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Nucleocapsid protein of porcine reproductive and respiratory syndrome virus antagonizes the antiviral activity of TRIM25 by interfering with TRIM25-mediated RIG-I ubiquitination

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Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV), and is characterized by respiratory diseases in piglet and reproductive disorders in sow. Identification of sustainable and effective measures to mitigate PRRSV transmission is a pressing problem. The nucleocapsid (N) protein of PRRSV plays a crucial role in inhibiting host innate immunity during PRRSV infection. In the current study, a new host-restricted factor, tripartite motif protein 25 (TRIM25), was identified as an inhibitor of PRRSV replication. Co-immunoprecipitation assay indicated that the PRRSV N protein interferes with TRIM25–RIG-I interactions by competitively interacting with TRIM25. Furthermore, N protein inhibits the expression of TRIM25 and TRIM25-mediated RIG-I ubiquitination to suppress interferon β production. Furthermore, with increasing TRIM25 expression, the inhibitory effect of N protein on the ubiquitination of RIG-I diminished. These results indicate for the first time that TRIM25 inhibits PRRSV replication and that the N protein antagonizes the antiviral activity by interfering with TRIM25-mediated RIG-I ubiquitination. This not only provides a theoretical basis for the development of drugs to control PRRSV replication, but also better explains the mechanism through which the PRRSV N protein inhibits innate immune responses of the host.

1. Introduction

The tripartite motif protein 25 (TRIM25) is an E3 ubiquitin ligase involved in various cellular processes, including regulating the innate immune response against viruses (Heikel et al., 2016). Immediately after a viral infection, pathogen-associated molecular patterns are sensed by host pattern recognition receptors (PRRs), thereby allowing cells to distinguish self from non-self and activate an immune response (Sparrer and Gack, 2015). For RNA viruses, the main cytoplasmic PRR is retinoic acid–inducible gene I (RIG-I), which can directly recognize and bind viral 5′-PPP RNA and short double-stranded RNA (Chan and Gack, 2016). After recognition, the N-terminal caspase recruitment domains (CARDs) of RIG-I are modified by ubiquitin which is mediated by TRIM25. Such modification is essential for activating a signaling cascade, ultimately resulting in the transcriptional activation of type I and III interferons (IFNs), mediating viral clearance, and inhibiting viral replication and spread (Gack et al., 2009; Martin-Vicente et al., 2017). Various viruses are inhibited by TRIM25, *e.g.*, Coxsackie B virus and poliovirus (Schoggins et al., 2014).

Porcine reproductive and respiratory syndrome (PRRS) is endemic in most pig-producing countries (Han and Yoo, 2014), and is caused by PRRS virus (PRRSV). In 2006, a highly pathogenic PRRSV (HP-PRRSV) emerged in China, causing high fever, high morbidity, and high mortality (Tian et al., 2007).

PRRSV is an enveloped, single-stranded positive-sense RNA virus. Its genome is approximately 15.4 kb and encodes at least 10 open reading frames (ORFs) (Han and Yoo, 2014). ORF1a and ORF1b encode two large nonstructural polyproteins, ppla and pplb, which are processed into at least 14 nonstructural proteins (Snijder and Meulenberg, 1998; Ziebuhr et al., 2000). ORF2a–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) (Dea et al., 2000; Snijder and Meulenberg, 1998). Among these, nsp1, nsp2, nsp4, nsp11, and N, have

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been identified and characterized as IFN antagonists (Lunney et al., 2016). Further, the N protein is the most abundant protein during infection, accounting for approximately 40% of virion proteins (Snijder and Meulenberg, 1998). It plays essential roles in the virus life cycle, including encapsidation of viral RNA (Spilman et al., 2009). Currently, it is only known that N protein suppresses IFN-β induction by antagonizing IRF3 activation (Sagong and Lee, 2011). However, other mechanisms through which N protein inhibits IFN-β production are not clear. The nucleocapsid protein of severe acute respiratory syndrome (SARS) inhibits type I IFN production by interfering with TRIM25-mediated RIG-I ubiquitination (Hu et al., 2017). Whereas PRRSV and SARS both belong to the Nidovirales order, whether PRRSV N protein inhibits the host innate immune response by interfering with TRIM25-mediated RIG-I ubiquitination is not clear.

Herein, we show that TRIM25 plays an important role in inhibiting PRRSV replication. The N protein interacts with TRIM25 and suppresses the ubiquitination of RIG-I. Further, TRIM25 expression is reduced upon co-transfection with the N protein or PRRSV infection. Together, these findings suggest a novel mechanism through which PRRSV N protein inhibits host innate immunity, and provide improved understanding of the mutual regulatory mechanism between PRRSV and the host.

2. Materials and methods

2.1. Cells and viruses

Human embryonic kidney (HEK293T) cells and African green monkey kidney (Marc-145) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA). Cells were maintained at 37 °C with 5% CO_2. The highly pathogenic PRRSV strain HuN4 (GenBank no. EF635006) was used for all the experiments(Tong et al., 2007).

