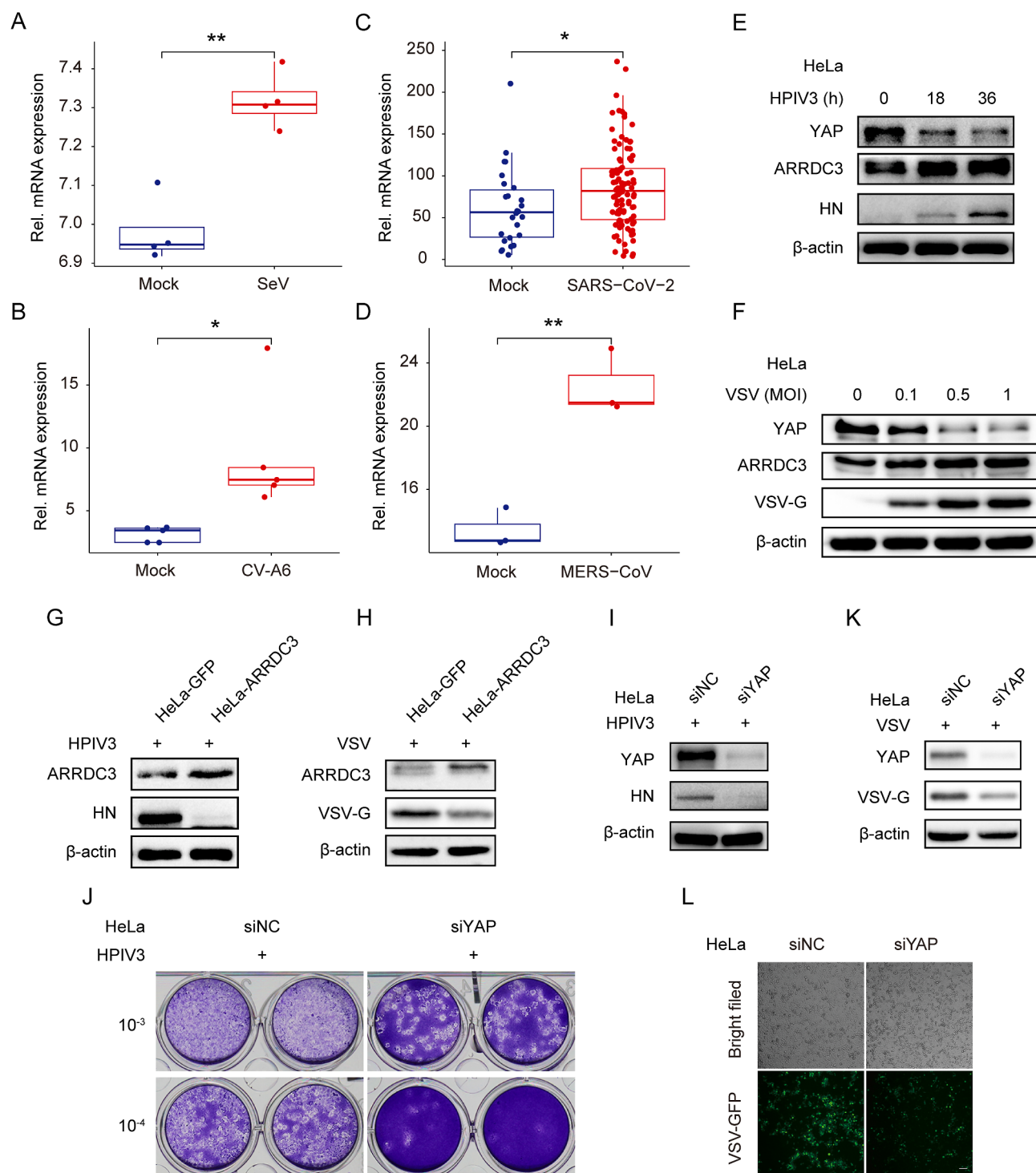


Fig. 5. The ARRDC3-YAP pathway emerges as a broad host response to viral infection. **A–D** ARRDC3 mRNA expression upon SeV, CV-A6, SARS-CoV-2 and MERS-CoV infection. Data from GEO: GSE157103, GSE139516, GSE243200, and GSE67198. **E** Western blot analysis of YAP, ARRDC3 and HPIV3 HN protein levels in HeLa cells infected with HPIV3 at MOI of 0.1 for 0, 18, 36 h. **F** Western blot analysis of YAP, ARRDC3 and VSV-G protein levels in HeLa cells infected with VSV at MOI of 0, 0.1, 0.5, 1 for 24 h. **G, H** HeLa-GFP cells or HeLa-ARRDC3 cells were infected with HPIV3 at MOI of 0.1 for 36 h (**G**) or VSV at MOI of 0.1 for 24 h (**H**), and then ARRDC3 and HPIV3 HN or VSV-G protein levels were analyzed. **I–K** HeLa cells were transfected with siYAP or siNC for 30 nM. After 48 h, cells were infected with HPIV3 at MOI of 0.1 for 36 h or VSV at MOI of 0.1 for 24 h. YAP and viral protein levels were analyzed (**I, K**). Viral titers were quantified by plaque assay (**J**). **L** HeLa cells were transfected with siYAP (30 nM) for 48 h. Then cells were infected with GFP-expressing VSV at MOI of 0.1. After 12 h, the cells were observed under brightfield microscopy (upper row) and fluorescence microscopy (lower row). Scale bar: 100 μm. Data are representative of results from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$.

virus type 3 (HPIV3) and vesicular stomatitis virus (VSV) infection. Our results demonstrated that, in HeLa cells infected with HPIV3, ARRDC3 expression increased in a time-dependent manner, accompanied by reduced YAP protein levels (Fig. 5E). Likewise, heightened VSV infection led to elevated ARRDC3 levels and downregulation of YAP (Fig. 5F). Furthermore, in HeLa-ARRDC3 cells, the expression levels of HPIV3 haemagglutinin-neuraminidase (HN) and VSV glycoprotein (G) were significantly diminished (Fig. 5G and H), suggesting that ARRDC3 inhibits the replication of both HPIV3 and VSV. Conversely, siRNA-mediated knockdown of YAP in HeLa cells markedly suppressed HPIV3 HN protein expression (Fig. 5I) and reduced viral titers (Fig. 5J). Knocking down of YAP also repressed VSV replication, as shown by decreased VSV G protein levels (Fig. 5K) and fewer GFP-positive cells (Fig. 5L). These findings demonstrate that YAP facilitates the replication of HPIV3 and VSV. Taken together, these observations underscore the ARRDC3-YAP pathway as a novel broad-spectrum antiviral mechanism.

DISCUSSION

