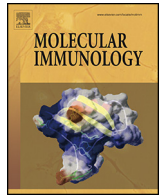




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The nonstructural protein 11 of porcine reproductive and respiratory syndrome virus inhibits NF- κ B signaling by means of its deubiquitinating activity



Dang Wang^{a,b,1}, Jinxiu Fan^{a,b,1}, Liurong Fang^{a,b}, Rui Luo^{a,b}, Haiping Ouyang^{a,b},
Chao Ouyang^{a,b}, Huan Zhang^{a,b}, Huanchun Chen^{a,b}, Kui Li^c, Shaobo Xiao^{a,b,*}

^a State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China

^b The Cooperative Innovation Center for Sustainable Pig Production, Wuhan 430070, China

^c Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee 38163, USA

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ABSTRACT

Since its emergence in the late 1980s, porcine reproductive and respiratory syndrome (PRRS) has been devastating the swine industry worldwide. The causative agent is an *Arterivirus*, referred to as PRRS virus (PRRSV). The pathogenic mechanisms of PRRS are poorly understood, but are believed to correlate with the ability of PRRSV to inhibit immune responses of the host. However, precisely how the virus is capable of doing so remains obscure. In this study, we showed that PRRSV infection led to reduced ubiquitination of cellular proteins. Screening all of the 12 nonstructural proteins (Nsps) encoded by PRRSV revealed that, apart from the Nsp2 which contains the deubiquitinating (DUB) ovarian tumor (OTU) domain, Nsp11, which encodes a unique and conserved endoribonuclease (NendoU) throughout the *Nidovirus* order, also possesses DUB activity. *In vivo* assay demonstrated that Nsp11 specifically removed lysine 48 (K48)-linked polyubiquitin chains and the conserved sites C112, H144, D173, K180, and Y219 were critical for its DUB activity. Remarkably, DUB activity was responsible for the capacity of Nsp11 to inhibit nuclear factor κ B (NF- κ B) activation. Mutations abrogating the DUB activity of Nsp11 toward K48-linked polyubiquitin chains of $\text{I}\kappa\text{B}\alpha$ nullified the suppressive effect on NF- κ B. Our data add Nsp11 to the list of DUBs encoded by PRRSV and uncover a novel mechanism by which PRRSV cripples host innate immune responses.

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1. Introduction

Protein ubiquitination is a reversible process that plays a vital role in nearly every aspect of cellular physiology, including protein degradation, protein trafficking, transcription, cell-cycle control, and cell signaling; (Liu et al., 2005; Pickart, 2001). Not surprisingly, ubiquitination is targeted for manipulation by a wide range of microbial pathogens (Randow and Lehner, 2009). In particular, many viruses have evolved elaborate strategies to inhibit or redirect the ubiquitination machinery of the host for their survival (Viswanathan et al., 2010). For example, human immunodeficiency virus 1 (HIV-1) prevents antiviral interferon response via

Vpr- and Vif-directed, ubiquitin-mediated proteasomal degradation of interferon regulatory factor 3 (IRF-3) (Okumura et al., 2008); the papain-like protease (PLpro) domains of many coronaviruses, such as severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV), human coronavirus NL63 (HCoV-NL63), and mouse hepatitis virus A59 (MHV-A59), have deubiquitinating (DUB) activity that blocks type I interferons (IFNs) induction (Barretto et al., 2005; Chen et al., 2007b; Clementz et al., 2010; Devaraj et al., 2007; Frieman et al., 2009; Lindner et al., 2005; Zheng et al., 2008); the leader proteinase (L^{pro}) of Foot-and-mouth virus (FMDV) acts as a deubiquitinase that cleaves ubiquitin chains from retinoic acid-inducible gene I (RIG-I), TANK-binding kinase 1 (TBK1), TNF receptor associated factor 6 (TRAF6), and TRAF3, thereby inhibiting the activation of type I IFN signaling (Wang et al., 2011); the N-terminal protease (Npro) of bovine viral diarrhea virus interacts with IRF-3 and promotes its polyubiquitination and subsequent degradation through the proteasome (Chen et al., 2007a); the latency associated protein ORF73 of murine herpesvirus-4 (MuHV-4) associates with the host ubiquitin-ligase complex to promote

* Corresponding author at: Laboratory of Infectious Diseases, College of Veterinary Medicine, Huazhong Agricultural University, 1 Shi-zi-shan Street, Wuhan 430070, China. Fax: +86 27 8728 2608.

E-mail address: vet@mail.hzau.edu.cn (S. Xiao).

¹ These authors contributed equally to this work.

poly-ubiquitination and subsequent proteasomal degradation of p65/RelA, which inhibits the activity of nuclear factor κ B (NF- κ B) to facilitate the establishment of MuHV-4 latency (Rodrigues et al., 2009). Collectively, these previous findings reveal that hijacking of the cellular ubiquitin system is an emerging, central theme around virus replication. In this regard, studying ubiquitination events in virus-infected cells holds great promise to unravel important modulators of the intricate relationship between host and pathogen. Not less important, understanding the mechanisms by which viral products interact with the ubiquitin system provides novel insights into viral pathogenesis and informs approaches to antiviral drug development.

Porcine reproductive and respiratory syndrome (PRRS) is a relatively new viral infectious disease of the swine (Rossow, 1998). It is characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs. Since it was first reported in the United States in 1987 and in Europe in 1990, PRRS has devastated the swine industry worldwide, causing tremendous economic losses. As such, PRRS is now considered to be one of the most important diseases in countries with intensive swine industries (Meulenbergh, 2000; Murtaugh et al., 2010; Neumann et al., 2005). The causative agent, PRRS virus (PRRSV), is a single-stranded positive-sense RNA virus classified within the order *Nidovirales*, family *Arteriviridae*, which also includes equine arteritis virus (EAV), murine lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (Cavanagh, 1997). At least nine open reading frames (ORFs) have been identified in the PRRSV genome. ORF1a and ORF1b, which are situated in the 5'-proximal three quarters of the genome, encode the viral non-structural proteins (Nsps): Nsp1 α , Nsp1 β , and Nsp2 to Nsp12. ORF2a, ORF2b, and ORF3 to ORF7 are located at the 3' end of the genome and encode the viral structural proteins GP2, E, GP3, GP4, GP5, M, and N, respectively. Because the Nsps constitute ~80% of the PRRSV genome coding capacity, much attention has been garnered in studying the functions and immuno-modulatory roles of PRRSV Nsps (Dokland, 2010). To date, several Nsps, including Nsp1 α/β , Nsp2, Nsp4, and Nsp11 have been reported to have inhibitory effect on activation of the IFN- β promoter (Beura et al., 2010; Kim et al., 2010; Li et al., 2010; Shi et al., 2011; Song et al., 2010). More recently, the cysteine protease domain (CP) of PRRSV Nsp2 was identified as a member of the ovarian tumor domain (OTU) family of deubiquitinating (DUB) enzymes. It was shown that the OTU domain of PRRSV Nsp2 antagonizes the induction of type I IFNs by interfering with the NF- κ B pathway (Sun et al., 2010). However, it remains unclear whether other Nsps encoded by PRRSV have DUB activity. In the present study, we screened the 12 Nsps of PRRSV and found both Nsp11 and Nsp2 possess DUB activity. Interestingly, Nsp11 specifically targeted lysine 48 (K48)-linked but not K63-linked ubiquitination chains for cleavage. This attenuated ligand-induced degradation of inhibitor of NF- κ B alpha (I κ B α), thereby inhibiting the activation of NF- κ B. Our data identify Nsp11 as a second DUB encoded by PRRSV and describe a novel mechanism by which PRRSV antagonizes innate immunity of the host.