2.2. Construction of plasmids

Total RNA was extracted from porcine alveolar macrophages (PAMs) using RNeasy mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Reverse transcription reactions were performed at 25 °C for 5 min and 42 °C for 1 h using the M-MLV reverse transcription polymerase system (TaKaRa, Dalian, China). Full-length TRIM25- and RIG-I-encoding sequences were amplified with specific indicated primers. The sequences were then cloned into pCAGGS vector to generate pCAGGS-TRIM25-HA, pCAGGS-TRIM25-Flag, pCAGGS-TRIM25-Myc, pCAGGS-RIG-I-HA, pCAGGS-RIG-I-Flag, or pCAGGS-^{2}CARD-Flag. ORF7 of PRRSV HuN4 was cloned into pCAGGS to generate pCAGGS-N-HA, pCAGGS-N-Flag, or pCAGGS-N-Myc. All plasmids were constructed by homologous recombination using the NEBuilder® HiFi DNA assembly master mix (New England Biolabs; Ipswich, MA) according to the manufacturer's instructions. Sequences of all primers used for gene amplification will be made available upon request.

2.3. Plasmid transfection and virus challenge

To investigate the effect of TRIM25 on PRRSV replication, Marc-145 cells cultured in 6-well plates were transfected with 2 μg of pCAGGS-TRIM25-Flag using X-treme GENE HP DNA reagent (Roche Applied Science, Penzberg, Germany). Next, 36 h post-transfection (hpt), the cells were infected with PRRSV HuN4 (multiplicity of infection, MOI, of 0.1). After inoculation for 1 h at 37 °C, the supernatants were discarded, and cells were washed three times with phosphate-buffered saline (PBS). The supernatant was harvested 12, 24, 36, and 48 h post-infection (hpi), and the cells were lysed using RIPA lysis buffer (Thermo Fisher Scientific). Viral titers in the supernatants were determined using a microtitration assay, according to the method of Reed and Muench (Reed and Muench, 1938). The amount of the N protein was then detected in cell lysates by western blotting (WB) using a mouse anti-N polyclonal antibody (1:1000) produced by the authors.

2.4. RNA interference

Small interfering RNAs (siRNAs) against macaque TRIM25 (GenBank no. XM_015119181.1) were synthesized by GenePharma (Shanghai, China). The siRNA molecules used were CCU GGA GUA UUA CGU UAA ATT (siRNA-TRIM25-1356) and GCA UCU ACC AUA GCA CCU UTT (siRNA-TRIM25-1007). The control siRNA (NC) sequence was UUC UCC GAA CGU GUC ACG UTT. Marc-145 cells were seeded in 6-well plates and transfected with 50 pM siTRIM25 or NC using Lipofectamine™ RNAiMAX transfection reagent (cat. no. 13,778,075; Thermo Fisher Scientific). The cells were lysed in RIPA lysis buffer after 36 h of transfection and the effects of siRNAs were analyzed by WB using an anti-TRIM25 monoclonal antibody (cat. no. 13,773; Cell Signaling Technology; Danvers, MA; 1:1000). The knockdown efficiency of siRNA was analyzed by grayscale scanning with Image J software. Next, efficient siRNA and NC were selected for transfection; 36 hpt, the cells were infected with PRRSV HuN4 at an MOI of 0.1. The supernatant and cells were harvested 12, 24, 36, and 48 hpi, and analyzed based on virus titers and by WB.

2.5. WB

Cell lysates were prepared by harvesting virus-infected or plasmid-transfected cells in RIPA or IP-lysate buffer(25 mM Tris•HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol) at 4 °C for 15 min containing 1 mM phenylmethylsulfonyl fluoride and 1 mg/ml of protease inhibitor cocktail (Roche). After centrifuging at 12,000 × g for 10 min, the supernatants of cell lysates were mixed with 5 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (Beyotime), and placed in boiling water for 5 min. The proteins were then separated by SDS-PAGE and transfected onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk at room temperature for 2 h and then incubated with the indicated primary antibody at 4 °C overnight. After washing three times with Tris-buffered saline with 0.1% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG(H + L) and/or goat anti-rabbit IgG(H + L) secondary antibody (1:5000) for 1 h at room temperature. The target proteins were visualized by treating the membrane with Pierce ECL WB substrate (Thermo Fisher Scientific). For the quantification of target proteins, their levels were normalized to the levels of β-actin.

2.6. Co-immunoprecipitation (Co-IP)

To detect the interaction between TRIM25 and PRRSV N protein, HEK293 T cells grown in 6-well plates were co-transfected with pCAGGS-TRIM25-Flag and pCAGGS-N-HA (2 μg each) using X-tremeGENE DNA transfection reagent. At 24 hpt, the cells were lysed with ice-cold IP lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and 1 mg/ml of protease inhibitor cocktail (Roche) at 4 °C for 15 min. Approximately 10% of the lysate supernatant was used as an input control and the remaining lysates were incubated with anti-HA agarose beads (cat. no. A2095; Sigma-Aldrich; St. Louis, MO) or EZview™ Red ANTI-FLAG® M2 affinity gel (cat.no. M2426; Sigma-Aldrich) for 6 h at 4 °C. The beads were washed five times with the IP lysis buffer and then analyzed by WB using rabbit anti-Flag monoclonal antibody (cat. no. F7425; Sigma-Aldrich; 1:5000) or mouse anti-HA monoclonal antibody (cat. no. H9658; Sigma-Aldrich; 1:5000). The interaction between TRIM25 and RIG-I was tested using the same methods with anti-HA agarose beads and WB using mouse anti-HA monoclonal antibody (cat. no. H9658; Sigma-Aldrich; 1:5000) and mouse anti-Myc monoclonal antibody (cat. no. 2276; Cell Signaling Technology; 1:1000). In order to investigate whether the N protein

