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Nuclear localization signal in TRIM22 is essential for inhibition of type 2 porcine reproductive and respiratory syndrome virus replication in MARC-145 cells

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection causes one of the most economically important swine diseases worldwide. Tripartite motif-containing 22 (TRIM22), a TRIM family protein, has been identified as a crucial restriction factor that inhibits a group of human viruses. Currently, the role of cellular TRIM22 in PRRSV infection remains unclear. In the present study, we analyzed the effect of TRIM22 on PRRSV replication in vitro and explored the underlying mechanism. Ectopic expression of TRIM22 impaired the viral replication, while TRIM22-RNAi favored the replication of PRRSV in MARC-145 cells. Additionally, we observed that TRIM22 deletion SPRY domain or Nuclear localization signal (NLS) losses the ability to inhibit PRRSV replication. Finally, Co-IP analysis identified that TRIM22 interacts with PRRSV nucleocapsid (N) protein through the SPRY domain, while the NLS2 motif of N protein is involved in interaction with TRIM22. Although the concentration of PRRSV N protein was not altered in the presence of TRIM22, the abundance of N proteins from simian hemorrhagic fever virus (SHFV), equine arteritis virus (EAV), and murine lactate dehydrogenase-elevating virus (LDV) diminished considerably with increasing TRIM22 expression. Together, our findings uncover a previously unrecognized role for TRIM22 and extend the antiviral effects of TRIM22 to arteriviruses.

Keywords Porcine reproductive and respiratory syndrome virus (PRRSV) · Nucleocapsid (N) protein · Tripartite motif-containing 22 (TRIM22) · PRRSV-host interaction · Replication

Introduction

Classified within the order *Nidovirales*, family *Arteriviridae*, porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive failures in sows and respiratory diseases in growing pigs, leading to enormous economic loss to the pork industry worldwide [1]. Current

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antiviral/vaccination strategies provide only limited protection from infections [2–5], due to suppression of the immune response by this virus [6–12]. The single-stranded positive sense RNA genome of this virus is approximately 15 kb in size and encodes at least 12 overlapping open reading frames (ORFs), including envelope glycoprotein, unglycosylated membrane protein, and nucleocapsid (N) protein [13–15].

As the most abundant protein of PRRSV, N protein has been recognized to be involved in both viral replication and pathogenesis [16, 17]. Besides encapsidation of viral RNA, N protein can also interact with nonstructural protein 2 (nsp2), nsp9, and N protein itself [18–20]. Most importantly, interplay between N protein and the host cell has been shown to modulate interferon (IFN), interleukin-10 (IL-10), IL-15 and sCD83 production and Tregs proliferation through activation of p38 MAPK (mitogen-activated protein kinases), NF-κB (Nuclear factor-κB) and Sp1 signal pathways, thus facilitating viral infection [21–27]. Given the suppression of innate immunity by N protein, it is essential to identify host

restriction factors and intrinsic immunity against PRRSV infection.

The tripartite motif (TRIM) family of proteins are defined by the highly conserved N-terminal RBCC (RING-finger B-Box Coiled-coil) signature domain [28, 29]. The RING-finger domain is associated with E3 ubiquitin ligase activity, whereas the B-Box and Coiled-coil region is crucial for homo- and hetero-oligomerization [28, 29]. Functionally, TRIM proteins are implicated in a broad array of cellular processes that ranging from apoptosis, transcription, differentiation, and autophagy to, more recently antiviral immunity [30–32]. A prime example is TRIM22 (also referred to as Staf50), which has previously been identified as a target gene for tumor suppressor p53 and involved in cellular proliferation [33]. As a member of TRIM family, the RING-finger domain was shown to be required for regulation of NF-κB [34, 35], apoptosis [36], NOD2 signaling [37], and autophagy-dependent *Mycobacterium tuberculosis* clearance [38]. However, TRIM22 also contains C-terminal SPRY domain to dictate the different subcellular distributions and specific functions which has not been fully clarified [39, 40].

Recently, the role of TRIM22 acting as a restriction factor to potentiate the intrinsic immunity has become increasingly clear [41]. Indeed, a broad antiviral spectrum has been described for TRIM22, including human immunodeficiency virus type 1 (HIV-1) [42, 43], DNA virus from the *Hepadnaviridae* family (hepatitis B virus, HBV) [44], positive-strand RNA viruses, including members of the family *Picornaviridae* (encephalomyocarditis virus, ECMV) [45], *Flaviviridae* (hepatitis C virus, HCV) [46], as well as negative-strand RNA viruses in the family *Orthomyxoviridae* (influenza A virus, IAV) [47]. Thus, the aim of present study is to seek evidence for a potential restrictive role for cellular TRIM22 in PRRSV infection.

Materials and methods

Cells, viruses, and antibodies

HEK293T and MARC-145 cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and then incubated at 37 °C in a humidified 5% CO_2 incubator. PRRSV strain HN1 (GenBank: AY457635.1) is a highly pathogenic type 2 (North American) PRRSV, which was isolated from the lung of pigs suffering from "high fever" syndrome in 2009 in Henan Province, China [48]. The virus was amplified and titered in MARC-145 cells.

Mouse monoclonal anti-hemagglutinin (HA), anti-Flag, and anti-actin antibodies were purchased from ABclonal Biotechnology. Anti-Flag, anti-HA polyclonal antibody (Macgene), TRIM22 polyclonal antibody (Abcam) were purchased and used according to the manufacturers' recommendations. HRP-conjugated anti-mouse and anti-rabbit IgG light (or heavy) chain specific antibodies (Abbkine Science, USA) were purchased and used according to the manufacturers' recommendations. A monoclonal antibody directed against PRRSV N protein was produced from hybridoma cells derived from Sp2/0 myeloma cells and spleen cells of BALB/c mice immunized with recombinant N protein [49]. HRP-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China).

