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Restriction of porcine reproductive and respiratory syndrome virus replication by galectin-1

Liwei Li[a,b,1], Kuan Zhao[a,1], Fei Gao[a,b], Yifeng Jiang[a,b], Tongling Shan[a,b], Wu Tong[a,b], Hao Zheng[a,b], Lingxue Yu[a,b], Guoxin Li[a,b], Zhiyong Ma[a], Guangzhi Tong[a,b,*]

[a] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, PR China
[b] Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Disease and Zoonose, Yangzhou University, Yangzhou, 225009, PR China

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) causes great economic losses to the swine industry globally; however, effective control measures for this virus are limited. Here, we screened a porcine alveolar macrophage (PAM) cDNA library with a yeast two-hybrid system to reveal that galectin-1 (Gal-1), an endogenous innate immune protein encoded by *LGALS1*, interacts with nonstructural protein 11 (Nsp11) of PRRSV. Western blotting and viral titer assays indicated that Gal-1 overexpression suppressed replication in multiple PRRSV strains ($P < 0.001$), whereas *Gal-1* knockdown or knockout increased viral titer and nucleocapsid protein expression. The Gal-1-specific anti-PRRSV effect was associated with the endoribonuclease domain of Nsp11 through inactivation of interferon-antagonist function and stimulation of interferon-stimulated gene expression. Additionally, Gal-1 interacted with PRRSV E protein but not with PRRSV glycoproteins, and recombinant Gal-1 treatment inhibited PRRSV in PAMs and MARC-145 cells. Furthermore, Gal-1 inhibited replication in multiple viruses, including equine arteritis virus, porcine epidemic diarrhea virus, pseudorabies virus, Japanese encephalitis virus, and classical swine fever virus, suggesting its potential broad application for antiviral strategies. Our findings provide insight into the important role of Gal-1 in PRRSV pathogenesis and its potential use as a novel therapeutic target against PRRSV infection.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) affects the swine industry worldwide, manifesting as reproductive problems in sows and respiratory problems in piglets (Chand et al., 2012). Despite the existence of commercial vaccines, PRRS virus (PRRSV) constantly evolves, allowing it to evade existing immunity in vaccinated herds in China (Han and Yoo, 2014; Jiang et al., 2015).

PRRSV has a 15-kb RNA genome containing at least 10 open reading frames (ORFs), with ORF1a and ORF1b producing at least 14 nonstructural proteins (Nsps) (Fang and Snijder, 2010). ORF2a through ORF7 encode structural proteins, including four membrane-associated glycoproteins (GP2a, GP3, GP4, and GP5), three unglycosylated membrane proteins (E, ORF5a, and M), and a nucleocapsid protein (N) (Sun et al., 2013). PRRSV infection causes type I interferon (IFN) suppression in porcine alveolar macrophages (PAMs) and in the lungs where PRRSV actively replicates (Buddaert et al., 1998). Several PRRSV proteins downregulate IFN induction, including Nsp1, Nsp2, Nsp4, Nsp11, and N (Lunney et al., 2016). Of these, only Nsp11 has RNA nuclease activity (Sun et al., 2016).

Nsp11 is a 223-amino acid protein produced from a large polyprotein (PP1ab) and contains a highly conserved endoribonuclease (EndoU) domain in its C-terminal region, which is unique to *Nidovirales* viruses (Nedialkova et al., 2009). The EndoU domain consists of two subdomains. Subdomain A maintains nuclease activity critical for both viral replication and IFN antagonism (Shi et al., 2011; Sun et al., 2016). PRRSV Nsp11 inhibits host innate immune responses, such as type I IFN transcription, RNAi innate immune response, and interleukin-1β production, thereby playing an important role in PRRSV infection (Wang et al., 2015a, b).

Galectins are a widely distributed protein family that modulates antiviral immunity and direct host–virus interactions (Vasta, 2009). Galectin-1 (Gal-1), encoded by *LGALS1*, is a highly conserved protein (Camby et al., 2006) with antiviral activity verified in infections caused

* Corresponding author at: Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences., No. 518, Ziyue Road, Minhang District, Shanghai, 200241, PR China.
E-mail address: gztong@shvri.ac.cn (G. Tong).
[1] These authors contributed equally to this work.

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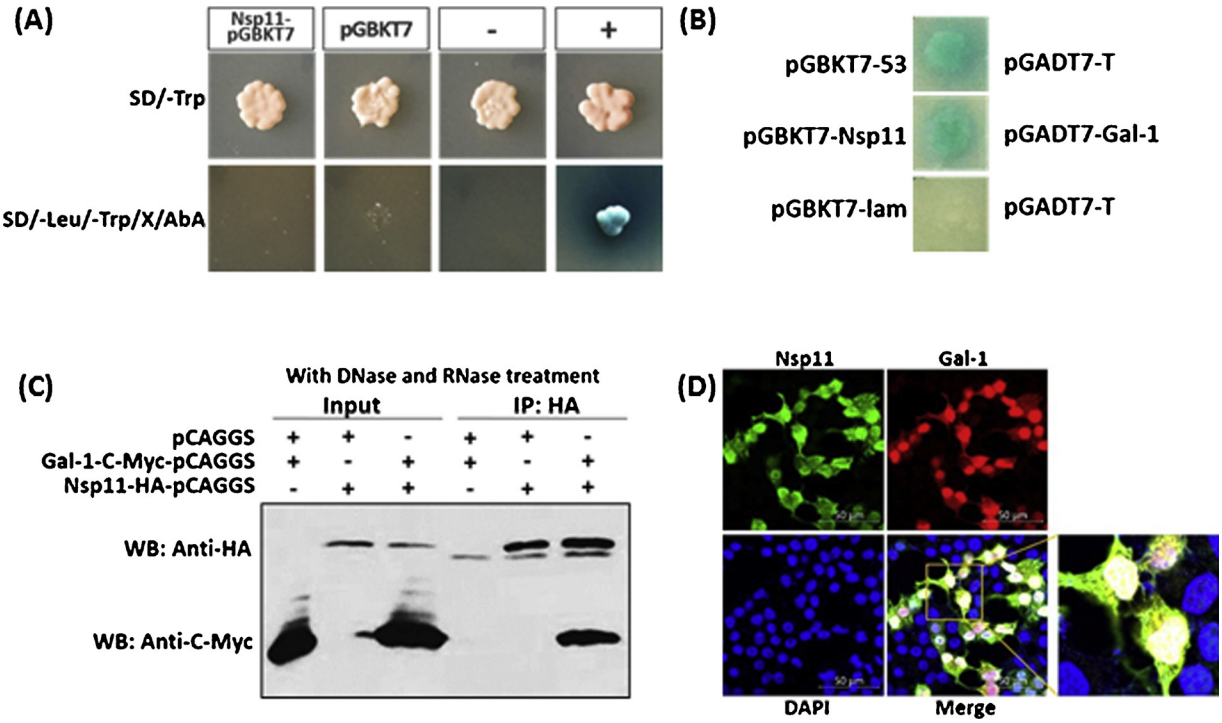

Fig. 1. Interaction between Gal-1 and PRRSV Nsp11. (A) Y2HGold yeast cells were transformed with pGBKT7-Nsp11 and grown on SD medium with –Trp or –Ade/–His/–Leu/–Trp supplemented with X-α-gal and ABA, respectively. Positive control: pGBKT7-53; negative control: pGBKT7-Lam. (B) pGADT7-Gal-1 co-transfected with pGBKT7-Nsp11 into Y2HGold yeast cells on QDO plates containing X-α-gal and ABA. The positive interactors (blue) were identified by reporter gene activation. (C) Interaction of Gal-1 with Nsp11 by Co-IP. HEK293 T cells were co-transfected with Gal-1-C-Myc-pCAGGS and Nsp11-HA-pCAGGS in the presence of DNase and RNase. Cell lysates were precipitated with an anti-HA mAb in conjunction with protein A Sepharose and further assessed by WB with anti-HA and anti-C-Myc antibodies, respectively. (D) HEK293 T cells were co-transfected and double stained with a rabbit anti-HA antibody and a mouse anti-Myc antibody, followed by FITC-conjugated anti-rabbit IgG (green) and Alexa Fluor-conjugated anti-mouse IgG (red). Cell nuclei were counterstained with 1 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

by Nipah virus (Levroney et al., 2005), influenza virus (Yang et al., 2011), dengue virus (Toledo et al., 2014), and human simplex virus 1 (Rajasagi et al., 2012). By contrast, previous studies report that Gal-1 promotes infections caused by human immunodeficiency virus 1 (Ouellet et al., 2005), human T-lymphotropic virus (Gauthier et al., 2008), and enterovirus 71 (Lee et al., 2015). However, its ability to modulate PRRSV infection has not been reported.

