



# The antiviral protein viperin interacts with the viral N protein to inhibit proliferation of porcine epidemic diarrhea virus

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## Abstract

In the early stage of virus infection, the pattern recognition receptor (PRR) signaling pathway of the host cell is activated to induce interferon production, activating interferon-stimulated genes (ISGs) that encode antiviral proteins that exert antiviral effects. Viperin is one of the innate antiviral proteins that exert broad-spectrum antiviral effects by various mechanisms. Porcine epidemic diarrhea virus (PEDV) is a coronavirus that causes huge losses to the pig industry. Research on early antiviral responses in the gastrointestinal tract is essential for developing strategies to prevent the spread of PEDV. In this study, we investigated the mechanisms of viperin in PEDV-infected IPEJ-C2 cells. Increased expression of interferon and viperin and decreased replication of PEDV with a clear reduction in the viral load were observed in PEDV-infected IPEC-J2 cells. Amino acids 1–50 of porcine viperin contain an endoplasmic reticulum signal sequence that allows viperin to be anchored to the endoplasmic reticulum and are necessary for its function in inhibiting PEDV proliferation. The interaction of the viperin S-adenosylmethionine domain with the N protein of PEDV was confirmed via confocal laser scanning microscopy and co-immunoprecipitation. This interaction might interfere with viral replication or assembly to reduce virus proliferation. Our results highlight a potential mechanism whereby viperin is able to inhibit PEDV replication and play an antiviral role in innate immunity.

## Introduction

Porcine epidemic diarrhea virus (PEDV), the causative agent of PED, is a member of the genus *Alphacoronavirus*, family *Coronaviridae* [27]. In pigs, PEDV first infects the Peyer's patch, a small area of intestinal lymph nodes [31]. It then proliferates and spreads to intestinal epithelial cells, eventually leading to infection of the entire small intestine [37]. Injury of intestinal organelles causes cell dysfunction and a reduction or loss of related enzyme activities. Impaired nutrient absorption due to enzyme inactivation can lead to osmotic diarrhea, dehydration, and death [18].

PEDV is a common coronavirus that has caused huge economic losses to the pig industry and its related peripheral industries worldwide [32]. Research on the natural immune responses to PEDV is still in its infancy, and there have been few reports in the literature about this topic [6].

Viperin is a broad-spectrum antiviral protein that has important antiviral effects, and its full potential remains to be explored. Its role in PEDV infection remains unclear, but its possible involvement in preventing PED is potentially significant for the stable and healthy development of the pig industry.

Previous studies have shown that virus-infected cells activate various signaling pathways to produce interferons [19]. When type I interferon is released, it binds to specific receptors on the cell surface and activates more than 300 downstream IFN-stimulated genes (ISGs) through a signal cascade reaction [11]. Many ISGs have been found to significantly limit viral replication and participate in a variety of antiviral processes, including presentation of viral antigens, apoptosis, and interference with viral replication and assembly [22]. Products of interferon-stimulated genes with antiviral activity are also known as “innate immune factors”. At present, only a small number

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of proteins encoded by interferon-stimulated genes have been reported, such as protein kinase R (PKR), ribonuclease L (RNase L), and viperin [9].

Host stress or overexpression of certain proteins can inhibit virus proliferation during viral infection [34]. The antiviral effect of IFN is usually indirect. It induces host cells to produce antiviral proteins and then exerts antiviral effects on transcription and translation through protein kinases, 2'-5'A synthase, and 2-phospholipase [15]. Viperin is a broad-spectrum antiviral protein that is usually overexpressed at a low level in many types of normal healthy cells [5, 40]. However, when induced by interferon, double-stranded DNA, double-stranded RNA, lipopolysaccharide, poly(I:C), or various viruses, the expression of viperin increases significantly [4, 42]. There are two main pathways by which the expression of viperin is induced. Sendai virus, pseudorabies virus, and Sindbis virus are all capable of inducing the expression of interferon-stimulated genes [8, 16, 35]. These viruses are first recognized by pattern recognition receptors (PRRs), such as the Toll-like receptors TLR3 and TLR4, the retinoic-acid-inducible gene RIG-1, and the cytoplasmic DNA sensor. The interferon regulatory factors IRF3 and IRF7 are then activated to produce IFN- $\beta$ , which binds to type I interferon receptors on the cell surface through autocrine or paracrine pathways, resulting in the synthesis of the complex ISGF3, which binds to the viperin promoter to activate its expression [36]. In addition, there are some other viruses, such as vesicular stomatitis virus and human cytomegalovirus, whose dsRNA stimulates RLRs and interaction with the adaptor protein MAVS can activate the production of IRF1 and IRF3, which in turn can induce viperin expression [10].

Viperin plays an important role in the production of type I interferon in plasmacytoid dendritic cells (pDCs) [30], which are immune cells derived from bone marrow. These cells are capable of rapidly activating responses to non-self nucleic acids to produce interferons in large amounts [23]. The main reason for this ability is that pDCs continuously produce the endogenous Toll-like receptors TLR7 and TLR9. Activated TLR7/9, combined with IRAK1 and TRAF6, can induce viperin expression [21]. In pDCs, viperin is necessary for producing type I interferon [28]. In viperin-deleted mouse cells, activation of the Toll-like receptors TLR7 and TLR9 does not lead to the production of interferon. Recruiting IRAK1 and TRAF6 in lipid droplets can activate IRF7, and viperin is also crucial for this process. Thus, the induction of viperin in pDCs depends on the activation of the TLR7 and TLR9 signaling pathways. IRAK1 and TRAF6 need to be recruited to lipid droplets through viperin, and IRF7 is then activated to induce the expression of type I interferon [29].

The role of porcine viperin in PEDV infection is still not well understood. In this study, the mechanisms by

which porcine viperin regulates PEDV proliferation were investigated in detail.

