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GTPase-activating Protein-binding Protein 1 Plays an Antiviral Role in Porcine Epidemic Diarrhea Virus (PEDV) Replication

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GTPASE-ACTIVATING PROTEIN -BINDING PROTEIN 1 PLAYS AN ANTIVIRAL ROLE IN PORCINE EPIDEMIC DIARRHEA VIRUS REPLICATION

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences degree and is accepted for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

APN	Aminopeptidase -N
ADCC	Antibody Dependent Cellular Cytotoxicity
AIF	Apoptosis Inducing Pathway
BHK-21	Baby hamster kidney-21
CCoV	Canine Coronavirus
CHIKV	Chikungunya Virus
CPE	Cytopathic Effect
3CL Pro	3-C of Protease
dd-Ct	Delta-Delta-Ct
DDX6	DEAD-box helicase 6
DENV	Dengue virus
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle's Medium
DAPI	4',6-diamidino-2-phenylindole
ER	Endoplasmic reticulum
EVD	Enteric Viral Disease
ELISA	Enzyme-Linked Immunosorbent Assay
eIF2α	Eukaryotic translation initiation factor alpha

FECV	Feline enteric coronavirus
FIPV	Feline infectious peritonitis virus
FBS	Fetal Bovine Serum
FFN	Fluorescent focus neutralization
FMIA	Fluorescent microsphere immunoassay
GCN2	General control non-depressible 2
GSH	Glutathione
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
G3BP1	GTPase-activating protein SH3 domain-Binding Protein 1
HRI	Heme-regulated Inhibitor
HSV	Herpes simplex virus
HCV	Hepatitis C virus
HDAC6	Histone deacetylase
IFA	Immunofluorescence Assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IHC	Immunohistochemistry

IF	Immunofluorescence
IFN-α/β	Interferon- alpha/beta
IFN-γ	Interferon- gamma
IRF3	Interferon Regulatory Factor-3
IL-8	Interleukin-8
IL-β	Interleukin-Beta
IRES	Internal Ribosome Entry Site
JNK	Jun N-terminal kinase
kDA	Kilo-Dalton
MRV	Mammalian Ortho-reovirus0
MFI	Median Fluorescent Intensity
MDA-5	Melanoma Differentiation Associated protein-5
mRNA	Messenger RNA
mL	Milliliter
mM	Millimolar
MCV	Mink Coronavirus
MOI	Multiplicity of Infection
NK cells	Natural killer cells

NsP1	Non-structural protein 1
NF-κβ	Nuclear factor kappa beta
NEMO	NF-κβ essential modulator
ORF	Open reading frame
PAMP	Pathogen Associated Molecular Pathway
PBS	Phosphate Buffer Serum
PV	Poliovirus
PABP	Poly(A)-binding protein
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene difluoride
pAPN	Porcine aminopeptidase-N
PED	Porcine Epidemic Diarrhea
PEDV	Porcine Epidemic Diarrhea Virus
PRCV	Porcine Respiratory Coronavirus
PB	Processing Body
PKR	Protein Kinase-R
PERK	PKR- like Endoplasmic Reticulum Kinase
RT-qPCR	Real-time reverse transcriptase quantitative PCR

ROS	Reactive Oxygen Species
RT-PCR	Reverse transcriptase PCR
RSV	Respiratory Syncytial Virus
RIG-1	Retinoic acid-inducible gene-1
RV	Rotavirus
SFV	Semliki Forest Virus
siRNA	Small interfering RNA
SA	Sodium Arsenite
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SDSU	South Dakota State University
SGs	Stress Granules
TIA-1	T-cell intracellular antigen
TIA-1R	TIA-1-related protein (TIAR)
TCID50	Tissue Culture Infective Dose-50
TNF-α	Tumor necrosis factor-alpha
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
USP-10	Ubiquitin specific peptidase 10
UV	Ultraviolet

VN	Virus Neutralization
WNV	West Nile Virus

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ABSTRACT

GTPASE-ACTIVATING PROTEIN -BINDING PROTEIN 1 PLAYS AN ANTIVIRAL ROLE IN PORCINE EPIDEMIC DIARRHEA VIRUS REPLICATION

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Porcine Epidemic Diarrhea Virus (PEDV) is a non-segmented virus which uses one-stranded, positive-sense RNA as genetic material. PEDV falls under *Coronaviridae* family and extensive diarrhea and dehydration in nursing piglets are the major clinical signs. This emerging disease leads to huge economic loss in the pig farming. Very less studies are done towards the role of innate immunity in PEDV infection. The formation of Stress granules (SGs) are seen when cells are introduced to different stressful conditions, which also includes viral infections. SGs are formed when one of the four kinases: double-stranded RNA (dsRNA)-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control nonderepressible 2 (GCN2) kinase and heme-regulated inhibitor kinase (HRI), causes phosphorylation of eukaryotic translation initiation factor (eIF2α). The alternative pathway of SG formation is eIF4A RNA helicase inhibition. Phosphorylation of eIF2α by PKR activation is the most common pathway of SGs formation. SGs are considered as an indication of an antiviral innate response of the host that limits translation of the viral genes. Ras-GTPase-activating protein (SH3 domain) binding protein 1 (G3BP1) is one of the important stress granule-resident protein that nucleates stress granule assembly. The objective of the study was to investigate the role of G3BP1 in PEDV replication. We found that 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. Similarly, overexpression of exogenous G3BP1 lowered virus replication by approximately 100-fold compared to vector control. We also observed that PEDV-infected cells are resistant to SGs formation upon sodium arsenite treatment. An enhancement in the levels of mRNAs of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor - α (TNF-α) was also observed in PEDV-infected G3BP1 knockdown cells compared to PEDV-infected control cells. Taken together, our results demonstrate that PEDV induces transient SGs formation and G3BP1 plays an antiviral role in virus replication.

Chapter 1: Literature Review

1. Porcine Epidemic Diarrhea Virus

1.1 Introduction

Porcine epidemic diarrhea virus (PEDV) is a non-segmented RNA virus which uses positive sense and one-strand RNA as genome. PEDV is a coronavirus of Alphacoronavirus genus and Nidoviridae order [44, 59]. The symptoms seen in PEDV infections are diarrhea with vomiting that ultimately results in dehydration. The condition is Porcine Epidemic Diarrhea (PED) that causes the death of piglets [23]. PEDV infects the pigs of any ages, including neonates, sows, boars to old age hogs [98]. Infected old hogs usually lose weight but in newborn piglets, mortality is seen within five days of infection [23, 98]. PEDV infection is non-transferable to humans since no zoonotic cases have been reported.

PEDV was not recognized until it was first seen as enteric diarrhea mostly in growing piglets. The symptoms were much like that of Transmissible Gastroenteritis Virus (TGEV), but later it was found that TGEV affects only nursing ones and effects were milder than PEDV infection. In 1976, the disease emerged with similar clinical signs but to a different age group; the suckling piglets. Thus, the condition where the infection was seen in growing and fattening pigs were named as EVD Type-1 and the latter condition was named as an EVD Type-2 [14, 114]. In 1978, researchers from Ghent University, Belgium found PED was caused by Corona virus-like agent (CV777). This agent was unique to TGEV and Hemagglutinating Encephalomyelitis virus and it was then called as Porcine Epidemic Diarrhea Virus [23, 86]. In 1982, PEDV outbreaks were seen Asian countries like Thailand and China causing a huge financial impact on pork producing

industries[16].PEDV outbreak was seen in USA in 2013, which then spread more than 40 states, in major swine producing areas. Within a year, it caused the destruction above 8 million neonatal piglets and then became transboundary[75, 80]. The disease recurred in various Asian countries like Japan, South Korea, and Taiwan [62, 67]. PEDV is one of the devastating swine virus which has caused a huge economic loss in the global pork industry.