Exploration of host–virus interactions enables discovery of potential antiviral proteins and the development of effective alternative strategies to combat multiple circulating PRRSV strains. Numerous recent studies have investigated host–virus interactions associated with PRRSV pathogenesis (Jin et al., 2017; Li et al., 2018; Zhao et al., 2019, 2015); however, few studies have investigated the association between host–virus interaction and Nsp11. Therefore, we performed yeast two-hybrid screening using Nsp11 as bait to identify interacting elements in a cDNA library of PAMs in order to screen attractive targets for the development of anti-PRRSV therapies.

2. Materials and methods

2.1. Cells and virus

PAMs, MARC-145 cells, HEK293 T cells, BHK-21 cells, and PK-15 cells were cultured as described previously (Gao et al., 2018; Zhao et al., 2018; Zheng et al., 2018). The highly pathogenic PRRSV vHuN4 (GenBank accession no. EF635006), attenuated vaccine virus vHuN4-F112, vJX143 (GenBank accession no. EU708726), vJXM100 (GenBank accession no. GQ475526), classic type 2 strain vAPRRS (GenBank accession no. GQ330474), a classic type 1 strain vSHE (GenBank accession No. GQ461593), equine arteritis virus (EAV) strain vEAV030 (GenBank accession No. NC002532), porcine epidemic diarrhea virus (PEDV) strain JS-2013, pseudorabies virus (PRV) strain JS-2012 (GenBank accession No. KP257591), Japanese encephalitis virus (JEV) strain HEN0701 (GenBank accession No. FJ495189), and a swine fever virus (CSFV) Shimen strain (GenBank accession No. AF092448) were used.

2.2. Construction of plasmids

Nsp11 from vHuN4 was cloned into pGBKT7 or pCAGGS to generate pGBKT7-Nsp11 or Nsp11-HA-pCAGGS, respectively. *LGALS1* was cloned into pGADT7 or pCAGGS to generate pGADT7-Gal-1 or Gal-1-C-Myc-pCAGGS, respectively. Three fragments of *Nsp11* were inserted downstream of three Flag tags, generating Nsp11-N (aa 1–123)-pCAGGS, Nsp11-C (aa 124–223)-pCAGGS, and Nsp11-A (aa 124–183)-pCAGGS. The plasmids expressing GP2a, GP3, GP4, GP5, and E were constructed by cloning the corresponding fragments into pLOV.CMV (modified version of pLOV.CMV.GFP). Primers are listed in Table S1.

2.3. Yeast two-hybrid screening

Yeast two-hybrid screening was performed using pGBKT7-Nsp11 as bait to screen a PAM cDNA library using the Matchmaker Gold Yeast two-hybrid system (Clontech, TaKaRa, Dalian, China). Briefly, the yeast strain Y2HGold was transformed with pGBKT7-Nsp11, mated with Y187, and selected on quadruple dropout (QDO) plates for 7 days. Positive colonies were re-selected on high-stringency QDO plates containing 0.04 mg/ml 5-bromo-4-chloro-3-indoyl-α-D-galactopyranoside (X-α-gal) and 0.07 μg/ml aureobasidin A (ABA), and confirmed by sequencing. Sequence alignments were performed using BLAST (https://blast.ncbi.nlm.nih.gov/). The candidate gene was cloned into pGADT7 to perform a one-to-one yeast hybridization assay. pGBKT7-53 and

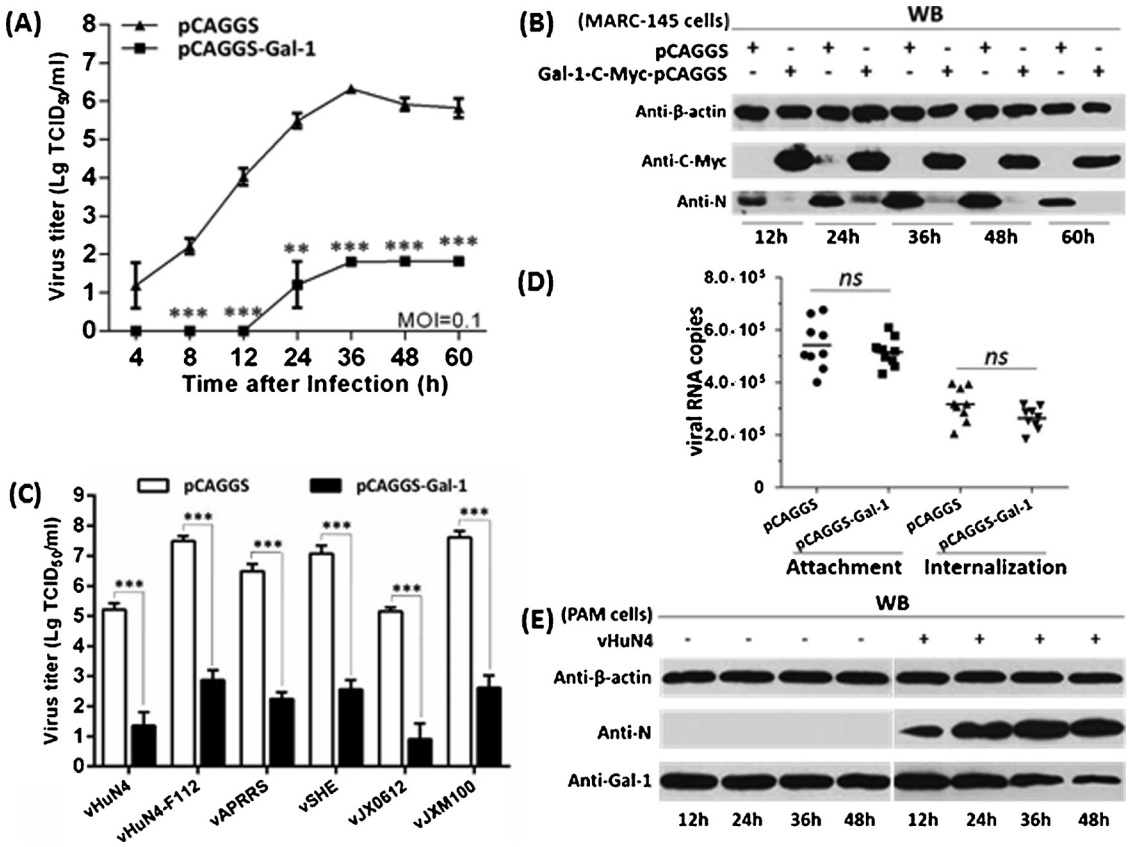

Fig. 2. Gal-1 overexpression exerts strong anti-PRRSV activity, and Gal-1 is downregulated in response to PRRSV *in vitro*. (A) PRRSV proliferation in MARC-145 cells transfected with Gal-1-C-Myc-pCAGGS or control plasmids. Supernatant was collected at the indicated times and titrated. (B) Time course of transfected Gal-1 and N protein expression after PRRSV infection of MARC-145 cells according to WB analysis. (C) Viral titers for MARC-145 cells transfected with Gal-1-C-Myc-pCAGGS or control plasmids 36 h prior to PRRSV (vHuN4, vHuN4-F112, vAPRRS, vSHE, vJX0612, or vJXM100) infection (MOI = 0.1). (D) Viral attachment and internalization were analyzed by qPCR. Data represent the mean ± standard deviation of three independent experiments. Statistical significance was analyzed using *t* tests; ***$P < 0.001$, *ns*, not significant. (E) Time course of endogenous *Gal-1* expression following infection of PAMs with PRRSV vHuN4 at an MOI of 0.1. Cells were collected at the indicated times, and WB was performed to detect Gal-1 levels in PRRSV-infected cells and mock cells.

pGBKT7-lam were used as positive and negative controls, respectively.

2.4. Co-immunoprecipitation (Co-IP) and confocal imaging

HEK293 T cells were co-transfected with the indicated plasmids using X-tremeGENE DNA transfection reagent (Roche Applied Science, Penzberg, Germany). IP and confocal imaging assays were performed as described previously (Zhao et al., 2018), except that mouse anti-E protein polyclonal antibody (pAb; 1:100) produced in our laboratory, rabbit anti-HA monoclonal antibody (mAb; Cell Signaling Technology; Danvers, MA, USA; 1:1000), and mouse anti-C-Myc mAb (Cell Signaling Technology; 1:1000) were used.

