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A novel PCV2 ORF5-interacting host factor YWHAB inhibits virus replication and alleviates PCV2-induced cellular response

Kangkang Guo a,1, Xiuping Zhang a,b,1, Yufeng Hou a,1, Jing Liu a, Quanwen Feng a, Kai Wang a, Lei Xu c,d*, Yanming Zhang a,**

a College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China
b College of Animal Science, Tarim University, Alar, Xinjiang, 843300, China
c College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China

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ABSTRACT

Porcine circovirus type 2 (PCV2) infection causes porcine circovirus associated diseases (PCVAD) worldwide. Identification of host factors that interact with viral proteins is a fundamental step to understand the pathogenesis of PCV2. Our previous study reported that ORF5, a newly identified PCV2 viral protein supports PCV2 replication and interacts with multiple host factors. Here, we showed that a host factor YWHAB is an ORF5-interacting protein and plays essential roles during PCV2 infection. By using protein-protein interaction assays, we confirmed that YWHAB directly interacts with PCV2-ORF5 protein. We further showed that YWHAB expression was potently induced upon ORF5 overexpression and PCV2 infection. Remarkably, we found that the YWHAB strongly inhibited PCV2 replication, suggesting its role in defending PCV2 infection. By using the ectopic overexpression and gene knockdown approaches, we revealed that YWHAB inhibits PCV2-induced endoplasmic reticulum stress (ERS), autophagy, reactive oxygen species (ROS) production and apoptosis, suggesting its vital role in alleviating PCV2-induced cellular damage. Together, this study demonstrated that an ORF5-interacting host factor YWHAB affects PCV2 infection and PCV2-induced cellular response, which expands the current understanding of YWHAB biological function and might serves as a new therapeutic target to manage PCV2 infection-associated diseases.

1. Introduction

Porcine circovirus type 2 (PCV2), with a 1.76 kb single-stranded circular DNA genome, is a member of the genus Circovirus in the family Circoviridae, causes porcine circovirus-associated diseases (PCVAD) worldwide (Lv et al., 2014; Meng, 2013). As the smallest virus that can infect mammals, the PCV2 genome contains 11 predicted open reading frames (ORFs), with 6 of them have been well-characterized (Li et al., 2018). Our previous study showed ORF4 suppresses PCV2-induced reactive oxygen species (ROS) accumulation in host cells (Lv et al., 2016). The ORF5 was initially characterized as a novel viral protein that induces Endoplasmic Reticulum Stress (ERS) and activates NF-κB cascade (Lv et al., 2015). By using the ectopic overexpression approach, we further showed that the PCV2 ORF5 also induces Unfolded Protein Response (UPR) (Ouyang et al., 2019). Our recent work demonstrated that PCV2 ORF5 induces autophagy in a AMPK-ERK1/2-mTOR signaling pathways dependent manner (Lv et al., 2020). In addition, it is also reported that PCV2 ORF5 inhibits type I interferon (IFN) expression by inhibiting the genes required for IFN production and thus enhances PCV2 replication (Choi et al., 2018). However, the underlying mechanism of how PCV2 ORF5 affects these host cellular responses remains enigmatic.

To investigate which host factors that interact with PCV2 ORF5, yeast two-hybrid assay was performed and several host proteins including transmembrane glycoprotein NMB (GPNNM), cytochrome P450 1A1 (CYP1A1), 14-3-3/α (YWHAB), zinc finger protein 511 isoform X2 (ZNF511) and serine/arginine-rich splicing factor 3 (SRSF3) were characterized (Lv et al., 2015). By using transcriptome analysis, we found GPNNMB expression was downregulated in PCV2-infected and PCV2 ORF5-transfected cells, which suggests its important role in PCV2...

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However, the interaction between PCV2 ORF5 and other characterized host factors were still unknown.

