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Hyper-phosphorylation of nsp2-related proteins of porcine reproductive and respiratory syndrome virus

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ABSTRACT

Viruses exploit phosphorylation of both viral and host proteins to support viral replication. In this study, we demonstrate that porcine reproductive and respiratory syndrome virus replicase nsp2, and two nsp2-related −2/−1 frameshifting products, nsp2TF and nsp2N, are hyper-phosphorylated. By mapping phosphorylation sites, we subdivide an extended, previously uncharacterized region, located between the papain-like protease-2 (PLP2) domain and frameshifting site, into three distinct domains. These domains include two large hypervariable regions (HVR) with putative intrinsically disordered structures, separated by a conserved and partly structured interval domain that we defined as the inter-HVR conserved domain (IHCD). Abolishing phosphorylation of the inter-species conserved residue serine918, which is located within the IHCD region, abrogates accumulation of viral genomic and subgenomic RNAs and recombinant virus production. Our study reveals the biological significance of phosphorylation events in nsp2-related proteins, emphasizes pleiotropic functions of nsp2-related proteins in the viral life cycle, and presents potential links to pathogenesis.

1. Introduction

As the most common form of post-translational modification, phosphorylation is involved in every aspect of cellular processes, including signal transduction, gene transcription, protein translation, and substrate protein regulation, among others (Humphrey et al., 2015; Hunter, 1995; Johnson, 2009; Tarrant and Cole, 2009; Ubersax and Ferrell, 2007). It is estimated that up to 30% of the constituent proteins of the human proteome are modified by kinases (Ubersax and Ferrell, 2007). Protein kinases mediate the transfer of the γ-phosphate from ATP to specific residues (Ser, Thr, or Tyr) of target proteins (Humphrey et al., 2015; Hunter, 1995; Johnson, 2009; Tarrant and Cole, 2009; Ubersax and Ferrell, 2007). As intracellular pathogens that replicate exclusively in live cells, viruses modulate phosphorylation-regulated signaling pathways in host cells. Viruses also utilize host phosphorylation machinery to modify viral proteins in order to establish a productive life cycle (Keating and Striker, 2012; Keck et al., 2015). Phosphorylation of specific viral proteins regulates viral protein functionality, virus replication, and control of host cellular responses (Keating and Striker, 2012; Keck et al., 2015). In nidoviruses, nucleocapsid (N) proteins of coronaviruses and arteriviruses have been shown to be phosphorylated, with potential impacts on viral RNA transcription (McBride et al., 2014; Wu et al., 2009; White et al., 2007; Surjit et al., 2005; Stohlman and Lai, 1979; Spencer et al., 2008; Peng et al., 2008; Mohandas and Dales, 1991; Chen et al., 2005; Chang et al., 2009; Calvo et al., 2005; Zeegers et al., 1976). However, whether other nidoviral proteins are phosphorylated and the functional consequences of such phosphorylation events remain largely uncharacterized.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a leading viral pathogen that has caused significant economic loss to the global swine industry (Fang and Snijder, 2010; Snijder et al., 2013). PRRSV belongs to order Nidovirales, family Arteriviridae. Previously, PRRSV was divided into two distinct genotypes, European genotype (type 1) and North American genotype (type 2). Due to the substantial genetic difference in their sequences, the two genotypes were recently assigned to two distinct species, PRRSV-1 and PRRSV-2 (Kuhn et al., 2016). PRRSV contains a positive-sense (+) single-stranded (ss) RNA genome (Fang and Snijder, 2010; Snijder et al., 2013). The viral genome is approximately 15 kilobases (kb) in length and contains eleven known open reading frames (ORFs). The 3′-end ORFs encode
four membrane-associated glycoproteins (GP2a, GP3, GP4 and GP5), two non-glycosylated membrane proteins (E and M) and the nucleocapsid protein (N) (Fang and Snijder, 2010; Snijder et al., 2013). The 5′ proximal three-quarters of the genome contain ORF1a and ORF1b, which encode two long polyproteins, pp1a and pp1ab (Fang and Snijder, 2010; Snijder et al., 2013). The pp1a and pp1ab polyproteins are further proteolytically processed into 14 mature nonstructural proteins (nsps) after synthesis from the genomic RNA template (Fang and Snijder, 2010; Snijder et al., 2013). Four virus-encoded protease domains direct the proteolytic processing. These include two papain-like proteases (PLP1α and PLP1β) located in nsp1α and nsp1β, a papain-like protease (PLP2) domain located at the N-terminus of nsp2, and a serine protease (SP) located in nsp4. PLP1α autocleaves between nsp1α/nsp1β, PLP1β autocleaves between nsp1β/nsp2, and PLP2 cleaves between nsp2/nsp3. These autocleavage processes induce the rapid release of nsp1α, nsp1β and nsp2 from the polyprotein. Recently, we identified two novel nsp2-related proteins, nsp2TF and nsp2N (Fang et al., 2012; Li et al., 2014). The nsp2TF is expressed through a ~2 programmed ribosomal frameshift (PRF) mechanism, which accesses an alternative ORF (TF) through a frameshifting site that overlaps the nsp2-encoding region. The resulting transframe fusion protein contains the N-terminal two thirds of nsp2 followed by a unique C-terminal domain that is translated from the novel TF ORF. At the same frameshifting site, ~1 PRF also occurs, in which the ~1 PRF encounters an immediate stop codon to generate a truncated nsp2 protein, termed nsp2N. Nsp2, nsp2TF and nsp2N all contain the PLP2 domain and the following region located between the PLP2 domain and the frameshifting site. Other cleaved nsp2 variants of smaller protein size have also been reported (Han et al., 2010).