2.5. Virus challenge

To determine the effect of Gal-1 on PRRSV replication, MARC-145 cells were transfected with pCAGGS or Gal-1-C-Myc-pCAGGS, followed by vHuN4 infection (multiplicity of infection, MOI = 0.1). Cells were harvested at 12-, 24-, 36-, 48-, and 60-h post-infection (hpi) using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by western blotting (WB) using an anti-N protein pAb (1:1000) produced in our laboratory. Recombinant Gal-1 (rGal-1) and rGal-3 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in serum-free DMEM (Sigma-Aldrich). MARC-145 cells were pretreated with rGal-1 or rGal-3 for 2 h prior to vHuN4 infection. Viral titers were measured by standard $TCID_{50}$ assay using the Reed and Muench method (Reed and Muench, 1938). Viral attachment and internalization experiments were performed according to a previous study (Zhao et al., 2018).

2.6. RNA interference

Small-interfering RNA (siRNA) were used to knockdown *Gal-1* expression, with SiGal-1 and control siRNA (SiCon) synthesized by GenePharma (Shanghai, China). PAMs and MARC-145 cells were transfected with siRNAs (100 nM) using X-tremeGENE siRNA transfection reagent (Roche), followed by PRRSV infection. Gal-1 and N protein levels were determined at 24 hpi.

2.7. Construction of gene-knockout cell lines

The CRISPR/Cas9 system was used to generate *Gal-1*-knockout (KO) MARC-145 cells. The knockout strategy involved introducing frameshift mutations into the coding sequence, as described previously (Li et al., 2018). WB with a specific Gal-1 antibody (D608 T; Cell Signaling Technology; 1:1000) was performed to confirm the absence of the target protein.

2.8. Luciferase-reporter assays

HEK293 T cells were plated in 12-well plates and transfected with a mixture of IFN-β-luc and pRL-TK-Renilla luciferase plasmids and appropriate control or protein-expressing plasmid(s). Cells were infected with Sendai virus (SeV) and collected at 24 hpi. Luciferase activity was

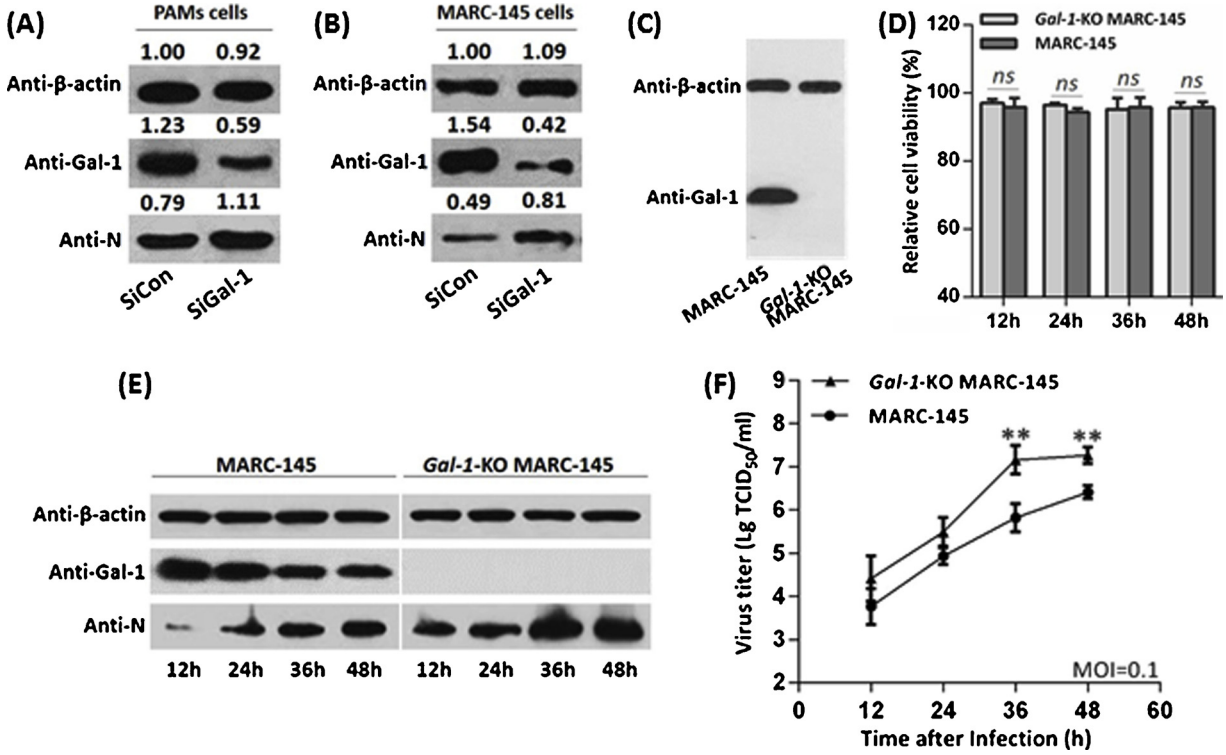

Fig. 3. Effect of *Gal-1* knockdown or knockout on PRRSV replication. (A) PAMs and (B) MARC-145 cells were transfected with SiCon or SiGal-1 for 30 h, infected with vHuN4 for 24 h, and collected for WB analysis of endogenous Gal-1 and N protein levels. (C) WB analysis of endogenous Gal-1 levels in *Gal-1*-KO MARC-145 cells. β-actin level was used as a loading control. (D) Evaluation of the effect of *Gal-1*-KO on cell proliferation and viability at the indicated times. (E) Time course of endogenous Gal-1 and N protein levels following PRRSV infection of *Gal-1*-KO MARC-145 cells or wild-type MARC-145 cells at the indicated times. (F) Viral growth in *Gal-1*-KO MARC-145 and wild-type MARC-145 cells. Supernatant was collected, as indicated, and titrated. Data are the mean ± standard deviation of three independent experiments. Statistical significance was analyzed using t-tests; **, $P < 0.01$. *ns*, not significant.

measured with a dual-luciferase assay (Promega, Madison, WI, USA). Reporter gene activity was determined by normalization of firefly luciferase activity against Renilla luciferase activity.

2.9. Quantitative real-time PCR (qPCR)

Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific), and a PrimeScript first strand cDNA synthesis kit (Takara) was used for reverse transcription. SYBR Premix Ex Taq (Takara) was used to quantify levels of *interferon-stimulated gene 15* (*ISG15*), *2′-5′-oligoadenylate synthetase 1* (*OAS1*), and *MX1* mRNA. Relative expression levels were analyzed using the ΔΔCt method (Bookout et al., 2006), with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA used as a control. Primers are listed in Table S1.

2.10. Bimolecular fluorescence complementation (BiFC) assay

BiFC assays were conducted as previously described, with minor modifications (Zhang et al., 2012). *LGALS1*, *ORF2b*, and four fragments of *ORF2b* were amplified by PCR using gene-specific primers and cloned into the N-terminus of VN or VC with a flexible linker and designated E-VC, Gal-1-VN, E(aa 1–48)-VC, E(aa 1–21)-VC, E(aa 21–48)-VC, and E (aa 48–73)-VC, respectively. HEK293 T cells were co-transfected with 500 ng of each BiFC plasmid, and at 24-h post-transfection (hpt), cells were visualized using an Olympus inverted fluorescence microscope (Olympus, Tokyo, Japan).

2.11. Statistical analysis

All experiments were performed at least three times independently. Statistical significance was analyzed using Student's *t* test, and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Gal-1 interacts with PRRSV Nsp11 in both the yeast system and HEK293 T cells

pGBKT7-Nsp11 transfection into Y2HGold yeast cells showed no toxicity affecting yeast colony size and did not induce self-activation of reporter genes (Fig. 1A). Therefore, pGBKT7-Nsp11 was used to screen the cDNA library. Sequence alignments were performed using BLAST (https://blast.ncbi.nlm.nih.gov/), and the candidate interacting protein was identified as Gal-1. A one-to-one yeast hybridization assay was performed to verify interaction between Nsp11 and Gal-1 on QDO plates containing X-α-gal and ABA (Fig. 1B). The results demonstrated that Gal-1 interacted with PRRSV Nsp11 in the yeast hybridization system.

We then validated the interaction by Co-IP, revealing that Nsp11 efficiently co-immunoprecipitated with Gal-1 in HEK293 T cells (Fig. 1C). In confocal imaging assays, green fluorescence (Nsp11) was superimposed on areas of red fluorescence (Gal-1) in both the nucleus and cytoplasm (Fig. 1D), thereby demonstrating Gal-1 co-localization with Nsp11 in HEK293 T cells.

