The Superimposed Deubiquitination Effect of OTULIN and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Nsp11 Promotes Multiplication of PRRSV

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ABSTRACT  Linear ubiquitination plays an important role in the regulation of the immune response by regulating nuclear factor κB (NF-κB). The linear ubiquitination-specific deubiquitinase ovarian tumor domain deubiquitinase with linear linkage specificity (OTULIN) can control the immune signaling transduction pathway by restricting the Met1-linked ubiquitination process. In our study, the porcine OTLLIN gene was cloned and deubiquitin functions were detected in a porcine reproductive and respiratory syndrome virus (PRRSV)-infected-cell model. PRRSV infection promotes the expression of the OTULIN gene; in turn, overexpression of OTULIN contributes to PRRSV proliferation. There is negative regulation of innate immunity with OTULIN during viral infection. The cooperative effects of swine OTULIN and PRRSV Nsp11 potentiate the ability to reduce levels of cellular protein ubiquitin associated with innate immunity. Importantly, PRRSV Nsp11 recruits OTULIN through a nonenzymatic combination to enhance its ability to remove linear ubiquitination targeting NEMO, resulting in a superimposed effect that inhibits the production of type I interferons (IFNs). Our report presents a new model of virus utilization of the ubiquitin-protease system in vivo from the perspective of the viral proteins that interact with cell deubiquitination enzymes, providing new ideas for prevention and control of PRRSV.

IMPORTANCE  Deubiquitination effects of swine OTULIN were identified. The interaction between porcine OTULIN and PRRSV Nsp11 is dependent on the OTU domain. PRRSV Nsp11 recruits OTULIN through a nonenzymatic combination to promote removal of linear ubiquitination targeting NEMO, resulting in a superimposed effect that inhibits the production of type I IFNs.

KEYWORDS  porcine OTULIN, porcine reproductive and respiratory syndrome virus (PRRSV), Nsp11, superimposed deubiquitination, linear ubiquitination

Porcine reproductive and respiratory syndrome virus (PRRSV) is a critical pathogen in pigs. It is a variety of capsule single-stranded positive-chain virus of arteritis virus (1). Infections by PRRSV often result in delayed, low-level induction of antiviral cytokines, thereby destroying the early endogenous immune response, while PRRSV can also inhibit the normal function of B cells and T cells (2).

When virus infects the natural host cells, the ubiquitin (Ub)-proteasome system (UPS) is an important target for the virus to help itself better achieve its life cycle (3–5). The UPS is involved in the replication of a broad range of viruses (6). Hindering the activity of the cellular ubiquitin proteasome system, the physiological processes of many viruses are blocked, including invasion by the virus (7), nucleic acid synthesis (8–11), and the release of virus particles (12). On the other hand, virus infection also affects the host’s ubiquitin proteasome system (9).

Among various types of ubiquitination modifications, linear ubiquitylation is a
newly identified posttranslational modification (13). Linear ubiquitinylation plays an important role in the regulation of the immune system (14). The linear ubiquitin chain assembly complex (LUBAC) is composed of two RING E3 ubiquitin ligases, HOIL-1L and HOIP (15). The complex displays the activity of ubiquitin ligases, thereby identifying the substrate for extension of poly-linear ubiquitin chains (16).

Some PRRSV encoding proteins possess ubiquitin- or deubiquitin-related functions, which enable them to block the production of beta interferon (IFN-β) (17). For example, Du et al. (18) reported that the highly pathogenic porcine reproductive and respiratory syndrome virus Nsp1α replicating protein can target swine leukocyte antigen class I molecules for proteasomal degradation. Jing et al. (19) found that LUBAC-induced NF-κB and proinflammatory cytokine expression can be inhibited in the early phase of PRRSV infection. Mechanistically, Nsp1α binds to HOIP and HOIL-1L and impairs the interaction between HOIP and SHARPIN, thus reducing the LUBAC-dependent linear ubiquitination of NEMO. Sun et al. (20) showed that PRRSV Nsp2 contained a cysteine protease domain belonging to the ovarian tumor (OTU) protease family at its N terminus and demonstrated that the Nsp2 OTU domain antagonizes the induction of type I interferon by interfering with the NF-κB signaling pathway. Further analysis showed that the Nsp2 OTU domain had ubiquitin debinding activity. Zhang et al. found that PRRSV NSP4 cleaved the host protein NEMO and inhibited the production of IFN-β in pulmonary alveolar macrophages (PAMs) (21). PRRSV infection significantly reduced cellular protein ubiquitination, whereas Nsp11 was previously shown to be a key nonstructural protein that regulates ubiquitination levels during PRRSV infection (22). Nsp11 was related to the deubiquitination that regulates host type I IFN signaling.