competitively affects the interaction between RIG-I and TRIM25, HEK293 T cells grown in 6-well palate were co-transfected with pCAGGS-RIG-I-HA (1 μg) and pCAGGS-TRIM25-Myc (1 μg) with or without pCAGGS-N-Flag (1 μg) for immunoprecipitation. Further, to detect the interaction between endogenous (host) TRIM25 and N protein in PRRSV-infected cells, PAM cells were grown in 5–cm-diameter dishes and infected with PRRSV at an MOI of 0.5 for 24 h. The cells were lysed in 500 μL of IP lysis buffer and the supernatants were precleared with protein G-agarose beads (cat. no. 11243233001; Roche). The supernatant was incubated with 2 μg of anti-N polyclonal antibody for 8 h. Next, 20 μL of protein G-agarose beads was added, followed by an additional 3 h incubation. IP pellets were washed five times with 500 μL of IP lysis buffer, boiled in SDS-PAGE sample loading buffer, and analyzed by WB with rabbit anti-TRIM25 monoclonal antibody (cat. no. ab167154; Abcam; 1:2000) and anti-N polyclonal antibody.

2.7. Confocal imaging

HEK293 T cells were co-transfected with pCAGGS-N-HA and pCAGGS-TRIM25-Flag, or pCAGGS-RIG-I-HA and pCAGGS-TRIM25-Flag. At 24 hpt, the cells were fixed in 4% paraformaldehyde for 30 min, blocked with 3% bovine serum albumin for 1 h, and permeabilized with 0.1% Triton X-100 for 15 min. The transfected cells were incubated with mouse anti-HA monoclonal antibody (cat. no. H9658; Sigma-Aldrich; 1:2000) and rabbit anti-Flag monoclonal antibody (cat. no. F7425; Sigma-Aldrich; 1:2000) for 1 h at 37 °C, and then washed three times with PBS. The cells were then incubated at 37 °C for 1 h with donkey anti-mouse IgG(H + L) antibody conjugated with Alexa Fluor 596 (cat. no. 1692912; Life Technologies; 1:1000) and goat anti-rabbit IgG(H + L) antibody labeled with Alexa Fluor 488 (cat. no. 1674651; Life Technologies; 1:1000). Samples were then stained with 1 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) for 10 min and examined using a Zeiss confocal system. Besides, in order to investigate the colocalization of endogenous (host) TRIM25 and N protein in PRRSV-infected cells, the PAM cells infected with PRRSV were send to colocalization detection with anti-TRIM25 and anti-N protein antibody.

2.8. Dual luciferase assays

HEK293 T cells seeded in a 24-well plate were co-transfected with control plasmids or the indicated expression plasmids (0.25 μg per well) together with 100 ng of a luciferase reporter plasmid; pRL-TK plasmid (10 ng) was used as a control of transfection efficiency (Huang et al., 2014). Next, 24 hpt, cells lysates were harvested and luciferase activity in the lysates was analyzed using a dual-luciferase reporter assay kit (Promega, Madison, WI), following the manufacturer's instructions. The results are expressed as the means ± standard deviation (SD) from three independent experiments.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, La Jolla, CA). The measured values are expressed as the mean with SD. The statistical significance was assessed using Student's *t*-test, with $p < 0.05$ considered statistically significant.

3. Results

3.1. TRIM25 acts as an inhibitor of PRRSV

To examine the effects of TRIM25 on PRRSV replication, specific siRNA molecules were synthesized to knockdown *TRIM25* expression in Marc-145 cells and efficiently reduce TRIM25 expression. Using siRNA-1356, the knockdown efficiency was approximately 65% (Fig. 1A). This siRNA molecule was used in the subsequent interference experiments. As shown in Fig. 1B, N protein levels increased upon transfection with siRNA-1356, especially 36 and 48 hpi, compared with those in NC-transfected cells. Virus titers in the culture supernatants of cells transfected with siRNA-1356 were also increased, which was consistent with the expression levels of the N protein, with a significant difference 36 hpi ($p < 0.05$; Fig. 1C). By contrast, when TRIM25 was overexpressed, the N protein levels of HuN4 were lower than those in the control cells (Fig. 1D). Furthermore, there was a significant difference in virus titers between cells transfected with pCAGGS-TRIM25-Flag or pCAGGS, with an approximate 1.0 log decrease in virus titers from 24 to 36 hpi ($p < 0.05$; Fig. 1E). These observations suggested that TRIM25 is a cellular antiviral factor that represses PRRSV infection.

3.2. TRIM25 is down-regulated in response to PRRSV infection and N protein accumulation

As shown in Fig. 1D, the expression of TRIM25-Flag decreased gradually upon PRRSV infection. Therefore, we speculated that the virus might inhibit the expression of TRIM25. To verify this hypothesis, Marc-145 cells infected with PRRSV or uninfected cells were lysed 12, 24, 36, and 48 hpi, and analyzed by WB. The analysis revealed that the expression of TRIM25 decreased in PRRSV-infected cells at every time point, as compared with the uninfected cells (Fig. 2A). Furthermore, TRIM25 expression was significantly suppressed when pCAGGS-TRIM25-Myc and pCAGGS-N-HA were co-transfected into HEK293 T cells. These findings indicated that the N protein inhibits the expression of TRIM25. Furthermore, the N protein inhibited TRIM25 expression in a dose-dependent manner (Fig. 2B).