3.2. Gal-1 exhibits anti-PRRSV activity

To determine the effect of Gal-1 on PRRSV replication, induction of PRRSV infection was conducted at 36 hpt. The viral titer in Gal-1-C-Myc-pCAGGS-transfected cells was significantly lower ($P < 0.001$) than that in corresponding control cells at different time points (Fig. 2A). WB showed that Gal-1 overexpression strongly reduced

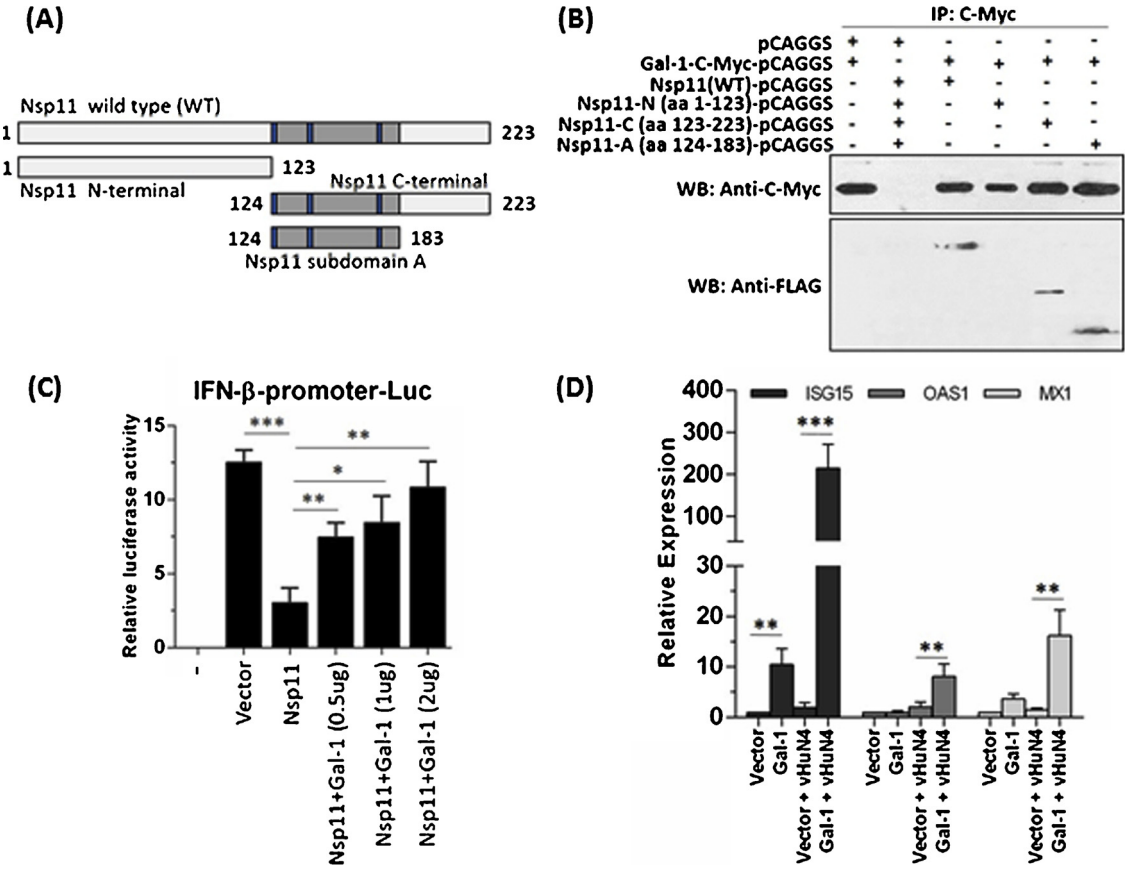

Fig. 4. Gal-1 inactivates the IFN-antagonist function of Nsp11 and stimulates ISG secretion. (A) Schematic diagrams of Nsp11 (wild-type), Nsp11-N (aa 1–123), Nsp11-C (aa 124–223), and Nsp11-A (aa 124–223). (B) Determination of the binding regions of Nsp11 with Gal-1 by Co-IP. HEK293 T cells were co-transfected with the indicated plasmids, and cell lysates were precipitated with an anti-C-Myc mAb in conjunction with protein A Sepharose and subjected to WB with mouse anti-C-Myc and rabbit anti-FLAG antibodies, respectively. (C) HEK293 T cells were co-transfected with the IFN-β-Luc firefly luciferase reporter plasmid, Renilla luciferase control reporter plasmid pRL, pCAGGS vector, Nsp11-HA-pCAGGS, and Gal-1-C-Myc-pCAGGS at the concentrations indicated. Cell-lysate luciferase activities were analyzed using the dual luciferase-reporter assay system. (D) qPCR analysis of *ISG15*, *OAS1*, or *MX1* expression in MARC-145 cells transfected with Gal-1-C-Myc-pCAGGS or control plasmids, followed by infection with vHuN4 for 36 h at an MOI of 0.1 or left untreated. Data were normalized to *GAPDH* expression and are the mean ± standard deviation of three independent experiments. Statistical significance was analyzed using t-tests; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

accumulation of N protein, confirming the restriction activity of Gal-1 (Fig. 2B). We then investigated the antiviral activity of Gal-1 against other PRRSV strains, including vHuN4-F112, vAPRRS, vSHE, vJX0612, and vJXM100. Gal-1 overexpression significantly reduced viral titers of all strains in MARC-145 cells (Fig. 2C; $P < 0.001$). Collectively, these data show that Gal-1 strongly reduces PRRSV translation and replication in multiple strains and two genotypes. We reasoned that Gal-1-specific inhibition of PRRSV replication might be caused by interference with PRRSV attachment and internalization. To test this, we performed PRRSV-attachment and -internalization experiments. As shown in Fig. 2D, Gal-1 overexpression did not affect PRRSV attachment and internalization, indicating that Gal-1 exerts inhibitory effects after PRRSV entry.

3.3. PRRSV infection leads to endogenous Gal-1 reduction

We then investigated the consequences of PRRSV infection on endogenous cellular Gal-1 expression. PAMs, the primary target cell for PRRSV replication *in vivo*, were used to analyze changes in Gal-1 expression. As shown in Fig. 2E, Gal-1 was substantially downregulated in response to PRRSV infection, especially at 36 hpi and 48 hpi.

3.4. Gal-1 knockdown or knockout facilitates PRRSV replication

We designed an siRNA (siGal-1) against *Gal-1*, finding that Gal-1 expression was clearly inhibited by siGal-1 in PAMs and MARC-145 cells as compared to levels observed in controls (Fig. 3A, B). Cells transfected with siGal-1 were then infected with vHuN4, and we found that compared with controls, viral N protein levels were increased at 24 hpi (Fig. 3A, B), indicating that Gal-1 knockdown promoted viral translation and replication in PAMs and MARC-145 cells. To confirm this finding, the *Gal-1* gene was knocked out in MARC-145 cells, and Gal-1 was subsequently confirmed as absent (Fig. 3C). An MTT assay performed to analyze the potential effect of *Gal-1* knockout on cell proliferation and viability revealed no appreciable effect (Fig. 3D), indicating successful generation of *Gal-1*-KO MARC-145. Compared with MARC-145 cells, we found that N protein levels in *Gal-1*-KO MARC-145 cells increased significantly following PRRSV infection (MOI = 0.1; Fig. 3E). Similarly, the effect of *Gal-1*-KO on viral growth was confirmed, revealing that *Gal-1* KO significantly increased viral titer as compared with that in MARC-145 cells, especially at 36 hpi and 48 hpi (Fig. 3F). Overall, knockdown or knockout of endogenous *Gal-1* not only reversed the inhibitory effect but also facilitated PRRSV replication, confirming the antiviral effect of Gal-1.

