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Proteomic profiling of pseudorabies virus-infected PK-15 cells based on 4D label free analysis

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Abstract

Pseudorabies virus (PRV) heavily depends on host machinery to support its life cycle. Investigating the interaction between PRV and host could aid in the understandings of viral pathogenesis. In this study, we performed a 4D label free proteomic method to examine the differentially expressed proteins in porcine kidney PK-15 cells with PRV infection. The results showed that the levels of 661 proteins were significantly elevated and 693 proteins were markedly reduced. Furthermore, these altered proteins were primarily enriched in spliceosome, protein processing in endoplasmic reticulum (ER), RNA transport, and protein export. To ensure the reliability of the proteomic results, the protein levels of formin binding protein 11 and wolfram syndrome 1as components of spliceosome and ER were verified via western blotting and the results were consistent. Together, our data shed light on a new protein profiling induced by PRV infection and highlighted the importance of spliceosome and ER in PRV replication which could promote understandings of host-PRV interplay.

Introduction

One of the most well-known swine diseases, pseudorabies (PR), also referred to as Aujeszky's disease, causes the pig-raising sector to suffer significant financial losses.[1] Nervous system abnormalities, respiratory distress and sow reproductive failures are typical clinical manifestations of PR. Pseudorabies virus (PRV), known as suid herpesvirus 1, is the virus that causes PR.[2] It is a member of the *Alphaherpesvirus* subfamily within the *Herpesviridae* family. The approximately 175 kb genome of PRV encodes more than 70 viral proteins that are involved in immunological regulation and neural latent infection.[3] Over the past 70 years, PRV has expanded throughout China since the first report of a PR epidemic was made in the 1950s. The PR eradication is facilitated by the intensive herd immunization with the attenuated live vaccine Bartha-K61; however, intense immunological pressure may hasten the viral evolution and open the door for the generation of variations. Large-scale PR outbreaks brought on by PRV variations ravaged China in 2011.[4] Bartha-K61, the usual vaccine, offered only a restricted level of protection against infections caused by PRV mutations. Thus, it is necessary to further explore the pathogenic mechanism of PRV which could provide new insights into the development of effective vaccines.

As numerous proteins and pathophysiological processes are involved, and the interactions between viruses and host cells are incredibly complex. To facilitate their infection, viruses can manipulate the expression of host proteins. At the same time, host cells also have developed a variety of defense mechanisms to prevent viral reproduction and create an antiviral state.[5] The pathogenic mechanisms of PRV are still unclear after years of continuous investigation. Nevertheless, the growing body of evidence highlights the increasing prevalence of proteomic techniques in screening the differentially expressed proteins linked to viral replication.[6] This approach is beneficial in elucidating the molecular mechanism underlying the interactions between the viruses and host cells that are involved in viral pathogenesis.[7] Mass spectrometry-based high-throughput

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proteomics is an effective method for examining how virus infections alter host protein composition.

It has been published on the proteome study of PRV infection on host cells. Numerous biological processes were found to be altered after PRV infection. For instance, it was discovered that an epithelial cell line infected with PRV produces a unique cluster of viral microRNAs in addition to a diverse set of host microRNAs, which may aid in PRV replication in cells.[8,9] A recent report has shown that PRV-induced proteins were mostly enriched in chaperonin-containing T-complex and NF-κB-Inducing Kinase (NIK)/Nuclear Factor kappa-light-chain-enhancer of activated B cells(NF-κB) signaling pathway.[10] In addition, activator factor-2 was shown to be required for PRV replication based on phospho-proteomics.[11] Furthermore, we previously observed that PRV infection reduced the global levels of succinylation in PK-15 cells which may in turn control PRV replication.[12] Despite extensive research, many questions remain regarding the pathophysiology of PRVs including the consequences of post-translational modification of viral proteins. Therefore, proteomics and even modification omics must be carried out to study the intricate network of interactions between the virus and host.