## Materials and methods

### Ethics statement

The study was approved by Tianjin University Institutional Animal Care and Use Committee (TJIACUC) (protocol number: SYXK-Jin 2014-0004). All BALB/c mice were kept in well-ventilated cages and were anesthetized for blood collection. The Guide for the Care and Use of Laboratory Animals of the Tianjin Government Authority was strictly followed.

### Cells, viruses and antibodies

BALB/c mice were obtained from the Tianjin Laboratory Animals Center. Cells of the porcine alveolar macrophage (PAM) cell line CRL2843-CD163 (3D4/21 cells) were kindly donated by Professor Han Jun of China Agricultural University. HEK-293T cells were cryopreserved in our laboratory. Cells of the porcine intestinal epithelial cell line IPEC-J2 and human embryonic kidney (HEK) 293T cells were cultured in high-sugar DMEM medium supplemented with 10% (V/V) fetal bovine serum (FBS) and antibiotic-antifungal solution. Both cell lines were cultured in a humidified 5% CO<sub>2</sub> incubator at 37 °C. PEDV strain CV777 was used in our study, and the titer was 10<sup>4</sup> PFU/ml [41]. Polyclonal antibodies against viperin were prepared by immunizing BALB/c mice with recombinant His-viperin with a mineral oil adjuvant. Labeled antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA, USA) and Applied Biological Materials Inc. (ABM, Vancouver, Canada). The internal reference antibody and HRP-conjugated antibody were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

### RT-PCR amplification of the complete porcine viperin CDS

Total RNA was extracted from PAMs using TRIzol Reagent (TaKaRa, China) and transcribed into cDNA using reverse transcriptase (TaKaRa). Viperin was amplified using oligonucleotide primers (Table 1) designed based on a porcine viperin sequence (NM\_213817.1) obtained from the GenBank database. The amplified fragment was cloned into pGEM-T Easy Vector (Transgen, Beijing).

**Table 1** Primers for cloning the viperin gene

Primer set	Accession no.	Primer sequence (5'-3')	T <sub>m</sub> (°C)
Viperin-T	NM_213817.1	F: ACCTGCTGCCATGTGGACTGGTA R: GCTCAGCTCTCAGCTTACCAGTCC	56

**Table 2** Primers for construction of eukaryotic expression vectors

Primer set	Primer sequence (5'-3')	Product (bp) size	T <sub>m</sub> (°C)
Flag-Viperin	F: ACCAGTCGACTC TAGAGGATCCATG TGGACTGGTA CCTG R: ACAGGGATGCCA CCCGGGATCCTCA CCAGTCCAGCTT CAGG	1089	58

### Plasmid construction

A FLAG-viperin construct for expression in eukaryotic cells was constructed as follows: The viperin gene was amplified using a specific primer pair (Table 2) with a sequence in common with the vector was ligated to the pFlag-CMV2 vector using a one-step cloning kit (Vazyme, Nanjing, China). The prokaryotic expression plasmid pET-28a-Viperin was constructed using the same method to express the recombinant protein His-viperin.

### Virus titration

The 50% tissue culture infective dose (TCID<sub>50</sub>) method was used to determine virus titers. IPEC-J2 cells were seeded in 96-well plates (1 × 10<sup>4</sup> cells/well). After the cells had adhered to the plate and grown to about 50% confluency, a serial dilution (1-10<sup>-6</sup>) of PEDV was inoculated onto the cells. Mock-infected cells were used as a control. The cells were incubated at 37°C for 7 days, and TCID<sub>50</sub> values were calculated by the Reed-Muench method [41].

### Real-time reverse transcription quantitative PCR (RT-qPCR)

Primers were designed based on available sequences in the GenBank database (Table 3). Relative gene expression was analyzed using TransStart Top Green qPCR SuperMix (dye I + dye II) reagents on a real-time PCR instrument (ABI 7500) with the following cycling conditions: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 45 s; 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. The cDNA was obtained by RNA extraction and reverse transcription. Three replicates were performed for each sample. The relative transcription level of each gene was calculated using GraphPad Prism software with β-actin as an internal reference.

### Confocal immunofluorescence

The procedure used for confocal microscopy was described previously [20]. IPEC-J2 cells were cultured overnight at 37 °C in 12-well plates (Corning Inc., Corning, NY, USA) and transfected either with plasmids containing the viperin gene or with empty vector (0.5 g). After the cells had adhered to the plate and grown to about 50% confluency, PEDV was inoculated onto the cells (100 mL/well), and an uninfected blank control (NC) was also included. Twenty-four hours later, cells were fixed with 4% paraformaldehyde solution and permeabilized with PBS containing 0.3% Triton X-100. Five hundred microliters of 0.5% Triton X-100 was then added to each well to permeabilize the cells for 20 minutes at room temperature. The cells were blocked with 1% bovine serum albumin (BSA) at room temperature for 30 minutes and incubated with

**Table 3** Primers for real-time PCR

Gene	Accession no.	Primer sequence (5'-3')	Product (bp) size	T <sub>m</sub> (°C)
Viperin	NM_213817.1	F:TCATCAATCGCTTCAATGTGG R:CGTCGCTGATAACAACTGCT	292	57.4
PEDV-N	AF353511.1	F:CTATGCTCAGATCGCCAGT R:GTAGATGGCCTTTCATGCT	275	55.6
PEDV-M	AF353511.1	F:ATGTCTAACGGTTCTATCCC R:AGAATAGCCATCTTGACACC	285	56.0
IFN-β	NM_002176.3	F:CAACAAGTGTCTGCTCGAAAT R:TCTCCTCAGGGATGTCAAAG	188	56.5
β-actin	DQ452569.1	F:GAATCCTGCGGCATCCACGA R:CTCGTCTACTCCTGCTTGCT	230	55.0

anti-Flag and anti-Myc or anti-GFP antibody at 4°C overnight. Secondary antibodies (fluorescein-isothiocyanate-conjugated anti-mouse IgG or phycoerythrin-conjugated anti-rabbit IgG) were used, and nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI). The cells were examined under an inverted fluorescence and phase-contrast microscope (Olympus) to determine the subcellular localization of viperin protein.