3.5. Gal-1 inactivates the IFN-antagonist function of Nsp11 and stimulates secretion of ISGs

To identify the interacting regions, three truncated Nsp11 variants [Nsp11-N (aa 1–123), Nsp11-C (aa 124–223), and Nsp11-A (aa 124–183)] were constructed (Fig. 4A), and each Nsp11 domain interacting with Gal-1 was examined using Co-IP. Nsp11-C and Nsp11-A

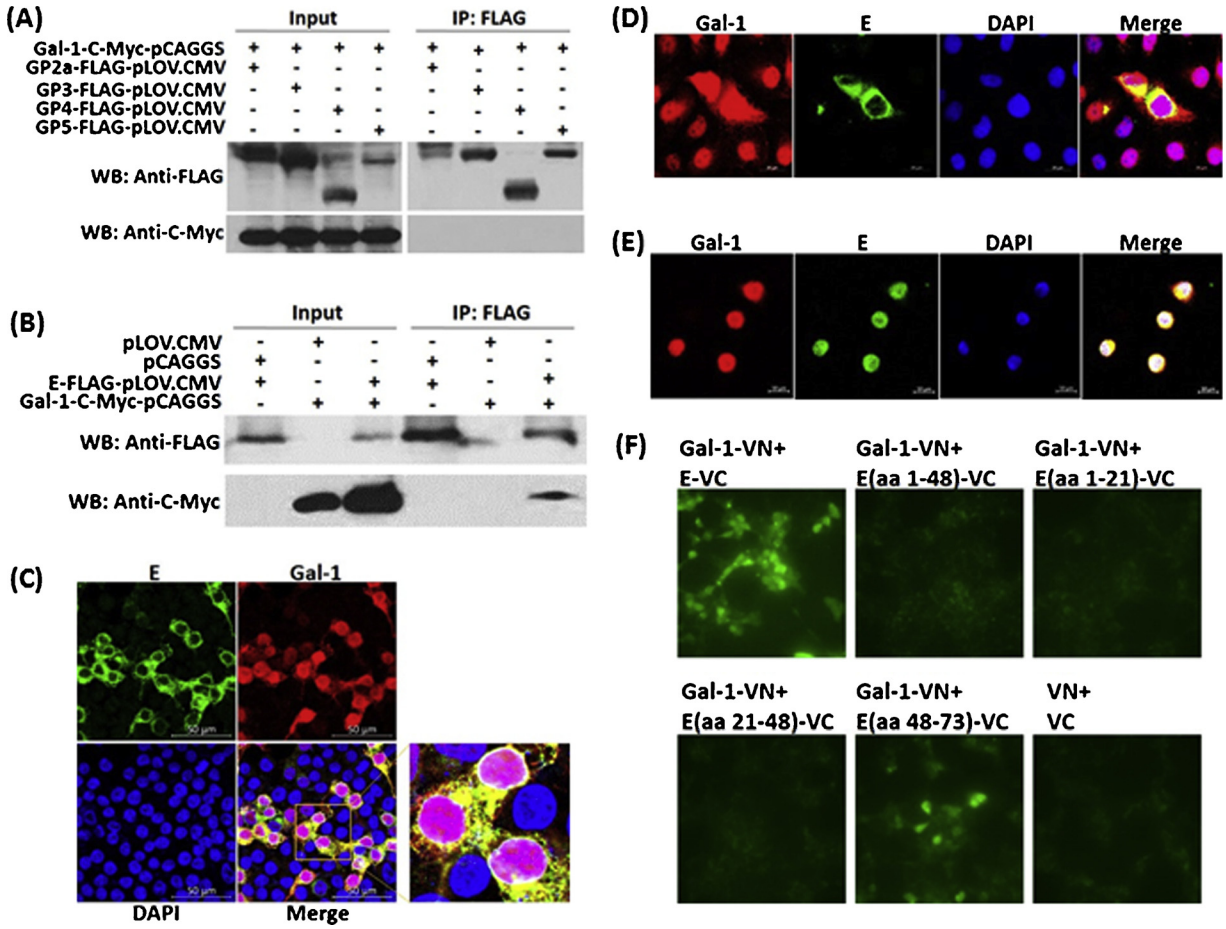

Fig. 5. Gal-1 interacts with PRRSV E protein. (A) Interaction of Gal-1 with PRRSV GP2a, GP3, GP4, and GP5 protein by Co-IP. (B) Interaction of Gal-1 with PRRSV E protein by Co-IP. HEK293 T cells were co-transfected with Gal-1-C-Myc-pCAGGS and E-FLAG-pLOV.CMV. Cell lysates were precipitated with an anti-FLAG mAb in conjunction with protein A Sepharose and further assessed by WB with an anti-FLAG antibody and anti-C-Myc antibody, respectively. (C) HEK293 T cells were co-transfected, and cells at 24 hpt were fixed and double stained with rabbit anti-FLAG and mouse anti-C-Myc antibodies, followed by FITC-conjugated anti-rabbit IgG (green) and Alexa Fluor-conjugated anti-mouse IgG (red). Cell nuclei were counterstained with DAPI (blue). (D, E) Co-localization of PRRSV E and endogenous Gal-1 in MARC-145 cells and PAMs. Cells infected with PRRSV were fixed at 24 hpi and then subjected to indirect immunofluorescence to detect E protein and Gal-1. (F) BiFC analysis of Gal-1 interaction with E protein. HEK293 T cells were co-transfected with the indicated plasmids, and BiFC fluorescence was observed at 24 hpt (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

interacted with Gal-1, whereas Nsp11-N did not (Fig. 4B), indicating Nsp11 subdomain A (aa 124–183) maintaining EndoU activity as the Gal-1-binding region. Because Nsp11 is an IFN antagonist with EndoU activity essential for inhibiting induction of IFN-β (Shi et al., 2011), we examined activation of the *IFN-β* promoter by Nsp11 and Gal-1. As shown in Fig. 4C, Nsp11 inhibited SeV-induced activation of the *IFN-β* promoter ($P < 0.001$) but was substantially rescued by increasing Gal-1 expression in a dose-dependent manner. Furthermore, Gal-1 overexpression significantly upregulated mRNA levels of *ISG15*, *OAS1*, and *MX1* in PRRSV-infected MARC-145 cells (Fig. 4D). Moreover, *ISG15* levels increased by 10- or 200-fold in mock or PRRSV-infected MARC-145 cells, respectively, indicating that transfection of Gal-1 into MARC-145 cells in the absence of PRRSV infection enhanced *ISG15* expression. Meanwhile, *OAS1* and *MX1* levels did not differ in mock cells but increased by ~8- and 16-fold in PRRSV-infected MARC-145 cells, respectively, confirming that Gal-1 overexpression mediated the induction of innate immune responses in PRRSV-infected cells, leading to the inhibition of viral infection.

3.6. Gal-1 interacts with the PRRSV E protein but not with PRRSV glycoproteins

We then analyzed interactions between Gal-1 and five membrane-associated proteins, including GP2a, E, GP3, GP4, and GP5. Co-IP showed that viral E protein but not PRRSV GP2a, GP3, GP4, or GP5, interacted with Gal-1 (Fig. 5A, B). To confirm co-localization of Gal-1 and E protein, HEK293 T cells were co-transfected, and green fluorescence (E) superimposed on areas of red fluorescence (Gal-1) in the cytoplasm (Fig. 5C) demonstrated co-localization of Gal-1 and E protein in HEK293 T cells. To examine the interaction between E protein and endogenous Gal-1 in the context of PRRSV infection, virus-infected MARC-145 cells or PAMs were stained with anti-E pAb and an anti-Gal-1 monoclonal antibody, revealing that endogenous Gal-1 co-localized with E protein (Fig. 5D, E). These observations confirmed that endogenous Gal-1 interacts with viral E protein in PRRSV-infected cells. Further, HEK293 T cells were co-transfected with BiFC pairs, resulting in detectable BiFC fluorescence in cells co-transfected with Gal-1 and E (aa 48–73) (Fig. 5F), suggesting that the C-terminal domain of E (aa 48–73) is required for its interaction with Gal-1.

3.7. rGal-1 but not rGal-3 treatment suppresses PRRSV replication

We assessed the functional consequence of Gal-1 treatment by evaluating the impact of soluble rGal-1 on PRRSV infection in primary and permissive cells. Our previous work showed that Gal-3, another member of the galectin family, inhibits PRRSV replication in MARC-145