Ubiquitination and deubiquitination are now considered to be important processes in the regulation of intracellular signals. Ovarian tumor domain deubiquitinase with linear linkage specificity (OTULIN) is the only known linear ubiquitination-specific deubiquitinase. It can restrict Met1-linked ubiquitination to control innate immune signaling (23). OTULIN depletion leads to enhanced formation of linear Ub chains, resulting in local recruitment of NEMO and activation of IκB kinase alpha (IKKα) or IKKβ and, ultimately, NF-κB (24). Further analysis showed that binding of OTULIN to the PUB domain of HOIP controls NF-κB signaling (25). The OTULIN PIM domain can be combined with the HOIP-conserved PUB domain of the LUBAC complex. Interestingly, unphosphorylated OTULIN is an important component of the LUBAC complex, but phosphorylated OTULIN cannot interact with LUBAC (26). Additionally, a previous study showed that OTULIN in the human body and in mice can inhibit tumor necrosis factor (TNF)-related systemic inflammation, acting as a key molecule in the regulation of immune homeostasis, inflammation, and autoimmune responses (27).

In this study, the relationship between OTULIN and PRRSV replication was investigated. The cooperative action of swine OTULIN and PRRSV Nsp11 contributed to the reduction of the level of the cellular protein ubiquitin associated with innate immunity. Our report presents a new model of virus utilization of a ubiquitin-protease system in vivo from the perspective of the interaction of viral proteins with cell deubiquitination enzymes, providing new ideas for the prevention and control of PRRSV.

RESULTS

Upregulation of OTULIN accumulation of 3D4/21 by PRRSV. PRRSV infection can lead to upregulation or downregulation of many genes in cells (28, 29). A transcriptome sequencing analysis derived from 3D4/21 cell results was carried out to look for genes that showed significant changes in transcription after PRRSV infection, and OTULIN was found to be significantly upregulated after viral infection (Fig. 1a). As it is a novel deubiquitinating enzyme specifically targeting the linear ubiquitin chain among these genes and specifically participating in the innate immune signaling pathway of the linear ubiquitination, analyses of the functions of porcine OTULIN were performed in this study. To investigate the levels of OTULIN accumulation during PRRSV infection, we infected 3D4/21 cells with PRRSV at a multiplicity of infection (MOI) of 0.5. Total RNA was extracted from the cells at different time points postinfection and analyzed for the
abundance of OTULIN by quantitative reverse transcription-PCR (qRT-PCR). Compared to the control group results, PRRSV infection resulted in a significant increase in the mRNA levels of OTULIN, especially at the late stage of virus infection (Fig. 1b). Interestingly, the trend of expression of OTULIN after Sendai virus (SeV) infection was similar to that seen after PRRSV infection (Fig. 1c). Furthermore, cell lysates were collected at 24 h postinfection and subjected to Western blot analysis with antibody (Ab) to OTULIN. There was an increase in cellular levels of OTULIN after PRRSV infection in comparison to the results seen with mock infection (Fig. 1d). In line with that finding, the results of flow cytometry analysis further confirmed that the PRRSV infection could cause upregulation of endogenous OTULIN expression in cells (Fig. 1e). Together, the results described above showed that PRRSV infection upregulated OTULIN expression in 3D4/21 cells.

**OTULIN contributes to PRRSV proliferation.** Previous research found that the deubiquitinase OTULIN is an essential negative regulator of inflammation and autoimmunity (27, 30). To figure out the effect of OTULIN on PRRSV proliferation, Flag-OTULIN expression plasmids or empty vector was transfected into 3D4/21 cells. 3D4/21 cells were infected by PRRSV at 12 h posttransfection, and total RNA was extracted from the cells at 12 h or 24 h postinfection and then analyzed for the mRNA level of the PRRSV N gene by qRT-PCR. Compared to the control sample, the overexpression of OTULIN contributed to the mRNA level of the PRRSV N gene while the result seen at 12 h postinfection was more remarkable (Fig. 2a). The results of Western blot analysis were consistent with those obtained using qRT-PCR (Fig. 2b). Meanwhile, the virus titer in cells after transfection of the OTULIN plasmid was higher than the titer seen with the
control cells (Fig. 2c). In order to further clarify the effect of OTULIN on PRRSV proliferation, we designed OTULIN small interfering RNA (siRNA). As previously speculated, proliferation of PRRSV was inhibited to some extent when the expression of intracellular OTULIN was disturbed (Fig. 2d to f). These data indicate that the changes in cellular OTULIN expression were consistent with PRRSV proliferation and that OTULIN expression can promote PRRSV proliferation.