Among these ORF5-interacting host factors, 14-3-3ζ/α (also known as YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) piqued our interest for its role in viral infection (Chen et al., 2018). The 14-3-3ζ/α protein belongs to the highly conserved 14-3-3 protein family that widely distributed from plants to mammals (Obsilova et al., 2008). The 14-3-3 family consists of seven isoforms (ζ, ε, η, γ, θ, ζ and α), which were encoded by seven distinct genes (YWHAB, YWAHE, YWHAH, YWHAG, YWHAQ, YWHAZ, and SFN) (Rosenquist et al., 2000). The 14-3-3 family members are involved in various cellular processes including signal transduction, cell cycle, cell proliferation, apoptosis, differentiation and survival (Fu et al., 2000; Nathan and Lal, 2009). Accumulating evidence suggests that the isoforms of 14-3-3 in cells are very diverse, with more than 300 different proteins have been identified and these proteins were involved in diverse essential cellular processes including cell-cycle regulation, apoptosis, metabolism, protein trafficking and signal transduction (Morrison, 2009). The target mechanisms of PCV2 and represents a novel therapeutic target in constraining PCV2 infection.

Porcine kidney PK-15 cells (ATCC: CCL-33) and human embryonic kidney HEK293 (ATCC: CRL-1573) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Solarbio, China), supplemented with 10 % fetal bovine serum (FBS) (Gibco, United Kingdom) and penicillin (100 U/mL) and streptomycin (100 μg/mL). Porcine alveolar macrophages 3D4/2 (PAMs) (ATCC: CRL-2845) were grown in RPMI 1640 medium (Solarbio, China) with 10 % FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL).

**Fig. 1.** Interaction of YWHAB with PCV2 ORF5 protein.

(A) Exogenous co-IP analysis the binding of ORF5 and YWHAB. HEK293 cells were co-transfected with CMV-Flag-YWHAB and pEGFP-ORF5 plasmids for 48 h. Cells co-transfected with pEGFP-C1 and CMV-Flag-YWHAB were used as negative controls. A quarter of the cell extract was subjected to the input assay to assess β-actin, Flag-fusion and GFP-fusion protein levels. The rest of the extract was subjected to IP assay. Western blot detected proteins with a mouse anti-GFP mAb and a mouse anti-Flag pAb.

(B) GST pull-down assay analysis the interaction of ORF5 and YWHAB. The GST and GST-YWHAB proteins were produced from Escherichia coli Rosetta (DE3) cells and were immobilized on a glutathione agarose beads for 2 h at 4 °C, followed by incubation of the resin with the cell lysates containing GFP-ORF5 protein (HEK293 cells were transfected with pEGFP-ORF5 plasmid for 48 h).

(C) HEK293 cells were co-transfected with pDsRed-YWHAB and pEGFP-ORF5 plasmids for 48 h. Cells co-transfected with pEGFP-C1 and pDsRed-N1 were used as negative control. Cells were fixed and stained with DAPI (blue) for 10 min at room temperature. Scale bar, 10 μm. Data in (A, B and C) are one representative of those from three independent experiments.
and streptomycin (100 μg/mL). Delivery of eukaryotic expression plasmids into cells was performed by using the TurboFect Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions.

### 2.2. Recombinant plasmid construction

The YWHAB gene (14-3-3β/α, GenBank No. XP-005673018.1) was amplified from the cDNA of PK-15 cells by using the reverse transcription-PCR (RT-PCR) with primer pairs listed in Table S1 and then subcloned into the pCDH-CMV-MCS-EF1 vector with a Flag tag to generate CMV-Flag-YWHAB. The PCR product was also inserted into the pDsRed-N and pGEX-6P-1 vectors to generate pDsRed-YWHAB-N1 and pGEX-6P-1-YWHAB, respectively. Three pairs of shRNAs targeting to YWHAB gene and a random sequence negative control naming shN were predicted and designed (http://rnaidesigner.thermofisher.com). The fragments were cloned into pCDH-U6-MCS-EF1GreenPuro vector after annealing to generate shYWHAB and shN lentivectors. The integrity of all plasmids was confirmed by sequencing. All primers were listed in Table S1.
2.3. Virus infection

The wild-type PCV2 Yangling strain (wPCV2) was kept in our laboratory (Tang et al., 2011). PK-15 or PAMs cells were infected with PCV2 with an indicated multiplicity of infection (MOI) for 1 h, then replaced with fresh RPMI 1640 medium with 2% FBS.

