Virology 532 (2019) 55–68

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

E3 ligase ASB8 promotes porcine reproductive and respiratory syndrome virus proliferation by stabilizing the viral Nsp1α protein and degrading host IKKβ kinase

Ruiqiao Li[a,1], Chen Chen[a,1], Jing He[b], Lilin Zhang[a], Lei Zhang[a], Yanyu Guo[a], Wentao Zhang[a], Kun Tan[a], Jinhai Huang[a,*]

[a] School of Life Sciences, Tianjin University, Tianjin, 300072, China
[b] Tianjin Heping District Hospital of Obstetrics and Gynecology, 300041, China

ARTICLE INFO

Keywords:
Ubiquitination
Ankyrin repeat and SOCS box–containing 8 (ASB8)
IκB kinase β (IKKβ)
Porcine reproductive and respiratory syndrome virus (PRRSV)
Nonstructural protein 1α (Nsp1α)
NF-κB signaling

ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) can potently suppress type I interferon production and escape from innate immune responses. PRRSV nonstructural protein 1α (Nsp1α) can inhibit IFN-β and NF-κB gene promoter activities, but the precise mechanisms are largely unclear. In this study, we demonstrated that PRRSV Nsp1α interacted with the host E3 ubiquitin ligase ankyrin repeat and SOCS box–containing 8 (ASB8). Specifically, porcine ASB8 promoted K63–linked ubiquitination and increased stability of Nsp1α and boosted PRRSV replication. Moreover, we found that ASB8 was phosphorylated at the N-terminal Ser-31 by host IκB kinase β (IKKβ). In turn, ASB8 facilitated K48–linked ubiquitination and degradation of IKKβ via the ubiquitin–proteasome pathway, resulting in remarkable inhibition of I-kappa-B-alpha (IκBα) and of p65 phosphorylation, consequently suppressing NF-κB activity. Our results provide evidence that PRRSV Nsp1α hijacks up-regulated host ASB8 to escape from intrinsic antiviral immunity.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped, single-stranded RNA virus, causes complex clinical symptoms and great economic losses to worldwide swine industry (Albina, 1997; Wenhui et al., 2012). PRRSV has a potent ability to escape from immune surveillance (Ke and Yoo, 2017; Rascon-Castelo et al., 2015; Wang and Zhang, 2014). Although some commercial vaccines against PRRSV have been provided, unfortunately, neither commercial vaccines nor traditional control strategies provide sustainable control of PRRSV (Meng, 2000; Zuckermann et al., 2007). A key step toward the induction of innate immunity against viral infections, including PRRSV, is the production of type I interferon (Huang et al., 2014; Kim et al., 2010; Wang and Zhang, 2014). The detection of virus can be mediated by Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and other pattern recognition receptors (PRRs). The involved PRRs triggers downstream signaling pathways through different adaptor proteins and further transmit downstream signals to the IκB (IKK) and IKK-related kinases for the activation of nuclear factor kappa B (NF-κB) and the subsequent synthesis of proinflammatory cytokines. The IKK-related kinases (TBK1/IKKε) also activate the production of IFN type I by phosphorylating the transcription factors IFN-regulatory factor 3 (IRF3) and IRF7 (Garcia-Sastre, 2017; Goubau et al., 2013; Kawasaki et al., 2011).

To date, several PRRSV Nsps (non-structure proteins), Nsp1, Nsp2, Nsp4, and Nsp11 have been respectively reported to display the inhibitory effect on the activation of the IFN-β and NF-κB promoter (Huang et al., 2014; Song et al., 2018). Nsp1(Nsp1α subunit and Nsp1β subunit), containing two papain-like cysteine protease (PCP) domains, is indispensable for IFN inhibition by degrading intracellular CREB-binding proteins(Kim et al., 2010). The ovarian tumor domain of Nsp2 inhibits NF-κB activation through degrading the IκBα protein (Sun et al., 2010). Nsp4, a viral 3C-like serine protease, significantly suppressed IFN-β expression by targeting the NF-κB essential modulator (NEMO)(Huang et al., 2014). Nsp11 blocks the phosphorylation of nuclear translocation of IRF3 and IκBα, resulting in the repression of IFN production(Sun et al., 2016).

The ubiquitin–proteasome system (UPS) is the cardinal intracellular pathway for degradation and functional modification of proteins, which has emerged as a key mechanism in the regulation of many fundamental cellular functions, including immune signaling, apoptosis and

* Corresponding author. School of Life Sciences, Tianjin University, Tianjin 300072, China. No. 92, Weijin road, Nankai District, Tianjin, 300072, China.
E-mail address: jinhaih@tju.edu.cn (J. Huang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.virol.2019.04.004
Received 12 February 2019; Received in revised form 30 March 2019; Accepted 12 April 2019
Available online 15 April 2019
0042-6822/

cell cycle regulation. In many cases viruses developed the evasion mechanisms from UPS-dependent protein degradation and hijack the ubiquitin system to enhance their own proliferation (Davis and Gack, 2015; Isaacson and Ploegh, 2009; Luo, 2016a; Rudnicka and Yamauchi, 2016). Ubiquitination involves in a sequential enzymatic reaction mediated by ubiquitin-activating enzyme E1, ubiquitin-binding enzyme E2 and ubiquitin ligase E3. E3 ligases contribute to the complexity and flexibility of protein in UPS and are consequently the chief targets of viruses (Hatakeyama, 2017; Zhang et al., 2018b). PRRSV relies on host protein to complete its life cycle and UPS is required for its proliferation (Zhang et al., 2018a). For example, PRRSV Nsp2 and Nsp11 utilize the host ubiquitin system to prevent IκBα degradation by interfering with polyubiquitination process, thereby inhibiting the NF-κB activation (Sun et al., 2010; Wang et al., 2015). However, host ubiquitinate protein (especially E3 ubiquitin ligase) participated in PRRSV infection has not yet been elucidated.

In the present study, the differentially expressed genes of E3 ubiquitin ligase were examined in PRRSV-infected 3D4/21 cells, suggesting the involvement possibility of ubiquitination in PRRSV infection. One type of novel E3 ubiquitin ligase-containing protein-8 (ASB8) is obviously upregulated in PRRSV-infected cells. The predicted porcine ASB8 protein harboring ankyrin repeats, a protein-protein interaction domain, and a suppressor of cytokine signaling (SOCS) box containing a BC motif. SOCS domain inhibits cytokine action by interacting directly with Janus kinases or activating cytokine receptors. And it can form an E3 ubiquitin ligase complex with the Elongin BC complex, Cullin5, and Rbx1 to stimulate polyubiquitination (Kamura et al., 1998; Li et al., 2011; Thomas et al., 2013). SOCS family also can act as a negative regulator of cytokine signal (Krebs and Hilton, 2001). SOCS1 would be up-regulated to promote virus production in influenza virus, Japanese encephalitis virus and duck tembusu virus infection (Kundu et al., 2013; Sun et al., 2017; Wei et al., 2016). SOCS3 also involves in signaling suppression and production of IFN in herpes simplex virus infection process (Yokota et al., 2004).

The function knowledge of ASB8 is limited, and its roles in viral infection are poorly understood, too. In our study, we demonstrated the critical role of ASB8 as a negative regulator in innate immunity. The ASB8 is manifested in its capacity to enhance virus proliferation and inhibit the expression level of inflammatory cytokines in HEK293T and 3D4/21 cells. Mechanically, ASB8 interacted with PRRSV Nsp1α and promoted its ubiquitination, which stimulates virus proliferation. Moreover, we identified that ASB8 could directly bind the kinase domain of IKKβ and mediated K48-linked ubiquitination and degradation of IKKβ, consequently suppressing NF-κB signaling pathway. Our results revealed a novel posttranslational mechanism of ASB8 in regulating viral proliferation.

2. Results

2.1. PRRSV infection induces ASB8 expression

To hunt the intrinsic antiviral molecules of host cell resist virus infection, RNA-Seq was performed to screen out the host factors involved in the PRRSV-infected 3D4/21 cells, and then E3 ubiquitin ligase associated genes on the pathogenesis of PRRSV were concerned (Fig. 1A). Notably, one novel type of E3 ubiquitin ligase-ASB was found and their expression in PRRSV-infected 3D4/21 cells was verified by qPCR subsequently (Fig. 1B). We noticed that the ASB8 transcription level was sharply upregulated at 24 h in PRRSV-infected 3D4/21 cells (Fig. 1C). Western blotting and flow cytometry results further confirmed that the upregulation of endogenous ASB8 protein expression after PRRSV infection (Fig. 1D–F). These results indicated that the mRNA transcription and protein expression of porcine ASB8 were upregulated in PRRSV-infected cells. The ASB8 expression were also upregulated after inoculation of DNA-virus Herpes simplex virus (HSV) or Porcine circovirus (PCV2) (Supplementary Fig. 1A). Together, these data indicated that ASB8 participated in viral infection and played a role in antiviral innate immunity.

2.2. Overexpression ASB8 contributes to PRRSV proliferation

To examine whether ASB8 affects PRRSV proliferation, we transfected 3D4/21 cells with Flag-ASB8, and then infected the cells with 1 MOI PRRSV. qPCR suggested that ASB8 overexpression promoted the transcription of PRRSV Nsp2 and N gene (Fig. 2A). The expression of Nsp2 protein by Western blot was statistically higher in ASB8 overexpressed PRRSV inoculated cells (Fig. 2B). Meanwhile, the virus titer in ASB8-transfected cells was higher than that of control (Fig. 2C). The results indicated that the up-regulated ASB8 contributed to the proliferation of PRRSV in 3D4/21 cells.

To further characterize the influence of ASB8 on PRRSV proliferation, a small RNA interfering assay for silencing ASB8 expression was performed. As shown in Fig. 2D, the ASB8 expression was inhibited significantly by these two siRNAs and siASB8-690 was used for following study due to obvious interference effect. The results showed that the virus titers in the ASB8 knockdown cells were lower than those in the cells with negative control (Fig. 2E–G), indicating that the silencing of ASB8 obviously inhibits the proliferation of PRRSV in 3D4/21 cells.

2.3. Overexpression of ASB8 inhibits RLR-mediated signaling and promotes RNA virus proliferation

Published studies have suggested that SOCS family is a pivotal negative regulator for cytokine signaling(Pothlichet et al., 2008). To obtain further insight into the potential regulatory roles of ASB8 in antiviral signaling, we examined the effects of overexpressed ASB8 on virus-induced reporter gene activation. As shown in Fig. 3A and B, SeV-induced IFN-β, ISRE and NF-κB activation were inhibited by ASB8 in a dose-dependent manner, implicating a negative regulation role for ASB8 in SeV-induced antiviral responses. qPCR analysis indicated that overexpression of ASB8 inhibited the mRNA levels of IFNβ, CCL5 and CXCL10 genes after treated with poly (I:C) and SeV or VSV infection (Fig. 3C). In line with that finding, the ectopically expressed ASB8 cells showed lower IFN-β transcription and secretion (Fig. 3D). In addition, ASB8 overexpression attenuated HSV-induced activation of IFN-β and NF-κB in HEK293T cells, compared to control vector (Supplementary Fig. 1B). Conversely, knockdown of ASB8 facilitated RLR-induced transcription of IFNβ, CCL5 and CXCL10 genes (Fig. 3E).

In order to further understand the roles of ASB8 in PRRSV infection, the expression of IFN-β, IL-6, IL-1β, and TNF-α in ASB8 upregulated or downregulated of PRRSV-infected 3D4/21 cells were examined, and the results found that ASB8 significantly inhibited the expression of IFN-β and proinflammatory cytokines IL-6, IL-1β, and TNF-α as expected in response to PRRSV infection (Fig. 3F). Consistent with the overexpression of ASB8, deficiency of ASB8 significantly enhanced mRNA expression of IFN-β, TNF-α and IL-6 in 3D4/21 cells upon infection with PRRSV (Fig. 3G). These data showed that ASB8 inhibited innate immune responses in PRRSV infection.

In addition, the expression of VSV and SeV specific mRNA were enhanced in HEK293T cells by qPCR in overexpressed-ASB8 cells (Fig. 3H). In line with that, the replication of VSV and SeV were much lower after ASB8 knockdown. The VSV-GFP inoculated HEK293T cells showed a reduced fluorescence intensity, which demonstrated that the knockdown of ASB8 substantially inhibited viral proliferation (Fig. 3I).