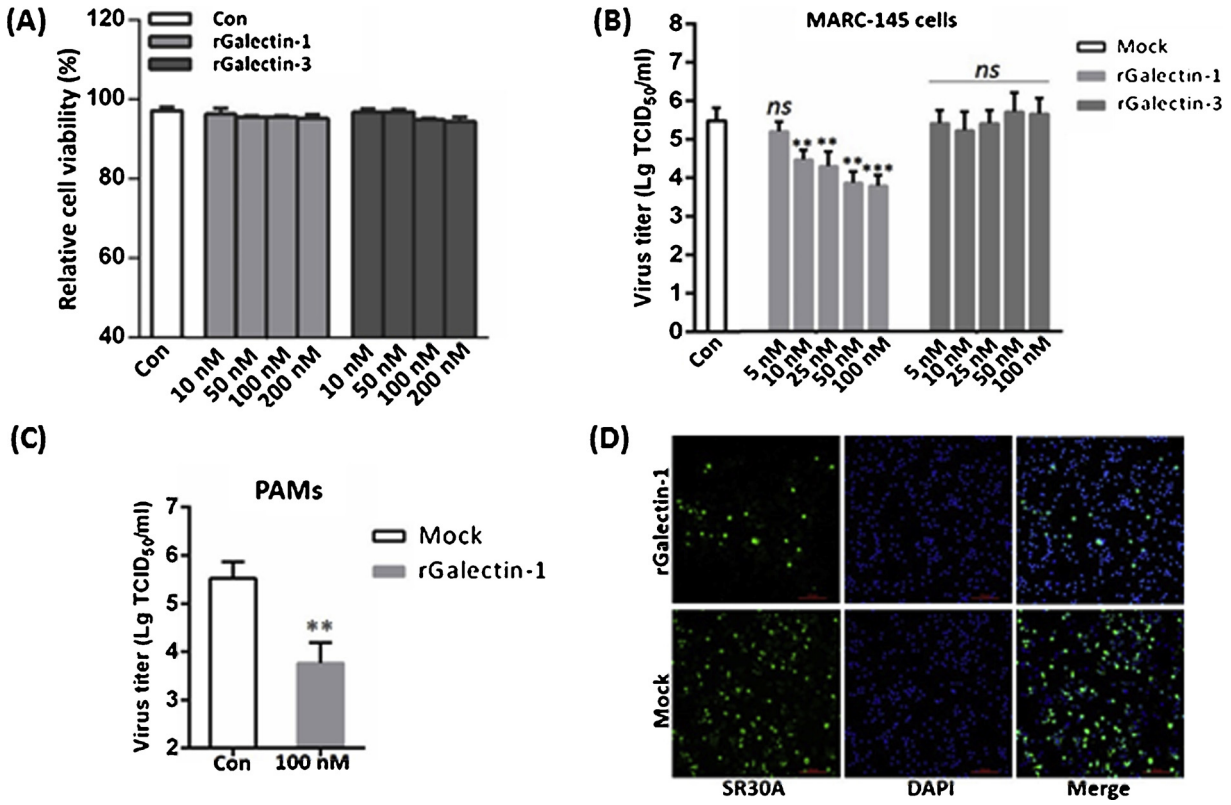

Fig. 6. rGal-1 but not rGal-3 treatment suppresses PRRSV replication. (A) Determination of cytotoxicity of rGal-1 and rGal-3 by MTT assay at the indicated concentrations. (B) MARC-145 cells infected with vHuN4 (MOI = 0.1) with or without 2 h of rGal-1 (5–100 nM) or rGal-3 (5–100 nM) pretreatment. At 36 hpi, the supernatants were collected and titrated. (C) PAMs seeded onto 6-well plates and pretreated with rGal-1 (100 nM) and infected with vHuN4. The supernatants were collected at 36 hpi to determine viral titers. Data are the mean ± standard deviation of three independent experiments. Statistical significance was analyzed using *t* tests; $^{**}P < 0.01$; $^{***}P < 0.001$. (D) Experiments were performed as described for panel B, except that cells were fixed and immunostained with the mouse SR30A mAb against viral N protein and FITC-conjugated goat anti-mouse IgG (green). Cell nuclei were counterstained with DAPI (blue) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

cells (Li et al., 2018). As shown in Fig. 6A, no appreciable cytotoxicity was observed at concentrations ranging from 10 nM to 200 nM. Therefore, we pretreated MARC-145 cells with rGal-1 or rGal-3 at a range of concentrations (5–100 nM) for 2 h prior to infection with vHuN4. As shown in Fig. 6B, rGal-1 but not rGal-3 restricted viral replication in a dose-dependent manner, and there was a significant effect on rGal-1-treated MARC-145 cells, with an approximately 1.78-log decrease in viral titers at a dose of 100 nM ($P < 0.001$). PAMs were then pretreated with rGal-1 (100 nM) and infected with vHuN4, resulting in a significantly lower viral titer (Fig. 6C). Additionally, N protein expression in PAMs was suppressed by rGal-1 (Fig. 6D), consistent with the viral titer data. These results suggested that Gal-1 is capable of inhibiting PRRSV replication through host-cell binding, and that this is not a general property of all galectin protein family members.

3.8. Gal-1 overexpression inhibits the replication of multiple viruses, including EAV, PEDV, JEV, PRV, and CSFV

To determine whether Gal-1 and Gal-3 have antiviral effects on other viruses, cells were transfected with the respective expression plasmids and infected with a variety of viruses maintained in our laboratory, including EAV vEAV030, PEDV JS-2013, JEV HEN0701, PRV JS-2012, and CSFV Shimen. As shown in Fig. 7A, vEAV030 viral titer in Gal-1-overexpressing MARC-145 cells was reduced, whereas there was no significant difference in viral titers between mock and Gal-3-overexpressing cells. In the case of PEDV, both Gal-1 and Gal-3 displayed antiviral activity, with Gal-1 reducing viral titers more efficiently than Gal-3 (Fig. 7B). The cytopathic effect (CPE) in Gal-1-overexpressing MARC-145 cells was significantly lower than that in the control (Fig. 7C), consistent with the viral titer results. For JEV HEN0701, viral titers in Gal-1-overexpressing MARC-145 cells were significantly lower at two infection doses (Fig. 7D). For PRV JS-2012, viral titers in the Gal-1- or Gal-3-overexpressing MARC-145 cells were lower at an MOI of 0.01 (Fig. 7E), and CPE in Gal-1-overexpressing MARC-145 cells was obviously lower than that in the control (Fig. 7F). In the case of the CSFV Shimen strain, Gal-1 but not Gal-3 showed antiviral activity. These results demonstrated that Gal-1 exhibited antiviral activity against EAV, PEDV, JEV, PRV, and CSFV.

4. Discussion

PRRSV is a pathogen of significant economic importance and that has adversely impacted the global swine industry for almost 30 years. Currently, vaccination is the most common method for controlling PRRS; however, commercially available vaccines fail to provide sustainable protection against the disease due to the rapid evolution of the viral genome and the number of epidemic variants in China. Antiviral therapies provide creative insights to guide future PRRSV control and prevention efforts, especially for cases in which existing vaccines fail to match the circulating virus. Here, we show that Gal-1 overexpression strongly inhibited PRRSV replication, whereas *Gal-1* knockdown or knockout facilitated PRRSV replication (Figs. 2 and 3). Moreover, soluble rGal-1 but not rGal-3 treatment inhibited PRRSV in both primary and permissive cells (Fig. 6), suggesting that Gal-1 might have potential use as a novel approach to control PRRSV infection, and that this is not a general property of all galectins.