### Western blot analysis

Cells were lysed and homogenized in RIPA cell lysate in the presence of 1% phenylmethylsulfonyl fluoride (PMSF) to inhibit proteases. The protein samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose (NC) filter membranes (ExPro), and blocked. The NC membrane was treated overnight at 4°C with antibodies against Flag (1:5000), Myc (1:5000), viperin (1:500) or  $\beta$ -actin (1:5000) and then washed and incubated with HRP-conjugated antibody for 1 h at room temperature. Immunoreactive bands were visualized using a chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA) and a Gel Doc XR imaging system (Bio-Rad, USA).

### Immunoprecipitation

IPEC-J2 cells cultivated in 60-mm-diameter plates were transfected with Flag-viperin and Myc-N, or related expression plasmids. Twenty-four hours after transfection, the cells in each plate were treated with 400  $\mu$ L of lysis buffer containing 1% protease inhibitor (PMSF). The supernatant of the cell lysate was incubated overnight with anti-Myc-labeled or anti-Flag-labeled beads (Sigma) at 4 °C. The beads were washed three times for 10 min with lysis buffer and boiled for 5 min. The proteins bound to the beads were separated by SDS-PAGE and detected by Western blot.

### RNA interference

A small interfering RNA (siRNA) against viperin was synthesized by GenePharm (Table 3). A tube containing the RNA Oligo dry powder was centrifuged at 3000 rpm for 2 min and diluted with diethyl pyrocarbonate (DEPC)-treated water. When the cells in a 12-well plate had grown to 70% confluency, they were transfected with the siRNA using Lipofectamine 3000 at a final concentration of 50 nmol. After transfection with siRNA-Viperin for 16 hours, the cells were collected to measure the level of interference and then infected with PEDV. The cells were then collected for real-time quantitative RT-PCR and western blotting (Table 4).

**Table 4** Primers for siRNA of viperin

siRNA	Abbreviation	Primer sequence (5'- 3')
Negative control	NC	F:UUCUCCGAACGUGUCACG UTT
		R:ACGUGACACGUUCGGAGA ATT
siViperin-1	Sus-242	F:CCC GCCAGUGCAACUACA ATT
		R:UUGUAGUUGCACUGGCGG GTT
siViperin-2	Sus-732	F:CCG CUGGAAGGUCUCCA ATT
		R:UUGGAAGACCUCCAGCG GTT

### Statistical analysis

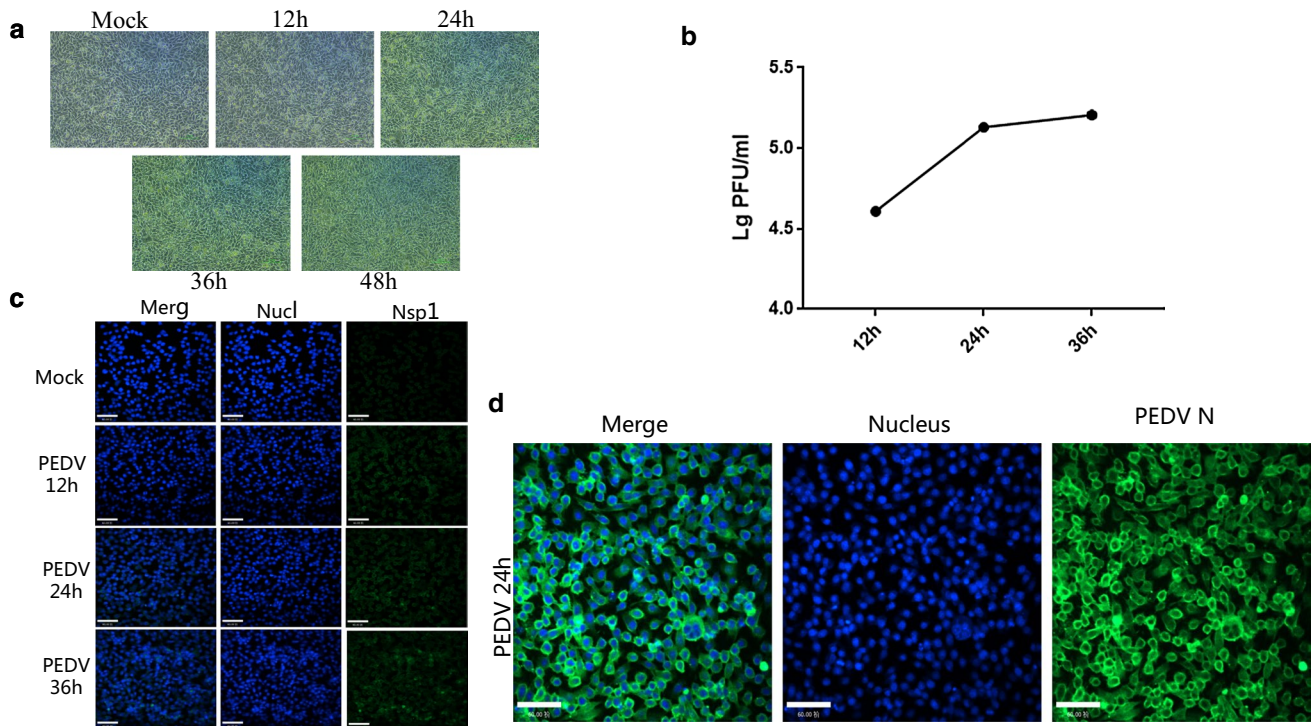
Statistical analysis was performed using GraphPad Prism software, and the significance of changes in gene expression was analyzed by two-way ANOVA. Statistical results are presented as mean  $\pm$  standard deviation (mean  $\pm$  SEM).  $P < 0.05$  (\*) indicates a statistically significant difference.