1.2 Viral structure and genome

PEDV is positive- sense, single- stranded RNA. The size of virus is approximately 28 kb and the genome runs from 5′ cap to the 3′ poly A tail. PEDV genome has seven open reading frames (ORFs) that represents for viral proteins [54]. ORF1a and ORF1b represent the enzymes for virus replication and transcription, and ORF3 represents a nonstructural protein that has unidentified function [103]. ORFs (2-6) have specific names based on the proteins that are present in these regions, i.e., spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins [39, 62, 100].

The S protein (150–220 kDa) helps in the interaction of virion with the cellular receptor during virus entry [24]. It is also responsible to induce neutralizing antibodies in the natural host and identification of the genetic relationships in between PEDV strains [61]. The M protein (20–30 kDa), present in the highest amount in the genome, helps in the assembly of virus and antibodies production [26, 118]. E protein helps in virus budding [7, 27]. Protein N helps in viral replication and pathogenesis [73].

1.3 PEDV and its association with other Coronaviruses

Initially, there was a confusion between PEDV and TGEV but later it was found that PEDV and TGEV share the same kind of pathogenesis only [23]. PEDV and TGEV cannot be distinguished based on their clinical signs or histopathology. But advanced

development in Enzyme-Linked Immunosorbent Assays (ELISA) and Immunohistochemistry has made it easy to identify.

PEDV has also antigenic similarities between other viruses of the same genus *Alphacoronavirus,* such as Feline Infectious Peritonitis Virus (FIPV), TGEV, Porcine Respiratory Coronavirus (PRCV), Canine Coronavirus (CCoV) and Mink Coronavirus (MCV). The main reason behind the similarity may be the presence of the receptor, aminopeptidase N (APN), for virus attachment and entry.

1.4 Heterogeneity

Coronaviridae family has a slow revolution because of the presence of 3'to-5' proofreading exoribonuclease in their non-structural proteins. Enzyme enables them to maintain the stability and diversity in genetic material [99]. To study PEDV, different genetic and phylogenetic tree analysis was conducted among PEDV isolates. The S-gene was much studied in this regard and it was found that the PEDV exists in two groups: Genogroup 1 (G1) and Genogroup 2 (G2). Genogroup 1 can be further divided into subgroups 1a and 1b, and Genogroup 2 can be divided into subgroup 2a and 2b. Genogroup 1a includes PEDV strain CV777, vaccine strains, and other cell culture-adapted strains whereas G1b includes strains from Asian countries like South Korea and China, United States, and other European countries. [66, 80, 108].

1.5 Transmission, Pathogenesis and Clinical Signs

PEDV is transmitted through direct and indirect contact, airborne route, contamination through diarrhea or vomiting including other environmental outcomes from infected pigs and farm equipment [70]. Once PED outbreak occurs on the farm, it remains in the farm premises if proper hygiene is not maintained. Inadequate maternal antibodies,

inappropriate vaccination, and stress may lead the virus to infect piglets. PED can be seen in two kinds of the syndrome: Porcine Epidemic Diarrhea I which infects weaned to adult pigs and has low morbidity and low mortality. Porcine Epidemic Diarrhea II which infects nursing pigs and has high mortality and morbidity. But in young piglets due to less developed immunity, they result in the fatal death. PEDV infects enterocytes and destroys them. The loss of cell results causes dehydration and lethargy in infected piglets, so they become weak [25]. Macroscopic examinations are intestinal walls are sloughed intestines and fecal contents. Accumulation of fluid in the intestinal lumen results in the distended stomach [23]. Histopathology reveals severe enteritis, swelling of the intestine, necrotic enterocytes.

1.6 PEDV host-virion interaction

Porcine amino-peptidase N (pAPN), the receptors that are present on the surface of epithelial cells in small intestine, help PEDV to enter the intestinal epithelial cells. [64, 77]. The spike protein recognizes the pAPN receptor, which then helps PEDV bind to target cells and internalizes by direct membrane fusion [58].

Vero and MARC-145, African green monkey kidney cell lines, are the most common cells for the PEDV propagation [37, 57]. There is an ongoing controversy on whether APN acts as the functional receptor for PEDV because pAPN enables virus to infect cells when they are expressed exogenously [77]. A recent study has also revealed that cell-surface heparan sulfate acts as the attachment factor of PEDV in Vero cells [38]. Trypsin has a major role in entering PEDV in Vero cells [63, 81]. Trypsin cleaves S protein into S1 and S2 subunits for efficient viral replication [113].PEDV shows cytopathic effects by cell fusion, syncytium, and detachment after killing cells [60].

1.7 Immune Response to PEDV

Like other viruses, PEDV infections also induce both innate and adaptive immunity. Innate immunity acts first in the defense with virus and if the virus wins, then clinical signs appear. The innate immunity not only controls the preliminary virus infection but also activates adaptive immunity and also plays a significant role in controlling primary viral infections [2]. Cytokines, interferons dendritic cells, natural killer (NK) cells and macrophages come under innate immunity components [94]. When Pathogen-associated molecular pattern (PAMP) recognizes viral nucleic acid, the host body immune system gets activated and they initiate an antiviral response. Retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation associated protein 5 (MDA-5), Toll-Like Receptors (TLRs are specific to double-stranded RNA. They recognize it and pass the signal of recognition to the host's cells which then activates transcription factors like interferon regulatory factor 3 (IRF3), activating transcription regulatory factor c-Jun (AT-F-2/c-Jun), and nuclear factor κβ (NF-κβ). Activated transcription factors ultimately express type 1 interferons: IFN-α and Interferon- β [49]. PEDV's structural protein and non-structural protein are responsible to inhibit IFN- β production. NSP1 and PEDV N protein inhibit IRF3 activation whereas NSP5 inhibits activation of NF-κβ [117],[109]. Also, the IL-8 expression is enhanced by PEDV N protein [115]. NK IFN-γ and TNFα are produced by NK cells that helps to combat viruses.[30], [10].

1.8 Diagnosis

Due to the similarities between different viruses in Coronavirus family, it is difficult to differentiate between them. Therefore, a proper laboratory diagnosis is important to

characterize PEDV. Broadly, we classify diagnostic methods into Virological assays and serological assays.

1.8.1 Virological Assays

Vero cells are the cell lines that are suitable for the propagation of PEDV [17, 44, 81]. Immunofluorescence Assay (IFA) and RT-PCR are the best techniques for PEDV isolation from cell culture [17, 81]. Other cell lines like the Primary intestinal epithelial cell or any other cells that express porcine aminopeptidase N (APN), can help to propagate PEDV in cell line [64, 77, 97].

Immunohistochemistry (IHC) is a technique for the detection of PEDV [35, 102]. They use antibody-antigen and enzyme-substrate reactions [91]. Through IHC, PEDV can be visualized on target cells [26]. IHC has another advantage too because the samples which we prepared during IHC long-term has long-term storage.

Immunofluorescence (IF) assay helps in the identification of PEDV antigens infecting the cells[17, 81]. IFA detects viral antigens (antibody-antigen reaction) in cell culture. Intra-fluorophore-conjugated PEDV-specific antibodies are used to detect viral antigens. Under a fluorescent microscope, viral antigens, are visualized in the cytoplasm of infected cells during various phases of infection.

ELISA detects virus antigen using PEDV-specific antibodies [12, 13]. Firstly, the plate is coated with the PEDV-specific capture antibody overnight and incubated with the sample. The antibody-antigen binding, and the reaction is developed by adding an enzyme-linked secondary antibody [5]. Limitations may be how and when the samples were collected and stored [43, 93].

Real-time Reverse transcriptase-polymerase chain reaction (r RT-PCR) assays are methods of choice for the diagnosis of PEDV [33, 51]. PEDV genome in feces, rectal swabs or intestinal samples from animals can be used as a sample [17, 44]. PCR detects PEDV genes based on one of the structural proteins [42, 101]. In conventional RT-PCR, the virus-specific primers target the PEDV genome and amplify it to the thousands of copies. The process is then followed by electrophoresis in agarose gels [40, 55]. RT-PCR detects genes by using non-specific fluorescent dyes for double-stranded DNA [4]. The major advantages of RT-PCR assays are that they are highly sensitive and able to detect the target sequences simultaneously with the amplification reaction [4].