In this work, we examined the global alterations of proteins caused by PRV infection based on a 4D label-free proteomics. The enriched pathways that could have an impact on viral replication were further analyzed. Additionally, we highlighted the spliceosome and endoplasmic reticulum (ER) and confirmed the levels of their components formin binding protein 11 (FBP11) and wolfram syndrome 1 (WFS1). We hope that this research will advance our knowledge of the interactions between the host and PRV.

Materials and Methods

Cells and viruses. Porcine kidney PK-15 cells were raised at 37.00 ˚C with 5.00% CO_2 in Dulbecco's modified Eagle's medium (DMEM; BasalMedia, Shanghai, China) which was augmented with 10.00% fetal bovine serum (FBS; Gibco, Waltham, USA). The PRV RA strain was preserved in our lab.[12] At a multiplicity of infection of 1.00, the virus was used to infect PK-15 cells. The unattached viruses were eliminated using phosphate buffered saline following a 1-hr incubation period at 37.00 ˚C. After that, the cells were kept in DMEM with 2.00% FBS.

Antibodies. Antibodies against tubulin were purchased from Beyotime Biotechnology (Shanghai, China). The horseradish peroxidase conjugated antibody against mouse immunoglobulin G or rabbit immunoglobulin G, antibodies against FBP11 and antibodies against WFS1 were obtained from Proteintech Group Inc. (Rosemont, USA).

Western blotting. Western blotting was performed as previously reported.[13] Briefly, PK-15 cells were lysed, eluted in sodium dodecyl sulfate loading buffer (Beyotime Biotechnology), and denatured following separation with sodium dodecyl sulfate - polyacrylamide gel electrophoresis (GenScript, Nanjing, China). Then, the proteins in the gel were transferred to nitrocellulose membranes (GE Healthcare, Chicago, USA) on the eBlot system (GenScript, Piscataway, USA) and followed by incubation with quick blocking buffer (Beyotime Biotechnology). After that, the membranes underwent incubation with primary antibodies and secondary antibodies. Finally, after washing with Tris-buffered Saline with Tween 20 (Beyotime Biotechnology), the proteins were detected using enhanced chemiluminescence (Beyotime Biotechnology).

Sample collection. At 36 hr post-infection, PK-15 cells were collected from two groups: The mock group and the PRV group, each with three duplicates. Cells were sonicated three times in lysis buffer supplemented with protease inhibitor on ice using a high intensity ultrasonic processor (Scientz, Ningbo, China). The debris was removed. After that, the supernatant was collected and the Easy II Protein Quantitative Kit (TransGen Biotech, Beijing, China) was used to measure the protein concentration.

Mass spectrum. The tryptic peptides were initially dissolved in an aqueous solution comprising 0.10% formic acid (Life Sciences, Shanghai, China) and 2.00% acetonitrile (Life Sciences, Shanghai, China), followed by loading onto a homemade reversed phase analytical column. Peptide separation was achieved through a three-phase elution gradient: the first phase linearly increased the proportion of acetonitrile modified with 0.10% formic acid from 6.00% to 24.00% over 70 minutes; the second phase elevated this acidified acetonitrile component to 35.00% within 14 minutes; and the final phase rapidly escalated the organic phase concentration to 80.00% in 3 minutes to complete the elution process. Ultimately, it was maintaining 80.00% for the final 3 min on a nanoElute UHPLC system (Bruker Daltonics, Billerica, USA) at a steady flow rate of 450 nL *per* min. The trapped ion mobility spectrometry time-of-flight mass spectrometer (timsTOF Pro; Bruker Daltonics, Bremen, Germany) and capillary source were then used to investigate these peptides. On a TOF detector, precursors and fragments were examined within the 100 - 1,700 mass-to-charge ratio (m/z) scan range. The parallel accumulation serial fragmentation mode was used to process the timsTOF Pro. Ten parallel accumulation serial fragmentation-MS/MS images were acquired *per* cycle from precursors with charge states 0.00 to 5.00. These precursors were then chosen for fragmentation. Thirty sec was the dynamic exclusion setting.