## Results

### Stable proliferation of PEDV in IPEC-J2 cells

Through cell culture and inoculation, we found that PEDV can stably proliferate in porcine intestinal epithelial cells (IPEC-J2), providing a cell model for further studies. At 36 h postinfection, the cells gradually became detached from the culture vessel, indicating that infection had occurred (Fig. 1a). Fluorescence-based quantitative analysis also showed that the number of PEDV virus particles stabilized and peaked at 24–36 h postinfection (Fig. 1b). We selected the structural protein N and the non-structural protein Nsp1 of PEDV for immunofluorescence experiments and observed their expression to reflect the replication of the virus. In the early stage of PEDV replication, genes encoding viral non-structural proteins were transcribed first, which could reflect viral replication to some extent. The amount of Nsp1 protein gradually increased with time (Fig. 1c). In the late stage of PEDV replication, the viral structural protein genes began to be transcribed and expressed. Expression of the structural protein N was also be used to trace the proliferation of PEDV (Fig. 1d), and the PEDV CV777 strain was found to stably proliferate in IPEC-J2 cells, reaching a peak at 36 hours postinfection.





**Fig. 1** Effect of PEDV infection on IPEC-J2 cells. (a) IPEC-J2 cells were observed at 12, 24, 36 and 48 h after inoculation with strain CV777. Compared with the negative control, the infected cells showed obvious fusion and eventually peeled off the surface of the

Petri dish. (b) Growth curve of PEDV in IPEC-J2 cells. (c) Expression of PEDV nsp16 in IPEC-J2 cells after infection with PEDV (bar: 60  $\mu$ m) (d) Expression of PEDV N in IPEC-J2 cells after infection with PEDV (bar: 60  $\mu$ m)

### Interferon production and viperin accumulation in IPEC-J2 cells after PEDV infection

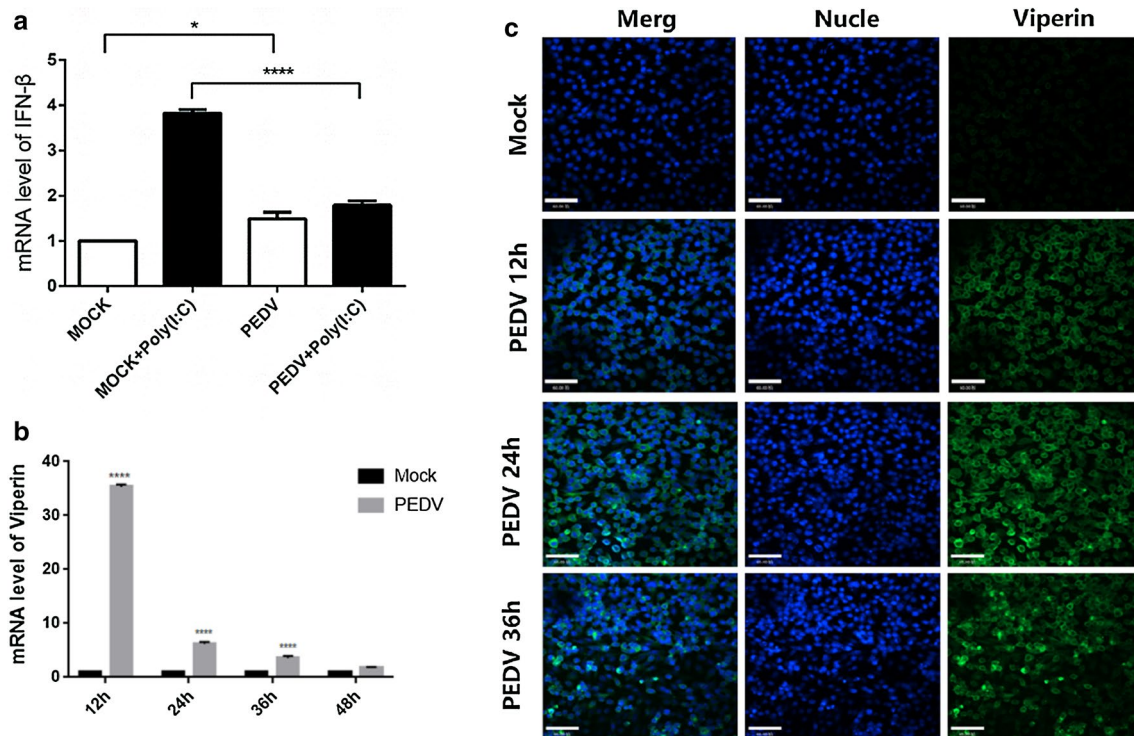
The innate immune system is evolutionarily conserved to defend against pathogens [26]. After virus infection, pattern recognition receptors recognize released viral nucleic acids and activate downstream signaling pathways to promote the production of specific transcription factors and type I interferons [25]. To measure interferon levels during PEDV infection, we infected IPEC-J2 cells with PEDV at a multiplicity of infection (MOI) of 0.5. Using quantitative reverse transcription PCR (qRT-PCR), we found that PEDV infection resulted in a significant increase in the level of IFN- $\beta$  mRNA compared to mock-infected cells. Interferon production was lower in PEDV-infected cells than in cells that were stimulated with poly(I:C) (Fig. 2a).

When IPEC-J2 cells were infected with PEDV, the transcription level of viperin increased significantly and reached a peak after 12 hours. After that, as the virus continued to replicate, the cells began to die and the transcription level of viperin began to decline. However, compared to control cells, the transcription level of viperin remained high in the infected cells (Fig. 2b). An immunofluorescence assay also showed that the expression of

viperin protein increased significantly infection (Fig. 2c). These results indicate that PEDV infection upregulates the expression of interferon and viperin in IPEC-J2 cells.