2.4. ASB8 interacts with the PRRSV Nsp1α

PRRSV Nsp1, Nsp4 or Nsp11, each of which is the major expression protein related to suppression activation of IFN-β and NF-κB promoter. The effect relationship between ASB8 and PRRSV non-structural protein is unclear. Co-immunoprecipitation (IP)-Western blot analysis was performed to understand the interaction between ASB8 and PRRSV

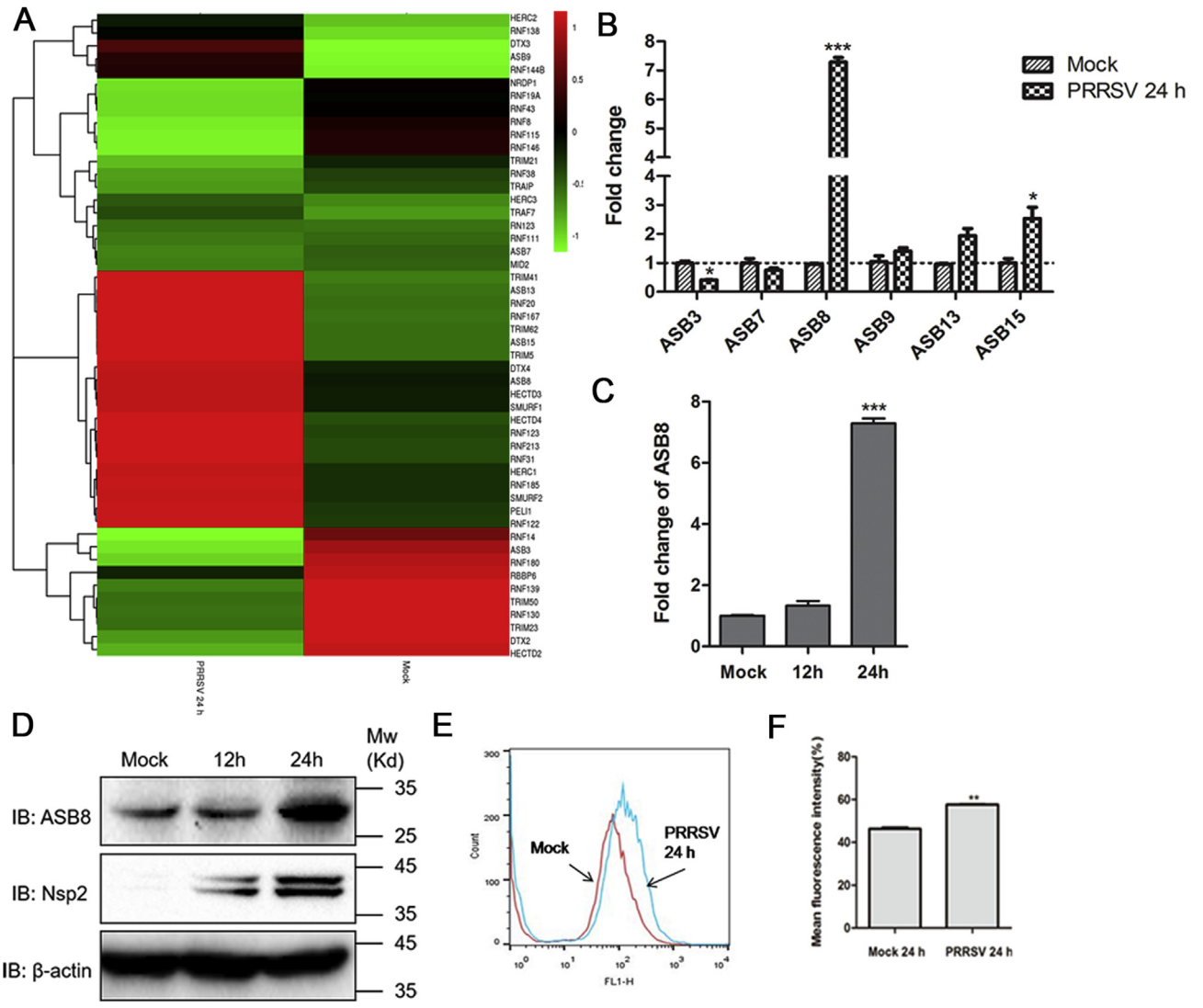


Fig. 1. PRRSV infection induces ASB8 expression. (A) Transcriptomics analysis of E3 ubiquitin ligase expression in 3D4/21 cells infected with PRRSV at a multiplicity of infection (MOI) of 1 for 24 h. (B) PRRSV JXwn06 infected the 3D4/21 cells 24 h, cells were subjected to qRT-PCR to quantify ASB family mRNA levels. (C) qRT-PCR to quantify ASB8 mRNA levels in PRRSV-infected 3D4/21 cells at different time points of 12, and 24 h postinfection. (D) Immunoblot analysis of ASB8 and Nsp2 protein expression in 3D4/21 cells infected with PRRSV an MOI of 1. β-actin was used as loading control. (E, F) 3D4/21 cells were mock infected or infected with PRRSV at an MOI of 1. At the indicated time points, the cells were harvested and subjected to FACS analysis with mouse polyclonal antibody against ASB8. The data are expressed as the mean ± SEM of three independent experiments.

nonstructural proteins. The plasmids expressing individual Myc-tagged Nsp1, 1α, 1β, 4, 11 protein were co-transfected with Flag-tagged ASB8 plasmid. The results indicated that Nsp1α interacts with ASB8 (Fig. 4A and B). The interaction between ASB8 and other PRRSV Nsp proteins were not detected in transfected cells. Moreover, Nsp1α co-precipitated with endogenous ASB8, also certified the interaction between ASB8 and Nsp1α (Fig. 4C). Confocal immunofluorescence microscopy was further verified the interaction of Nsp1α with ASB8 in 3D4/21 cells (Fig. 4D). Importantly, we found ASB8 was co-localized well in PRRSV infected 3D/4/21 cells (Fig. 4E). The results suggested that ASB8 could interacted with PRRSV Nsp1α.

2.5. Nsp1α and ASB8 inhibitory effects on IFN-β and NF-κB were superimposed

PRRSV Nsp1α suppresses IFN-β promoter activity and NF-κB signaling (Han et al., 2013; Kim et al., 2010). We thus investigated whether ASB8 and Nsp1α could synergistically inhibit IFN-β and NF-κB pathway. For this purpose, HEK293T cells were co-transfected with Flag-tagged ASB8 plasmid and equalizing amount of Myc-tagged Nsp1α-encoding plasmids. The ASB8, also PRRSV Nsp1α inhibited the formation of IFN-β and NF-κB, and the suppress effect on IFN-β and NF-κB was further strengthened in Nsp1α and ASB8 co-expressed cells (Fig. 5A and B). After viral infection, phosphorylated P65 translocate from the cytoplasm to the nucleus to activate proinflammatory cytokines transcription(Lawrence, 2009). We therefore examined the phosphorylation of P65 in Nsp1α and ASB8 co-expressed cells. As shown in Fig. 5C, co-expression of ASB8 and Nsp1α enhanced the inhibitory effect of p-P65 in HEK293T cells after SeV inoculation. Knockdown of ASB8 did not abolish the inhibitory effect of Nsp1α on IFN-β and NF-κB, suggesting that more than one host proteins were involved in Nsp1α inhibition of IFN-β, which is a complex process (Fig. 5D). Together, these results suggested that ASB8 promoted the inhibitory effect of Nsp1α on IFN-β and NF-κB production.

2.6. ASB8 catalyzes K63-linked polyubiquitination of Nsp1α

ASB8 is a SOCS family member containing a SOCS domain with E3

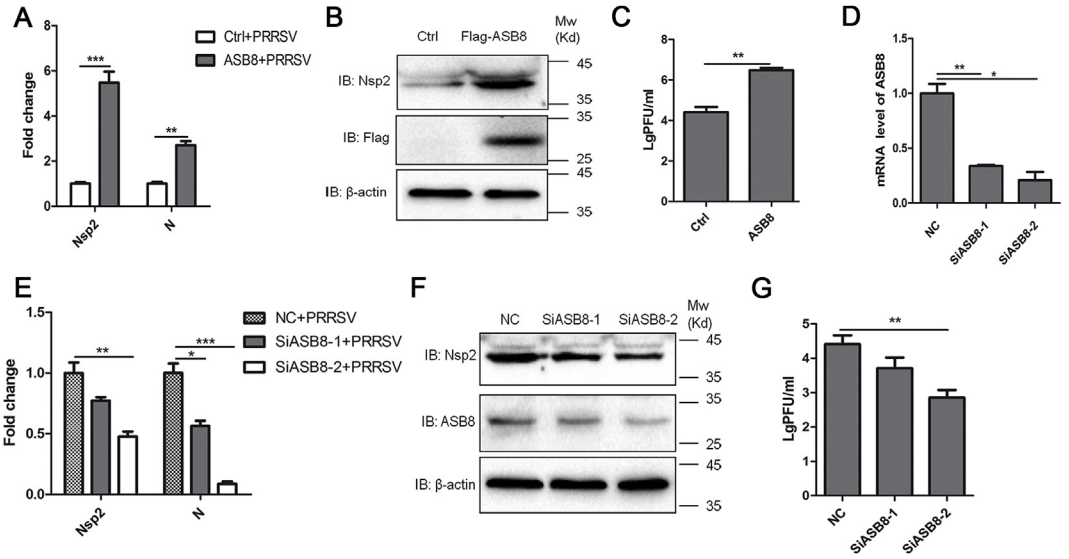


Fig. 2. ASB8 promotes PRRSV proliferation. (A, B) Flag-ASB8 or the control vectors were transfected into 3D4/21 cells, 24 h later, 1 MOI PRRSV was inoculated the cells, mRNA of PRRSV Nsp2 and N gene by qRT-PCR, and Nsp2 protein expression were detected by Western blot. (C) PRRSV-titer were measured by plaque assay. (D) 50 nM of ASB8 siRNA or negative control siRNA were transfected into 3D4/21 cells. 24 h later, PRRSV infected the cells for 24 h qRT-PCR was used to measure ASB8 expression. (E, F) 3D4/21 cells were transfected with siRNA of ASB8 or of negative control (NC); 24 h after transfection, cells were infected with I MOI PRRSV. The infected cells were collected at 24 h postinfection, mRNA loads of PRRSV N and Nsp2 were measured by qRT-PCR, and PRRSV Nsp2 levels were tested by WB. (G) 3D4/21 cells were transfected with ASB8 or control siRNA at a final concentration of 50 nM, and then infected with PRRSV JXwn06 at MOI of 1. At 24 h post-infection, PRRSV titer in the supernatants were measured by plaque assay in 3D4/21 cells. The data are expressed as the mean ± SEM of three independent experiments.

ubiquitin ligase activity, whether ASB8-mediated Nsp1α protein ubiquitination depended on the SOCS box domain was further investigated. Myc-Nsp1α with HA-Ub (WT) plasmid plus full-length ASB8 plasmid or ASB8 mutant deleting C-terminal SOCS box domain (ASB8dSOCS) were co-transfected into HEK293T cells. Co-immunoprecipitation experiments showed that Nsp1α ubiquitination was markedly increased in the presence of ASB8 expression plasmid. Notably, SOCS box domain deleted mutant of ASB8 did not increase Nsp1α polyubiquitination, which indicating that the E3 ligase activity of ASB8 was required for the polyubiquitination of Nsp1α (Fig. 6A).

To investigate the type of ASB8-mediated polyubiquitination of Nsp1α, we used ubiquitin mutants in which all lysine residues except K48 or K63 were mutated to arginine (K48 or K63) and an ubiquitin mutant in which only the K63 residue was mutated to arginine (K63R). Ubiquitination assay confirmed that addition of ASB8 dramatically promotes the wild type and K63-linked ubiquitination of Nsp1α, while there is no effect on the ubiquitin lacking the K63 residue(K63R) (Fig. 6B and C). Nsp1α has three lysine residues (K117, K150, K169). To identify the lysine residues responsible for ASB8-mediated polyubiquitination, we first generated the Nsp1α-KO mutant, in which all of the lysine residues in Nsp1α were replaced with arginine. Then, we reintroduced individual lysine residues into Nsp1α-KO to generate the single-lysine mutants. The ASB8 imposed the polyubiquitination effect of Nsp1α (WT) on the Nsp1α K117 mutant were identified by co-immunoprecipitation analyses in HEK293T cells (Fig. 6D).