1.8.2 Serological Assays

Serological examination measures the level of exposure of the animal herd to the virus. Detection of PEDV specific antibodies helps to know about the sow immunity and to track the neonatal immunity level. The principle is based on antibody-antigen reaction where PEDV infected Vero cell cultures are used as antigen and antibodies are already present in serum samples from suspect animals. The antibodies are detected with fluorophore-labeled anti-porcine secondary antibodies and visualized under a fluorescence microscope [105]. The main advantage of IFA is that it is less time consuming and easier to perform [59].

Virus neutralization assays (VNs) are also one of the serological assays that are widely used in detection of PEDV [82]. It is the process to check viral infectivity. The neutralizing antibodies bind virus(antigens) and block one or more steps of the virus formation [53]. Formation of CPE is used in detection of neutralizing antibodies against PEDV.

In indirect ELISA, the viral antigen is coated in the plate and the test sample is added to the plate. Antibody-antigen complex is formed when the sample is incubated using an enzyme-linked secondary antibody [5]. Competitive ELISAs works on the principle that when the antigen is added to microplates coated with the antibodies, a reaction is developed based on the PEDV-specific monoclonal or polyclonal antibodies [13, 82]. The blocking ELISA is more specific when compared to the indirect ELISA [13].

1.9 Prevention and Control Measures

1.9.1 Biosecurity

Proper disinfection and biosecurity are the most important measures to prevent acute PED outbreaks. PEDV can survive in feces, feed, and water for extended periods. PEDV can survive in fresh feces up to 1 week at 40ºC, dry feed for 1 week at 25ºC, wet feed for 4 weeks at 25ºC, drinking and recycled water for 1 week at 25ºC and slurry for 2 weeks at 25°C [95]. The proper and strict application of disinfection blocks the entry of pathogens to the farm. Disinfection must be thoroughly applied to all fomites, personnel, and external visitors that could be contaminated with PEDV. PEDV is inactivated by most viricidal disinfectants but sometimes PEDV RNA can be detected by RT-PCR even though disinfection is done [9]. Firstly, cleaning should be done under high pressure and water at high temperature. Secondly, disinfection by an appropriate disinfectant and thirdly, drying it overnight. The crowd people should be restricted between fattening and farrowing such that there will be less contact between trailers and the farm interior New animals should be quarantined before they are shifted to the farm.

1.9.2 Vaccines

Vaccination to the dams is the fundamental control strategy for PED because it makes them immune and helps to transfer their maternal antibodies via colostrum to their piglets. Sows should be vaccinated to stimulate lactogenic immunity to PEDV. To boost up the maternal antibodies, vaccination is also prescribed to the weaned pigs. [15]. Cell culture attenuated virus CV777strains have been used to produce vaccines. Modified Live Vaccine and killed strain SM98 have higher neutralizing titer [85]. Killed virus vaccine from Zoetis, and an RNA particle vaccine from Harris Vaccines are available in the US. PEDV vaccines need to induce good IgG and IgA immune responses in herds after vaccination. Modified live virus vaccines are based on US PEDV strains.

2. Stress Granules

2.1 Formation and Composition

Whenever cells are exposed to environmental stress like heat shock, UV irradiation, hypoxia, endoplasmic reticulum (ER) stress, and viral infection, the cells pass the vibes of stress to the nucleus and rapid translational arrest occurs leading to polysome disassembly [45]. The affected cell must decide the fate of mRNA that it will decay or remain translationally silent [47]. Eukaryotic cells can contain multiple types of cytoplasmic mRNA like processing bodies(PBs) and stress granules (SGs) [31] [11]. SGs and PBs both are found in most of the cells where they contribute to the regulation messenger RNA. Both of them are formed in response to the stress condition in cells but vary in these conditions: PBs are also observed in unstressed cells and PBs have proteins that are involved in mRNA deterioration, whereas SGs only contain proteins from the translation initiation complex [29].

Stress Granules starts to get induced as soon as initiation of translation starts getting impaired [48], or due to the treatment with the drugs that block translation [21]. Knockdown of some initiation factors [74] or overexpression of RNA binding proteins that repress translation may also help to induce stress granules. Stress granules ,for the first time, were reported in tomato cells when they were treated with heat [79]. SGs include 40S ribosome subunits, eIF2, eIF3, eIF4A, eIF4B, eIF4E, eIF4G and eIF5 [45]. The pathway for SG formation is the addition of phosphoryl group to the eukaryotic translation initiation factor (eIF2α) by the kinases double-stranded RNA (dsRNA)-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), General control nonderepressible 2 (GCN2) kinase and heme-regulated inhibitor kinase (HRI) [45]. The alternative pathway is the inhibition of eIF4A RNA helicase [72].

PKR which is one of the element of the interferon response, is regulated by RNA viruses producing double-stranded RNA. PERK/PEK is activated by unfolded proteins present in endoplasmic reticulum (ER). GCN2 is activated during insufficient amino acids in cells and UV irradiation [41] and heme-deprivation and oxidative stress activate HRI [36]. These kinases cause the phosphorylation of the α-subunit of eIF2 at Ser52, binds tightly with eIF2B and inhibits the exchange of GDP for GTP. Therefore, there is a decrease in translation tertiary complex assembly (eIF2/GTP/Met tRNA) which suppresses the initiation of translation and promotes SGs assembly [45]. There is also another mechanism that follows different pathway than eIF2α phosphorylation. Drugs like Hippuristanol and Pateamine A inhibit the helicase activity of eIF4A kinase and induce the assembly of SGs [21, 72]. Some researchers also support that the overexpression of some of the SGs markers [47] like TIA1 [48] or G3BP-1 [107] helps in the gathering of stress

granules. The mechanism behind the formation of SGs has not understood properly. Self-oligomerization of TIAR and G3BP is important during early SGs aggregation and then the overexpression of these proteins induces SGs [107]. The post-translational modification of the several factors is also responsible for SGs formation. This involves post-translational modifications of several other proteins that regulate SGs dynamics. Modification of the O-linked N-acetylglucosamine, methylation, acetylation, and phosphorylation are responsible. Also, the breakdown of the microtubules formation due to any drug treatment disrupts the formations of SGs and extends their recovery from any stress in the cells [18].

2.2 Interaction with Virus

SGs suppress translation which then ultimately affect virus replication. Since viruses' interrupt hosts translation and transcription processes, they will induce stress responses on several levels. In case of RNA viruses, SGs are controlled and directed by viruses themselves such that SGs help in RNA silencing and storage. There are some DNA viruses which also control SGs responses. When viruses interact with the SGs pathway, they show different changes at the cellular level. In general, most viruses are opposite of SGs formation whereas some induce and may exploit portions of SGs responses during infection. Some viruses may inhibit the SGs formation, some may induce and later inhibit whereas, some may exploit SGs.

West Nile Virus (WNV), a neurotropic virus, interacts with two SGs markers, TIA-1, and TIAR [65]. The other viruses from the same family as WNV, Dengue Virus, was found to interact with TIA-1/TIAR at viral replication complexes (dsRNA and NS3). Proteins like G3BP1, caprin1, and USP10 colocalize with dsRNA that marked viral

replication and hence block SGs formation. Hepatitis-C virus (HCV) is also a virus from family *Flaviviridae* which promotes SGs assembly during infection [3]. After 48 h post-infection, G3BP-1 and DDX6, components of SGs co-localize with the HCV non - structural protein 5B and the 5' end of the HCV minus-strand RNA and inhibit SGs assembly [116]. Rotavirus (RV) infection activates phosphorylation of eIF2α but not SGs formation and also the infected cells do not form SGs in response to arsenite treatment [76]. Theiler's murine encephalomyelitis virus (TMEV) leader protein (L) inhibits SGs formation in entire infection as well as to exogenous stress [8]. Acute Junin virus infection inhibits phosphorylation of eIF2α in response to arsenite [69]. Influenza A virus infection fails to induce SGs. Although, when NS1 mutant viruses are used, SGs are formed by PKR pathway [50]. Human immunodeficiency virus 1 (HIV-1) inhibits the formation of SGs when treated with arsenite treatment but it upregulates eIF2a phosphorylation. Protein Staufen1 interacts with the viral Gag protein and forms HIV-1 ribonucleoproteins (RNPs) [1]. Human T-cell leukemia virus type-1 (HTLV-1) also inhibits SGs due to Tax protein because it interacts with histone deacetylase 6 (HDAC6) [56]. Similarly, DNA viruses such as Herpes simplex virus 1 (HSV1) mutant viruses only induce SGs [22].