### Inhibition of PEDV proliferation in IPEC-J2 cells by viperin

We constructed a Flag-viperin eukaryotic expression plasmid and verified the expression of this protein in 293T cells (Fig. 3a). To investigate the effect of viperin on PEDV replication, the viperin gene was overexpressed in IPEC-J2 cells, and the cells were infected with PEDV 24 h later. The viral load was measured to investigate the effect of viperin overexpression on PEDV proliferation. Overexpression of the viperin gene was found to reduce the levels of PEDV produced ( $P < 0.05$ ) (Fig. 3b). For further validation, a specific siRNA targeting viperin was designed. Transfection of J2 cells with this siRNA resulted in a decrease in the expression of viperin (Fig. 3c), which was accompanied by an increase in PEDV proliferation that was proportional to the degree of interference of viperin expression (Fig. 3d). This suggests that viperin inhibits the proliferation of PEDV in IPEC-J2 cells.



**Fig. 2** PEDV infection promotes viperin expression in IPEC-J2 cells and inhibits interferon production in virus-infected cells. (a) PEDV inhibits the production of interferon in virus-infected cells. (b)

Changes in the level of viperin transcription at 12, 24, 36 and 48 h after viral infection. (c) Expression of viperin protein at 12, 24, 36 and 48 h after viral infection (bar: 60  $\mu$ m)

### Distribution of porcine viperin in the cytoplasm and localization in the endoplasmic reticulum

It has been reported that human viperin can be localized in the endoplasmic reticulum [38]. It has been proposed that viperin forms a dimer through its C-terminal domain and its N-terminal alpha helical domain to mediate the endoplasmic reticulum crystallization [13]. Since the amino acid sequences of porcine and human viperin are 83% identical, we investigated the location of porcine viperin in IPEC-J2 cells. Fluorescence microscopy showed that the viperin protein was distributed in the cytoplasmic region (Fig. 4a) and had significant aggregation and colocalization with an endoplasmic reticulum marker protein. At the same time, no co-localization of viperin with Golgi and lysosome markers was observed (Fig. 4b).

### Interaction between porcine viperin and the PEDV N protein

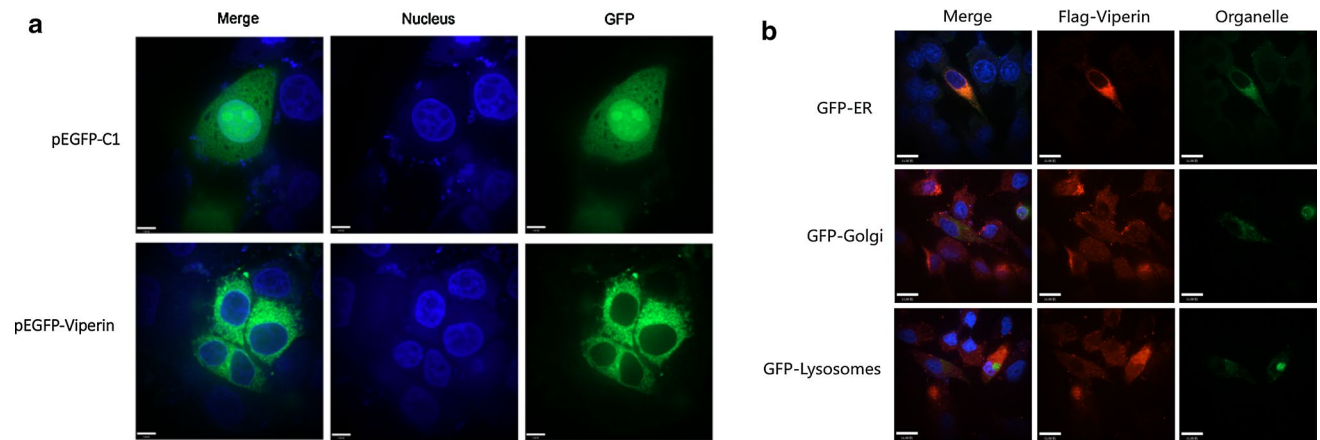
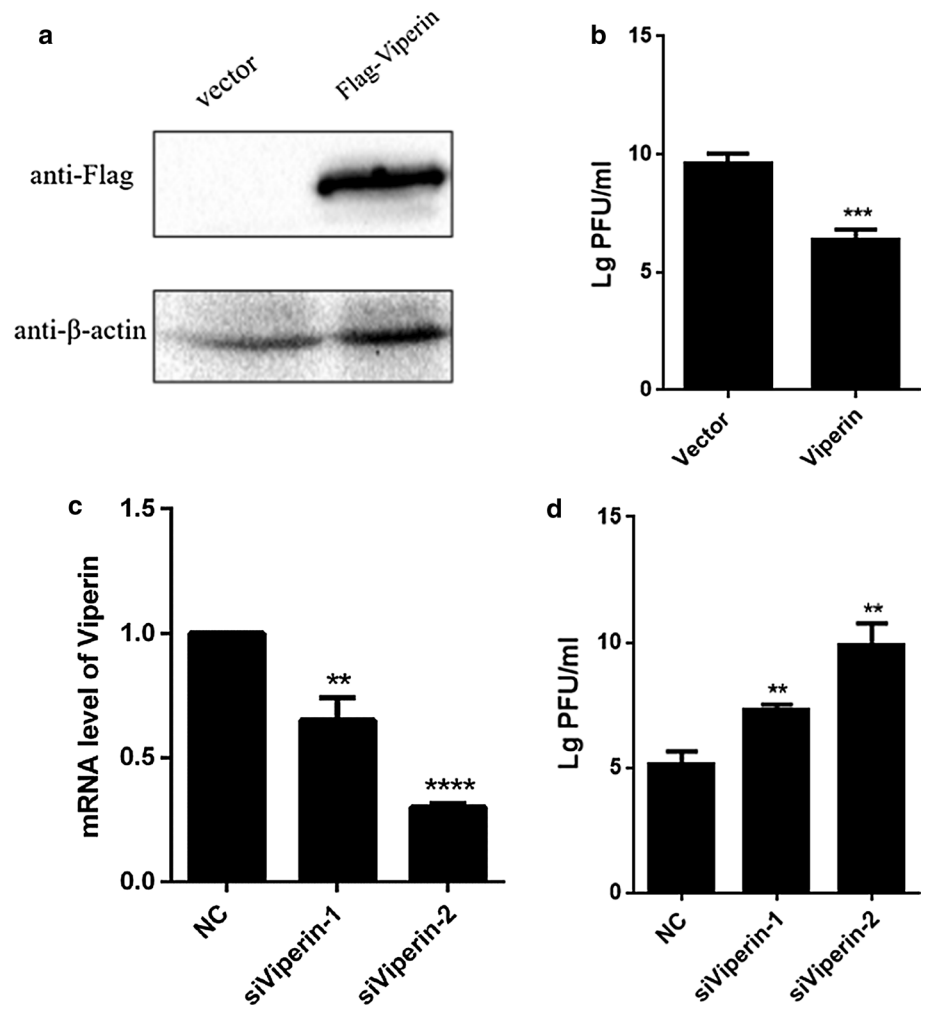
When PEDV infects cells, structural proteins and non-structural proteins encoded by the virus use the endoplasmic reticulum for their synthesis, processing, and modification, which makes us suspect that viperin interacts with certain proteins in PEDV. We therefore chose the major

