



Nucleotide-binding oligomerization domain-like receptor X1 restricts porcine reproductive and respiratory syndrome virus-2 replication by interacting with viral Nsp9

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) causes one of the most economically important diseases of swine worldwide. Current antiviral strategies provide only limited protection. Nucleotide-binding oligomerization domain-like receptor (NLR) X1 is unique among NLR proteins in its functions as a pro-viral or antiviral factor to different viral infections. To date, the impact of NLRX1 on PRRSV infection remains unclear. In this study, we found that PRRSV infection promoted the expression of NLRX1 gene. In turn, ectopic expression of NLRX1 inhibited PRRSV replication in Marc-145 cells, whereas knockdown of NLRX1 enhanced PRRSV propagation in porcine alveolar macrophages (PAMs). Mechanistically, NLRX1 was revealed to impair intracellular viral subgenomic RNAs accumulation. Finally, Mutagenic analyses indicated that the LRR (leucine-rich repeats) domain of NLRX1 interacted with PRRSV Nonstructural Protein 9 (Nsp9) RdRp (RNA-dependent RNA Polymerase) domain and was necessary for antiviral activity. Thus, our study establishes the role of NLRX1 as a new host restriction factor in PRRSV infection.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important viral diseases affecting the swine industry worldwide since it was first reported in 1987 (Harding et al., 2017; Snijder et al., 2013). The etiological agent of this devastating disease, PRRS virus (PRRSV), is a positive-sense single-stranded RNA virus that belongs to the family *Arteriviridae*, order *Nidovirales* (Loving et al., 2015). The virus engages with the host cellular protein interaction network during infection, facilitating virus hijacking of the host molecular machinery to fulfill the viral life cycle (Ke and Yoo, 2017; Lunney et al., 2016; Rahe and Murtaugh, 2017; Shi et al., 2015; Sun et al., 2012). Based on their genetic and antigenic differences, PRRSV strains are classified into 2 distinct genotypes, PRRSV-1 (European type) and PRRSV-2 (North American type) (Han and Yoo, 2014; Murtaugh et al., 2010). The ~15 kb viral genome contains at least 10 open reading frames (ORFs), which encode at least 14 nonstructural proteins (Nsp) and 8 structural proteins (Han and Yoo, 2014).

Among the nonstructural proteins, Nsp9 contains an RNA-dependent RNA polymerase (RdRp) domain in its C-terminal portion, which is critical for viral RNA synthesis, and replication efficiency (Fang and Snijder, 2010; Yang et al., 2015; Zhou et al., 2011). In this regard, recent advances show that amino acids at positions 519, 544, 586 and 592 in Nsp9 contribute to enhanced pathogenicity and determine the fatal virulence of the virus (Xu et al., 2018; Zhao et al., 2018). In addition, two highly conserved T-cell epitopes have been identified in Nsp9, which may provide broad cross-protection against diverse PRRSV strains (Parida et al., 2012; Rascon-Castelo et al., 2015). Noteworthy, multiple novel antiviral treatments, including Nsp9 specific Camel single-domain antibodies, and siRNA targeting Nsp9, as well as vaccine strategy of de-optimization of codon pair bias in Nsp9, significantly decreased PRRSV replication *in vitro* and *in vivo* (Gao et al., 2015; Liu et al., 2015; Xie et al., 2014; Zheng et al., 2015; Zhu et al., 2018). Finally, Nsp9 was found to associate with at least 9 PRRSV encoded proteins, including Nsp1 α , Nsp1 β , Nsp3, Nsp7 α , Nsp7 β , Nsp8, Nsp11, Nsp12, and nucleocapsid (N) protein, validating the notion of this

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protein as a core component of the viral replication/transcription complex (RTC) (Chen et al., 2017; Liu et al., 2016; Nan et al., 2018). Further exploring the interaction of Nsp9 with host cellular proteins and analyzing the biological significance are necessary for fully understanding the replication mechanisms and pathogenesis of PRRSV (Wen et al., 2017).

Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) X1 (also known as CLR11.3 and NOD9), a member of the NLR family proteins, is initially identified as key mediators of immune defense and inflammation (Arnoult et al., 2009; Coutermarsh-Ott et al., 2016; Eitas et al., 2014; Imbeault et al., 2014; Kang et al., 2015; Kanneganti, 2010; Koblansky et al., 2016; Li et al., 2016; Lupfer and Kanneganti, 2013; Moore et al., 2008; Philipson et al., 2015; Singh et al., 2015; Soares et al., 2014; Tattoli et al., 2016; Ting et al., 2008; Wang et al., 2013). To date, accumulated evidence indicated that NLRX1 inhibits NF- κ B (Nuclear factor-kappa B) signaling, inflammatory activation, double-stranded RNA (dsRNA) activated kinase PKR and type I interferon (IFN) production but potentiates reactive oxygen species (ROS) production and autophagy (Abdul-Sater et al., 2010; Allen et al., 2011; Feng et al., 2017; Guo et al., 2016; Hung et al., 2018; Kim et al., 2017; Lei et al., 2012; Li et al., 2016; Moore et al., 2008; O'Neill, 2008; Qin et al., 2017; Stokman et al., 2017; Tattoli et al., 2008; Theus et al., 2017; Xia et al., 2011; Yin et al., 2017; Zeng et al., 2017). Recently, NLRX1 was also identified to facilitate Human Immunodeficiency Virus 1 (HIV-1) (Guo et al., 2016), herpes simplex virus 1 (HSV-1) (Guo et al., 2016), hepatitis C virus (HCV) (Qin et al., 2017) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Ma et al., 2017) infection, whereas restricts influenza A virus (IAV) (Jaworska et al., 2014), and hepatitis A virus (HAV) (Feng et al., 2017) replication. These conflicting reports about whether NLRX1 functions as a pro-viral or antiviral factor imply that the role of NLRX1 in viral infection is more complex than previously thought and remains to be clarified. Until now, the specific role of NLRX1 in PRRSV infection was scarcely known. Thus, the aim of the present study is to seek evidence for a potential role of NLRX1 in PRRSV infection.

