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Host cell protein PSMB10 interacts with viral NS3 protein and inhibits the growth of classical swine fever virus

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ABSTRACT

Classical swine fever (CSF) is a major infectious disease of pigs caused by classical swine fever virus (CSFV). NS3 is one of the non-structural proteins of CSFV and plays an important role in the infection process. However, the NS3-interacting cellular proteins involved in viral replication are poorly documented. In this study, proteasome subunit beta 10 (PSMB10) was identified as a novel NS3-interacting partner using yeast two-hybrid screening of a porcine peripheral blood mononuclear cell (PBMC) cDNA library. The PSMB10-NS3 interaction was confirmed by co-immunoprecipitation, glutathione S-transferase pulldown, and laser confocal microscopy. Overexpression of PSMB10 inhibited CSFV replication. Conversely, CSFV infection inhibited PSMB10 expression. Furthermore, we demonstrated that NS3 is degraded by PSMB10 through the ubiquitin-proteasome system and that CSFV inhibits the expression of MHC class I antigen presentation-related transporter proteins, whereas PSMB10 can restore the function of MHC class I antigen presentation and inhibit CSFV proliferation.

1. Introduction

Classical swine fever (CSF) is a major infectious disease of pigs with high morbidity and mortality, caused by classical swine fever virus (CSFV) (Kleiboeker, 2002; Moennig, 2000a). The clinical symptoms are fever, diarrhea, neurological signs, and even death. With the high risk of spreading, with serious economic and public health consequences, CSF is listed by the OIE (World Organisation for Animal Health), as a class A animal disease that requires reporting (Moennig, 2000b; Stegeman et al., 2000).

Non-structural protein 3 (NS3) is one of the non-structural proteins of CSFV that has a variety of functions, including nucleoside triphosphatase activity (Tamura et al., 1993), serine protease activity (Wiskerchen et al., 1991), and RNA helicase activity (Sheng et al., 2007), which are essential for CSFV transcription and translation (Agapov et al., 2004; Wen et al., 2007). The accumulation of NS3 protein in cells can lead to a cytopathic effect (Meyers et al., 1992, 1996; Xu et al., 2008) and induce high levels of antibody (Brown et al., 2002), while the antibody is not protective (Corapi et al., 1990).

Proteasome subunit beta 10 (PSMB10), also known as low-molecular-mass protein 10 (LMP10) or multicatalytic endopeptidase complex subunit (MECl-1), is one of the immune proteasome subunits that replaces the standard proteasome subunit β2 when induced by γ-IFN and that participates in the process of MHC class I antigen presentation (Angeles et al., 2012). PSMB10 is associated with the occurrence and development of various diseases, e.g., high blood pressure caused by high salt concentration can promote the expression of immunoproteasome subunits PSMB10 and LMP7 in the mouse heart, thus promoting immunity, while knockout of PSMB10 significantly reduces high blood pressure (Yan et al., 2017).

In this study, we studied the interaction between the host cell protein PSMB10 and CSFV NS3 and the related mechanisms.

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Table 1
Potential binding partners of the CSFV NS3 protein.

Gene	Protein	NCBI Accession	Function
HAUS2	HAUS augmin-like complex, subunit 2	XM_003121567	Microtubule generation, mitotic spindle assembly
CTSV	Cathepsin V	NM_213892	Cysteine protease
ACTB	Actin, beta	XM_003124280	Involved in cell motility, structure and integrity
PSMB10	Proteasome subunit β10	NM_001044565	Multicatalytic proteinase
LDHB	Lactate dehydrogenase B	XM_013988347	catalyse the reversible Interconversion of pyruvate and lactate
MARC1	Mitochondrial amidoxime reducing component 1	XM_003357633	Prodrugs activation and detoxification pathways
ASB3	Ankyrin repeat and SOCS box containing 3	XM_013992808	Degradation of tumor necrosis factor receptor II
ETFA	Electron-transfer-flavoprotein, alpha polypeptide	XM_013989021	catalyse The mitochondrial fatty acid β oxidation

2. Results

2.1. Construction and evaluation of a PBMC cDNA library

The Y2H-compatible PBMC cDNA library was generated using pGADT7 vector in strain Y187. The cDNA library represented 6.0×10^8 independent clones. The insert size of the library varied from 500 bp to 3000 bp. Of the 23 colonies screened, 22 were positive, and the recombination rate was estimated to be 95.65%.

2.2. Y2H screening

Y2H screening identified eight proteins as potential binding partners of the CSFV protein NS3 (Table 1). The biological functions of the identified proteins included cellular immunity, cell migration, cellular signal transduction, and metabolism of glucose, lipid, and protein.

2.3. CSFV NS3 interacts with PSMB10 in vivo and in vitro

PSMB10, one of the potential binding partners of the CSFV NS3 protein identified in the Y2H screening, was selected for further study considering its involvement in diverse cellular processes, such as protein degradation and the antigen presentation pathway. To confirm the interaction between NS3 and PSMB10, AD-PSMB10 and BD-NS3 were cotransformed into yeast strain Y2HGlod and grown on synthetically defined media. Yeast cells cotransformed with AD-PSMB10 and BD-NS3 could grow on three kinds of synthetically defined media and formed blue colonies on QDO/X/A medium (Fig. 1), which indicated that the host cell protein PSMB10 interacts with CSFV NS3 protein in yeast cells. For the Co-IP assay, 3D4/2 cells were transformed with HA-NS3, and cell lysates were immunoprecipitated with antibody against HA or antibody against PSMB10 followed by Western blot analysis. Coimmunoprecipitation assay demonstrates that endogenous PSMB10 binds HA-NS3 in transfected 3D4/2 cells (Fig. 2). To further verify the interaction between NS3 and PSMB10 in vitro, GST pulldown assay was conducted with GST-tagged NS3 protein expressed in *E. coli* and p3 × Flag-tagged PSMB10 protein expressed in HEK293T cells. Western blot results indicated that p3 × Flag-tagged PSMB10 protein was captured by GST-NS3 (Fig. 3). Since the direct interaction between PSMB10 and CSFV NS3 has been determined, we further explore whether CSFV NS3 protein colocalizes with PSMB10.3D4/2 cells co-expressing HA-NS3 and p3 × Flag-PSMB10 were analyzed by laser-scanning confocal microscopy. Confocal images showed that PSMB10 was expressed in the cytoplasm with a perinuclear accumulation, with NS3 in the same cytoplasmic distribution showing exact co-localization with PSMB10 (Fig. 4). These results along with a previous yeast two-hybrid screen and the co-immunoprecipitation and the GST pulldown assay indicated that CSFV NS3 interacts with PSMB10 in vitro and in vivo.

