Label-Free Quantitative Phosphoproteomic Analysis Reveals Differentially Regulated Proteins and Pathway in PRRSV-Infected Pulmonary Alveolar Macrophages

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Supporting Information

ABSTRACT: Porcine reproductive and respiratory syndrome virus (PRRSV) is an important pathogen of swine worldwide and causes significant economic losses. Through regulating the host proteins phosphorylation, PRRSV was found to manipulate the activities of several signaling molecules to regulate innate immune responses. However, the role of protein phosphorylation during PRRSV infection and the signal pathways responsible for it are relatively unknown. Here liquid chromatography−tandem mass spectrometry for label-free quantitative phosphoproteomics was applied to systematically investigate the global phosphorylation events in PRRSV-infected pulmonary alveolar macrophages. In total, we identified 2125 unique phosphosites, of which the phosphorylation level of 292 phosphosites on 242 proteins and 373 phosphosites on 249 proteins was significantly altered at 12 and 36 h pi, respectively. The phosphoproteomics data were analyzed using ingenuity pathways analysis to identify defined canonical pathways and functional networks. Pathway analysis revealed that PRRSV-induced inflammatory cytokines production was probably due to the activation of mitogen-activated protein kinase and NF-κB signal pathway, which were regulated by several protein kinases during virus infection. Interacting network analysis indicated that altered phosphoproteins were involved in cellular assembly and organization, protein synthesis, molecular transport, and signal transduction in PRRSV infected cells. These pathways and functional networks analysis could provide direct insights into the biological significance of phosphorylation events modulated by PRRSV and may help us elucidate the pathogenic mechanisms of PRRSV infection.

KEYWORDS: PRRSV, PAMs, quantitative phosphoproteomic, label-free, pathway analysis

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), which is characterized by severe reproductive failure in sows and acute respiratory distress in pigs of all ages, is one of the most economically important diseases impacting swine industry worldwide.1 The etiological agent, PRRS virus (PRRSV), is an enveloped, positive-strand RNA virus and belongs to the genus Arteriviridae together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV).2 The approximate 15 kb genome encodes at least 10 open reading frames (ORFs): ORF1a, ORF1b, ORF2a, ORF2b, ORF3−7, and the newly identified ORF5a,3,4 ORF1a and ORF1b encode two large polyproteins, which are predicted to be cleaved into 14 nonstructural proteins by the papain-like cysteine protease (PCP) in Nsp1,5,6 while the other eight ORFs encode seven membrane proteins (GP2, E, GP3, GP4, GP5, M, and the newly identified GP5a) and a nucleocapsid (N) protein.7 Pulmonary alveolar macrophages (PAMs) are the most permissive to PRRSV infection and multiplication in the natural host.8,9 After PRRSV infection, PAMs are functionally damaged and destroyed, eventually leading to pigs more susceptible to secondary infections.10,11

Quantitative proteomics approaches have been widely used in the analysis of host cellular responses to virus infection, such as influenza A virus,12 pseudorabies virus,13 infectious bronchitis virus,14 human respiratory syncytial virus,15 porcine circovirus type 2 (PCV2),16 and foot-and-mouth disease virus (FMDV), to discover significant insights into viral pathogenesis.17 To date, several quantitative approaches of proteomics have been applied to analyze the gene expression profiles during PRRSV infection. For example, using two-dimensional electrophoresis (2-DE) coupled to mass spectrometry (MS) approach, Zhang et al.18 identified 23 cellular proteins associated with metabolism, stress response, and
ubiquitin-proteasome pathway, which were significantly changed in PRRSV-infected PAMs. In addition, 2-D fluorescence difference gel electrophoresis (2D-DIGE) was also employed to investigate differentially expressed proteins in PAMs after PRRSV infection.20 Recently, Lu and coworkers21 utilized isobaric tags for relative and absolute quantification (iTRAQ) technique and identified 160 cellular proteins in PAMs that are remarkably altered at different times of postinfection.