2.4. Reverse transcription-PCR (RT-PCR) and real-time PCR

Total cell RNA was isolated using TRIzol Reagent (Takara, Japan), then reverse transcribed into cDNA using Fasking RT kit (Takara, Japan) according to the manufacturers’ instructions. To determine the expression level of targeted genes, quantitative real-time PCR was conducted using SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa). The primers were designed and synthesized by AUGCT Biotech sequences (Beijing, China) (Table S1). Finally, the relative mRNA expression level of each gene was assessed using the 2\(^{-\Delta\Delta Ct}\) instructions. To determine the expression level of targeted genes, quantitative real-time PCR was conducted using SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa). The primers were designed and synthesized by AUGCT Biotech sequences (Beijing, China) (Table S1). Finally, the relative mRNA expression level of each gene was assessed using the 2\(^{-\Delta\Delta Ct}\) method and normalized to the housekeeping gene \(\beta\text{-actin}\). A non-treatment (Mock) PK-15 cells served as the negative control.

2.5. Western blot assay

Cells were lysed by radioimmunoprecipitation assay (RIPA) buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Sigma-Aldrich, United States) on ice for 30 min. The protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Fisher, USA). The cells were treated with trypsin without EDTA and resuspended to 5 \(\mu\text{L}\) 7-aminoactinomycin D (7-AAD) for 10 min in the dark at room temperature or the cells were incubated with 2',7'-dichlorofluorescein diacetate (DHE, 5 \(\mu\text{M}\), Beyotime, China) at 37 °C for 30 min. The apoptotic cell populations and the ROS production level were determined by chemotherapy microscopy and flow cytometry. Fluorescence intensity was collected at 518 nm and 605 nm under the logarithmic mode. The data were analyzed using FlowJo v10 software.
2.7. Co-immunoprecipitation (Co-IP) assays

HEK293 cells were co-transfected with pEGFP-ORF5 and CMV-Flag-YWHAB plasmids and harvested at 48 h post-transfection by using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) containing PMSF for 30 min at 4°C. Cell lysate was collected by centrifuging for 30 min at 12,000 rpm and then incubated with anti-Flag A+G-agarose beads (Sigma-Aldrich, United States) at 4°C overnight. The beads were collected by centrifugation and washed 3 times with ice-cold TBST. Immunoprecipitated proteins were extracted from the agarose beads by boiling for 10 min in 5× loading buffer and then subjected to SDS-PAGE and incubated with anti-GFP antibody as described above.

2.8. GST pull-down assays

The GST-YWHAB protein was produced from Escherichia coli BL21 and treated with pull-down lysis buffer (Thermo Fisher Scientific) containing PMSF for 30 min at 4°C. Cell lysate was collected by centrifuging for 30 min at 12,000 rpm and then incubated with anti-Flag A+G-agarose beads (Sigma-Aldrich, United States) at 4°C overnight. The beads were collected by centrifugation and washed 3 times with ice-cold TBST. Immunoprecipitated proteins were extracted from the agarose beads by boiling for 10 min in 5× loading buffer and then subjected to SDS-PAGE and incubated with anti-GFP antibody as described above.

2.9. Confocal fluorescence microscopy

PAMs cells were co-transfected with pEGFP-ORF5 and pDsRed-YWHAB plasmids by TurboFect Transfection Reagent (Thermo Fisher Scientific). As a negative control, pEGFP-C1 and pDsRed-N1 plasmids were subjected to same experimental conditions. At 36 h post-infection, the cultured cells were washed with PBS and then fixed with 4% paraformaldehyde diluted in PBS. Cell nuclei were stained with 4′,6′-diamino-2-phenylindole (DAPI) for 10 min at room temperature. The cells were imaged under laser confocal scanning microscopy (Carl Zeiss, Germany).

2.10. Statistics

All data were presented as mean ± SD. Comparisons between groups were determined with the Student’s t-test. One-way analysis of variance (ANOVA) difference test was used to compare the data from pairs of treated or untreated groups. Statistical significance is indicated as *P < 0.05; **P < 0.01; ***P < 0.001.