2.4. CSFV infection inhibits PSMB10 expression

To investigate the effects of CSFV infection on PSMB10 expression, 3D4/2 cells were infected with CSFV strain Shimen at a MOI of 0.1. At

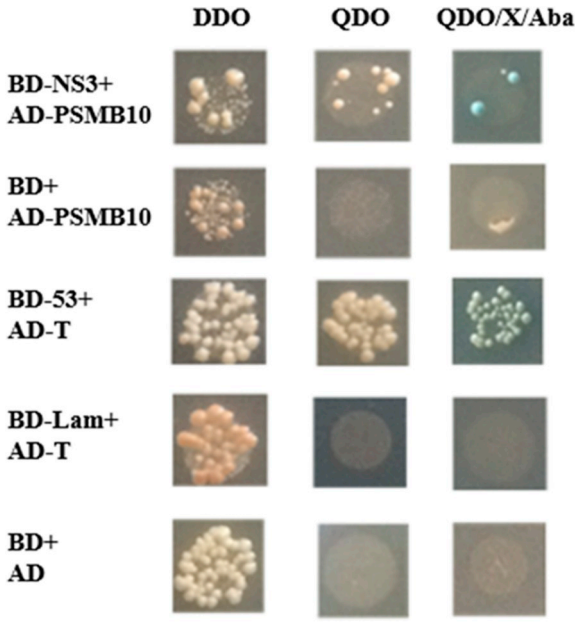

Fig. 1. Identification of the interaction between PSMB10 and CSFV NS3 by Y2H assay. The Y2HGold yeast strain was cotransformed with pGBKT7-NS3(BD-NS3)/pGADT7-PSMB10 (AD-PSMB10), pGBKT7-p53 (BD-53)/pGADT7-T (AD-T) (positive control), or pGBKT7-Lamin (BD-Lamin)/AD-T (negative control).

24 hpi and 48 hpi, PSMB10 mRNA expression was analyzed by RT-qPCR, and PSMB10 protein expression was analyzed by Western blot using anti-PSMB10 antibody. The analyses revealed that PSMB10 mRNA and protein expression in CSFV-infected 3D4/2 cells was decreased at 24 hpi and 48 hpi compared with the control (Fig. 5), which demonstrated that CSFV infection inhibits PSMB10 expression.

2.5. Overexpression of PSMB10 inhibits CSFV replication

Previous studies have shown that NS3 plays an important role in the transcription and translation of CSFV. As we found that CSFV infection inhibits PSMB10 expression, we tested the functional relevance of PSMB10 in CSFV propagation. p3 × Flag-PSMB10 was transfected into 3D4/2 cells followed by CSFV infection at 24 hpt. At 24 hpi and 48 hpi, CSFV genome copies were quantified by qRT-PCR and CSFV titers by IFA. The results showed that after transfection with p3 × Flag-PSMB10, CSFV genome copies and titers were significantly decreased compared with the controls (Fig. 6). These results indicated that overexpression of PSMB10 inhibited CSFV proliferation.

2.6. shRNA-mediated PSMB10 knockdown

To detect the efficacy of the three shRNAs targeting PSMB10, the plasmids harboring the PSMB10 shRNAs and shRNA-NC control were transfected into 3D4/2 cells, respectively. At 48 hpt, transfection efficiency was evaluated using fluorescence microscopy and by RT-qPCR

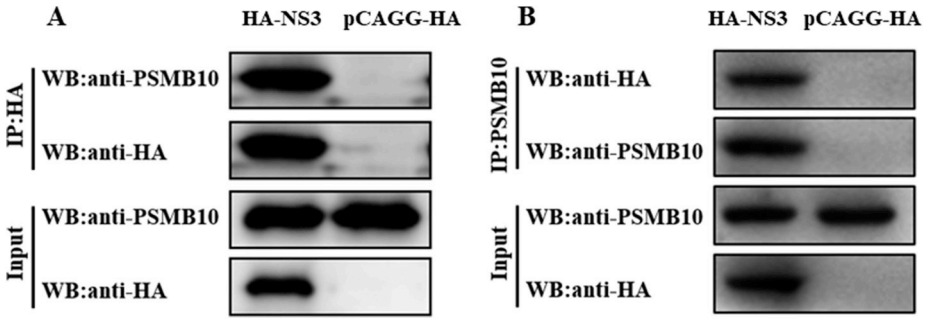

Fig. 2. Identification of the interaction between PSMB10 and CSFV NS3 by co-IP assay. Coimmunoprecipitation assay demonstrates that endogenous PSMB10 binds HA-NS3 in transfected 3D4/2 cells. 3D4/2 cells were transfected HA-NS3 plasmid for 48 h and harvested. Cell lysates were immunoprecipitated with antibody against HA(A) or antibody against PSMB10(B) followed by Western blot analysis.

and western blotting for PSMB10. Green fluorescence was observed in all transfected cells, indicating that plasmid transfection was successful (Fig. 7A). PSMB10 mRNA and protein expression in 3D4/2 cells transfected with shRNA-PSMB10-3 plasmid were decreased significantly compared with the levels in cells transfected with shRNA-NC (Fig. 7B–D). The interference effect of shRNA-PSMB10-3 was the best among the three shRNAs, and thus, this plasmid was selected for further functional experiments.

2.7. Knockdown of PSMB10 enhances CSFV replication

By overexpressing PSMB10 protein, we preliminarily determined that PSMB10 inhibits CSFV proliferation. To confirm the effect of PSMB10 on CSFV proliferation, shRNA-PSMB10 plasmid and shRNA-NC negative control plasmid were transfected into 3D4/2 cells respectively, and at 24 hpt, the cells were infected with CSFV Shimen at a MOI of 0.1. The cell culture medium was collected at 24 hpi and 48 hpi, and CSFV genome copies were detected by RT-qPCR and virus titers by IFA. The results showed that CSFV genome copies and titers in 3D4/2 cells transfected with shRNA-PSMB10 were significantly increased compared with those in 3D4/2 cells transfected with shRNA-NC (Fig. 8). These results indicated that knockdown of PSMB10 enhances CSFV replication.

2.8. PSMB10 degrades NS3 via the ubiquitin-proteasome system

Since PSMB10 inhibited CSFV replication, and PSMB10 interacted with CSFV NS3, we further investigated whether PSMB10 inhibits CSFV proliferation through NS3 protein degradation. We cotransfected 0, 0.5, 1, or 2 μg p3 × Flag-PSMB10 plasmid with 2 μg HA-NS3 plasmid into 3D4/2 cells. The results showed that with increasing amount of p3 × Flag-PSMB10 plasmid, the expression of CSFV NS3 protein gradually decreased (Fig. 9A and B), indicating that PSMB10 inhibited the expression of CSFV NS3 protein.

PSMB10 is a subunit of the immunoproteasome and participates in the ubiquitin-proteasome system. To determine whether PSMB10 inhibits NS3 expression through the ubiquitin-proteasome system, an IP assay was conducted to detect the ubiquitination of NS3 in 3D4/2 cells. As shown in Fig. 9C, ubiquitination of NS3 was detected in cells treated with MG132, and the rise in NS3 was associated with its ubiquitination (Fig. 9C). Thus, PSMB10 inhibited the expression of CSFV NS3 protein through the ubiquitin-proteasome system in 3D4/2 cells.

2.9. Effects of PSMB10 and CSFV on MHC-I antigen processing-related transporter proteins

Endogenous antigens, including viral proteins, are degraded by the ubiquitin-proteasome system, thus generating polypeptides for MHC-I antigen presentation (Glickman and Ciechanover, 2002; Lecker et al., 2006; Pickart, 2004). We found that PSMB10 inhibits the expression of CSFV NS3 protein via the ubiquitin-proteasome system and inhibits the proliferation of CSFV.