2. Materials and methods

2.1. Cells, viruses, and antibodies

HEK293 T and PAMs cells (Jing et al., 2017b) were cultured and maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS). Hela and Marc-145 cells were cultured and maintained in DMEM supplemented with 10% heat-inactivated FBS. PRRSV strain HN1 (GenBank: AY457635.1) is a highly pathogenic PRRSV-2 (North American) strain, which was isolated from the lung of pigs suffering from “high fever” syndrome in Henan Province, China (Cao et al., 2015). The virus was amplified and titered in Marc-145 cells. UV-inactivated PRRSV was generated by irradiating the virus with short-wave UV light (254 nm) for 1 h. The loss of infectivity was confirmed by the inability of the UV-exposed virus to produce a cytopathic effect on monolayer of Marc-145 cells.

Mouse monoclonal anti-HA, anti-Flag, and anti- β -actin antibodies were purchased from ABclonal Biotechnology (China). Anti-Flag polyclonal antibody (Macgene, China), anti-NLRX1 polyclonal antibody (Proteintech, China), were purchased and used according to the manufacturers' recommendations. HRP-conjugated anti-mouse and anti-rabbit IgG light (or heavy) chain specific antibodies (Abbkine Science, USA) were purchased and used according to the manufacturers' recommendations. A monoclonal antibody directed against PRRSV N protein was produced from hybridoma cells derived from Sp2/0 myeloma cells and spleen cells of BALB/c mice immunized with recombinant N protein (Jing et al., 2017b).

Table 1

The sequences of primers used for construction of NLRX1, and Nsp9 protein mutants.

Primer names	Sequence (5'-3')
NLRX1-F	TTTGAATTCATGAGGTGGGGCCACCATTGCCCAG
NLRX1-R	TTTCTCGAG TCAGCTTCCAGAGCTTCCAGCTGCT
NLRX1- Δ X-F	TTTGAATTCGCGCGGGTGAGACAGTGGTGCTGTA
NLRX1-LRR-F	TTTGAATTCCTCAACCAGCCGTACTGCGGGTATGC
NLRX1- Δ L-R	TTTCTCGAGGCGGAGCTGGGAAGTAGAGCTTCTGCA
Nsp9-F	TTTGAGCTCGGAGCAGTGTAACTGCTAGCCGCC
Nsp9-R	TTTCTCGAGCTCATAATTGGACCTGAGTTTTCAC
Nsp9N-R	TTTCTCGAGCAGGACCGGAGTCTGTAGCTCCTAAAC
Nsp9C-F	TTTGAGCTCGGAGGTGCCTTGAAGCTGATCTCGCAT

The restriction enzyme sites used for cloning are underlined in italics.

2.2. Plasmids constructions and mutagenesis

The Flag or HA epitope tag was amplified by PCR and cloned into the pCAGGS-MCS vector to generate the pCAGGS-Flag or pCAGGS-HA plasmid, encoding an N-terminal Flag or HA tag (Jing et al., 2017b). Expression plasmids for Flag-tagged Nsp9 (full-length and truncated) were constructed by PCR amplification of the cDNA from PRRSV-infected Marc-145 cells. Plasmids encoding full-length and truncated NLRX1 (NM_024618) were constructed by PCR amplification using the specific primers listed in Table 1. All constructs were confirmed by DNA sequencing.

2.3. Transfection and NLRX1 gene silencing by siRNA

The cells were transfected with expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Where necessary, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. For siRNA knockdowns, cells plated in 6-well plates and transfected with 30 nM the indicated siRNAs twice over a 48-h period by using Lipofectamine 2000. siRNA sequences used are as follows: si-NLRX1-1[#], 5'-UUGUCAAUCUGCUGCGCAA-3'; si-NLRX1-2[#], 5'-GUGCUGGGC UUGCGGAAGA-3'; si-NLRX1-3[#], 5'-GCAUGUCUCCGCCGGGAU-3'; Negative control siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3' (Table 2).

2.4. TCID₅₀ assay for PRRSV

PRRSV titers were expressed as the tissue culture infectious dose 50 (TCID₅₀) per milliliter using the Reed–Muench method as previously described (Jing et al., 2017a). Briefly, Marc-145 cells were seeded in 96-well plates, following, infected with serial 10-fold dilutions of PRRSV samples in eight replicates. Plates were incubated for 72–96 h before virus titers were calculated.

2.5. Quantitative RT-PCR (qPCR)

Total RNA was isolated at the indicated time points using TRIzol reagent. qPCR was performed using SYBR Green Real Time PCR Master Mix (Toyobo Biologics, Osaka, Japan) in a LightCycler 480 (Roche

Table 2

The sequences of primers used for real-time PCR.

Primer names	GeneBank	Sequence (5'-3')
NlrX1-F	NM_001204769.1	CAGACCCTCACAAGCATCTA
NlrX1-R		CACGGACATCCTCTTCAGA
β -actin-F	XM_021086047.1	TGAGAACAGCTGCATCCACTT
β -actin-R		CGAAGGCAGCTCGGAGTT
Gapdh-F	NM_001195426.1	TCATGACCAAGTCCATGCC
Gapdh-R		GGATGACCTTGCCACAGCC

Molecular Biochemicals). Individual transcripts in each sample were assayed three times. The PCR conditions were as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C and 40 s at 72 °C. The fold change in gene expression relative to normal was calculated using the delta delta cycles to threshold ($\Delta\Delta CT$) method. Gene expression in Marc-145 cells and PAMs was normalized to that of GAPDH and β -actin, respectively. Primers were designed using the Primer Express software (version 3.0; Applied Biosystems, Carlsbad, CA). Absolute quantitative mRNA levels were calculated using standard curves as previously described (Ke et al., 2017).