To further verify the effect of ASB8 on the stability of Nsp1α protein, ASB8 and Nsp1α were co-transfected in HEK293T cells. After 24 h, the cells were treated with a protein synthesis inhibitor, cycloheximide (CHX), and collected after treatment of 0, 4, 8, and 16 h. The results showed that ASB8 can potentiate the stability of Nsp1α protein after 8 h of treatment compared to the control (Fig. 6E). The Nsp1α expression level was reduced 0.5-fold in the cells treated with the Nsp1α and empty vector after 8 h of cycloheximide treatment, while it remained at 0.8-fold in the co-transfection of ASB8 and Nsp1α treatment. These data suggested the expression of ASB8 induced an increase of the Nsp1α half-life from approximately 8 h–16 h.

2.7. ASB8 targets IKKβ

To further explore the interaction molecules reacted with ASB8 and participated in IFN signaling pathway, the blank vector or Flag-ASB8 transfected HEK293T cells were inoculated with SeV and cultured for 12 h, and then the whole-cell lysed supernatants were collected and affinity purified by anti-Flag affinity column. The bound proteins were eluted with excess Flag peptides after extensive buffer washing. The resolved proteins were visualized by silver staining on SDS-PAGE and the individualistic protein bands on gels were recovered and analyzed by mass spectrometry. A number of proteins interacted with ASB8, including IKKβ, HSP70 and LRRC10B were identified. Interestingly, an serine kinase(IKKβ)which plays an essential role in the NF-κB signaling pathway was also detected in the ASB8-containing protein complex (Fig. 7A).

During viral infection, PRRs transmit signals to IκB kinases(IKK)via different adaptor proteins, including canonical kinases IKKα, IKKβ and its essential regulatory subunit NEMO, which lead to the phosphorylation and degradation of IκBα that provide a sensitive trigger for NF-κB signaling pathway (Hayden and Ghosh, 2008). We next examined the interaction between ASB8 and IKKα or IKKβ in HEK293T cells. Transient transfection and co-immunoprecipitation experiments showed that ASB8 interacted with IKKβ but not IKKα (Fig. 7B). This interaction of endogenous ASB8 and IKKβ was also observed by using ASB8 specific antibody (Fig. 7C). Deletion of the IKKβ kinase domain ablated the interaction between IKKβ and ASB8 (Fig. 7D). Moreover, colocalization between ASB8 and IKKβ was also observed in HeLa cells (Fig. 7E). Together these data demonstrated that ASB8 interacted with the kinase domain of IKKβ.

The activation of NF-κB mediated by IKKβ was attenuated in ASB8 overexpression cells (Fig. 7F). Notably, SeV-induced phosphorylation of IκBα and p65 was markedly inhibited in ASB8-transfected HEK293T cells than in those transfected with empty vector (Fig. 7G). Together these data demonstrated that ASB8 might specifically target IKKβ to negatively regulate NF-κB signaling pathway.

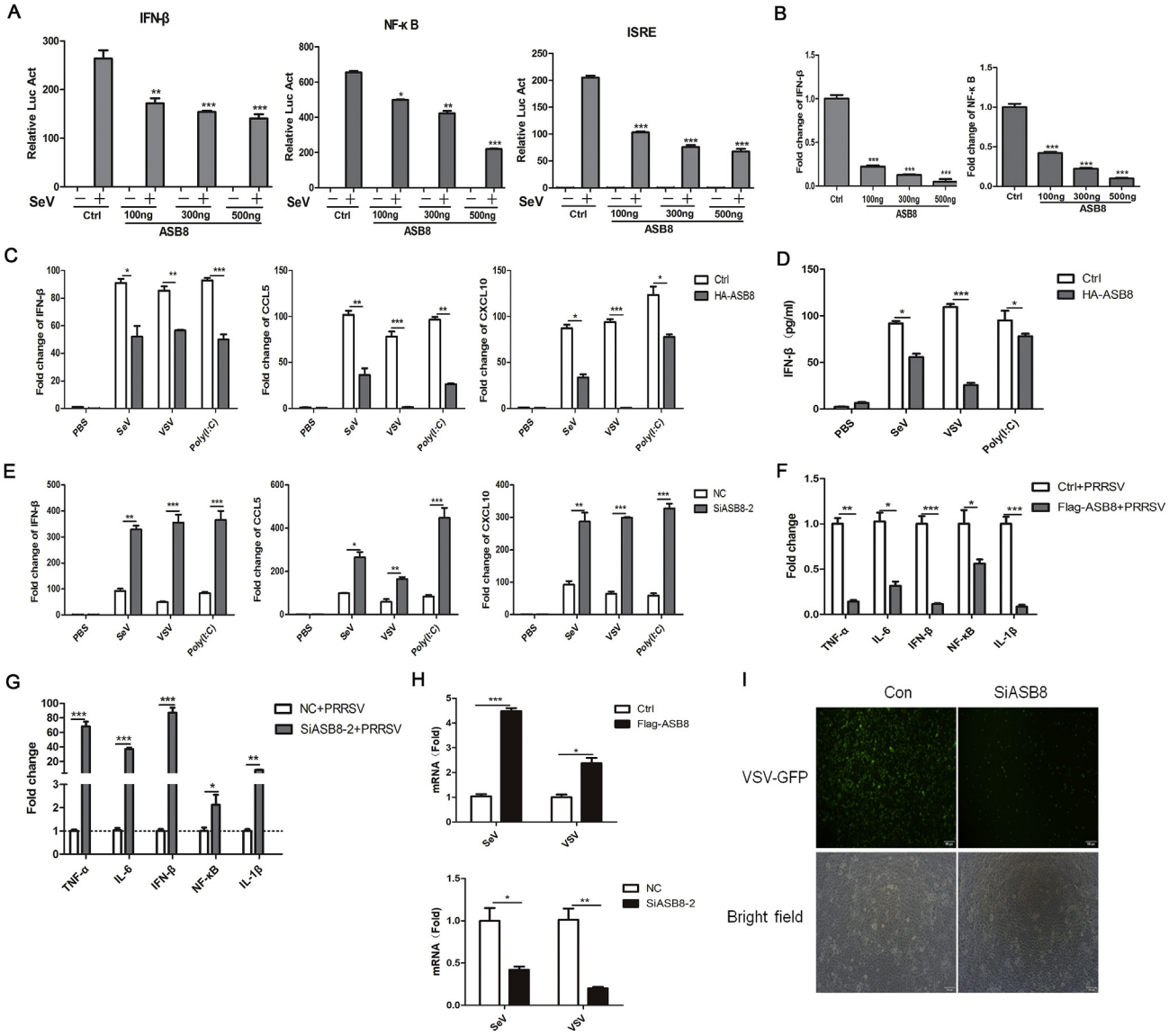


Fig. 3. ASB8 regulates RLR-induced signaling. (A, B) HEK293T cells were seeded on 24-well plates and transfected with 100 ng IFN-β luciferase reporter or ISRE and NF-κB reporter and 100 ng pRL-SV40 plasmid, plus the indicated amount of ASB8 plasmids. 16 h after transfection, cells were infected with SeV or left uninfected for 16 h before luciferase assays and qRT-PCR were performed. (C) Expression of IFNβ, CXCL10, CCL5, mRNA in HEK293T cells transfected with HA-ASB8 stimulated with poly(I:C) or infected with SeV or VSV for 12 h. (D) ELISA quantification of IFN-β secretion in HEK293T cells treated as in C. (E) Expression of IFNβ, CXCL10, CCL5, mRNA in HEK293T cells transfected with control siRNA (NC) or ASB8 siRNA for 24 h stimulated with poly(I:C) or infected with SeV or VSV for 12 h. (F, G) Flag-ASB8 and si-ASB8 were transfected into 3D4/21 cells for 24 h and PRRSV was inoculated for another 24 h, then the IFN-β, TNF-α, IL-1β, NF-κB and IL-6 mRNA expression level were measured by qRT-PCR. (H) qPCR analysis of VSV and SeV RNA in HEK293T cells transfected with Flag-ASB8 or si-ASB8 for 24 h, followed by infection for 12 h with VSV and SeV (MOI, 0.5). (I) Fluorescence intensity of VSV–GFP proliferation in HEK293T cells transfected with SiASB8. Scale bars, 100 μm. The data are expressed as the mean ± SEM of three independent experiments.

2.8. IKKβ phosphorylates ASB8 at Ser31

A mobility shift for ASB8 was detected after co-transfected ASB8 with wild-type IKKβ but not with IKK-KM. And we noticed that ASB8 also undergo mobility shift after transfected with SOCS domain deleted mutant (Fig. 8A). That mobility shift of ASB8 was reversed after incubation of the cell lysates with calf intestinal alkaline phosphatase (CIP) (Fig. 8B). The mass-spectrometry analysis of Flag-tagged ASB8 in the presence or absence of IKKβ were further performed. This ASB8 Ser31 as specific phosphorylation sites targeted by IKKβ was identified (Fig. 8C). We subsequently constructed a series of ASB8 phosphorylation site mutants and found that Ser31 was the most crucial residue for the mobility shift induced by IKKβ (Fig. 8D). Luciferase assay results confirmed that Ser31 phosphorylation of ASB8 was essential for inhibition of the NF-κB signaling pathway (Fig. 8E). Thus, we concluded that phosphorylation of ASB8 at Ser31 by IKKβ was required for the regulation of NF-κB signaling pathway.

2.9. ASB8 induces K48-linked ubiquitination and degradation of IKKβ

To investigate whether ASB8 regulates IKKβ signaling through its E3 ligase activity, we assessed ASB8-mediated ubiquitination of IKKβ. Altogether, our data suggested that ASB8 induces ubiquitination of IKKβ in the presence of ubiquitin (WT) or K48 ubiquitin but not in the presence of K48R and K63 ubiquitin (Fig. 9A and B). While disturbing the expression of ASB8, the IKKβ K48 ubiquitination level was down-regulated (Fig. 9C). The ubiquitin conjugation of IKKβ were observed in the HA-Ub (WT) and K48-only groups after MG132 treatment, but not