Plasmids constructions and mutagenesis

Expression plasmids for human TRIM22 (NM_006074) were purchased from Vigene Bioscience and were cloned into the pCMV–Tag2B vector (Stratagene). The HA epitope tag was amplified by PCR and cloned into the pCAGGS–MCS vector to generate the pCAGGS–HA plasmid, encoding an N-terminal HA tag. To construct pCAGGS–HA–N, the cDNA fragment encoding full-length PRRSV N protein was amplified by PCR and inserted into the pCAGGS–HA plasmid [49]. Plasmids encoding truncated human TRIM22 or N protein muants were constructed by overlapping PCR amplification using the specific primers listed in Table 1. N protein expression plasmids for simian hemorrhagic fever virus (pCAGGS–HA–N/SHFV, NC_003092.2), equine arteritis virus (pCAGGS–HA–N/EAV, NC_002532.2), and lactate dehydrogenase-elevating virus (pCAGGS–HA–N/LDV, NC_001639.1) were purchased from TsingKe Biotechnology. All constructs were confirmed with DNA sequencing.

Transfection and TRIM22 gene silencing by siRNA

The HEK293T cells were transiently transfected with expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. MARC-145 cells were transfected with expression plasmids using Lipofectamine 3000. Where necessary, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. For siRNA knockdowns, cells plated in 6-well plates were transfected with 30 nM the indicated siRNAs twice over a 48 h period by using Lipofectamine 2000. SiRNA sequences targeting monkey TRIM22 (NM_001113359.1) used are as follows: si-TRIM22, 5′-CACCAAACAUUCCGCAUAATT-3′; Negative control-siRNA, 5′-UUCUCCGAACGUGUCACG UTT-3′. The cells were infected with PRRSV 24–36 h after transfection when indicated and incubated for the indicated times. Whole-cell lysates were then prepared and subjected to Western blotting assays.

Table 1 The sequences of primers used for construction of TRIM22 (GenBank: NM_006074), and N (GenBank: KY964305.1) protein mutants

Name	Primer sequence (5′-3′)
TRIM22-F	TTT*AAGCTT*ATGGATTTCTCAGTAAAGGTAGACATA
TRIM22-R	TTT*CTCGAG*GGAGCTCGGTGGGCACACAGTCATGGG
ΔRING-F	TTT*AAGCTT*CAGACCAGATTCCAGCCTGGGAACCTC
SPRY-F	TTT*AAGCTT*AAGAAGCCAAAATCTGTTTCCAAGAAA
ΔSPRY-R	TTT*CTCGAG*GTACTGGACATCTGTCAGCTCTTTAAG
ΔNLS-F	GAGTGAAAGCTGGACATTGAGTGTATTCCGAGTACCAG
ΔNLS-R	CTGGTACTCGGAATACACTCAATGTCCAGCTTTCACTC
ORF7-F	TTT*GAATTC*ATGCCAAATAACAACGGCAAGCAGC
ORF7-R	TTT*AGATCT*TCATGTTGGGGGGTGATGCTGTGGCG
K10-4A-F1	AGCAAG<u>CGGCAGCGGCG</u>GGGAATGGCCAGCCAGTCGA
K10-4A-F2	ATAACAACGGCAAGCAGCAAG<u>CGGCAGCGGCG</u>GGGAATG
C23A-F1	AGCCAGTCGATCAGCTG<u>GCC</u>CAAATGCTGGGTAAGATCA
C23A-F2	AAAGAAAAAGAAGGGGAATGGCCAGCCAGTCGATCAGCTGG
C23A-F3	ATAACAACGGCAAGCAGCAAAAGAAAAAGAAGGGGAA
Q32-4A-F	CATCGCC<u>GCAGCAGCCGCG</u>TCCAGAGGCAAGGGACCG
Q32-4A-R	CTCTGGA<u>CGCGGCTGCTGC</u>GGCGATGATCTTACCCAG
K43-2A-F	ACCGGGGG<u>CGGCA</u>AATAGGAAGAAAAACCCG
K43-2A-R	CTTCCTATT<u>TGCCG</u>CCCCCGGTCCCTTGCCTCTG
P50-3A-F	<u>CGG</u>AGAAGG<u>GCC</u>CATTTC<u>GCT</u>CTAGCGACTGAAGATGACG
P50-3A-R	<u>GC</u>GAAATG<u>GGG</u>CCTTCTCCGCGTTTTTCTTCCTATTTTTC

The restriction enzyme sites used for cloning are underlined in italics. Locations of mutations are underlined

TCID$_{50}$ assay for PRRSV

PRRSV titers were expressed as the tissue culture infectious dose 50 (TCID$_{50}$) per milliliter using the Reed–Muench method as previous described [12]. Briefly, MARC-145 cells were seeded in 96-well plates, following, infected with serial 10-fold dilutions of PRRSV samples in eight replicates. Plates were incubated for 72–96 h before virus titers were calculated.

Quantitative RT-PCR (qPCR)

Total RNA was isolated from MARC-145 cells using TRIzol Reagent (Invitrogen). qPCR was performed with SYBR® Green Realtime PCR Master Mix (Toyobo Biologics, Japan) in a ABI 7500 system. The individual transcripts in each sample were assayed three times. The PCR conditions were as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C, and 40 s at 72 °C. The fold change in gene expression relative to the normal control was calculated with the ΔΔCT method. Primers (Table 2)

were designed using the Primer Express software (version 3.0; Applied Biosystems, Carlsbad, CA). Absolute quantitative mRNA levels were calculated using standard curves as previously described [50].

Western blotting analysis

Cytoplasm and nuclear protein extracts from PRRSV-infected MARC-145 cells were prepared with the cytoplasmic and nuclear protein extraction kit (Aidlab Biotechnologies) according to the manufacturer's protocols. Cells cultured in 60-mm dishes were prepared by adding 120 μL of 2×lysis buffer A (65 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate (SDS), 3% DL-dithiothreitol, and 40% glycerol). The cell extracts were boiled for 10 min, and then resolved with 8–12% SDS-PAGE. The separated proteins were electroblotted onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Billerica, MA). Run for 1–2 h at 100 V on ice. The Western blotting was probed with specific antibodies. The expression of β-actin was detected with an mouse monoclonal antibody

Table 2 The sequences of primers used for real-time PCR

Name	Forward primer (5′-3′)	Reverse primer(5′-3′)
TRIM22 (NM_001113359.1)	TCAGTGACCATCTCAAGAGG	CACAAACCCAGCAAATGAC
GAPDH (NM_001195426.1)	TCATGACCACAGTCCATGCC	GGATGACCTTGCCCACAGCC
Viral total RNA	AAACCAGTCCAGAGGCAA	CGGCAAACTAAACTCCACA

to demonstrate equal protein sample loading. Densitometry quantification of protein bands of interest was performed using ImageJ software.