Formation of SGs are not always related to the shut-off of host protein synthesis [52] but they sometimes sequester apoptotic factors and helps in the cell survival if exposed to some stress [52]. Thus, some viruses mediated induction of SG assembly which represents a strategy employed by some viruses to ensure replication. Mammalian orthoreovirus (MRV) induces SGs in at starting phase but the SGs gradually disappear as the infection progress [88]. Also, MRV infection do not produce SGs when they cells are treated with arsenite [89]. Semliki Forest virus (SFV) also induces SGs by phosphorylating

eIF2α during early infection [84]. Another, Poliovirus (PV) also induces SGs containing G3BP and eIF4GI in some cells at early time points during infection in an eIF2-independent pathway. The number of SGs decline as infection proceeds [110] and it inhibits the formation of canonical SGs in response to arsenite. PV cleaves G3BP by a viral 3Cprotease enzyme with separates the G3BP RNA-binding and protein-interaction domains.

2.3 Immune Responses

When a virus infects host cells, the innate immune sensors in cells that trigger signaling pathways downstream result in the activation of immune responses. The factors of innate immunity that detect viruses are Toll-like receptors-3, 7, 8 and 9, retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and DNA sensors like DNA-dependent activator of IFN-regulatory factors (DAI), stimulator of IFN genes (STING), DEAD box polypeptide 41 (DDX41), and cGMP/cAMP synthase (cGAS) recognize cytoplasmic viral RNA [34]. These antiviral effects include double-stranded (ds) RNA dependent protein kinase (PKR)-dependent inhibition of mRNA translation, and 20, 50 -oligoadenylate synthetase (OAS)/RNase L-mediated RNA degradation

Formation of SGs can be considered as the antiviral response due to the collective response of the host cells to stress. SGs contains different cellular components that are important for viral replication. TIA-1 and TIAR are the cellular protein which is required for flavivirus replication by binding a 30stem loop that is complementary to minus strand RNA [28]. Another cellular component, G3BP is also utilized by HCV [3]. Different eukaryotic initiation factors as well as 40S subunits are part of SGs, that can negatively impact virus replication. Further, internal ribosome entry site (IRES) transactivating factors such as

PTB, PCBP2, and UNR are also important components in picornavirus translation which are sequestered in SGs [111].

2.4 Ras-GTPase-activating protein (SH3 domain) binding protein 1 (G3BP1)

Ras-GTPase-activating protein (SH3 domain) binding protein 1 (G3BP1) is retained in SGs and helps in the nucleation of the stress granule assembly [46]. Silencing G3BP1 inhibits SGs formation in the presence of different stress conditions[83, 110] whereas overly expressed G3BP1 nucleates SGs formation. G3BP1 induced SGs by eIF2α phosphorylation-dependent pathway where PKR is involved [92]. G3BP1 PXXP domain is extremely important for the activation of PKR [83]. In case of Influenza A virus, the SGs formations are disrupted by the Influenza virus NS1 protein that also triggers in innate response and production of beta interferon (IFN-β). Encephalomyocarditis virus infection in Hela Cells cleaves G3BP1 and aborts the formation of SGs[78].

Chapter 2

3. GTPase-activating protein-binding protein 1 (G3BP1) plays an antiviral role in Porcine Epidemic Diarrhea Virus (PEDV) replication

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3.1 Abstract

Porcine Epidemic Diarrhea Virus (PEDV) is a single-stranded, positive-sense RNA virus that belongs to the *Coronaviridae*. PEDV causes severe diarrhea and dehydration in nursing piglets, which leads to significant economic loss 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 serve as an 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. Similarly, overexpression of exogenous G3BP1 lowered virus replication by approximately 100-fold compared to vector control. We also observed that PEDV-infected cells are resistant to SGs formation upon sodium arsenite treatment. An increase in the levels of mRNAs of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor - α (TNF-α) was also observed in PEDV-infected G3BP1 knockdown cells compared to PEDV-infected control cells. Taken together, our results demonstrate that G3BP1 plays an antiviral role in virus replication.

Key Words: PEDV, Stress granules, G3BP1, Antiviral

3.2 Introduction

PEDV is a one stranded RNA virus, positive- sense, that belongs genus *Alphacoronavirus* and family *Coronaviridae* [44, 59]. The size of the genome is ~ 28 kb and contains 5′ cap and a 3′ poly A tail. There are 7 open reading frames (ORFs) encoding viral proteins [54]. The PEDV genome contains Nucleocapsid protein (N), that forms a long and helical coil structure. The N protein is wrapped by 3 surface associated structural proteins: Surface (S), Membrane(M), and Envelope (E). The disease caused by PEDV is Porcine Epidemic Diarrhea (PED) inducing diarrhea with vomiting that ultimately results in dehydration [23]. PEDV can infect pigs of any ages, including neonates, sows, boars and old age hogs [98].

PEDV is a re-emerging epizootic swine disease. It was first seen in the United Kingdom , and gradually spread to other areas of Europe [14]. Clinically, PEDV started disappearing from Europe in the 1980s, only persisting in few farms [87]. PEDV then spread globally, affecting Asian countries including Japan, South Korea, China, Thailand, and Vietnam [104]. In 2013, the disease suddenly appeared in the United States, and then to Mexico and Canada. In a very short duration, it led to huge economic loss in pork industry globally [75, 80].

When cells undergo any kind of environmental stress, one of the four kinases namely, double-stranded RNA (dsRNA)-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control nonderepressible 2 (GCN2) kinase and hem-regulated inhibitor kinase (HRI) is activated [45, 72]. Their activation causes the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) to phosphorylate, which then ultimately dephosphorylates RNA binding proteins to form RNA granules [45].

Mammalian cells form two major kinds of cytoplasmic RNA granules which store suppressed messenger ribonucleoproteins (mRNPs): stress granules (SGs) and processing bodies (PBs) [11, 31]. SGs form as a result of various environmental stresses and serve as a location for mRNA storage. However, PBs are usually present in unstressed cells and exposure to stress increases its size and quantity [112]. SGs contain translationally silent mRNAs along with 40S ribosomal subunits, eukaryotic initiation factors and RNA binding proteins (e.g., G3BP1, TIA1, TIAR) [11]. Among these all components, RNA binding protein TIA1 and G3BP1 are the key markers of SGs[45].

Ras-GTPase-activating protein (SH3 domain) binding protein 1 (G3BP1), is a component of stress granule, helps to form the stress granule assembly[46]. G3BPs are proteins with conserved acidic domain, several SH3 domain binding motifs, and an RNA recognition motif. When we deplete G3BP1 from the cells, it inhibits SGs formation and if overexpressed starts forming SGs assembly [83, 110]. G3BP1 induce SGs by eIF2α phosphorylation-dependent pathway, that brings the repression of translation machinery [92]. G3BP1 is an antiviral protein that is a component for innate immune response through Nuclear Factor Kappa Beta (NF-κB) and Jun N-terminal kinase (JNK) transcription [92].