The nsp2 protein is the largest replicase cleavage product and contains multiple domains that serve multifunctional roles. Besides cleaving the nsp2/nsp3 site, the nsp2 functions as a cofactor for the SP to process the nsp3-12 region (Fang and Snijder, 2010). In addition, PLP2 is capable of removing ubiquitin (Ub) and Ub-like modifiers like ISG15 (an Interferon-stimulated gene) from host cell substrates (Sun et al., 2010, 2012; van Kasteren et al., 2012; Frias-Staheli et al., 2007). The C-terminus of nsp2 contains a highly conserved Cys-rich domain of unknown function as well as a multi-spanning transmembrane domain that plays a role in the formation of membranous structures that facilitate viral replication (Fang and Snijder, 2010; Snijder et al., 2013). The central region of nsp2 (which is also present in nsp2TF and nsp2N) contains hypervariable sequences, in which various deletions/insertions have been reported (Fang and Snijder, 2010; Snijder et al., 2013). Significantly, a cluster of immuno-dominant B cell epitopes and potential T-cell epitopes was identified within this region (Oleksiwicz et al., 2001; Fang et al., 2004). In particular, highly pathogenic PRRSV variants exhibit specific signature-like variations, raising the possibility of connections between nsp2 sequence hyper-variability and viral pathogenicity (Fang and Snijder, 2010; Snijder et al., 2013). However, the central region of nsp2 is poorly characterized in terms of its structure and function. In this study, we demonstrate that the PRRSV nsp2 and two nsp2-related PRF products, nsp2TF and nsp2N, are hyper-phosphorylated. By combining phosphorylation site mapping, disorder analysis and protein structural prediction, we divided the central region of nsp2 into three distinct domains, located between the PLP2 domain and PRF site. We determined that the central region contains two large hypervariable regions (HVRs) with putative intrinsically disordered structures, separated by a conserved and partially structured interval domain that we defined as the IHCD (Inter-HVR Conserved Domain). Importantly, we identified a highly conserved phosphorylation site (Serine918) in the IHCD that is critical for viral replication. The results of our study expand the nidovirus phospho-proteome and reveal important roles for the phosphorylation of nsp2-related proteins in the regulation of virus replication, as well as potential effects on viral pathogenesis.

2. Results

2.1. PRRSV nsp2-related proteins are hyper-phosphorylated

Based on the protein sequences, the predicted molecular weights of PRRSV-1 (strain SD01-08) nsp2-related proteins, nsp2, nsp2TF, and nsp2N, are 114 KDa, 96 KDa, and 76 KDa, respectively. The corresponding molecular weights in PRRSV-2 (strain SD95-21) are 129 KDa, 110 KDa, and 92 KDa, respectively (Fig. 1). However, in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we observed that the apparent sizes of nsp2-related proteins were significantly larger than the predicted molecular weights (Fang et al., 2010; van Kasteren et al., 2012; Frias-Staheli et al., 2007). We therefore investigated whether post-translational modifications, in particular phosphorylation, affect the mobilities of nsp2-related proteins in SDS-PAGE. Nsp2-related proteins were immuno-precipitated (IP) from PRRSV-1 SD01-08-infected cells using a monoclonal antibody (mAb) that recognizes the N-terminal PLP2 domain region, which is common to all three proteins (Fang et al., 2012;
Li et al., 2014). Immunoprecipitated proteins were treated with λ-phosphatase (λPP) and compared to their untreated counterparts by SDS-PAGE and western blotting analysis. Dephosphorylation by λPP increased the apparent mobilities of all three nsp2-related proteins relative to the untreated samples (Fig. 2A), suggesting that the nsp2-related proteins are phosphorylated. Subsequently, we used a panel of anti-phosphoserine monoclonal antibodies (mAbs) to confirm the phosphorylation states of nsp2-related proteins. Anti-pSer mAbs #4A9 and #7F12 specifically recognized nsp2 protein, indicating that serine residues within nsp2 were phosphorylated. After λPP treatment, nsp2 bands with increased mobility were still recognized by mAbs #4A9 and #7F12 (Fig. 2B); however, the corresponding protein band intensities of the higher-mobility species of nsp2 were reduced by ~63% and ~49%, respectively (Fig. 2C), indicating that the complement of phosphorylated serine/threonine residues of nsp2 were partially dephosphorylated after λPP treatment, yielding hypo-phosphorylated nsp2-related proteins. It is noted that a new protein band appeared between serine687, serine838, serine859, serine863, serine897, serine918, and serine969 in nsp2, or between serine1027 and threonine1029 in nsp2TF. Among the identified sites, up to fifteen phosphorylated residues are located between the PLP2 domain and the –2/−1 programmed frameshifting site. This region is present in all three nsp2-related proteins. Residues serine867, serine838, serine859, serine863, serine902 and serine1010 are unambiguously phosphorylated in both nsp2 and nsp2TF. Tyrosine1253, threonine1373 and serine1440 are located in regions found only in nsp2, which include the predicted transmembrane (TM) domain and C-terminal domain. We assessed the conservation of these phosphorylation sites by quantifying their percentage of change in the genomes of other PRRSV isolates deposited in Genbank (Table 1). Serine879, serine827, serine838, serine859, serine863, serine897, serine918, tyrosine1253 and serine1440 showed high conservation with less than 10% of changes (Table 1).

2.2. Mapping phosphorylation sites to functional domains of nsp2-related proteins

To obtain insights into potential consequences and effects of nsp2-related protein phosphorylation on viral replication, we closely analyzed the sequences of nsp2-related proteins. We focused in particular on identifying predicted functional and structural features of regions that contain key phosphorylated sites. The nsp2, nsp2TF and nsp2N proteins contain a relatively conserved PLP2 domain, a small hypervariable region (HVR-1) N-terminal to the PLP2 domain, and two large hypervariable regions (HVR-2 and HVR-3) between the PLP2 domain and the –2/−1 PRF site. The HVRs, by definition, diverge widely between species (Fang and Snijder, 2010; Snijder et al., 2013), but can be defined as distinct domains because they are separated by a more conserved domain. Sequence analysis and structural modeling of HVR-2 and HVR-3 showed that these regions have low sequence...
In contrast, a conserved region between HVR-2 and HVR-3 exhibits several consistent structural features (Fig. 3). Based on structure prediction and sequence variation analysis, we define this region as an independent structured domain, termed the inter-HVR-conserved domain (IHCD). The N-terminal portion of the IHCD is predicted to primarily adopt random coil conformations, with relatively little predicted secondary structure (Fig. 4A). The C-terminal portion of the IHCD is predicted to contain a structured fold putatively consisting of 4 or 5 helices (α1-α4 or α4'). Moreover, three short peptides with uncharacteristically high inter-species conservation are present within the structured core (Fig. 4A; highlighted in grey color). Homology-based and ab initio structural modeling of the C-terminal portion of the IHCD using I-TASSER and Quark (Xu and Zhang, 2012; Xu et al., 2014), yields a number of helical bundle models with distinctive topologies (Fig. 4B). A lack of proteins/domains of known structure and with high sequence similarity to the IHCD prevents us from determining their precise location in the IF-1 reading frame after mass spectrometry identification by mass spectrometry.

Amino acids with bold font: identified phosphorylation sites in peptide analysis; #: ambiguously identified phosphorylation sites; #: phosphorylation sites that only exist in ORF1a reading frame; & Epitope sites were identified from previous study (de Lima et al., 2006). Percentage of conservation is generated by comparing all the available PRRSV-1 sequences in GenBank as of November 26, 2019.