ARRDC3 participates in various physiological and pathological processes by modulating receptor trafficking (Wedegaertner et al., 2022). However, its role in viral infection remains poorly defined. In this study, we observed that multiple viruses, including enterovirus as well as HPIV3 and VSV, induce ARRDC3 expression. RNA sequencing data also showed that ARRDC3 expression was upregulated after infection with SeV, CV-A6, SARS-CoV-2 or MERS-CoV, consistent with previous reports (Wyler et al., 2021). Our data further demonstrated that ARRDC3 functions as a novel broad-spectrum antiviral gene. In addition to ARRDC3, other research has shown that ARRDC4 recruited E3 ubiquitin ligase TRIM65 to facilitate K63-linked poly-ubiquitination of MDA5, thus suppressing EV-A71 replication by activating downstream inflammation-related genes (Meng et al., 2017). This suggests that α -arrestins family members play pivotal roles in innate antiviral immunity, which deserves to be further explored. Meanwhile, the mechanism by which ARRDC3 is upregulated during viral infection is poorly understood. Further studies, such as the investigations into ARRDC3 promoter activation, may shed light on this issue.

As the key effector of Hippo pathway, YAP is phosphorylated at Ser381 by the upstream kinases LATS1/2, which triggers its ubiquitination and proteasome-dependent degradation (Zhao et al., 2010). In addition, IKK ϵ mediates the phosphorylation of YAP at Ser403, thereby inducing lysosome-mediated degradation of YAP to relieve its inhibitory effect on IFN- β (Wang et al., 2017). During herpes simplex virus type 1 (HSV-1), SeV, and VSV infections, serine deficiency reduces S-adenosyl methionine-dependent H3K27me3 occupancy at the promoter, leading to increased expression of the V-ATPase subunit ATP6V0d2, which further promotes YAP lysosomal degradation (Shen et al., 2021). In our study, YAP protein levels are obviously downregulated, whereas its mRNA levels remained unchanged, indicating that YAP is negatively regulated in a post-translational manner rather than transcriptional manner, which is supported by previous researches. Furthermore, we identified that ARRDC3 interacted with YAP and facilitates its lysosomal degradation, which is a novel regulatory mechanism of YAP during viral infection and the precise mechanism of how YAP is targeted to lysosome will be carried out in our future research. Our results aligned with prior cancer studies indicating that ARRDC3 exerts its tumor suppressor function via modulating Hippo pathway signaling. For instance, ARRDC1 and ARRDC3 bind to the WW domain of the oncoprotein YAP via the PPXY motif in clear cell renal cell carcinoma (ccRCC), promoting E3 ubiquitin ligase Itch-mediated ubiquitination and degradation of YAP (Xiao et al., 2018). In colorectal cancer, ARRDC3 interacts with YAP and regulates its stability by facilitating lysosome-mediated degradation (Shen et al., 2018). Moreover, in *Drosophila*, Leash (the ortholog of ARRDC3) interacts with Yki (the ortholog of YAP) to promote its degradation and regulate the Hippo pathway (Kwon et al., 2013), suggesting that the negative regulation of YAP by ARRDC3 is highly

