Porcine circovirus type 2 ORF4 protein binds heavy chain ferritin

QIZHUANG LV, KANGKANG GUO, TAO WANG, CHENGCHENG ZHANG and YANMING ZHANG*
College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China

**Corresponding author (Fax, +86 2987091032; Email, zhangym@nwsuaf.edu.cn)*

Porcine circovirus type 2 (PCV2) is the primary infectious agent of PCV-associated disease (PCVAD) in swine. ORF4 protein is a newly identified viral protein of PCV2 and is involved in virus-induced apoptosis. However, the molecular mechanisms of ORF4 protein regulation of apoptosis remain unclear, especially given there is no information regarding any cellular partners of the ORF4 protein. Here, we have utilized the yeast two-hybrid assay and identified four host proteins (FHC, SNRPN, COX8A and Lamin C) interacting with the ORF4 protein. Specially, FHC was chosen for further characterization due to its important role in apoptosis. GST pull-down, subcellular co-location and co-immunoprecipitation assays confirmed that the PCV2 ORF4 protein indeed interacted with the heavy-chain ferritin, which is an interesting clue that will allow us to determine the role of the ORF4 protein in apoptosis.

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1. Introduction

Porcine circovirus (PCV), as a member of the Circovirus genus within the *Circoviridae* family (Pringle 1999), is classified into porcine circovirus type 1 (PCV1) and type 2 (PCV2). Both are small, spherical, non-enveloped viruses with a single-stranded (ss), ambisense and closed circular DNA genome of 1759 bp in the case of PCV1 and 1766–1768 bp for PCV2 (Finsterbusch & Mankertz 2009; Lv *et al*. 2014). PCV1 is non-pathogenic (Tischer *et al*. 1995), while PCV2 is an important porcine pathogen causing severe economic losses to the swine production worldwide (Clark 1997; Allan *et al*. 1998; Segales *et al*. 2005; Ge *et al*. 2012).

The overall DNA sequence identity between PCV1 and PCV2 isolates is only approximately 68% to 76% (Hamel *et al*. 1998; Cheung 2003a), whereas the genomic organization of the two genotypes of PCV is similar. The PCV2 genome contains 11 predicted overlapping open reading frames (ORFs) (Hamel *et al*. 1998), of which four have been characterized in detail. ORF1 encodes the two replicases Rep and Rep', which are indispensable for viral replication (Mankertz & Hillenbrand 2001; Mankertz *et al*. 2003; Cheung 2003b). ORF2 encodes the capsid protein Cap, which is the major structural protein and the dominant immunogenic agent of PCV2 (Nawagitgul *et al*. 2002). ORF3 encodes an apoptotic protein, which is not essential for viral replication in cultured cells but is involved in the virulence and spread of the virus (Liu *et al*. 2005; Karuppannan, *et al*. 2009; Karuppannan & Kwang 2011). The newly characterized ORF4 protein, which appears to be implicated in inhibiting apoptosis, is likely also not essential for PCV2 replication (He *et al*. 2013; Gao *et al*. 2014). Previous studies have identified numerous porcine proteins that interact with the viral protein Rep/Rep' (ORF1), Cap (ORF2) and the ORF3 protein using a yeast or bacteria-based two-hybrid assay (Timmusk *et al*. 2006; Liu *et al*. 2007; Finsterbusch *et al*. 2009), although the details of interactions between these viral and cellular proteins remain poorly understood. However, there are no data concerning any host binding partners for the ORF4 protein, and the intracellular regulatory mechanism of the ORF4 protein in apoptosis is still unknown.

Due to PCV2, the ORF4 protein can enter the nucleus of infected PK15A cells (Gao *et al.* 2014), which is a requirement for the yeast two-hybrid model (Gladue *et al*. 2014). We attempted to screen for cellular partners

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of ORF4 protein by means of the yeast two-hybrid approach using the Matchmaker Gal4 Two-Hybrid system 3, with the expectation of advancing the current understanding of the functions of the ORF4 protein in the biology of PCV2 infection. Our results indicated that PCV2 ORF4 protein interacted with four different porcine proteins. The possible biological significance of the identified porcine proteins was discussed. Particllarly, the physical interaction between ORF4 and FHC was verified by GST pull-down, subcellular co-location and co-immunoprecipitation assays. Development of this work may significantly improve the understanding of the roles of the ORF4 protein during PCV2 infection.