Bioinformatic analysis. Annotation of Gene Ontology (GO) was done using the UniProt-Gene Ontology Annotation (GOA) database (https://www.ebi.ac.uk/GOA). Identified protein Identifier (ID) was transformed

into UniProt ID and then mapped by protein ID to GO IDs. Where the UniProt-GOA database did not annotate the detected proteins, the GO functional of the proteins would be annotated using the InterProScan tool. A conserved region of a certain protein sequence and structure that may exist, function, and evolve separately from the rest of the protein chain is called a protein domain. The detected protein domains were annotated using the InterProScan and InterPro domain database (https://www.ebi.ac.uk/interpro/) in accordance with the protein sequence alignment approach. Proteins' Kyoto Encyclopedia of Genes and Genomes (KEGG) database descriptions (https://www.kegg.jp/) were annotated and the annotation result was mapped onto the KEGG pathway database using the KEGG online service tools KAAS (https://www.genome.jp/tools/kaas/). Subcellular localization was finally predicted using the WoLF PSORT (https://wolfpsort.hgc.jp/).

Results

Overview of this study. To dissect how PRV infection modulates the global levels of proteins in PK-15 cells, we employed a 4D label free proteomic analysis. Initially, the principal component analysis (PCA) results showed that these two groups were distinct from each other (Fig. 1A) indicating a remarkable change induced by PRV infection. As shown in Figure 1B, the determined mass primarily ranged from 10.00 to 110 kDa and the results from Figure 1C showed the peptides in each protein. Specifically, a total number of 59,646 peptides and 6,639 proteins were identified (Fig. 1D). The proteomic data have been uploaded to ProteomeXchange and assigned identifier PXD040521. These data indicated that PRV infection induced a pronouncedly change of proteins in PK-15 cells.

Large-scale analysis of proteomics against PRV infection. Next, we analyzed how host proteins responded to PRV infection. As shown in Figure 2A, the volcano plots showed the upregulated proteins (Orange) and down-regulated proteins (Green). Also, there were many unchanged proteins (Grey). Specifically, a total number of 693 proteins were reduced, while 661 proteins were increased (Fig. 2B). Consistently, the heat map also revealed the altered proteins induced by PRV infection (Fig. 2C). Some of the upregulated proteins were trans-cription termination factor 2, nuclear factor of activated T cells 2, progesterone receptor membrane component 2, CGG triplet repeat-binding protein 1, and zinc finger and BR-C, ttk, and bab (BTB) domain containing 7B. While some proteins were downregulated, including ATP-binding cassette sub-family C member 4, X-ray repair cross-complementing protein 5, sortilin-related receptor 1, dystroglycan 1 and cadherin 16. We next analyzed their subcellular locations. Interestingly, many altered proteins were located in mitochondria. The results from Figure 2D demonstrated these proteins were mainly associated with RNA processing and modification, transcription, signal transduction mechanisms and metabolism. As seen in Figure 3, most of them were located in nucleus (31.54%) and cytoplasm (25.48%). Proteins located in different places may have various functions. For example, proteins in nucleus may primarily regulate gene expression, while proteins in mitochondria are mostly linked to metabolism.