Table 1
PRRSV-1 nsp2 and nsp2TF phosphorylation sites identification by mass spectrometry.

| Nsp2 residues (position in pp1a/pp2a) | Mass spec identified peptide | Nsp2TF residues (position in pp1a/pp2TF) | Mass spec identified peptide | Percentage of conservation | Localization in B-cell epitopesa |
|--------------------------------------|-----------------------------|-----------------------------------------|-----------------------------|---------------------------|---------------------------------|
| S687/S302                            | GFEGTASEAEQSGHK             | TLDKMTSPPERSGF                         | S667/S282                   | 3.4%                       |                                 |
|                                      |                             | S678/S302                               | SPGAVALGSPDAK               | 37.4%                      |                                 |
|                                      |                             | T665/T300                               | SFDRKFGTASEAEQSGHK          | 77.5%                      |                                 |
|                                      |                             | S687/S302                               | GFEGTASEAEQSGHK             | 84.7%                      |                                 |
| S685/S474                            | FSDGDSA                    | LLSLAAW                                | S827/S442                   | 1.7%                       |                                 |
|                                      |                             |                                       |                             |                           |                                 |
| S838/S453                            | TADPGWVR                   | AAWPVTAKATDGPW                         | S838/S453                   | 0%                         |                                 |
|                                      |                             | KATAIDPGWVR                            |                             |                           |                                 |
|                                      |                             | TADPGWVR                               |                             |                           |                                 |
|                                      |                             | FSDGDSAQ                               | S687/S302                   | 0%                         |                                 |
| S863/S478                            | FSDGDSAQFQGESL             | FLKPRKAFSDGDA                          | S687/S302                   | 23.1%                      |                                 |
| T896*/T511*                          | DAPVDTTSNEALSA             |                                       |                             |                           |                                 |
| S897*/S512*                          | DAPVDTTSNEALSA             | DAPVDTTSNEALSAADVP                    | S897/S512                   | 6.0%                       |                                 |
| S902/S517                            | DAPVDTTSNEALSAADVP        | DAPVDTTSNEALSAADVP                    | S902/S517                   | 10.3%                      |                                 |
| S1010*/S625                          | RAADSAGLQKVL               | RAADSAGLQKVL                           | S918/S533                   | 0%                         |                                 |
|                                      |                             | S1010*/S625                            | S1010*/S625                 | 25.6%                      |                                 |
| T1029*/T644                          | RWDKDSLVTPPPKSA            |                                       |                             |                           |                                 |
|                                      |                             | RWDKDSLVTPPPKSAGL                      |                             |                           |                                 |
|                                      |                             | SVTPPKSAGL                             |                             |                           |                                 |
|                                      |                             | KLVTPPPK                              |                             |                           |                                 |
|                                      |                             | LVVTPPPK                              |                             |                           |                                 |
| Y1253*/Y988*                         | ASSLYVVSSQGR               |                                       |                             |                           |                                 |
|                                      |                             | VLOAGGAVDQPTPEVVR                      |                             |                           |                                 |
| S1440*/S1055*                        | NQYPLRSASTXTG             |                                       |                             |                           |                                 |
|                                      |                             | LNQYPLRSASTXTTGG                      |                             |                           |                                 |

Table 2
Comparative analysis on intrinsically disordered regions between PLP2 and -2/-1 PRF

| HVR approximate positions (in pp1a) | Positions of putative IDRs between PLP2 and TM1 (in pp1a) |
|--------------------------------------|-----------------------------------------------------------|
|                                      | PPRRSV-1 nsp2 (SD01-08 strain)                          |
|                                      | HVR-2: 665aa-825aa                                       |
|                                      | 657aa-819aa                                             |
|                                      | 661aa-781aa                                             |
|                                      | 680aa-913aa                                             |
|                                      | 728aa-758aa                                             |
|                                      | 655aa-710aa                                             |
|                                      | 727aa-780aa                                             |
|                                      | 799aa-814aa                                             |
|                                      | 863aa-900aa                                             |
|                                      | 1003aa-1066aa                                           |
|                                      | 610aa-1085aa                                            |
|                                      | 968aa-1077aa                                            |
|                                      | 998aa-1075aa                                            |
|                                      | 1005aa-1079aa                                           |
|                                      | 1013aa-1064aa                                           |
|                                      | 842aa-993aa                                             |
|                                      | 752aa-1014aa                                            |
|                                      | 628aa-661aa                                             |
|                                      | 683aa-739aa                                             |
|                                      | 809aa-856aa                                             |
|                                      | 899aa-936aa                                             |
|                                      | 948aa-979aa                                             |
|                                      | 655aa-1213aa                                            |
|                                      | 1185aa-1222aa                                           |
|                                      | 1108aa-1227aa                                           |
|                                      | 1115aa-1205aa                                           |
|                                      | 1156aa-1221aa                                           |
|                                      | 1149aa-1213aa                                           |

Amino acids with bold font: identified phosphorylation sites in peptide analysis; #: ambiguously identified phosphorylation sites; #: phosphorylation sites that only exist in ORF1a reading frame; & Epitope sites were identified from previous study (de Lima et al., 2006). Percentage of conservation is generated by comparing all the available PRRSV-1 sequences in GenBank as of November 26, 2019.
serine\(^{827}\), serine\(^{838}\), serine\(^{859}\), serine\(^{863}\), serine\(^{897}\), and serine\(^{902}\), while serine\(^{918}\) is located in the C-terminal structured core of the IHCD (Fig. 3). Further in-depth sequence analysis showed that Serine\(^{918}\) is located within the first inter-species short conserved motif (Fig. 4A).

2.3. Serine\(^{918}\)-phosphorylation regulates viral infectious particle production and accumulation of subgenomic RNAs

To determine whether nsp2-related protein phosphorylation is involved in critical viral functions, we introduced phospho-ablatant mutations into a full-length cDNA infectious clone of PRRSV-1 SD01-08 (Li et al., 2013). We mutated the relevant serines or threonines to alanines and replaced tyrosines with phenylalanines. Each residue was changed individually, while adjacent mutations were also combined (Fig. 5). The phosphorylation site Tyr\(^{1253}\) is located in a sequence that overlaps with the \(-2\) ORF, although it is located within the nsp2-coding region. To avoid non-target mutation in the corresponding amino acid of nsp2TF, we introduced the Y1253F mutation into a previously constructed mutant (KO2), in which the expression of ns2TF and nsp2N was knocked out, but the nsp2 sequence was not affected (Fang et al., 2012).