To further explore the mechanism of how PSMB10 inhibits CSFV proliferation, we evaluated the effects of PSMB10 and CSFV on MHC-I antigen processing-related transporter proteins. The relative mRNA expression of TAP1, TAP2, and Tapasin was decreased in 3D4/2 cells

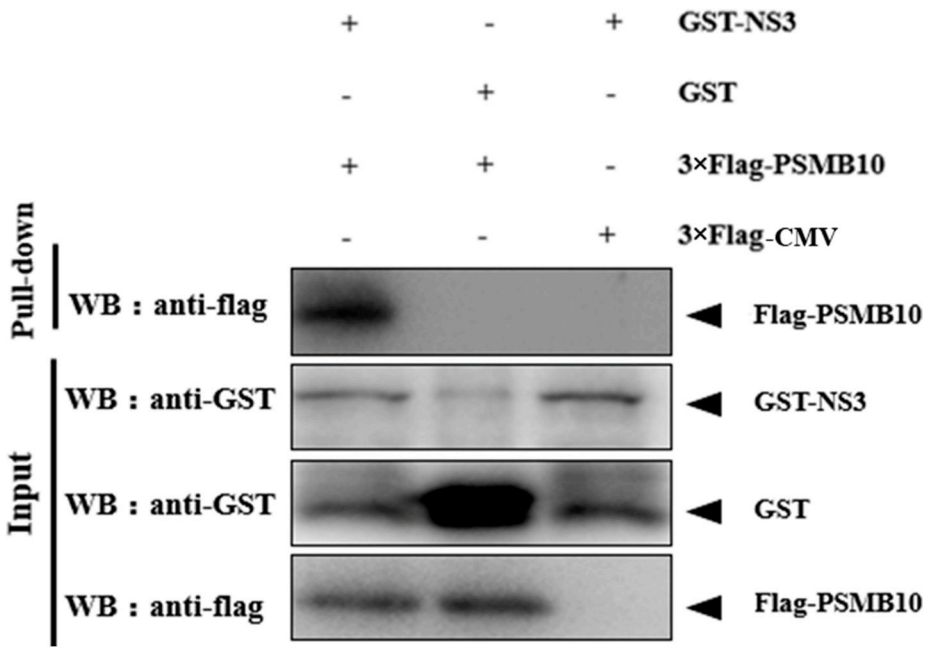

Fig. 3. Identification of the interaction between PSMB10 and CSFV NS3 by GST-pulldown assay. Glutathione beads conjugated to GST or the GST-NS3 protein fusion protein were incubated with recombinant Flag-PSMB10 protein. After washing, proteins were eluted from the beads and SDS-PAGE was performed. The expression of PSMB10 was detected by immunoblotting with anti-Flag mAb. GST and GST-NS3 protein expression was confirmed by immunoblotting.

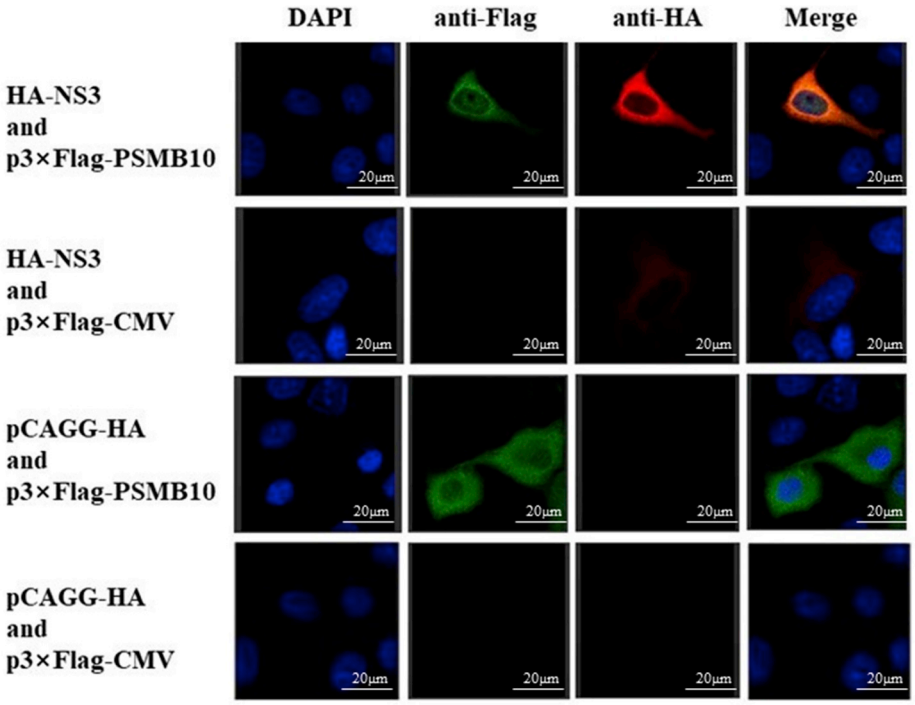

Fig. 4. Identification of the colocalization of PSMB10 and NS3 in transiently transfected 3D4/2 cells by confocal microscopy. 3D4/2 cells co-expressing HA-NS3 and p3 × Flag-PSMB10 were analyzed by laser-scanning confocal microscopy. Results show that PSMB10 was expressed in the cytoplasm with a perinuclear accumulation, with NS3 in the same cytoplasmic distribution showing exact co-localization with PSMB10.

infected with CSFV compared with mock-infected cells (Fig. 10A). In addition, relative mRNA expression of TAP1, TAP2, and Tapasin was increased in 3D4/2 cells transfected with p3 × Flag-PSMB10, but decreased in 3D4/2 cells transfected with shRNA-PSMB10 (Fig. 10B and C). These findings indicated that CSFV infection suppresses the mRNA expression of the MHC-I antigen processing-related transporter proteins TAP1, TAP2, and Tapasin, while PSMB10 has the opposite effect.

2.10. PSMB10 reduces the inhibitory effect of CSFV on MHC-I antigen presentation-related proteins

3D4/2 cells were transfected with 3 × Flag-PSMB10 and 3 × Flag-CMV plasmids, respectively, and then infected with CSFV at 24 hpt. The protein expression of PSMB10 and TAP1, TAP2, Tapasin was detected by western blotting at 48 hpi. CSFV inhibited the expression of PSMB10 and TAPs, while overexpression of PSMB10 in 3D4/2 cells reduced the inhibitory effect of CSFV on antigen presentation-related protein expression, and even induced TAP1 and TAP2 protein expression to levels