3.3. The N protein of PRRSV interacts with TRIM25

Since we demonstrated that PRRSV infection and N protein accumulation suppress the expression of TRIM25, we next investigated whether the N protein of PRRSV exerts an inhibitory effect on TRIM25 expression by interacting with it. To test this, the interaction between TRIM25 and N protein was investigated by Co-IP. Based on precipitation with anti-Flag agarose, Flag-tagged TRIM25 interacted with HA-tagged N protein (Fig. 3A). Furthermore, N protein was efficiently co-immunoprecipitated with TRIM25 using anti-HA agarose (Fig. 3B). We also investigated whether TRIM25 co-localizes with the N protein in HEK293 T cells co-transfected with pCAGGS-TRIM25-Flag and pCAGGS-N-HA. TRIM25 was mainly located in the cytoplasm (Fig. 3C–a), whereas the N protein localized in the cytoplasm and nucleus (Fig. 3C–b). As shown in Fig. 3C–d, TRIM25 and N protein co-localized in the cytoplasm.

To examine the interaction of N protein and endogenous TRIM25 in the context of PRRSV infection, virus-infected PAM cells were stained with anti-N polyclonal antibody and anti-TRIM25 monoclonal antibody or immunoprecipitated using anti-N polyclonal antibody and the pellets were detected with anti-TRIM25 and N antibody. As shown in Fig. 3D and 3E, endogenous TRIM25 was co-localized with N protein and co-immunoprecipitated with the N protein. These observations confirmed that the endogenous TRIM25 indeed interacts with the N protein of PRRSV in PRRSV-infected cells.

3.4. The PRRSV N protein competitively interferes with TRIM25–RIG-I interactions

In the current study, we demonstrated that the N protein of PRRSV could interact with TRIM25. Further, it has been reported that TRIM25 interacts with RIG-I N-terminal CARDs and E2 ubiquitin-conjugating enzymes (Gack et al., 2007). Whether the N protein can competitively regulate the interaction between TRIM25 and RIG-I was unknown. We first detected the interaction between TRIM25 and RIG-I by Co-IP, confirming that TRIM25 could be immunoprecipitated with RIG-I (Fig. 4A). In addition, co-localization analysis demonstrated that RIG-I and TRIM25 co-localize in the cytoplasm (Fig. 4B). Further, we

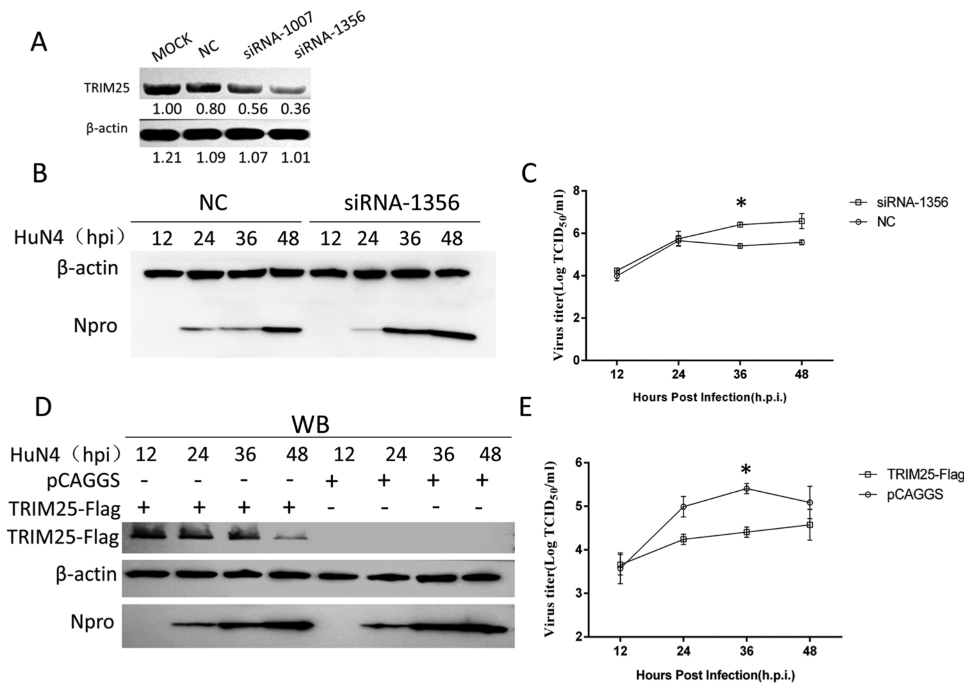

Fig. 1. TRIM25 affects the replication of PRRSV. (A) Marc-145 cells were transfected with siRNA-1007, siRNA-1356, or negative control (NC) (50 pM per well). The knockdown efficiency of siRNA was analyzed by WB 36 hpt and the knockdown efficiency analyzed with grayscale is showed below the figure panel. (B and C) Marc-145 cells were transfected with NC or siRNA-1356 (50 pM pre well). The cells were infected with PRRSV HuN4 at an MOI of 0.1 36 hpt. The cells and the supernatant were harvested at the indicated times, and the replication of PRRSV was evaluated by WB (B) and $TCID_{50}$ assay (C). (D and E) Marc-145 cells transfected with pCAGGS-TRIM25-Flag (2 μg) or pCAGGS (2 μg) were inoculated with PRRSV at an MOI of 0.1. The cells and supernatant were harvested at the indicated times, and the replication of PRRSV was evaluated by WB with an anti-N polyclonal antibody (D) and $TCID_{50}$ assay (E). The data are presented as the mean ± SD from three experiments. The statistical significance of differences was determined using Student's *t*-test ($^{*}p < 0.05$).

validated the effect of N protein on the interaction between RIG-I and TRIM25. When RIG-I-HA and TRIM25-Myc were co-transfected with N-Flag, the interaction between TRIM25 and RIG-I was markedly diminished. Meanwhile, when TRIM25-Myc and RIG-I-HA were co-transfected without N-Flag, the interaction between TRIM25 and RIG-I was not affected (Fig. 4C). These results suggested that the N protein inhibits the interaction between TRIM25 and RIG-I via competitive binding to TRIM25.