The enhancement of OTULIN on PRRSV is related to innate immunity. IFN-β plays critical roles in the innate immune responses to viral infection (31, 32). Our previous studies showed that OTULIN is beneficial to PRRSV proliferation, maybe due to the negative regulation function of porcine OTULIN in innate immunity. To test that possibility, we examined the mRNA levels of transcription factors and cytokines related to innate immunity. Overexpression of OTULIN decreased the levels of PRRSV-induced TNF-α, NF-κB, and interferon regulatory transcription factor 3 (IRF3) activation in 3D4/21 cells, in which NF-KB and IRF3 are important transcription factors for interferon production (Fig. 3a). Overexpression of OTULIN also attenuated levels of IFN-β induced by PRRSV (Fig. 3c). While expression of OTULIN was disturbed by the introduction of siRNA, the mRNA levels of NF-κB and IFN-β were upregulated (Fig. 3b and d). To directly investigate the effect of OTULIN on antiviral responses, vesicular stomatitis virus (VSV) was used. 3D4/21 cells transfected with OTULIN expression plasmid showed more green fluorescence under a fluorescence microscope than empty vector (Fig. 3e). Correspondingly, levels of VSV RNA replicates were increased in OTULIN-transfected cells (Fig. 3f), and Western blot analysis showed that expression of VSV green fluorescent protein (VSV-GFP) was also increased in the OTULIN group (Fig. 3g). Taken together, these data demonstrated that OTULIN negatively regulates IFN-β production and antiviral immune responses, resulting in the enhancement of OTULIN levels on PRRSV.

OTULIN enhances the PRRSV deubiquitination effect. Previous reports indicated that PRRSV infection led to reduced ubiquitination of cellular proteins (22). As OTULIN FIG 2 Contribution of PRRSV replication by OTULIN overexpression in 3D4/21 cells. (a and b) 3D4/21 cells were transfected with Flag-OTULIN or with empty vector or left untransfected. At 12 h posttransfection, cells were infected with PRRSV (MOI of 0.5). The infected cells were collected at 12 h (a) or 24 h (a and b) postinfection, mRNA loads of PRRSV N were tested by qRT-PCR, and PRRSV Nsp2 levels were tested by WB. (c) PRRSV-containing samples were tested by plaque assay. (d and e) 3D4/21 cells were transfected with siRNA of OTULIN or of the negative control (NC) or were left untransfected; 12 h after transfection, cells were infected with PRRSV (MOI of 0.5). The infected cells were collected at 12 h (d) or 24 h (e) postinfection, mRNA loads of PRRSV N and OTULIN were tested by qRT-PCR, and PRRSV Nsp2 levels were tested by WB. (f) PRRSV-containing samples were tested by plaque assay. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (two-way ANOVA followed by Bonferroni posttest). Data are representative of results from three independent experiments.
is the only known linear ubiquitination-specific deubiquitinase, we tested whether OTULIN could help PRRSV further decrease intracellular ubiquitination. PRRSV infection inhibited ubiquitination caused by wild-type ubiquitin (WT-Ub) in 3D4/21 cells, while overexpression of OTULIN further decreased the ubiquitination (Fig. 4a). Similarly, OTULIN and PRRSV doubly regulated endogenous ubiquitin levels (Fig. 4b). These data indicated that the deubiquitination relationship between OTULIN and PRRSV is not competitive or, at least, that they do not interfere with each other.

**OTULIN interaction with Nsp11.** We hypothesized that there is an unknown effect of the relationship between OTULIN and PRRSV Nsp1 or Nsp11, each of which is a major protein related to ubiquitination or deubiquitination (19, 22). We confirmed that Flag-tagged porcine OTULIN interacted specifically with Nsp11 but only interacted with Nsp1 at a much lower level (Fig. 5a). In agreement with the immunoprecipitation results, His-OTULIN immobilized on agarose beads efficiently bound to Nsp11-Myc in cell lysates, whereas His alone did not (Fig. 5b). In addition, PRRSV Nsp11 was able to
coprecipitate with endogenous OTULIN, which further demonstrated the interaction between OTULIN and PRRSV Nsp11 (Fig. 5c). Confocal microscopy showed that Flag-OTULIN was expressed throughout the cytoplasm and partially coexisted with Myc-Nsp11 in HeLa and 3D4/21 cell lines, while no such results were seen with the control cells (Fig. 5d and f). Importantly, we found that OTULIN and NSP11 were able to form endogenous complexes when PRRSV infected cells (Fig. 5e). Previous reports showed that the ubiquitination activity of Nsp11 was essential for the activation of its inhibition of NF-κB whereas the binding of OTULIN to the PUB domain of HOIP was found to control NF-κB signaling, and our results indicated that OTULIN and Nsp11 directly interacted with each other.