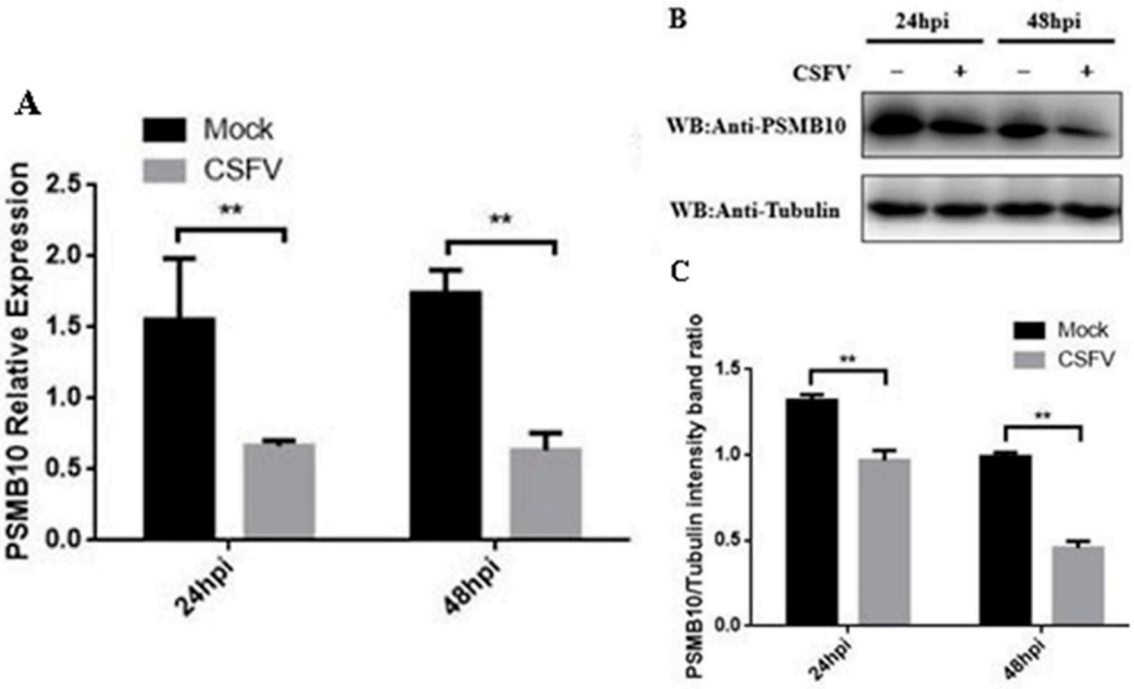

Fig. 5. CSFV infection inhibits PSMB10 expression. (A) PSMB10 mRNA expression was analyzed by RT-qPCR; (B) Western blot showing PSMB10 protein expression; (C) Quantification of PSMB10 protein expression (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

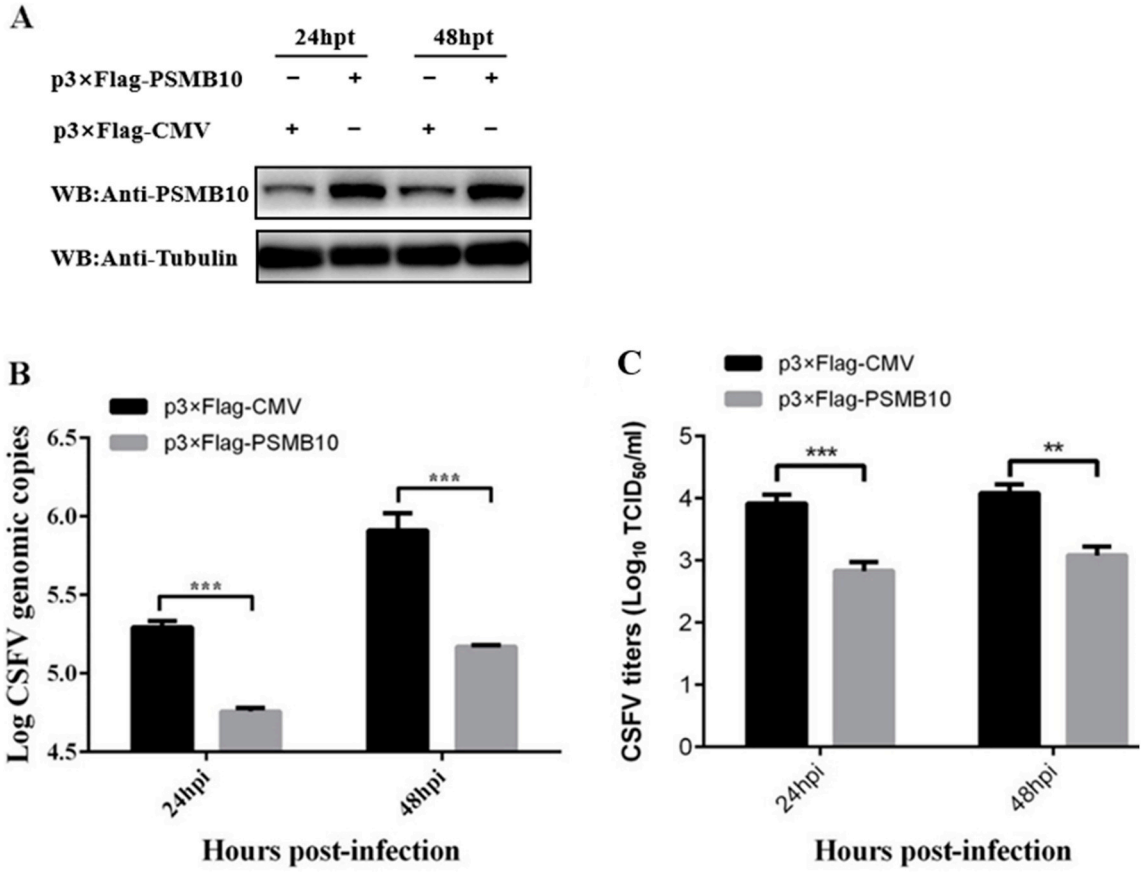

Fig. 6. Overexpression of PSMB10 suppresses CSFV growth. (A) PSMB10 expression in 3D4/2 cells detected by Western blot after transfected with p3 × Flag-PSMB10; (B) Genome copies of CSFV as analyzed by qRT-PCR. (C) Virus titers of CSFV as analyzed by IFA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

higher than those in normal cells (Fig. 11).

3. Discussion

The interactions between host cell proteins and viral proteins are the basis of virus infection. However, how protein interactions affect the infection process of CSFV and the mechanism underlying the antiviral response are poorly documented. Zhang has reported that heat shock protein 70 is associated with CSFV NS5A protein and enhances viral RNA replication (Zhang et al., 2015), and Wang found that mitogen-activated protein kinase kinase 2 is a novel E2-interacting protein, which promotes the growth of CSFV via attenuation of the JAK-STAT signaling pathway (Wang et al., 2016). In this study, the interaction between NS3 and PSMB10 protein was confirmed and we explored how PSMB10 influences CSFV proliferation at the molecular level. Our findings provide a scientific basis for the prevention and control of CSF.

NS3 protein plays an important role in the transcription and translation of CSFV. However, few studies have focused on the interaction between NS3 protein and host cell proteins. It was reported that TNF receptor-associated factor interacts with NS3 protein to inhibit CSFV replication (Lv et al., 2017). Y2H has been used to screen host cell proteins interacting with CSFV NS3 proteins in some studies (Lv et al., 2017); however, our results were not consistent the findings in these studies, may be due to differences in the libraries used in the screening; different types and expression levels of the proteins contained in the libraries can lead to different results.