structural protein N and two non-structural proteins, Nsp13 and Nsp16, for experiments. Immunofluorescence images of IPEC-J2 cells 24 hours after infection with PEDV showed that viperin and N showed clear co-localization, but no obvious co-localization with Nsp13 and Nsp16 was observed (Fig. 5a). Considering that the antibodies used in previous experiments were polyclonal antibodies, we cotransfected IPEC-J2 cells with recombinant plasmids encoding viperin and N, Nsp13 or Nsp16 and carried out the immunofluorescence experiments again. The results showed that there was significant co-localization between viperin and N, but no co-localization between viperin and Nsp13 or Nsp16 (Fig. 5b). A co-immunoprecipitation assay further demonstrated that viperin interacted with N (Fig. 5c) but not Nsp13 or Nsp16 (Fig. 5d). These experiments showed that porcine viperin interacts with the N protein, but not with the non-structural proteins Nsp13 and Nsp16.

### Localization of truncated porcine viperin in IPEC-J2 cells and its interaction with the PEDV N protein

Studies have shown that the N-terminal alpha-helical structure of human viperin protein helps to anchor it to the endoplasmic reticulum. In order to explore the specific mechanism of interaction between viperin and the PEDV N

**Fig. 3** Viperin inhibits the proliferation of PEDV in IPEC-J2 cells. (a) Verification of intracellular viperin protein expression with beta-actin as an internal reference. (b) Virus proliferation measured by TCID<sub>50</sub> after transfection of IPEC-J2 cells with a viperin overexpression plasmid and infection with PEDV 24 h later. (c) Expression of viperin, detected by RT-qPCR after transfection of IPEC-J2 cells with an siRNA targeting viperin. (d) Virus proliferation measured by TCID<sub>50</sub> assay in IPEC-J2 cells infected with PEDV 24 h after transfection with an siRNA targeting viperin