2. Materials and methods

2.1. Cells and viruses

Marc-145 cells, PK-15-CD163 cells and HEK293 cells were maintained in Dulbecco's modified eagle media (DMEM, Invitrogen) supplemented with 10% heated-inactivated fetal calf serum (FBS), 100 U/mL penicillin, 10 μ g/mL streptomycin sulfate at 37°C in a humidified 5% CO₂ incubator. PK-15-CD163 cells, a porcine kidney cell line stably expressing the receptor CD163 of PRRSV, were gifts of Dr. En-min Zhou (Northwest A&F University, China) (Wang

et al., 2013). The WUH3 strain of PRRSV (Li et al., 2009), which was isolated from the brains of pigs that contracted the "high fever" syndrome in China at the end of 2006, was used in this study. PRRSV was propagated in Marc-145 cells or PK-15-CD163 cells, and the supernatants of infected cells were clarified and stored at -80°C in aliquots. Poly(I:C) (Sigma-Aldrich) and tumor necrosis factor α (TNF- α) (Sigma-Aldrich) were also used to stimulate cells.

2.2. Plasmids

Full-length HA-tagged ubiquitin (Ub) plasmid (HA-Ub) and HA-Ub mutants in which all but one Lys residue (HA-K48-Ub or HA-K63-Ub) were substituted with Arg were gifts of Dr. T. Ohta (St. Marianna University School of Medicine, Japan) (Nishikawa et al., 2004). pCDNA3.1-Flag-Ub was previously described (Clementz et al., 2010). The luciferase report plasmid pNF- κ B-Luc was purchased from Stratagene.

The hemagglutinin (HA) or V5 epitope tag was amplified by PCR and cloned into the pCAGGS-MCS vector (Niwa et al., 1991) to generate pCAGGS-HA or pCAGGS-V5 plasmid with N-terminally HA or V5 tag, respectively. For construction of the mammalian expression plasmids encoding various nonstructural proteins of PRRSV, cDNA fragments encoding full-length Nsp1 α , Nsp1 β , Nsp2-5, Nsp7-12 of PRRSV strain WUH3 (GenBank accession no. HM853673) were amplified by PCR and inserted into the pCAGGS-HA or pCAGGS-V5 plasmid. C64A, C112A, H129A, H144A, K173A, D180A, D204A, and Y219A mutants of Nsp11 were generated by overlap extension PCR in the pCAGGS-V5-Nsp11 backbone. Detailed sequences of the mutagenesis primers are available upon request. All mutants were validated by DNA sequencing. The mammalian expression plasmid for I κ B α was constructed by PCR amplifying the cDNA of I κ B α (GenBank accession no. NM020529) from HEK293 cells, followed by cloning into the pCMV-Tag 2B vector (Stratagene).

2.3. Antibodies

The monoclonal antibody (mAb) A2F1 used for detection of PRRSV Nsp2 was produced from hybridoma cells derived from Sp2/0 myeloma cells and spleen cells of BALB/c mice immunized with recombinant Nsp2 protein of PRRSV strain WUH3. The A2F1 mAb specifically recognized PRRSV Nsp2 in Western blot and indirect immunofluorescence assays (unpublished data). The anti-beta-actin (Biotechnology, China), anti-Flag (Macgene, China), anti-HA (MBL, Japan), anti-V5 (MBL, Japan), and anti-ubiquitin (Santa Cruz, CA) antibodies were used to detect the indicated proteins. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

2.4. PRRSV infection and polyubiquitination analysis

Marc-145 cells and PK-15-CD163 cells were infected with PRRSV strain WUH3 at different MOIs or sham-infected with DMEM. At different time points post infection, cell lysates were collected and subjected to Western blot analysis with an anti-ubiquitin antibody to measure the abundance of ubiquitinated proteins in the cell.

2.5. Luciferase reporter gene assay

HEK293 cells grown in 24-well plates were co-transfected with 0.1 μ g/well of luciferase reporter plasmid pNF- κ B-Luc along with 0.05 μ g/well of pRL-TK plasmid (Promega, for normalization of transfection efficiency) and various viral Nsp-encoding plasmids or an empty control plasmid. In some experiments, cells were further transfected with poly(I:C) (1.0 μ g/well) at 24 h after the

initial co-transfection. Cells were harvested 12 h later and firefly luciferase and *Renilla* luciferase activities were determined using the Dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol. Data represent relative firefly luciferase activity normalized to *Renilla* luciferase activity and are representative of three independently conducted experiments. Data are presented as means \pm standard deviation (SD). A *P*-value of less than 0.01 was considered highly statistically significant.

2.6. RNA extraction and quantitative real-time RT-PCR

To determine the effect of Nsp11 on expression of IL-6, IL-8, and CCL5, HEK293 cells in 24-well plates were transfected with 1 μ g of empty vector or a plasmid encoding V5-Nsp11. 24 h later, cells were mock-transfected or transfected with 1 μ g of poly(I:C) for 24 h. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, U.S.A.). One microgram of this total RNA was reverse transcribed to cDNA using AMV reverse transcriptase (Toyobo, Japan), which (1 μ l of 20 μ l cDNA) was subsequently used in a SYBR green PCR assay (Applied Biosystems, U.S.A.). The abundance of individual mRNA transcript in each sample was assayed three times and normalized to that of porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (as an internal control). The primers were designed by Primer Express software v.3.0 (Applied Biosystems, U.S.A.). Detailed sequences of the primers used are available upon request.

2.7. Assay of DUB activity

HEK293 cells cultured in 60-mm dishes were cotransfected with 1.2 μ g of HA-Ub, HA-K48-Ub, or HA-K63-Ub plus appropriate amounts of vector encoding wild-type PRRSV Nsp11 or the indicated mutant using Lipofectamine 2000 (Invitrogen). Where applicable, the empty vector was supplemented to keep the total amount of DNA transfected constant. At 30 h post transfection, cells were harvested by adding 250 μ L lysis buffer A (LBA) (65 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 3% DL-dithiothreitol, and 40% glycerol) containing 20 mM *N*-ethylmaleimide (NEM) (Sigma) and 20 mM iodoacetamine (Sigma). Cell lysates were then analyzed for expression of ubiquitin-conjugated proteins by Western blot with an anti-HA antibody (1:1,000) (MBL, Japan). To verify the expression levels of Nsp11 and the mutants, an anti-V5 antibody (MBL, Japan) was used to detect the V5-tagged proteins. Beta-actin was immunoblotted with anti-beta-actin mAb (Beyotime, China) to demonstrate equal protein sample loading.