PSMB10 was one of the eight host cell proteins we identified as potential interactors with NS3 by Y2H screening, and was selected for further exploration because it plays an important role in various diseases (Li et al., 2018). As its most important functions, PSMB10 is an immunoproteasome subunit and participates in the ubiquitin-proteasome system and MHC-I antigen presentation process, which execute viral protein degradation and antigen presentation, respectively, to eventually produce an antiviral effect. Therefore, this study mainly focused on the role of the interaction between PSMB10 and NS3 proteins in the proliferation of CSFV and the underlying mechanism. In this light, we found that PSMB10 inhibits CSFV proliferation by stimulating NS3 protein degradation via the ubiquitin-proteasome pathway and promotes the expression of MHC-I antigen presentation-related transporter proteins.

Antigen presentation refers to the process by which cells present processed antigens to the surface of cells for T cell recognition. It is an effective mechanism to control virus infection.

Transporter associated with antigen processing (TAP, which composed of two subunits, TAP1 and TAP2) provide energy for transporting endogenous antigen peptide by degrading ATP. TAP-associated protein (Tapasin) binds to the cytoplasmic side of TAP pore to make endogenous antigen enters ER. Some viruses can continue to infect by blocking the function of TAP protein and interfering with the presentation process of MHC-I antigen, thus escaping the antiviral effect of host cells. For example, bovine herpesvirus and pseudorabies virus can encode protein UL49.5 to induce proteasome to degrade TAP protein and inhibit the function of TAP protein to escape the host antigen presentation process (Koppers-Lalic D. et al., 2005).

In this study, In order to explore the mechanism of PSMB10 inhibiting CSFV proliferation during CSFV infection in 3D4/2 cells, we first examined the effects of CSFV and PSMB10 on MHC-I antigen presentation-related transporters TAP1, TAP2 and Tapasin, respectively. The results showed that the expression of TAP1, TAP2 and Tapasin in 3D4/2 cells infected with CSFV was inhibited, while the overexpression of PSMB10 significantly reduced the inhibition, and

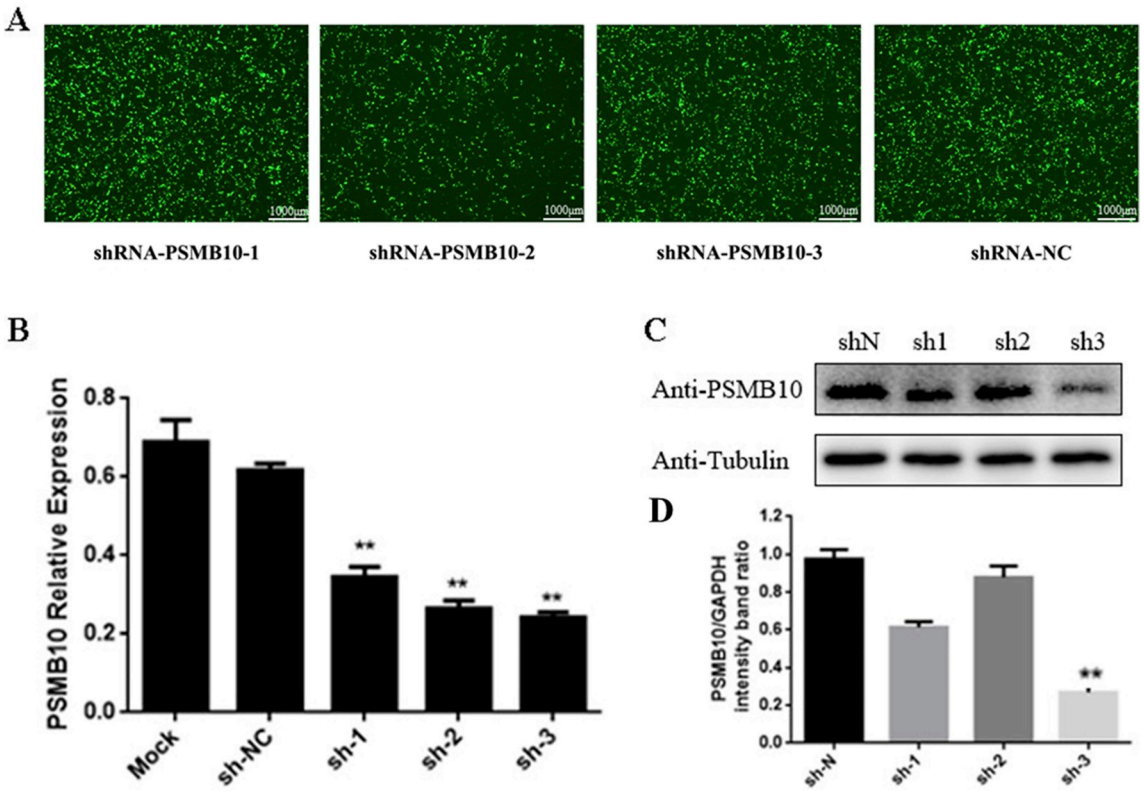

Fig. 7. Screening of PSMB10 shRNA plasmids. (A) The transfected cells glowed green, transfection effects as observed by fluorescence microscopy. (B) Relative mRNA expression of PSMB10 as evaluated by qRT-PCR. (C) PSMB10 protein expression as detected by western blotting. D. Quantification of PSMB10 protein expression (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

even increased the expression of TAP1 and TAP2 protein, which may restoring the function of MHC-I antigen presentation pathway. so that the process of MHC-I endogenous antigen presentation can proceed normally, presenting CSFV antigen peptide, and finally being recognized by $CD8^+$ T cells, differentiating into cytotoxic T cells, preventing the replication and proliferation of CSFV.

However, the inhibitory mechanism of CSFV on PSMB10 was not determined. In addition, the mechanism underlying the inhibitory effect CSFV on TAP1, TAP2, and Tapasin expression requires further study. These issues require clarification as they might have significance to the pathogenesis of CSFV. We expect our results to aid in the elucidation of the pathogenic mechanism of CSFV and the antiviral response of host cells, and to provide a theoretical basis for the prevention and control of CSF.

In summary, our study revealed the interaction between the host cell protein PSMB10 and CSFV NS3 and we further explored how PSMB10 inhibits CSFV proliferation. As a subunit of the immunoproteasome, PSMB10 degrades NS3 protein via the ubiquitin-proteasome pathway, and promotes the expression of MHC-I antigen presentation-related transporter proteins.

4. Material and methods

4.1. Cells, virus, and antibodies

3D4/2 porcine cells were cultured in RPMI 1640 medium (Gibco, UK) with 10% fetal bovine serum (FBS) (Gibco, UK) in 37 °C constant temperature incubator. Porcine kidney epithelial cells (PK-15) cells and human embryonic kidney (HEK293T) cells were grown in Dulbecco's minimal essential medium (Gibco) with 10% FBS in 37 °C constant temperature incubator. The virulent CSFV strain Shimen used in this study was stored in our laboratory and was propagated in PK-15 cells. The method used for determining virus titer was previously described (Pei et al., 2014). The following primary antibodies were used in the study: rabbit polyclonal anti-PSMB10 (ab183506; Abcam Biotechnology, USA), mouse monoclonal anti-tubulin (AT819, Beyotime, Shanghai, China), rabbit polyclonal anti-Tap1 (bs-2789R, Bioss Antibodies, Beijing, China), rabbit polyclonal anti-Tap2 (D162339, Sangon Biotech, Shanghai, China), rabbit polyclonal anti-Tapasin (AP18702a, Abgent, Suzhou, China).