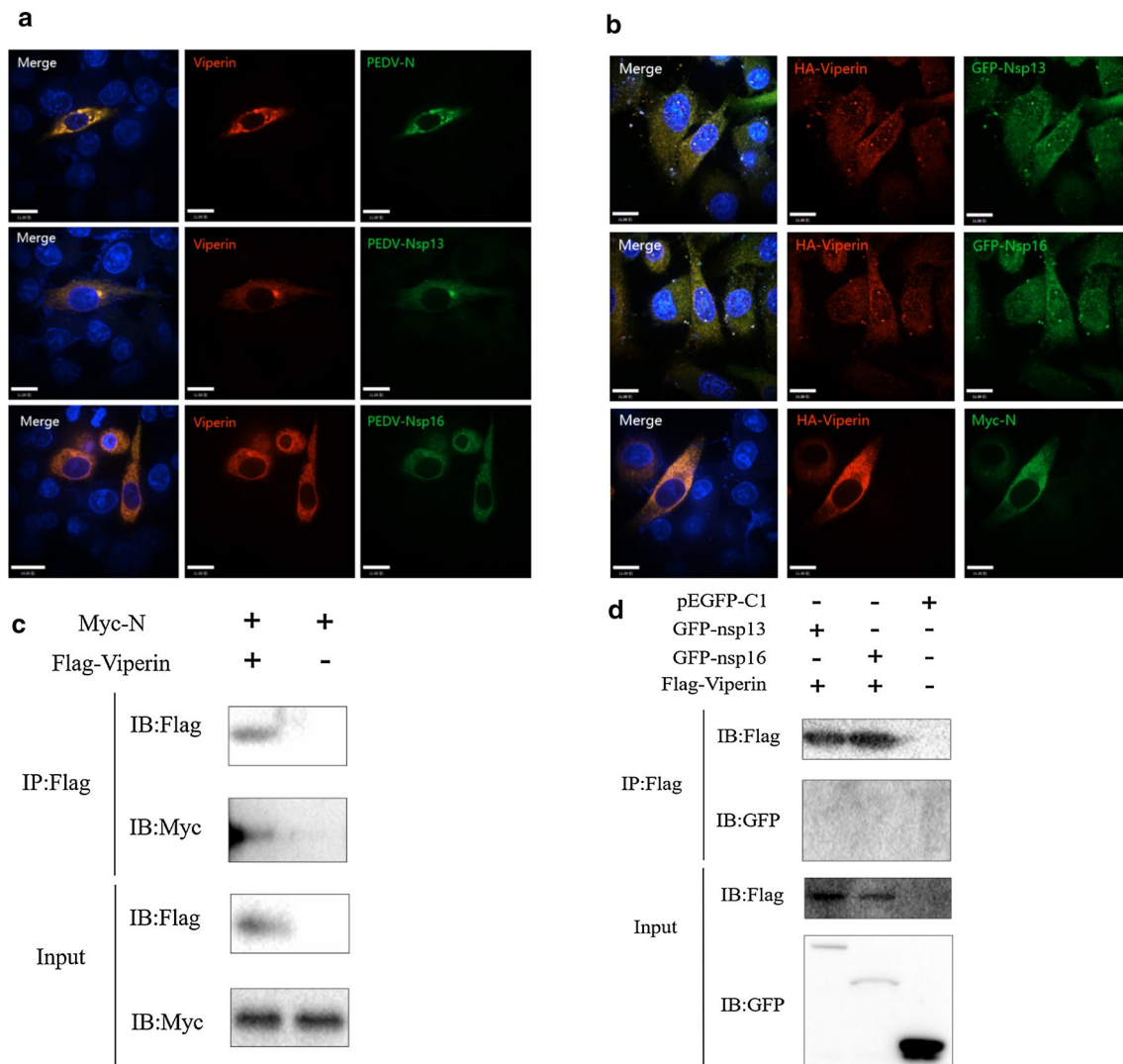


**Fig. 4** Subcellular localization of porcine viperin in IPEC-J2 cells. (a) Distribution of viperin in the cytosol (bar: 11 μm) (b) Subcellular localization of porcine viperin in IPEC-J2 cells (bar: 11 μm)

protein, the domains of viperin was analyzed and a truncated form of the gene was constructed. Viperin is approximately 42 kDa in size and can be divided into three distinct domains

[12], an N-terminal alpha-helical domain, a C-terminal conserved domain, and a middle S-adenosyl methionine domain (SAM domain) (Fig. 6a). We made three truncated forms





**Fig. 5** Interaction between porcine viperin and the PEDV N protein. (a) Co-localization of viperin and N after infection of IPEC-J2 cells with PEDV (bar: 11  $\mu$ m). (b) Co-localization of viperin and N (bar: 11  $\mu$ m) detected by confocal microscopy after transfection of IPEC-J2 cells with viperin and PEDV N overexpression plasmids. (c)

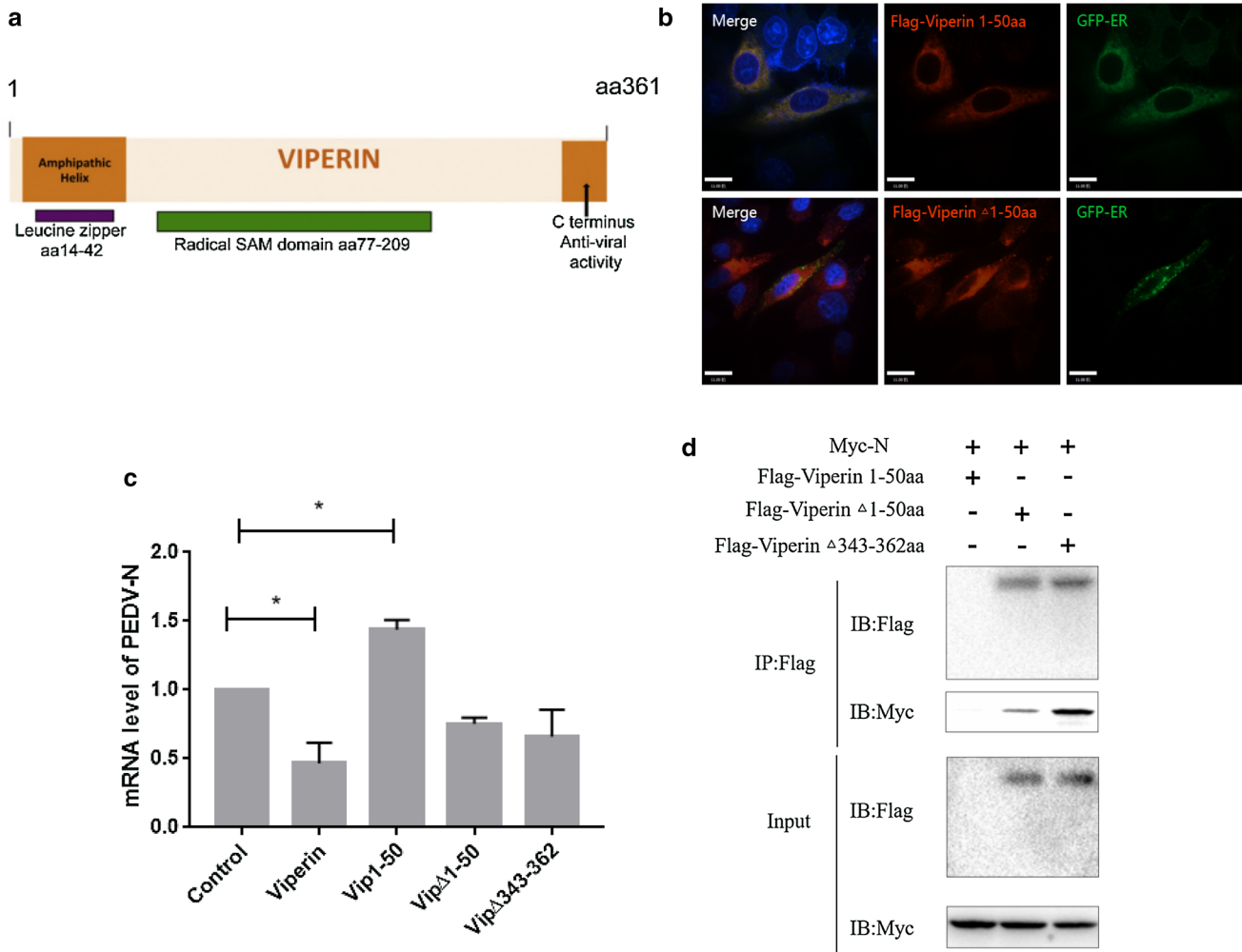
Interaction between viperin and N detected by immunoprecipitation after transfection of IPEC-J2 cells with overexpression plasmids for viperin and PEDV N. (d) IPEC-J2 cells transfected with viperin and PEDV N overexpression plasmids. No interaction between viperin and Nsp13 or, Nsp16 was detected by immunoprecipitation