Indirect immunofluorescence assay (IFA)

MARC-145 cells seeded on microscope coverslips and placed in 24-well dishes were infected with PRRSV (MOI = 0.5). At 24 hpi, the cells were fixed with 4% paraformaldehyde for 10 min, and then permeated with 0.1% Triton X-100 for 10 min at room temperature. After three washes with PBS, the cells were sealed with PBS containing 5% bovine serum albumin for 1 h, and then incubated separately with rabbit polyclonal antibody directed against TRIM22 (1:200) and mouse monoclonal antibody directed against PRRSV N protein (1:200) for 1 h at 37 °C. The cells were then treated with fluorescein-isothiocyanate-labeled goat anti-rabbit or Cy3-labeled goat anti-mouse antibodies (Invitrogen) for 1 h, followed by 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. After the samples were washed with PBS, fluorescent images were acquired with a confocal laser scanning microscope (Fluoview ver. 3.1; Olympus, Tokyo, Japan).

Co-immunoprecipitation (Co-IP) and immunoblotting analyses

To investigate the interactions between proteins, HEK293T or MARC-145 cells were lysed in immunoprecipitation lysis buffer (RIPA). After the lysates were incubated for 1 h at 37 °C with DNase (100 µg/mL) and RNase A (100 µg/mL), the lysate proteins were incubated overnight at 4 °C with the indicated antibodies. Protein A + G agarose beads (30 µL; Beyotime) were then added to each immunoprecipitation reaction for another 6 h. The agarose beads were then washed three times and the captured proteins resolved on

8–12% SDS-PAGE, transferred to PVDF membrane, and analyzed with immunoblotting.

Statistical analysis

The results represent the means and standard deviations from three independent experiments. GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for data analysis using a two-tailed unpaired t test. Differences between groups were considered statistically significant when the P value was less than 0.05 (*$P = 0.05$; **$P = 0.01$).

Results

Ectopic expression of TRIM22 inhibited propagation of PRRSV

Accumulating data suggest that TRIM22 is a restriction factor of several human viral infection [41]. However, whether TRIM22 participates in host defense against PRRSV is largely unknown. We first examined the impact of TRIM22 over-expression on PRRSV infection. To this end, MARC-145 cells grown in 24-well plates were transfected with the Flag–TRIM22 (2 µg) and followed by infected with PRRSV strain HN1 at an multiplicity of infection (MOI) of 0.5. Supernatants collected at the 24, 36, and 48 h post-infection (hpi) were analyzed for virus titers with $TCID_{50}$. As shown in Fig. 1a, over-expression of TRIM22 resulted in a significantly decrease of virus titers in supernatants of PRRSV-infected MARC-145 cells at 24 and 36 hpi (Fig. 1a). This antiviral effect of TRIM22 persisted at later time points (48 hpi). To further corroborate this finding, the effect of TRIM22 over-expression on the expression of corresponding viral total RNA in PRRSV-infected MARC-145 cells were
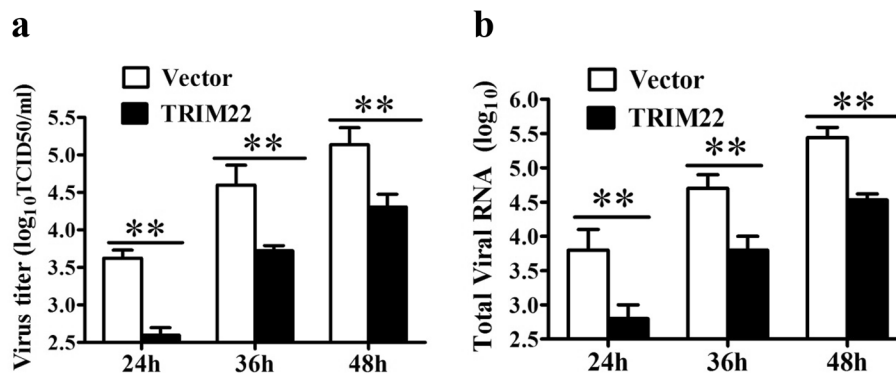

Fig. 1 Ectopic of TRIM22 impairs PRRSV replication in MARC-145 cells. MARC-145 cells transfected with plasmid expressing Flag–tagged TRIM22 or an empty vector control were infected with PRRSV at an MOI of 0.5. **a** Progeny virus production were analyzed by $TCID_{50}$ at the indicated time points post-infection. **b** The mRNA expression level of viral total RNA by qPCR were analyzed at the time points shown

examined by qPCR. As expected, RNA qPCR results were in accordance with the TCID$_{50}$ data (Fig. 1b), suggesting that the over-expression of TRIM22 impairs the replication efficiency of PRRSV.

Knockdown of TRIM22 augmented the replication of PRRSV

Next, the effect of TRIM22 knockdown on PRRSV propagation was dissected by using siRNAs. To this end, MARC-145 cells were transfected with the siRNAs targeting TRIM22 and were then harvested for evaluating the knockdown efficiency of TRIM22 by real-time PCR and Western blotting analyses. As shown in Fig. 2a and b, compared with cells transfected with control siRNAs

(NC), the cells transfected with TRIM22-specific siR-NAs exhibited a significantly decreased level (~85%) of TRIM22 expression (Fig. 2a, b).