**The OTU domain of OTULIN interacts with PRRSV Nsp11.** Next, to figure out the specific mechanism of the interaction of OTULIN with Nsp11, we first mapped the domains of OTULIN and constructed the corresponding truncated body, which consisted of amino acids (aa) 1 to 79, aa 49 to 353, and aa 80 to 353 (Fig. 6a). Roughly, OTULIN consisted of a PIM domain and an OTU domain; the PIM domain is responsible for the combination with LUBAC, while the OTU domain containing the deubiquitinating enzyme active site is responsible for binding to NEMO. The results showed that Flag-OTULIN could be coprecipitated with Nsp11-Myc and that the key to the binding was the OTU domain (Fig. 6b). Given the presence of enzyme sites in the OTU domain, we next constructed several mutants to find out whether this example of binding represents enzyme-competitive binding. The results of ubiquitination showed that the deubiquitination effect of several mutants was weakened to some extent compared with the wild-type (WT) results, and Flag-OTULIN (H339Y/N341Y) mutants had the lowest deubiquitination ability (Fig. 6c). The effects of these enzyme site mutants on type I interferons also differed (Fig. 6e). Immunoprecipitation analysis showed that the mutation of the enzyme site did not affect the binding of NSP11 to OTULIN (Fig. 6d). The analysis also demonstrated that PRRSV Nsp11 could interact with the OTU domain of OTULIN but that the interaction had nothing to do with the OTULIN enzyme sites, indicating the possibility that Nsp11 is associated with OTULIN targeting NEMO to remove linear ubiquitin chains.

**PRRSV Nsp11 interaction with NEMO.** Previous studies have shown that human OTULIN can remove linear ubiquitination targeting NEMO (23). So far, the results have shown that there may be some connection between PRRSV Nsp11 and linear ubiquitination targeting NEMO. To verify that PRRSV Nsp11 is associated with OTULIN targeting...
Identification of the interaction between OTULIN and PRRSV Nsp11. (a) 293T cells were cotransfected with Flag-OTULIN and Nsp1-Myc, Nsp11-Myc, or empty vector. At 24 h after transfection, the cell lysates were precipitated with an anti-Myc monoclonal antibody (MAb) in conjunction with protein A Sepharose and were further detected by Western blotting performed with an anti-Flag MAb antibody and an anti-Myc antibody, respectively. (b) The interaction of OTULIN with PRRSV Nsp11 by pulldown is shown. Bacterial purified His-OTULIN protein interacted with Nsp11-Myc in cell lysates. (c) Detection of the interaction between endogenous OTULIN and PRRSV Nsp11 by coimmunoprecipitation. (d) HeLa or 3D4/21 cells were cotransfected with Nsp11-Myc and Flag-OTULIN. The cells were fixed at 24 h posttransfection and doubly stained with a rabbit anti-Myc MAb and a mouse anti-Flag antibody followed by (Continued on next page)
NEMO to remove linear ubiquitin chains, 293T cells were cotransfected with Nsp11-Myc and Flag-IKKα, Flag-IKKβ, or Flag-NEMO. The results showed that Nsp11 could be coprecipitated with NEMO, IKKβ, and IKKα (Fig. 7a). Furthermore, we found that PRRSV Nsp11 has a recruitment effect on endogenous OTULIN targeting NEMO (Fig. 7b), further degrading linear ubiquitination in cells (Fig. 7c). Moreover, colocalization between PRRSV Nsp11 and NEMO was observed in both HeLa cells (Fig. 7d) and 3D4/21 cells (Fig. 7e) cotransfected with Flag-NEMO and Nsp11-Myc. Additionally, Nsp11 did not affect the total abundance of OTULIN protein (Fig. 7b), suggesting that there may be other molecular mechanisms responsible for OTULIN overexpression in PRRSV infection. These data suggested that Nsp11 recruits extra OTULIN, resulting in an abnormally reduced linear ubiquitination level in targeting NEMO.

The superimposed effects of OTULIN and Nsp11 on deubiquitination. Nsp11 is one of the key proteins involved in reduction of ubiquitin levels when cells are infected by PRRSV (22). Thus, we tested the effects of OTULIN and Nsp11 on intracellular ubiquitin levels. Overexpression of Nsp11 in HEK293T cells decreased the ubiquitination caused by WT-Ub in HEK 293T cells, which was in agreement with the previous reported data. Interestingly, OTULIN further inhibited ubiquitination in a dose-dependent manner (Fig. 8a). Similarly, OTULIN and Nsp11 also had a synergistic reduction effect on endogenous ubiquitin levels in cells (Fig. 8b). OTULIN specifically targets linear ubiquitin chains to reduce ubiquitination; thus, while expression of OTULIN was disturbed, the linear ubiquitination level was upregulated (Fig. 8c). Importantly, the linear ubiquitination-specific deubiquitinase OTULIN had no effect on K48 or K63 polyubiquitination (Fig. 8d). Previous studies have shown that PRRSV Nsp11 specifically removed K48 ubiquitination but had no effect on K63 ubiquitination, and our results were consistent with that finding. Meanwhile, Nsp11 has no significant effect on linear ubiquitination levels, suggesting that Nsp11 does not directly affect linear ubiquitin chains. Those data indicated that OTULIN and Nsp11 have superimposed effects on intracellular ubiquitination. Nsp11 has a recruiting effect on the delinear ubiquitination of OTULIN. However, the effect of OTULIN on Nsp11 removal of ubiquitination at K48 was not statistically significant.