2.8. Coimmunoprecipitation and immunoblot analysis

Transient transfection of HEK293 cells with the indicated plasmids was performed routinely using Lipofectamine 2000 as per the manufacturer's instructions (Invitrogen). Transfected HEK293 cells from each 100-mm dish were lysed by adding 1 ml of lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 20 nM phenylmethylsulfonyl fluoride [PMSF]), and the protein concentration was measured and adjusted. For immunoprecipitation, 500 μ g of total cell lysates were incubated with 0.5 μ g of the indicated antibody and 25 μ l of protein + G-agarose (Beyotime, China) overnight at 4 °C. The agarose beads were then washed three times with 1 ml of lysis buffer. The immunoprecipitates were subjected to 10% SDS-PAGE and subsequent immunoblot analysis using the indicated antibodies.

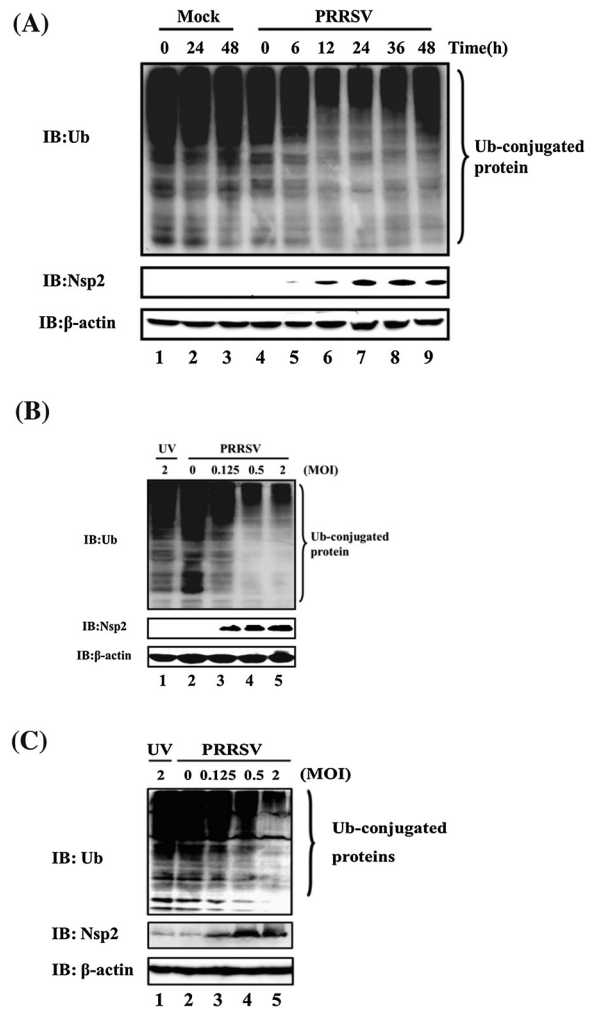


Fig. 1. PRRSV infection results in decreased cellular protein polyubiquitination. (A) Marc-145 cells cultured in 100-mm dishes were infected with PRRSV (MOI = 0.1). Cells were collected at 0, 6, 12, 24, 36, and 48 h post-infection and subjected to Western blot with monoclonal antibodies against ubiquitin, Nsp2, and beta-actin. Mock-infected cells were collected at 0, 24, 48 h for negative controls. (B–C) Marc-145 cells (B) and PK-15-CD163 cells (C) were infected with PRRSV at different MOIs or UV-inactivated PRRSV. Cells were collected at 24 h postinfection and Western blot was performed to detect cellular protein polyubiquitination.

3. Results

3.1. PRRSV infection results in decreased protein ubiquitination

To investigate the levels of ubiquitinated cellular proteins during PRRSV infection, we infected Marc-145 cells (a clone of the African green monkey kidney cell line MA-104) with PRRSV strain WUH3 at a multiplicity of infection (MOI) of 0.1. Cell lysates were collected at different time points post infection and subjected to Western blot analysis with anti-ubiquitin. As shown in Fig. 1A, while the level of ubiquitinated cellular proteins was steady in mock-infected cells (lanes 1–3), it varied dynamically during the course of PRRSV infection. A decrease in ubiquitination was first observed at 6 h post infection (h.p.i.) (compare lanes 5 vs. 4), reaching a plateau phase between 12 and 36 h.p.i. (lanes 6–8 vs. 4). Although there was a slight rebound at 48 h.p.i. (lane 9), the level of ubiquitinated cellular proteins remained substantially lower than that of mock-infected cells (lane 4). When Marc-145 cells were infected with PRRSV at increasing MOIs, the levels of ubiquitinated cellular proteins were reduced in a dose-dependent manner (Fig. 1B). Importantly, infection with UV-inactivated PRRSV, which

is capable of receptor binding and internalization but not viral gene synthesis, did not alter the cellular level of ubiquitinated protein conjugates (Fig. 1B). Similar results were observed in PRRSV-infected PK-15-CD163 cells (Fig. 1C). These data demonstrate that the level of ubiquitinated cellular proteins is reduced in PRRSV-infected cells and that this is a result of active viral replication. Of note, the latter notion was also supported by the Western blot data that measured expression of PRRSV Nsp2 protein at different time points post infection (Fig. 1A).

3.2. PRRSV Nsp11 possesses DUB activity

Because the decreased cellular protein ubiquitination depended on PRRSV replication and it has been shown that PRRSV Nsp2 has DUB activity, we sought to determine whether other Nsp(s) also contributes to protein deubiquitination. To this end, all of the Nsps of PRRSV strain WUH3, except Nsp6 (which encodes a very short peptide of 16 amino acids), were cloned into a mammalian expression vector pCAGGS-HA, such that they would be expressed as N-terminal HA-tagged fusion proteins. These Nsp constructs were transiently transfected into cells and their expression were verified by Western blot using an anti-HA antibody (data not shown). Subsequently, each of these Nsp constructs was co-transfected with a plasmid encoding Flag-tagged ubiquitin (pcDNA3.1-Flag-Ub) into HEK293 cells (human embryonic kidney epithelial cells) and Western blot was performed to detect the ubiquitin-conjugated proteins. As a negative control, the empty pCAGGS-HA plasmid was used in place of the Nsp-encoding vectors. As shown in Fig. 2A, of the 12 tested Nsps, ectopic expression of Nsp2 or Nsp11 resulted in markedly reduced levels of ubiquitin-conjugated proteins. Notably, the effect of Nsp11 appeared to be stronger than that of Nsp2 (compare lanes 13 vs. 5). Since, the DUB activity of Nsp2 had been characterized in previous studies, we focused on Nsp11 in subsequent experiments.

