



GTPase-activating protein-binding protein 1 (G3BP1) plays an antiviral role against porcine epidemic diarrhea virus

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ABSTRACT

Porcine epidemic diarrhoea virus (PEDV) is a single-stranded, positive-sense RNA virus that belongs to the *Coronaviridae*. PEDV causes severe diarrhoea and dehydration in nursing piglets, which leads to significant economic losses to the swine industry worldwide. Stress granules (SGs) are sites of mRNA storage that are formed under various stress conditions including viral infections. Increasing evidence suggests that SGs function in antiviral innate immunity of host cells to limit virus replication. Ras-GTPase-activating protein (SH3 domain) binding protein 1 (G3BP1) is a key stress granule-resident protein that nucleates stress granule assembly. Depletion of G3BP1 inhibits SGs formation and overexpression of G3BP1 nucleates SGs assembly. We observed that knockdown of G3BP1 by silencing RNA significantly increased PEDV replication. Overexpression of exogenous G3BP1, on the other hand, lowered virus replication by 100-fold compared to vector control. An increase in the levels of mRNAs of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) was also observed in PEDV-infected G3BP1 depleted cells compared to PEDV-infected control cells. Taken together, our results suggest that G3BP1 plays an antiviral role and impairs PEDV replication.

1. Introduction

PEDV is a positive-sense, single-stranded RNA virus in the family of *Coronaviridae*. PEDV causes acute diarrhea, vomiting, dehydration and high mortality in nursing piglets (Jung et al., 2015; Stevenson et al., 2013). PEDV infection is one of the most devastating emerging viral diseases of swine in the world, leading to significant financial losses to the global pork industry (Lee, 2015).

Stress granules (SGs) are dynamic cytoplasmic foci that form in response to different stress conditions including virus infections (Kedersha and Anderson, 2002). Some virus infections cause SGs and the formation of SGs is considered as an indication of an antiviral innate response that limits translation of the viral genes (Onomoto et al., 2014). Many viruses have evolved strategies to overcome the antiviral effect of SGs by degrading or sequestering its key components such as G3BP1 or TIA-1/TIAR to prevent the formation of SGs (Emara and Brinton, 2007; Humoud et al., 2016; Le Sage et al., 2017; Nelson et al., 2016; White et al., 2007; White and Lloyd, 2011). The nonstructural protein 1 (NS1) of influenza A virus (IAV) inhibits eIF-2 α

phosphorylation mediated SGs by blocking PKR activation (Khapersky et al., 2012). Some viruses have been reported to benefit from SGs formation. For instance, respiratory syncytial virus (RSV) replication was impaired in cells with a reduced level of G3BP1 expression (Lindquist et al., 2010).

In the present study, we examined the role of G3BP1 in PEDV replication and the interaction between G3BP1 and PEDV. We observed that knockdown of G3BP1 significantly promoted viral replication and PEDV induced transcriptional activation of pro-inflammatory cytokines. Overexpression of G3BP1 in Vero-76 cells reduced virus replication.

2. Materials and methods

2.1. Cells and viruses

Vero-76 cells (ATCC[®] CRL-1587[™]), a monkey kidney cell line highly permissive for PEDV infection, were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL

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penicillin, and 100 µg/mL streptomycin sulfate in a humidified 37 °C, 5% CO₂ incubator. Cells were passaged in 6-well, 12-well, 48-well and 96-well plates at a seeding concentration of 4×10^5 , 2×10^5 , 2×10^4 and 5×10^3 cells/per well, respectively. PEDV-CO (Wang et al., 2017) stocks were prepared by infecting Vero-76 cells at a MOI of 1 in a 6-well plate in medium supplemented with 3 µg/ml of TPCK trypsin (Sigma) whereas the stock of recombinant icPEDV-ΔORF3-EGFP virus was prepared by infecting Vero-76 cells at a MOI of 0.05 in a T75 cell culture flask in medium supplemented with 10 µg/ml of trypsin (Invitrogen). The icPEDV-dORF3-EGFP was generated by replacing the PEDV ORF3 gene with an EGFP gene, using the infectious clone icPC22A (Hou et al., 2017). Supernatants were harvested at 72 h post infection after more than 80% of cytopathic effects (CPE) was observed. Virus infectivity was quantified by TCID₅₀ assay. A MOI of 1 or 5 was used to infect Vero-76 cells in the experiments described below.