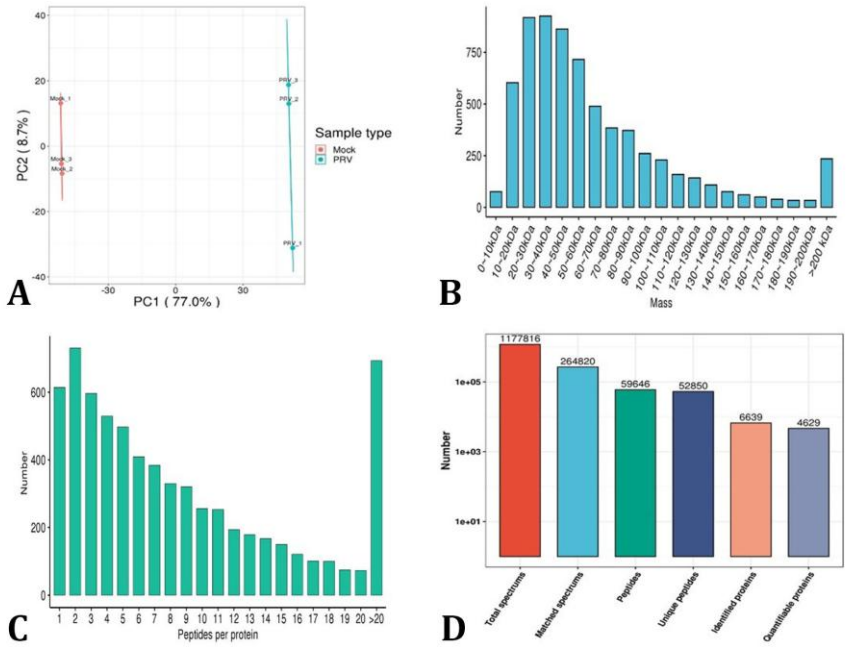

Fig. 1. Overview of the study. **A)** Principal component analysis. **B)** The number of mass distribution. **C)** The number of peptides *per* protein. **D)** The number of detected peptides and proteins. PRV: Pseudorabies virus