To investigate the role of endogenous TRIM22 on PRRSV replication, MARC-145 cells were treated with control-siRNA or si-TRIM22 followed by PRRSV infection, then viral titers were measured by TCID$_{50}$, and viral total RNA were quantified by qPCR at 36 hpi. Compared to control cells, we observed that there was a statistically significant increase of the virus yields in the supernatants of si-TRIM22-transfected cells (Fig. 2c). At the same time, a slight increase of PRRSV total RNA level were observed after silencing of TRIM22 (Fig. 2d), suggesting that the knockdown of endogenous TRIM22 favors the multiplication of PRRSV in MARC-145 cells.
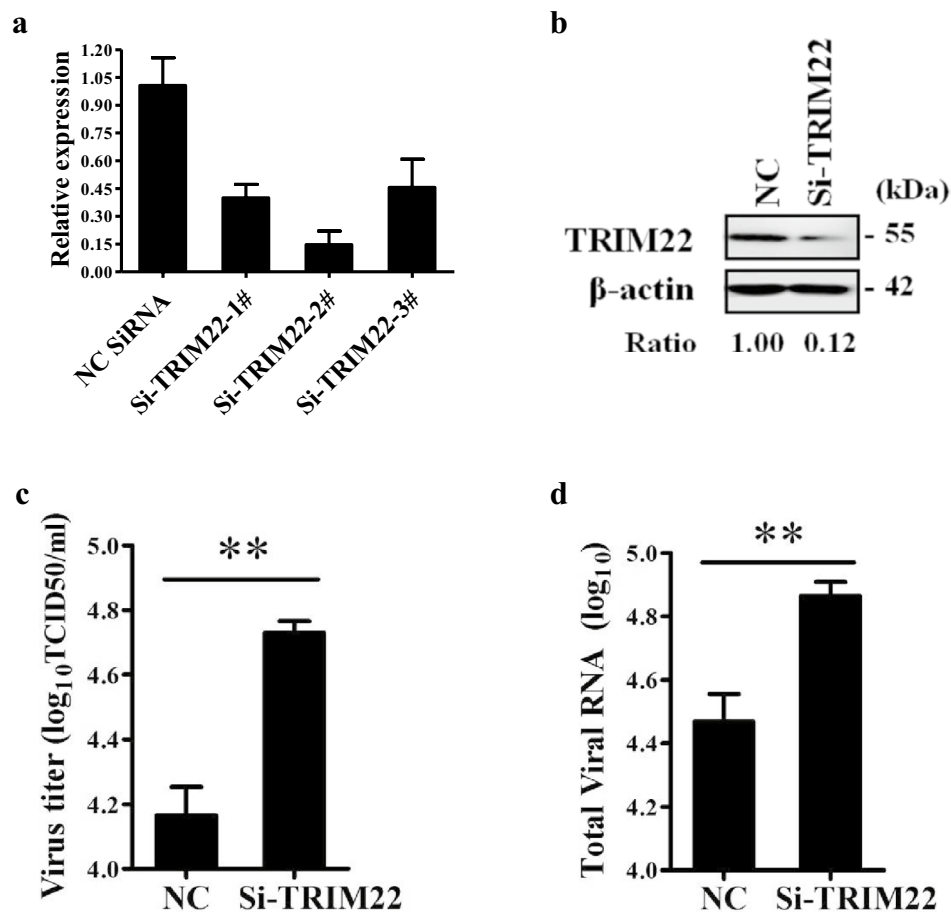

Fig. 2 Enhancement of PRRSV replication by TRIM22 knockdown. **a** TRIM22 mRNA level were examined by real-time PCR in negative control-siRNA (NC) or si-TRIM22 treated MARC-145 cells to confirm the knockdown efficiency of endogenous TRIM22. **b** MARC-145 cells were transfected with siRNA targeting TRIM22 or NC siRNA. The cells were harvested at 36 h post-transfection and the silencing efficiency of TRIM22 was examined by Western blotting with an anti-TRIM22 antibody. The relative levels of TRIM22 were shown below the images after normalization with β-actin in densi-

tometry analysis. **c** MARC-145 cells transfected with the si-TRIM22 or NC siRNA for 24 h were infected with PRRSV at MOI of 0.1 and the virus titers were examined at 36 hpi. **d** PRRSV RNA replication in TRIM22-silenced MARC-145 cells. MARC-145 cells transfected with the si-TRIM22 or NC siRNA for 24 h were infected with PRRSV at MOI of 0.1 and collected at 36 hpi. The total cellular RNA was extracted and the mRNA levels of PRRSV N gene were determined by qPCR

RING domain was not required for restriction of PRRSV

In the case of ECMV, HBV, IAV, and HCV, the N-terminal RING domain of TRIM22 was invariably shown to be essential for viral restriction [44–47]. To investigate the exact antiviral mechanism of TRIM22 against PRRSV, three truncations of TRIM22 lacking only the RING domain, the RING, B-box and coiled-coil domains, or the SPRY domain were constructed and tested for repression activity (Fig. 3a). Unexpectedly, the results presented in Fig. 3b showed that the propagation of PRRSV was still restricted by ectopic expression of TRIM22ΔRING. However, when challenged with PRRSV, an inhibitory effect on progeny virus production were observed in TRIM22 WT, and SPRY, except in ΔSPRY expression cells (Fig. 3b). Concordant with this, over-expression of TRIM22 SPRY domain led to a sharp decline in PRRSV total RNA expression compared with cells transfected with empty vector (Fig. 3c). Collectively, these data implied that TRIM22 exerts its antiviral function towards viruses of distinct families via distinct molecular determinants, and the SPRY domain is involved in PRRSV suppression.

Nuclear localization signal of TRIM22 is essential for PRRSV inhibition

The localization of TRIM22 has been associated with its antiviral function, as exemplified by TRIM22 restriction of HIV [51, 52]. To analysis the localization of TRIM22 after PRRSV infection, MARC-145 cells were infected with PRRSV, and confocal immunofluorescence assay were performed. As shown in Fig. 4a, endogenous TRIM22 localized in both the nucleus and the cytoplasm in MARC-145 cells. However, TRIM22 obviously redistributed from cytoplasm into the nucleus in PRRSV-infected cells, where they partially colocalized with N protein.

To further confirm the localization of TRIM22, the cytoplasmic and nuclear extracts of PRRSV-infected MARC-145 cells were prepared and endogenous TRIM22 was detected by Western blotting using anti-TRIM22 antibody. In parallel, Western blotting showed TRIM22 was upregulated in the nucleus, but was downregulated in the cytoplasm of PRRSV-infected MARC-145 cells. This indicated that PRRSV-induced translocation of TRIM22 into the nucleus.