2.2. Knockdown of G3BP1 by silencing RNA

G3BP1-specific silencing RNA (siRNA) and control siRNA were purchased from Santa Cruz Biotechnology. Silencing RNA transfection was performed by using Lipofectamine 2000 RNAiMAX Reagent (Life Technologies) based on the manufacturer's instructions. Briefly, Vero-76 cells were seeded in a 12-well plate one day before transfection. G3BP1 specific silencing RNA and control silencing RNA were each transfected at a final concentration of 45 nM. Cells were infected with 1 MOI of PEDV-CO at 48 h after transfection. Supernatants were collected at 24 h after virus inoculation and used for virus titration using the TCID₅₀ method. Cells were harvested and used in either real-time reverse transcription (RT)-PCR to quantify viral RNA copies and inflammatory gene transcripts or Western blotting analysis to examine the expression of G3BP1, beta-actin, and PEDV-N proteins.

2.3. Immunofluorescence staining (IFA)

For virus titration, Vero-76 cells were seeded in a 96-well plate one day before addition of virus. Virus was diluted 10-fold serially and incubated for 3–5 days until cytopathic effects were observed. Then, cells were washed three times with PBS and fixed with 80% acetone for 15 min at room temperature. Cells were then incubated with FITC-labeled monoclonal antibody (SD6-29, 1:100) against PEDV N for one hour at 37 °C. Cells were washed with PBS and viewed under a fluorescence microscope.

For induction of stress granules, Vero-76 cells were treated with 0.5 mM sodium arsenite (AS) for 30 or 50 min. Cells were washed with phosphate-buffered saline (PBS) 3 times and fixed in 4% (v/v) paraformaldehyde. Cells were permeabilized in 0.2% Triton X-100 for 15 min and blocked in blocking buffer (5% bovine serum albumin and 5% goat serum in PBS) for 1 h, followed by incubation with primary antibodies and secondary antibodies. The primary antibody used was anti-G3BP1 rabbit antibody (Sigma, CA, USA, 1:200). The secondary antibody was Alexa-Fluor-546-conjugated goat anti-rabbit-IgG (R&D, MN, 1:200). PEDV was stained with a FITC-labeled monoclonal antibody against PEDV N protein (1:100). DAPI was used to visualize the nuclei of cells. Alexa-546-anti-TIA-1 antibody (Santa Cruz Biotechnology, 1:50) was used in some experiments.

2.4. Western blotting

Cell lysates were mixed with equal volumes of sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were separated by SDS-PAGE gel (10% polyacrylamide gel) and transferred to a Polyvinylidene difluoride (PVDF) membrane (Hybond P; Amersham Pharmacia). Membranes were washed with PBST (1X PBS with 5% Tween 20), blocked with 5% non-fat dry milk for an hour and stained with indicated primary antibodies at 4 °C overnight. Rabbit anti-G3BP1 antibody (Sigma, CA, USA) and mouse

anti-beta actin monoclonal antibody (Sigma, 1:5000) and mouse against PEDV-N monoclonal antibody (1:500) were used as primary antibodies. The secondary antibodies were IRDye 800 CW Goat anti-rabbit or IRDye 680 Donkey anti-rabbit IgG obtained from LI-COR (NE, USA, 1:15,000). Images were scanned and captured using LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

2.5. Real-time RT-PCR

Total RNA was extracted from harvested cells using the RNeasy mini kit (Qiagen, Valencia, CA). The concentrations of RNA were determined using a Nano Drop ND-2000 spectrometer (Thermo Scientific). Complementary DNA (cDNA) synthesis was performed with the same amount of RNA using a High Capacity cDNA Synthesis Kit (Applied Biosystems Inc.) according to the manufacturer's instructions. Real-time PCR was then performed by using the Brilliant II SYBR green QPCR master mix (Stratagene, Lajolla, CA) on a Mx3000P Real-time thermocycler (Agilent Technologies). Primer sequences used in this study are available upon request. Beta-actin was included as a housekeeping gene to normalize the gene expression level. Relative fold changes in transcript levels were quantified using the ΔΔCT method.