Protein phosphorylation, a crucial post-translational modification, plays critical roles in the regulation of many cellular processes including cell proliferation, differentiation, apoptosis, and signal transduction pathways.22 Through regulating the host proteins’ phosphorylation and dephosphorylation, PRRSV controls the activities of signaling molecules and downstream target proteins to facilitate its replication. For example, PRRSV inhibits type-I IFN production by blocking IFN regulatory factor 3 (IRF3) phosphorylation to interfere with RIG-I signal pathway.23 At the same time, PRRSV modulates NF-κB activation by inducing p65 phosphorylation at 36 h post-inoculation (pi) to promote the expression of inflammatory cytokines.24 In recent years, high-throughput and quantitative phosphoproteomics based on mass spectrometry (MS) has emerged as a powerful tool to study the signal transduction pathway and cellular responses through analyzing the differentially phosphorylation level of proteins under different conditions.25−27 In this study, quantitative approach in conjunction with liquid chromatography-tandem mass spectrometry (LC−MS/MS) was applied to systematically investigate global cellular protein phosphorylation alteration during PRRSV infection with three independent biological replicates (Figure 1). By using proteomic tools, several inflammation-related signal pathways mapped by the altered phosphoproteins were found to be activated, and interacting networks of phosphoproteins were also constructed during PRRSV infection. Overall, this work is the first application of quantitative phosphoproteomic to analyze the global phosphorylation events upon PRRSV infection in porcine cells, which may provide valuable insight into the molecular mechanisms of PRRSV pathogenesis and promote a new generation of antiviral drug development.

## MATERIALS AND METHODS

### Virus and Preparation of Pulmonary Alveolar Macrophages

PRRSV WUH3 used for this study was isolated from the brains of pigs with “high fever syndrome” in China at the end of 2006 (GenBank accession no. HM853673). A stock of the virus was prepared in PAMs with a titer of 10^{6.60} TCID_{50}/mL. PAMs were isolated from 4-week-old specific-pathogen-free (SPF) piglets that were free of PRRSV, PCV2, pseudorabies virus, porcine parvovirus, classical swine fever virus, swine influenza virus, and Mycoplasma hyopneumoniae infections as previously described.28 The isolated PAMs were mixed and cultured in RPMI-1640 medium (GIBCO, Invitrogen, CA) containing 10% fetal bovine serum, 0.25 μg/mL fungizone, 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

### Virus Inoculation

PAMs were incubated in RPMI-1640 medium for 16 h at 37 °C in 5% CO2 incubator, and the nonadherent cells were removed by washing with RPMI-1640 medium gently before inoculation. Then, the cells were inoculated with 200 μL of 10^{6.60} TCID_{50}/mL PRRSV WUH3 and were collected at 12 and 36 h pi respectively. The uninfected cells served as mock-infected group. Either infected or mock groups had three independent biological replicates at different times of postinfection (pi). Viral propagation was confirmed by observation of the cytopathic effect and indirect immunofluorescence assay (IFA) using a monoclonal antibody against PRRSV Nsp2 protein (made in our laboratory).

### Protein Extraction, Digestion, and Phosphopeptide Enrichment

The cells were collected by cell scraper, centrifugated at 300g for 10 min, and washed twice with ice-cold PBS containing 1 mM p-mercaptobenzoic acid and 1 mM sodium fluoride at 12 and 36 h pi, respectively. Lysis was performed by using a denaturing buffer (1% SDS, 1 mM PMSF, 2 mM EDTA, 10 mM NaF, 5 mM β-glycerophosphate, 100 mM sodium pyrophosphate decahydrate, 10 mM DTT). Further protein solubilization was achieved by sonication. Lysate debris was moved by centrifugation at 25,000g for 20 min. Sample reduction and alkylation were performed with 10 mM DTT and 55 mM iodoacetamide in the dark and at room temperature. After centrifugation, the precipitate was washed twice with chilled 80% acetone, dried under vacuum, and resuspended in 0.5 M triethylammonium bicarbonate. After centrifugation, supernatant was quantified by Bradford assay and digested with sequencing-grade modified trypsin (Promega, Madison, WI) at 10 μg per mg of protein at 37 °C for 16 h. Peptides were desalted on a C18 Sep-Pak cartridge (Waters, Milford, MA) and dried under vacuum. Peptide elution was performed with 65% acetonitrile (ACN). 3.5% trifluoroacetic acid (TFA) solution saturated with glutamic acid.

Phosphopeptides were enriched by titaniuim dioxide (TiO2), as previously described.29,30 In short, 1 mg of peptides was added to 500 μg of equilibrated TiO2 for enrichment (GL Science, Saitama, Japan) and then incubated for 20 min with end-overend rotation. The column was washed with 65% ACN, 0.5% TFA and 65% ACN, 0.1% TFA, respectively. The peptides were finally eluted with a 0.3 M NH4OH solution in 50% ACN and vacuum-dried.