2. Materials and methods

2.1 *Library construction and generation of bait plasmid for yeast two-hybrid screening*

The porcine alveolar macrophages (PAMs) of a healthy, 2-week-old Landrace piglet obtained from a herd in the Shaanxi province of China, which tested negative for both PCV1 and PCV2, were used to generate a cDNA library for yeast two-hybrid screening. In brief, PAMs were collected from the lung of the piglet by bronchoalveolar lavage as described previously (Wensvoort et al. 1991). Total RNA was extracted from adherent cells using the Trizol RNA extract reagent (Invitrogen, Carlsbad, CA). After treatment with gDNA Eraser (TAKARA Biotechnology), first-strand cDNA was synthesized using the SMART system (Clontech) with a CDS III Primer. Subsequently, amplification of double-strand cDNA (dsDNA) was performed using long-distance PCR (LD-PCR). After purification of dsDNA using BD CHROMA SPIN-400 Columns, yeast strain Y187 (Clontech) was cotransformed with dsDNA and pGADT7-Rec and selected on SD/-Leu plates. Finally, transformants were harvested for subsequent screening by yeast mating. The constructed porcine cDNA library contained approximately 2×10^6 independent clones, and inserts were found in 95% of the tested colonies. All of the handling procedures of Matchmaker^{TM} Library Construction & Screening Kit (Clontech) were performed according to the manufacturer's instructions.

For generation of bait plasmid, the ORF4 gene of PCV2 was PCR-amplified from the DNA purified from PCV2 Yangling strain (Tang *et al*. 2011) using a primer pair (forward primer 5′-CGGAATTCATGACGTGTACATTAGTCTTCC-3′ and reverse primer 5′-CGGGATCCGTCAGGGACAACGGAGTG-3′) and subcloned into the *Eco*RI/*Bam*HI sites of the GAL4 DNA binding fusion vector pGBKT7. The obtained plasmid was named pGBKT7-ORF4 and confirmed by restriction analysis and sequencing.

2.2 *Yeast two-hybrid screen*

For yeast two-hybrid screening, the Matchmaker GAL4 Two-Hybrid system 3 (Clontech) was used as described elsewhere with slight modifications (Finsterbusch *et al*. 2009). Plasmids pGBKT7-53, pGBKT7-Lam and pGADT7-RecT were used as controls. Briefly, the yeast strain Y2H (Clontech) was first transformed with the bait plasmid pGBKT7-ORF4 and selected on SD/-Trp plates. After elimination of toxicity and autoactivation, the harvest positive colonies were then used to mate with the preselected positive Y187 transformants and selected on double dropout (DDO) plates lacking leucine and tryptophan. Successful mating colonies (~21.7%) were subsequently cultivated on quadruple dropout (QDO) plates lacking adenine, histidine, tryptophan and leucine for one week. Positive yeast colonies growing on QDO plates were restreaked on quadruple drop out plus X-α-Gal (QDO/X) plates to test α-galactosidase expression. After repeated inoculation on QDO/X plates three times, blue colonies were then grown in DDO liquid medium lacking tryptophan and leucine for 48 h at 250 rpm and 30°C. Plasmid DNA was isolated from yeast cultures using the Yeast Plasmid Mini Preparation Kit (Beyotime) and transformed into *Escherichia coli* DH5α cells. Positive clones growing on the Luria–Bertani (LB) agar plates were then grown in LB broth with ampicillin, the DNA was sequenced, and the identity of the interacting target was determined from databases using the NCBI BLASTP program. Results were confirmed by co-transformation of the respective bait and prey plasmids into the yeast strain Y2H and tested for the presence of growth on DDO and QDO/X plates. In addition, each putative interaction partner was tested for autoactivation to exclude its potential intrinsic DNA binding activity.

2.3 *Expression of recombinant proteins*

To obtain GST-ORF4 fusion proteins, the entire coding region of PCV2 ORF4 was inserted into the *Eco*RI/*Xho*I sites of the plasmid pGEX-6P-1 using the following primers: forward, 5′-CGGAATTCATGACGTGTACATTAGTCTTCC-3′, and reverse, 5′-CCGCTCGAGTCAGGGACAACGGAGTG-3′. After transformation into *Escherichia coli* BL21 and being sequenced, the resulting BL21 cells were induced to express GST-ORF4 proteins by addition of IPTG following the manufacturer's guide (GE Healthcare).