2.6. Western blotting analysis

Cells cultured in 60-mm dishes were prepared by adding 120 μ L of 2 \times lysis buffer A (65 mM Tris – HCl [pH 6.8], 4% sodium dodecyl sulfate (SDS), 3% DL-dithiothreitol, and 40% glycerol). The cell extracts were boiled for 10 min, and then resolved with 8%–12% SDS-PAGE. The separated proteins were electroblotted onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Billerica, MA). Run for 1–2 h at 100 V on ice. The western blotting was probed with specific antibodies. The expression of β -actin was detected with a mouse monoclonal antibody to demonstrate equal protein sample loading. Densitometry quantification of protein bands of interest was performed using ImageJ software (Table 3).

2.7. Indirect immunofluorescence assay (IFA)

Hela cells seeded on microscope coverslips and placed in 24-well dishes were transfected with Flag-tagged Nsp9 expression plasmid when the cells reached approximately 70–80% confluence. At 28 h after transfection, cells were fixed with 4% paraformaldehyde for 10 min, subsequently permeated with 0.1% Triton X-100 for 10 min at room temperature. After three washes with PBS, cells were sealed with PBS containing 5% bovine serum albumin for 1 h, followed by incubation with rabbit polyclonal antibody against NLRX1 and mouse monoclonal antibody against Flag tag for 1 h at room temperature separately. Successively, cells were treated with FITC-labeled goat anti-mouse and Cy3-labeled goat anti-rabbit (Invitrogen) antibodies for 1 h, with DAPI for 15 min at room temperature. After washing with PBS, fluorescent images were acquired using a confocal laser scanning microscope (OLYMPUS FLUOVIEW Ver.3.1, Japan).

2.8. Co-immunoprecipitation (Co-IP) and immunoblotting analyses

To investigate the interactions between proteins, HEK293 T cells were lysed in immunoprecipitation lysis buffer (RIPA). After the lysates were incubated for 1 h at 37 °C with RNase and DNase, the lysate proteins were incubated overnight at 4 °C with the indicated antibodies. Protein A + G agarose beads (30 μ L; Beyotime) were then added to each immunoprecipitation reaction for another 6 h. The agarose beads were then washed three times and the captured proteins were resolved on

8%–12% SDS-PAGE, transferred to PVDF membranes, and analyzed by immunoblotting.

2.9. Statistical analysis

The results represent the means and standard deviations from three independent experiments. GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for data analysis using a two-tailed unpaired *t*-test **P* < 0.05; ***P* < 0.01.

3. Results

3.1. PRRSV infection significantly upregulated NLRX1 expression

Our work has previously shown that PRRSV infection up-regulates the expression of NLRX1 in Marc-145 cells, a highly permissive cell line derived from epithelial cells of a monkey kidney (Jing et al., 2014). To ascertain this result in the main target cells of acute PRRSV infection, porcine alveolar macrophages (PAMs) were infected with PRRSV at an MOI of 0.1. Real-time PCR was performed at 12 h, 24 h, and 48 h post infection (hpi) to determine the expression levels of NLRX1. As shown in Fig. 1A, the data showed that NLRX1 expression was significantly up-regulated at 24 h, and increased at a steady-state level in PRRSV-infected PAMs when compared to non-infected cells (> 10-folds). Moreover, UV-inactivated PRRSV failed to induce NLRX1 mRNA expression, indicating that the up-regulation of NLRX1 depends on viral replication (Fig. 1A). Next, PAMs were infected at different MOI with PRRSV for 48 h before analysis for the mRNA expression of NLRX1. Result indicated that PRRSV up-regulated NLRX1 in a dose dependent manner (Fig. 1B).

Aiming to clarify whether PRRSV induces NLRX1 expression in protein level, PAMs were infected at an MOI of 0.1 with PRRSV or UV-inactivated PRRSV for the designated time. NLRX1 protein levels measured by Western blotting reflected the changes in mRNA observed, suggesting that endogenous NLRX1 expression was upregulated by PRRSV infection (Fig. 1C).

3.2. Ectopic expression of NLRX1 impaired PRRSV replication in Marc-145 cells

In order to investigate whether the upregulation of NLRX1 might have a correlation with PRRSV infection, we transfected Marc-145 cells with NLRX1 expression plasmid, followed by infection with PRRSV. NLRX1 activity on viral infection was monitored by measuring PRRSV titers in the culture supernatants collected at 12, 24 and 48 hpi. As shown in Fig. 2A, overexpression of NLRX1 consistently reduced viral titers of PRRSV at 12–48 hpi. Next, Marc-145 cells transfected with NLRX1 were infected with different MOI of PRRSV, a TCID₅₀ assay was used to determine the effect of NLRX1 on the production of infectious PRRSV particles. As expected, TCID₅₀ data suggested that overexpression of NLRX1 inhibited PRRSV proliferation, especially at low infection doses (Fig. 2B).

3.3. Enhancement of PRRSV replication by NLRX1 knockdown in PAMs

Since Marc-145 cells are monkey derived though they are PRRSV permissive, we wondered whether endogenous NLRX1 had the same effect on PRRSV replication in primary PAM cells. To this end, three pairs of siRNA duplexes against NLRX1 were individually transfected into PAMs. The knockdown efficiency of these siRNA was evaluated by real-time PCR. Result indicated that si-NLRX1-2[#] achieved the highest efficiency (Fig. 3A). Western blot analysis further showed that si-NLRX1-2[#] reduced the level of NLRX1 protein by ~87% compared to the controls cells transfected with NC siRNA (Fig. 3B).

To investigate the function of endogenous NLRX1 in PRRSV replication, PAMs were treated with NC siRNA or si-NLRX1-2[#] followed

Table 3

The sequences of primers used for quantification of total viral RNA and sgRNAs.