3.5. The PRRSV N protein suppresses RIG-I ubiquitination

To investigate whether TRIM25-mediated RIG-I ubiquitination is regulated by the PRRSV N protein, HEK293T cells grown in 6-well plates were co-transfected with pCAGGS-Flag-RIG-I (0.5 μg per well) and HA-ubiquitin (0.5 μg per well), and the indicated amounts of the Myc-N expression plasmids. Cells lysates were immunoprecipitated using mouse anti-HA monoclonal antibody or rabbit anti-Flag monoclonal antibody. The experiment revealed that TRIM25-mediated RIG-I ubiquitination was potentiated by Sendai virus (SEV) infection but was substantially suppressed by increasing the PRRSV N protein expression, in a dose-dependent manner (Fig. 5).

3.6. N protein-mediated inhibition of RIG-I activation and IFN production is rescued by TRIM25

When viral RNA is recognized by RIG-I, this protein is modified by ubiquitin, which is mediated by the E3 ligase TRIM25. Hence, this enzyme is essential for activating a signaling cascade that ultimately results in the transcriptional activation of type I and III IFNs. To examine regulation of RIG-I activity by the PRRSV N protein, a luciferase reporter under the control of the *IFN-β* promoter (IFN-*β*-Luc) was used to quantify promoter activation. Consistent with the inhibition of RIG-I ubiquitination by the N protein, *IFN-β* promoter activation induced by RIG-I or RIG-I CARD domain overexpression was significantly inhibited by PRRSV N expression, in a dose-dependent manner (Fig. 6A, B). However, co-expression of TRIM25 with PRRSV N significantly counteracted this inhibitory effect mediated by the N protein ($p < 0.05$) (Fig. 6C). In addition, suppression of RIG-I ubiquitination by the PRRSV N protein was partially rescued by TRIM25 overexpression (Fig. 6D). These observations indicated that the N protein inhibits RIG-I ubiquitination and that IFN production can be restored by TRIM25 overexpression.

4. Discussion

PRRSV has been a major threat to global industrial pig farming ever since its emergence in the late 1980s (Shi et al., 2010), and especially

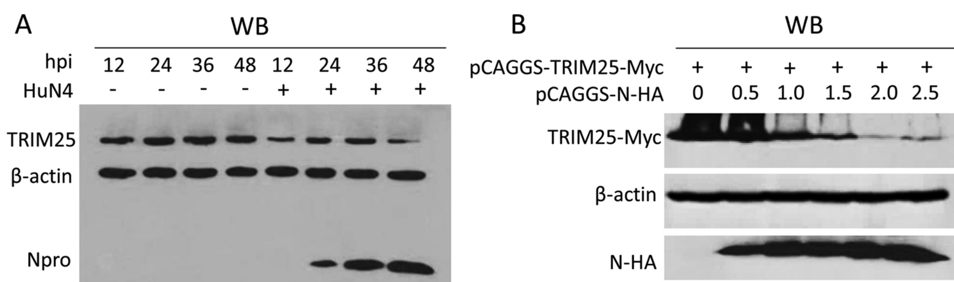

Fig. 2. PRRSV inhibits the expression of TRIM25. (A) Marc-145 cells were infected with PRRSV HuN4 at an MOI of 0.1, and the cells were harvested 12, 24, 36, and 48 hpi. The expression of TRIM25 was monitored by WB using rabbit anti-TRIM25 monoclonal antibody and mouse anti-N polyclonal antibody. (B) The N protein of PRRSV inhibits TRIM25 expression in a dose-dependent manner. For the experiment, pCAGGS-N-HA (0, 0.5, 1.0, 1.5, 2.0, or 2.5 μg) and pCAGGS-TRIM25-Myc (1 μg) plasmid were co-transfected into HEK293 T cells. The N protein and TRIM25 levels were detected by WB using anti-HA and anti-Myc monoclonal antibodies, respectively.