Using this panel of mutants, we first determined the impact of nsp2-related protein phosphorylation on viral production. The full-length cDNA infectious clone of each mutant was transfected into BHK-21 cells and cell culture supernatant was harvested at 48 h post transfection, followed by measurement of viral titer. Compared to the wild type virus, the mutant S918A showed the lowest viral yield, with the titer decreased by one log (Fig. 5).

We confirmed this result by determining the impact of serine\(^{918}\) mutation on accumulation of viral genomic RNA (gRNA) and subgenomic RNA (sgRNA). Initially, viral RNA was transcribed from the T7 promoter-driven cDNA infectious clone of the S918A mutant and transfected into BHK-21 cells. The expression levels of both plus (+) and minus (−) strand gRNA and sgRNA were measured by qRT-PCR at 18 hpt. In comparison to the WT virus, the S918A mutant showed lower levels of (+) and (−) gRNA, with (+) and (−) mutant gRNA/WT gRNA ratios of 69.5% and 58.6%, respectively (Fig. 6A and B). The relative accumulations of three representative sgRNAs, sgRNA 2, 6 and 7, were significantly reduced in S918A transfected cells. The ratio of (+) sgRNA2/6/7 to (+) gRNA was reduced by 62.3%, 57.3%, and 47.6%, respectively (Fig. 6A). Similarly, the relative ratios of (−) sgRNA2/6/7 were reduced by 78.4%, 51.2%, and 60.1%, respectively (Fig. 6B). To further confirm the importance of S918 phosphorylation on viral RNA expression, a phospho-mimetic substitution S918E was included in this assay. As shown in Fig. 6, the S918E mutation showed higher levels of viral gRNA and sgRNA expression compared to that of S918A mutant. We obtained a similar result from virus-infected MARC-145 cells, in which S918A mutant showed large reduction in viral RNA expression compared to that of WT virus, while S918E mutant showed much less decrease in the level of viral RNA expression compared to the WT virus (Fig. 7). In contrast, the accumulated levels of nsp1β and N protein were slightly decreased by the S918E mutation, with about 32% and 16% in S918E mutant infected cells compared to those in WT virus (Fig. 7).

Next, we compared the in vitro growth kinetics of S918 mutants to that of WT virus. PRRSV-permissive MARC-145 cells were infected with WT virus or a S918 mutant (passage 0). Cell cultural supernatants were harvested at selected time points post-infection and virus titers were determined. Compared to WT virus, S918A mutant showed significantly attenuated replication ability in MARC-145 cells (Fig. 8A). At 60 hpi, WT virus reached peak viral titer of 5.83 log\(_{10}\) FFU/ml, while S918A mutant reached a viral titer of 4.47 log\(_{10}\) FFU/ml. Consistent with the result of viral RNA expression levels, the S918E mutant showed viral
Fig. 4. Sequence analysis of inter-HVR conserved domain (IHCD) in PRRSV nsp2-related proteins. (A) IHCD regions from representative PRRSV strains were aligned in CLC Main Workbench 8.1 (CLC bio). Highly conserved sequences are highlighted by grey shading. Secondary structures predicted by I-TASSER modeling of PRRSV-1 and PRRSV-2 IHCD sequences are shown above or beneath corresponding amino acids (cylinders indicate $\alpha$-helices; arrows indicate $\beta$-sheets). Sequences putatively corresponding to $\alpha$-helix and $\beta$-sheet structures are shown in blue and magenta. Predicted structures with high confidence are highlighted in underlined bold font. Sequence conservation measure and logo obtained using CLC Main Workbench 8.1 (CLC bio) are shown underneath. Phosphorylation sites are labeled with red triangles. The critical phosphorylation site serine918 is indicated by a red frame. Sequences of representative PRRSV strains were obtained from Genbank, including SD01-08 (Genbank DQ489311.1), Lelystad virus (LV) (Genbank M96262.2), Iena (Genbank JF802085.1), EuroPRRSV (Genbank AY366525.1) and PRRSV-2 strains-VR-2332 (Genbank AY150564.1), MN184A (Genbank DQ176019.1), JXA1 (Genbank EF112445.1), NADC30 (Genbank JN654490.1), and NVSL 97–7895 (Genbank AY545985.1). (B) Representative structural predictions of the C-terminal structured region of the IHCD. B1 and B2 show homology models of PRRSV-1/2 sequences. Similar I-TASSER homology modeling predictions from multiple PRRSV-1 and/or PRRSV-2 family members are shown superimposed and colored in a blue-to-red spectrum (N-terminus to C-terminus of modeled sequence). B3 and B4 are ab initio predicted models including helical bundles and super-helical (helix-of-helices) arrangement for of PRRSV-1/2 sequences.
growth ability greater than that of the S918A mutant, with higher peak viral titer (5.01 log\textsubscript{10} FFU/ml) at 60 hpi. The cytolytic ability of mutant S918A was severely attenuated with no visible plaque detected in MARC-145 cells at 4 days post infection (Fig. 8B). IFA results confirmed that the S918A mutant infected the cells; however, this mutant resulted in smaller viral clusters than the WT virus. In contrast, S918E mutant exhibited higher growth ability with visible plaques and larger viral clusters than that observed for S918A mutant (Fig. 8B and C).

Fig. 5. Rescue efficiencies of phospho-ablatant mutants. Individual PRRSV cDNA infectious clones of phospho-ablatant mutation were individually transfected into BHK-21 cells. Cell culture supernatants were harvested 48 h post transfection and virus titers were determined. Each data point represents the mean value of triplicates.

Fig. 6. Phosphorylation at serine\textsuperscript{918} influences relative accumulation of viral RNAs. In vitro transcribed genomic RNA of WT virus and mutants S918A and S918E were transfected into BHK-21 cells. At 18 h post transfection, BHK-21 cells were lysed and qRT-PCR was performed using the specific primers and probes (Table S1, Fig. S3) to quantify the plus and minus genomic RNA and subgenomic RNA. Ratios of plus-strand (A) and minus-strand (B) RNA were calculated by \( \Delta \Delta Ct \) method (Pfaffl, 2007; Rao et al., 2013). Each data point represents the mean value of triplicates. *: p < 0.05.