### LC−MS/MS Analysis

For phosphoproteomic experiments, enriched phosphopeptides samples were dissolved in buffer A (2% ACN, 0.1% formic
acid) at the final concentration of 0.5 μg/μL measured by a nanodrop system (Thermo Scientific) and analyzed in a LC−MS/MS system. Then, 10 μL of supernatant was loaded on Shimadzu LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2 cm C18 trap column (inner diameter 200 μm, Waters), and the peptides were eluted onto a resolving 10 cm analytical C18 column (inner diameter 75 μm, Waters). The samples were loaded at 15 μL/min for 12 min, and then the gradient run at 400 nL/min was from 2 to 35% buffer B (98% ACN, 0.1% formic acid) in 91 min, followed by a 5 min wash at 80% B and a 10 min equilibration step at 2% B. After a precursor scan of intact peptides was measured in the LTQ−Orbitrap Velos (Thermo Fisher Scientific) by scanning from m/z 350−2000 (with a resolution of 60 000), the eight most intense multiply charged precursors were selected for collision-induced dissociation (CID) analysis in the linear ion trap. Activation times were 30 ms for CID fragmentation with normalized collision energy of 35.0. Automatic gain control targets were 100 000 ions for Orbitrap scans and 10 000 for MS/MS scans. Dynamic exclusion for 30s was used to prevent repeated analysis of the same components.

Data Analysis
The acquired MS raw data were loaded into Progenesis LC-MS (version 4.1, Nonlinear Dynamics, Newcastle, U.K.), and label-free quantification was performed for each condition after peak detection, automatic retention time calibration, and normalization in Progenesis LC−MS. The peaklist was created by Progenesis LC-MS and searched in Mascot (version 2.4.1, Matrixscience, London, U.K.). The Uniprot Sus Scrofa database (release Oct. 2013) was used, and the decoy option was selected in Mascot. The following parameters were considered for the searches: peptide mass tolerance was set to 10 ppm, for the searches: peptide mass tolerance was set to 10 ppm, fragment mass tolerance was set to 0.6 Da, and a maximum of two missed cleavage of trypsin was chosen. Carbamidomethyl (C) was set as fixed modification, and acetyl (Protein N-terminal), pyro-Glu (N-terminal), deamidation (NQ), oxidation (M), phosphor (ST), and phosphor (Y) were set as variable modifications. All search results was integrated in Scaffold (version 4.1.2, Proteomesoftware, Portland, OR), and peptide probability was set as 95%. These results in a peptide probability was set as 95%. These results in a peptide probability was set as 95%. These results in a peptide probability was set as 95%. These results in a peptide probability was set as 95%. These results in a peptide probability was set as 95%.
incubated with horseradish peroxidase (HRP) conjugated goat antirabbit IgG (Beyotime, China) at 37 °C for 1 h and detected using clarity-enhanced chemiluminescence (ECL) reagent (Bio-Rad, Hercules, CA).

## RESULTS

### Confirmation of PRRSV Multiplication in PAMs by IFA

To investigate the potential changes to the host cell phosphoproteomes during PRRSV infection and replication, we identified kinetics of PRRSV propagation in PAMs by IFA analysis using mAb against Nsp2 protein of PRRSV. Fluorescence microscopy revealed that a small amount of specific immunofluorescence was detected at 12 h pi, indicating that PRRSV began to replicate in PAMs at 12 h pi. Then, PRRSV titers remarkably increased during the first 24 h of infection, and most of the cells were infected at 36 h pi, but no fluorescence was detected in mock-infected PAMs (Figure 2). Meanwhile, typical CPE of PRRSV in PAMs was observed from 24 h pi (data not shown). Therefore, PAMs in the early stage of infection (12 h pi) and late stage of infection (36 h pi) were selected for phosphoproteomic analysis.