2.6. Statistical analysis

All data were presented as mean ± SD from three independent experiments in triplicate. Results were analyzed by Student's t-test. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Knockdown of G3BP1 expression by silencing RNA enhances PEDV replication

G3BP1 is a major component of SGs that nucleates their formation. To determine the role of G3BP1 in PEDV replication, we used silencing RNA to knockdown the expression of G3BP1. Depending on the individual experiment, we observed that silencing RNA could successfully knockdown the expression of G3BP1 by 50–80% compared to the non-target control silencing RNA. Accordingly, we observed that G3BP1 knockdown enhanced PEDV replication by 10-100-fold based on the G3BP1 knockdown efficiency. Representative results are shown in Fig. 1A and B, which showed a 50% reduction in G3BP1 expression and a 10-fold increase in virus titer compared to control silencing RNA. A 100-fold increase in virus titer was observed when 80% of G3BP1 knockdown was achieved (data not shown).

To validate the results, we used real-time RT-PCR to measure viral gene copies in control silencing RNA and G3BP1 silencing RNA transfected cells. Results showed that PEDV-N gene copies in cells transfected with G3BP1 silencing RNA are 1.5fold higher than those of the control silencing RNA (Fig. 1C). This agrees with the viral titer as determined by TCID₅₀ assay (Fig. 1B). In both experiments, an approximately 50% reduction in G3BP1 expression was observed compared to the control silencing RNA. Overall, the results suggest an anti-viral role of G3BP1 in PEDV replication.

We next examined whether G3BP1 knockdown has any impact on PEDV-induced pro-inflammatory cytokines in infected cells. Vero-76 cells were transfected with G3BP1-specific silencing RNA or control silencing RNA prior to PEDV infection. Western blotting analysis was used to determine the G3BP1 knockdown efficiency. A 50% knockdown of G3BP1 was achieved. The mRNA levels of pro-inflammatory cytokines IL-1β and TNFα were detected by real-time RT-PCR. As shown in Fig. 1D, both IL-1β and TNFα mRNA levels in the G3BP1 knockdown cells increased by 1.5-fold and 2-fold, respectively, when compared to the control silencing RNA transfected cells.