conserved across mammalian and *Drosophila*. Our finding further demonstrated that this negative regulation of YAP by ARRDC3 is crucial in antiviral immunity, thus highlighting the multifaceted roles of ARRDC3.

Accumulating evidence indicate that ARRDC3 regulates the endosome-lysosome degradation pathway of GPCRs and integrin β 4 (ITG β 4). Specifically, ARRDC3 interacts with neural precursor development downregulated protein 4 (NEDD4), the E3 ubiquitin ligase, through its conserved PPXY motifs, thus facilitating NEDD4-mediated ubiquitination and subsequent degradation of β 2AR (Nabhan et al., 2010). Besides, ARRDC3 promotes the ubiquitination of ALG-interacting protein X (ALIX), leading to the subsequent interaction between PAR1 and the endosomal complex required for transport (ESCRT-III) machinery, thereby directing PAR1 to lysosomes for degradation (Dores et al., 2015). ARRDC3 also induces NEDD4-dependent ITG β 4 ubiquitination and targets endosomal ITG β 4 to lysosomes, thereby inhibiting the ITG β 4 endocytic recycling (Soung et al., 2018). It would be of considerable interest to elucidate the detailed mechanism by which ARRDC3 mediates YAP lysosomal degradation and to examine the potential post-translational modifications of YAP involved in this process. Furthermore, because ARRDC3 binds to YAP through the interaction between its PPXY motifs and YAP WW domain to destabilize YAP (Xiao et al., 2018), PPXY motifs might be crucial for YAP degradation and viral replication. Further characterization of this regulatory mechanism will deepen our understanding of the Hippo pathway. Additionally, given that ARRDC3 localizes in the plasma membrane and endocytic vesicles (Tian et al., 2016; Han et al., 2013), whether ARRDC3 regulates the cellular receptors for viruses or directly participates in the viral infection process, such as viral internalization and viral uncoating, remains to be clarified.

Some studies have revealed that the transcriptional activity of YAP is critical for regulating certain viral infection. For instance, YAP collaborates with DNA-binding TEAD to repress the STING expression, thereby inhibiting HCMV replication (Lee et al., 2022). YAP also activates the Epstein-Barr virus (EBV) BZLF1 immediate-early promoter via TEAD binding motifs, inducing EBV immediate-early genes and reactivation (Van Sciver et al., 2021). Distinct from these researches, our findings suggested that the pro-viral effect is independent of its transcriptional activity, as evidenced by the lack of any reduction in viral replication following verteporfin treatment and the robust pro-viral effect of YAP4, which are consistent with other reports (Zhang et al., 2017). It is likely that the function of YAP transcriptional activity may vary depending on the viral species.

YAP has been reported to antagonize the IFN pathway to inhibit innate antiviral response (Wang et al., 2017). However, previous reports generally suggested that enterovirus significantly suppress the IFN pathway (Rasti et al., 2019; Lei et al., 2010, 2013). EV-D68 3C protease mediates the cleavage of TIR domain-containing adaptor inducing beta interferon (TRIF), a key adaptor downstream of TLR3, thus abolishing the subsequent activation of NF- κ B and IFN- β signaling (Xiang et al., 2014). RIG-I receptor signaling is also suppressed by EV-D68 3D polymerase, leading to the inhibition of IFN- β expression (Yang et al., 2021). In line with these reports, we observed that EV-D68 infection does not significantly upregulate IFN- β during early stages. Consequently, the inhibitory effect of YAP overexpression on IFN is not significant during this early infection stage. However, in the mid to late stages of infection (12–24 h), IFN- β levels are markedly upregulated and YAP overexpression significantly reduces its levels, suggesting that YAP antagonizes the interferon pathway to inhibit enterovirus replication at later stage of viral infection. Moreover, YAP contains a TEAD binding domain, one or two WW domains, an SH3 binding motif, a transcriptional activation domain (TAZ) and a PDZ binding motif (Iglesias-Bexiga et al., 2015). Future studies are needed to pinpoint the specific region within YAP that confers its pro-viral effect.