SGs formation is considered as a host defense mechanism to suppress translation and impact virus replication. Since, viruses interrupt hosts translation and transcription processes, they will induce stress responses on multiple levels. Different RNA viruses are reported to manipulate SGs such that SGs are involved in RNA silencing and storage. There are some DNA viruses which also modulate SGs responses. When viruses interact with the SGs pathway, they show different changes at the cellular level. In general, most viruses antagonize SGs formation during infection whereas some induce and may exploit portions

of SGs responses as part of the infectious cycle. Some viruses may inhibit the formation of SGs, some may induce and later inhibit whereas, some may exploit SGs.

Rotavirus, Influenza A virus (IAV) and Theiler's murine encephalomyelitis virus (TMEV) inhibit SG formation [8, 50, 76]. Similarly, viruses like Respiratory syncytial virus (RSV), Encephalomyocarditis virus, Poliovirus and Mammalian Orthoreovirus (MRV) also induce SGs that suppress viral infections [68, 89, 111]. IAV mutant strain, that lacks nonstructural protein 1 (NS1), produces stress granules which first inhibits virus replication but later helps in enhancement of IAV replication [83]. There are some viruses that are benefitted from SGs formation. RSV replication is impaired when G3BP1 expression levels are reduced from the cells [68].

The main goal of my study was to find the role of SGs and G3BP1 in PEDV replication. PEDV has caused huge economic loss to the pig farming. It is an emerging disease and there are no preventive measures for it. Only limited information is available regarding the role of innate immunity against PEDV infection. Our findings show that when we infect Vero cells with PEDV-CO, transient stress granules appear at 24 hours post infection. Overexpression of G3BP1 in Vero cells decreases virus replication while knockdown of G3BP1 significantly promoted viral genome replication, mRNA gene expression and pro-inflammatory cytokines produced by PEDV. Taken together, our results show that the principal stress granule marker, G3BP1 may play major role in antiviral immune response against PEDV.

3.3 Materials and Methods

3.3.1 Cells, Viruses and Drugs

Vero-76 cells from African Green monkey kidney cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate in a humidified 37◦C, 5% CO2 incubator. Cells were passaged in 6-well, 12-well, 24-well,48 well and 96-well plates at 1×10^5, 5×10^5, 2×10^5, 5×10^4 and 1×10^4 cells/per well, respectively. Vero-76 cells were seeded in a six well plate a day before inoculation. Multiplicity of Infection (MOI) refers to the total virions that we add to infect one cell. For example, if we have one million cells, we need to add one million virions, so that the MOI is one. The next day, MOI of 1 was used to infect the cells in the plate after the number of cells in the plate. Since, PEDV needs trypsin for infection, we used modified trypsin treated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). 3 μg/ml of TPCK, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate were mixed to 50ml of raw DMEM media. Virus was aliquoted after MOI calculation and diluted into the prepared TPCK media. Virus suspension was used to infect the cells and then incubated in 37◦C for 3-7 days. The inoculated cell cultures were checked daily under microscope for cytopathic effects (CPE). After 5-7 days of incubation, cells and supernatant fluids were frozen and thawed three times to release intracellular virus into the medium. The fluid was clarified by centrifugation 1200 x g for 5 mins and was separated into separate tubes to store back. Titers of the virus were checked by using the TCID50 assay.

TCID50 assay is an end -point dilution assay that measures infectious virus titer. It measures the amount of the virus that is required to produce 50 % of the cytopathic effects

in the inoculated cells. Vero-76 cells were plated, and the virus stocks were added in serial dilutions. The cells were incubated for 3-5 days and stained for immunofluorescence. We used FITC labeled antibody specific to PEDV N to stain PEDV-CO and counted the wells with staining. Then we calculated the value of TCID50.

The stocks of recombinant icPEDV-ΔORF3-EGFP virus was prepared by infecting Vero-76 cells at an MOI of 0.05 on T75 cell culture flask. Growth medium was DMEM supplemented with 5% FBS (Sigma) and antibiotics (100 IU of penicillin and 100 pg of streptomycin per ml). Maintenance medium were prepared fresh each time before infection. Maintenance medium consisted DMEM supplemented with 0.3% tryptose phosphate broth (Sigma) and containing 10 ug of trypsin per ml. Vero-76 cells were cultured in Growth media in T75 flask till it reaches to 90% confluent. Virus stocks were diluted in freshly prepared Supernatants were harvested at 72 hours post infection (hpi) after more than 80% of cytopathic effects (CPE) were observed. Virus infectivity was quantified by TCID50 assay on Vero-76 cells. A MOI of 1 or 5 of was used to infect Vero-76 cells in the experiments.

For stress treatments, Sodium arsenite (SA) (Sigma, 50 mM) were prepared at the concentration of 0.5 mM in 10% FBS media. SA were added 30 mins before processing for indirect immunofluorescence assays, supernatants were collected at 24 hpi and titrated by TCID50 assay.

3.3.2 Knockdown of G3BP1 by silencing RNA

G3BP1-specific silencing RNA (siRNA) and control siRNA (Santa Cruz Biotechnology, Catalogue No: sc-75076) were used for transfection. Lipofectamine 2000 RNAiMAX Reagent (Life Technologies) was used according to the manufacturer's

instructions. Vero-76 cells were grown in a 12-well plate a day before transfection. The si-G3BP1 and control si-RNA were each transfected at a final concentration of 45nM using Lipofectamine 2000 reagent according to the manufacturer's instructions. Cells were infected with PEDV-CO at an MOI of 1, 48 hours (h) after transfection and incubated. The supernatants were collected 24 h after virus infection and used for virus titration using the TCID50 method. Cells were harvested for real-time RT-qPCR to quantify viral gene transcription or for Western blotting analysis to examine the expression of G3BP1, B-actin, and PEDV-N proteins.

3.3.3 Immunofluorescence staining (IFA)

We did immunofluorescence staining for TCID50 assay and for proteins. Vero-76 cells were cultured in a 96-well plate the day before so that the cells are confluent. After calculation of MOI of 1, virus aliquots were taken and added at 10 folds serial dilutions. The plate was then incubated for 3-5 days until CPE were observed. Cells were washed three times with PBS and fixed with 80 % acetone for 15 min at room temperature. Cells were then incubated with FITC for one hour at 37°C. PBS was used to wash cells and viewed under a fluorescence microscope. Ten random fields were selected, and the virus-positive cells in each field were counted.

For stress induced treatments Vero-76 cells were seeded into 48 -well plates a day prior to infection. Cells were infected with virus at MOI of 5 using N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin or were incubated at 37 ℃ for 30 minutes with 0.5 mM arsenite just before staining. On the day of staining cells were washed in phosphate-buffered saline (PBS) 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 (Catalog no. G6046, Sigma, CA, USA) against G3BP1. The secondary antibody was Alexa-Fluor-546-conjugated goat anti-rabbit-IgG (R&D, MN). PEDV was stained with FITC antibody. Nucleus were then stained with 0.2 mg/ml DAPI (Invitrogen, CA) and visualized under fluorescence microscope. Ten random fields were selected, and virus infected cells were counted.

3.3.4 Western blotting

Cells were grown in 12 well plates or 6 well plates a day before infection. Next day, cells were checked for 90% confluency and infected or mock-infected with PEDV-CO at MOI 5. Cells were incubated and then harvested at indicated time points. Scrapper was used while harvesting the cells. The cell lysates were used in western blot analysis to detect viral proteins. The harvested cells were lysed by mixing equal volume of Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Cell lysates were separated by SDS-PAGE gel (10% polyacrylamide gel) and transferred to a Polyvinylidene difluoride (PVDF) membrane (Hybond P; Amersham Pharmacia) in a dry system. 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 and kept at 4°C overnight. The secondary antibodies were diluted in PBST and incubated with the membranes for 1 h at room temperature. Proteins were visualized by ECL detection (GE Biosciences) and detected by using the Bio-Rad ChemiDoc XRS Imaging System (Bio-Rad). Image analysis was performed using (Bio-Rad) Quantity One analysis software (Bio-Rad). The expression of beta-actin detected by an anti-beta-actin mouse monoclonal

antibody as a loading control (Sigma) at 1:5000 dilution. Anti-G3BP1 Rabbit antibody (Catalog no. G6046, Sigma, CA, USA) against G3BP1 at 1:5000 and PEDV-NP Mouse monoclonal antibody at 1:500 dilution was used as primary antibody. The secondary antibodies IRDye 800 CW Goat anti-rabbit or Donkey anti -Rabbit IRDye 680 were obtained from LI-COR (NE, USA).