Primer names	Sequence (5'-3')
5'UTR-F	CACCTTGCTTCCGGAGTTG
gRNA-R	GAGAGACCGTGCACTGAGACATC
sgRNA2-R	CAGCAACCGCGGATTTGTGAA
sgRNA3-R	GCAAAGCGGCATACCGTGT
sgRNA4-R	ACGAAGTCTGATGCTGCGGTG
sgRNA5-R	CTGGCGTTGACGAGCAGCA
sgRNA6-R	CATCACTGGCGTGTAGGTAATGGA
sgRNA7-R	GGCTTCTCCGGGTTTTCTTCCTA
total vRNA-F	AAACCAAGTCCAGAGGCAA
total vRNA-R	CGGCAAACTAAATCCACA

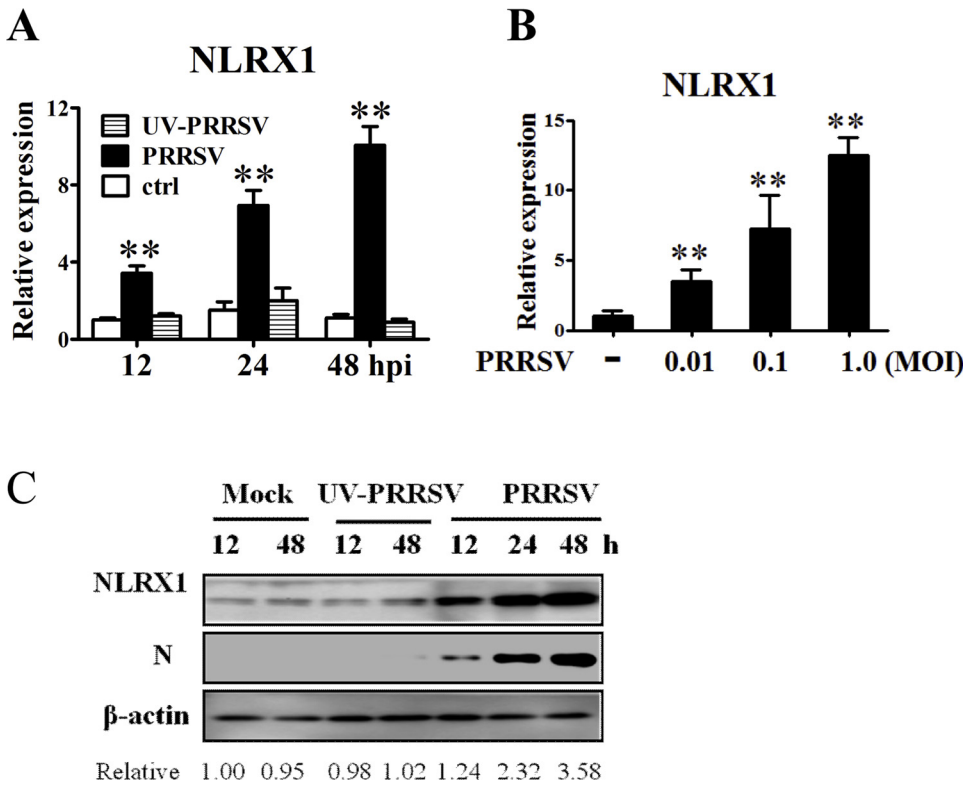


Fig. 1. PRRSV infection up-regulates NLRX1 expression in PAMs. **(A)** PAMs were infected at an MOI of 0.1 with PRRSV or UV-inactivated PRRSV for the designated time. Total RNA was extracted, and real-time PCR was performed to determine the relative levels of NLRX1. Gene expression was normalized to that of β -actin. Significant differences from the uninfected cells are denoted by asterisks (**) for $P < 0.01$. **(B)** PAMs were infected at the indicated MOI with PRRSV for 48 h. Total RNA was extracted, and real-time PCR was performed to determine the relative levels of NLRX1. **(C)** PAMs were infected at an MOI of 0.1 with PRRSV or UV inactivated PRRSV for the designated time. Cell lysates were blotted with the indicated antibodies.

by PRRSV infection. Then viral titers were measured by TCID₅₀ at 12–48 hpi. We observed that reduced NLRX1 protein expression was correlated with increased PRRSV in PAMs (Fig. 3C). In aggregate, these data suggested that NLRX1 can potentially inhibit replication of PRRSV in PAMs.

3.4. NLRX1 suppressed the synthesis of viral subgenomic RNAs

NLRX1 contains a carboxy-terminal LRR domain, a central NOD domain and a unique N-terminal CARD-related X domain (Moore et al., 2008) (Fig. 4A). To better understand how NLRX1 impaired PRRSV replication, we mapped the domains of NLRX1 required for this restriction. To this end, we transfected Marc-145 cells with plasmids encoding either the full-length NLRX1 or its mutants prior to PRRSV infection. Progeny virus in culture supernatants was determined by TCID₅₀ assay. The results showed that constructs lacking the X domain (Δ X) impaired PRRSV replication. Whereas constructs lacking the LRR domain (Δ L) did not. As shown in Fig. 4B, construct encoding LRR alone or LRR in combination with NOD domain also impaired PRRSV

replication, suggesting that LRR is the key domain of NLRX1 that impairs PRRSV replication.

The C-terminal LRR domain of NLRX1 has been shown to bind RNA, and participate in regulation of steady-state levels of a subset of mitochondrial RNA (Feng et al., 2017; Hong et al., 2012; Singh et al., 2018). The observations in Fig. 4B led to our working hypothesis that NLRX1 modulated viral RNA synthesis. To test this, increasing dose of NLRX1 was transfected into Marc-145 cells followed by PRRSV infection, qPCR was then performed to test the total viral RNA levels. Result in Fig. 4C showed that NLRX1 inhibited the synthesis of viral RNAs in a dose dependent manner. Furthermore, knockdown of endogenous NLRX1 in PAMs increased the levels of viral total RNA (Fig. 4D).