For expression of the prey protein FHC, the coding sequence of the FTH1 gene was PCR-amplified from the genomic DNA of PK15 cells with a primer pair (forward primer 5′-CGGGATCCATGACGACCTCG TGCTCCTCG-3′ and reverse primer 5′-CCGCTCGAGTTACTTATC GTCGTCATCCTTGTAATCGCTCTCACTGCTCCCCAG-GGTG-3′), which adds a flag tag at the C-terminal of FHC.

After purification, the PCR product digested with *Bam*HI and *Xho*I was cloned into the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). As negative control in co-immunoprecipitation (Co-IP) assays, the pcDNA-EGFP construct, consisting of the coding sequence of EGFP from pEGFP-C1 (Clontech) in pcDNA3.1(+), was used. After confirmation by restriction analysis and DNA sequencing, the recombinant plasmids were transfected into HEK293T cells using TurboFect™ Transfection Reagent (Thermo) according to the manufacturer's guide. The HEK293T cells were cultured in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, UK) at 37°C with 5% CO_2 (Zhang *et al.* 2015).

2.4 *Pull-down experiment*

For GST pull-down assays, the Pierce® GST Protein Interaction Pull-Down Kit (Thermo) was used according to the manufacturer's instructions. In brief, GST-ORF4 proteins were extracted from IPTG-induced *E.coli* culture using Pull-Down Lysis Buffer under the protection of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (Beyotime). After immobilization of GST-ORF4 fusion proteins on the equilibrated Glutathione Agarose resin, FHC proteins prepared from HEK293T cell lysates was added. After incubation at 4°C for 2 h and five washes, protein samples were eluted with Glutathione Elution Buffer. Finally, the glutathione agarose plus fusion proteins were boiled in SDS-loading buffer and subjected to SDS-PAGE and Western blot analyses. As negative control, mock GST protein was used.

2.5 *Immunoblotting analysis*

Western blot analysis was performed as described elsewhere with some modifications (He *et al.* 2013). Briefly, protein samples were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). After blocking with 5% skim milk, the membranes were probed with indicated primary antibodies at room temperature for 2 h, followed by HRP-conjugated secondary antibodies. Finally, immunoreactive bands were visualized by chemiluminescence using an enhanced ECL western blotting analysis system (Thermo). Detection of cellular protein GAPDH served as an internal control.

2.6 *Confocal microscopy*

To examine the intracellular distribution of ORF4 and FHC proteins, ORF4 and FTH1 genes were inserted into the eukaryotic expression vector pDsRed-Monomer-C1 and pEGFP-C1 with primer pairs F-*Eco*RI 5′-CGGAATTCTATGACGTGTACATTAGTCTTCC-3′/R-*Bam*HI 5′-ATTGGATCCTCAGGGACAACGGAGTG-3′ and F-*Eco*RI 5′-CGGAATTCTATGACGACC TCGTGCTCCTCG-3′/R-*Bam*HI 5′-CGGGAT CCTTAGCTCTCACTGCTCCCCAGGGTG-3′. HEK293T cells grown on glass bottom dishes (35 mm) were simultaneously transfected with the combination of pDsRed-ORF4 and pEGFP-FTH1 plasmid. After incubation at 37 °C for 48 h, the cells were washed thrice with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 10 min and finally incubated with Hoechst 33342 for 10 min at 37°C. The images were viewed under a laser confocal microscope (Model LSM510 META, Zeiss).

2.7 *Co-immunoprecipitation assays*

To verify whether the ORF4 protein interacts with FHC inside cells, *in vitro* co-expression of PCV2 ORF4 and porcine FHC was conducted using HEK293T cells. Cells grown in 6-well plates were simultaneously transfected with a combination of 2 μg of pDsRed-ORF4 and 2 μg of pcDNA-FTH1 plasmids. As negative controls, the empty pDsRed-Monomer-C1 and pcDNA-FTH1 co-transfected cells, as well as pDsRed-ORF4 and pcDNA-EGFP co-transfected cells, were used. At 48 h post-transfection, the cells were washed twice with PBS and treated with lysis buffer (containing PMSF) for 30 min on ice. After centrifugation, the supernatants were subjected to immunoprecipitation using ANTI-FLAG® M2 Affinity Gel (Sigma) according to the manufacturer's instructions. In brief, the ANTI-FLAG M2 affinity resin was equilibrated by centrifugation and rinses with TBS (50mM Tris HCl, with 150mM NaCl, PH 7.4). After the addition of cell lysates at 4°C overnight with gentle rocking motion on a rotating platform, the resin was washed thrice with TBS and resuspended with 2×SDS sample buffer and then subjected to SDS-PAGE and immunoblot assays. For reverse IP, the supernatants were subjected to IP with rabbit anti-DsRed antiserum for 2 h at 4°C. After the addition of 30 μl of protein G Sepharose (GE healthcare) at 4°C for 2 h, the immuno complex was washed three times with ice-cold lysis buffer and subjected to SDS-PAGE followed by Western blot analysis.