3.3.5 Real-time RT-PCR

Viral gene transcription level was measured by real-time RT-PCR. G3BP1-specific silencing RNA (siRNA) and control siRNA were the two groups used. The cells were extracted with scrapper for RNA isolation. RNeasy Kit RNeasy protect mini kit (Qiagen) was used and the procedures according to manufacturers was followed. The concentrations of RNA were determined using a Nano Drop ND-2000 spectrometer (Thermo Scientific). Complementary DNA (cDNA) synthesis was performed using a High Capacity cDNA Synthesis Kit (Applied Biosystems Inc.) according to the manufacturer's instructions. The same amount of RNA in each treatment group was reverse transcribed into (cDNA) using high-capacity cDNA reverse transcription kits (Applied Biosystem, Foster City, CA) by following the manufacturer's instructions. Real-time PCR was then performed on a Mx3000P Real-time thermocycler (Agilent Technologies). Primer sequences used in this study are shown in Table 1.

Four µl of cDNA was added to a mixture containing 10 µl of SYBR green master mix (Brilliant II SYBR green QPCR master mix), 0.3 µl of ROX reference dye (Stratagene), 0.25 µl of forward primer (50 pmol/µl) and 0.25 µl of reverse primer (50 pmol/µl). We duplicated all the samples. The condition for cycling were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Threshold

values (Ct) were obtained at the end of the cyles. B-actin transcription were examined to normalize the amount of input RNA. Relative transcript levels were quantified by the ΔΔCT method.

3.3.6 Statistical analysis

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

3.4 Results

3.4.1 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 folds based on the G3BP1 knockdown efficiency. Representative results are shown in Figure 1a, 1b, 1c, which showed a 50% reduction in G3BP1 expression and a 10-fold decrease in virus titer compared to control silencing RNA. A 100-fold decrease 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 1.5 folds higher than those of the control silencing RNA (Figure 1d). This agrees with the viral titer as determined by

TCID50 assay (Figure 1c). In both experiments, a 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 Figure 2, 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.

3.4.2 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. At 48hr after transfection, cells were infected with 1 MOI of PEDV CO. Culture supernatants were collected for virus titration at 24h post virus infection. As shown in Figure 3, overexpression of G3BP reduced PEDV titer by 100-fold compared to that of control plasmid

3.4.3 PEDV induces transient SGs and PEDV-infected cells fail to form SGs after sodium arsenite treatment

To determine whether PEDV infection induces SGs, Vero-76 cells were mock infected or infected with 5 MOI of PEDV CO or icPEDV-ΔORF3-EGFP for 24-48 hr. Then cells were stained with antibody specific for G3BP and PEDV N specific antibody. We

observed some G3BP positive cytoplasmic granules in some PEDV-infected cells at 24 and 48 hr post infection (Figure 4a). Approximately 30% of infected cells showed SGs (Figure 4c). To examine whether PEDV-infection interferes with SGs induced by sodium arsenite treatment, we treated cells with 0.5 mM sodium arsenite for 30 min prior to immunofluorescence staining. As shown in Figure 4B and 4C, SGs appeared in mock-infected cells after treatment with sodium arsenite, whereas PEDV-infected cells did not show SGs after sodium arsenite treatment. Approximately 45 % of cell exhibits such granules. To further confirm this observation, we repeated the experiment by using antibody specific for TIA-1 as a marker for SGs, similar results were observed (data not shown).

3.5 Discussion

G3BP1 is one of the key components of SGs. Overexpression of G3BP1 induce SG formation [71]. Cleavage of G3BP1 by viral protease or sequestering of G3BP1 by viral proteins inhibits formation of SGs. We observed that knockdown of G3BP 1 enhanced PEDV replication. Overexpression of G3BP1 reduced PEDV replication. Our data suggested an antiviral role of G3BP1 against PEDV possibly through SGs. Similar observations were reported for other viruses including mammalian orthoreovirus (MRV) [19] and sindbis virus [20]. G3BP1 appeared to play no role in PRRSV replication. A proviral role of G3BP1 in Hepatitis C Virus (HCV) [106], chikungunya virus [96] and Respiratory syncytial virus [68] was reported.

Different viruses or even viruses within the same family exhibit unique features in their interaction with key components of SGs and in forming different patterns of SGs. At least three distinct patterns of SGs including stable, transient, oscillating have been

described following infections with either RNA or DNA viruses [78]. We observed that PEDV-Co induced transient SGs in some infected Vero-76 cells at 24 and 48 h of infection, but not at 6 and 12 h after infection. This contrasts with poliovirus, which causes transient SG formation during the early phase of infection and disperse SGs by cleavage of G3BP1 during later stages of infection [110]. RSV has stable SG formation [68]. Hepatitis C virus-infected cells form oscillating SG corresponding with the levels of growth arrest DNA-damage-inducible 34 (GADD34) and the phosphorylated form of eIF-2α [32]. In contrast, 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 [8]. Influenza virus restricts SG accumulation [50]. Mouse hepatitis coronavirus (MHV), a member of coronaviridae, induced SG formation at early infection [90]. The percentages of SGs positive cells increases as PRRSV infection progresses, suggesting that PRRSV induces stable SGs [119].

West Nile Virus inhibits SGs upon arsenite treatment by up-regulating glutathione S-transferase (GSH) in cells to counteract the reactive oxygen species (ROS) [6]. We observed that PEDV-infected cells failed to form SGs when exposed to arsenite. Cells infected with PEDV and treated with SA induced significantly less stress granules than that of the non-infected cells treated with arsenite. Since virus infections typically induce both ROS and antioxidant response simultaneously [6], it is reasonable to speculate that activation of antioxidant pathway by PEDV leads to the depletion of inhibition of SGs that are induced by arsenite treatment.

We observed an increase in the transcription of inflammatory cytokines in G3BP1 knockdown cells after PEDV infection compared to control cells. Either PEDV is replicating much in G3BP1 knockdown cells or presence of G3BP1 may impact the inflammatory cytokine signaling pathway. A previous has also shown that knockdown of G3BP1 caused increment in NF-kB and transcription of inflammatory cytokines in Porcine Reproductive and Respiratory Virus infected cells [119]. The detailed mechanisms by which G3BP1 knockdown affects virus induced inflammatory cytokines need to be addressed in future studies.

One limitation of this study was that we used Vero-76 cells, which are not the natural host cells for PEDV. It would be more accurate and interesting to examine the role of G3BP in PEDV replication in porcine primary intestinal epithelial cells in future study.

In summary, we have shown that PEDV infection induces SGs and interferes with the formation of SGs induced by arsenite treatment. Knockdown of G3BP1 significantly enhanced viral replication and transcription of inflammatory cytokines. Overexpression of exogenous G3BP1 reduced virus replication. Furthermore, studies are needed to uncover the mechanisms by the virtue of which G3BP1 impacts PEDV replication.

Acknowledgements

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Figure legends

Figure 1. Knockdown of G3BP1 reduces PEDV replication. A: Western blotting analysis showing the successful knockdown of G3BP1 protein expression by silencing RNA. Three replicates are shown. An average of approximately 50% knockdown efficiency is achieved. G3BP1 knockdown efficiency was calculated based on the band densities after normalization with protein loading control beta-actin. B: PEDV replication is reduced in G3BP1 knockdown cells compared to control cells. * indicates $p < 0.05$. The average and standard deviations of three experiments are shown. C: The nucleocapsid (N) gene copies are decreased in cells with G3BP1 knockdown compared to control cells as determined by real-time RT-PCR. The average and standard deviations of three experiments are shown.