3. Results

3.1. YWHAB interacts with PCV2 ORF5

In our previous study, by using the yeast two-hybrid assay, several proteins (YWHAB, GPNMb, CYP1A1, ZNF511 and SRSF3) were identified as ORF5-interacting host factors. To further validate the interaction between YWHAB (also known as 14-3-3β/α) and ORF5 protein, the CMV-Flag-YWHAB and pEGFP-ORF5 plasmids were co-transfected into HEK293 cells. The result showed a clear binding between YWHAB and
PCV2 ORF5 (Fig. 1A), suggesting the potential interaction between ORF5 and YWHAB. Next, the GST pull-down assay also confirmed a specific interaction between YWHAB and PCV2 ORF5 protein (Fig. 1B), which purified GST-YWHAB protein and cell lysis from ORF5-overexpressed cells were employed. Furthermore, colocalization of YWHAB and PCV2 ORF5 protein was observed in the cytoplasm of pEGFP-ORF5 and pDsRed-YWHAB vectors co-transfected porcine alveolar macrophages 3D4/2 (PAMs) cells (Fig. 1C), which further fostered an interaction between ORF5 and YWHAB. Together, these results confirmed that PCV2 ORF5 interacted with the host factor YWHAB.

3.2. PCV2 infection and ORF5 transfection induces YWHAB expression

We have already shown that PCV2 ORF5 interacts with YWHAB. However, whether PCV2 infection or ORF5 transfection could affect the expression of YWHAB remains unknown. To this aim, the endogenous YWHAB levels were measured upon PCV2 infection and ORF5-overexpressed cells were employed. Furthermore, colocalization of YWHAB and PCV2 ORF5 protein was observed in the cytoplasm of pEgfp-ORF5 and pDsRed-YWHAB vectors co-transfected porcine alveolar macrophages 3D4/2 (PAMs) cells (Fig. 1C), which further fostered an interaction between ORF5 and YWHAB. Together, these results confirmed that PCV2 ORF5 interacted with the host factor YWHAB.

3.3. YWHAB inhibits PCV2 replication

Although has been shown that YWHAB expression was greatly induced by PCV2 infection, the role of YWHAB during PCV2 infection is still enigmatic. To investigate the potential function of YWHAB in affecting PCV2 infection, the lentiviral-based overexpression and knockdown approaches were employed. As shown in Fig. 3A, a successful overexpression of YWHAB was observed in LV-YWHAB cell line. Remarkably, the overexpression YWHAB significantly suppressed the replication of PCV2 (Fig. 3B). Consistently, the protein expression of PCV2 Cap protein and mRNA expression of ORF5 was strongly decreased in YWHAB-overexpressed cells (Fig. 3C, D and E). As shown in Fig. 3F, a successful knockdown of YWHAB was obtained and following studies were performed on the shYWHAB-3 cell lines (Fig. 3F). Conversely, the PCV2 replication level was significantly increased in YWHAB-knockdown cells (Fig. 3G). The Cap protein expression and ORF5 gene expressions were also up-regulated in YWHAB silenced cells (Fig. 3H, I and J). Together, these results convincingly demonstrated that YWHAB inhibits PCV2 replication.

3.4. YWHAB alleviates the PCV2-induced ERS

PCV2 infection and ORF5 protein induce the Endoplasmic Reticulum Stress (ERS) (Lv et al., 2015). To investigate whether the YWHAB protein could affect the PCV2-induced ERS, we measured the expression of GRP78 and GRP94, which are the hall markers of ERS, in YWHAB-overexpressed or silenced cells upon PCV2 infection. Both mRNA expressions of GRP78 and GRP94 were significantly decreased in YWHAB overexpressed cells at 24 and 48 h post PCV2 infection (Fig. 4A and B). Consistently, the protein expression levels of GRP78 and GRP94 was also induced to a lower level by PCV2 in YWHAB overexpressed cells.
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In contrast, the gene expression of GRP78 and GRP94 was elevated in YWHAB silenced cells at 24 h and 48 h post PCV2 infection (Fig. 5 A and B). Importantly, similar results were obtained for the protein expression level of GRP78 and GRP94 in YWHAB silenced cells (Fig. 5 C, D and E). Together, we revealed that YWHAB alleviated the PCV2-induced endoplasmic reticulum stress.

3.5. YWHAB inhibits the PCV2-induced autophagy

Our previous work reported that ORF5 protein induces autophagy through PERK-eIF2a-ATF4 and AMPK-ERK1/2-mTOR pathways to promote viral replication (Lv et al., 2020). To determine whether YWHAB affects the PCV2-induced autophagy, the autophagy markers Beclin1 and LC3-II were measured in YWHAB overexpressed or silenced cells. Importantly, similar results were obtained for the protein expression level of GRP78 and GRP94 in YWHAB silenced cells (Fig. 5C, D and E). Together, we revealed that YWHAB alleviated the PCV2-induced endoplasmic reticulum stress.