2.8 *Other reagents*

Mouse anti-Flag, anti-GAPDH monoclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody were purchased from Sigma. Rabbit anti-GST antisera, in conjunction with HRP-conjugated goat anti-rabbit antiserum, were purchased from Santa Cruz. Rabbit anti-DsRed polyclonal antibody was purchased from Clontech.

3. Results

3.1 *The PCV2 ORF4 protein interacts with FHC, SNRPN, COX8A and Lamin C*

To identify cellular partners of the PCV2 ORF4 protein, we screened a PAM cDNA library using the yeast two-hybrid approach. As a bait protein may possess intrinsic transactivating properties, autoactivation was tested before screening the library. Afterwards, the ORF4 protein was used as bait to screen approximately 2×10^6 cDNA library clones. A total number of 22 positive clones were selected, sequenced and compared to general databases using BLAST programs. To validate the interaction, the bait protein was separately co-transformed with the rescued prey plasmids encoding putative interacting partners into the yeast strain Y2H and selected on DDO and QDO/X plates. Four porcine proteins identified as interacting with the PCV2 ORF4 protein and the coding potential of each DNA sequence are listed in table 1. They are the ferritin heavy chain (FHC), the small nuclear ribonucleoprotein polypeptide N (SNRPN), the cytochrome c oxidase subunit 8A (COX8A), and the lamin C. Interactions of ORF4 and its cellular partners were reflected by growth of blue colonies on QDO/X plates (figure 1).

3.2 *Verification of the interaction between the ORF4 protein and FHC by GST pull-down assay*

To verify interactions identified by the yeast two-hybrid assay *in vitro*, GST pull-down experiments were performed with the FHC and ORF4 proteins. The constructed plasmid pGEX-6P-ORF4 was used to transform *E. coli* BL21 and induced to express GST-ORF4 proteins with IPTG. To validate the binding of the ORF4 protein to FHC, the GST-tagged fusion protein (GST-ORF4) was added to the Pierce Spin Column (Thermo) prior to incubating with Flag-tagged FHC produced by *in vitro* expression. The eluted protein samples were analyzed by SDS-PAGE and Western blot assays. As shown in figure 2, FHC was captured by GST-ORF4 rather than GST only, confirming the interaction between ORF4 and FHC suggested by the yeast mating test.

3.3 *Subcellular localization of the ORF4 protein and FHC*

Because the function of a protein will usually correlate with its intracellular localization and functional interaction between proteins may be reflected by their co-localization, the subcellular localization of ORF4 protein, in conjunction with FHC, was examined by laser confocal microscopy. As shown in figure 3A (panel a and b), EGFP-FHC expressed in HEK293T cells was located predominantly in the cytoplasm (speckle-like structures) and, to a lesser degree, in the nucleus (evenly distributed). Conversely, DsRed-Monomer-ORF4 was primarily located in the nucleus and, to a lesser degree, in the cytoplasm of the infected cells (figure 3A, panel c and d). To compare the localization of FHC with and without ORF4 expression, co-localization and reallocation of the proteins was investigated after co-transfection of the plasmids pDsRed-ORF4 and pEGFP-FTH1. As shown in figure 3B (panel e to h), when co-expressed with the ORF4 protein, FHC co-localized clearly with ORF4, and the original localization pattern of FHC was slightly altered, as green fluorescent signal was observed only in the cytoplasm but not in the nucleus. In contrast, both EGFP and DsRed-Monomer proteins were distributed evenly throughout the whole cell (figure 3B, panel a to d). These data suggested that the ORF4 protein and FHC were spatially accessible.

Table 1. Identified cDNA clones coding for PCV2 ORF4 interacting proteins.