Finally, we validated the broad-spectrum antiviral potential of the ARRDC3-YAP pathway. In addition to enterovirus, infections caused by

HPIV3, VSV, SeV, CV-A6, SARS-CoV-2 and MERS-CoV all significantly induce ARRDC3 expression. Our further investigations proved that ARRDC3 negatively regulates YAP to inhibit HPIV3 and VSV replication. Interestingly, ARRDC3 demonstrated a more potent inhibitory effect against HPIV3 compared to VSV at equivalent expression levels. The precise mechanisms underlying this differential antiviral activity warrant further investigation.

CONCLUSIONS

In summary, our finding identify ARRDC3 as a novel broad-spectrum antiviral gene, acting by promoting the lysosomal degradation of YAP. Moreover, YAP facilitates enterovirus replication by antagonizing the IFN- β pathway, independent of its transcriptional regulation. These results broaden our understanding of the roles of α -arrestins family and YAP in antiviral immunity, and offer potential therapeutic targets for future antiviral interventions (Fig. 6).

MATERIALS AND METHODS

Cell lines and reagents

Human rhabdomyosarcoma cells (RD), human embryonic kidney 293T cells (293T), and human cervical cancer cells (HeLa) were kept in our lab. ARRDC3 stable overexpressed cell line (RD-ARRDC3 and HeLa-ARRDC3) and the control cells (RD-GFP and HeLa-GFP) were purchased from Biorun (Wuhan, China). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (HyClone, USA) at 37 °C in the presence of 5% CO₂.

The proteasome inhibitor MG-132 (HY-13259) and verteporfin (HY-B0146) were both purchased from MedChem Express (Shanghai, China). The lysosomal inhibitor NH₄Cl (A116373) was obtained from Aladdin (Shanghai, China).

Viruses and infections

EV-D68 (Fermon strain) and EV-A71 was kept in our lab and propagated in RD cells. VSV and VSV-GFP were also kept in our lab. HPIV3 (NIH47885) was kindly provided by Professor Mingzhou Chen of Hubei University and propagated in HeLa cells by inoculation at a multiplicity of infection (MOI) of 0.1. Cells were seeded in 6-well plates. When the

cell density reached 40%–50%, the cells were inoculated with virus at indicated MOI and incubated at 37 °C with 5% CO₂. After 2 h, the medium was replaced by DMEM containing 4% FBS. Cell samples were collected at the indicated time based on the various experimental requirements. For the HPIV3 and VSV infections, the same operation were performed on HeLa cells.

TCID₅₀

Cells were cultured in 96-well plates. The virus stock for testing was diluted in 10-fold gradient in DMEM medium from 10⁻³ to 10⁻⁸, followed by the addition of the diluted samples into cells when the cell density reached 30%–40%. Each dilution was replicated for three wells. After incubation for 2 h at 37 °C with 5% CO₂, the supernatant was replaced with DMEM medium contains 4% FBS. The cells were incubated for another 3–5 days at 37 °C with 5% CO₂ and the cytopathic effect (CPE) was observed by light microscopy. TCID₅₀ was calculated using the Reed-Muench method.

Plaque assay

HeLa cells in 24-well plates were cultured to 60%–70% confluency and then infected with the virus samples which were serially 10-fold diluted up to 10⁻⁵. After 2 h, the supernatant was replaced with methylcellulose and the plates were incubated for 3–5 days at 37 °C with 5% CO₂ until visible viral plaques were observed. Then plates were stained with 0.5% crystal violet for 4 h at room temperature and washed. The plaques were counted and the viral titers were calculated.