of the viperin gene, Vip1-50, Vip $\Delta$ 1-50, and Vip $\Delta$ 343-362. These truncated genes were overexpressed in IPEC-J2 cells, which were then infected with PEDV. The transcription level of N was measured by fluorescence quantification (Fig. 6b). As described previously, viperin inhibited the proliferation of PEDV. This inhibition was not observed with the N-terminal fragment Vip1-50 alone, but inhibition was still observed with the deletion mutants Vip $\Delta$ 1-50, and Vip $\Delta$ 343-362 (Fig. 6c), suggesting that the interaction region should be the middle part, and this was confirmed by immunoprecipitation (Fig. 6d). These data indicated that aa 1-50 of viperin play an important role in its anchoring to the endoplasmic reticulum, while the middle SAM domain of viperin The specific region interacts with N protein.

## Discussion

This study showed that the expression of type I interferon increases and that of viperin is also upregulated in IPEC-J2 cells infected with PEDV. Overexpression of viperin and interference with its expression showed that viperin inhibits PEDV proliferation. Porcine viperin was found to be localized in the endoplasmic reticulum, and aa 1-50 of this protein were found to be indispensable for its ability to anchor itself to the endoplasmic reticulum. Furthermore, there was colocalization and interaction between porcine viperin and the structural N protein of PEDV. The S-adenosyl methionine domain in the middle of the viperin protein was found to be the key region for its interaction with the N protein.





**Fig. 6** Distribution of truncated porcine viperin in cells and its interaction with viral proteins. (a) Complete viperin protein. (b) Localization of truncated viperin in cells (bar: 11µm). (c) Effects of different truncations on PEC-J2 cells transfected with an overexpression plasmid containing a truncated viperin gene and then infected with

PEDV. PEDV N gene transcription was measured by RT-qPCR. (d). Interaction between truncated viperin and the PEDV N protein in IPEC-J2 cells transfected with constructs with a truncated viperin gene and PEDV N overexpression plasmids, detected by immunoprecipitation

Viperin may interfere with the assembly and proliferation of the virus by interacting with the N protein, thereby exerting an antiviral effect.

Viperin is a broad-spectrum antiviral protein that can inhibit the proliferation of multiple viruses [17]. Previous studies have shown that human viperin relies on its N-terminal amphipathic alpha-helical domain to anchor itself to the endoplasmic reticulum membrane, with its C-terminus protruding into the cytoplasm. Overexpression of viperin results in the formation of homodimers that crystallize the endoplasmic reticulum and destroy its structure [14]. Viperin can also be localized on lipid droplets [1]. It is noteworthy that lipid droplets have been shown to be sites of replication of many viruses, such as hepatitis C virus (HCV) and dengue virus (DENV). The amphiphilic alpha-helical domain at the N-terminus of viperin is also necessary for its localization

to lipid droplets [39]. Consistent with previous studies, we demonstrated that porcine viperin was also localized in the endoplasmic reticulum.

After entering the host cell by membrane fusion, PEDV releases its positive-strand RNA genome in the cytoplasm [25]. The viral polymerase uses the positive-strand genomic RNA as a template to generate negative-strand RNA, which in turn is used as a template for synthesis of subgenomic RNA and subgenomic mRNA of different lengths by the polymerase. mRNAs of different lengths are translated to produce viral proteins of different sizes [43]. The newly synthesized structural N protein forms a nucleocapsid by binding to the genomic RNA in a helical pattern [24]. The nucleocapsid then binds to the E and M proteins and to the modified S protein to form a complete mature virus particle in the endoplasmic reticulum-Golgi

intermediate compartment (ERGIC), which is then released by exocytosis [3]. Our experiments showed that viperin can inhibit the proliferation of PEDV. In order to investigate the specific mechanism of this inhibition, we analyzed interactions between viperin and different viral proteins of PEDV. The experiments showed that viperin colocalized and interacted with the N protein, but there was no obvious co-localization or interaction with the Nsp13 or Nsp16 protein.

Viperin differs slightly among different species although it is highly conserved. In porcine viperin, the N-terminal amphipathic alpha-helical domain consists of approximately aa 1-50. The highly conserved C-terminal domain consists of approximately aa 340-361. The middle part consists of aa 71 to 182 in which there are four relatively conserved modules (M1, M2, M3, M4). The M1 module contains a conserved CxxxCxxC sequence, a feature of enzymes that use *S*-adenosyl methionine as a cofactor that binds to iron-sulfur clusters [2, 7]. Viperin decreases flavivirus virulence by promoting the secretion of unproductive noninfectious virus particles via a GBF1-dependent mechanism [33]. The iron-sulfur clusters are also important for its antiviral function. In order to identify the specific site of interaction between viperin and N, we constructed truncation forms of viperin. Co-immunoprecipitation assays showed that the loss of the N-terminus and C-terminus did not affect the interaction between viperin and the N protein, indicating that the key residues responsible for interaction between viperin and N are in the middle region of the *S*-adenosyl methionine domain.

In conclusion, this study showed that viperin regulates the proliferation of PEDV, and our results suggest a possible role of viperin in PEDV infection, and its antiviral activity was confirmed. This provides new ideas for research on the natural immune response to PEDV infection and strategies for prevention of PED. However, the molecular mechanism by which viperin is upregulated by PEDV infection remains unclear, and still needs to be investigated whether viperin plays other roles in PEDV infection.

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**Author contributions** Conceived and designed the experiments: JHH. Performed the experiments: JQW, YLF, HC, APC, DPH, LLZ and JXS. Analyzed the data: YLF, JQW and JHH. Contributed reagents/materials/analysis tools: JHH Wrote the paper: JQW, and JHH.

## Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

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