3.6. YWHAB inhibits PCV2-induced intracellular ROS

Intracellular ROS production is an important indicator of cellular stresses. Previous studies showed that PCV2 infection induces the production of intracellular ROS (Sun et al., 2020; Zhang et al., 2019). To investigate the role of YWHAB in regulating PCV2-induced ROS production, we measured the intracellular ROS in YWHAB overexpressed and knockdown cells by using flow cytometry. In line with a previous study (Zhang et al., 2019), we confirmed that PCV2 infection strongly induced intracellular ROS level. Importantly, the intracellular ROS was significantly decreased in YWHAB upregulated cells (Fig. 7). Conversely, the intracellular ROS level was significantly elevated in the cells that have lower YWHAB expression (Fig. 7). This result indicated that YWHAB inhibits PCV2-induced ROS.

3.7. YWHAB inhibits PCV2-induced apoptosis

It has been demonstrated that PCV2 infection induces apoptosis in both cell culture model and animal model (Chang et al., 2007; Resendes et al., 2011). To explore whether the YWHAB affects the PCV2-induced apoptosis, the apoptosis in PCV2-infected YWHAB overexpressed or knockdown cells was measured at 24 h post-infection. As shown in Fig. 8A, the PCV2-induced apoptosis was strongly attenuated in YWHAB overexpressed cells compared to that in Lv control cells. As expected, the PCV2 infection activated apoptosis was potentiated in YWHAB silenced cells (Fig. 8B). Together, this result suggested that YWHAB inhibits cells from PCV2-induced apoptosis.

4. Discussion

The identification of host factors that interact with viral proteins is essential to understand the pathogenesis of virus. In our previous study,
five host proteins (YWHAB, GPNMB, CYP1A1, ZNF511 and SRSF3) were identified as PCV2 ORF5-interacting factors by used yeast two-hybrid assay (Lv et al., 2015). The interaction between GPNMB and PCV2 ORF5 was confirmed through different approaches and we surprisingly found that GPNMB inhibits PCV2 replication and ORF5 expression by regulating the Cyclin A expression in host cells (Guo et al., 2018). This study validated our previous screening approach and prompt us to investigate the role of other ORF5-interacting proteins in PCV2 infection. Intrigued by its role in regulating diverse cellular processes and affecting virus replication, we focused on the interaction between YWHAB and PCV2 in present study. A specific interaction between PCV2-ORF5 and YWHAB was validated via different approaches (Fig. 1). Then, we surprisingly found that PCV2 infection and ORF5 transfection strongly activated YWHAB expression. This is consistent with a previous study that showed pancreatic necrosis virus (IPNV) induced the expression of a large number of genes including YWHAB (14-3-3β), but they did not illustrate the role of YWHAB against IPNV infection (Vilgalba et al., 2017). In this study, we further showed that YWHAB inhibits PCV2 replication. Several isoforms of 14-3-3 proteins have been reported to affect viral infection (Aoki et al., 2000; Diao et al., 2001; Kino et al., 2005). In particular, a study shows that the M protein of paramyxovirus 5 (PIV5) interacts with host protein 14-3-3 β and the production of PIV5 particles was negatively affected (Schmitt and Lamb, 2004). Together, we believe that YWHAB is an anti-PCV2 host factor, in which the expression is induced upon viral infection. However, the molecular mechanism of how the YWHAB is induced needs further investigation.