Number of cDNA clones found	Description of cDNA clones and best hits of homolog proteins identified in databases
3	Partial cDNA encoding aa 1-177 of porcine ferritin heavy chain, (*Sus scrofa* NP_999140.1, E value=2e−117; *Homo sapiens* AAI05803.1, E value=1e−118)
1	Partial cDNA encoding aa 1-196 of porcine small nuclear ribonucleoprotein polypeptide N, (*Sus scrofa* NP_001230628.1, E value=8e−71; *Homo sapiens* NP_003082.1, E value=3e−70)
2	Complete cDNA encoding aa 1-69 of porcine cytochrome c oxidase subunit 8A, mitochondrial-like isoform 1, (*Sus scrofa* XP_003122631.1, E value=2e−41; *Homo sapiens* NP_004065.1, E value=5e−26)
4	Partial cDNA encoding aa 4-218 of porcine lamin C, (*Sus scrofa* AAY44742.1, E value=3e−115; *Homo sapiens* NP_005563.1, E value=4e−152)

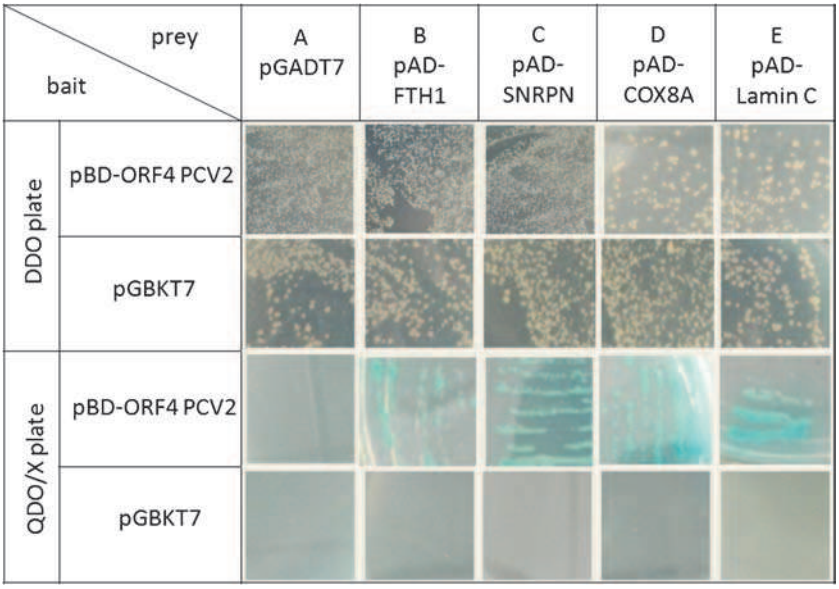

Figure 1. Yeast two-hybrid assay with PCV2 ORF4 protein and host proteins. Gold yeast strain Y2H transformed with pGBKT7-based bait and yeast strain Y187 transformed with pGADT7-based prey plasmids mate with each other and were selected for the presence of both plasmids on SD/-Leu/-Trp plates. Interaction of the ORF4 protein with the four cellular proteins FHC, SNRPN, COX8A and Lamin C is indicated by growth of blue colonies on SD/-Leu/-Trp/-His/-Ade/ X-α-Gal plates.

3.4 *Co-immunoprecipitation of the ORF4 protein with FHC*

To substantiate the interaction between the ORF4 protein and FHC within cells and lend further support to the validation of the yeast two-hybrid method, Co-IP was applied as described. Expression of Flag-tagged FHC and EGFP was detected by immunoblotting with a mouse anti-Flag monoclonal antibody (figure 4C, lower panel) and the DsRed-Monomer-tagged ORF4 and DsRed-Monomer with a rabbit anti-DsRed polyclonal antibody (figure 4C, upper panel). Complex formation between DsRed-Monomer-ORF4 and FHC was demonstrated by simultaneously detection of precipitation of cell lysates with both a mouse anti-Flag monoclonal antibody and a rabbit anti-DsRed polyclonal antibody. As shown in figure 4A, Flag-tagged FHC (FHC-Flag) was co-precipitated with the DsRed-Monomer-ORF4, while no signal was observed with DsRed-Monomer instead of DsRed-Monomer-ORF4. Similarly, Flag-tagged EGFP (EGFP-Flag) did not precipitate the DsRed-Monomer-ORF4. Reciprocal co-IP experiments also showed that the DsRed-Monomer-ORF4 precipitated FHC-Flag but not EGFP-Flag, whilst the DsRed-Monomer did not precipitate FHC-Flag (figure 4B). These findings provided strong evidence for the physical binding of FHC with the ORF4 protein.