Cell transfection

Small interfering RNA (siRNA) specific for YAP (si-YAP) and ARRDC3 (si-ARRDC3) were synthesized by Sangon Biotech (Shanghai, China). All sequences were presented in the [Supplementary Table S1](#). The plasmid pcDNA3.1-YAP-HA was generated by fusing YAP fragment amplified from RD cell-derived cDNA (with primers YAP-F and YAP-R) to the pcDNA3.1-HA plasmid (with primers HA-F and pcDNA3.1-R) via In-Fusion cloning. For plasmid pcDNA3.1-YAP4-HA, the YAP4 fragment amplified with primers YAP4-F and YAP-R replaced the wild-type YAP sequence using identical plasmid amplification and cloning procedures. The pCAGGS-ARRDC3-Myc plasmid was constructed by cloning the ARRDC3-Myc fragment (with primers ARRDC3-pCAGGS-F

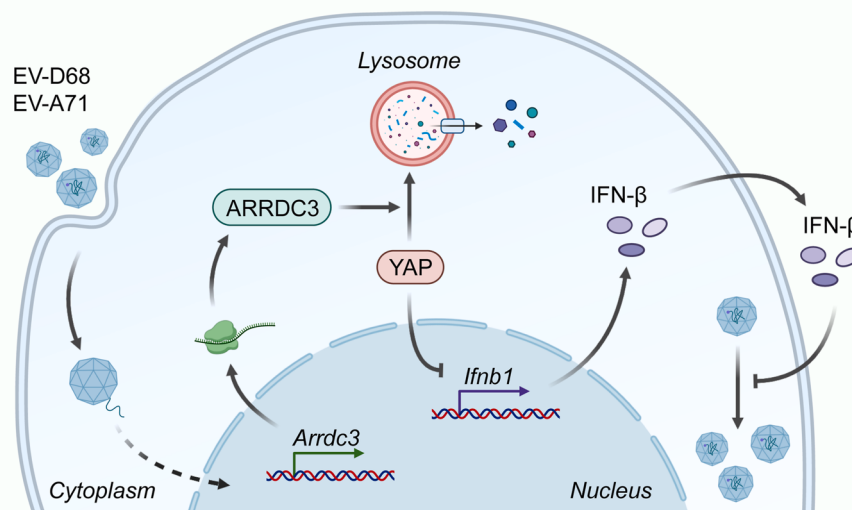


Fig. 6. Schematic diagram of ARRDC3 facilitates the lysosome degradation of YAP to inhibit enterovirus replication. EV-D68 and EV-A71 infection upregulate the expression of ARRDC3, which promotes lysosome-mediated YAP degradation. YAP inhibits IFN- β mRNA levels at later stage of infection to facilitate enterovirus replication, which is independent of its transcriptional activity. Illustration was generated using BioRender.

and ARRDC3-Myc-R) into the pCAGGS plasmid (with primers pCAGGS-Myc-F and pCAGGS-R) through In-Fusion cloning. Similarly, plasmid pcDNA3.1-ARRDC3-Flag was assembled by fusing the ARRDC3-Flag fragment (with primers ARRDC3-pcDNA3.1-F and ARRDC3-Flag-R) to the pcDNA3.1-3× Flag plasmid (with primers pcDNA3.1-Flag-F and pcDNA3.1-Flag-R). All the primer sequences were described in the [Supplementary Table S2](#). The plasmids mCherry-LAMP1 were kindly provided by Professor Tianle Gu of Chongqing Medical University. All plasmids were verified by Sanger sequencing before functional experiments. When cell confluency reached 60%, cells were transfected with siRNAs or plasmids for 48 h using lipofectamine 3000 reagent (Invitrogen, USA), according to the manufacturer's instructions.

RT-qPCR analysis

Total cellular RNA was extracted using TRIzol reagent (Beyotime, China). According to the manufacturer's instructions, reverse transcription was performed by PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Japan) and quantitative PCR was performed using BioRun ChemoHS qPCR Mix (SYBR) (Biorun, China). The mRNA levels of ARRDC3 and YAP were calculated after normalization to the house-keeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) by using the $2(-\Delta\Delta CT)$ method. All the primer sequences were described in the [Supplementary Table S3](#).