Fig. 7. Effect of serine\textsuperscript{918} phosphorylation on viral nonstructural and structural protein expression. In vitro transcribed genomic RNA of WT virus and mutants S918A and S918E were transfected into BHK-21 cells. Expression of viral nsp1\( \beta \) and N protein in BHK-21 cells at 18 h post transfection was detected by western blotting using \( \alpha \)-nsp1\( \beta \) m Ab #22–28 and \( \alpha \)-N mAb #14–126. Pixel intensity was quantified using Image Studio 5.2 (Li-Cor Biosciences). Normalization was performed using a monoclonal antibody specific for the host cell housekeeping protein Tubulin.
Stabilities of these mutants were further studied by serially passing the viruses in cell culture. At passage 2, we observed a significant increase of viral titer for the S918A mutant (4.57 log\(_{10}\) FFU/ml at P1 versus 5.47 log\(_{10}\) FFU/ml at P2), which reached levels similar to those of WT virus at P3 and P4 (Fig. 9A). At P3, the S918A mutant showed similar plaque size as that of the WT virus in infected cells (Fig. 9B), apparently recovering from its initially impaired growth ability caused by mutating the S918 phosphorylation site. Sequencing analysis of the nsp2 region showed that the S918A mutation is stable through different passages. However, we detected a second site mutation at amino acid 917 of nsp2 (H917Y, next to S918A), starting from passage 2 (Fig. 9C). The sequence RRPRYAAQA generated from the second site mutation is a predicted phosphorylation site of Tyrosine kinase, suggesting that the H917Y mutation may provide an alternative, functionally redundant phosphorylation site to the site eliminated by S918A mutation. These results further confirm the important role of S918 phosphorylation in the viral replication.

3. Discussion

As the most ubiquitous form of post-translational modification, protein phosphorylation modulates essentially all fundamental biological activities in a cell (Humphrey et al., 2015; Hunter, 1995; Johnson, 2009; Tarrant and Cole, 2009; Ubersax and Ferrell, 2007). Viruses exploit host phosphorylation mechanisms to carry out critical viral functions and processes, including survival and replication (Keating and Striker, 2012; Keck et al., 2015). The effects of phosphorylation of viral proteins on viral replication are broadly reported and have been characterized in both RNA and DNA viruses (Keating and Striker, 2012; Keck et al., 2015). Previous characterization of protein phosphorylation in nidoviruses, including murine hepatitis virus (MHV), infectious bronchitis virus (IBV), transmissible gastroenteritis coronavirus (TGEV), severe acute respiratory syndrome coronavirus (SARS-CoV), equine arteritis virus (EAV), and PRRSV focused exclusively on the N proteins of these viruses (McBride et al., 2014; Wu et al., 2009; White et al., 2007; Surjit et al., 2005; Stohlman and Lai, 1979; Spencer et al., 2008; Peng et al., 2008; Mohandas and Dales, 1991; Chen et al., 2005; Chang et al., 2009; Calvo et al., 2005; Zeegers et al., 1976). The
biological consequences of such phosphorylation events on viral replication were extensively investigated in coronaviruses (McBride et al., 2014). For example, the phosphorylation state of the IBV N protein regulates its affinity for the viral leader sequence, suggesting that phosphorylation regulates RNA transcription in IBV (Spencer et al., 2008; Chen et al., 2005). Phosphorylation by the host GSK3 kinase affects conformational transitions of the MHV N protein and regulates its interaction with the DDX1 helicase to fine-tune the discontinuous transcription of sgRNAs (Wu et al., 2014). Phosphorylation of the N protein of PRRSV has also been reported (Wootton et al., 2002; Yoo et al., 2003; Chen et al., 2018, 2019). Phospho-ablating mutations on two PRRSV N protein phosphorylation sites attenuated viral replication capability both in vitro and in vivo (Chen et al., 2018, 2019). However, specific biological roles of PRRSV N protein phosphorylation remain largely unclear.

In our study, we demonstrate for the first time the importance of phosphorylation of PRRSV proteins other than N protein in viral replication. We determined that PRRSV nsp2-related protein phosphorylation critically modulates viral RNA expression, especially expression levels of sgRNAs. Previous studies demonstrated that nsp2 plays a primary role in viral replication by proteolytically processing replicase polyproteins through its PL2P domain (Wassenaar et al., 1997). Arterivirus nsp2 was also reported to play an important role in the formation of double membrane vesicles (DMVs) that anchor the replication transcription complex (Snijder et al., 2001, 2013; Posthuma et al., 2008). The identification of two additional frameshifting products, nsp2TF and nsp2N, increases the functional versatility and also the complexity of potential roles played by nsp2 (Snijder et al., 2013; Fang et al., 2012; Li et al., 2014, 2018). We observed that all three nsp2-related proteins (nsp2, nsp2TF and nsp2N) are phosphorylated. Mutations introduced into the phosphorylation site serine918 (S918A) attenuated viral replication in vitro. In particular, disrupting the phosphorylation status of serine918 significantly downregulates steady-state levels of viral sgRNAs accumulation. Whether nsp2-related proteins, working as trans-acting factor(s), directly regulate arterivirus discontinuous transcription warrants further investigation. In PRRSV-infected cells, nsp2TF exhibits a different subcellular localization pattern than that of nsp2 (Fang et al., 2012). This suggests that the phosphorylation state of these proteins may regulate viral replication in different contexts, by different mechanisms, or that not all of the nsp2-related proteins are involved in sRNA synthesis.

Compared with other conserved domains in nsp2-related proteins, the region between the PL2P domain and −2/−1 PRF site is poorly defined in terms of structure and function. Based on sequence and structural prediction analysis, we subdivided this large region into two HVRs separated by a relatively conserved inter-HVR region that contains a highly conserved domain (IHCD) in both PRRSV-1 and PRRSV-2. Previous studies examined the consequences of deletions introduced into segments that overlap with the HVRs and the IHCD. In PRRSV-1 (strain SD01-08), HVR-2 contains two known immuno-dominant epitopes, ES3 (residues 691–722 of pp1a) and ES4 (residues 736–790 of pp1a). HVR-3 contains the epitope ES7 (residues 1015–1040 of pp1a), while another epitope, ES5 (residues 822–832 of pp1a) is located within the N-terminal portion of the IHCD. In addition, the epitope ES6 (residues 895–923 of pp1a) is located at the junction of two portions of the IHCD (Oleksiewicz et al., 2001; Chen et al., 2010) (Fig. 4). Our previous studies demonstrated that deletion of the ES3 epitope caused enhanced cytolysis and more vigorous in vitro growth kinetics, while ES4 and ES7 deletion mutants exhibited attenuated in vitro growth properties. In contrast, deletion of epitopes ES5 and ES6, located within the IHCD, was lethal and no viable virus was rescued. In vivo characterization of ES3, ES4, and ES7 deletion mutants in infected pigs showed levels of viremia consistent with the in vitro effects of epitope deletion. In addition, mRNA and protein expression levels of IL-1β and TNF-α were downregulated in ES3 deletion mutant-infected macrophages (Chen et al., 2010). Another study identified specific regions of nsp2 that are dispensable for replication of PRRSV-2 (strain VR-2932) (Han et al., 2007). Deletion of residues 707–906 in pp1a (residues 324–523 in nsp2) and 926–1015 in pp1a (residues 543–632 in nsp2), which overlap with HVR-2, resulted in viable recombinant viruses, but incurred severe fitness loss. Similarly, deletion of 1016–1109 in pp1a (residues 633–726 in nsp2), and 1110–1196 in pp1a (residues 727–813 in nsp2), which respectively overlap with the IHCD and partially overlap with HVR-3, also had severe effects on viral fitness (Han et al., 2007). Furthermore, a deletion in the IHCD C-terminal portion (residues 1011–1130 of pp1a) that occurred naturally during an in vitro passage attenuated a highly pathogenic PRRSV-2 strain to an avirulent vaccine strain-TJM (F92) (Leng et al., 2012). These results indicate that HVR-2/HVR-3 and IHCD potentially interact with the host immune system and that these regions are associated with viral fitness and replication ability.