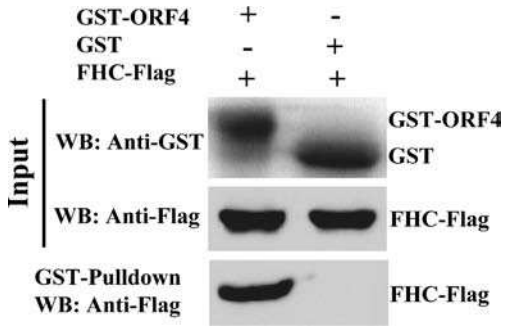

Figure 2. GST pull-down assay results. Glutathione beads conjugated to GST or GST-ORF4 fusion protein were incubated with Flag-tagged FHC protein. Proteins eluted from the beads were analyzed directly by SDS-PAGE and Western blot. The expression of input proteins (Flag-tagged FHC, GST and GST-ORF4 protein) was confirmed by immunoblotting using mouse anti-Flag monoclonal antibody and rabbit anti-GST polyclonal antibody, respectively. For each lane, the sampling amount of input proteins was 20 μl (~5% of total).

4. Discussion

Currently, the pathogenesis of PCV2 is still largely unknown, especially concerning its communication with the host and induction of disease. As a newly identified viral protein of PCV2, the ORF4 protein aroused our interest and inspired us to explore something deeper in this field. To obtain insight into the underlying mechanism of the ORF4 protein in regulating apoptosis and