Peptide sequence analysis of the TRIM22 protein using the PSORT program has identified two nuclear localization signal (NLS), a pat-4, 265-KKPK-268, and a pat-7 269-PVSKKLK-275 [53]. Given that the NLS was shown to be necessary for nuclear localization [39], these amino

Fig. 3 RING domain was not required for restriction of PRRSV. **a** Full-length and serial truncations of TRIM22 with deletion (Δ) of various domains. Numbers indicate the residues where deletions begin or end. MARC-145 cells transfected with Flag–TRIM22 (WT or domain lacking mutants) were infected with 0.5 MOI of PRRSV for 48 h. **b** Production of progeny virus were determined using TCID$_{50}$ assay. **c** The total cellular RNA was extracted and the mRNA levels of PRRSV N gene were determined by qPCR
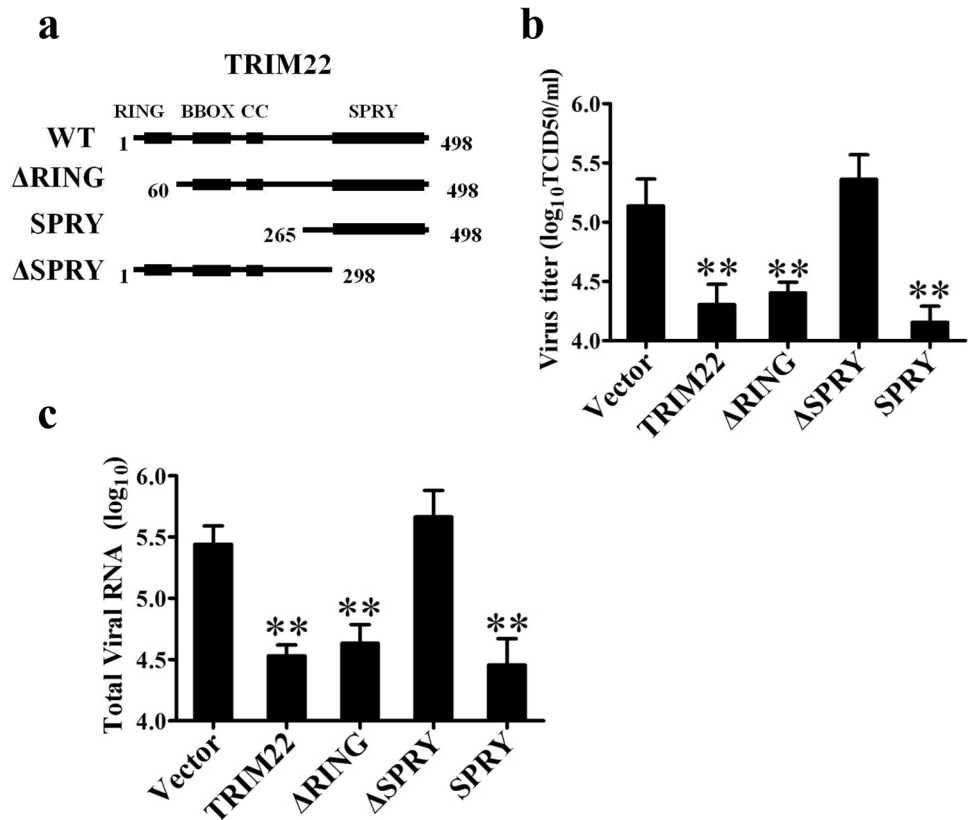
Fig. 4 Nuclear localization signal of TRIM22 is essential for PRRSV inhibition. **a** Subcellular distribution of TRIM22 after PRRSV infection in MARC-145 cells. MARC-145 cells were infected with PRRSV HN1 at a MOI of 1, and were fixed for immunofluorescence analysis of TRIM22 (green), N protein (red) and nucleus marker DAPI (blue) localization. **b** Nuclear and cytoplasmic fractionation of MARC-145 cells infected with PRRSV for 36 h. Each nuclear and cytosolic fraction was prepared and subjected to Western blotting analysis with an antibody specific for TRIM22, LaminA+C as a nuclear protein marker, HSP90 as a cytosolic protein marker, or the PRRSV N protein. **c** The position and sequence of the NLS in TRIM22. MARC-145 cells transfected with Flag–TRIM22 (WT or NLS lacking mutants) were infected with 0.5 MOI of PRRSV for 48 h. **d** Production of progeny virus were determined using $TCID_{50}$ assay. **e** The total cellular RNA was extracted and the mRNA levels of PRRSV N gene were determined by qPCR (Color figure online)
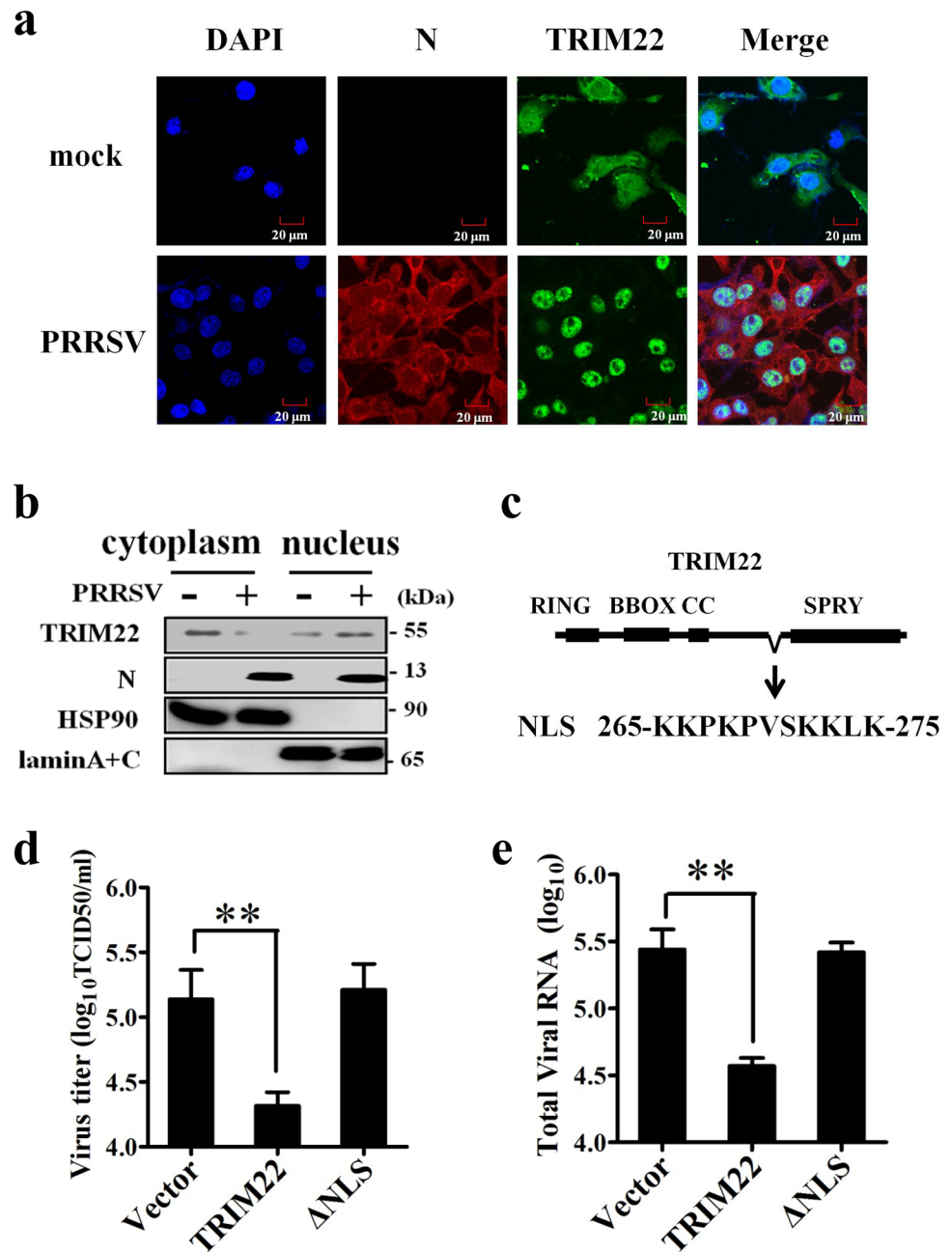
acids were deleted to give rise TRIM22 NLS mutant (Fig. 4c). $TCID_{50}$ assay was then performed to determine whether the NLS of TRIM22 is related to PRRSV antagonism. As shown in Fig. 4d, the cells transfected with the wild-type Flag–TRIM22 expressing construct exhibited a significant reduction in virus production, as compared with cells transfected with the empty vector. However, the NLS deleted TRIM22 showed minimal or no effect on PRRSV replication. Consistent with the results of $TCID_{50}$, the PRRSV total RNA level in transfected cells was significantly suppressed by TRIM22 as determined by qPCR (Fig. 4e). This result directly suggested that the NLS is