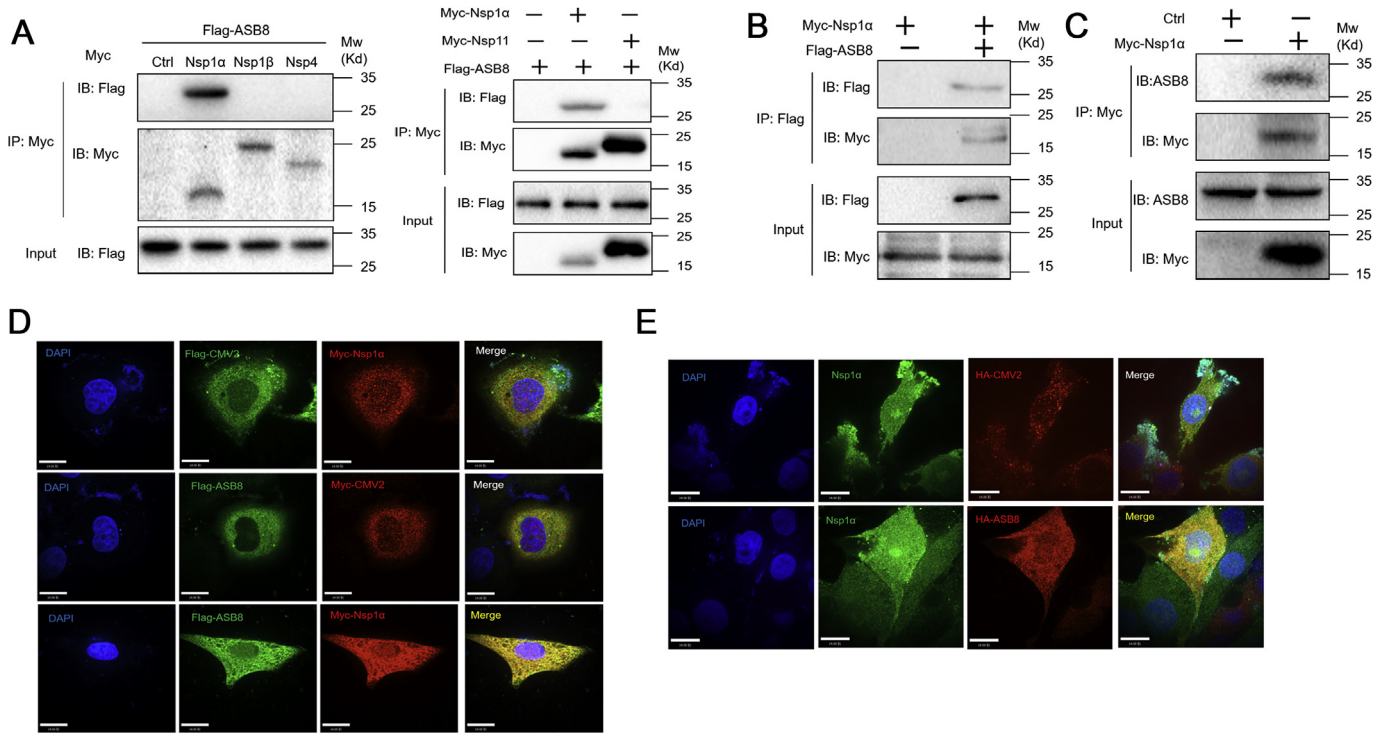


Fig. 4. ASB8 interacts with the PRRSV Nsp1α. (A, B) HEK293T cells were co-transfected with Flag-ASB8 expression plasmid plus the indicated plasmids expressing Myc-tagged PRRSV viral proteins. The cells were harvested at 24 h posttransfection and co-immunoprecipitation was performed. (C) Detection of the interaction between endogenous ASB8 and Nsp1α by co-immunoprecipitation. (D) 3D4/21 cells were cotransfected with Myc-Nsp1α and Flag-ASB8. The cells were fixed at 24 h posttransfection and doubly stained with a rabbit anti-Myc antibody and a mouse anti-Flag antibody followed by FITC-conjugated anti-mouse IgG (green) and Cy5-conjugated anti-rabbit IgG (red). Nuclei were stained with DAPI (blue). Cells were observed under a laser confocal imaging analysis system. (E) 3D4/21 cells were transfected with the HA-ASB8 or HA-expressing plasmid, 16 h later PRRSV infected the cells, and were examined at 16 h post-transfection with confocal immunofluorescence microscopy. Scale bars, 10 μm. Image shown is representative of three independent experiments.

in K48 mutant (K48R) group. Collectively, these data illustrated that ASB8 acts as an E3 ubiqutin ligase and specific catalyzed K48-linked polyubiquitination of IKKβ. (Fig. 9D).

To verify whether IKKβ protein undergoes ubiquitination and degradation, the HEK293T cells were transfected with Flag-IKKβ plasmid in condition of upregulation the amounts of HA-ASB8 were performed. The immunoblot analysis result showed that ASB8 could decrease IKKβ protein in a dose-dependent manner (Fig. 9E), and the degradation of IKKβ was also blocked by proteasome inhibitor MG132 (Fig. 9E and F). The results illustrated that ASB8 mediated IKKβ degradation via proteasome pathway.

We next investigated the relationship between ASB8 ubiquitination and phosphorylation. We noted that the ubiquitination-defective mutant ASB8(dSOCS) could readily undergo phosphorylation (Fig. 8A). These data suggested that ASB8 phosphorylation did not require ubiquitination. Conversely, ubiquitination of the phosphorylation-defective mutant S31A was noted to be significantly impeded, indicating that phosphorylation was a prerequisite for IKKβ ubiquitination (Fig. 9G).

3. Discussion

Emerging evidence places ubiquitination functions at the core of a multitude of regulatory processes ranging from signal transduction to inflammatory response and viral replication (entry, transcription, translation, and release)(Davis and Gack, 2015; Luo, 2016a). Some E3-ubiquitin ligase proteins have been demonstrated to have direct antiviral activity. For example, TRIM22 restricts hepatitis C virus (HCV) and influenza A virus (IAV) infection by degrading HCV viral NS5A and IAV viral nucleoprotein (NP)(Di Pietro et al., 2013; Yang et al., 2016). While TRIM32 restricts IAV proliferation by ubiquitination and degradation of PB1 polymerase (Fu et al., 2015). In addition, some E3 ubiquitin ligases have been demonstrated to display an activity of promoting virus replication. TRIM6 promotes VP35 ubiquitination and is important for Ebola virus replication (Bharaj et al., 2017). PJA2, is a RING finger E3 ligase that polyubiquitinates Tat, activating HIV-1 viral transcription. Additionally, ubiquitin ligase CNOT4 medicates ubiquitination of IAV NP, resulting in the enhancement of viral RNP activity (Lin et al., 2017). Our results indicated that silencing expression of E3 ubiquitin ligases ASB8 could inhibit the replication of PRRSV, on the contrary, the upregulated ASB8 expression promoted the replication of PRRSV, suggesting that ASB8 might function as a positive cellular cofactor to boost PRRSV replication. Few SOCS box domain proteins, including SOCS1, SOCS2 and SOCS3, directly enhance virus infection (Wei et al., 2016; Zheng et al., 2015). In addition, as a novel negative feedback regulator of NF-κB activation, intracellular ASB8 worked as a critical step to reduce excessive inflammatory responses by IKKβ degradation. Moreover, ASB8 directly interacted with PRRSV Nsp1α and catalyzed K63-linked polyubiquitination of Nsp1α on Lys117, which strengthens the stability of Nsp1α protein and promotes proliferation of PRRSV. Present results demonstrated that ASB8 acts as an identified E3 ligase for Nsp1α providing a mechanistic rationale for PRRSV replication.

As the largest family of SOCS box-containing proteins, with 18 murine and human ASBs have been identified (Debrincat et al., 2007). However, no identified reports were available related to *Sus scrofa,* especially in ASB8, yet their biological functions are largely unknown. Screening data from transcriptomics, we found differences in the six ASB proteins following PRRSV infection, including ASB3, 7, 8, 9, 13, 15, among which ASB8 was remarkably upregulated rather than others. In this study, the critical role of ASB8 in innate immune response to viruses was identified. Overexpression of ASB8 significantly inhibited virus-induced transcription of downstream antiviral genes and renders

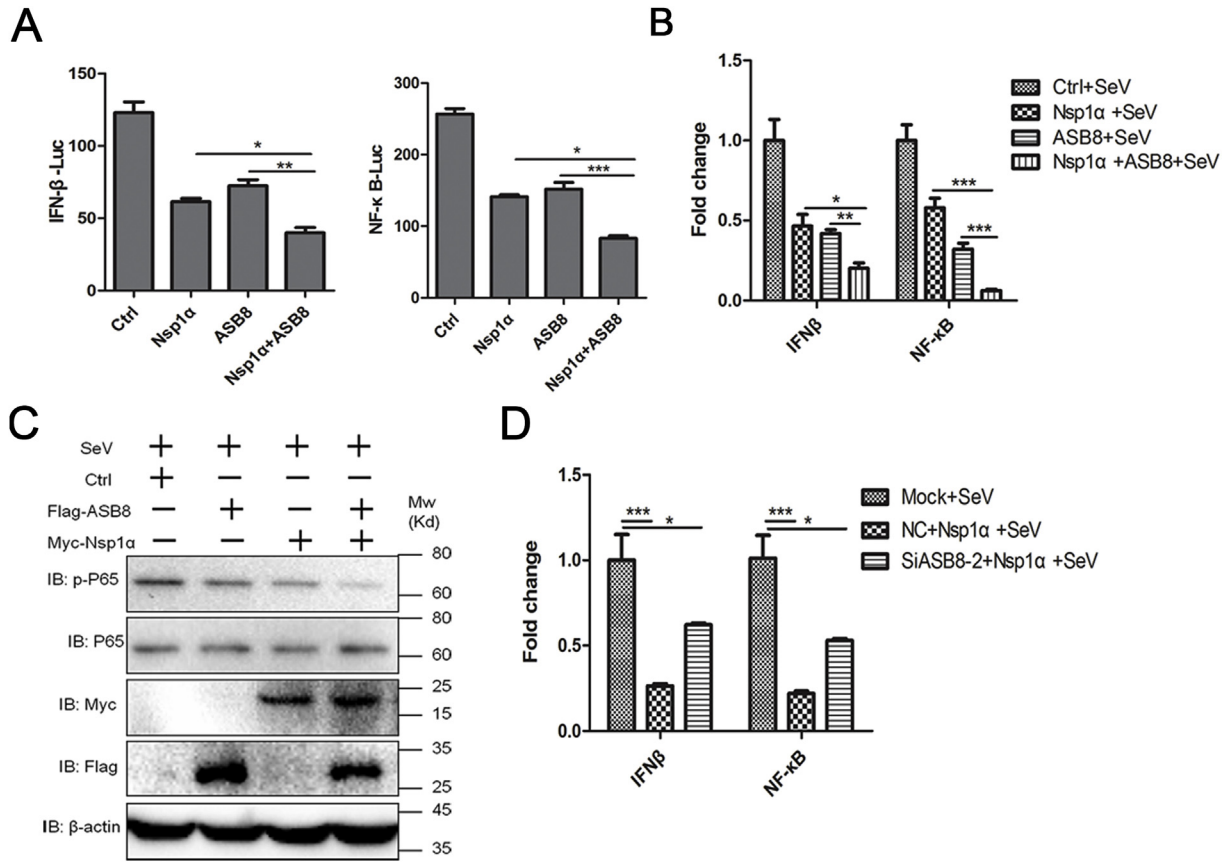


Fig. 5. ASB8 and Nsp1α jointly inhibit IFN-β and NF-κB production. (A, B) HEK293T cells were transfected with ASB8, Nsp1α plasmids or co-transfected with two plasmids. 16 h later, cells were inoculated with 0.5 MOI SeV for 16 h before luciferase and qPCR analysis were performed. (**C**) HEK293T cells were transfected with Flag-ASB8 and Myc–Nsp1α for 16 h before the phosphorylation of p65 was tested by Western blot after the cells were inoculated with 0.5 MOI SeV for 16 h. (**D**) qPCR analysis in HEK293T cells transfected with NC or SiASB8 and Myc-Nsp1α for 24 h, followed by infection for 12 h with SeV (MOI, 0.5). The data are expressed as the mean ± SEM of three independent experiments.

the cells more susceptible to virus infection. Consistently, in PRRSV infection, upregulated ASB8 also significantly inhibited the expression of IFN-β and proinflammatory cytokines IL-6, NF-κB, IL-1β, and TNF-α, which is consistent with SOCS-1 negative regulation of cytokine signaling during influenza virus infection(Wei et al., 2014).

NF-κB activation has been believed to play key roles in pathogenic mechanisms. The suppression of NF-κB signal pathway is also evident in virus-infected MARC-145 and PAM cells (Duan et al., 2014; Fu et al., 2012; Kim et al., 2010). Nsp1α possesses the potential to inhibit the NF-κB promoter activity by blocking the phosphorylation of IκBα(Kim et al., 2010; Rascon-Castelo et al., 2015; Wang and Zhang, 2014; Wang et al., 2016), but the precise mechanism was still unclear. The current study identified that ASB8 is involved in Nsp1α protein-mediated NF-κB repression. We proposed that the PRRSV Nsp1α hijacks ASB8 to promote virus replication via ubiquitination. Both ASB8 and Nsp1α protein possessed a demonstrated capacity to inhibit NF-κB pathway, implying that manipulating ASB8 levels may serve as a switch for controlling NF-κB signaling mediated through Nsp1α. Therefore, ASB8 can be a critical host factor targeted by multiple viruses to enhance their proliferation. However, knockdown of ASB8 did not eliminate the inhibitory effect of Nsp1α on IFN-β and NF-κB, implying that the involvement of other host factors in this process. So we have proposed the further research necessity to understand the detailed process of virus Nsp1a hijacking ASB8 to facilitate PRRSV proliferation.

IκB kinase β (IKKβ) functions as a fine-tuning controller of the nuclear factor NF-κB pathway (Maubach et al., 2017; Scheidereit, 2006). Published studies have established Kelch-like ECH-associated protein 1 (KEAP1) is responsible for IKKβ ubiquitination and degradation (Lee et al., 2009). In our study, we demonstrated that ASB8 functions as an IKKβ E3 ubiquitin ligase, consequently suppressing NF-κB antiviral response by promoting proteasome degradation of IKKβ. Moreover, IKKβ phosphorylated ASB8 at Ser31, which is critical for suppression of NF-κB pathway. The ubiquitinated defective ASB8 variant is readily phosphorylated, confirming that ASB8 phosphorylation does not require ubiquitination, but rather the opposite. Thus, our results suggested that ASB8 as an antagonist of IKKβ and signaling via NF-κB, can be phosphorylated by IKKβ to further regulate innate antiviral immunity in viral infection.