In the current study, mass-spectrometric analysis of PRRSV-1 identified that most of the phosphorylation sites are located in the HVRs and inter-HVR regions, in which nsp2 contains 6 phosphorylated sites and nsp2TF contains 7 sites. As described above, PRRSV nsp2-related proteins are enriched with immune-dominant B-cell epitopes, as well as predicted T-cell epitopes (Snijder et al., 2013; Oleksiewicz et al., 2001; Chen et al., 2010; de Lima et al., 2006; Fang et al., 2004). Interestingly, serine827 is located in epitope ESS; threonine896 (or serine897), serine902 and serine918 are located in epitope ES6, and serine902 and threonine1028 are located within epitope ES7 (Table 1). We previously demonstrated that the epitopes ESS, E56, and ES7 in IHCD must be fully intact for the viability or fitness of the virus (Chen et al., 2010). The functional importance of nsp2 phosphorylation sites other than serine918 needs to be further elucidated.

Our sequence analysis showed that the region between the PL2P domain and PRF site contains intrinsically disordered regions (IDRs), which have only low levels of sequence similarity to any other known proteins or protein domains. To investigate whether the hypervariable and potentially disordered nature of these regions in PRRSV apply to other arteriviruses, we used Pondr VL-XT software (Bomma et al., 2012) to analyze the sequences of representative species of five genera in the family Arteriviridae (Gulyaeva et al., 2017). In all of these species, regions between the PL2P and the putative transmembrane region TM1 are predicted to contain IDRs of variable length (Fig. S2). Structural flexibility in the segments between PL2P and TM1 seems to represent a general property of the nsp2-related proteins of the arterivirus family. In general, IDRs and IDPs (intrinsically disordered proteins) lack hydrophobic residues to support formation of well-defined and folded hydrophobic cores and do not adopt stable tertiary structures in the absence of binding partners (Wright and Dyson, 2015; Van Der Lee et al., 2014; Oldfield and Dunker, 2014; Uversky, 2013; Dyson and Wright, 2005). Conformations of IDPs/IDRs are more typically represented as dynamic ensembles. Such disordered regions can present highly diversified binding sites for other macromolecular partners. As intracellular parasites, the life cycle of viruses depends critically upon interactions between viral proteins and host components. The absence of strong structural constraints correlates with less restriction on sequence selection and higher tolerance to sequence substitution, promoting sequence variability (Van Der Lee et al., 2014; Oldfield and Dunker, 2014; Uversky, 2013; Xue et al., 2014; Gitlin et al., 2014). Furthermore, looser constraints on conformation permit dynamic display of post-translational modification sites, including phosphorylation sites, to facilitate transient interactions with modifying enzymes. Similarly, structural flexibility allows for ready access and recognition of modified sites for downstream interactions (Van Der Lee et al., 2014; Oldfield and Dunker, 2014; Uversky, 2013).

The functional consequences of structural disorder are commonly found in the proteomes of viruses, especially for RNA viruses (Wright and Dyson, 2015; Van Der Lee et al., 2014; Oldfield and Dunker, 2014; Uversky, 2013; Dyson and Wright, 2005; Xue et al., 2014). Structural flexibility can endow viral proteins from compact RNA genome with the
ability to carry out multiple functions in viral life cycles, and is compatible with the low-fidelity transcription typically mediated by viral RNA polymerases (Van Der Lee et al., 2014; Xue et al., 2014). For example, the Hepatitis C virus nonstructural protein NS5A contains two disordered domains (D2 and D3), which are believed to serve as protein-protein interaction (PPI) hubs (Shanmugam et al., 2018; Badillo et al., 2017; Sólyom et al., 2015). The D2 domain interacts with the RNA-dependent RNA polymerase NSSB and the host innate immune receptor Protein Kinase R (Shanmugam et al., 2018; Badillo et al., 2017; Sólyom et al., 2015). Both D2 and D3 domains mediate the interaction between NS5A and the critical host factor cyclophilin A (CypA), facilitating the formation of an NS5A-NSSB transcription and replication complex. The disordered D2 domain tolerates large deletions without obvious fitness loss. In alphaviruses, the c-terminal region of nsP3 proteins contain a large hypervariable domain, which is also mostly disordered and hyper-phosphorylated (Götte et al., 2018; Frolov et al., 2017; Rupp et al., 2015). The nsP3 plays critical roles in the formation of viral replication complexes (vRCs) and also regulates RNA transcription. The HVD of nsP3 is enriched with functional PPI motifs, including a Src homology 2 (SH2) domain-binding YXXM motif that interacts with the PI3K-Akt-mTOR pathway and an SH3 domain-binding PXPxPR motif that interacts with host proteins of the amphiphysin family (Götte et al., 2018; Rupp et al., 2015). Importantly, repetitive motifs in the nsP3 HVD mediate critical binding to host stress granule (SG) proteins including G3BP1/2 and/or FXR family proteins (Götte et al., 2018; Frolov et al., 2017; Rupp et al., 2015). A growing number of studies show that disordered HVDs serve as PPI hubs, contributing to viral adaptation to the host environment, viral fitness and pathogenicity (Götte et al., 2018; Frolov et al., 2017; Chetan et al., 2018). In the case of PRRSV nsP2-related proteins, the two large putative IDRs associated with the HVRs and hyper-phosphorylation sites support the notion of a functional connection between sequence variability and mechanistic roles. Given that nsP2-related proteins contain the most apparent disordered regions in the viral proteome, the hyper-phosphorylated IDRs in nsP2-related proteins may function as evolutionary “hot-spots” for rapid adaptation to changing host environments, including immunological and physiological conditions, and to the very diverse range of host genetic backgrounds.