Nsp11 further reduces type I interferon production by recruiting OTULIN. In order to figure out the physiological changes caused by the cooperation of OTULIN and Nsp11, HEK 293T cells were transfected with Flag-OTULIN or Nsp11-Myc and were then infected with SeV, and the mRNA levels of NF-κB, IFN-β, and IRF3 were detected by qRT-PCR. PRRSV Nsp11 and OTULIN downregulated the expression of NF-κB, and the downregulation effect on NF-κB was more obvious in cells coexpressed with Nsp11 and OTULIN (Fig. 9a). Moreover, PRRSV Nsp11 and OTULIN could also downregulate the expression of IFN-β (Fig. 9b). Meanwhile, OTULIN inhibited IFN production together with PRRSV Nsp11, and the downregulation effect on IFN-β was more obvious when cells were coexpressed with Nsp11-Myc and Flag-OTULIN (Fig. 9c). Interestingly, when OTULIN deubiquitination was inactivated, this additive effect disappeared (Fig. 9a to c). Western blot analysis showed that expression of PRRSV Nsp11 and OTULIN blocked NF-κB passage into the nucleus and that the inhibitory effects were superimposed (Fig. 9d). These data suggested that Nsp11 could further reduce type I interferon production by recruiting OTULIN and that the level of influence depends on the deubiquitination of OTULIN.

DISCUSSION

Ubiquitination generally regulates cellular function by timely and selective addition of the ubiquitin molecule to its substrate (33). There are eight different types of
FIG 6 The OTU domain of OTULIN interacts with PRRSV Nsp11. (a) Mapping of OTULIN domains. OTULIN is consisted of a PIM domain and an OTU domain. (b) 293T cells were cotransfected with Nsp11-Myc and Flag-OTULIN (aa 1 to 79), Flag-OTULIN (aa 80 to 353), Flag-OTULIN (aa 49 to 363), or Flag-OTULIN (aa 1 to 353). At 24 h posttransfection, the cell lysates were precipitated with an anti-Myc MAb in conjunction with protein A Sepharose and were further detected by Western blotting with an anti-Flag MAb antibody and an anti-Myc antibody, respectively. (c) 293T cells were cotransfected with an HA knockout (HA-KO) (ubiquitin mutants which all lysines mutated to arginine) and Flag-OTULIN mutant plasmid, and the effects of different mutants on the linear ubiquitination were detected by WB. (d) Detection of the interaction between Nsp11-Myc and different Flag-OTULIN mutant by coimmunoprecipitation. (e) Effects of different OTULIN mutants on the type I interferon signaling pathway. HEK 293T cells were transfected with Flag-OTULIN or mutants. At 12 h posttransfection, cells were

(Continued on next page)
connection methods. The first seven types include K6, K11, K27, K29, K33, K48, and K63, which are associated with the internal lysine K and the glycine G at the C terminus of ubiquitin molecules (34–36). Most of the studies have investigated K48 and K63 polyadenylation modification, in which the polyubiquitination modification at position K48 mainly plays a role in degradation (37, 38) and the polyubiquitination modification at position K63 mainly plays a role in signal transduction DNA repair function (39, 40). In recent years, the eighth type of ubiquitin chain linkage connection method, linear ubiquitination, was found. The ubiquitin chain is linked by the amino group of ubiquitin methionine Met1 and the carboxyl group of another ubiquitin glycine.

The ubiquitin proteasome pathway is important for the degradation of intracellular proteins in eukaryotic cell organisms and can regulate the physiological balance of proteins (41). Ubiquitin is essential for regulation of various proteins, and deubiquitination of proteases plays a very important role in the regulation of the first critical response factor in the innate immune response to viral infection (34). The human OTULIN gene (Human Genome Organisation [HUGO] Gene Nomenclature Committee [HGNC]-approved gene nomenclature) is located on 5p15.2 and encodes an OTULIN protein consisting of 352 amino acids (42). OTULIN specifically binds to Met l-Ub and LUBAC and hydrolyzes the Met l-Ub chain bound to the substrate specifically (not acting on the nonubiquitinated protein), allowing ubiquitination and deubiquitination of the substrate to maintain homeostasis for participation in a variety of biological functions in the organism. Research has shown that homozygous mutations in the human OTULIN gene can lead to potentially lethal autoinflammatory states, known as OTULIN-related autoimmune syndrome (ORAS) (43).

The ubiquitin-proteasome pathway (UPP) is an important target for the virus to help itself better achieve its life cycle (5, 44). Many viruses can encode proteins to modify the host’s ubiquitin regulatory mechanisms, and some viruses can even encode some of the ubiquitination or deubiquitination proteases. Such activity may represent a unique protective mechanism established by the virus for long periods of confrontation with host innate immunity. In addition, early replication of the viruses depends on the nonstructural protein, which exhibits a large amount of enzyme activity, including polymerization and replicase activity (45, 46). Exploitation of such activity has become an effective strategy for research on nonstructural proteins involved in virus deubiquitination.