Ubiquitin chains linked through different lysine display variable cellular functions. Most of studies have investigated lysine of K48-linked and K63-linked polyubiquitination. K48-linked polyubiquitination are usually involves in regulation function of target protein degradation, while K63-linked polyubiquitination mainly plays a role in activation of antiviral signaling pathway (Hatakeyama, 2017; Luo, 2016b; Rajsbaum et al., 2014). SOCS is one of the substrate recognition modules of Cullin5/Rbx2 ubiquitin ligases (Heuze et al., 2005; Kohroki et al., 2005). The SOCS box is necessary for stabilization and/or degradation of SOCS proteins by recruitment of the ubiquitin transferase system (Chung et al., 2005; Li et al., 2011). The ubiquitin ligase Cullin5^{SOCS2} mediated regulation of TNFα−NF−κB signaling through NDR1 ubiquitination and degradation(Paul et al., 2017). Additionally, creatine kinase B (CKB) interacts with Asb-9, which prompts the polyubiquitylation of CKB and reduces total CKB levels within the cell (Debrincat et al., 2007). In the present study, ASB8 catalyzed the K48-linked polyubiquitin and proteasomal degradation to IKKβ and thereby restrain immune responses after viral infection. Meanwhile, in response

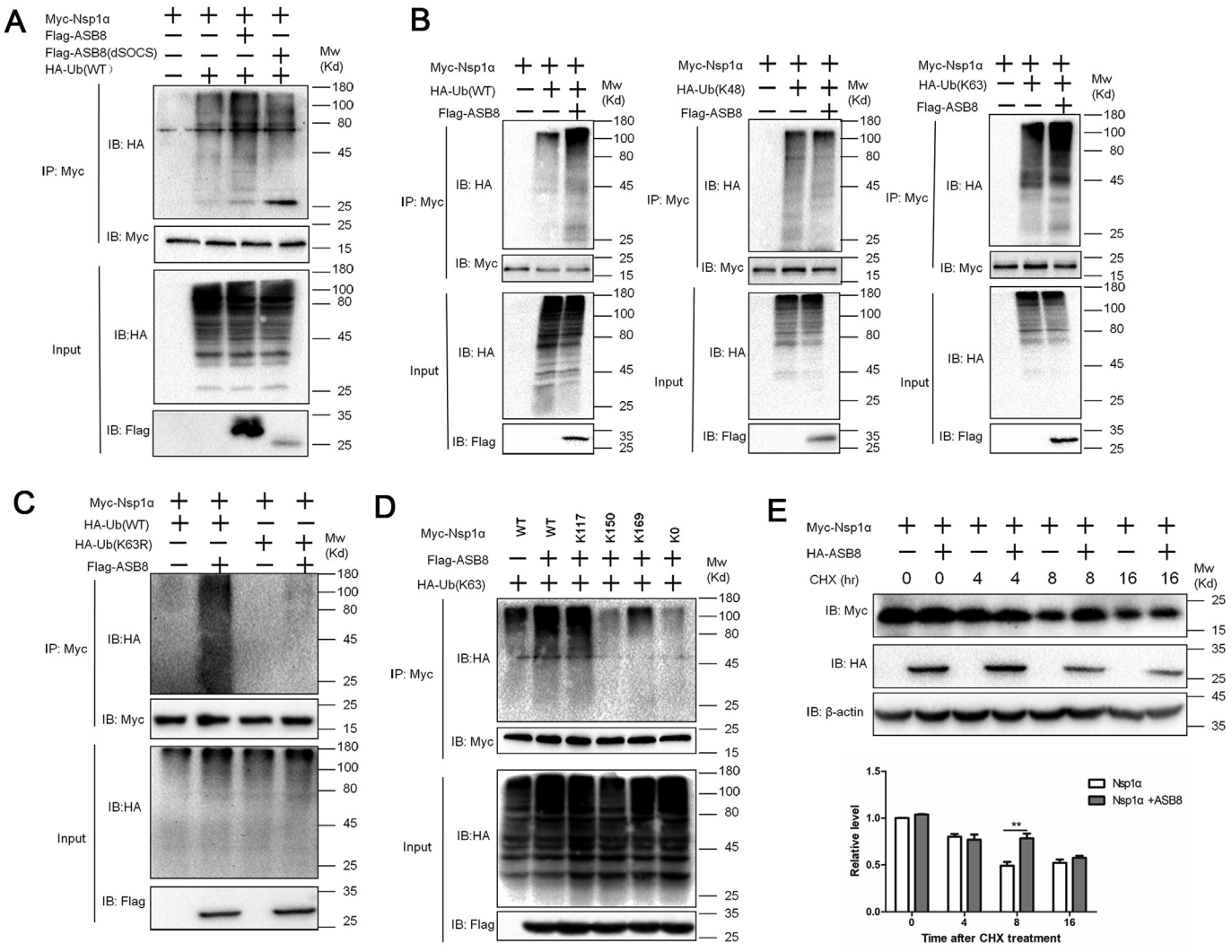


Fig. 6. ASB8 catalyzes the K63-linked polyubiquitination of Nsp1α. (**A**) Co-immunoprecipitation analysis of the ubiquitination of Nsp1α in HEK293T cells transfected with Myc–Nsp1α and HA–Ub (WT), as well as Flag–ASB8 or Flag–ASB8(dSOCS). (**B**) Co-immunoprecipitation analysis of the ubiquitination of Nsp1α in HEK293T cells transfected with plasmids expressing Myc–Nsp1α, Flag–ASB8, and HA–Ub (WT), HA–Ub (K48) or HA–Ub (K63). (**C, D**) HEK293T cells were transfected with the indicated plasmids. After 24 h, cell lysates were immunoprecipitated using anti-Myc antibody, followed by Western blot analysis using the indicated antibodies. (**E**) HEK293T cells were transfected with control vector or HA-ASB8, as well as Myc–Nsp1α. After 24 h, 100 μg/ml CHX was added for different time and Western blot was used to detect the level of Nsp1α protein. Densitometry analysis showing fold changes of Nsp1α levels after cycloheximide (CHX) addition, normalized with β-actin. Data are representative of results from three independent experiments.

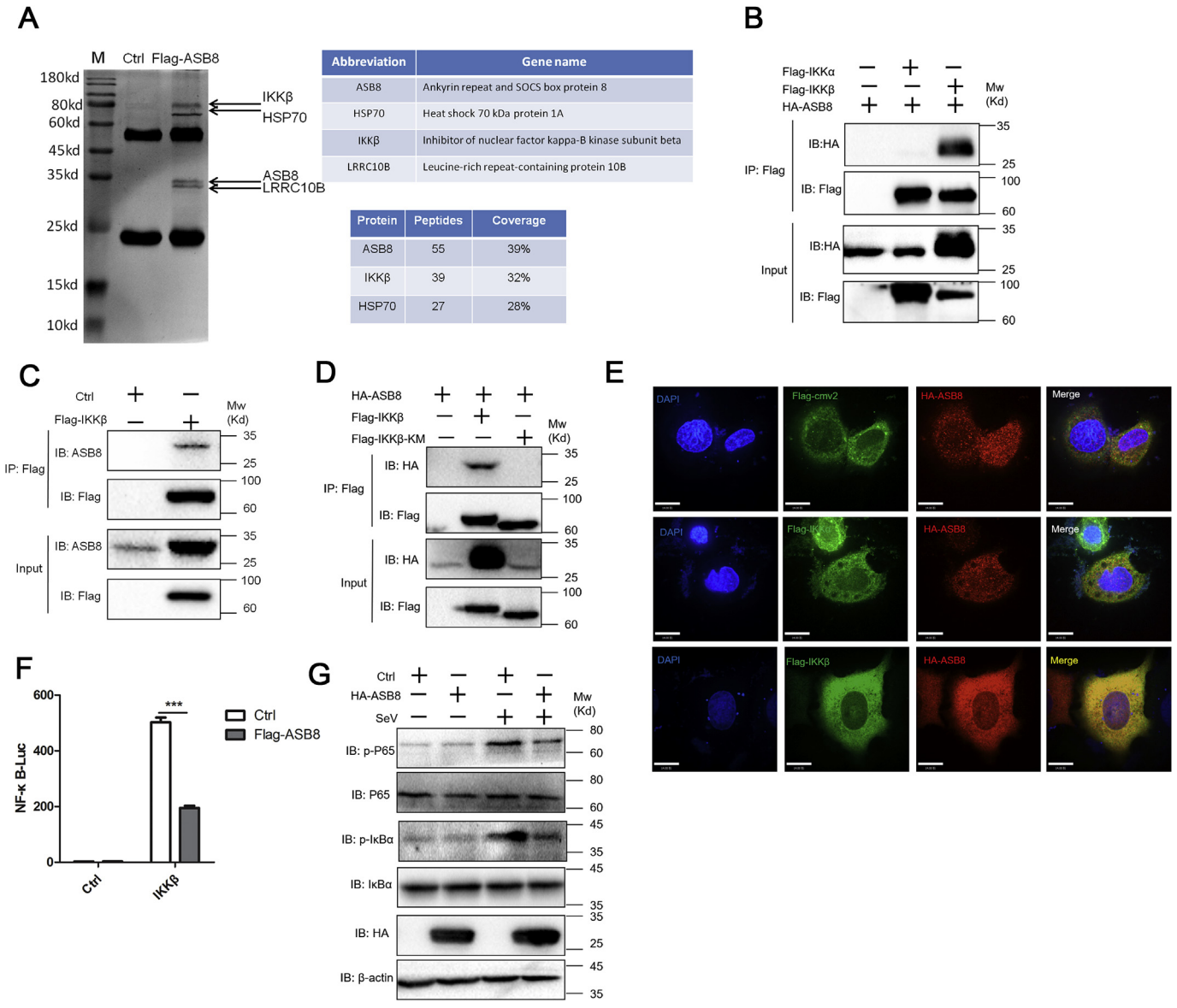
to PRRSV infection, ASB8 facilitated K63-linked polyubiquitination of PRRSV Nsp1α on Lys117, which increases the protein stability of Nsp1α and promotes PRRSV proliferation. Therefore, ASB8 may mediate both K48 and K63-linked ubiquitination leading to the degradation or activation of respective target protein. Similarly, RNF128 catalyzed both K48-linked and K63-linked ubiquitination, in which RNF128 degrades T cell antigen receptor and activates of TBK1, respectively (Song et al., 2016; Su et al., 2009).

In summary, we have identified an E3 ubiquitin ligase ASB8 as a negative regulator of IKKβ and a host factor directed against RNA virus infection. Firstly, ASB8 interacted with IKKβ but not recognized IKKα in our study. And the competition combination of ASB8 with IKKβ blocked the formation of IKKα-IKKβ-NEMO compounds, which further inhibits NF-κB pathway and IFN-β production. Secondly, ASB8 was phosphorylated by IKKβ at Ser31, ASB8 bound to IKKβ, which leads to IKKβ K48-linked ubiquitination and degradation, thereby inhibiting phosphorylation of IκBα and p65. Thirdly, ASB8 interacted with the PRRSV Nsp1α and involved in Nsp1α protein-induced NF-κB signaling repression. This work provides a new perspective of PRRSV to suppress the NF-κB signaling, but also defines a previously unknown, yet critical role of ASB8. The more precise roles of ASB8 in NF-κB signal pathway remains under investigation.

4. Materials and methods

4.1. Viruses and cells

The Chinese highly pathogenic PRRSV strain JXwn06 was used in this study (Du et al., 2016; Su et al., 2018). 3D4/21 cells (CRL-2843) were porcine alveolar macrophage cell line and were grown in RPMI 1640 medium containing 10% fetal bovine serum (Biological Industries), 100 U/ml penicillin and 10 μg/ml streptomycin sulfate at 37 °C in a humidified 5% CO_2 incubator(Zhao et al., 2015). Human embryonic kidney 293T cells (HEK293T) and HeLa cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum.