4.2. Construction of expression vectors

To construct plasmids for recombinant protein expression, including pGBKT7-NS3 (BD-NS3), pCAGG-HA-NS3 (HA-NS3), pET-N-GST-NS3 (GST-NS3), and p3 × Flag-PSMB10, PSMB10 and CSFV NS3 were amplified by PCR and cloned into the respective vectors. The primers used for amplifying PSMB10 and NS3 are listed in Table 2.

4.3. Construction of a peripheral blood mononuclear cell (PBMC) cDNA library

Monocytes were isolated from swine periphery blood, and total RNA was extracted using Trizol reagent (R0016, Beyotime, Shanghai, China). First-strand cDNA and double-stranded (ds) cDNA were prepared using SMART technology. After purification over CHROMA SPIN + TE-400 columns, the ds cDNA was transformed into competent cells of the yeast strain Y187 with linearized pGADT7-Rec vector to construct a monocyte cDNA library. The titer and capacity of the monocyte library were evaluated using Make Your Own “Mate & Plate™” Library System (630490; Clontech, USA) and PCR.

4.4. Yeast two-hybrid (Y2H) screening

After we confirmed that BD-NS3 does not have self-activating activity and is not toxic to yeast strain Y2HGold, the bait BD-NS3 was

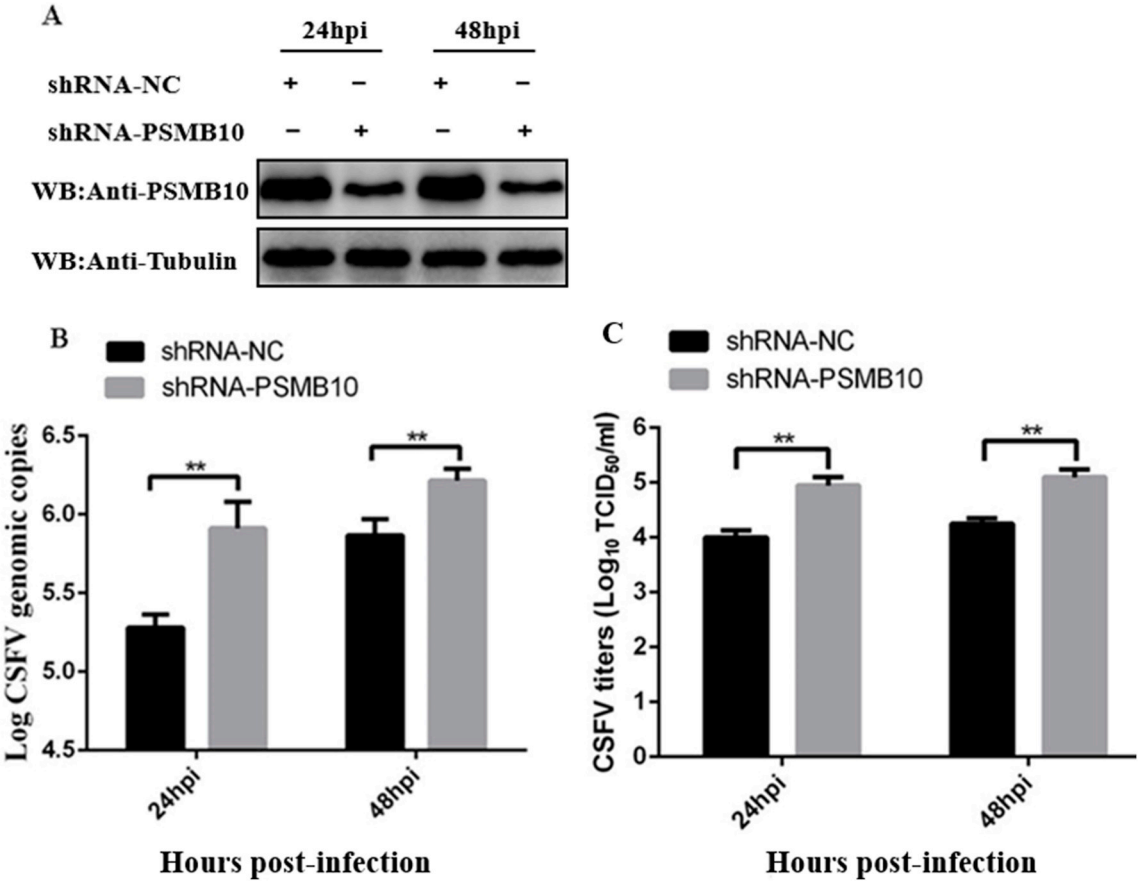

Fig. 8. Knockdown of PSMB10 enhances CSFV replication. (A) PSMB10 expression in 3D4/2 cells detected by Western blot after transfected with shRNA-PSMB10; (B) CSFV genome copies as determined by qRT-PCR. (C) CSFV titers as determined by IFA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

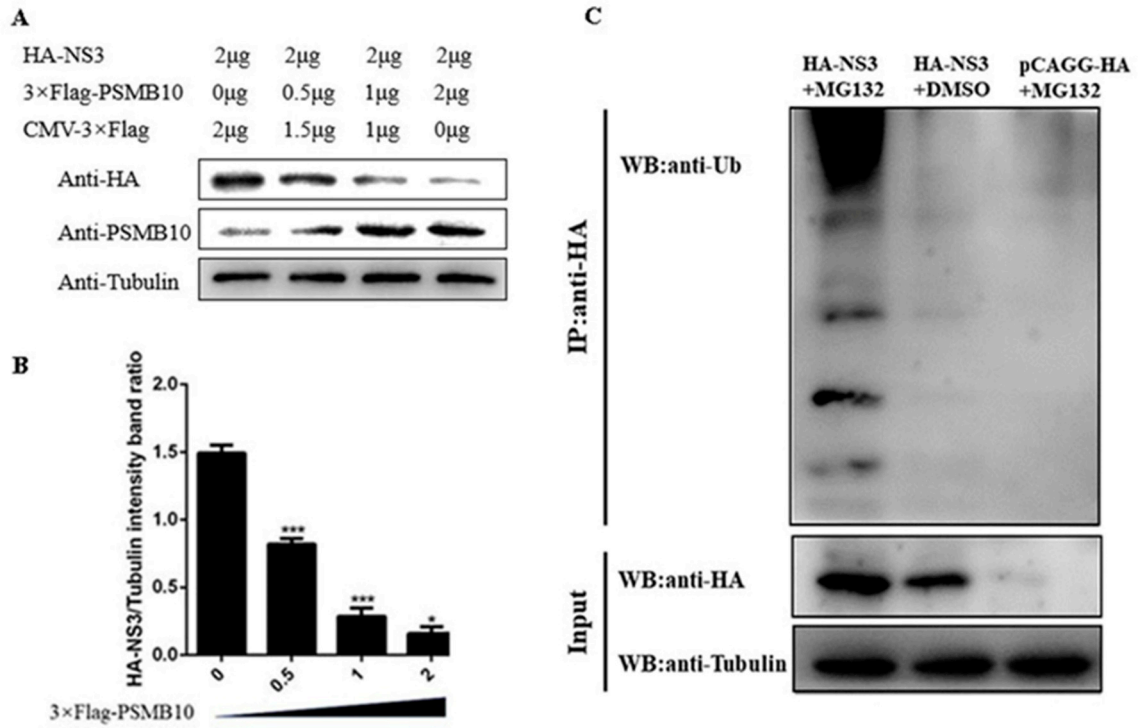

Fig. 9. PSMB10 inhibits CSFV NS3 protein expression and ubiquitination. (A) Protein expression of NS3 and PSMB10 as detected by western blotting. (B) Quantification NS3 protein expression. (C) Ubiquitination of NS3 in 3D4/2 cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