The PRRSV genome is a single-strand positive-sense RNA flanked by the 5' and 3' un-translated regions (UTRs). A set of 3'-coterminal subgenomic RNAs (sgRNAs) are produced during infection (Han and Yoo, 2014). Thus, we evaluated the role of NLRX1 in viral sgRNAs synthesis. These synthesized viral sgRNAs were then quantified by qPCR after the infection of the Marc-145 cells with PRRSV. As shown in Fig. 4E, the relative levels of viral gRNA and sgRNAs, including gRNA,

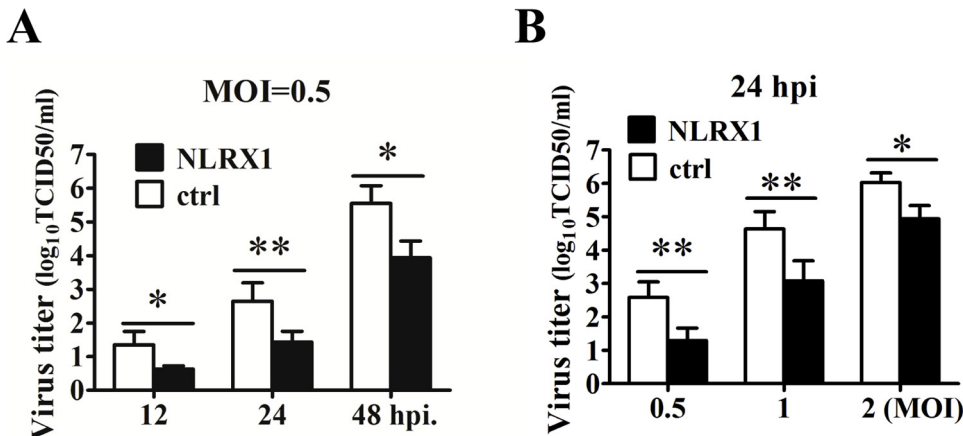


Fig. 2. Ectopic expression of NLRX1 impairs PRRSV replication in Marc-145 cells. **(A)** Marc-145 cells were transfected with pCAGGS-HA vector or HA-NLRX1 for 24 h and then infected at an MOI of 0.5 with PRRSV. After the designated hour postinfection, virus titers were determined by TCID₅₀ assay. **(B)** Marc-145 cells were transfected with vector or HA-NLRX1 for 24 h and then infected at the indicated MOI with PRRSV for 24 h. Virus titers were determined by TCID₅₀ assay.

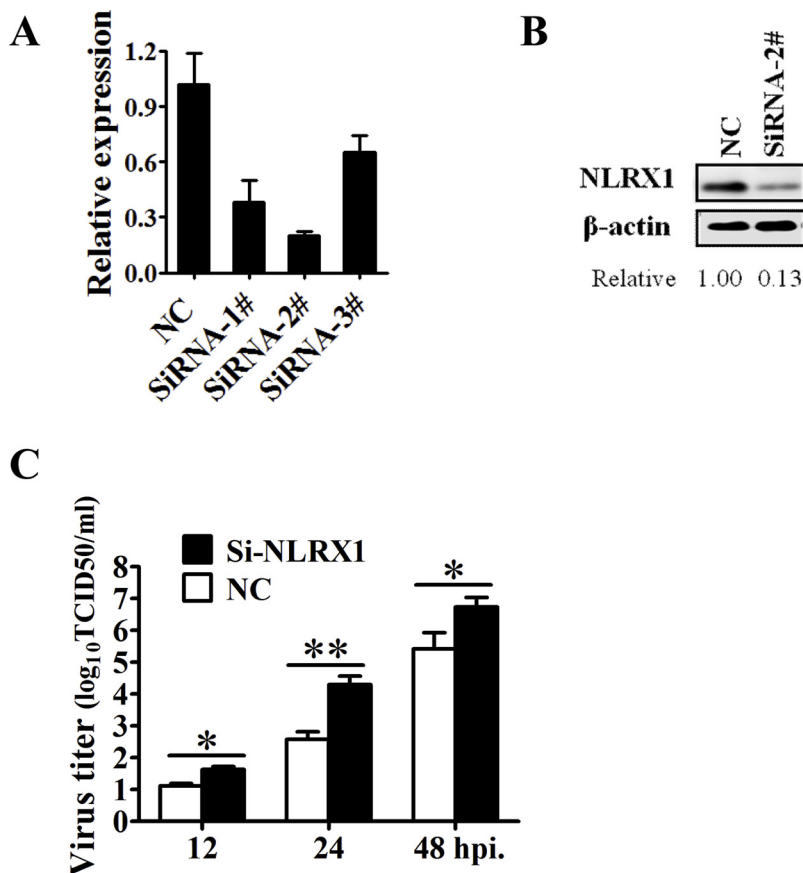


Fig. 3. Enhancement of PRRSV replication by NLRX1 knockdown in PAMs. **(A)** PAMs were transfected with a scrambled control siRNA (NC) or three different siRNA duplexes against NLRX1 for 48 h. Total RNA was extracted, and real-time PCR was performed to determine the relative levels of NLRX1. β -actin level was monitored as an internal control. **(B)** PAMs were transfected with NC siRNA or siRNA 2# against NLRX1. After 48 h, cell lysates were blotted with anti-NLRX1 or anti- β -actin antibody. **(C)** PAMs were transfected with the control siRNA or the NLRX1 siRNA for 48 h and then infected at an MOI of 0.1 with PRRSV. Virus titers were determined by TCID₅₀ assay at 12, 24, and 48 h post infection.

sgRNA2 to 4, apparently declined in NLRX1-overexpressing cells compared to the control, but the relative levels of viral sgRNA5-7 had no significant differences. This result suggested the inhibitory role of NLRX1 in the process of synthesizing long sgRNAs and genomic RNA.