important for TRIM22 to curtail the replicative activity of PRRSV.

TRIM22 interacted with the N protein of PRRSV

Since TRIM22 exerts its antiviral function in the nucleus, we then tested whether TRIM22 might interact with nuclear-localized proteins encoded by PRRSV, such as the nucleocapsid protein. To this end, HEK293T cells were transfected with Flag–TRIM22-expressing plasmid and HA–N-expressing plasmid individually or together. The cell lysates were immunoprecipitated with an anti-HA mAb

and were then subjected to Western blotting. It was shown that N protein could be precipitated in the cells that were co-transfected with the plasmids pCAGGS–HA–N and pCMV–Flag–TRIM22 (Fig. 5a). The reciprocal IP assay was also performed and the same results were observed (Fig. 5a). We also tested whether TRIM22 could interact with three PRRSV proteins: Nsp1α, Nsp1β, and Nsp11, but no interaction were detected by Co-IP (data not shown).

We further examined the interaction of endogenous TRIM22 with the N protein in the context of PRRSV infection. MARC-145 cells were infected with PRRSV for 30 h before harvested and immunoprecipitated with an anti-TRIM22 mAb. As shown in Fig. 5b, by Co-IP assay suggested that endogenous TRIM22 could interact with the N protein of PRRSV in MARC-145 cells.

The regions responsible for the interaction of TRIM22 with N protein

Several functional motif in N protein has been reported to interacts with the host cellular proteins, including NLS1, NLS2, N–N interaction motif and an SH3 binding motif (Fig. 6a) [54–57]. To determine the regions of N protein responsible for the interaction with TRIM22, a serial of truncated mutants, as indicated, were co-transfected with pCMV–Flag–TRIM22 in HEK293T cells. The cell lysates were precipitated with an anti-HA or anti-Flag mAb. As shown Fig. 6b, TRIM22 was strongly co-precipitated with HA–N, K10-4A, C23A, Q32-4A, and P50-3A. No co-precipitation was detectable with extracts from K43-2A expressing cells or cells tranfected with the empty vector, indicating that the NLS2 of N protein is involved in interaction with TRIM22.

We next identified the domain of TRIM22 that is essential for the interaction between TRIM22 and N protein. Four truncations of TRIM22 lacking only the RING domain, the RING, B-box and coiled-coil domains, the SPRY domain, or the NLS, were constructed to identify the domains required for the interaction between TRIM22 and N protein. The results showed that the truncated TRIM22 which lack the RING domain or B-box and coiled-coil domains retained the ability to interact with N protein, while TRIM22 mutants which lack the SPRY domain or NLS were unable to interact with N protein, indicating that the SPRY domain and NLS are important for the interaction between TRIM22 and N protein (Fig. 6c).

TRIM22 destabilizes the N proteins from the SHFV, EAV, and LDV

Finally, we examined whether TRIM22-N protein interaction could be expanded to other members in family *Arteriviridae*, such as simian hemorrhagic fever virus (SHFV), equine arteritis virus (EAV), and murine lactate dehydrogenase-elevating virus (LDV). For this purpose, HEK293T cells were co-transfected with Flag-tagged TRIM22-expressing plasmid and HA-tagged N protein-expressing plasmids, Co-IP experiments were then performed to assess possible interactions. Unexpectedly, the N protein levels of SHFV (Fig. 7a), EAV (Fig. 7b), and LDV (Fig. 7c) decreased sharply upon co-transfection with TRIM22. As shown Fig. 7a–c, evident down-regulation of SHFV, EAV and LDV N protein could be detected, indicating TRIM22 targets arteriviruses N protein in a species-specific manner.