To further confirm the DUB activity of Nsp11, HEK293 cells were transfected with the empty vector or increasing amounts of V5-tagged Nsp11 expression plasmid along with HA-tagged ubiquitin vector (HA-Ub) and the levels of ubiquitin-conjugated proteins were monitored at 36 h post-transfection. As shown in Fig. 2B, compared to the control vector transfected cells (lane 2), the degree of deubiquitination directly correlated with the amount of Nsp11 expressed (lanes 3–6). These data strongly suggest that Nsp11 is directly responsible for the decrease in ubiquitinated cellular proteins.

3.3. PRRSV Nsp11 processes K48-linked, but not K63-linked polyubiquitin

Protein ubiquitination is an important posttranslational modification that has an essential role in the positive and negative regulation of NF- κ B signal transduction pathway, among different ubiquitination types K48- and K63-linked polyubiquitin chains are of great significance (Wertz and Dixit, 2010). To further identify which Ub linkage type is targeted by Nsp11, HEK293 cells were transfected with HA-K48-Ub or HA-K63-Ub in place of HA-Ub. These constructs allow solely the formation of K48- or K63-linked polyubiquitin chains, respectively. As shown in Fig. 2C and D, while the accumulation of K48-linked ubiquitinated proteins was reduced by Nsp11 in a dose-dependent manner (Fig. 2C), K63-linked Ub moieties were left intact upon Nsp11 coexpression (Fig. 2D). These data indicate that PRRSV Nsp11 specifically targets K48-linked polyubiquitin chains.

3.4. The C112 residue is a potential catalytic site for Nsp11 DUB activity

There are five families of DUBs characterized by specific structural domains: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins and JAB1/MOV34 metalloenzymes (JAMMs). UCHs, USPs, OTUs and Josephins function as cysteine proteases, whereas JAMMs are zinc dependent metalloproteases. Based on the structure of their catalytic domains, the human DUBs are classified into five subfamilies, most of which are cysteine proteases characterized by a cysteine (Cys, C) active site located within the amino terminus (Nijman et al., 2005; Wilkinson et al., 1995). To identify the potential cysteine catalytic site for Nsp11 DUB activity, the amino acid sequences of Nsp11 from various PRRSV strains were aligned using the Clustal W program. Sequence alignment showed that Cys112 of Nsp11 is highly conserved across different genotypes of PRRSV. However, Cys64 is only identical among type 2 genotype PRRSV (Fig. 3A). To determine whether these two cysteines are critical residues involved in the DUB activity of Nsp11 of PRRSV WUH3 strain, we constructed C64A and C112A Nsp11 mutants and compared them with WT Nsp11 for the ability to reduce protein ubiquitination. As shown in Fig. 3B, overexpression of WT Nsp11 or the C64A mutant significantly inhibited K48-linked ubiquitination of cellular proteins (compare lanes 3 and 4 vs. 2, respectively). In contrast, the C112A mutant had no such effect (compare lanes 5 vs. 2). These data suggest that the C112 residue is pivotal for the DUB activity of PRRSV Nsp11.

3.5. Residues D144, K173, D180 and Y219 are also associated with the DUB activity of Nsp11

PRRSV Nsp11 encodes an endoribonuclease (NendoU), which is conserved throughout the *Nidovirales* order but has not been identified in RNA viruses of other families (Ivanov et al., 2004; Nedialkova et al., 2009). Nedialkova et al. (2009) compared the amino acid sequences of the NendoU domain of *Arterivirus* (in Nsp11) and its counterpart in Nsp15 of SARS-CoV and found that at least 6 residues, corresponding to H129, H144, K173, D180, D204, and Y219 in PRRSV Nsp11, are highly conserved among PRRSV strain VR2332, SARS-CoV strain Frankfurt 1, and EAV strain Bucyrus (Nedialkova et al., 2009). Considering that PRRSV is divided into distinct genotypes and even exhibits remarkable genetic diversity within each genotype, we compared the amino acid sequences of Nsp11 of several representative PRRSV strains isolated from different geographical regions in different years. As shown in Fig. 3A, the six residues reported by Nedialkova et al. (2009) are indeed highly conserved among different genotypes of PRRSV and EAV. To determine whether these residues contribute to the DUB activity of Nsp11, we performed Alanine substitution at each site. Each of these mutants was co-transfected with HA-Ub vector into HEK293 cells and Western blot was performed to detect the expression of ubiquitin-conjugated proteins and the Nsp11 mutant. As shown in Fig. 3C, while mutants bearing the H129A or D204A substitution (lanes 4 and 8, respectively) acted as effective as WT Nsp11 (lane 3) in preventing the accumulation of ubiquitination of cellular proteins, mutants harboring H144A, K173A or Y219A substitution remarkably lost the DUB activity (lanes 5, 6 and 9), as compared to vector-transfected cells (lane 2). Intermediate DUB activity was observed for the D180A mutant (lane 7). Collectively, these data suggest that residues H144, K173, D180 and Y219 are also associated with the DUB activity of Nsp11. Given that mutation at H129 or D204 destroys the NendoU activity (Nedialkova et al., 2009), our data also indicate that the DUB activity of Nsp11 is uncoupled from its NendoU activity.