3.5. NLRX1 interacted with PRRSV Nsp9

Nucleocapsid (N)-Nsp9 interaction has been shown to be involved in the production of PRRSV sgRNAs (Liu et al., 2016). To explore the mechanism by which NLRX1 suppresses viral sgRNAs synthesis, we investigated whether NLRX1 interacts with PRRSV Nsp9/N protein. To this end, HEK293 T cells were transfected with plasmids expressing Flag-Nsp9/N and HA-NLRX1 and co-immunoprecipitated with Flag antibody or HA antibody. Even though no co-immunoprecipitation of N protein was detectable with overexpressed NLRX1 in HEK293 T cells (data not shown), HA-NLRX1 was co-precipitated with Flag-tagged Nsp9 and vice versa (Fig. 5A).

Next, we investigated whether NLRX1 could colocalize with Nsp9. Hela cells were transfected with plasmids expressing Flag-Nsp9. Confocal data revealed ectopically expressed Flag-Nsp9 and endogenous NLRX1 partially co-localized with each other in the cytoplasm (Fig. 5B).

To identify the binding region within NLRX1 involved in the Nsp9-NLRX1 interaction, HEK293 T cells were co-transfected with various combinations of HA-tagged full-length or deleted versions of NLRX1 and Flag-tagged Nsp9. Mapping studies by Co-IP revealed that Nsp9 interacted with the C-terminal LRR domain of NLRX1, but not with its central NOD domain or N-terminal X domains (Fig. 5C).

PRRSV Nsp9 contains N-terminal RdRp domain and a C-terminal domain of unknown function (Wen et al., 2017; Zhao et al., 2015). To further investigate which domain within Nsp9 is required for binding with NLRX1, plasmids expressing Nsp9 mutants were constructed and their ability to interact with NLRX1 was assessed by Co-IP. Result

showed that WT Nsp9 and Nsp9C, but not the Nsp9N, bound to NLRX1 (Fig. 5E). The results showed that the RdRp domain of Nsp9 is responsible for interaction with NLRX1.

4. Discussion

NLRX1 was the first NLR shown to reduce type-I IFN production and facilitate viruses infection, by binding to MAVS (mitochondrial antiviral signaling protein) on mitochondria and STING (stimulator of interferon genes) on endoplasmic reticulum (ER), as demonstrated for Sindbis-virus, HIV-1, HSV-1 and KSHV (Guo et al., 2016; Ma et al., 2017; Moore et al., 2008; Qin et al., 2017). However, all of these functions reported for NLRX1 are not without controversy. For example, NLRX1 prevented IAV-induced macrophage apoptosis. Its deficiency led to increased pulmonary viral titer, inflammation, and reduced pulmonary function during IAV infection (Jaworska et al., 2014). Analogously, NLRX1 was required for immediate IRF1 (interferon regulatory factor 1)-directed antiviral responses. Replication of both hepatitis A virus (HAV) and hepatitis C virus (HCV) was enhanced in NLRX1-deficient hepatocytes (Feng et al., 2017). Adding to these similarities, we show here that NLRX1 acts to restrict PRRSV replication. Based on these observations, it is possible that the effect of NLRX1 during viral infection is a double-edged sword, potentially weakening IRF3 mediated responses as well as presenting a hurdle to be overcome by the virus.

NLRX1 and IAV PB1-F2 interaction is critical for the control of IAV replication and inflammation, indicating that interaction between NLRX1 and viral component might be a key factor in determining the outcome of viral infections (Jaworska et al., 2014). Our study shows that NLRX1 inhibits PRRSV replication by suppression its RNA synthesis. This is achieved by the association of PRRSV Nsp9 with LRR domain of NLRX1. Strikingly, C-terminal LRR domain of NLRX1 has been shown to possess a RNA binding site (Hong et al., 2012). In this regard, NLRX1 interacts with Rhinovirus (RV) RNA in polarized airway