In this study, we successfully amplified the complete coding sequence (CDS) of OTULIN and performed domain analyses with results that indicated that OTULIN shares a high level of amino acid sequence identity with Homo sapiens and includes a PIM domain and an OTU domain.

The scientific results of our work can be summarized as follows. First, PRRSV infection upregulated intracellular OTULIN gene expression; in turn, upregulation of the OTULIN gene further promoted virus proliferation. Moreover, proliferation of PRRSV was inhibited when OTULIN gene expression was disturbed. We found that the levels of OTULIN expression and PRRSV proliferation level are consistent to some extent. Second, the level of ubiquitin in 3D4/21 cells decreased after PRRSV infection, and the overexpression of OTULIN further removed the ubiquitination. Third, further research showed that PRRSV Nsp11 interacts with OTULIN and that the two colocalize with each other; the key of the interaction between PRRSV Nsp11 and OTULIN is the OTU domain. It is noteworthy that this binding represents a combination of nonenzymatic live sites and that the interaction therefore does not affect OTULIN deubiquitination capabilities. Previous data showed that PRRSV inhibits the ability of type I IFNs to induce an antiviral response in MARC-145 and PAM cells and that Nsp11 is an important interferon antagonist. We found that Nsp11 removes the K48 ubiquitin chain and has no direct

FIG 6 Legend (Continued)
infected with SeV (MOI of 0.5) or left uninfected. After 12 h, the infected cells were collected and the mRNA levels of NF-κB, IRF3, and IFN-α were analyzed by qRT-PCR. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (two-way ANOVA followed by Bonferroni posttest). Data are representative of results from three independent experiments.
FIG 7 PRRSV Nsp11 interact with NEMO. (a) HEK 293T cells were cotransfected with Nsp11-Myc and Flag-IKKα, Flag-IKKβ, or Flag-NEMO. At 24 h posttransfection, the cell lysates were precipitated with an anti-Flag MAb in conjunction with protein A Sepharose and further detected by Western blotting with an anti-Flag MAb antibody and an anti-Myc antibody, respectively. (b) (c) 293T cells were cotransfected with Flag-NEMO, HA-KO, and Nsp11-Myc or blank vector. At 24 h posttransfection, the cell lysates were precipitated with an anti-Flag MAb in conjunction with protein A Sepharose and further detected by Western blotting with an anti-Myc MAb and an anti-HA antibody, respectively. (d and e) HeLa cells (d) or 3D4/21 cells (e) were cotransfected with Nsp11-Myc and (Continued on next page)
effect on the linear ubiquitin chain but instead recruits OTULIN to enhance its ability to remove linear ubiquitination targeting NEMO.

PRRSV Nsp11 targets \( \text{I}_{\lambda} \text{B9260} \) to remove ubiquitination of K48, thereby inhibiting IFN-\( \beta \) production (22, 47). Our study showed that Nsp11 can influence linear ubiquitination targeting NEMO by recruiting OTULIN and causing superimposition of the effect of inhibition of production of type I IFNs. The results imply that the related proteins play

**FIG 7** Legend (Continued)

Flag-IKK\( \alpha \), Flag-IKK\( \beta \), or Flag-NEMO. The cells were fixed at 24 h posttransfection and doubly stained with a rabbit anti-Myc MAb and a mouse anti-Flag antibody followed by FITC-conjugated anti-mouse IgG (green) and PE-conjugated anti-rabbit IgG (red). Nuclei were stained with DAPI (blue). Cells were observed under a laser confocal imaging analysis system. Bar, 7 \( \mu \)m.
a role in the innate immunity of the host when the virus invades. Interestingly, the antagonism of the viral proteins may not be directed at single molecules but may act on the synergy of multiple molecules.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a critical pathogen of swine (47). Infection of PRRSV significantly reduced cellular protein ubiquitination, whereas Nsp11 was found to be a key nonstructural protein that regulates ubiquitination levels during PRRSV infection. Our study explored the role of OTULIN in PRRSV infection and the effect of PRRSV infection on intracellular OTULIN. We found that the cooperative effect of swine OTULIN and PRRSV Nsp11 can enhance the ability of Nsp11 to reduce cellular levels of the protein ubiquitin, which is associated with innate immunity. In brief, PRRSV infection upregulates OTULIN expression. Nsp11 is produced during PRRSV replication, and it recruits more OTULIN molecules to reduce ubiquiti-
nation levels in interferon signaling pathways, thus inhibiting the production of type I interferon and promoting the proliferation of the virus (Fig. 9e). Our report provides a new model of virus utilization of the ubiquitin-protease system in vivo from the perspective of the interaction of viral proteins with cell deubiquitination enzymes. However, whether OTULIN plays another role in PRRSV infection needs to be further studied. The molecular mechanism by which PRRSV infection leads to the upregulation of OTULIN expression remains unclear. The specific sites of molecular interaction still need further exploration.