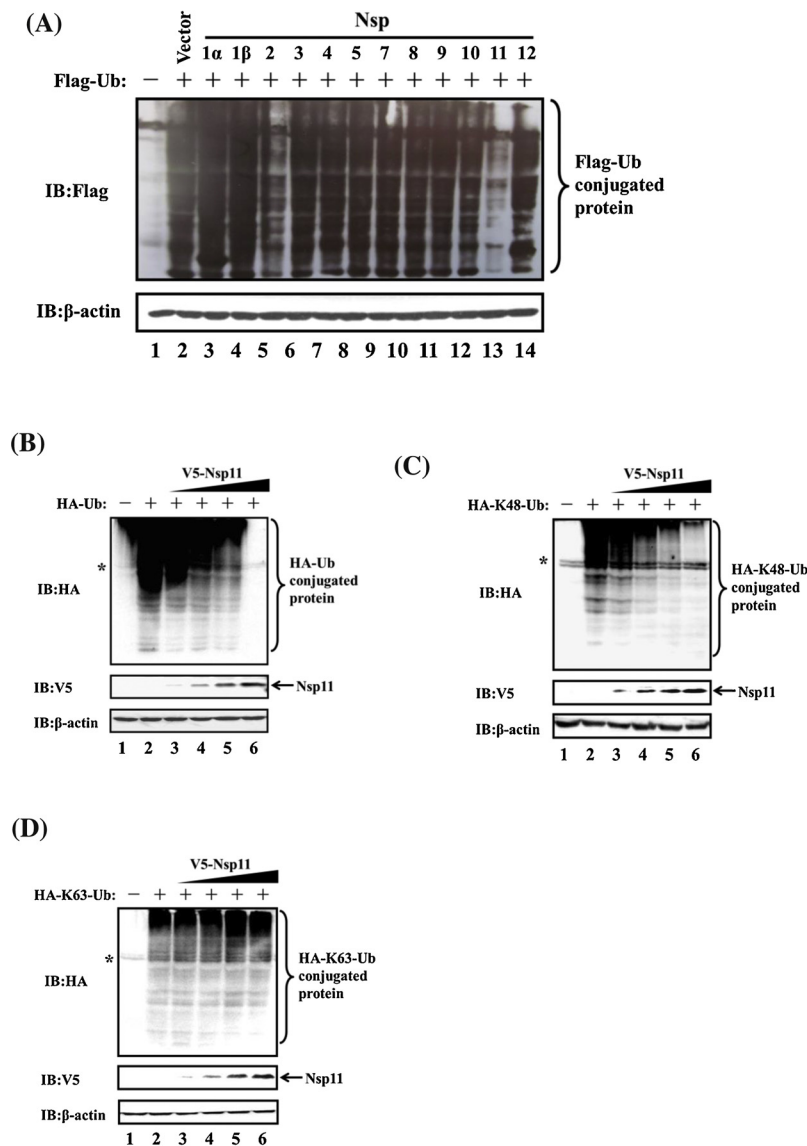


Fig. 2. PRRSV Nsp11 possesses DUB activity.

(A) HEK293 cells grown in 60-mm dishes were cotransfected with pcDNA3.1-Flag-Ub (1.0 μ g) and the indicated PRRSV Nsp expression plasmids (pCAGGS-HA-Nsp1 α , 1 β , 2, 3, 4, 5, 7, 8, 9, 10, 11, 12) or empty vector (2.0 μ g). Cell lysates were prepared at 30 h posttransfection and analyzed for Ub-conjugated proteins by Western blot with an anti-Flag antibody. Western blot with anti-beta-actin serves as a protein loading control. (B) HEK293 cells grown in 60-mm dishes were transfected with HA-tagged Ub expression plasmids (1.2 μ g), along with increasing quantities (0, 0.2, 0.4, 0.8, or 1.6 μ g) of plasmid encoding V5-Nsp11, using Lipofectamine 2000. Cell lysates were prepared at 30 h posttransfection and analyzed for Ub-conjugated proteins by Western blot with an anti-HA antibody. Western blot with anti-V5 antibody shows expression of Nsp11, and Western blot for beta-actin serves as a protein loading control. (C–D) The experiments were performed similarly to those in panel B, except that the HA-K48-Ub or HA-K63-Ub plasmid was used in lieu of HA-Ub.

3.6. PRRSV Nsp11 substantially attenuates the transcription of NF- κ B-target genes

Ubiquitination is an important regulatory mechanism of NF- κ B signaling (Wertz and Dixit, 2010). The discovery that Nsp11 has DUB activity would imply that Nsp11 may affect NF- κ B activity. Indeed, it has been shown that ectopic expression of PRRSV Nsp11 inhibits NF- κ B activation through unknown mechanism(s). In agreement with these previous reports (Beura et al., 2010), our luciferase reporter assays showed that Nsp11 down-regulated poly(I:C)-induced activation of a synthetic NF- κ B-dependent promoter in a dose-dependent fashion (Fig. 4A). We next asked the question whether Nsp11 also attenuates expression of poly(I:C)-induced, endogenous NF- κ B-responsive genes. To this end, HEK293 cells were transfected with a plasmid encoding V5-Nsp11 or the empty control vector. Twenty-four hours post-transfection, cells

were further transfected with poly(I:C) or mock-transfected. Total RNA was extracted from the cells and analyzed for the abundance of endogenous interleukin-6 (IL-6), IL-8 and chemokine (C–C motif) ligand 5 (CCL5, also known as RANTES), mRNAs by SYBR Green real-time RT-PCR. While poly(I:C) robustly induced the expression of IL-6 (Fig. 4B), IL-8 (Fig. 4C), and CCL5 (Fig. 4D) in cells transfected with control vector, it was substantially less effective in cells expressing Nsp11. These results show that Nsp11 negatively regulates the induction of NF- κ B-target genes following stimulation by intracellular dsRNA.

3.7. Nsp11 removes K48-linked ubiquitin chains from I κ B α to inhibit NF- κ B activation

Generally, attachment of K-48 linked Ub chains promotes the degradation of a protein by the Ub-proteasome system (UPS), one