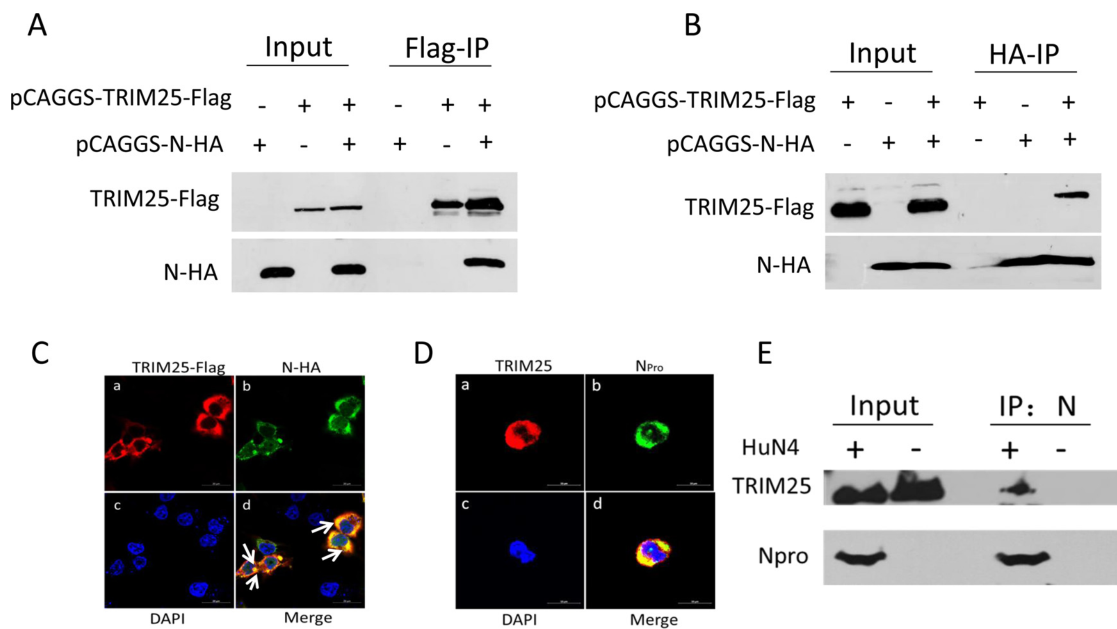

Fig. 3. TRIM25 interacts with the PRRSV N protein. (A) HEK293 T cells were co-transfected with pCAGGS-TRIM25-Flag and pCAGGS-N-HA. Co-IP experiment was performed using agarose beads conjugated with anti-Flag monoclonal antibody or (B) agarose beads conjugated with anti-HA monoclonal antibody. The precipitated proteins were analyzed by WB using antibodies against the Flag and HA tags. (C) Co-localization of the N protein with TRIM25. HEK293 cells were transfected with plasmids expressing HA-N protein and Flag-TRIM25. After 24 h, the cells were fixed and analyzed by IFA to detect tagged N protein and TRIM25, using mouse anti-HA and rabbit anti-Flag monoclonal antibodies, respectively. The nucleus is stained with DAPI (blue) in the merged images. (D and E) Colocalization and interaction of PRRSV N and endogenous TRIM25. PAM cells infected with PRRSV were fixed 24 h after infection, and then subjected to indirect immunofluorescence to detect N protein and TRIM25. In addition, cell lysates from PRRSV-infected or mock-infected PAM cells were Co-IP with mouse anti-N polyclonal antibody, and the precipitates were immunoblotted with the indicated antibodies (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

since the outbreak of HP-PRRSV in 2006. To date, although there is some understanding of the mechanisms through which PRRSV escapes innate immunity, very little is known about how viral proteins of PRRSV antagonize the host innate immune response. Therefore, understanding the interactions between PRRSV and host proteins will be beneficial for the development of effective therapies to control outbreaks of this disease in the future.

In the current study, we demonstrated that TRIM25 functions by restricting PRRSV replication, and that it could act as a host-derived inhibitor of the virus. Previously, many host factors that result in cellular resistance to PRRSV via different mechanisms were identified (Li et al., 2018; Song et al., 2017; Wang et al., 2016; Zhao et al., 2018, 2017). For example, cholesterol 25-hydroxylase protects against PRRSV infection by converting cholesterol to 25-hydroxycholesterol, which can be used as a natural antiviral agent to combat PRRSV infection (Song et al., 2017). Further, galectin-3 inhibits replication PRRSV by interacting with viral Nsp12 in vitro (Li et al., 2018). In the current study, using Marc-145 cells, we demonstrated that the overexpression of TRIM25 restricts the replication of PRRSV, whereas knocking down this protein promotes the replication of PRRSV (Fig. 1). Therefore, TRIM25 acts as a novel host factor that inhibits the replication of PRRSV.

TRIM25 is an E3 ubiquitin ligase enzyme that can regulate the innate immune response against viruses (Martin-Vicente et al., 2017). TRIM25-mediated ubiquitination of the cytosolic PRR RIG-I is an essential step for the initiation of an intracellular antiviral response (Gack et al., 2007). Data presented herein revealed that upon TRIM25 overexpression, the N protein-mediated inhibition of RIG-I ubiquitination and *IFN-β* promoter activity were diminished (Fig. 6). The host innate immunity was hence activated, leading to a series of signaling cascades and thereby inhibiting PRRSV replication.