Abbreviation	Gene name
ASB8	Ankyrin repeat and SOCS box protein 8
HSP70	Heat shock 70 kDa protein 1A
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
LRRC10B	Leucine-rich repeat-containing protein 10B

Protein	Peptides	Coverage
ASB8	55	39%
IKKβ	39	32%
HSP70	27	28%

Fig. 7. ASB8 interacts with IKKβ. (**A**) Cellular extracts from HEK293T cells with expression of Flag-ASB8 were immunopurified with anti-FLAG affinity beads and eluted with Flag peptides. The eluates were resolved on SDS-PAGE and silver-stained followed by mass spectrometry analysis. Peptide coverage of the indicated proteins were shown. (**B**) Immunoblot analysis of total cell lysates and proteins immunoprecipitated, with antibody to anti-Flag, from HEK293T cells transfected with expression plasmids for HA-ASB8 and Flag-tagged IKKα and IKKβ. (**C**) Detection of the interaction between endogenous ASB8 and IKK β by co-IP. (**D**) Interaction between the IKKβ kinase domain and ASB8 as examined by co-IP. (**E**) HeLa cells were cotransfected with HA-ASB8 and Flag-IKKα or Flag-IKKβ. The cells were fixed at 24 h posttransfection and doubly stained with a rabbit anti-HA antibody and a mouse anti-Flag antibody followed by FITC-conjugated anti-mouse IgG (green) and Cy5-conjugated anti-rabbit IgG (red). Nuclei were stained with DAPI (blue). Cells were observed under a laser confocal imaging analysis system. Scale bars, 10 μm. (**F**) HEK293T cells were seeded on 24-well plates and transfected the next day with control or corresponding plasmids. 24 h later, luciferase assays were performed. (**G**) Immunoblot analysis of phosphorylated (p-) p65 and IκBα in lysates of HEK293T cells transfected for 24 h with appropriate plasmid, followed by SeV infection for 12 h. Data are representative of results from three independent experiments.

4.2. Antibodies and reagents

Mouse anti-Flag monoclonal antibody (MAb) and anti-Flag M2 affinity gel were all purchased from Sigma-Aldrich. Anti-Myc-tag mouse MAb (agarose conjugated) was purchased from Abmart. The antibodies specific to Myc, HA and phospho-IκBα were from Cell Signaling Technology (CST). Anti-phosphor-p-p65 polyclonal antibodies (ImmunoWay) were purchased and used according to the manufacturers' instructions. Antibody to ASB8 was prepared by immunizing mice with the purified recombinant protein. Anti-PRRSV Nsp1α was a gift from Huazhong Agricultural University of China. The rabbit polyclonal antibody specific for PRRSV Nsp2 were generously provided by Prof. Jun Han of China Agricultural University. FITC-conjugated goat anti-rabbit antibody and Cy5-conjugated goat anti-mouse antibody were purchased from Invitrogen. The horseradish peroxidase (HRP)-conjugated antibodies including goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Sungene Biotech Co. Cycloheximide (CHX), NH_4Cl and MG132 were purchased from Sigma. Poly (I:C) was purchased from Invitrogen and was used at a final concentration of 1 μg/ml for cell transfection.

4.3. Plasmids constructs

The cDNA encoding ASB8 was amplified from porcine PBMCs and cloned into the vector pFlag-CMV2/pHA-CMV2/pMyc-CMV2(Clontech) via *Eco*RI/*Xba*I sites to construct a recombinant plasmid Flag-ASB8/

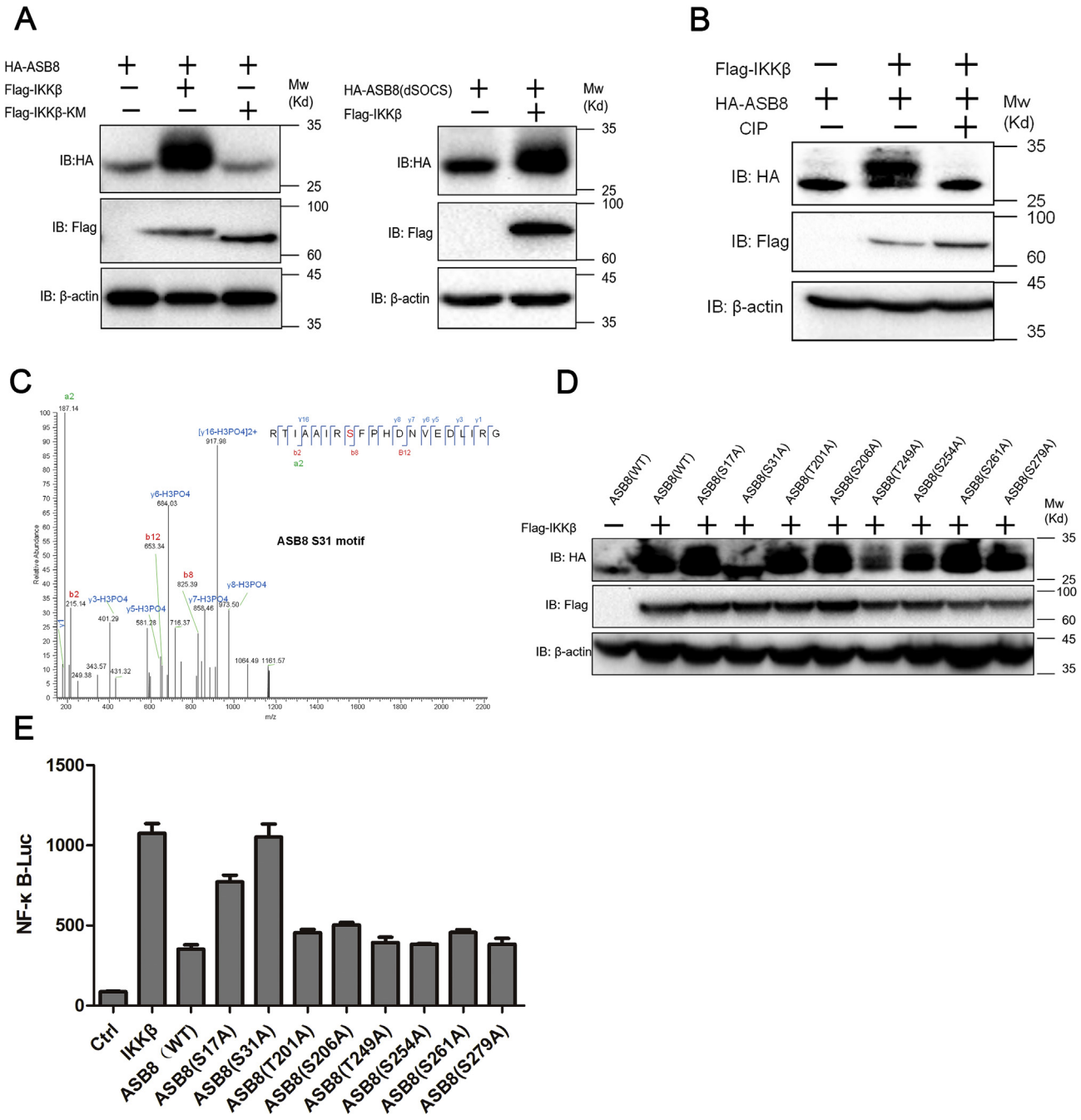


Fig. 8. IKKβ phosphorylates ASB8 at Ser31. (**A**) Immunoblot analysis of lysates of HEK293T cells transfected to express HA-ASB8 and no IKKβ or Flag-tagged IKKβ or IKKβ-KM. (**B**) Immunoblot analysis of lysates of HEK293T cells transfected to express HA- ASB8 and no IKKβ or Flag-tagged IKKβ and untreated or treated with CIP. (**C**) Mass-spectrometry analysis of an IKKβ-phosphorylated ASB8 Ser31 peptide. (**D**) Immunoblot analysis of lysates of HEK293T cells transfected to express HA-ASB8 or eight phosphorylation mutants with or without an expression vector for Flag- IKKβ. (**E**) HEK293T cells were seeded on 24-well plates and transfected the next day with control or corresponding plasmids. 24 h later, luciferase assays were performed. Data are representative of three independent experiments with three biological replicates.

HA-ASB8. cDNA encoding full-length nonstructural protein (Nsp) from PRRSV strain JXwn06 were subcloned into expression vector pMyc-CMV2. Truncation and point mutations were generated by the fast multisite mutagenesis system (TransGen Biotech, China) using the WT protein plasmid as the template. The primer of all constructs were provided in Table 1.

4.4. RNA extraction, reverse transcription (RT) and quantitative PCR (qPCR)

Total RNA from the PRRSV-infected 3D4/21 cells were extracted using the RNAiso Plus reagent (Takara) according to the manufacturer's protocols. qPCR was carried out to determine the transcription levels of specific genes, using DBI Bioscience-2043 Bestar SybrGreen qPCR master mix on ABI 7500 Real-Time PCR System. Table 2 listed the specific primers for qPCR detection. The relative quantities of mRNA accumulation were calculated based on the $2^{-\Delta\Delta Ct}$ method and the data

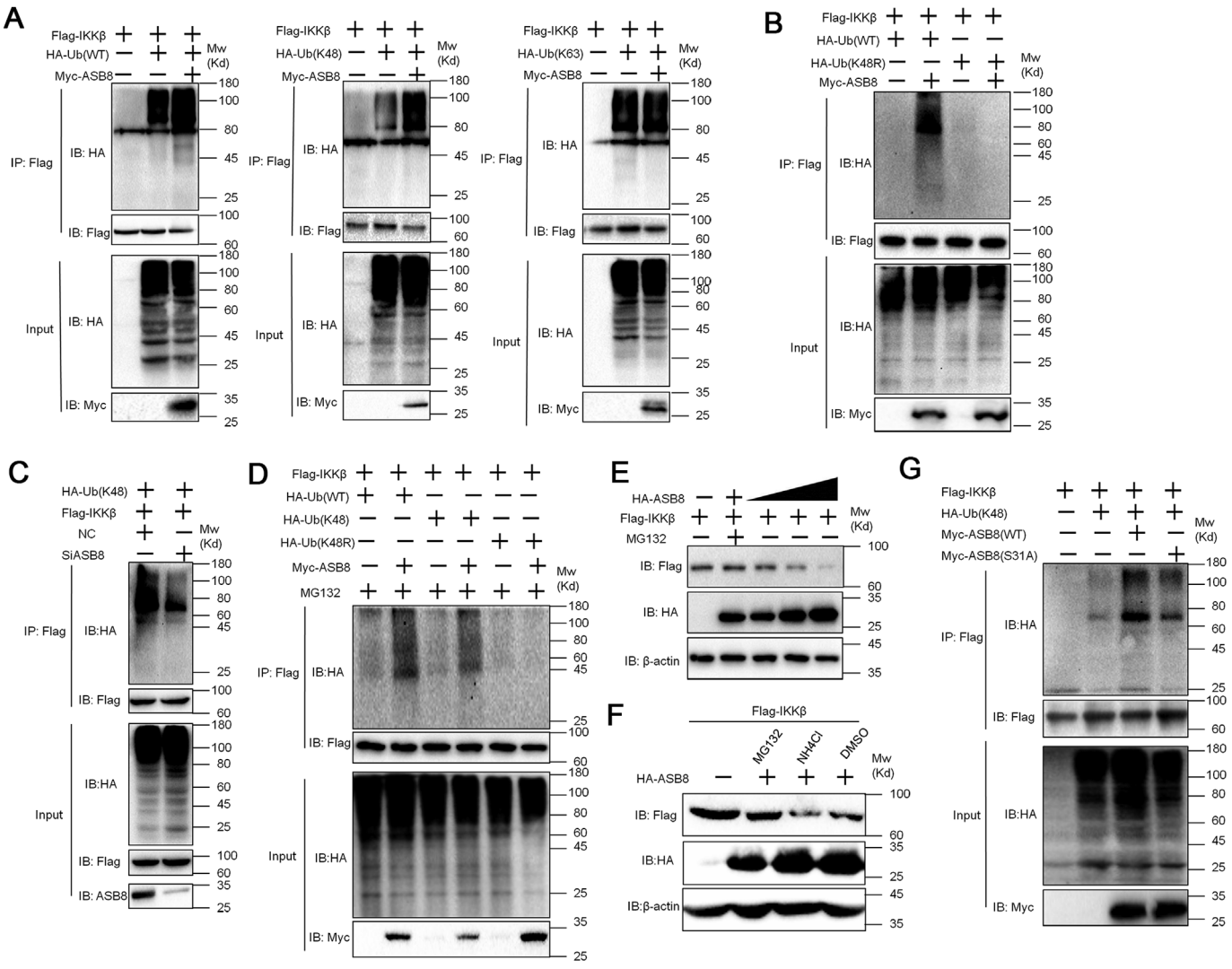


Fig. 9. ASB8 facilitates the K48-linked ubiquitination and degradation of IKKβ. (**A, B**) Co-immunoprecipitation analysis of the ubiquitination of IKKβ in HEK293T cells transfected with the indicated plasmids. (**C**) HEK293T cells were transfected with Flag-IKKβ, HA–Ub (K48) and NC or SiASB8 plasmids. After 24 h, cell lysates were immunoprecipitated using anti-Flag antibody, followed by Western blot analysis using the indicated antibodies. (**D**) HEK293T were transiently transfected with Flag-IKKβ, HA–Ub (K48), HA–Ub (K48R) along with Myc-ASB8. After 24 h, cells were treated for 10 h with 10 μM MG132, then immunoprecipitated with anti-Flag and probed with the indicated antibodies. (**E**) HEK293T cells were transfected with IKKβ plasmid, along with increasing amounts of plasmid expressing ASB8, and then treated with MG132 (10 μM) for 10 h. (**F**) HEK293T cells were transfected with IKKβ and ASB8 plasmids for 24 h and treated with vehicle (DMSO), NH4Cl (10 mM), or MG132 (10 μM) for 10 h. Cell lysates were immunoblotted with the indicated antibodies. (**G**) Co-immunoprecipitation analysis of the ubiquitination of IKKβ in HEK293T cells transfected with plasmids expressing Flag-IKKβ, Myc-ASB8 or its mutants, and HA–Ub (K48). Data are representative of three independent experiments.