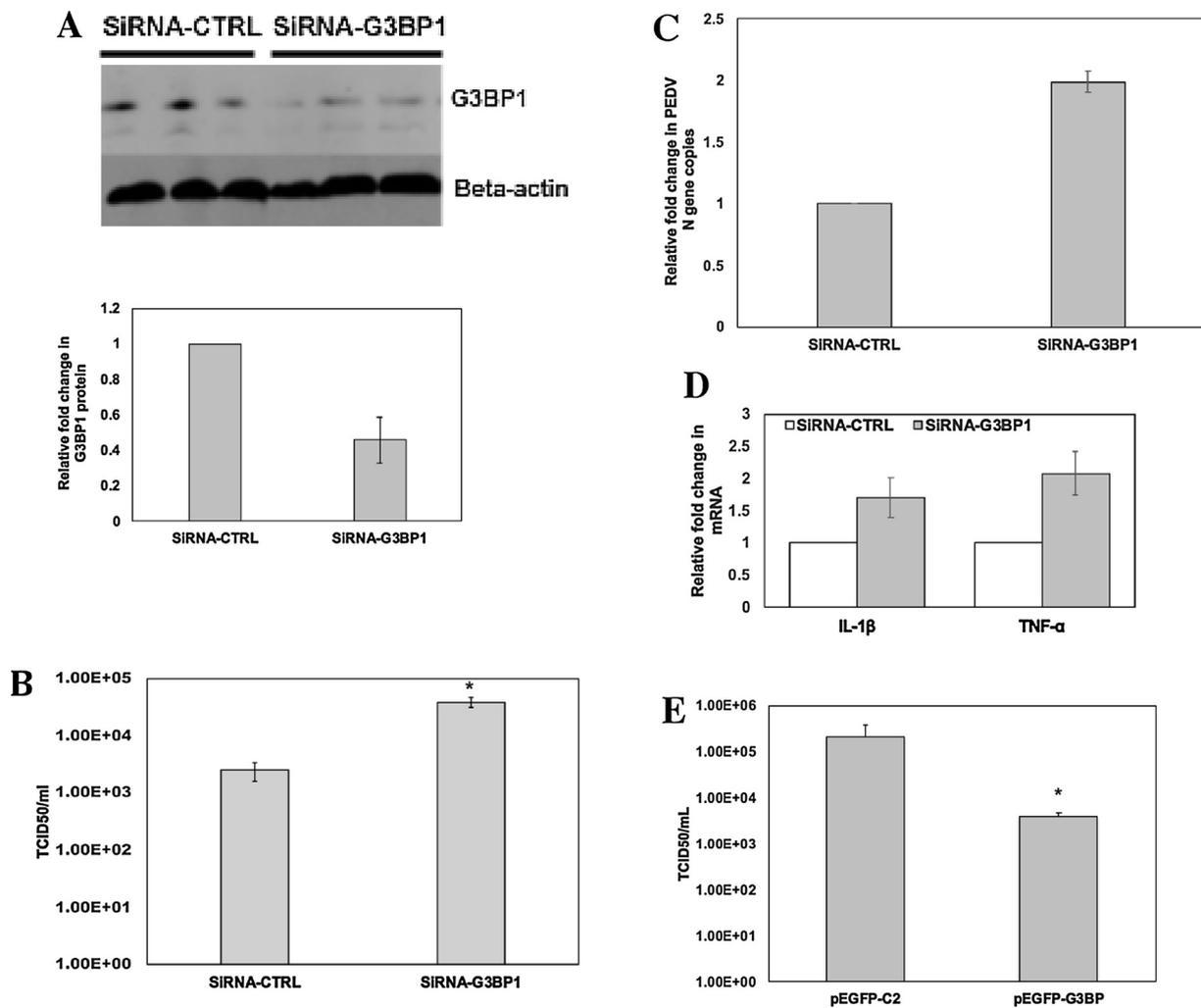


Fig. 1. Role of G3BP1 in PEDV replication. **A:** Western blotting analysis showing the successful knockdown of G3BP1 protein expression by silencing RNA. Three replicates are shown. An average of 50% knockdown efficiency was achieved. G3BP1 knockdown efficiency was calculated based on the band densities after normalization with protein loading control beta-actin. **B:** PEDV replication was enhanced in G3BP1 knockdown cells compared to control cells. * indicates $p < 0.05$. **C:** The PEDV nucleocapsid (N) gene copies were enhanced in cells with G3BP1 knockdown compared to control cells as determined by real-time RT-PCR. **D:** The mRNA levels of pro-inflammatory cytokines were increased in cells with G3BP1 knockdown after PEDV infection as determined by real-time RT-PCR. **E:** Overexpression of exogenous G3BP reduced PEDV replication. * indicates $p < 0.05$. The average and standard deviations of three experiments are shown.

3.2. Overexpression of G3BP in vero-76 cells reduces viral titer

To further confirm the role of G3BP in PEDV replication, we transfected Vero-76 cells with either pEGFP-C2 or pEGFP-G3BP. The successful expression of EGFP-G3BP fusion protein was confirmed by fluorescent microscopy (data not shown). At 48 h after transfection, cells were infected with 1 MOI of PEDV-CO. Culture supernatants were collected for virus titration at 24 h post infection. As shown in Fig. 1E, overexpression of G3BP reduced PEDV titer by 100-fold compared to that of control plasmid.

3.3. PEDV induces SGs and PEDV-infected cells are resistant to sodium arsenite (AS) induced SG formation

To determine whether PEDV infection induces SGs, Vero-76 cells were mock infected or infected with 5 MOI of PEDV-CO or icPEDV- Δ ORF3-EGFP. Then cells were stained with antibody specific for G3BP, TIA-1, and PEDV N specific antibody at 12, 24, and 48 h after infection. We observed G3BP and TIA-1 positive cytoplasmic granules in some PEDV-infected cells as early as 12 h post infection, but not in mock cells (data not shown). Approximately 35% of the PEDV-infected cells showed two or more SGs at 48 h (Fig. 2A) after infection. To examine

whether PEDV-infection interferes with the formation of SGs induced by AS treatment, we treated cells with 0.5 mM AS for 30 min prior to immunofluorescence staining. As expected, SGs appeared in mock-infected cells after treatment with AS, whereas PEDV-infected cells did not show an increased SGs after AS treatment (Fig. 2B).