Discussion

To date, the most intensively studied antiviral TRIM protein may be TRIM5α, especially to retrovirus [58]. Being one of its closest paralogs, TRIM22 was first identified independently by several laboratories to possess antiviral

Fig. 5 Interaction of the N protein with TRIM22. **a** HEK293T cells were co-transfected with Flag–TRIM22 and HA–N. The cell lysates were immunoprecipitated with an anti-HA (or Flag) antibody and detected with an anti-HA or anti-Flag antibody. Whole-cell lysates (WCL) were probed with Flag and HA antibody. **b** The interaction of N protein with endogenous TRIM22. MARC-145 cells were infected with PRRSV. The cell lysates were immunoprecipitated with an anti-TRIM22 antibody and detected with an anti-N protein antibody
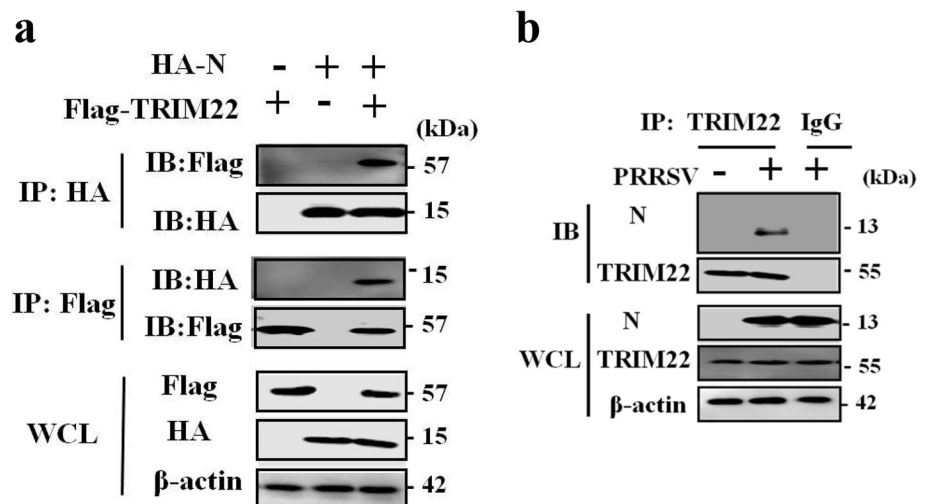
Fig. 6 The regions responsible for the interaction of TRIM22 with N protein. **a** The schematic diagram of N protein individual mutants investigated in this study. **b** The interaction of TRIM22 with N protein mutants by Co-IP. HEK293T cells were co-transfected with the indicated plasmids. The cell lysates were immunoprecipitated with an anti-HA mAb and further probed with an anti-HA or anti-Flag antibody. **c** The interaction of N protein with TRIM22 deletion mutants by Co-IP. HEK293T cells were co-transfected with the indicated plasmids. The cell lysates were immunoprecipitated with an anti-Flag mAb and further probed with an anti-HA or anti-Flag polyclonal antibody
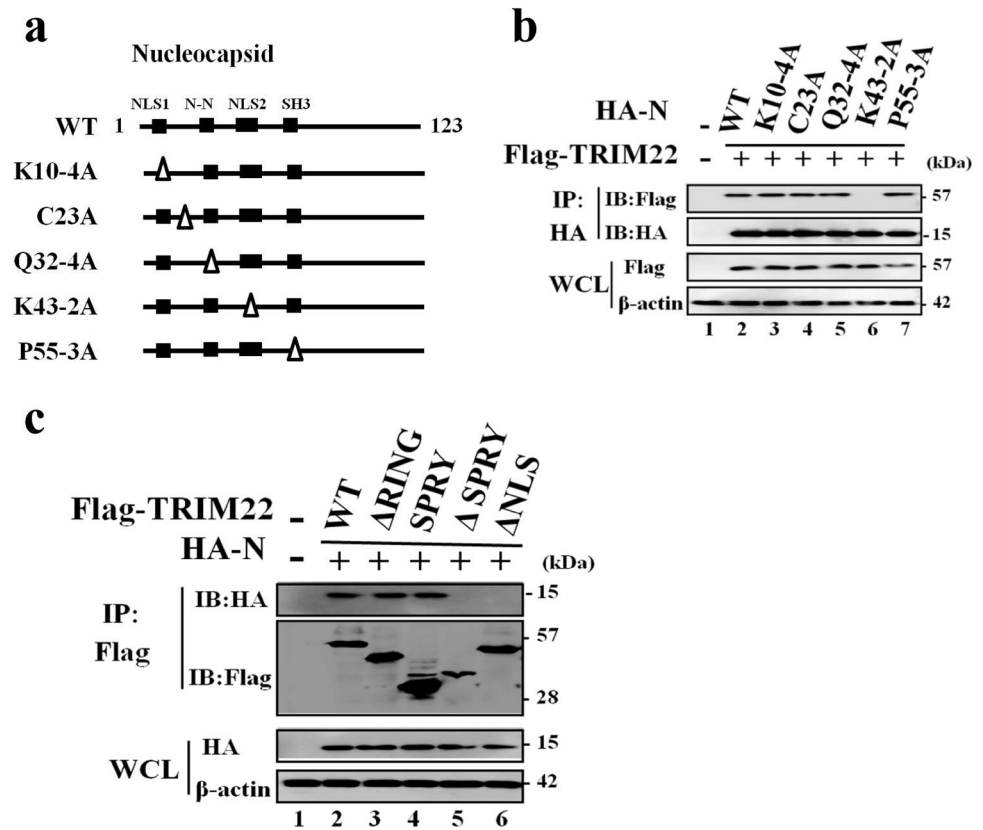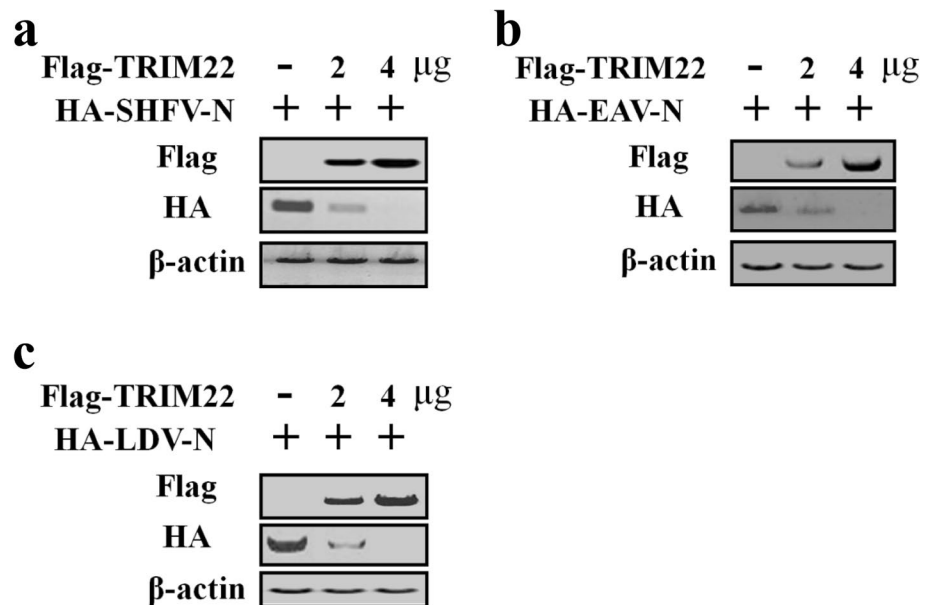