Table 1
Primers used for PCR amplification.

Gene	GenBank accession No.	Primer (5′-3′)
ASB8	XM_005664169.2	**F:** GTCAAGTTCCAGTTTGGAGCATGT **R:** ATCCAGAGCTGCCAGCATGCTGGT
Flag/HA/Myc-ASB8	XM_005664169.2	**F:** CGGAATTCAATGAGTTCCAGTATGTGG **R:** GCTCTAGACTATTCTACAAGTAACAGGTAT
Myc-Nsp1α	ABR37297.1	**F:** CGGAATTCAATGTCTGGGATACTTGAT **R:** GCTCTAGATCAAGCCATAGCACACTC
Myc-Nsp1β	ABR37297.1	**F:** CGGAATTCCATGGACGTCTATGACAT **R:** GCTCTAGATCAACCGTACCACTTATG
Myc-Nsp4	ABR37297.1	**F:** CGGAATTCAATGGGCGCTTTCAGAACT **R:** GCTCTAGATCATTCCAGTTCGGGTTT
Myc-Nsp11	ABR37297.1	**F:** CGGAATTCAATGGGGTCGAGCTCCCCG **R:** GCTCTAGATCATTCAAGTTGAAAATAG

were normalized to the expression level of β-actin gene. The reported values were from three independent tests.

4.5. Flow cytometry analysis

3D4/21 cells infected with PRRSV strain JXwn06 at 1 MOI (multiplicity of infection) for 1 h or mock treated with serum free RPMI 1640. Then the cells were washed once again and maintained in RPMI 1640 medium supplemented with 2% fetal calf serum for 24 h, 3D4/21 cells were dissociated from the plates with 0.25% EDTA PBS and washed with cold PBS containing 2% bovine serum albumin (BSA). Then cells were stained with ASB8 (1:200) antibody for 30 min at 4 °C, followed by incubation with 1:200 goat anti-mouse IgG FITC conjugate for 30 min at 4 °C. A total of 1×10^5 cells were used for fluorescence-activated cell sorter (FACS) analysis, and the expression level of ASB8 molecules was presented as the mean fluorescence intensity (MFI).

Table 2
Primers used for qRT-PCR amplification.

Gene	GenBank accession No.	Primer (5′-3′)
Q-ASB8	XM_005664169.2	**F:** GAGAAGGGAGCTGAGGTG **R:** AGCGGAGTGTCATTGTTG
IFN-β	NM_001003923.1	**F:** GCAGTATTGATTATCCACGAGA **R:** TCTGCCCATCAAGTTCCAC
NF-κB	EU399817.1	**F:** GTGTGTAAAGAAGCGGGACCT **R:** CACTGTCACCTGGAAGCAGAG
IL-6	NM_214399	**F:** ACTGGCAGAAAACAACCTGA **R:** CCTCGACATTTCCCTTATTGCT
IL-1β	M86725	**F:** TGTTCTGCATGAGCTTTGTG **R:** TCTGGGTATGGCTTTCCTTAG
TNF-α	NM_214022	**F:** GAGATCAACCTGCCCGACT **R:** CTTTCTAAACCAGAAGGACGTG
β-actin	XM_003124280	**F:** CAAATGCTTCTAGGCGGACT **R:** TGCTGTCACCTTCACCGTTC
Nsp2	ABR37297.1	**F:** CAGCCTTATGACCCCAACCAG **R:** TGGGCAAAGTCCCCTGTACCAA
N	ABR37297.1	**F:** CAGTCAATCAGCTGTGCCAAA **R:** ATCTGACAGGGCACAAGTTCCA
CCL5	NM_001278736.1	**F:** AATTATTCCTGCAAGCCAA **R:** AGTAAATTCTTGATGGCCTT
CCL10	NM_001565.3	**F:** CTCCCCATATTCCTCGGACACCA **R:** GGAAATCCTGCCAGACTTGCT
SeV	NC_001552.1	**F:** GAAAGAGATACCGAACCCAGAG **R:** GCTTGAGGGAGTGTATTGTAGG
VSV	AM689874.1	**F:** CTCCATATGAAGACGTGGAA **R:** ATCGGAGTCCAACATACCA

4.6. Small RNA interfering assay

To analyze the effects of knockdown ASB8 on PRRSV replication and cytokine expression, a small RNA interfering (siRNA) assay was performed by siASB8 RNA targeting for ASB8 gene and scrambled control siRNA which were synthesized by GenePharma (Suzhou, China) (Table 3).

4.7. Dual luciferase assays

To evaluate IFN-β and NF-κB expression, HEK293T cells were seeded into 24-well plates and transiently cotransfected with control plasmids or ASB8 expression plasmids together with the luciferase reporter plasmid were carried out by following vendor's instruction. A 100 ng of the pRL-null (Renilla luciferase control reporter vector) plasmid DNA (Promega) was included as the control. The transfected cells were cultured for 16 h before inoculated with 0.5 MOI Sendai virus (SeV) for 16 h and then the lysed cell supernatants were subjected to luciferase activity analysis using the dual luciferase reporter assay system (Promega).

4.8. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IFN-β in cell culture supernatants were measured by ELISA Kits (R&D Systems).

Table 3
Sequences used in small RNA interfering assay.

Sequence name	Trans- and anti-sense sequences (5′-3′)
SiASB8-363	F: GCUUCACUGGGCAGCCUUUTT R: AAAGGCUGCCCAGUGAAGCTT
SiASB8-690	F: GGCCAAAGACCAGCAGCUUTT R: AAGCUGCUGGUCUUUGGCCTT
Negative control	F: UUCUCCGAACGUGUCACFUTT R: ACGUGACACGUUCGGAGAATT

4.9. Ubiquitination assay

For analysis of the ubiquitination of Nsp1α and IKKβ, HEK293T cells were transfected with plasmids expressing Myc–Nsp1α or Flag-IKKβ, HA-ubiquitin(WT), HA-ubiquitin(K48), HA-ubiquitin(K63) and Flag-ASB8/Myc-ASB8 or its mutants, and then lysed cell supernatants were immunoprecipitated with the Myc/Flag-specific antibody and analyzed by immunoblot with anti-HA probe.

4.10. Immunofluorescence

HeLa or 3D4/21 cells grown in 12-well plates were transiently transfected with Lipofectamine 3000 Reagents (Invitrogen), ASB8 expressing plasmid or Nsp1α plasmid by following manufacturer's protocol. For endogenous fluorescence detection, 3D4/21 cells transfected with plasmid were inoculated with 1 MOI PRRSV. After 24 h post-transfection or virus infection, the cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 at RT for 10 min. The cells were incubated with rabbit anti-HA or anti-Myc and mouse Nsp1α antibody for 1 h before being washed twice with PBS. After three washes with PBS, the cells incubated with FITC-conjugated goat anti-mouse antibodies and Cy5-conjugated goat anti-rabbit antibodies for 1 h. The cells were continue washed for three times and then incubated with 4′, 6-diamidino-2-phenyl-indole (DAPI, Sigma) for 5 min. Finally, the images were visualized under an Olympus confocal microscope.

4.11. Western blot and co-immunoprecipitation assays

HEK293T cells were cultivated separately in 60-mm-diameter plate transfected with either singly or doubly appropriate expression plasmid. The harvested cells were lysed with cell lysis buffer (Solarbio) after 24 h transfection. The cell debris were removed by centrifugation at 13,000 rpm for 15 min. The supernatants were used in immunoprecipitation with anti-Myc/Flag agarose beads. The beads were boiled for 10 min before being washed three times with lysis buffer, and then the proteins bound to the beads were separated by SDS-PAGE, and then transferred onto polyvinylidene difluoride (PVDF) membranes; probed with proper antibodies against Flag or Myc, finally exposed by using an ECL Western blotting kit (Pierce) and a chemiluminescence apparatus (Gel Doc XR + Imaging System, BIO-RAD, USA) were performed.

4.12. Statistical analysis

The various treatments were compared using an unpaired, two-tailed Student's *t*-test using GraphPad Prism software (Version 6, USA) (Wang et al., 2019). $P < 0.05$ was considered statistics significantly. In addition, $P < 0.01$ and $P < 0.001$ were marked with two (**) and three (***) asterisks, respectively.

Competing financial interests

The authors declare no competing interests.

Author contributions

Conceived and designed the experiments: JH H. Performed the experiments: RQ L, C C, J H, YX S, LL Z, YY G, WT Z, K T and L Z. Analyzed the data: RQ L and J H. Contributed reagents/materials: JH H. Wrote the paper: RQ L and JH H.

Acknowledgments

We thank Prof. Shaobo Xiao for providing Nsp1α monoclonal antibody. This work was supported by The National Key Research and

Development Program of China (2018YFD0500500), National Natural Science Foundation of China (31272540) and the underprop project of Tianjin Science and Technology in China (16YFZCNC00640). Dr. JH H received the three funding.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.04.004.

References

Albina, E., 1997. Epidemiology of porcine reproductive and respiratory syndrome (PRRS): an overview. Vet. Microbiol. 55, 309–316.

Bharaj, P., Atkins, C., Luthra, P., Giraldo, M.I., Dawes, B.E., Miorin, L., Johnson, J.R., Krogan, N.J., Basler, C.F., Freiberg, A.N., Rajsbaum, R., 2017. The host E3-ubiquitin ligase TRIM6 ubiquitinates the Ebola virus VP35 protein and promotes virus replication. J. Virol. 91.

Chung, A.S., Guan, Y.J., Yuan, Z.L., Albina, J.E., Chin, Y.E., 2005. Ankyrin repeat and SOCS box 3 (ASB3) mediates ubiquitination and degradation of tumor necrosis factor receptor II. Mol. Cell Biol. 25, 4716–4726.

Davis, M.E., Gack, M.U., 2015. Ubiquitination in the antiviral immune response. Virology 479–480, 52–65.

Debrincat, M.A., Zhang, J.G., Willson, T.A., Silke, J., Connolly, L.M., Simpson, R.J., Alexander, W.S., Nicola, N.A., Kile, B.T., Hilton, D.J., 2007. Ankyrin repeat and suppressors of cytokine signaling box protein asb-9 targets creatine kinase B for degradation. J. Biol. Chem. 282, 4728–4737.

Di Pietro, A., Kajaste-Rudnitski, A., Oteiza, A., Nicora, L., Towers, G.J., Mechti, N., Vicenzi, E., 2013. TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. J. Virol. 87, 4523–4533.

Du, J., Ge, X., Liu, Y., Jiang, P., Wang, Z., Zhang, R., Zhou, L., Guo, X., Han, J., Yang, H., 2016. Targeting swine leukocyte antigen class I molecules for proteasomal degradation by the nsp1alpha replicase protein of the Chinese highly pathogenic porcine reproductive and respiratory syndrome virus strain JXwn06. J. Virol. 90, 682–693.