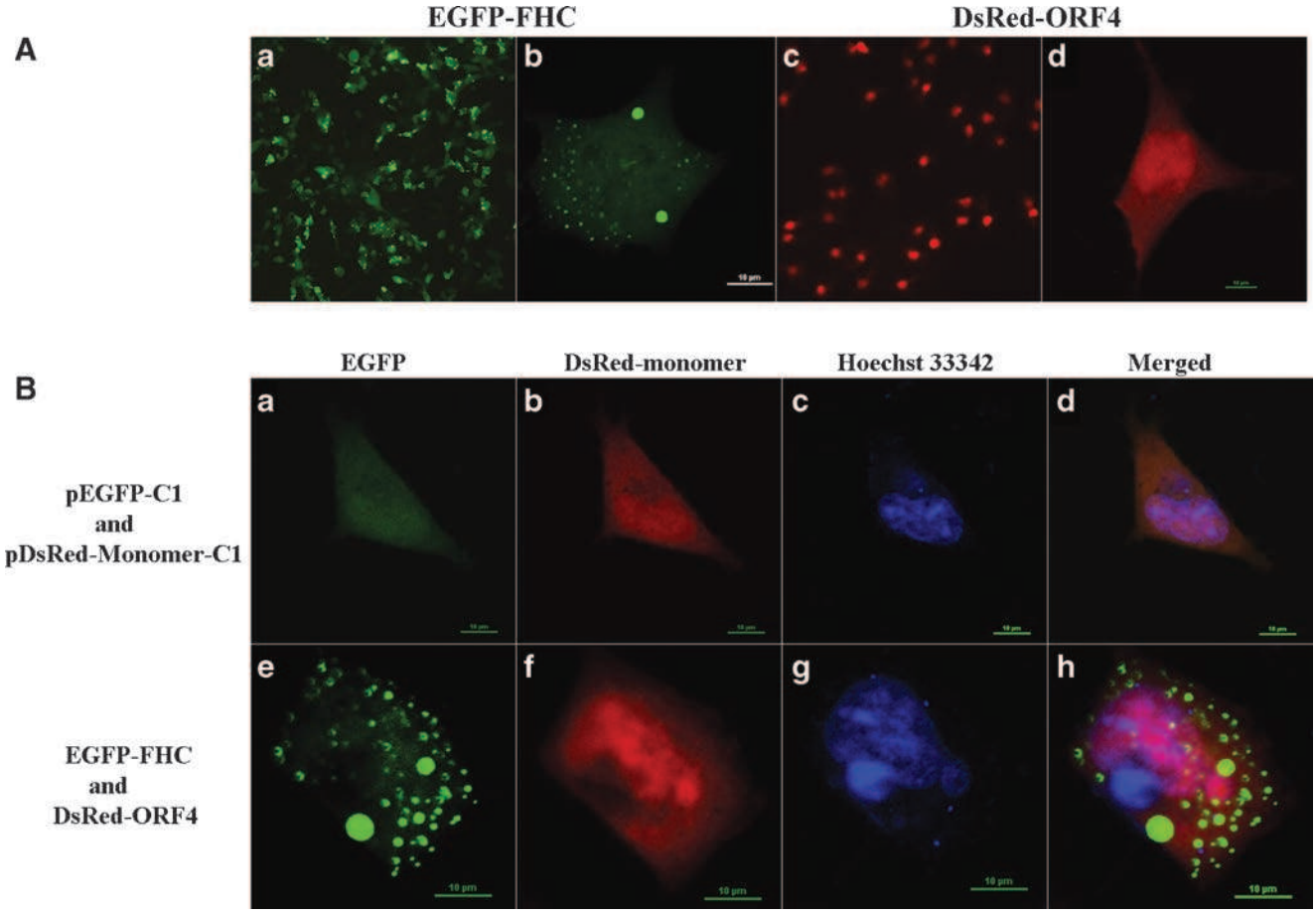

Figure 3. Intracellular distribution of DsRed-Monomer-ORF4 and EGFP-FHC in HEK293T cells. HEK293T cells grown on glass bottom dishes were co-transfected with pDsRed-ORF4 in combination with pEGFP-FTH1. Cells transfected with only one plasmid and cells co-transfected with pDsRed-Monomer-C1 and pEGFP-C1 were used as controls. At 48 h post transfection, cells were fixed and photographed. (**A**) EGFP-FHC expressed alone (panel a, green, 100×; panel b, enlarged inset from panel a, bar = 10 μm) and DsRed-ORF4 (panel c, red, 100×; panel d, enlarged inset from panel c, bar = 10 μm) were detected by fluorescence microscopy. (**B**) After co-transfection, all the cells were stained with Hoechst 33342, and each fluorescent label was recorded in independent scans. Merged images are shown in panel d and h, which showed overlap of the DsRed-monomer-ORF4 and EGFP-FHC in the cytoplasm. Images were taken by confocal laserscanning microscopy. Bar = 10 μm for all the figures.

further discern the virus host interplay, we have used a yeast mating test to screen for porcine host proteins that interact with the PCV2 ORF4 protein. Visibly, four porcine protein fragments or full-length proteins were identified as cellular interacting partners for the ORF4 protein as presented in figure 1. To corroborate the interactions identified by the yeast two-hybrid approach, FHC was chosen for further identification using GST pull-down, subcellular co-location and co-immunoprecipitation assays. Results confirmed that the PCV2 ORF4 protein could bind with FHC inside and outside cells, which further demonstrated the validity of the yeast two-hybrid assay. Even so, due to intrinsic limitations of the yeast two-hybrid system, the identified host binding partners for the ORF4 protein are not exhaustive, and any definitive conclusion regarding the virus life cycle could not be drawn unless each interaction identified is confirmed by independent experiments.

Despite each identified partner being annotated with multifunctionality in the literature, a convincing common ontology has yet to appear. Hence, the potential roles of each partner regarding PCV2 infection are discussed below. SNRPN encoded by the *SNRPN* gene is one polypeptide of a small nuclear ribonucleoprotein complex and belongs to the snRNP SMB/SMN family (Schmauss *et al*. 1992). It has been suggested that the protein plays a role in pre-mRNA splicing, possibly tissue-specific alternative RNA processing events (Ozgelik *et al*. 1992). In a previous report, weak interactions between the ORF1 associated Rep protein and