Western blotting

Cells were washed and harvested with cold phosphate buffered saline (PBS), then centrifuged at $16100 \times g$ for 1 min and lysed in TNE buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% 2-mercaptoethanol and protease inhibitor cocktail] for 30 min at 4 °C. The cell lysates were centrifuged at $16100 \times g$ for 30 min at 4 °C. 5× SDS-PAGE loading buffer was added into the supernatant and boiled at 100 °C for 10 min. The samples were loaded onto 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, USA). After blocking with 5% skim milk for 30 min, the membrane was incubated with the primary antibody for 1.5 h. After washing three times with PBS-Tween (PBST), the membrane was incubated with the secondary antibody for 45 min. Visualization was achieved with chemiluminescence. The primary antibodies used were rabbit anti-YAP (1:2500, ET1608-30, Huabio, China), rabbit anti-ARRDC3 (1:500, 34169, SAB, USA), rabbit anti-HA (1:4000, 51064-2-AP, Proteintech, China), rabbit anti-beta-actin (1:1000, 81115-1-RR, Proteintech, China), mouse anti-EV-D68 VP1 (1:2000, GTX633770, Gene Tex, China), rabbit anti-EV-A71 VP1 (1:2000, GTX132339, Gene Tex, China), rabbit anti-Myc (1:1000, M20002F, Abmart, China), rabbit anti-GAPDH (1:1000, D264398, Sangon, China), goat anti-HPIV3 HN (1:2500, ab28584, Abcam, United Kingdom) and mouse anti-VSV-G (1:1000, sc-365019, Santa Cruz Biotechnology, USA). The secondary antibodies included HRP-conjugated goat anti-mouse IgG (1:5000, D110087, Sangon, China), HRP-conjugated goat anti-rabbit IgG (1:5000, D110058, Sangon, China) and HRP-conjugated donkey anti-goat IgG (1:5000, D110115, Sangon, China).

Immunofluorescence analysis

Cells in 24-well plates grown to 30%–50% confluency on glass coverslips were transfected with the plasmids or infected with EV-D68 for indicated time. Then cells were washed three times with pre-chilled PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 (Solarbio, China) for 25 min. After blocking with 3% bovine serum albumin (BSA) (Solarbio, China) for 30 min, the cells were incubated with primary antibody for 1.5 h. Then cells were washed three times with 1% BSA and incubated with secondary antibody for 45 min. Nuclei were stained with DAPI (Solarbio, China). Microscopic analysis was performed with immunofluorescence microscopy (Nikon, Ts2-FL,

Japan). The primary antibody were mouse anti-Flag (1:1000, 66008-4-Ig, Proteintech, China), rabbit anti-HA (1:200, 51064-2-AP, Proteintech, China) and mouse J2 anti-dsRNA (1:200, 10010500, Scicons, Netherlands). The secondary antibody were Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:1000, A-11008, Thermo, USA) and Alexa Fluor 568 conjugated goat anti-mouse IgG (1:1000, A-11004, Thermo, USA).

Co-immunoprecipitation

Cells were harvested as described above and lysed in 300 µL of TNE buffer for 15 min. Cell lysates (50 µL) were taken out for input analysis. The remaining lysates were incubated with 200 µL of TNE buffer and 10 µL of protein A + G magnetic beads (Beyotime, China) with rotation for 2 h and beads were removed. Protein A + G Beads (20 µL) were incubated with 2 µg anti-Myc antibody (Proteintech, China) for 4 h with gentle rotation. The pre-cleared lysate were then incubated with antibody-conjugated beads for 6 h and the beads were collected and washed 3 times with PBS. The immunocomplexes were eluted by boiling with 50 µL of TNE buffer and 12.5 µL of 5× SDS-PAGE loading buffer and analyzed by Western blotting as described above.

Protein-protein interaction network analysis

The GeneMANIA database (<http://genemania.org>) was utilized to screen protein molecules that interact with ARRDC3. First, the target gene “ARRDC3” was input and the human species (*Homo sapiens*) was selected. The association types of Physical interaction, Genetic interaction, Co-expression, Co-localization and Pathway sharing in the advanced options were checked and the 21 high-confidence associated proteins were obtained.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 and expressed as means ± standard error of mean (SEM). Student's *t*-test was used to compare two groups; a one-way or two-way analysis of variance was followed by Tukey's post hoc test to compare multiple groups. $P < 0.05$ were considered significantly different and indicated by asterisks in the figures.

DATA AVAILABILITY

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

Xia Huang: investigation, project administration, writing-original draft. Siyuan Wang: data curation, formal analysis, project administration. Yan Huang: data curation, methodology, validation. Yue Wang: project administration, writing-original draft. Guangchao Zang: resources. Yan Liang: methodology. Juntong Liu: software. Xinyue Han: formal analysis. Jingjing Liao: investigation. Tingting Chen: conceptualization, investigation, project administration, supervision. Nan Lu: conceptualization, funding acquisition, supervision, writing-review and editing. Guangyuan Zhang: conceptualization, funding acquisition, project administration, supervision, writing-review and editing. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this work.

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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.2025.07.009>.

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