TRIM25 can activate the host innate immune system and simultaneously induce a series of antiviral responses by promoting the ubiquitination of RIG-I and activation of *IFN-β* promoter activity. However, in the course of natural infection, PRRSV can complete the replication cycle and efficiently spread. Hence, PRRSV has evolved several general strategies to evade the innate immune response. It has been reported that some viral proteins interact with TRIM25 and inhibit RIG-I activation. For example, the non-structural protein 1 (NS1) of influenza A virus interacts with the CC domain of TRIM25 preventing its dimerization and the K63-linked ubiquitination of RIG-I CARDs, thereby suppressing RIG-I signal transduction (Gack et al., 2009). Further, TRIM25 interacts with the N protein of SARS-CoV, thereby inhibiting the activation of RIG-I (Hu et al., 2017). In the current study, we found that the N protein of PRRSV inhibits the ubiquitination of RIG-I by competitively interfering with the interaction between RIG-I and TRIM25. This might be the mechanism through which PRRSV inhibits the antiviral effect of TRIM25. Furthermore, TRIM25 levels decreased when the cells were infected with PRRSV. In addition, when plasmids expressing TRIM25 and the N protein of PRRSV were co-transfected into cells, the expression of TRIM25 was significantly suppressed. Based on this, it would be difficult for TRIM25 to exert an antiviral effect upon PRRSV infection. This might represent another mechanism through which PRRSV antagonizes the antiviral response of TRIM25. Besides, the N protein of PEDV, another coronavirus, is also able to antagonize IFN-β production(Ding et al., 2014). Since PRRSV, SARS, and PEDV all belong to Nidovirales, we speculate that the respective N proteins may exert a similar effect of inhibiting TRIM25-mediated ubiquitination of RIG-I. However, the effect of PEDV N protein on the inhibition of RIG-I ubiquitination requires further research.

In the present study, we confirmed that TRIM25 inhibits PRRSV replication. Further, PRRSV can antagonize the antiviral activity of this protein by decreasing its expression and modulating the TRIM25-mediated ubiquitination of RIG-I. In addition, the N protein of PRRSV inhibits IFN-β production. All these mechanisms improve the understanding of the effect of TRIM25 on PRRSV replication and will further

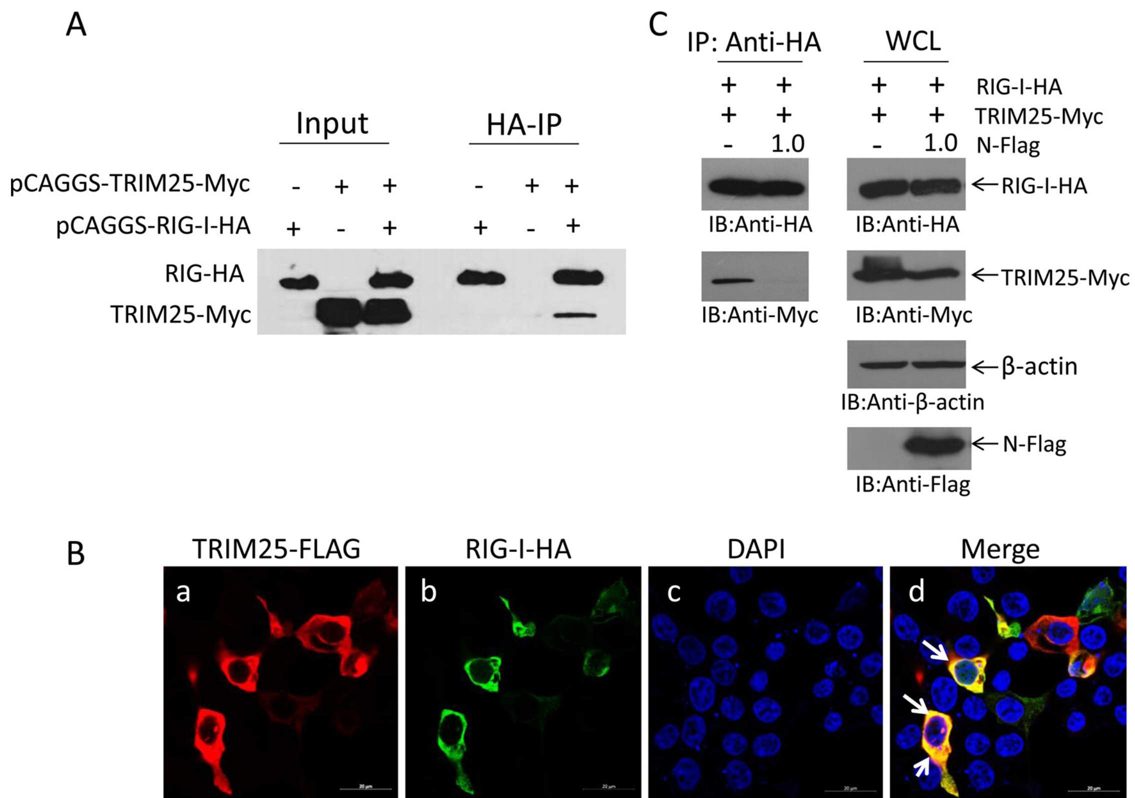

Fig. 4. The N protein interferes with the interaction between TRIM25 and RIG-I. (A) HEK293 T cells were co-transfected with pCAGGS-TRIM25-Myc and pCAGGS-RIG-I-HA (2 μg per well). Co-IP was performed using agarose beads conjugated with anti-HA monoclonal antibody. The precipitated proteins were analyzed by WB using antibodies against the Myc and HA tags. (B) Co-localization of RIG-I with TRIM25. Expression plasmids pCAGGS-TRIM25-Flag and pCAGGS-RIG-I-HA were used to co-transfect HET293 T cells, and analyzed by confocal microscopy using rabbit anti-Flag and mouse anti-HA antibodies. (C) RIG-I-HA and TRIM-25-Myc were co-transfected, with or without N-Flag, into HEK293 T cells. The interaction between TRIM25-Myc and RIG-I was then probed by immunoprecipitation.