Duan, E., Wang, D., Luo, R., Luo, J., Gao, L., Chen, H., Fang, L., Xiao, S., 2014. Porcine reproductive and respiratory syndrome virus infection triggers HMGB1 release to promote inflammatory cytokine production. Virology 468–470, 1–9.

Fu, B., Wang, L., Ding, H., Schwamborn, J.C., Li, S., Dorf, M.E., 2015. TRIM32 senses and restricts influenza a virus by ubiquitination of PB1 polymerase. PLoS Pathog. 11, e1004960.

Fu, Y., Quan, R., Zhang, H., Hou, J., Tang, J., Feng, W.H., 2012. Porcine reproductive and respiratory syndrome virus induces interleukin-15 through the NF-kappaB signaling pathway. J. Virol. 86, 7625–7636.

Garcia-Sastre, A., 2017. Ten strategies of interferon evasion by viruses. Cell Host Microbe 22, 176–184.

Goubau, D., Deddouche, S., Reis e Sousa, C., 2013. Cytosolic sensing of viruses. Immunity 38, 855–869.

Han, M., Du, Y., Song, C., Yoo, D., 2013. Degradation of CREB-binding protein and modulation of type I interferon induction by the zinc finger motif of the porcine reproductive and respiratory syndrome virus nsp1alpha subunit. Virus Res. 172, 54–65.

Hatakeyama, S., 2017. TRIM family proteins: roles in autophagy, immunity, and carcinogenesis. Trends Biochem. Sci. 42, 297–311.

Hayden, M.S., Ghosh, S., 2008. Shared principles in NF-kappaB signaling. Cell 132, 344–362.

Heuze, M.L., Guibal, F.C., Banks, C.A., Conaway, J.W., Conaway, R.C., Cayre, Y.E., Benecke, A., Lutz, P.G., 2005. ASB2 is an Elongin BC-interacting protein that can assemble with Cullin 5 and Rbx1 to reconstitute an E3 ubiquitin ligase complex. J. Biol. Chem. 280, 5468–5474.

Huang, C., Zhang, Q., Guo, X.K., Yu, Z.B., Xu, A.T., Tang, J., Feng, W.H., 2014. Porcine reproductive and respiratory syndrome virus nonstructural protein 4 antagonizes beta interferon expression by targeting the NF-kappaB essential modulator. J. Virol. 88, 10934–10945.

Isaacson, M.K., Ploegh, H.L., 2009. Ubiquitination, ubiquitin-like modifiers, and deubiquitination in viral infection. Cell Host Microbe 5, 559–570.

Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin Jr., W.G., Conaway, R.C., Conaway, J.W., 1998. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. Genes Dev. 12, 3872–3881.

Kawasaki, T., Kawai, T., Akira, S., 2011. Recognition of nucleic acids by pattern-recognition receptors and its relevance in autoimmunity. Immunol. Rev. 243, 61–73.

Ke, H., Yoo, D., 2017. The viral innate immune antagonism and an alternative vaccine design for PRRS virus. Vet. Microbiol. 209, 75–89.

Kim, O., Sun, Y., Lai, F.W., Song, C., Yoo, D., 2010. Modulation of type I interferon induction by porcine reproductive and respiratory syndrome virus and degradation of CREB-binding protein by non-structural protein 1 in MARC-145 and HeLa cells. Virology 402, 315–326.

Kohroki, J., Nishiyama, T., Nakamura, T., Masuho, Y., 2005. ASB proteins interact with Cullin5 and Rbx2 to form E3 ubiquitin ligase complexes. FEBS Lett. 579, 6796–6802.

Krebs, D.L., Hilton, D.J., 2001. SOCS proteins: negative regulators of cytokine signaling. Stem Cell. 19, 378–387.

Kundu, K., Dutta, K., Nazmi, A., Basu, A., 2013. Japanese encephalitis virus infection modulates the expression of suppressors of cytokine signaling (SOCS) in macrophages: implications for the hosts' innate immune response. Cell. Immunol. 285, 100–110.

Lawrence, T., 2009. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 1, a001651.

Lee, D.F., Kuo, H.P., Liu, M., Chou, C.K., Xia, W., Du, Y., Shen, J., Chen, C.T., Huo, L., Hsu, M.C., Li, C.W., Ding, Q., Liao, T.L., Lai, C.C., Lin, A.C., Chang, Y.H., Tsai, S.F., Li, L.Y., Hung, M.C., 2009. KEAP1 E3 ligase-mediated downregulation of NF-kappaB signaling by targeting IKKbeta. Mol. Cell. 36, 131–140.

Li, J.Y., Chai, B., Zhang, W., Wu, X., Zhang, C., Fritze, D., Xia, Z., Patterson, C., Mulholland, M.W., 2011. Ankyrin repeat and SOCS box containing protein 4 (Asb-4) colocalizes with insulin receptor substrate 4 (IRS4) in the hypothalamic neurons and mediates IRS4 degradation. BMC Neurosci. 12, 95.

Lin, Y.C., Jeng, K.S., Lai, M.M.C., 2017. CNOT4-Mediated ubiquitination of influenza a virus nucleoprotein promotes viral RNA replication. mBio 8.

Luo, H., 2016a. Interplay between the virus and the ubiquitin-proteasome system: molecular mechanism of viral pathogenesis. Curr Opin Virol 17, 1–10.

Luo, H.L., 2016b. Interplay between the virus and the ubiquitin-proteasome system: molecular mechanism of viral pathogenesis. Current Opinion in Virology 17, 1–10.

Maubach, G., Schmadicke, A.C., Naumann, M., 2017. NEMO links nuclear factor-kappab to human diseases. Trends Mol. Med. 23, 1138–1155.

Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.

Paul, I., Batth, T.S., Iglesias-Gato, D., Al-Araimi, A., Al-Haddabi, I., Alkharusi, A., Norstedt, G., Olsen, J.V., Zadjali, F., Flores-Morales, A., 2017. The ubiquitin ligase Cullin5(SOCS2) regulates NDR1/STK38 stability and NF-kappaB transactivation. Sci. Rep. 7, 42800.

Pothlichet, J., Chignard, M., Si-Tahar, M., 2008. Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-dependent pathway. J. Immunol. 180, 2034–2038.

Rajsbaum, R., Garcia-Sastre, A., Versteeg, G.A., 2014. TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. J. Mol. Biol. 426, 1265–1284.

Rascon-Castelo, E., Burgara-Estrella, A., Mateu, E., Hernandez, J., 2015. Immunological features of the non-structural proteins of porcine reproductive and respiratory syndrome virus. Viruses 7, 873–886.

Rudnicka, A., Yamauchi, Y., 2016. Ubiquitin in influenza virus entry and innate immunity. Viruses 8.

Scheidereit, C., 2006. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. Oncogene 25, 6685–6705.

Song, G., Liu, B., Li, Z., Wu, H., Wang, P., Zhao, K., Jiang, G., Zhang, L., Gao, C., 2016. E3 ubiquitin ligase RNF128 promotes innate antiviral immunity through K63-linked ubiquitination of TBK1. Nat. Immunol. 17, 1342–1351.

Song, J., Liu, Y., Gao, P., Hu, Y., Chai, Y., Zhou, S., Kong, C., Zhou, L., Ge, X., Guo, X., Han, J., Yang, H., 2018. Mapping the nonstructural protein interaction network of porcine reproductive and respiratory syndrome virus. J. Virol. 92 (24), e01112–e01118.

Su, L.L., Iwai, H., Lin, J.T., Fathman, C.G., 2009. The transmembrane E3 ligase GRAIL ubiquitinates and degrades CD83 on CD4 T cells. J. Immunol. 183, 438–444.

Su, Y., Shi, P., Zhang, L., Lu, D., Zhao, C., Li, R., Zhang, L., Huang, J., 2018. The superimposed deubiquitination effect of OTULIN and porcine reproductive and respiratory syndrome virus (PRRSV) Nsp11 promotes multiplication of PRRSV. J. Virol. 92, e00175–e00218.

Sun, L., Qin, J., Wang, K., Zhang, J., 2017. Expansion of pathogen recognition specificity in plants using pattern recognition receptors and artificially designed decoys. Sci. China Life Sci. 60, 797–805.

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

Sun, Z., Chen, Z., Lawson, S.R., Fang, Y., 2010. The cysteine protease domain of porcine reproductive and respiratory syndrome virus nonstructural protein 2 possesses deubiquitinating and interferon antagonism functions. J. Virol. 84, 7832–7846.

Thomas, J.C., Matak-Vinkovic, D., Van Molle, I., Ciulli, A., 2013. Multimeric complexes among ankyrin-repeat and SOCS-box protein 9 (ASB9), ElonginBC, and Cullin 5: insights into the structure and assembly of ECS-type Cullin-RING E3 ubiquitin ligases. Biochemistry 52, 5236–5246.

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

Wang, F., Zhao, K., Yu, S., Xu, A., Han, W., Mei, Y., 2019. RNF12 catalyzes BRF1 ubiquitination and regulates RNA polymerase III-dependent transcription. J. Biol. Chem. 294, 130–141.

Wang, R., Zhang, Y.J., 2014. Antagonizing interferon-mediated immune response by porcine reproductive and respiratory syndrome virus. BioMed Res. Int. 2014, 315470.

Wang, X., Shao, C., Wang, L., Li, Q., Song, H., Fang, W., 2016. The viral non-structural protein 1 alpha (Nsp1alpha) inhibits p53 apoptosis activity by increasing murine double minute 2 (mdm2) expression in porcine reproductive and respiratory syndrome virus (PRRSV) early-infected cells. Vet. Microbiol. 184, 73–79.

Wei, H., Wang, S., Chen, Q., Chen, Y., Chi, X., Zhang, L., Huang, S., Gao, G.F., Chen, J.L., 2014. Suppression of interferon lambda signaling by SOCS-1 results in their excessive production during influenza virus infection. PLoS Pathog. 10, e1003845.

Wei, H., Wang, S., Chen, Q., Chen, Y., Chi, X., Zhang, L., Huang, S., Gao, G.F., Chen, J.L., 2016. Correction: suppression of interferon lambda signaling by SOCS-1 results in

their excessive production during influenza virus infection. PLoS Pathog. 12, e1005402.
Wenhui, L., Zhongyan, W., Guanqun, Z., Zhili, L., JingYun, M., Qingmei, X., Baoli, S., Yingzuo, B., 2012. Complete genome sequence of a novel variant porcine reproductive and respiratory syndrome virus (PRRSV) strain: evidence for recombination between vaccine and wild-type PRRSV strains. J. Virol. 86, 9543.
Yang, C., Zhao, X., Sun, D., Yang, L., Chong, C., Pan, Y., Chi, X., Gao, Y., Wang, M., Shi, X., Sun, H., Lv, J., Gao, Y., Zhong, J., Niu, J., Sun, B., 2016. Interferon alpha (IFNalpha)-induced TRIM22 interrupts HCV replication by ubiquitinating NS5A. Cell. Mol. Immunol. 13, 94–102.
Yokota, S., Yokosawa, N., Okabayashi, T., Suzutani, T., Miura, S., Jimbow, K., Fujii, N., 2004. Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 contributes to inhibition of the interferon signaling pathway. J. Virol. 78, 6282–6286.
Zhang, H., Fang, L., Zhu, X., Wang, D., Xiao, S., 2018a. Global analysis of ubiquitome in PRRSV-infected pulmonary alveolar macrophages. J Proteomics 184, 16–24.
Zhang, Y., Li, L.F., Munir, M., Qiu, H.J., 2018b. RING-domain E3 ligase-mediated host-virus interactions: orchestrating immune responses by the host and antagonizing immune defense by viruses. Front. Immunol. 9, 1083.
Zhao, S., Ge, X., Wang, X., Liu, A., Guo, X., Zhou, L., Yu, K., Yang, H., 2015. The DEAD-box RNA helicase 5 positively regulates the replication of porcine reproductive and respiratory syndrome virus by interacting with viral Nsp9 in vitro. Virus Res. 195, 217–224.
Zheng, J., Yang, P., Tang, Y., Pan, Z., Zhao, D., 2015. Respiratory syncytial virus non-structural proteins upregulate SOCS1 and SOCS3 in the different manner from endogenous IFN signaling. J Immunol Res 2015, 738547.
Zuckermann, F.A., Garcia, E.A., Luque, I.D., Christopher-Hennings, J., Doster, A., Brito, M., Osorio, F., 2007. Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Vet. Microbiol. 123, 69–85.