The PCV2 ORF5 protein has been evidenced to induce the Endoplasmic Reticulum Stress (ERS) and unfolded protein response (UPR) (Ouyang et al., 2019). In the present study, we found that a host factor YWHAB alleviates PCV2-induced ERS. The hallmark of ERS is the upregulation of glucose-regulated protein 78 (GRP78) and 94 (GRP94) (Bailey and O’Hare, 2007). During ERS, Bip/GRP78 or GRP94 binds to misfolded proteins and releases PERK, ATF6, and IRE1, resulting in their activation and initiation of the UPR (Tu and Weissman, 2004). In this study, we found that YWHAB significantly inhibits GRP78 and GRP94 expression upon PCV2 infection (Figs. 3 and 4). PCV2 infection triggers autophagy to facilitate its replication (Zhai et al., 2019). Our previous work showed that ORF5 protein induces autophagy through PERK-eIF2α-ATF4 and AMPK-ERK1/2-mTOR pathways to promote viral replication (Lv et al., 2020). Given the importance of autophagy in regulating virus replication, it is not surprising that both PCV2 infection and host factors YWHAB can alter autophagy. Although no study has been reported that PCV2 ORF5 protein induces ROS production, PCV2 infection increases ROS production to facilitate PCV2 replication (Chen et al., 2012). PCV2 infection induced ROS production elicits dynamic relative protein1 (Drp1) phosphorylation and activation of the PINK1/Parkin pathway, which eventually activates mitophagy and mitochondrial apoptosis (Zhang et al., 2020). In this study, PCV2 infection induced ROS production is also observed (Fig. 6) and YWHAB potently decreases this effect. However, whether YWHAB inhibits PCV2 infection by suppressing GRP78/GRP94, autophagy or ROS production needs further investigation. Together, these results showed that the ORF5-interacting protein YWHAB potently alleviates the ORF5-elicited ERS, autophagy and ROS production. Nevertheless, further study is required to elucidate the underlying molecular mechanism of YWHAB in mitigating these cellular responses.

The 14-3-3 family proteins participate in versatile cell cascades through its phosphoserine and phosphothreonine binding activity (Berg et al., 2003; Weinert et al., 2019). In particular, recent studies revealed that 14-3-3 family proteins are involved in viral infection with diverse mechanisms (Chen et al., 2018; Hwang et al., 2018; Nathan and Lal, 2020; Toshima et al., 2001). The YWHAB protein interacting with the glycoprotein of spring viremia of carp virus positively affects viral attachment and entry (Chen et al., 2018). Some 14-3-3 family proteins directly act on the viral protein like the severe acute respiratory syndrome coronavirus (SARS-CoV) nucleocapsid (N) protein gets serine-phosphorylated and translocated to the cytoplasm by binding to 14-3-3 proteins (Surjit et al., 2005). On the other hand, some 14-3-3 proteins alter cellular responses to affect virus infection. For instance, 14-3-3ε stabilizes the interaction between RIG-I with TRIM25 to facilitate RIG-I ubiquitination, which confers innate immune response against hepatitis C virus and other pathogenic RNA viruses (Liu et al., 2012). Furthermore, binding of dengue virus NS3 protein to 14-3-3ε prevents the translocation of RIG-I to adaptor protein MAVS, thus blocking the antiviral signaling (Chan and Gack, 2016). Herein, we showed that the interaction of the YWHAB protein potently decreased the PCV2-induced cellular damages that could negatively affect PCV2 replication.

In summary, in this study we revealed that a host factor YWHAB interacts with PCV2-ORF5 protein and its expression is strongly induced by PCV2 infection and ORF5 protein. Mechanistically, YWHAB alleviates the PCV2 infection induced cellular damage including ERS, autophagy, ROS production and apoptosis. Our work reveals a novel role of YWHAB in defending PCV2 infection, which might serve as a new therapeutic target for the management of PCV2 infection-associated diseases.

Author contributions

K.G., Y.Z. and L.X. conceived the ideas and designed the experiments. Y.H. and K.G. performed the majority of experiments. J.L., Q.F and K.W. helped in cell culture, molecular biology and biochemical experiments. K.G., X.Z. and L.X. analyzed data and wrote the paper. X.Z. and K.G. critically revised the manuscript. All authors discussed the results and commented on the manuscript.
Fig. 8. YWHAB inhibits PCV2-induced apoptosis. Flow cytometry analysis of cell apoptosis in YWHAB overexpressed (A, CTR, untransduced control; LV, lentivirus control; LV-YWHAB, YWHAB overexpressed cells) or knockdown (B, CTR, untransduced control; shN, lentivirus control; shYWHAB, YWHAB knockdown cells) PK-15 cells infected with PCV2 at an MOI of 1. At 24 h post-infection, the cells were resuspended and stained with 5 μL Annexin V-FITC and 5 μL 7-aminoactinomycin D (7-AAD) for 10 min in the dark at room temperature. Fluorescent signals were detected by flow cytometry analysis. Data is one representative of those from three independent experiments.

Declaration of Competing Interest

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.vetmic.2020.108893.

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