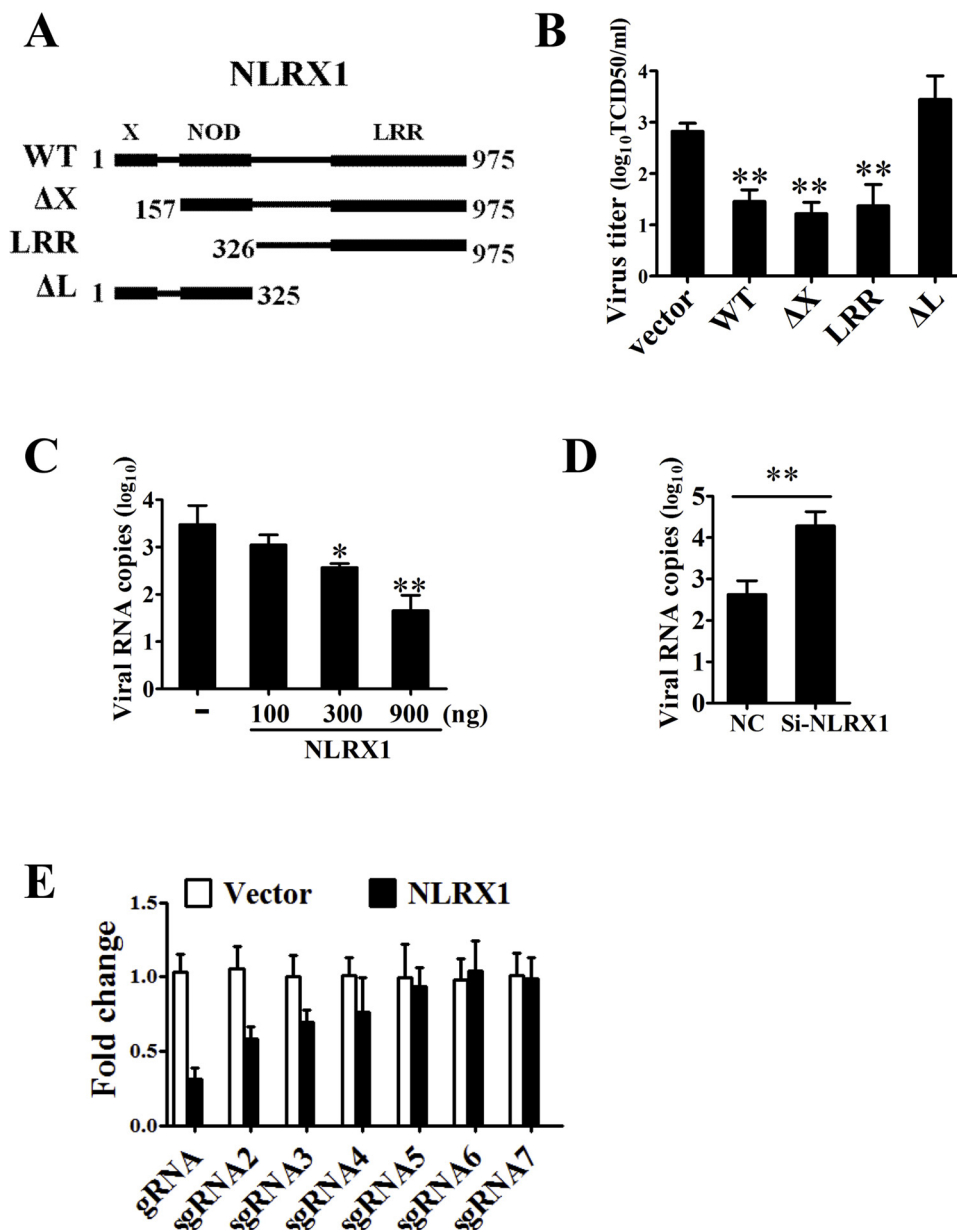


Fig. 4. NLRX1 suppresses the synthesis of viral subgenomic RNAs. **(A)** Schematic representation of full-length NLRX1 and corresponding deletion mutants is shown. Protein motifs are indicated. **(B)** Marc-145 cells were transfected with HA-tagged NLRX1, or its deletion mutants. After 24 h, cells were infected at an MOI of 0.5 with PRRSV for 24 h. Virus titers were determined by TCID₅₀ assay. **(C)** Marc-145 cells were transfected with an increasing amount of NLRX1 expression plasmid or control plasmid and then infected with PRRSV (MOI = 0.5) for 24 h. PRRSV total RNA were measured by qPCR. **(D)** PAMs were transfected with control siRNA or the NLRX1 siRNA for 48 h and then infected at an MOI of 0.1 with PRRSV. Viral total RNAs were measured by qPCR at 24 h post infection. **(E)** Marc-145 cells were transfected with the empty vector or NLRX1 expression plasmid for 24 h and then infected at an MOI of 0.5 with PRRSV. The levels of viral RNAs were monitored at 24 hpi by qPCR. The viral RNA levels in control cells were normalized to a value of 1.

epithelial cells (Unger et al., 2014). Likewise, NLRX1 also binds HAV genomic RNA to suppress PKR activation (Feng et al., 2017). Collectively, these findings support a model in which NLRX1 interacts with various viral factors to achieve an optimal immune state against diverse viruses. Future studies will be necessary to assess whether the Nsp9 of PRRSV-1 interacts with NLRX1.

Nsp9, the key enzyme encoded by ORF1b for RNA-templated RNA synthesis (RdRp) (Fang and Snijder, 2010), is closely related to the replication efficiency and pathogenicity for piglets (Li et al., 2014b; Music and Gagnon, 2010; Xu et al., 2018; Zhao et al., 2018). As a consequence, several host proteins have been reported to interact with PRRSV Nsp9. On the one hand, interaction between Nsp9 and cellular annexin A2, retinoblastoma protein (pRb), DDX5 positively regulates the replication of PRRSV *in vitro*, demonstrating that PRRSV heavily relies on host cellular proteins to complete life cycle (Dong et al., 2014; Li et al., 2014a; Zhao et al., 2015). On the other hand, recent literature indicated that the interaction of Nsp9 with SUMO E2 conjugating enzyme Ubc9 and cellular protein interleukin-2 enhancer binding factor 2 (ILF2) through its RdRp domain resulted in a significantly decrease of virus titers, indicating that cells utilize host antiviral factors as defense

mechanisms to limit PRRSV infection (Wang et al., 2017; Wen et al., 2017). Altogether, these results provide insights into the complex interactions of PRRSV RdRp with the host proteome. Noteworthy, the Nsp9 C-terminal fragment consisting of amino acid residues 599–646 was also identified as the N protein binding region (Liu et al., 2016), strengthening the concept that the C-terminal RdRp domain is a common protein interacting platform. Thus, the C-terminal RdRp domain of Nsp9 might be promising drug targets for interfering with PRRSV replication.