MATERIALS AND METHODS

**Cells, virus, and antibody.** Porcine alveolar macrophage (PAM) cell line 3D4/21 (CRL-2843) was grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Biological Industries). Human embryonic kidney (HEK) 293T cells, MARC-145 cells, and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal FBS, 100 U/ml penicillin, and 10 μg/ml streptomycin sulfate in a humidified incubator at 37°C and 5% CO2. A stock of porcine reproductive and respiratory syndrome virus (PRRSV-JXwn06) with a titer of 10⁴ PFU/ml was employed in this study. Antibody to OTULIN was prepared by immunizing mice with the purified protein. Anti-PRRSV Nsp11 was a gift from China Agricultural University. Labeled antibodies used in the experiments were purchased from Cell Signaling Technology (CST) and Applied Biological Materials Inc. (ABM). Internal reference antibody and secondary antibodies were purchased from Invitrogen.

**RT-PCR amplification of complete porcine OTULIN CDS.** A pair of oligonucleotide primers from the 5'-terminal coding sequence (CDS) and 3'-terminal CDS were designed based on the predicted OTULIN sequence as shown in Table 1. Intracellular total RNAs were extracted using TRIzol reagent. Total RNAs were reverse transcribed into cDNA using reverse transcriptase (TaKaRa).

**Determination of virus titer.** The virus titer was determined using the 50% tissue culture infective dose (TCID₅₀) method. Marc145 cells were seeded in 96-well plates (Corning Inc., Corning, NY, USA) at a ratio of 1 × 10⁴ cells/well, and a series of dilutions (approximately 10⁻² to 10⁻⁶) of a viral suspension in 2% DMEM were inoculated in triplicate onto Marc-145 monolayer cells and incubated for 1 h at 37°C. The normal cells were used as a mock control. The reaction mixtures were incubated at 37°C for 7 days, and TCID₅₀ values were calculated according to the Reed-Muench formula. The PFU levels of PRRSV on Marc145 cells were determined by a method using methyl cellulose overlay and crystal violet staining.

**Confocal microscopy.** The procedure used for confocal microscopy was described previously (48). Briefly, HeLa or 3D4/21 cells grown on coverslips in 12-well plates (Corning Inc., Corning, NY, USA) at 35% to 50% confluence were transfected with plasmids expressing OTULIN (0.5 μg) or Nsp11 (0.5 μg). The empty plasmid was used as a negative control (NC). For endogenous fluorescence detection, 3D4/21 cells transfected with plasmid were infected with PRRSV at an MOI of 0.5. At 24 h posttransfection or infection, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized for 10 min with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 2% bovine serum albumin (BSA), and then blocked with 5% BSA–PBS for 30 min. Next, cells were incubated with anti-Flag, anti-Myc, anti-hemagglutinin (anti-HA), and anti-PRRSV Nsp11 antibodies for 1 h at room temperature in a humid

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<td>pCMV-OTULIN-R</td>
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<td>pcDNA3.1-Nsp1-F</td>
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chamber. Following a wash performed with PBS 3 times for 5 min each time, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or phycoerythrin (PE)-conjugated anti-rabbit IgG secondary antibody for an additional hour. Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). The localization of OTULIN and Nsp11 was observed under an inverted fluorescence and phase-contrast microscope (Olympus). Images were taken at ×100 magnification.

**Western blot analysis.** Cell lysates were heated in buffer for 10 min and separated by SDS-PAGE. The separated proteins were transferred onto a nitrocellulose (NC) filter membrane (ExPro), and then membranes were blocked with PBST (1× PBS–0.05% Tween 20) mixed with 5% nonfat dry milk for 1 h at room temperature. The membranes were then stained with primary antibodies at 4°C overnight. The membranes were then washed with 0.05% PBST and stained with the proper horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The membrane was washed again and subjected to detection using a chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA) and, finally, was exposed to a chemiluminescence apparatus (Gel Doc XR+ imaging system; Bio-Rad, USA).

**Quantitative real-time PCR.** Quantification of the relative levels of gene expression was performed using qRT-PCR carried out on an ABI 7500 real-time PCR system and used the comparative cycle threshold (Ct) method, according to the instructions of the manufacturer (Applied Biosystems). The thermal cycler program consisted of 95°C for 10 min and then 45 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s and was performed using DBI Bioscience-2043 Bestar SYBR green qPCR Mastermix. All the primers used for quantitative real-time PCR are listed in Table 2.