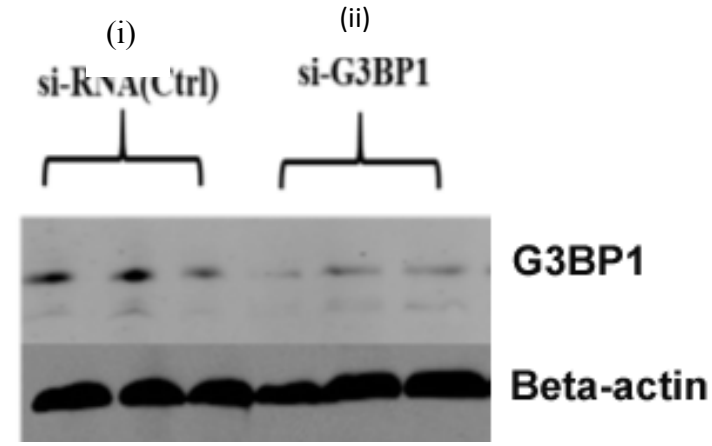
Figure 2. The mRNA levels of pro-inflammatory cytokines are increased in cells with G3BP1 knockdown after PEDV infection. The relative amount of IL-1β and TNF-α transcripts were determined by real-time RT-PCR. The average and standard deviations of three experiments are shown.

Figure 3. Overexpression of exogenous G3BP reduces PEDV replication. * indicates $p < 0.05$. The average and standard deviations of three experiments are shown.

Figure 4. PEDV induces SGs in some infected cells and PEDV-infected cells are resistant to SG formation induced by arsenite treatment. **A:** Some PEDV-infected cells showed cytoplasmic granules that are positive for G3BP at 24 or 48 hr post infection. Yellow arrows

indicate virus-infected cells with cytoplasmic SGs. **B:** PEDV-infected cells fail to show SGs after sodium arsenite treatment. Yellow arrows indicate virus-infected cell with little or no SGs. White arrows showed SGs in non-infected cells. **C:** PEDV infection reduces the % of SGs after sodium arsenite treatment. * indicates $p < 0.05$ when compared to Mock + AS. ** indicates $p < 0.05$ between PEDV and PEDV + AS group.

Figure 1A


(i)
si-RNA(Ctrl)
(ii)
si-G3BP1
G3BP1
Beta-actin

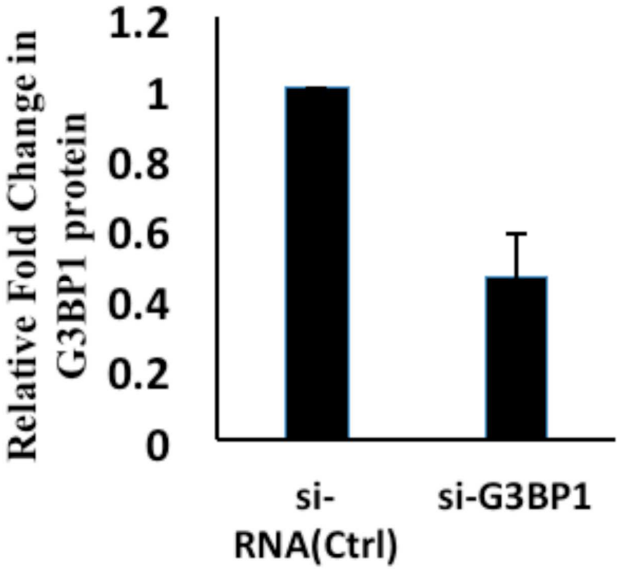

Relative Fold Change in G3BP1 protein
1.2
1
0.8
0.6
0.4
0.2
0
si-RNA(Ctrl)
si-G3BP1