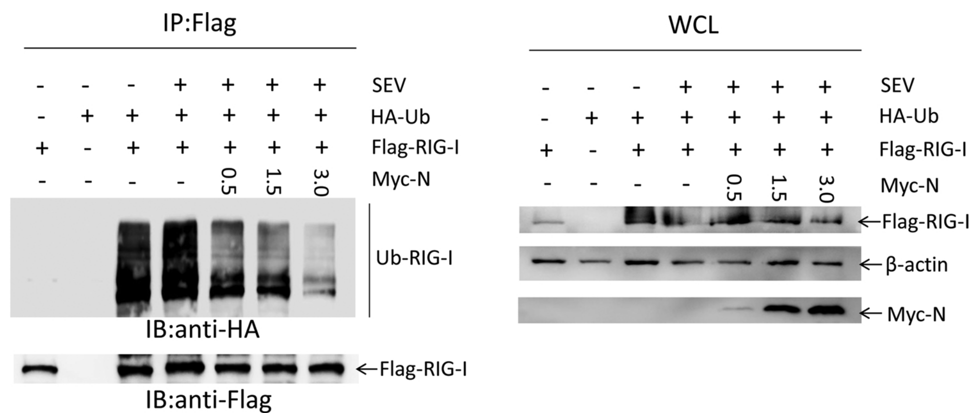

Fig. 5. The PRRSV N protein suppresses the TRIM25-mediated RIG-I ubiquitination. HEK293 T cells were transfected with the indicated plasmids for 36 h, and were infected (with or without SEV) for 12 h. Anti-Flag immunoprecipitates prepared from the cell extracts were analyzed by WB using the indicated antibodies.

help to understand how PRRSV evades the TRIM25-mediated innate immune response via the N protein. Hence, the current study not only offers a new target for the development of drugs to control PRRSV spread but also provides an explanation of the mechanism through which PRRSV N protein modulates host innate immune responses.

Declarations of interest

None.

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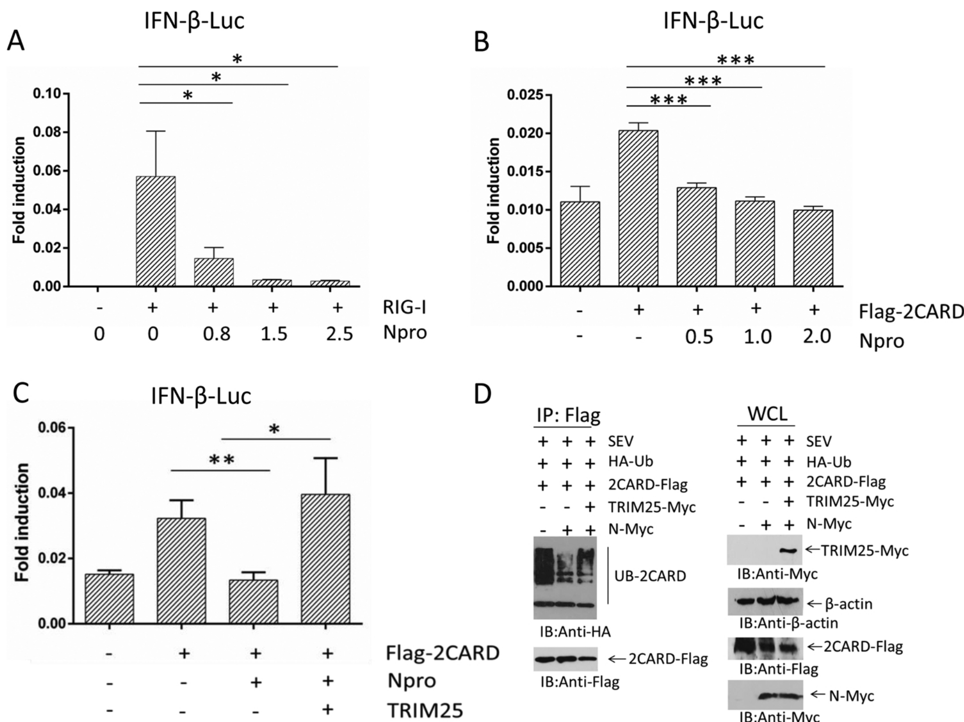

Fig. 6. The PRRSV N protein inhibits TRIM25-mediated RIG-I activation and IFN production. (A and B) HEK293 T cells seeded in 24-well plates were co-transfected using the *firefly* luciferase reporter plasmid IFN-*β*-Luc and the *Renilla* luciferase control reporter plasmid pRL-TK. For the experiment, pCAGGS-RIG-I-Flag (0.25 μg), or pCAGGS-^{2}CARD (0.25 μg), pCAGGS-N-HA were co-transfected. (C) pCAGGS-^{2}CARD-Flag (0.25 μg), pCAGGS-N-Falg (0.25 μg) and pCAGGS-TRIM25-Myc (0.5 μg) plasmids were cotransfected. The luciferase activity in cell lysates was analyzed using a dual luciferase reporter assay system. (D) HEK293 T cells grown in 6-well plates were co-transfected with plasmids encoding ubiquitin-HA (0.5 μg), Flag-2CARD (0.5 μg), N-Myc (1.0 μg), or TRIM25-Myc (1.0 μg). For the experiment, 24 hpt, the cells were infected with SEV, and 16 hpi, whole-cell lysates were analyzed by immunoprecipitation using the indicated antibodies to detect the ubiquitination of RIG-I-CARD. The data are presented as the mean ± SD from three experiments. The statistical significance of differences was determined using Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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