Gal-1 participates in antiviral defense in various ways, from the

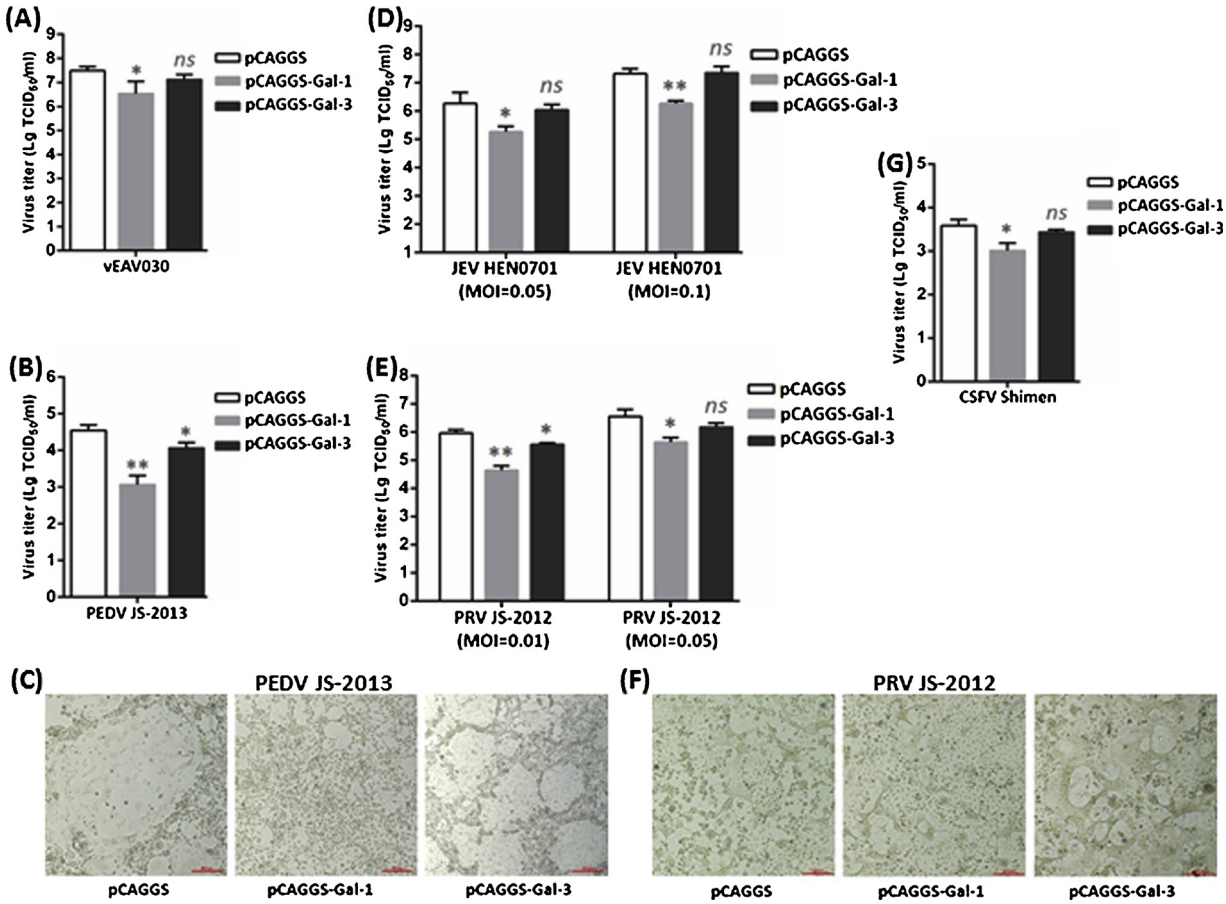

Fig. 7. Gal-1 overexpression inhibits the replication of multiple viruses, including EAV, PEDV, JEV, PRV, and CSFV. (A) Viral titers for MARC-145 cells transfected with Gal-1-C-Myc-pCAGGS, Gal-3-C-Myc-pCAGGS, or control plasmids for 36 h prior to vEAV030 infection (MOI = 0.1). (B) Viral titers and (C) CPE from MARC-145 cells transfected for 36 h prior to PEDV infection. (D) Viral titers from BHK-21 cells transfected for 36 h prior to JEV HEN0701 infection (MOI = 0.05 and 0.1). (E) Viral titers and (F) CPE from MARC-145 cells transfected for 36 h prior to PRV JS-2012 infection (MOI = 0.01 and 0.05). (G) Supernatants were collected from PK-15 cells transfected for 36 h prior to CSFV Shimen infection. Viral titers for CSFV were determined using an mAb against the CSFV E2 protein. Data represent the mean ± standard deviation of three independent experiments. Statistical significance was analyzed using *t* tests; $^{*}P < 0.05$; $^{**}P < 0.01$. *ns*, not significant.

recognition and blocking of surface glycoproteins to the activation of immune responses (Cedeno-Laurent and Dimitroff, 2012; Vasta, 2009). Previous reports found that Gal-1 can be upregulated by viral invasion (Gauthier et al., 2008; Yang et al., 2011); however, we found that Gal-1 is downregulated in response to PRRSV infection in PAMs and MARC-145 cells (Figs. 2D and 3E). Further, reducing the expression of endogenous Gal-1 promoted viral growth *in vitro* (Fig. 3F), indicating the important roles of Gal-1 in PRRSV infection. PRRSV infection appears to actively decrease *Gal-1* expression to promote viral replication, highlighting the complex interplay between PRRSV and its host.

As a soluble β-galactoside-binding lectin, the functions of Gal-1 are usually dependent on glycan binding (St-Pierre et al., 2011; Yang et al., 2011). In this study, Gal-1 binding to viral Nsp attracted our attention in the yeast system (Fig. 1). Nsp11 (aa 124–183) interacts with Gal-1 (Fig. 4B), which is thought to support EndoU activity and inhibit IFN-β induction. We found that suppression of *IFN-β*-promoter activity by PRRSV Nsp11 could be rescued by Gal-1 overexpression (Fig. 4C). Thus, we propose that the interaction between Gal-1 and Nsp11 might regulate IFN and ISG activation to facilitate their antiviral roles. In the case of human cytomegalovirus infection, Gal-9 acts as an ISG that can be induced by IFN-β, suggesting its potential as an important antiviral protein (Machala et al., 2019). We speculate that Gal-1 is a potential ISG, similar to Gal-9, which needs further validation.

Additionally, we assessed Gal-1 and PRRSV glycoprotein interactions, finding that Gal-1 does not bind to GP2, GP3, GP4, or GP5 (Fig. 5) and indicating that its anti-PRRSV activity might be unrelated to glycan binding. E protein, a minor envelope protein, is important for viral infectivity and induction of inflammasome activation in porcine macrophages (Du et al., 2010; Zhang et al., 2013). In the present study, we found that E protein interacts with Gal-1 (Fig. 5). Interestingly, we investigated this interaction not only in cells transiently transfected with the E-expressing plasmid but also during the course of viral infection (Fig. 5). Moreover, the C-terminal domain of E (aa 48–73) is required for Gal-1 binding, which is reportedly essential for its interaction with tubulin (Zhang and Zakhartchouk, 2017). It is possible that the interaction between Gal-1 and E protein might be related to Gal-1-specific anti-inflammatory activity (Vasta, 2009). Our findings suggest that the regulatory role of Gal-1 in immune responses might be orchestrated by its direct antiviral effect against PRRSV infection.

Gal-1 showed antiviral activity against five other viruses in our study (Fig. 7), indicating that it has an important and broad role in viral pathogenesis and potential application value in antiviral therapy. Of the viruses tested, PRV, like all other herpes viruses, establishes a lifelong latency; therefore, development of antiviral therapies offering a promising alternative to existing therapeutic agents is important (Machala et al., 2019). In the present study, we showed that Gal-1 bound to the EndoU domain of PRRSV Nsp11, which is a highly conserved genetic marker unique to *Nidovirales* viruses (Ivanov et al., 2004). We speculate that Gal-1 exerts similar mechanisms against EAV and PEDV; however, these hypotheses and the specific mechanisms underlying Gal-1-related antiviral activity require further validation in future work.

Overall, this study demonstrated that Gal-1 restricts PRRSV

replication in both PAMs and MARC-145 cells (Figs. 2 and 3) by binding to viral Nsp11 and E protein (Figs. 1 and 5), inactivating the IFN antagonist function of viral Nsp11, and stimulating the expression of ISGs (Fig. 4). Moreover, Gal-1 exhibited its antiviral effect against multiple PRRSV strains, as well as other viruses tested in our study (Fig. 7). These results establish a foundation for further exploration of host genes involved in resistance to PRRS transmission. Our study not only highlights the importance of host factors in PRRSV replication but also suggests potential antiviral therapies.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.07.024.

References

Bookout, A.L., Cummins, C.L., Mangelsdorf, D.J., Pesola, J.M., Kramer, M.F., et al., 2006. High-throughput real-time quantitative reverse transcription PCR. In: In: Ausubel, Frederick M. (Ed.), Current Protocols in Molecular Biology Volume 73 15.18.11-15.18.28.

Buddaert, W., Van Reeth, K., Pensaert, M., 1998. In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV). Adv. Exp. Med. Biol. 440, 461–467.

Camby, I., Le Mercier, M., Lefranc, F., Kiss, R., 2006. Galectin-1: a small protein with major functions. Glycobiology 16, 137R–157R.

Cedeno-Laurent, F., Dimitroff, C.J., 2012. Galectin-1 research in T cell immunity: past, present and future. Clin. Immunol. 142, 107–116.

Chand, R.J., Trible, B.R., Rowland, R.R., 2012. Pathogenesis of porcine reproductive and respiratory syndrome virus. Curr. Opin. Virol. 2, 256–263.

Du, Y., Zuckermann, F.A., Yoo, D., 2010. Myristoylation of the small envelope protein of porcine reproductive and respiratory syndrome virus is non-essential for virus infectivity but promotes its growth. Virus Res. 147, 294–299.

Fang, Y., Snijder, E.J., 2010. The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus Res. 154, 61–76.

Gao, F., Jiang, Y., Li, G., Zhou, Y., Yu, L., Li, L., Tong, W., Zheng, H., Zhang, Y., Yu, H., Shan, T., Yang, S., Liu, H., Zhao, K., Tong, G., 2018. Porcine reproductive and respiratory syndrome virus expressing E2 of classical swine fever virus protects pigs from a lethal challenge of highly-pathogenic PRRSV and CSFV. Vaccine 36, 3269–3277.

Gauthier, S., Pelletier, I., Ouellet, M., Vargas, A., Tremblay, M.J., Sato, S., Barbeau, B., 2008. Induction of galectin-1 expression by HTLV-I Tax and its impact on HTLV-I infectivity. Retrovirology 5, 105.

Han, M., Yoo, D., 2014. Engineering the PRRS virus genome: updates and perspectives. Vet. Microbiol. 174, 279–295.

Ivanov, K.A., Hertzig, T., Rozanov, M., Bayer, S., Thiel, V., Gorbalenya, A.E., Ziebuhr, J., 2004. Major genetic marker of nidoviruses encodes a replicative endoribonuclease. Proc. Natl. Acad. Sci. U.S.A. 101, 12694–12699.