Even though our knowledge on the unique discontinuous transcription of arterivirus and coronavirus has rapidly grown over the last years, the molecular mechanisms controlling template switch still remain unclear (Di et al., 2018; Sola et al., 2015). In coronavirus, RNA helicase DDX1 was reported to benefit the synthesis of longer sgRNAs by unwinding the hairpin loop structure of viral gRNA, implying that RNA secondary structure may hinder the balance between shorter and longer sgRNAs synthesis (Wu et al., 2014). In line with this, a recent study suggested that PRRSV sgRNA2-5, but not sgRNA6-7, were impaired in helicase DHX9-knockdown cells (Liu et al., 2016). In this study, we observed that ectopic expression of NLRX1 had no effects on

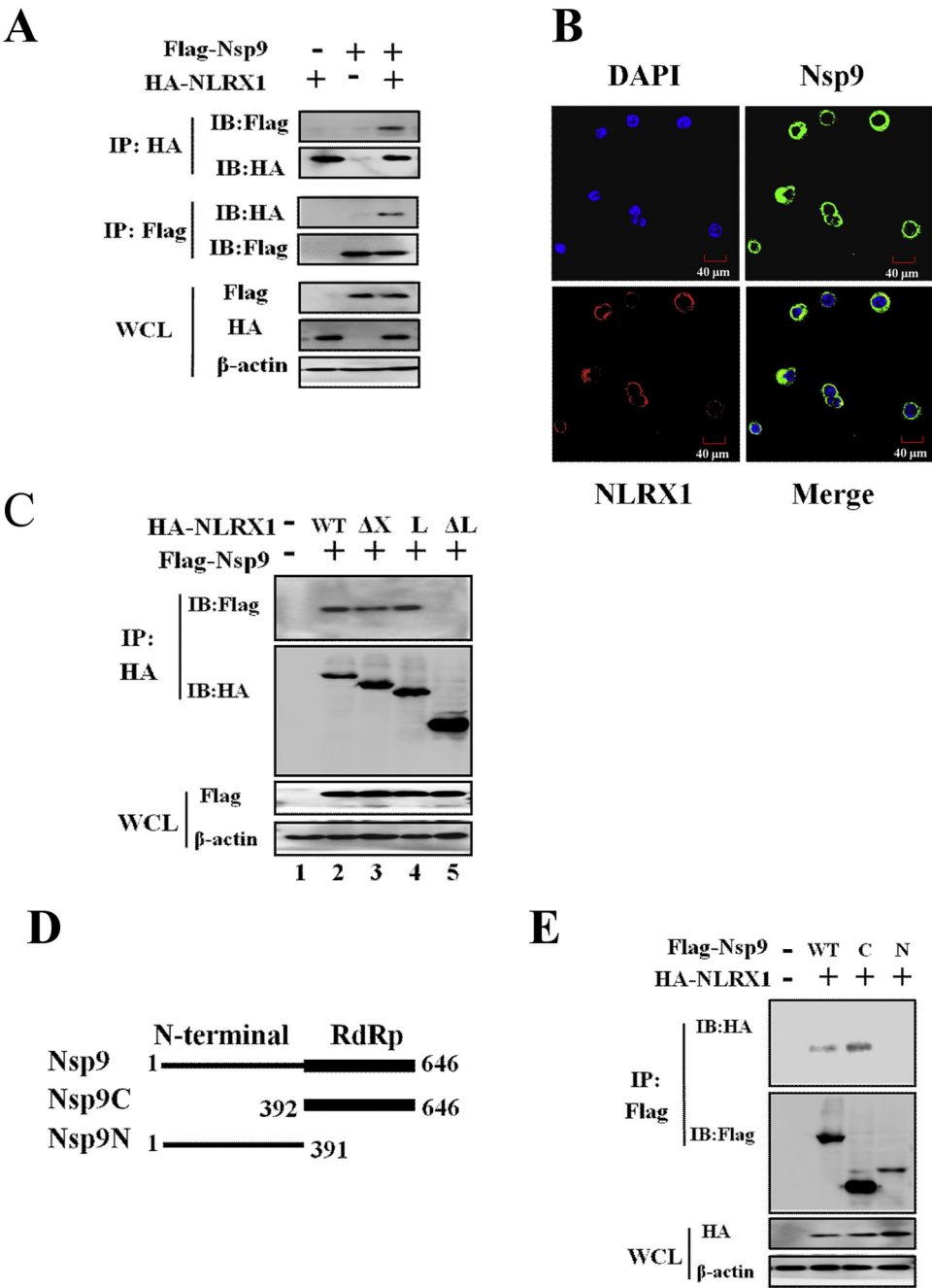


Fig. 5. Interaction of the Nsp9 with NLRX1. **(A)** HA-tagged NLRX1 was cotransfected with Flag-tagged Nsp9 into HEK293 T cells. Cell lysates were immunoprecipitated (IP) with anti-HA antibody or anti-Flag antibody and then blotted as indicated. **(B)** HeLa cells were transfected with Flag-tagged Nsp9 for 24 h. Cells were then fixed and incubated with anti-NLRX1 and anti-Flag antibodies. DAPI, 4'-diamidino-2-phenylindole. **(C)** Flag-tagged Nsp9 was cotransfected with the indicated HA-tagged NLRX1 mutants into HEK293 T cells. Cell lysates were immunoprecipitated with anti-HA antibody and blotted with the indicated antibodies. **(D)** The schematics of Nsp9 and corresponding truncation constructs. Numbers indicate the residues where deletions begin or end. **(E)** HA-tagged NLRX1 was cotransfected with the indicated Flag-tagged Nsp9 mutants into HEK293 T cells. Cell lysates were immunoprecipitated with anti-Flag antibody and blotted with the indicated antibodies.

the abundance of shorter sgRNA5-7. We therefore hypothesized that longer sgRNAs transcript could stop upon reaching NLRX1-Nsp9-RNA complex, thereby switching to produce shorter sgRNAs. Further investigations will be required to dissect the NLRX1-dependent mechanism for the effective control of continuous and discontinuous RNA synthesis.

In summary, these above findings indicate that NLRX1 inhibits the replication of PRRSV by interacting with viral Nsp9 *in vitro*, providing more knowledge for understanding the mechanisms associated with the replication regulation of PRRSV. Significant effort is still required to further uncover the potential interaction of Nsp9 with host cellular proteins and translate the current understanding of RdRp biology into effective antiviral drug development.

Conflict of interest

None.

Acknowledgments

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