4. Discussion and conclusion

G3BP1 is one of the key components of SGs. Overexpression of G3BP1 induces SG formation (Tourriere et al., 2003). Cleavage of G3BP1 by viral protease or sequestering of G3BP1 by viral proteins inhibits formation of SGs (Le Sage et al., 2017; Nelson et al., 2016; White et al., 2007). Here we observed that while knockdown of G3BP1 enhanced PEDV replication, overexpression of G3BP1 reduced PEDV replication in Vero cells. Our data suggested an antiviral role of G3BP1 against PEDV. Similar observations were reported for other viruses including mammalian orthoreovirus (MRV) (Choudhury et al., 2017) and sindbis virus (Cristea et al., 2010). G3BP1 appeared to play no role in porcine reproductive and respiratory syndrome virus (PRRSV) replication (Catanzaro and Meng, 2019; Zhou et al., 2017). A pro-viral role of G3BP1 in hepatitis C Virus (HCV) (Garagorta et al., 2012), chikungunya virus (Scholte et al., 2015), and respiratory syncytial virus

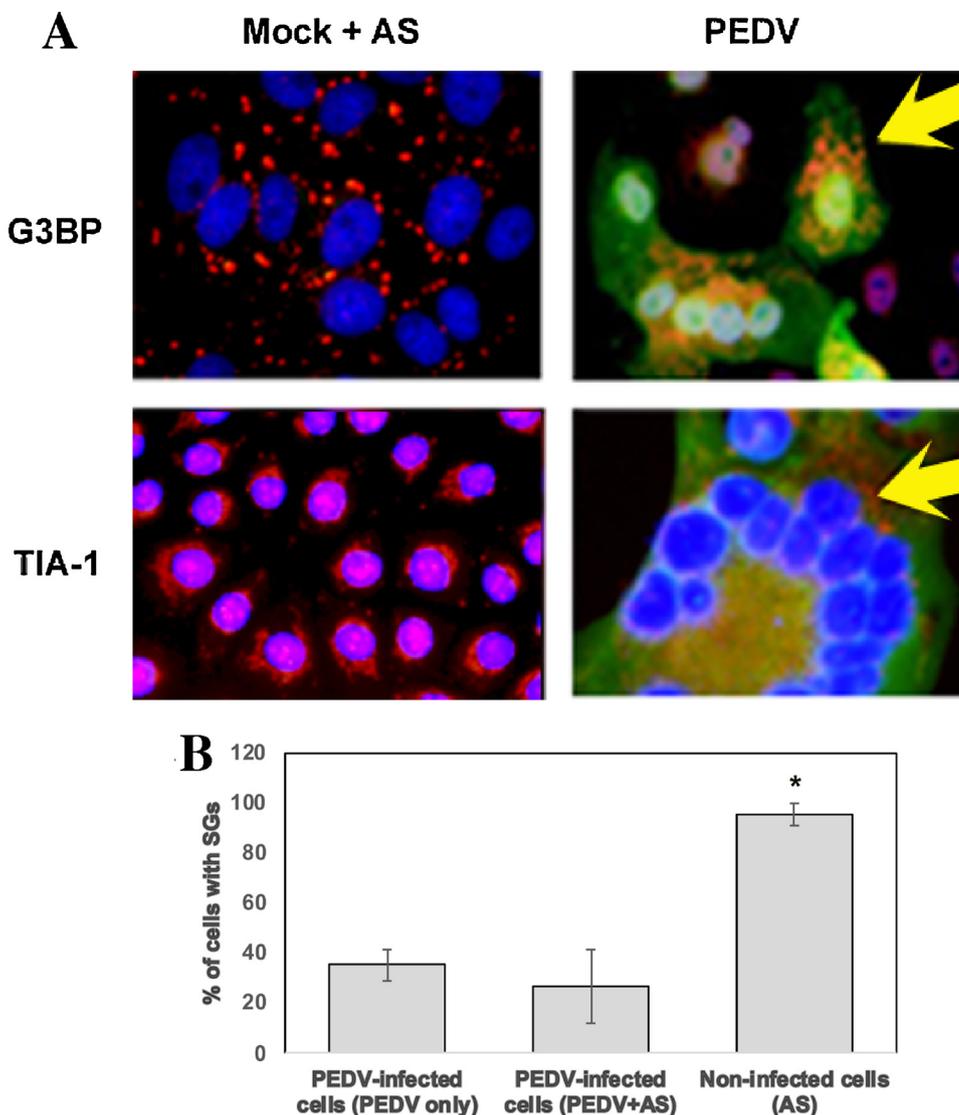


Fig. 2. PEDV induced SGs in infected cells. **A:** PEDV infected cells showed cytoplasmic granules that are positive for G3BP or TIA-1 at 48 h post infection. Yellow arrows indicate virus-infected cells with cytoplasmic SGs. Representative merged pictures of G3BP (TIA-1) (Red)/PEDV (Green)/DAPI (Blue) are shown. **B:** There were no significant difference in the % of cells showing SGs between PEDV-infected cells with or without AS treatment. The average numbers of SGs positive cells were determined from three to four random pictures taken for each experiment. The experiment was repeated three to four times. * indicates significant difference between non-infected + AS with both PEDV ($p = 0.0005$) and PEDV + AS groups ($p = 0.0016$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Lindquist et al., 2010) has been reported.

Different viruses or even viruses within the same family exhibit unique features in the type of SGs they cause following virus infection, which includes stable, transient, and oscillating SGs (Lindquist et al., 2010; Ruggieri et al., 2012; White et al., 2007). We observed that PEDV – CO induced SGs as early as 12 h after infection. Approximately 35% PEDV infected Vero-76 cells exhibited two or more SGs at 24 and 48 h of infection. This is in contrast to poliovirus, which causes transient SG formation during the early phase of infection and disperse SGs by cleavage of G3BP1 during later stages of infection (White et al., 2007). We did not observe any cleavage products of G3BP1 during PEDV infection (data not shown). RSV triggers stable SG formation throughout the viral replication cycle (Lindquist et al., 2010). Cells infected with Mengovirus