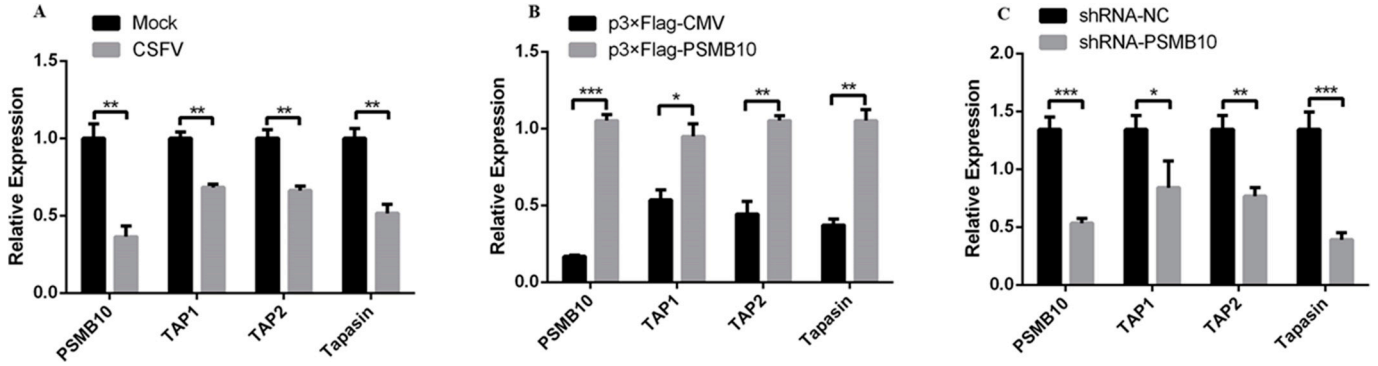

Fig. 10. mRNA expression of MHC-I antigen presentation-related transporter proteins. (A) CSFV infection suppresses the relative mRNA expression of MHC-I antigen presentation-related transporter proteins. (B) Overexpression of PSMB10 promotes the relative mRNA expression of MHC-I antigen presentation-related transporter proteins. (C) shRNA-mediated PSMB10 interferences suppress the relative mRNA expression of MHC-I antigen presentation-related transporter proteins (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

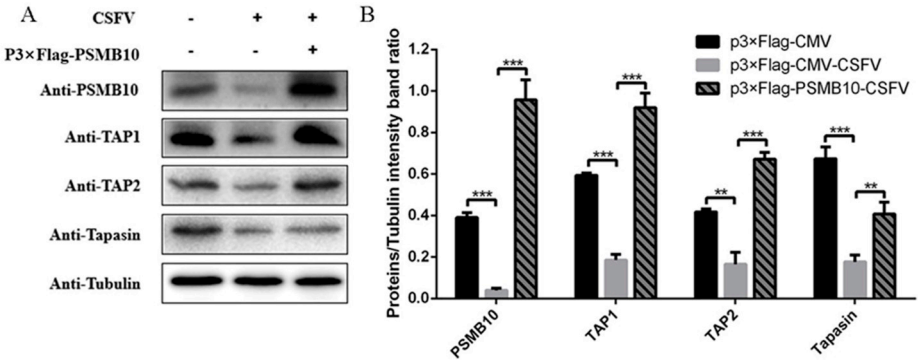

Fig. 11. PSMB10 reduces the inhibitory effect of CSFV on MHC-I antigen presentation-related proteins. (A) Protein expression of MHC-I antigen presentation-related proteins as determined by western blotting. (B) Quantification of protein expression of MHC-I antigen presentation-related proteins (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

transformed into Y2HGold and hybridized with the PBMC cDNA library using the Matchmaker Yeast Two-Hybrid System (630489; Clontech). Transformants were selected on synthetically defined (SD) medium lacking His, Leu, Trp, and Ade (SD/-4) for 3–6 days at 30 °C. Colonies that appeared were transferred to SD/-4 medium containing 5-bromo-4-chloro-3-indolyl-α-d-galactopyranoside (X-α-Gal) and aureobasidin A (Aba) (SD/-4/X-α-Gal/Aba) for 3–5 days at 30 °C. Blue colonies were inoculated in tubes containing 3 mL of SD/-4 medium and cultured at 30 °C for 1–3 days with shaking. Plasmids were extracted using a yeast plasmid kit (D3376; Omega, Guangzhou, China) and verified by sequencing. The bait and prey plasmids were cotransformed into the Y2HGold strain to verify that NS3 interacts with the cellular proteins using Yeast Transformation System 2 (630439, Clontech, Japan). Murine p53 and SV40 large T-antigen were cotransformed into Y2HGold as positive controls. Lamin C, which does not interact with SV40 large T-antigen, was used as a negative control.

4.5. Plasmid transfection and virus infection

HEK293T or 3D4/2 cells were transfected with plasmids using Lipofectamine™ 3000 Transfection Reagent (L3000015; Thermo Fisher, USA), according to the manufacturer's instructions. PK-15 or 3D4/2 cells were infected with CSFV at a multiplicity of infection (MOI) of 1.0 at 24 h post transfection. Cell culture supernatants were harvested

Table 2
Primers used in this study.

Primers	Sequences (5′-3′)
3 × Flag-PSMB10-FP	CGGAATTCCATGCAGAAGATAGCGCTAGAG
3 × Flag-PSMB10-RP	GAAGATCTTCACTCCACATCCATGGCCTG
BD-NS3-FP	CATGGAGGCCGAATTCGGGCCTGCCGTTTGCAAGAAGGTT
BD-NS3-RP	GCAGGTCGACGGATCCTAGACCAACTACTTGTTTTAGTGCTCTGCCAGCC
HA-NS3-FP	GAAGATCTTATGGGGCCTGCCGTTTGCAAG
HA-NS3-RP	GGGGTACCTAGACCAACTACTTGTTTTAG
GST-NS3-FP	AACTGCAGATGGGGCCTGCCGTTTGC
GST-NS3-RP	CCCTCGAGTGATAGACCAACTACTTGTTTTAGTGCTC
qPCR-β-actin-FP	GCT CGT TGT AGA AGG TGT GGT G
qPCR-β-actin-RP	CCTGACCCTCAAGTACCCCA
qPCR-PSMB10-FP	GATCCTAAGTGACCTGGGCTCTG
qPCR-PSMB10-RP	ATCTTTCTGTGGGCTTTGTGGGA
qPCR-TAP1-FP	GATGTTGCTGAAGGTGGGAAT CC
qPCR-TAP1-RP	TTCGGTGAACTGGATCTGGTAGAG
qPCR-TAP2-FP	TCGGACACCAAACTGATGAGTCTC
qPCR-TAP2-RP	CAGAGAGAGGAGGGTGAGTCGA
qPCR-Tapasin-FP	TCATCACCCACACACCCGCC
qPCR-Tapasin-RP	TCGCCACTCCAGCCCAAAGG

at 24 h post infection (hpi) and 48 hpi, and viral RNA was isolated detect CSFV genomic copies by quantitative (q)PCR. The transfected cells were harvested at 24 hpi and 48 hpi and analyzed by western blotting.