Figure 1B

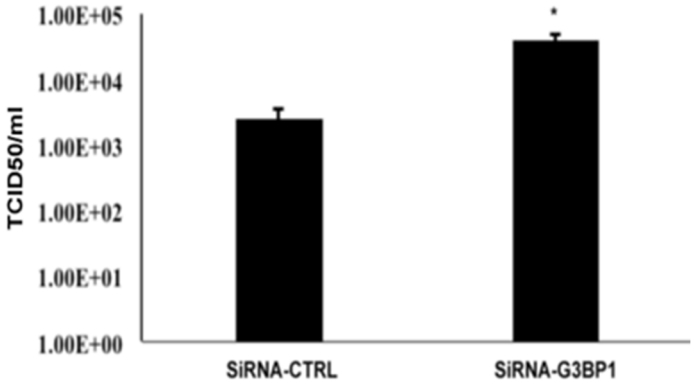


Figure 1C

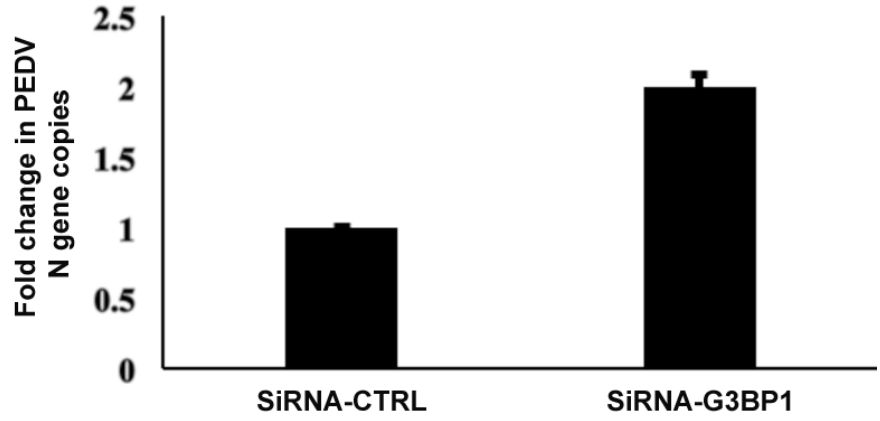


Figure 2

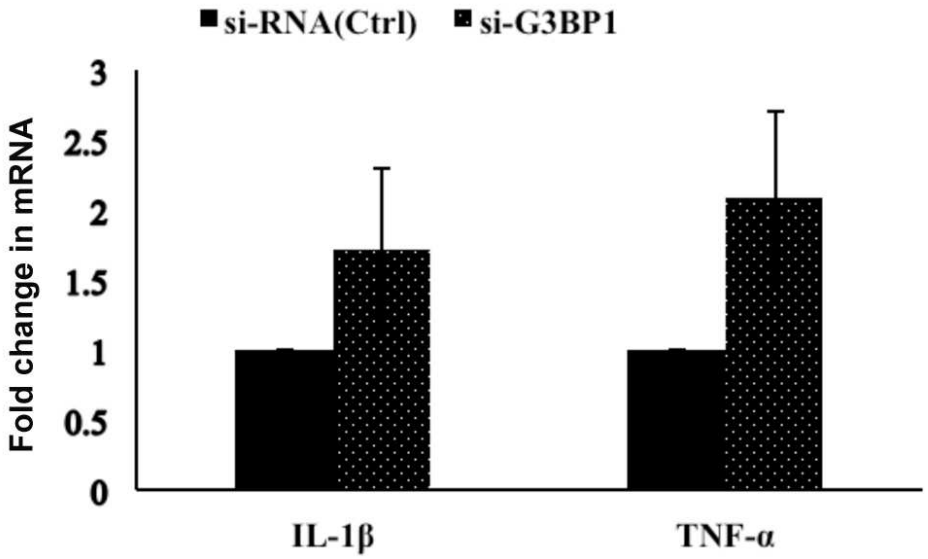


Figure 3

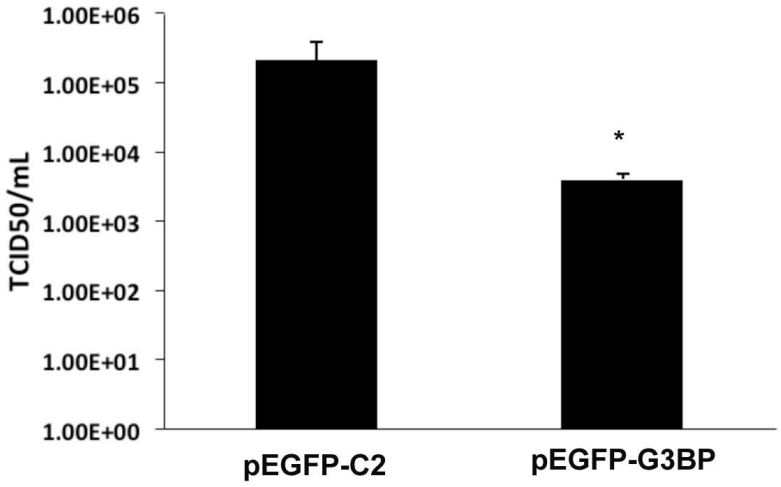
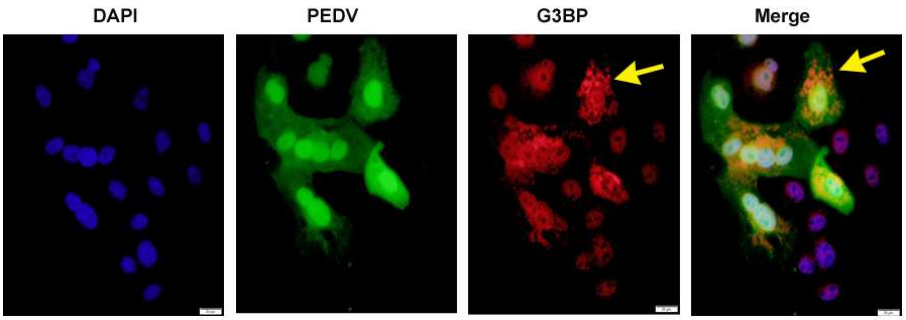


Figure 4A



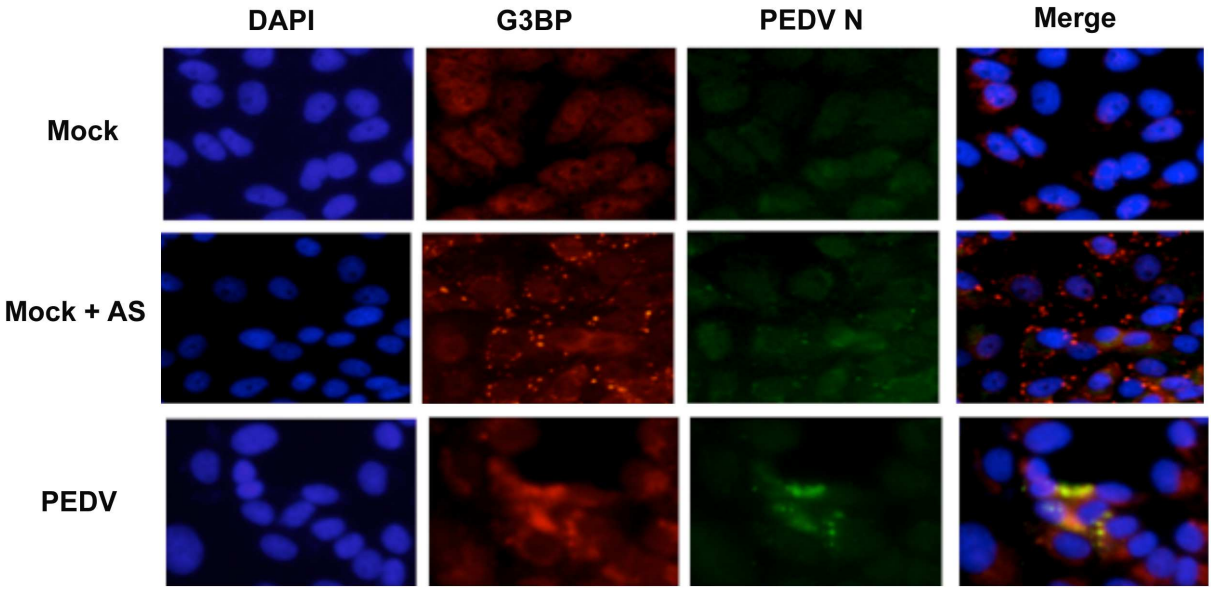


Figure 4B

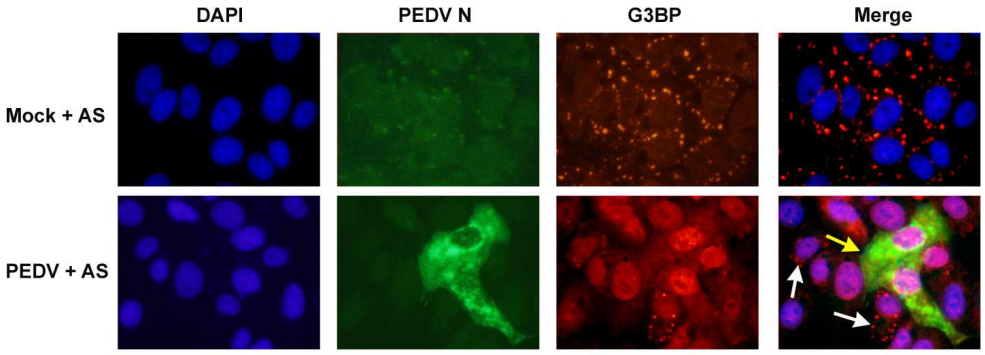


Figure 4C

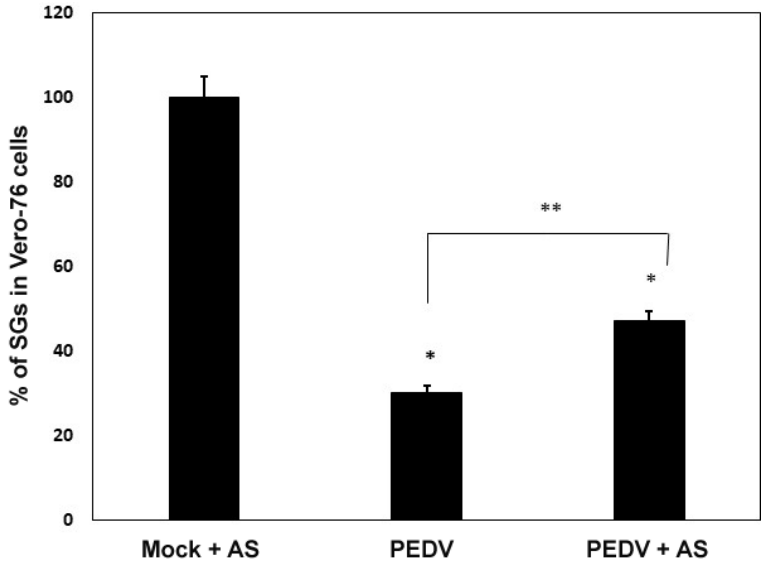


Figure 5. Primers used for real time RT-qPCR .

Primers	**5′-3′ sequence**
β-Actin FP	5’-TTG CTG ACA GGA TGC AGA AGG AGA-3’
β-Actin RP	5’-ACT CCT GCT TGC TGA TCC ACA TCT-3’
PEDV-N FP	5’-GAA TTC CCA AGG GCG AAA AT-3’
PEDV-N RP	5’-TTT TCG ACA AAT TCC GCA TCT-3’
IL-1β FP	5’-CGG CAT CCA GCT ACA AAT CT-3’
IL-1β RP	5’-CCA GCA TCT TCC TTA GCT TCT C-3’
TNF-α FP	5’-CAT CTA CTC CCA GGT CCT CTT-3’
TNF-α RP	5’-TTG ACC TTG GTC TGG TAG GA-3’

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