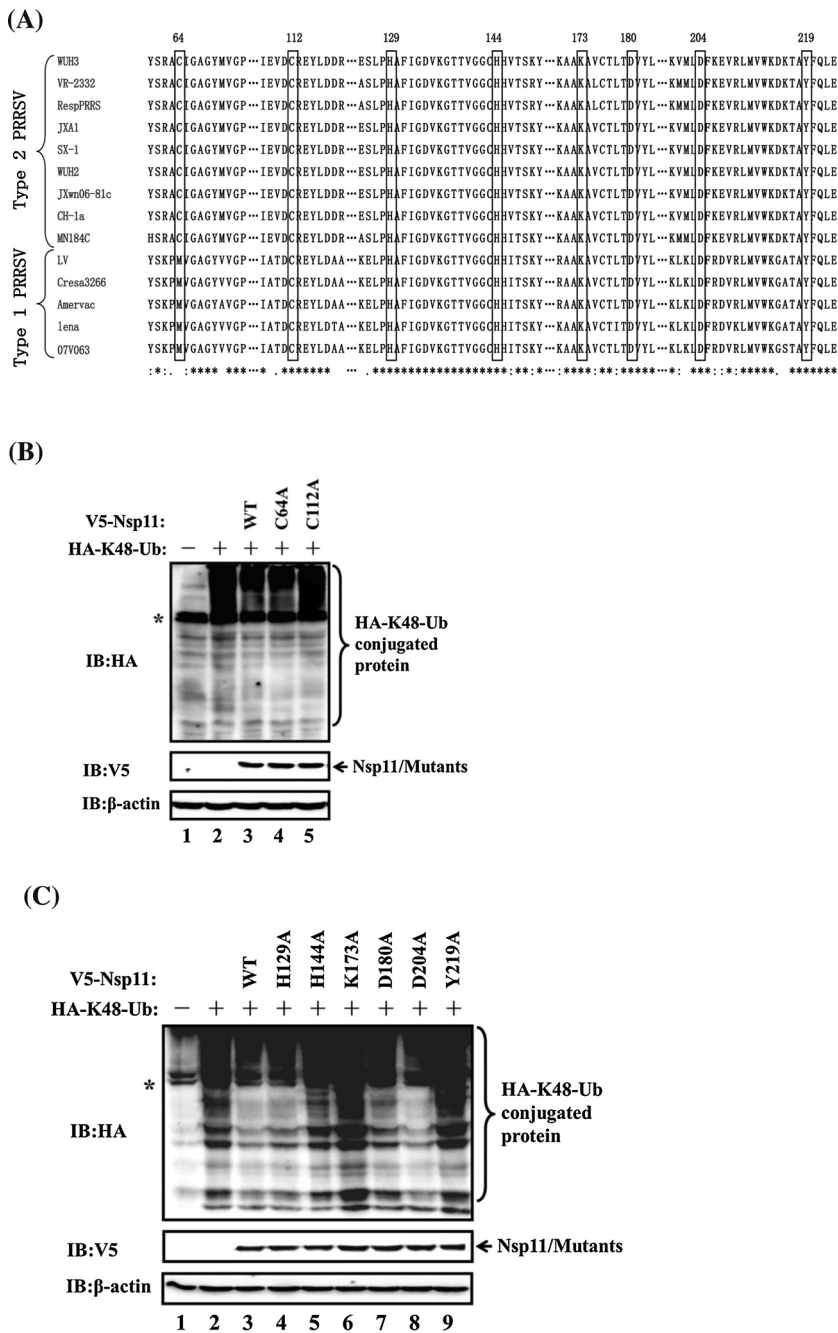


Fig.3. Mapping of the putative sites are associated with the DUB activity of Nsp11. (A) Amino acid alignment of the conserved region in the Nsp11 proteins of the different PRRSV strains. Black boxes indicate conserved residues tested in this study. The sequences were derived from GenBank entries with the following accession numbers: PRRSV WUH3, HM853673; PRRSV JXA1, FJ548853; PRRSV SX-1, GQ857656; PRRSV JXwn06-81c, HQ233604; PRRSV WUH2, EU678352; PRRSV CH-1a, AY032626; PRRSV VR-2332, AY150564; PRRSV MN184C, EF488739; PRRSV RespPRRS MLV, AF066183; PRRSV LV, NC.002533.2; PRRSV Amervac, GU067771; PRRSV Cresa3266, JF276434; PRRSV 07V063, GU737264.2; and PRRSV lena, JF802085. (B–C) HEK293 cells grown in 60-mm dishes were transfected with HA-tagged Ub expression plasmids HA-K48-Ub (1.2 μg), along with the indicated Nsp11 expression plasmids (1.6 μg). Cell lysates were prepared at 30 h posttransfection and analyzed for Ub-conjugated proteins by Western blot with an anti-HA antibody. Western blot with anti-V5 antibody shows expression of Nsp11, and Western blot for beta-actin serves as a protein loading control.

of the most important machineries for non-lysosomal degradation of cytoplasmic and nuclear proteins, while modification by K63-linked ubiquitination mediates largely non-proteolytic functions such as protein trafficking or kinase and phosphatase activation (Ikeda and Dikic, 2008). A key step that leads to NF-κB activation in response to many extracellular stimuli is the degradation of IκBα, which sequesters NF-κB proteins in the cytoplasm in resting cells (Wertz and Dixit, 2010). Because PRRSV Nsp11 selectively removes K48-linked ubiquitin moieties (Fig. 2C), we hypothesized that it

may inhibit NF-κB activation by interfering with the ubiquitination of IκBα and subsequent proteasomal degradation. To test our hypothesis, HEK293 cells were transfected with an Nsp11-encoding vector or the empty vector (as a control), followed by stimulation by TNF-α. At 30 and 60 min post-stimulation, cell lysates were collected to detect IκBα protein by Western blot. As shown in Fig. 5A and 5B, TNF-α-induced degradation of IκBα was significantly attenuated in cells expressing Nsp11, compared to cells transfected with the empty vector, indicating that Nsp11 inhibits ligand-induced

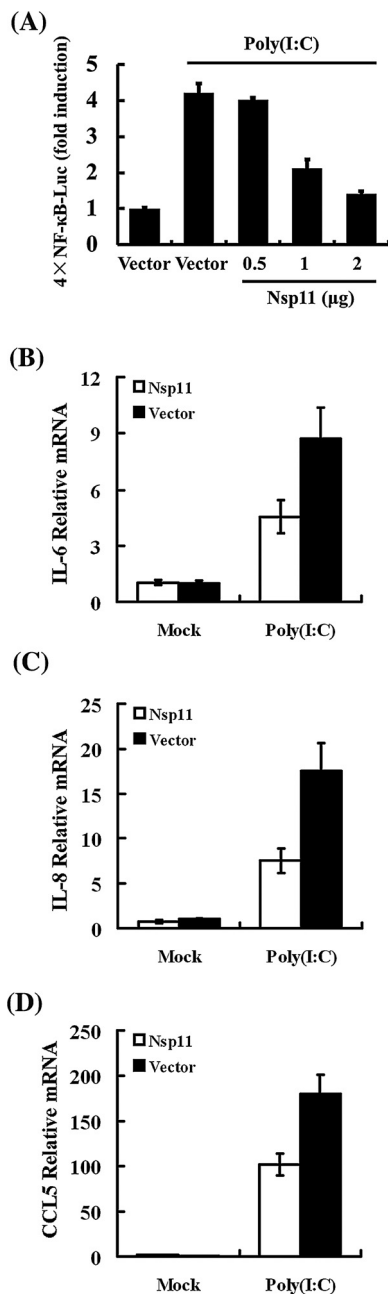


Fig. 4. Nsp11 significantly reduced the transcription of multiple NF-κB-responsive genes.

(A) HEK293 cells grown in 24-well plates were transfected with 0.1 μg/well of 4× NF-κB-Luc reporter plasmid, along with 0.1 μg/well of pRL-TK plasmid and increasing quantities (0, 0.5, 1.0, or 1.5 μg) of plasmid encoding V5-Nsp11, using Lipofectamine 2000. Twenty-four hours after the initial transfection, the cells were further treated with poly(I:C) or mock treated. Luciferase assays were performed at 12 h after infection. (B–D) HEK293 cells were transfected with 1 μg of plasmid encoding V5-Nsp11 or an empty vector, and, 24 h later, the cells were transfected with 1 μg of poly(I:C). Twenty-four hours after the second transfection, total RNA was extracted and the expression of IL-6 (B), IL-8 (C) and CCL5 (D) and GAPDH genes were evaluated by quantitative real-time RT-PCR. Results are expressed as increases in mRNA levels relative to those in cells transfected in the absence of poly(I:C) and were normalized by using GAPDH housekeeping gene expression. Results are representative of those from three independent experiments.