or Theiler's murine encephalomyelitis virus (TMEV), which belong to the *Cardiovirus* genus within the family of *Picornaviridae*, do not exhibit SGs because the viral nonstructural protein, leader (L) protein inhibits SGs (Borghese and Michiels, 2011). Mouse hepatitis coronavirus (MHV), a member of *Coronaviridae*, induced SG formation early in infection (Raaben et al., 2007; Sola et al., 2011). The percentages of SG positive cells increases as PRRSV infection progresses, suggesting that PRRSV induces stable SGs (Catanzaro and Meng, 2019; Zhou et al., 2017).

West Nile Virus inhibits SGs upon AS treatment by up-regulating glutathione S-transferase (GST) in cells to counteract the reactive

oxygen species (ROS) (Basu et al., 2017). We observed that cells infected with PEDV and treated with AS induced significantly less stress granules than that of the non-infected cells treated with AS. Since virus infections typically induce both ROS and antioxidant response simultaneously (Basu et al., 2017), it is reasonable to speculate that activation of antioxidant pathway by PEDV may mediate the inhibition of SGs induced by AS treatment. The exact mechanism by which PEDV prevents the AS induced SG formation needs to be examined in future studies.

We observed an increase in the transcription of proinflammatory cytokines in G3BP1 knockdown cells after PEDV infection compared to control cells. This could be due to the increased virus replication that we observed in the G3BP1 knockdown cells. Alternatively, G3BP1 may impact inflammatory cytokine signaling pathways. A previous study has shown that knockdown of SG markers such as G3BP1 increased NF- κ B activation and transcription of proinflammatory cytokines induced by PRRSV, which seems to be independent of virus replication (Zhou et al., 2017). The detailed mechanisms by which G3BP1 knockdown affects virus induced proinflammatory cytokines remain to be determined. One limitation of the study is that we used Vero-76 cells, which are not the natural host cells for PEDV. It would be interesting to examine the role of G3BP in PEDV replication in porcine intestinal epithelial cells in future studies.

In summary, we have shown that PEDV infection induces SGs and

interferes with the formation of SGs induced by AS treatment. Knockdown of G3BP1 significantly enhanced viral replication and transcription of inflammatory cytokines. Similarly, overexpression of exogenous G3BP1 reduced virus replication. Further studies are needed to uncover the detailed mechanisms by which G3BP1 impacts PEDV replication.

Declaration of Competing Interest

The authors declare no conflict of interest.

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