4.6. Co-immunoprecipitation (co-IP)

3D4/2 cells were transfected with HA-NS3 and pCAGG-HA as a control group, respectively. Then the transfected cells infected with CSFV at a multiplicity of infection (MOI) of 1.0 at 24 h post transfection. The cells were harvested at 24 h post infection (hpi), washed three times with cold PBS (pH 7.4), and lysed with Western and IP lysis buffer (P0013; Beyotime, Shanghai, China) containing 1 mM Phenyl methane sulfonyl fluoride (PMSF) (ST506; Beyotime) at 4 °C for 30 min. Clarified extracts were incubated with an anti-Flag monoclonal antibody (mAb) for 12 h, incubated with 30 μL Protein A/G beads for 1 h, washed with IP wash buffer five times, boiled in loading buffer, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with anti-HA and anti-PSMB10 mAbs.

4.7. Glutathione S-transferase (GST)-pulldown assays

The NS3 gene was subcloned into the pET-N-GST vector to create GST-NS3. GST or GST-NS3 protein expressed in *Escherichia coli* BL21 (DE3) cells were purified with GST-tag Purification Resin (P2250; Beyotime), according to the manufacturer's instructions. Briefly, the expression of GST or GST-NS3 protein was induced by addition of 1 mM isopropylthiogalactoside (ST098; Beyotime). The bacterial cells were harvested and resuspended in cold PBS containing 1 mM PMSF, followed by mild sonication. The GST or GST-NS3 protein was incubated with the GST-tag Purification Resin at 4 °C for 4 h. The resin was then washed five times with cold PBS and incubated with 200 μL of the lysates of HEK293T cells transfected with p3 × Flag-PSMB10 for 2 h at 4 °C. The resin was again washed five times with cold PBS, followed by protein detection by SDS-PAGE and immunoblotting.

4.8. Confocal microscopy

HA-NS3 and p3 × Flag-PSMB10 were transiently cotransfected into 3D4/2 cells and cultured in a confocal laser microscopy-dedicated culture vessel. While setting three control groups: 3D4/2 cells respectively cotransfected with HA-NS3 and empty vector p3 × Flag-CMV, p3 × Flag-PSMB10 and empty vector pCAGG-HA, or empty vector pCAGG-HA and p3 × Flag-CMV. The transfected cells were fixed with 1 mL absolute ethanol and permeabilized with 1 mL of 0.1% Triton X-100. The cells were then incubated with rabbit anti-HA Mab and mouse anti-Flag MAb at 37 °C for 1 h, followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody and tetramethyl rhodamine isocyanate-conjugated goat anti-rabbit IgG antibody. Subsequently, the cells were incubated with 4,6-diamidino-2-phenylindole at room temperature for 10 min. Fluorescence was observed with a confocal laser-scanning microscope (TCS SP5, Leica, Germany).

4.9. PSMB10 RNA interference

Three short hairpin (sh)RNAs against the porcine PSMB10 gene were designed and synthesized by Sangon (Shanghai, China). 3D4/2 cells were grown in 6-well plates and transfected with PSMB10-specific shRNAs or shRNA-NC using Lipofectamine™ 3000 Transfection Reagent. The cells were harvested at 48 hpt and analyzed by quantitative reverse transcription (RT-q)PCR and western blotting.

4.10. RNA isolation and cDNA preparation

Viral RNA was isolated from CSFV-infected cell culture supernatant using the Viral RNA Kit, according to the manufacturer's protocol (R6874-02; Omega Bio-tek, USA). Reverse transcription was performed using the random primer 6 N (3801; Takara Bio Inc. Dalian, China) and the PrimeScript RT Master Mix (RR036A; Takara Bio Inc.), according to the manufacturer's protocol.

4.11. Detection of NS3 ubiquitination

3D4/2 cells were grown in 6-well plates and transfected with plasmid HA-NS3, 10 μM MG132 (Proteasome inhibitors) was added at 24 h after transfection. Two control groups was set up at the same time: 3D4/2 cells transfected with plasmid HA-NS3 and add 10 μM DMSO; 3D4/2 cells transfected with pCAGG-HA empty carriers plasmid and add 10 μM MG132. After 12 h of continuous culture, the cells were lysed and the total protein was extracted, and immunoprecipitation test was carried out using anti-Ub antibody, anti-HA antibody and anti-Tubulin antibody.

4.12. RT-qPCR

RT-qPCR primers were designed for β-actin, PSMB10, TAP1, TAP2, and Tapasin based on published sequences; the primers are listed in Table 2. All primers were selected on the basis of specificity as determined by melting curve analysis. The CSFV-specific primers and reaction conditions have been described previously (Liu et al., 2015). qPCR was performed using Fast qPCR Mix (SYBR Green I) (TSE202; Tsingke Bio Inc., Beijing, China) on an iQ5 iCycler detection system (Bio-Rad). Known positive and negative controls were used throughout, and all assays were performed in triplicate.

4.13. Western blotting

Total cellular proteins were extracted with cell lysis buffer containing 1 mM PMSF, and the concentrations were determined with a BCA protein assay kit (23227; Thermo Fisher Scientific). Protein samples were boiled in 5 × SDS-PAGE loading buffer for 5 min. Equal amounts of protein were separated by 12% SDS-PAGE and then transferred to polyvinylidene membranes (IPVH00010; Millipore, Germany). Membranes were blocked with 5% skim milk dissolved in PBS plus Tween-20) at 37 °C for 1 h. Next, the membranes were incubated with primary antibodies at 4 °C overnight and the corresponding secondary antibodies conjugated to HRP at 37 °C for 1 h, at appropriate dilutions. The ECL Plus kit (P0018, Beyotime) and a chemiluminescence imaging system (Fine-do X6; Tanon) were used for visualizing the protein bands. Image-Pro plus 6.0 software (Media Cybernetics) was used to quantify protein band intensities according to the user's guide.

4.14. Statistical analysis

Statistical analysis using an unpaired Student's t-test was performed using GraphPad Prism 6 software (GraphPad software, USA).

Ethics statement

The authors declare that the animal breeding, care and all experiments were performed in adherence to the guidelines of the Laboratory Animal Center of South China Agricultural University and approved by the Animal Ethics Committee.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.05.017.

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