IkBα degradation. To further test whether Nsp11 removes K48-Ub from IkBα, HEK293 cells were transiently co-transfected with an Nsp11 expression construct, HA-K48-Ub, and an IkBα-encoding vector. At 24 h post transfection, MG132 (a proteasome inhibitor) was added for 6 h to retain ubiquitinated IkBα. Cell lysates were

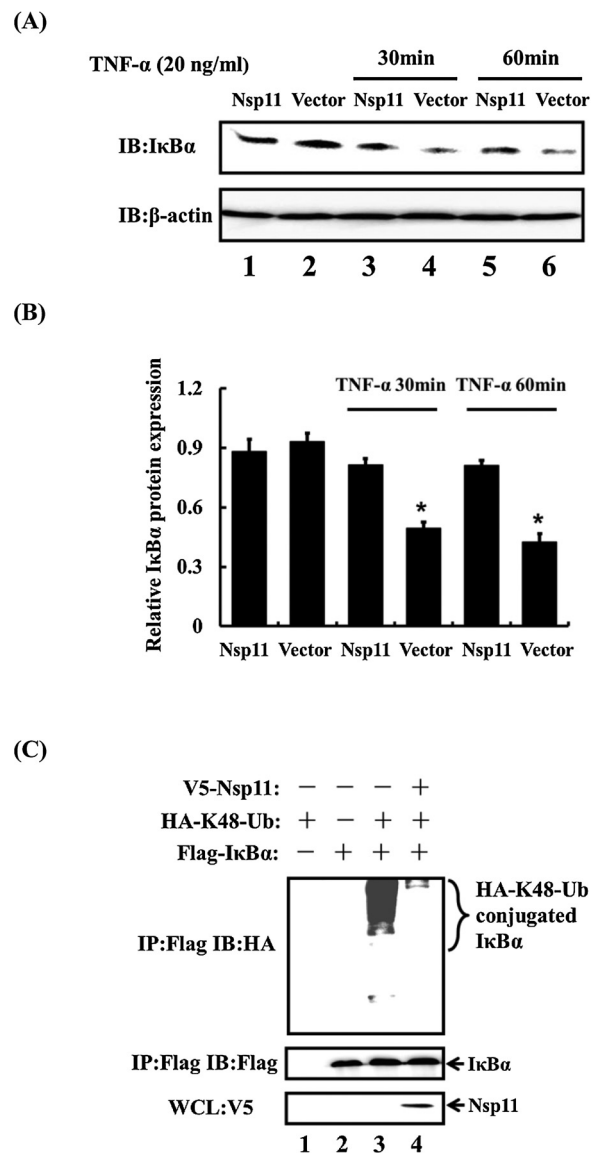


Fig. 5. Nsp11 inhibits NF-κB through degradation of K48-linked polyubiquitination of IkBα.

(A) HEK293 cells in 60-mm dishes were cotransfected with the expression plasmids encoding HA-Nsp11 or the pCAGGS-HA (Vector) empty plasmid (3 μg). Transfected cells were treated with TNF-α (20 ng/ml) for the amounts of time (30 min, 60 min) and subsequently immunoblotted. Western blots were analyzed for total IkBα. The beta-actin was detected as a loading control. (B) Relative levels of IkBα were estimated by densitometric scanning after normalization against beta-actin and are shown as bar diagrams. Data represent means of three replicates. (C) HEK293 cells grown in 100-mm dishes were transfected with the expression plasmids encoding V5-Nsp11 (2.5 μg), HA-K48-Ub (1 μg), pCMV-Tag-IkBα (2.5 μg) using Lipofectamine 2000. MG132 (20 nM) was treated at 30 h after transfection. Cell lysates were prepared at 4 h after treatment and immunoprecipitated with anti-Flag antibody and ubiquitin conjugation of protein was verified by immunoblotting with anti-HA antibody. The input tagged proteins were verified with indicated antibodies.

then collected for co-IP assay. As shown in Fig. 5C, IkBα was conjugated with K48-Ub in cells without Nsp11 coexpression (lane 3). However, in cells expressing Nsp11, there were very few K48-Ub moieties attached to IkBα, despite the latter being expressed at comparable levels (lane 4). In aggregate, these results suggest that Nsp11 inhibits NF-κB through removing K48-linked polyubiquitin chains from IkBα and thus preventing subsequent IkBα degradation.

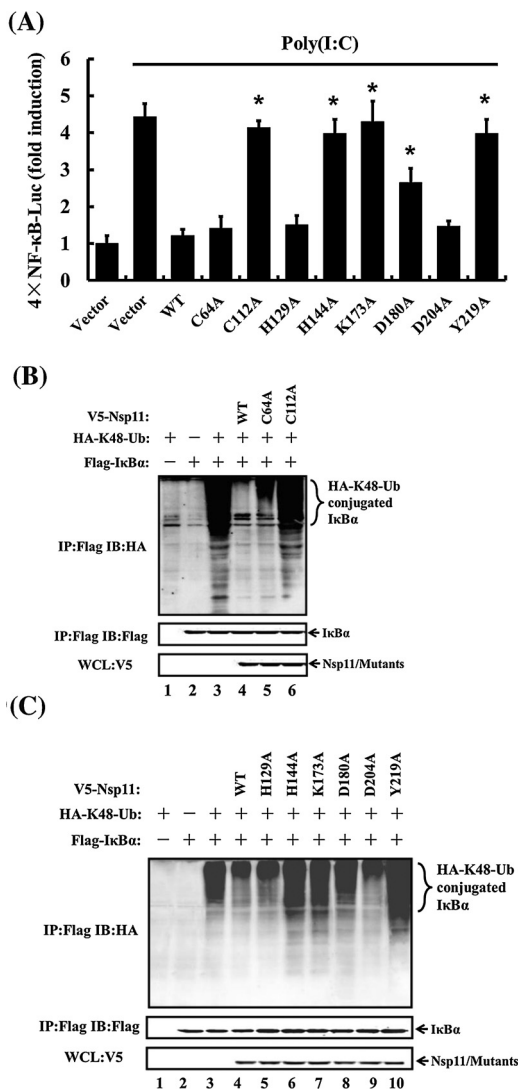


Fig. 6. DUB activity is essential for PRRSV Nsp11 to block NF- κ B activation. (A) HEK293 cells were cotransfected with the 4x NF- κ B-Luc reporter plasmid (0.1 μ g), pRL-TK plasmid (0.1 μ g), and the designated Nsp11 expression plasmids (0.8 μ g), using Lipofectamine 2000. Twenty-four hours after the initial transfection, the cells were further treated with poly(I:C) or mock treated. Luciferase assays were performed at 12 h after treatment. (B-C) HEK293 cells in 100-mm dishes were transfected with the expression plasmids encoding V5-Nsp11 and mutants (2.5 μ g), HA-K48-Ub (1 μ g), pCMV-Tag-I κ B α (2.5 μ g), using Lipofectamine 2000. MG132 (20 nM) was treated at 30 h after transfection. Cell lysates were prepared at 4 h after treatment and immunoprecipitated with anti-Flag antibody and ubiquitin conjugation of protein was verified by immunoblotting with anti-HA antibody. The input tagged proteins were verified with indicated antibodies.