### Label-Free LC−MS/MS for Quantitative Analyses of the PRRSV-Infected Cells Phosphoproteome

Through regulating the host proteins phosphorylation, PRRSV modulates the activities of signaling molecules and downstream target proteins to facilitate its survival. Until now, no previous study analyzed the global phosphorylation events in PRRSV-infected cells through phosphoproteomic approach. In this study, we digested the whole cellular proteins and enriched phosphopeptides by TiO2 from infected and mock-infected PRRSV cells and finally compared the differences in phosphorylation level of proteins by label-free LC−MS/MS technique. In total, 2125 unique phospho-modified sites on 1864 phosphopeptides corresponding to 966 nonredundant phosphorylated proteins were identified. Significant up/down-regulations between samples were determined by a fold change >1.5 or <0.67 and a p value <0.05 for peptide quantification. In addition, a cutoff of 90% confidence was set to filter high site-confident different expressed phosphopeptides. Among all identified phosphopeptides, 227 phosphopeptides containing 253 phosphosites were markedly up-regulated, and 39 phosphopeptides comprising 39 phosphosites were significantly down-regulated at 12 h pi, while 281 phosphopeptides embodying 344 phosphosites were distinctly up-regulated, and 24 phosphopeptides containing 29 phosphosites were obviously down-regulated at 36 h pi (Figure 3A and Supporting Information (SI) Table 1). The proportions of serine, threonine, and tyrosine phosphorylation were 85, 13, and 2% respectively (Figure 3B), which were comparable to previous observations. Meanwhile, monophosphosites held apparent superiority against multiphosphosites and made up ~78% of all phosphosites (Figure 3C).

### Subcellular and Functional Characterization and Bioinformatics Analysis of the Phosphoproteome

These proteins exhibiting phosphorylation level alteration during PRRSV infection were performed to analyze the biological process and subcellular locations based on the underlying biology evidence from the UniProt and the Gene Ontology database to gain functional insights into the cellular phosphoproteome. As expected, most proteins involved in RNA splicing, gene expression, apoptosis regulation, and signal transduction were identified. According to the subcellular locations, the differential phosphoproteins at 12 and 36 h pi were classified, respectively. Among these proteins of phosphorylation level alteration, most up-regulated and down-regulated phosphoproteins in infected cells were mainly located in the cytoplasm and nucleus, whereas ~28% down-regulated phosphoproteins were distributed in plasma membrane at 36 h pi (Figure 4).

Because of the poor annotation of the pig genome database compared with the human genome, many proteins were uncharacterized. Therefore, gene identifications of the identified phosphoproteins were converted to human protein gi numbers, followed by importing into the Ingenuity Pathways Analysis (IPA) tool, and function classifications, signal pathways, and interacting networks were analyzed and constructed based on the underlying biological evidence from the literature database.

According to the analysis of function classification by IPA, previously identified proteins of phosphorylation level alter-

![](figure3.png)

**Figure 3.** Phosphoproteome profiling during PRRSV infection. (A) Table summarized the total number of phosphoproteins, phosphopeptides and phosphosites, respectively, identified in 12 h or 36 h pi. (B) According to the identified phosphosites, the distribution of the individual residue was presented: S, serine; T, threonine; Y, tyrosine. (C) According to the number of phosphosites per peptide, two groups were presented: mono, single phosphosite per peptide; multi, no fewer than two phosphosites per peptide.
during PRRSV infection were divided into four distinctive functional sets: diseases and disorders, molecular and cellular functions, physiological system development and functions, as well as toxicity functions that were depicted in Figures 5 and 6 at statistically significant levels ($P < 0.05$), with additional data shown in SI Table 2. These differential phosphoproteins were involved in many biological functions including cellular growth and proliferation, cellular function and maintenance, RNA post-transcriptional modification, gene expression, protein synthesis, and cell-to-cell signaling and interaction. It is worth mentioning that several differential phosphoproteins were found to be involved in the occurrence of respiratory disease and reproductive system disease, which was the typical symptom of PRRS (SI Table 2).

To deeply explore the role of protein phosphorylation in innate immune responses, we applied the IPA tool to investigate the cell signal transduction pathways manipulated by PRRSV. Several transduction pathways related to inflammatory response were found to be significantly activated during virus infection, such as mitogen-activated protein kinase (MAPK) signaling pathway, NF-$\kappa$B signaling pathway, PI3K/AKT signaling pathway, PAK signaling, IL-6 signaling pathway, and IL-8 signaling pathway (SI Table 3). These data provide valuable insight into the molecular mechanisms of PRRSV-induced inflammatory response. Moreover, interacting signaling

![Figure 4. Subcellular locations of the differentially phosphorylated proteins in PAMs infected with PRRSV. More information is available in SI Table 1.](image1)