The disordered HVRs are separated by a conserved sequence region that we termed the inter-HVR conserved domain (IHCD). Unlike the HVRs, the C-terminal portion of the IHCD is likely to have a stable tertiary structure. Both homology modeling and ab initio structural predictions suggest that the C-terminal ~100 residues of this region are primarily alpha-helical and that the helices pack as a bundle. The topology of the overall fold cannot be unambiguously predicted as multiple topologies allow for plausible helix packing and a buried hydrophobic core. However, all the prediction software generated similar results for the PRRSV-1 and PRRSV-2 sequences, suggesting that this region likely adopts a common domain fold in both species. In contrast to the C-terminal structured domain, the N-terminal ~70–80 residues of the IHCD are predicted to contain little canonical secondary structure and may be conformationally dynamic or may adopt atypical tertiary structures. These sequences are relatively conserved within the PRRSV-1 and PRRSV-2 species. The conserved disorder and paucity of canonical secondary structure may be required for accessibility of the sequences to specific trans-acting factors and binding partners, including specific kinases that target the high density of phosphorylation sites in this region. It is noteworthy that the N-terminal portions of the IHCDs of PRRSV-1 and -2 sequences contain several instances of so-called PXDLS motifs. These linear motifs serve as binding sites for C-terminal binding proteins (CtBPs) (Chinnadurai, 2007), which may potentially interact with the PI3K-Akt-mTOR pathway and an SH3 domain-binding PXPxPR motif that interacts with host proteins of the amphiphysin family (Götte et al., 2018; Rupp et al., 2015).

4. Materials and methods

Cells, viruses: BHK-21 cells and MARC-145 cells were cultured in minimum essential medium (MEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) at 37°C with 5% CO2. For the maintenance of infected MARC-145 cells, 10% FBS was replaced by 2% horse serum (HyClone, Logan, UT). The PRRSV-1 strain (SD01-08) was used in the following experiments. SD01-08 was isolated in 2001 in the US and subsequently used to construct full-length cDNA infectious clones (Fang et al., 2006; Li et al., 2013).

Antibodies: All PRRSV-1 specific antibodies used in this study were targeted to the strain SD01-08. Monoclonal antibodies (α-nsP2 mAbs #36–19 and #58–46, α-N-Mab #14–126, and α-nsP1β mAb #22–28) and polyclonal antibody (pAb) against the PL2P domain were generated as described in our previous studies (Fang et al., 2012; Li et al., 2012; Guo et al., 2016). The pAb specifically recognizes the TF peptide of nsP2TF was generated by GenScript Biotech Corporation (Piscataway, NJ) as we described previously (Fang et al., 2012). A panel of six specific anti-phosphorylated serine (pser) mAbs was from a phosphoserine detection kit (Enzo Life Sciences, Ann Arbor, MI). The secondary antibodies, IRDye 800CW-conjugated goat anti-mouse IgG (H + L) and IRDye 680RD-conjugated goat anti-rabbit IgG (H + L), were purchased from Li-Cor Biosciences (Lincoln, NE), while Alexa-488 conjugated goat anti-mouse IgG and Alexa-594 conjugated goat anti-rabbit IgG were purchased from Jackson Immoneresearch (West Grove, PA). The anti-tubulin mAb (B-7) was obtained from Santa Cruz Biotechnology (Dallas, TX).

Mass spectrometric analysis: PRRSV nsP2-related proteins were pulled down from PRRSV-infected MARC-145 cells by immunoprecipitation (IP) as described previously (Fang et al., 2012). Virus-infected or mock-infected cells were harvested in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Carlsbad, CA). To remove non-specifically bound proteins, cell lysates were pre-cleared with protein A Sepharose CL-4B beads (GE Healthcare Biosciences, Pittsburgh, PA) and a non-specific mouse mAb at room temperature (RT) for 1 h. After centrifugation, cleared supernatant was transferred to a fresh tube containing mAb #36–19 and fresh Sepharose CL-4B beads. The mAb-protein complex was incubated with constant rotation at 4°C overnight. The sepharose beads were washed six times with wash buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate), followed by six washes with MiliQ water. Bound proteins were eluted and denatured in 2x Laemmli sample buffer (Bio-Rad, Hercules, CA) at 95°C for 10 min. PRRSV nsP2-related proteins were separated and analyzed by SDS-PAGE gel, fixed, and stained with Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Hercules, CA). Bands corresponding to PRRSV nsP2 and nsP2TF were excised from the gel and subjected to mass spectrometric analysis. Phosphorylation analysis was performed at the Harvard Microchemistry and Proteomics Analysis Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer.

Immuno-detection of phosphorylated nsP2-related proteins
and assessment of protein mobility in SDS-PAGE: Immunoprecipitated proteins were separated by SDS-PAGE and transferred onto nitrocellulose (NC) membranes (GE Healthcare Bio-Sciences, Pittsburgh, PA). A manufacturer recommended protocol was applied for phosphorylation immuno-detection using specific anti-pSer mAbs. Phosphate-buffered saline (PBS) with 1% BSA (MP Biological, Solon, OH), 1% PVP-10 (polyvinyl-pyrrolidone) (Sigma-Aldrich, St. Louis, MO), 1% PEG 3500 (Sigma-Aldrich, St. Louis, MO), and 0.2% Tween 20 (Sigma-Aldrich, St. Louis, MO) was used as blocking buffer and antibody diluent. NC membranes were blocked for 2 h at RT and incubated with diluted primary antibody for 1 h at RT. After three washes with 1x PBST (0.05% Tween-20), membranes were incubated with secondary antibodies for 45 min at RT. Following three washes with 1x PBST (0.05% Tween-20), protein bands were visualized using an Odyssey Fc imaging system (Li-Cor Biosciences, Lincoln, NE). Equivalent incubation and wash procedures were applied to membranes after a second incubation with antibodies against nsp2-related proteins. To assess differential mobilities of phosphatase-treated proteins in SDS-polyacrylamide gels, proteins bound to Seaphorose beads in the IP process were dephosphorylated using Lambda Protein Phosphatase (xPP) (New England Biolabs, Ipswich, MA) at 30°C for 2 h. In control samples, xPP was replaced by an equal volume of water. Migration of nsp2-related proteins after treatment was evaluated by SDS-PAGE and Western blot using the methods described previously (Li et al., 2014; Shang et al., 2017).