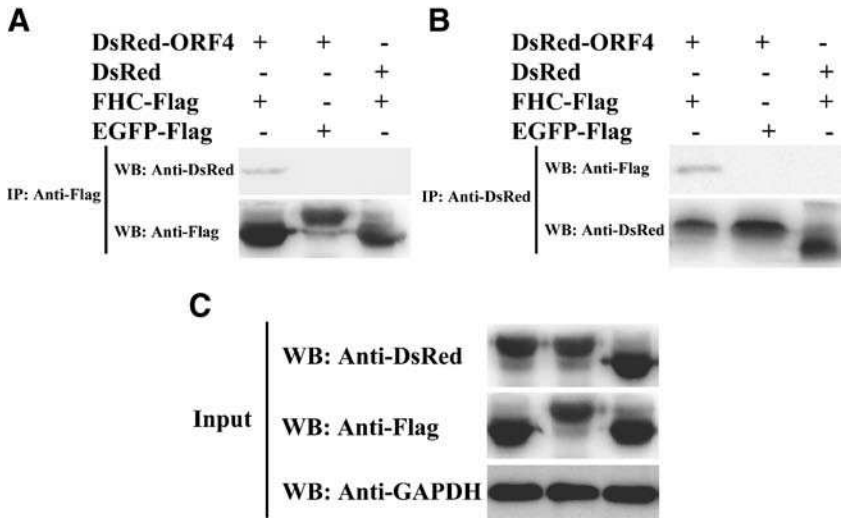

Figure 4. Co-immunoprecipitation analysis of ORF4 and FHC in HEK293T cells. Cells were co-transfected with plasmids expressing DsRed-Monomer-tagged ORF4 and plasmids expressing Flag-tagged FHC. DsRed-Monomer and Flag-tagged EGFP were used as negative controls. (**A**) Extracts were subjected to IP using ANTI- FLAG® M2 Affinity Gel and the eluate were separated by SDS-PAGE, blotted and detected with mouse anti-Flag and rabbit anti-DsRed antibodies. (**B**) Extracts were precipitated with rabbit anti-DsRed antiserum and protein G Sepharose followed by immunoblotting with mouse anti-Flag and rabbit anti-DsRed antibodies. (**C**) Expression control of cell lysates was conducted by immunoblotting using mouse anti-Flag and rabbit anti-DsRed antibodies. Detection of GAPDH with a mouse anti-GAPDH antibody served as an internal control.

the ORF4 protein were also observed (Timmusk *et al*. 2006), which may be correlated with the higher expression level of ORF1 mRNA in ORF4-deficient PCV2 mutants (Gao *et al*. 2014). More coincidentally, Rep was believed to be the primary transcript that gives rise to Rep', Rep3a, Rep3b, and Rep3c by alternate splicing (Cheung 2003c). We therefore attempt to deduce that there may be a complex relationship among SNRPN, ORF4 and ORF1 in co-regulating RNA splicing during PCV2 replication. COX8A, found only in eukaryotes, is the smallest nuclear-encoded COX subunit, which is the terminal enzyme of the mitochondrial respiratory chain and may be involved in the modulation of the catalytic function of the three mitochondrially encoded subunits (Grossman and Lomax 1997; Huttemann *et al*. 2003). In consideration of the pivotal role of COX in aerobic metabolism, we were concerned whether PCV2 disturbs mitochondrial function via the ORF4 protein by interacting with COX8A. Lamin C, one of the major proteins encoded by the *LMNA* gene and produced by alternative splicing, has been described as an intermediate filament protein that serves several functions including stabilization of the nuclear membrane, regulation of gene expression, and cell cycle control (Pasotti *et al*. 2004; Ivorra *et al*. 2006; Sliwinska 2007). Moreover, viruses often transport viral proteins or particles to defined subcellular locations using cytoskeletal filaments (Sodeik *et al.* 1997; Smith & Enquist 2002; Suikkanen *et al.* 2003). Interaction of the ORF4 protein with lamin C could perhaps affect PCV2 replication on multiple levels.

FHC, the only one subunit of ferritin that has ferroxidase activity, has been suggested to prevent cells from undergoing apoptosis induced by a variety of stimuli (Brouard *et al*. 2000; Berberat *et al*. 2003). The anti-apoptotic function of FHC is linked to its ability to suppress reactive oxygen species (ROS) accumulation and is achieved through iron sequestration (Pham *et al*. 2004). Interestingly, there was a time-dependent increase in ROS following PCV2 infection (Chen *et al*. 2012). Because ORF4 has been described as an anti-apoptotic protein, the interaction between the ORF4 protein and FHC presented here will draw us a new outline of the molecular mechanisms of PCV2 ORF4 protein regulation of apoptosis. This will be important for future studies to enrich our knowledge about the virus host interplay during PCV2 infection.

In conclusion, we have identified four porcine proteins interacting with PCV2 ORF4 protein, which can be associated with distinct aspects of viral lifecycle. In particular, we isolated FHC as a cellular partner of ORF4 protein, which highlighted a good start point to pursue the possible mechanism through which ORF4 protein exerts an anti-apoptotic effect. Additionally, further investigation is needed to address the question what are the domains responsible for the interaction and how these interactions impact on PCV2 infection.

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