3.8. The DUB activity of Nsp11 is essential for its ability to inhibit NF- κ B activation

To delineate the molecular determinants of Nsp11 involved in NF- κ B inhibition, various Nsp11 mutants with differing DUB activities (Fig. 3B and C) were analyzed for their abilities to affect poly(I:C)-induced NF- κ B activation in HEK293 cells by luciferase reporter gene assay. As shown in Fig. 6A, the results showed that mutants with impaired DUB activity were attenuated for the ability to suppress NF- κ B activation. Notably, the C64A, H129A and D204A mutants with DUB activities comparable to that of WT Nsp11 were as effective as the latter in blocking activation of NF- κ B, while the C112A, H144A, K173A and Y219A mutants with little or no DUB activity were without inhibitory effect. The D180A mutant possessing intermediate DUB activity had a moderate inhibition on

NF- κ B. Taken together, these data show that DUB activity is proportional to the suppressive effect on NF- κ B activation. Next, we determined the abilities of these Nsp11 mutants to remove K48-linked polyubiquitin chains from I κ B α . As shown in Fig. 6B and C, the DUB activity toward polyubiquitinated I κ B α of the individual Nsp11 mutant directly correlated with the capacity to inhibit NF- κ B activation. Collectively, these results provide further support to the notion that PRRSV Nsp11 inhibits NF- κ B activation by means of its DUB activity toward polyubiquitinated I κ B α .

4. Discussion

In this study, we attempted to elucidate the mechanism(s) employed by PRRSV to reduce cellular protein polyubiquitination and accidentally found that PRRSV Nsp11 possesses DUB activity. We also demonstrated that Nsp11 specifically cleave K48-linked, but not K63-linked polyubiquitin chains. Importantly, our data showed the DUB activity is responsible for the ability of Nsp11 to inhibit NF- κ B activation, revealing a novel mechanism evolved by PRRSV to disarm host innate immune responses. At present, we favor a model in which Nsp11 removes K48-linked ubiquitin moieties from I κ B α , thereby preventing the proteasomal degradation of I κ B α and subsequent liberation of NF- κ B. This is based on our data that the inhibitory effects of Nsp11 mutants on NF- κ B correlated with their abilities to deubiquitinate I κ B α conjugated with K48-linked ubiquitin chains. However, our study do not rule out the possibility Nsp11 may also act on other cellular targets to regulate NF- κ B activity.

Previous studies have revealed that PRRSV Nsp11 contains endoribonuclease (NendoU) activity (Nedialkova et al., 2009). Although, NendoU is highly conserved throughout the *Nidovirales* order, thus far no NendoU homologs have been identified in RNA viruses of other families. In this regard, NendoU is considered to be a genetic marker of Nidoviruses (Ivanov et al., 2004). Adopting a catalytic mechanism resembling that of RNase A, NendoU acts independent of Mn²⁺ and with a preference for uridylyte, as has been demonstrated for NendoUs encoded by EAV, SARS-CoV and PRRSV (Nedialkova et al., 2009). Through as-yet-unknown mechanisms, NendoU facilitates viral RNA synthesis (Kang et al., 2007; Posthuma et al., 2006). In this study, we demonstrated that PRRSV Nsp11 also has DUB activity, ascribing a novel function to this enigmatic protein. In addition, our mutational analyses suggested that the DUB activity of Nsp11 is separated from its NendoU activity and that the former rather than the latter mediates the suppressive effect on NF- κ B. It will be interesting to investigate in future studies whether the Nsp11 counterparts encoded by other Nidoviruses also possess DUB and NF- κ B-inhibitory activities and whether these relate to the NendoU activity.

Lee et al. (2005) firstly demonstrated that PRRSV infection activates NF- κ B signaling in Marc-145 cells and PAMs through inducing I κ B degradation and p65 nuclear translocation. Our group also showed that PRRSV infection triggers activation of NF- κ B and that the nucleocapsid (N) protein of PRRSV could elicit this process in Marc-145 cells (Luo et al., 2011; Luo et al., 2008). However, activated NF- κ B could only be detected after 24 h post infection. Given the recent reports that several PRRSV Nsps, including Nsp1 α , Nsp1 β , Nsp2, and Nsp11, could function as negative regulators of NF- κ B (Beura et al., 2010; Song et al., 2010; Sun et al., 2010), it is plausible that the regulation of NF- κ B is a dynamic process during the course of PRRSV infection, and that PRRSV has developed sophisticated strategies to either activate or inhibit NF- κ B at various stages of the viral life cycle to facilitate viral replication and/or disrupting host innate immune responses, offering the virus a maximal survival advantage.

Previous studies have shown that the Nsp11s of PRRSV strains FL12 and BJ-4 inhibited IFN- β production in HeLa and Marc-145 cells (Beura et al., 2010; Shi et al., 2011). We also found that the Nsp11 encoded by the highly pathogenic WUH3 strain, which is prevalent in China and surrounding countries, could inhibit activation of IRF-3 and IFN- β transcription in HEK293 cells by luciferase reporter assays (data not shown). To our surprise, all of the Nsp11 mutants characterized in the current study were impaired for the ability to block SeV-induced IFN- β and IRF-3 activation (data not shown). This result contrasted with the effects on NF- κ B, in that only the Nsp11 mutants losing the DUB activity were attenuated (Fig. 6). Thus, while the DUB activity of Nsp11 mediates the inhibition on NF- κ B activation, other mechanisms are involved in the Nsp11 blockage of IFN- β production and IRF-3 activation.

Apart from regulating innate immune signaling, ubiquitination has been demonstrated to orchestrate aspects of viral life cycle (Isaacson and Ploegh, 2009; Randow and Lehner, 2009). For instance, the assembly and release of some viruses are subjected to ubiquitination-dependent regulation. A number of viral proteins, such as HIV Gag (Demirov et al., 2002; Garrus et al., 2001), Ebola virus VP40 (Yasuda et al., 2003), Epstein-Barr virus (EBV) LMP2A (Ikeda et al., 2000), and human papillomavirus (HPV) E7 (Kamio et al., 2004; Oh et al., 2004), can be directly modified by ubiquitin or ubiquitin-like proteins, and these modifications play an important role in virion egress. While the precise role(s) of ubiquitination and/or deubiquitination in PRRSV infection remains to be established, the findings that PRRSV encodes at least two DUBs, i.e., Nsp2 and Nsp11, signify that these posttranslational modifications may play an important part in PRRSV propagation and/or pathogenesis. For example, it is possible that Nsp11 may cleave K48-linked polyubiquitin chains attached to viral replicase proteins and/or structural components to control the half-lives of these viral products, providing a fine-tuned condition that allows viral replication and/or packaging/release at maximal efficiency. Considering the fact that the papain-like proteases of several coronaviruses (SARS-CoV, NL63 and MHV) also possess DUB activity (Barretto et al., 2005; Barretto et al., 2005b; Lindner et al., 2005; Zheng et al., 2008), the involvement of ubiquitination-dependent regulation in viral life cycle may represent a selective survival advantage during the evolution of Nidoviruses.

In summary, the present study uncovered that PRRSV Nsp11 possesses DUB activity and went extra miles to show that DUB activity correlates with the ability of Nsp11 to inhibit NF- κ B through removing K48-linked polyubiquitin chains from I κ B α . Not only do these findings describe a novel mechanism acquired by PRRSV to counteract host innate immune responses, but they open new avenues to develop novel therapeutics against PRRS by targeting the interaction of Nsp11 with host ubiquitination system.

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