Fig. 7 TRIM22 reduces the expression levels of SHFV, EAV and LDV N proteins. HEK293T cells were co-transfected with arteriviruses N protein-expressing plasmids (0.5 µg) and TRIM22-expressing plasmid as indicated. The SHFV (**a**), EAV (**b**) and LDV (**c**). N proteins expression were analyzed by Western blotting 30 h later in whole-cell lysates

activity, targeting HIV-1 in particular [42, 43, 52, 53]. The RING domain of several TRIM proteins has been shown to possess an E3 ubiquitin ligase activity [41], either by targeting specific viral components (protein) for degradation or through governing the ubiquitination state of cellular proteins in innate immune signaling [34, 35, 37, 59–61].

Recently published data support this notion, showing that TRIM22 restricts EMCV, IAV, and HCV by degrading viral 3C protease [45], nucleoprotein [47] and NS5A [46].

However, our efforts lead to the identification that the C-terminal SPRY domain of TRIM22 curtailed the propagation of PRRSV, whereas the N-terminal RING domain was

dispensable. In fact, approximately 60% of all known human TRIM also harbor a variable SPRY domain at the carboxy terminus, facilitating protein–protein interactions [62]. The TRIM22 SPRY domain was found to specifically targeting viral N protein, much resembling the interaction between TRIM22 and nucleoprotein of IAV [47]. As nucleocapsid protein of RNA viruses, both of them have an essential role in virus assembly, encapsidating the genomic RNA, viral replication and transcription [63]. Noteworthy, the RING domain dependent/independent restriction strategies of virus from different family by TRIM22 appears to be mechanistically similar to the antiviral effect exerted by TRIM56 against *Flaviviruses*, *Coronavirus,* and Influenza virus infection [32, 64], suggested that TRIMs have developed multiple strategy to inhibit distinct viral infection.

It is widely accepted that the antiviral activities of TRIM22 was demonstrated to coupled with the localization [41]. Of interest, regarding HIV suppression, TRIM22 acts both in the nucleus at the level of transcription of viral DNA by blocking the action of Sp1 and in the cytoplasma at the level of assembly of new viral particles [43, 51, 65]. Moreover, Gao and colleagues observed that TRIM22 was mainly localized in the nucleus of HepG2 cells when it suppresses HBV survival [44]. In this study, it is proposed that the interaction between TRIM22 and PRRSV N protein in the nucleus might be involved in restriction of PRRSV replication.

Although PRRSV replicates in the cytoplasm, as is the case of most RNA viruses, its N protein is found in both cytoplasmic and nuclear compartments during infection [63]. Furthermore, it has been purposed that the NLS2 at position 41–47 affects N protein nuclear localization during viral infection and nuclear localization of the N protein participates in the regulation of several host cell processes [55, 66–68]. PRRSV with mutation of the NLS2 is shown to induce lower viremia, while higher neutralizing antibody level than the wild-type virus [68]. We found that N protein NLS2 mutants K43-2A failed to interact with TRIM22, further supporting the functional significance of this motif. Several cellular proteins have so far been demonstrated to interact with the N protein, as exemplified by inhibitor of MyoD family-a domain-containing protein, Poly(A)-binding protein (PABP), myxovirus resistance 2 (Mx2), DExD/H-Box Helicase 9 (DHX9), DHX36, SUMO E2 conjugating enzyme Ubc9, and MOV10 [19, 49, 67, 69–72]. These interactions conferred to the regulation of PRRSV replication and immune evasion. Ongoing and future research will no doubt continue to strive to identify key interactions involved in the PRRSV-associated immune evasion.

Previous studies have shown that the pig, cow, and goat genome lose the TRIM22 gene during evolution, whereas the primates and horse genome contain TRIM22 gene [42]. Our findings provide evidence that human TRIM22 targets the N proteins from SHFV, EAV, and LDV for degradation, suggesting a broad antiviral mechanism of TRIM22 against arteriviruses. However, the expression of PRRSV N protein is resistant to TRIM22 over-expression (Fig. 5a). A possible explanation is that the N proteins of arteriviruses share only ~40% sequence similarity of the amino acid and TRIM22 adopts distinct mechanisms towards different viral N proteins. In this regard, a previous report has shown that differences between the amino acid sequences of NP from several IAV strains determine the sensitivity to TRIM22-mediated degradation [73].

Meanwhile, monkey kidney cell lines, including MARC-145 cells, are extensively used to propagate PRRSV isolates for vaccine production [5]. Therefore, the identification that knockdown of TRIM22 in MARC-145 cells increases PRRSV titer might have promising implications in reducing vaccine production costs. Recently, the CRISPR-based technology has been applied to the generation of viral disease-resistant pigs [74–76]. In this context, restoring expression of TRIM22 via the breeding of transgenic pigs may provide potential anti-PRRSV strategies and considerable productivity benefits to pork producers.

Author contributions TR and HYJ designed the study. ND, YTS, SFC, JHW, WTK, YL, and YZ performed the experiments in the study. HH and WD analyzed the data. HYJ wrote and edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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