**Immunoprecipitations.** Cells cultivated in 60-mm-diameter plates were either singly or doubly transfected with appropriate expression plasmid. At 24 h posttransfection, the cells were harvested in 500 μl radioimmunoprecipitation assay (RIPA) lysis buffer (with protease inhibitor cocktail added). Anti-Myc-labeled or anti-Flag-labeled beads (Sigma) were added to the cell lysis supernatant at 10 μl per tube. The reaction mixture was incubated at 4°C for 2 h overnight. The beads were washed three times with lysis buffer for 10 min each time and boiled for 5 min, and the proteins bound to the beads were separated by SDS-PAGE, transferred onto NC membranes, and probed with the proper antibodies against Flag, Myc, or β-actin.

**His tag pulldown.** First, a His-OTULIN fusion protein was purified using prokaryotic expression and BL21(DE3) cells with nickel-nitrilotriacetic acid (Ni-NTA) resin (49). The localization of OTULIN and Nsp11 was observed under an inverted fluorescence and phase-contrast microscope (Olympus). Images were taken at ×100 magnification.

**Flow cytometry analysis.** 3D4/21 cells were infected with PRRSV at a multiplicity of infection (MOI) of 0.5 or were subjected to mock infection with RPMI 1640. After 1 h of incubation, the inoculum was removed, and the cells were supplemented with fresh RPMI 1640 medium containing 2% FBS. At 24 h postinfection, cells were gently washed three times with 1× PBS, dissociated from the plates with 0.25% trypsin at 37°C for 5 min, and then washed with precooled PBS containing 1% bovine serum albumin (BSA). Cells were stained with mouse monoclonal anti-OTULIN mixed in 2% BSA–PBS for 30 min at room temperature. Following a wash with PBS, the cells were then incubated with FITC-conjugated goat anti-mouse IgG (1:200) secondary antibody for 30 min in darkness. For fluorescence-activated cell sorter (FACS) analysis, a total of 1 × 10^6 cells were analyzed.

**Small interfering RNA assay.** A small interfering RNA (siRNA) assay was performed by using siRNA targeting the OTULIN gene (siOTULIN) and a negative control (NC), which were synthesized by Gene-Pharma (Tianjin, China) (Table 3). Briefly, 3D4/21 cells grown to 80% confluence in 12-well plates were

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### TABLE 2 The primers used for qRT-PCR amplification

<table>
<thead>
<tr>
<th>Primer name</th>
<th>GenBank no.</th>
<th>Primer sequence (5’–3’)</th>
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<tbody>
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<td>PRRSV-N-F</td>
<td>KX286735.1</td>
<td>GCCGTGTTGGTGAGCAAGA</td>
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<td></td>
<td>CAGTGTGCTAACCTTATTAG</td>
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<td>OTULIN-F</td>
<td>XM_003133841.4</td>
<td>ATCTGAGCCGTGAATAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTGAGACACCCCTCTGAGCC</td>
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<tr>
<td>TNF-α-F</td>
<td>X57321</td>
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<td>CTCTTCAACAGAGCAGTG</td>
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<td>IFN-β-F</td>
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<td>β-Actin-F</td>
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TABLE 3 Primers used in small interfering RNA assay

<table>
<thead>
<tr>
<th>Primer name</th>
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<tr>
<td>Negative control</td>
<td>F:UUCUCGGACGUCGUGACCGUTT</td>
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<td>R:ACGUGACACGUUCGGAGAATT</td>
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<tr>
<td>siOTULIN-1</td>
<td>F:GCAUCAGAACCAGAUAAATT</td>
</tr>
<tr>
<td></td>
<td>R:UUAACUGGGGUUCUGAUGCTT</td>
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<tr>
<td>siOTULIN-2</td>
<td>F:GCUUCUGAGGAAACCACUATT</td>
</tr>
<tr>
<td></td>
<td>R:UAGAUGUUCCCUCAGCAGCTT</td>
</tr>
</tbody>
</table>

transfected with the siRNA at a final concentration of 50 nmol with Lipofectamine 3000 (Invitrogen). After 24 h, cells were infected with PRRSV at an MOI of 0.5, and the cells were incubated for additional 24 h. Quantitative real-time PCR (qRT-PCR) and Western blotting analyses were performed to confirm the expression levels.

Statistical analysis. The statistical significance of results of comparisons among different groups was determined by two-way analysis of variance (ANOVA) using GraphPad Prism software (version 6.0). A P value of less than 0.05 was considered statistically significant.

ACKNOWLEDGMENTS

We declare that we have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The article does not contain any individual personal data, and guidelines concerning individual consent to publish are not applicable.

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Jinhai Huang conceived and designed the experiments. Yanxin Su, Peidian Shi, Ruiqiao Li, Dong Lu, Chengxue Zhao, Lilin Zhang, and Lei Zhang performed the experiments. Yanxin Su and Lei Zhang analyzed the data. Jinhai Huang contributed reagents/materials/analysis tools. Yanxin Su and Jinhai Huang wrote the paper.

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Deubiquitinase OTULIN Promotes PRRSV Multiplication

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