![Figure 5. Functional characterization of up-regulated and down-regulated phosphorylated proteins at 12 h pi. (A) Diseases and disorders; (B) molecular and cellular functions; (C) physiological system development and functions; and (D) toxicity functions. More information is available in SI Table 2.](image2)
networks analysis was used to group these differential phosphoproteins into different functional networks to determine whether different cellular activities were altered in PRRSV-infected cells. In total, 8 and 13 specific functional networks were mapped at 12 and 36 h pi, respectively (SI Figure 1). Proteins in those networks mainly involved in cell assembly, organization, growth, signaling, and protein synthesis in PRRSV-infected cells (SI Table 4). MAP3K7 and PI3K seem to be critical kinases, interacting with several phosphoproteins in the network at 12 h pi (Figure 7 and SI Table 4), which participated in cellular function and maintenance, protein synthesis, and DNA replication in PRRSV-infected cells. Additionally, proteins involved in cell-to-cell signaling and interaction as well as cellular assembly and organization could be grouped together, and the phosphorylation level of these proteins was significantly increased in PRRSV-infected cells, except LEO1 at 36 h pi (Figure 8 and SI Table 4).

Validation of Phosphoprotein Identification and Quantification by Western Blotting

To confirm the dynamic phosphorylation alterations of phosphoproteins during PRRSV infection, three phosphoproteins (HSPB1, G3BP1, and PAK2), for which phosphorylation antibody against the phosphorylated sites corresponding to the identified sites was available, were chosen to validate the identification and label-free results. As shown in Figure 9, the Western blotting results showed that the ratios of the three representative proteins between infected and uninfected cells were in agreement with those obtained from label-free approaches.

DISCUSSION

Phosphorylation usually acts as a molecular switch regulating the activities of signaling molecules and downstream target proteins involved in control of a wide range of cellular processes such as cell proliferation, differentiation, apoptosis, and signal transduction pathways.21 Like many other viruses, PRRSV develops the ability to manipulate a variety of host-cell signal transduction pathways though regulating phosphorylation of cellular proteins to facilitate virus survival. For example, PRRSV infection triggers the MAPK family and PI3K phosphorylation32−34 to promote viral entry and replication and also induces p65 phosphorylation to promote inflammatory cytokines expression.35,36 In addition, PRRSV nonstructural...
protein 1β (Nsp1β) is found to inhibit both IFN regulatory factor 3 (IRF3) and signal transducer and activator of transcription 1/2 (STAT1/2) phosphorylation to interrupt type-I IFN and antiviral proteins production. To date, high-throughput and quantitative phosphoproteome have not yet been applied to analyze the interaction between PRRSV and host cells. In the present work, to gain insight into how PRRSV infection affects cellular phosphoprotein response, network, and signal pathway, we utilized the label-free quantitative phosphoproteomic approach to dissect the global cellular protein phosphorylation alteration in PRRSV-infected PAMs with three independent biological replicates of each group. From our present data, the phosphorylation levels of 292 phosphosites on 242 proteins and 373 phosphosites on 249 proteins were significantly altered at 12 h pi and 36 h pi, respectively, based on a fold change >1.5 or <0.67 and a p value <0.05 for peptide quantification. The results of immunoblotting were also consistent with those of the...
quantitative phosphoproteomic analysis. These results provided large-scale phosphoprotein-related information to investigate the mechanism of PRSSV infection and pathogenesis.

Isotope labeling and fluorescence labeling techniques have been widely adopted in quantitative proteomics research. However, more and more people are turning to label-free proteomics techniques for faster, cleaner, and simpler results. Label-free quantitative technology was considered to be ideally suited for our study because it can assess the relative change in phosphorylation for several thousand sites between infected and uninfected controls in a single experiment. The extent of the proteomic profile observed in this study was comparable to those with other proteomics research methods such as iTRAQ and 2D LC−MS/MS methods. In our phosphoproteomic study, dozens of signal transduction pathways related to inflammatory response were found to be significantly activated during virus infection, which may help us shed light on the pathogenic mechanisms of PRSSV infection.