**PRRSV reverse genetics for generating recombinant viruses:** Site-directed mutations were introduced into the coding region of nsp2-related proteins of a PRRSV SD01-08 full-length cDNA infectious clone, pCMV-SD01-08, as described previously (Li et al., 2013). To recover the cloned virus, BHK-21 cells were transfected with the cDNA infectious clone of the wild type (WT) virus or its mutant. Transfection was conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following growth characterization of recombinant viruses: Each recombinant virus was serially passaged on MARC-145 cells 4 times. At each passage, cells were infected by each virus at multiplicity of infection (MOI) of 0.01. Virus titers were measured by fluorescent-focus assay on MARC-145 cells and calculated as fluorescent focus units per ml (FFU/ml) using the method described previously (Sun et al., 2012). The specific modification was confirmed by Sanger sequencing of the entire nsp2 region. In vitro growth kinetics of WT and mutant viruses (P0) were determined by infecting MARC-145 cells at 0.01 moi. Supernatants of infected cells were collected at 0, 12, 24, 36, 48, 60, and 72 h post-infection (hpi) and virus titers were measured by fluorescent-focus assay as described previously (Sun et al., 2012). Plaque morphologies of the WT and mutant viruses were compared by a plaque assay as described previously (Fang et al., 2012). Fluorescent images were taken under a confocal microscope (Nikon A1R HD25, Nikon).

**In vitro transcription:** The plasmid DNA of full-length cDNA infectious clone pSD01-08 (Fang et al., 2006) was linearized by XbaI, and the digested DNA was extracted using phenol extraction and ethanol precipitation method. Linearized DNA (2 μg) was used as template for in vitro RNA transcription. In vitro transcription was performed in 50 μl reaction condition at 37°C for 2 h: 5 μl RNPoly Reaction Buffer (New England Biolabs, Ipswich, MA), 5 μl ATP (10 mM), 5 μl UTP (10 mM), 5 μl CTP (10 mM), 2.5 μl GTP (10 mM) (New England Biolabs, Ipswich, MA), 7.5 μl m7G(5′)ppp(5′)G RNA Cap Structure Analog (10 mM) (New England Biolabs, Ipswich, MA), 2 μl T7 RNA Polymersase (New England Biolabs, Ipswich, MA), 2 μg linearized infectious clone plasmid, 2 μl RNaseOUT™ RNase inhibitor (Invitrogen, Carlsbad, CA). Then, 2 μl TURBO™ DNase (Invitrogen, Carlsbad, CA) was added into the reaction system to digest DNA templates for 30 min at 37°C. Newly transcribed RNAs were purified by NucAway™ Spin Columns (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction. Subsequently, in vitro transcribed RNAs (1.5 μg) were transfected into BHK-21 cells using Lipofectamine™ MessengerMAX™ Transfection Reagent (Thermo Fisher Scientific) with a 1:4 ratio of genomic RNA to MessengerMAX. Cell lysate and supernatant were collected at 18 post transfection (hpt).

**Genomic and subgenomic viral RNA quantification:** For RNA quantification, cellular total RNAs were extracted using the SV Total RNA Isolation Kit (Promega, Madison, WI). Equal amounts (1.5 μg) of total RNAs were reverse transcribed using the Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific). Plus-strand genomic RNA, plus-strand subgenomic RNA, and minus-strand genomic/subgenomic RNA were reverse transcribed in three different reactions using three different primers (Table S1, Fig. S3). Real-time PCR was performed in 20 μl reactions containing 10 μl TaqMan™ Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA), 0.25 μl forward primer (40 μM), 0.25 μl reverse primer (40 μM), 0.4 μl Taqman probe (10 μM), 2 μl cDNA, 7.1 μl H2O. Primer/probe sets were synthesized by Biosearch Technologies (Petaluma, CA). PCR reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The PCR protocol was selected based on TaqMan™ Fast Advanced Master Mix user’s instructions: 50 °C UNG incubation for 2 min, 95 °C polymerase activation for 2 min, followed by 40 cycles of denaturation cycles at 95°C for 3 s and annealing/extension at 60°C for 30 s. The relative accumulation ratio of RNAs was quantified by the threshold cycle (∆∆CT) method (Pfaffl, 2007; Rao et al., 2013). To precisely quantify RNA accumulation and to rule out possible background interference from initial transfected RNA, we constructed a replication-defective mutant, in which the nucleotide sequence from 495 nt to 515 nt was replaced with 7 consecutive stop codons to terminate translation of nonstructural polyproteins. The (+) gRNA detected from BHK-21 cells transfected with the defective mutant (+) gRNA was designated as background. For (+) gRNA quantification, the background signal derived from transfection was first subtracted.

**Sequence analysis and protein structure prediction:** Multiple sequence alignments were performed using the built-in ClustalW program of CLC Main Workbench 8.1 (CLC bio, Aarhus, Denmark). Intrinsic disorder prediction was conducted using multiple online programs including Pondr-FIT (Xue et al., 2010), Pondr VL-XT (Bonma et al., 2012), DISOPRED3 (Jones and Cozzetto, 2014), MobiDB 3.0 (Piwayne et al., 2017), and metaPrDOS (Ishida and Kinoshita, 2008). Protein secondary structure profiles and tertiary structure predictions were generated by homology modeling using I-TASSER (Yang et al., 2015). For homology modeling, no template models were provided as input. Each PRRSV-1 and PRRSV-2 species member sequence was modeled independently, and a consensus secondary structure profile was generated based on these predictions. Models with C-scores better than –3.0 were assessed further and grouped by overall structural similarity. Ab initio models for each sequence were also generated using Quark (Xu and Zhang, 2012). Predictions were assessed according to I-Tasser/Quark scoring (1–10 scale), with individual residue scores of 4–5 designated as “low-confidence” and 6–10 as “high-confidence”. Predicted structural models of similar topology and overall structure were evaluated, aligned and figures were generated using open-source PyMol 1.7 (The PyMol Molecular Graphics System, Version 1.7 Schrödinger, LLC).

**Statistical significance analysis:** All data in current study were shown as mean values with standard deviations. Each data represents three replicates. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test using GraphPad Prism 6 (GraphPad, La Jolla, CA). P-values were indicated by asterisks in figures. *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001. CRediT authorship contribution statement

Pengcheng Shang: Conceptualization, Methodology, Formal analysis, Writing - original draft. Fangfeng Yuan: Methodology, Formal
analysis. Saurav Misra: Formal analysis, Writing - review & editing. Yanhua Li: Methodology, Formal analysis. Ying Fang: Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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