Jiang, Y.F., Xia, T.Q., Zhou, Y.J., Yu, L.X., Yang, S., Huang, Q.F., Li, L.W., Gao, F., Qu, Z.H., Tong, W., Tong, G.Z., 2015. Characterization of three porcine reproductive and respiratory syndrome virus isolates from a single swine farm bearing strong homology to a vaccine strain. Vet. Microbiol. 179, 242–249.

Jin, H., Zhou, L., Ge, X., Zhang, H., Zhang, R., Wang, C., Wang, L., Zhang, Z., Yang, H., Guo, X., 2017. Cellular DEAD-box RNA helicase 18 (DDX18) promotes the PRRSV Replication via Interaction with Virus nsp2 and nsp10. Virus Res. 238, 204–212.

Lee, P.H., Liu, C.M., Ho, T.S., Tsai, Y.C., Lin, C.C., Wang, Y.F., Chen, Y.L., Yu, C.K., Wang, S.M., Liu, C.C., Shiau, A.L., Lei, H.Y., Chang, C.P., 2015. Enterovirus 71 virion-associated galectin-1 facilitates viral replication and stability. PLoS One 10, e0116278.

Levroney, E.L., Aguilar, H.C., Fulcher, J.A., Kohatsu, L., Pace, K.E., Pang, M., Gurney, K.B., Baum, L.G., Lee, B., 2005. Novel innate immune functions for galectin-1: galectin-1 inhibits cell fusion by Nipah virus envelope glycoproteins and augments dendritic cell secretion of proinflammatory cytokines. J. Immunol. 175, 413–420.

Li, L., Zhou, Y., Jiang, Y., Gao, F., Shan, T., Zhao, K., Zhang, Y., Li, L., Tong, G., 2018. Galectin-3 inhibits replication of porcine reproductive and respiratory syndrome virus by interacting with viral Nsp12 in vitro. Virus Res. 253, 87–91.

Lunney, J.K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B., Renukaradhya, G.J., 2016. Porcine reproductive and respiratory syndrome virus (PRRSV): pathogenesis and interaction with the immune system. Annu. Rev. Anim. Biosci. 4, 129–154.

Machala, E.A., Avdic, S., Stern, L., Zajonc, D.M., Benedict, C.A., Blyth, E., Gottlieb, D.J., Abendroth, A., McSharry, B.P., Slobedman, B., 2019. Restriction of human cytomegalovirus infection by Galectin-9. J. Virol. 93.

Nedialkova, D.D., Ulferts, R., van den Born, E., Lauber, C., Gorbalenya, A.E., Ziebuhr, J., Snijder, E.J., 2009. Biochemical characterization of arterivirus nonstructural protein 11 reveals the nidovirus-wide conservation of a replicative endoribonuclease. J. Virol. 83, 5671–5682.

Ouellet, M., Mercier, S., Pelletier, I., Bounou, S., Roy, J., Hirabayashi, J., Sato, S., Tremblay, M.J., 2005. Galectin-1 acts as a soluble host factor that promotes HIV-1 infectivity through stabilization of virus attachment to host cells. J. Immunol. 174, 4120–4126.

Rajasagi, N.K., Suryawanshi, A., Sehrawat, S., Reddy, P.B., Mulik, S., Hirashima, M., Rouse, B.T., 2012. Galectin-1 reduces the severity of herpes simplex virus-induced ocular immunopathological lesions. J. Immunol. 188, 4631–4643.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hy. 27, 493–497.

Shi, X., Wang, L., Li, X., Zhang, G., Guo, J., Zhao, D., Chai, S., Deng, R., 2011. Endoribonuclease activities of porcine reproductive and respiratory syndrome virus nsp11 was essential for nsp11 to inhibit IFN-beta induction. Mol. Immunol. 48, 1568–1572.

St-Pierre, C., Manya, H., Ouellet, M., Clark, G.F., Endo, T., Tremblay, M.J., Sato, S., 2011. Host-soluble galectin-1 promotes HIV-1 replication through a direct interaction with glycans of viral gp120 and host CD4. J. Virol. 85, 11742–11751.

Sun, L., Li, Y., Liu, R., Wang, X., Gao, F., Lin, T., Huang, T., Yao, H., Tong, G., Fan, H., Wei, Z., Yuan, S., 2013. Porcine reproductive and respiratory syndrome virus ORF5a protein is essential for virus viability. Virus Res. 171, 178–185.

Sun, Y., Ke, H., Han, M., Chen, N., Fang, W., Yoo, D., 2016. Nonstructural protein 11 of porcine reproductive and respiratory syndrome virus suppresses both MAVS and RIG-I expression as one of the mechanisms to antagonize type I interferon production. PLoS One 11, e0168314.

Toledo, K.A., Fermino, M.L., Andrade Cdel, C., Riul, T.B., Alves, R.T., Muller, V.D., Russo, R.R., Stowell, S.R., Cummings, R.D., Aquino, V.H., Dias-Baruffi, M., 2014. Galectin-1 exerts inhibitory effects during DENV-1 infection. PLoS One 9, e112474.

Vasta, G.R., 2009. Roles of galectins in infection. Nature reviews. Microbiology 7, 424–438.

Wang, C., Shi, X., Zhang, X., Wang, A., Wang, L., Chen, J., Deng, R., Zhang, G., 2015a. The endoribonuclease activity essential for the nonstructural protein 11 of porcine reproductive and respiratory syndrome virus to inhibit NLRP3 inflammasome-mediated IL-1beta induction. DNA Cell Biol. 34, 728–735.

Wang, D., Fan, J., Fang, L., Luo, R., Ouyang, H., Ouyang, C., Zhang, H., Chen, H., Li, K., Xiao, S., 2015b. The nonstructural protein 11 of porcine reproductive and respiratory syndrome virus inhibits NF-kappaB signaling by means of its deubiquitinating activity. Mol. Immunol. 68, 357–366.

Yang, M.L., Chen, Y.H., Wang, S.W., Huang, Y.J., Leu, C.H., Yeh, N.C., Chu, C.Y., Lin, C.C., Shieh, G.S., Chen, Y.L., Wang, J.R., Wang, C.H., Wu, C.L., Shiau, A.L., 2011. Galectin-1 binds to influenza virus and ameliorates influenza virus pathogenesis. J. Virol. 85, 10010–10020.

Zhang, K., Hou, Q., Zhong, Z., Li, X., Chen, H., Li, W., Wen, J., Wang, L., Liu, W., Zhong, F., 2013. Porcine reproductive and respiratory syndrome virus activates inflammasomes of porcine alveolar macrophages via its small envelope protein E. Virology 442, 156–162.

Zhang, M., Zakhartchouk, A., 2017. Porcine reproductive and respiratory syndrome virus envelope (E) protein interacts with tubulin. Vet. Microbiol. 211, 51–57.

Zhang, R., Chen, C., Sun, Z., Tan, F., Zhuang, J., Tian, D., Tong, G., Yuan, S., 2012. Disulfide linkages mediating nucleocapsid protein dimerization are not required for porcine arterivirus infectivity. J. Virol. 86, 4670–4681.

Zhao, K., Li, L.W., Jiang, Y.F., Gao, F., Zhang, Y.J., Zhao, W.Y., Li, G.X., Yu, L.X., Zhou, Y.J., Tong, G.Z., 2019. Nucleocapsid protein of porcine reproductive and respiratory syndrome virus antagonizes the antiviral activity of TRIM25 by interfering with TRIM25-mediated RIG-I ubiquitination. Vet. Microbiol. 233, 140–146.

Zhao, K., Li, L.W., Zhang, Y.J., Jiang, Y.F., Gao, F., Li, G.X., Yu, L.X., Zhao, W.Y., Shan, T.L., Zhou, Y.J., Tong, G.Z., 2018. MOV10 inhibits replication of porcine reproductive and respiratory syndrome virus by retaining viral nucleocapsid protein in the cytoplasm of Marc-145cells. Biochem. Biophys. Res. Commun. 504, 157–163.

Zhao, S., Ge, X., Wang, X., Liu, A., Guo, X., Zhou, L., Yu, K., Yang, H., 2015. The DEAD-box RNA helicase 5 positively regulates the replication of porcine reproductive and respiratory syndrome virus by interacting with viral Nsp9 in vitro. Virus Res. 195, 217–224.

Zheng, X., Zheng, H., Tong, W., Li, G., Wang, T., Li, L., Gao, F., Shan, T., Yu, H., Zhou, Y., Qiu, Y., Ma, Z., Tong, G., 2018. Acidity/Alkalinity of japanese encephalitis virus e protein residue 138 alters neurovirulence in mice. J. Virol. 92.