PRRSV replicates in PAMs and induces several cytokines, such as IL-6, IL-8, and TNF-α, leading to pulmonary inflammation response. In this study, signal pathways mapped by identified phosphoproteins using the IPA tool showed that IL-6 and IL-8 signal pathways were both found to be activated in PRRSV-infected PAMs (SI Table 4). PRRSV infection could activate transcription factor NF-κB through I-κB degradation, resulting in the transcription of many inflammatory molecules including IL-6 and IL-8. However, the upstream pathways responsible for NF-κB activation are still not well known. In

Figure 8. Network pathway analysis of phosphoproteins identified in PRRSV-infected PAMs at 36 h pi that are primarily involved in cell-to-cell signaling and interaction and cellular assembly and organization. Red, up-regulated proteins; green, down-regulated proteins; and white, proteins known to be in the networks but not identified in our study. The color depth indicates the magnitude of the change in protein expression level. The shapes are indicative of the molecular class. Lines connecting between the molecules indicate molecular relationships. Solid lines, direct known interactions; dashed lines, suspected or indirect interactions; and arrows, specific molecular relationships and the directionality of the interaction.
general, phosphorylation events of upstream kinases are critical for NF-κB activation. Therefore, we analyzed phosphorylation alteration of the upstream molecules in PRRSV-infected PAMs and constructed the PRRSV-induced NF-κB signaling pathway using the IPA tool (SI Figure 2). From this Figure, we could conclude that NF-κB was activated due to the phosphorylation of upstream kinases TAK1 (also known as MAP3K7), NIK, and PI3K during PRRSV infection. In the phosphoproteome, PRRSV-infected cells showed an increase in phosphorylation of S423 on TAK1 (corresponding to S439 on human) at 12 h pi, which was also discovered in epidermal growth-factor-stimulated cells.31 Owing to the critical role on TAK1/IKK-pi, which was also discovered in epidermal growth-factor-stimulated cells,31 phosphorylation of S423 on TAK1 (corresponding to S439 on human) at 12 h pi (SI Figure 3), indicating that TLR’s signaling cascades may play a critical role in MAPK and NF-κB activation, leading to inflammatory cytokine production.

In addition, G3BP1 is also important in respiratory syncytial virus replication.48 However, G3BP1 is found to be cleaved at residue Q325 by encephalomyocarditis virus, resulting in the disruption of stress granule to facilitate its proliferation.49 G3BP1 is a phosphorylated protein, and this modification was essential to its endoribonuclease activity.50 Here we found that phosphorylated G3BP1 on serine residues was significantly up-regulated in PRRSV-infected cells, which was also validated by Western blot, indicating that PRRSV might manipulate stress granule formation through G3BP1 and promote its replication.

Given the structural and functional complexity of many viral proteins, they are also dependent on chaperones for their folding and assembly like cellular proteins. Chaperones such as heat shock protein (Hsp) Hsp90 and Hsp27 (HspB1) are likely involved in the folding of most viral proteins after translation.51,52 Hsp90 phosphorylation is considered to be closely related to its chaperoning function, while phosphorylation of Hsp27 has been shown to result in inhibiting cell proliferation and participate in intracellular virus transport.53,54 It was noted that the phosphorylation level of Hsp90 and Hsp27 was greatly enhanced in PAMs during PRRSV infection, indicating that increase in phosphorylated Hsp90 and Hsp27 induced by PRRSV may ensure correct folding, transport, and assembly of viral proteins.

In summary, global cellular protein phosphorylation alteration in PRRSV-infected PAMs was characterized by using a label-free approach combined with LC–MS/MS. This is the first time quantitative phosphoproteomic analysis has been used to explore the cellular phosphoproteins that coordinately regulate the replication of PRRSV and host response to viral infection. An understanding of their roles during PRRSV infection is helpful for us to elucidate

Figure 9. Confirmation of three differentially phosphorylated proteins (HSPB1, G3BP1, and PAK2) with immunoblot analysis. PAMs were mock infected or infected with PRRSV and then processed for Western blotting at 12 h and 36 h pi with indicated phospho-antibody. The fold change of HSPB1, G3BP1, and PAK2 phosphorylation is expressed as densitometric units (ImageJ 1.45s, National Institutes of Health, USA) of the band normalized to β-actin level relative to the mock infected group. Each experiment was repeated three times, and representative results are shown. The average ratios of triple replicates are shown on the left side and label-free ratios are shown on the right side.
pathogenetic mechanisms and provide clues for further drug development against PRRSV infection.

**ASSOCIATED CONTENT**

* Supporting Information

Information for significant differentially expressed phosphopeptides in PAMs infected with PRRSV, as identified by label-free approach. Functional characterization, functional networks and pathways in PRRSV-infected PAMs at 12 h pi and 36 h pi. Additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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**REFERENCES**