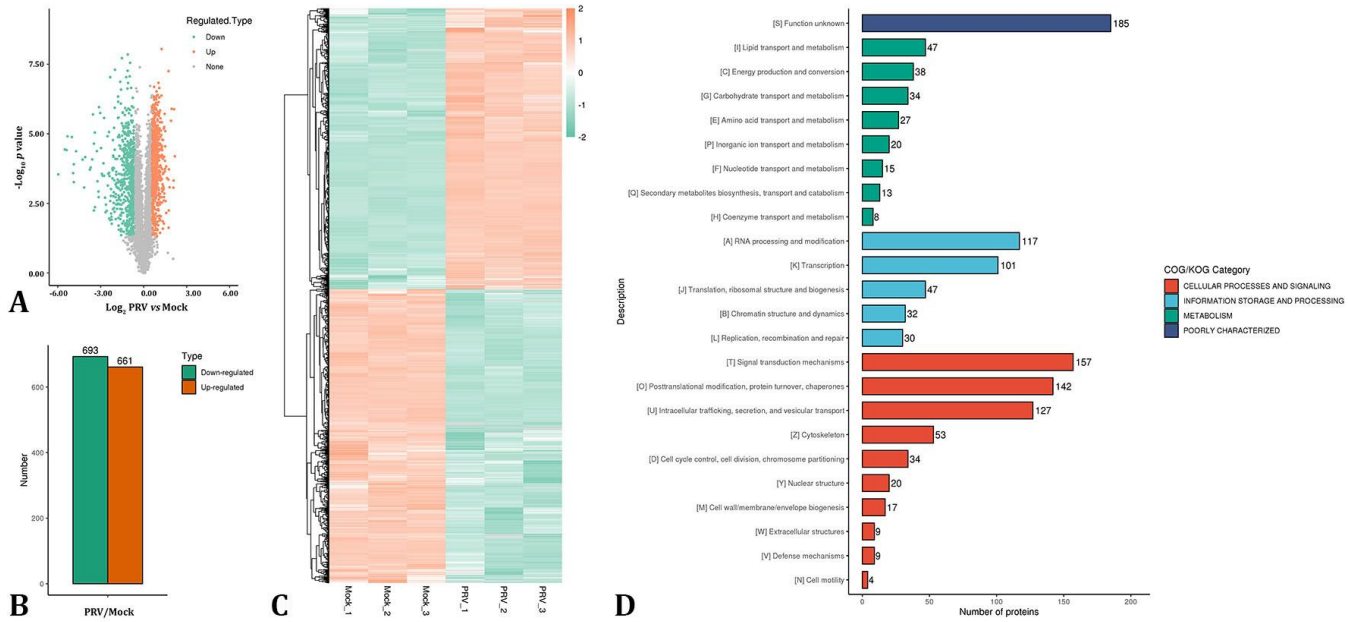

Fig. 2. Analysis of differentially expressed proteins. **A)** The volcano plots showed the downregulated proteins (Green), the upregulated proteins (Orange), and the unchanged proteins (Grey). **B)** Statistical analysis of changed proteins. **C)** The heat map showed the changes of proteins. **D)** Analysis of Clusters of Orthologous Groups (COG)/euKaryotic Orthologous Groups (KOG) pathways.

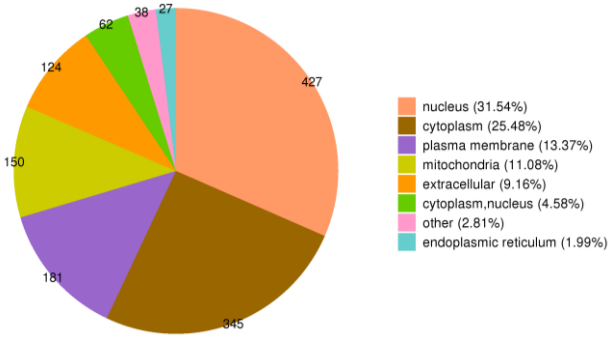

Fig. 3. The subcellular location illustrated the distribution of altered proteins.

Enriched pathways analysis of DEPs. Upon viral entry, cells immediately trigger various ways to defend against viruses, such as innate immunity and autophagy. However, PRV has also evolved strategies to antagonize cellular response. For example, viral proteins, Unique Long (UL) 24 and UL13 have been shown to suppress cellular innate immunity.[14] Thus, it is of great importance to dissect which pathways these proteins were enrich in, because these pathways could be the target of developing antiviral treatment to prevent virus spread. The results of biological process showed the altered proteins were related to the transport of the nucleus and cytoplasm (Fig. 4A). The cellular component revealed a major distribution of ER (Fig. 4B). Then, we analyzed the molecular function and found that most of the proteins were linked to the binding, like small nuclear ribonucleoprotein binding and U1 small nuclear RNA binding. Also, they may participate in enzymatic activity such as exonuclease activity and RNA polymerase core enzyme activity (Fig. 4C). The KEGG analysis revealed that they were largely enriched in spliceosome, RNA transport and protein processing in ER (Fig. 4D). Together, these data indicated that PRV infection induced various pathways in host cells.

Pronounced enrichment of spliceosome and ER pathways. The spliceosome, composed of small nuclear ribonucleoproteins, catalyzes the splicing of precursor messenger RNA (pre-mRNA). Many diseases are closely related to spliceosome, as mutations in key components of it occur frequently.[15] In recent years, more scientists have focused on the relationship between spliceosome and virus. For instance, dengue virus NS5 protein disrupts the cellular spliceosome, thus, reducing its ability of catalyzing pre-mRNA splicing.[16] Here, we observed that multiple spliceosome-related proteins were downregulated by PRV infection (Fig. 5A). The reduced levels of proteins in spliceosome may have some impacts on spliceosome, thus, affecting host cells and viral replication. The ER plays many roles in the biological processes, like protein synthesis and lipid metabolism.[17] In this study, we observed that a multitude of proteins associated with ER were upregulated (Fig. 5B). These upregulated proteins may promote the viral protein synthesis and enhance viral replication. To further confirm these results obtained by proteomic analysis, we detected the levels of FBP11 and WFS1, as components of spliceosome and ER, during PRV infection and found that the levels of both were reduced (Figs. 6A and 6B). These results highlighted the alterations of spliceosome and ER which needs to be further determined to clarify their effects on viral replication.

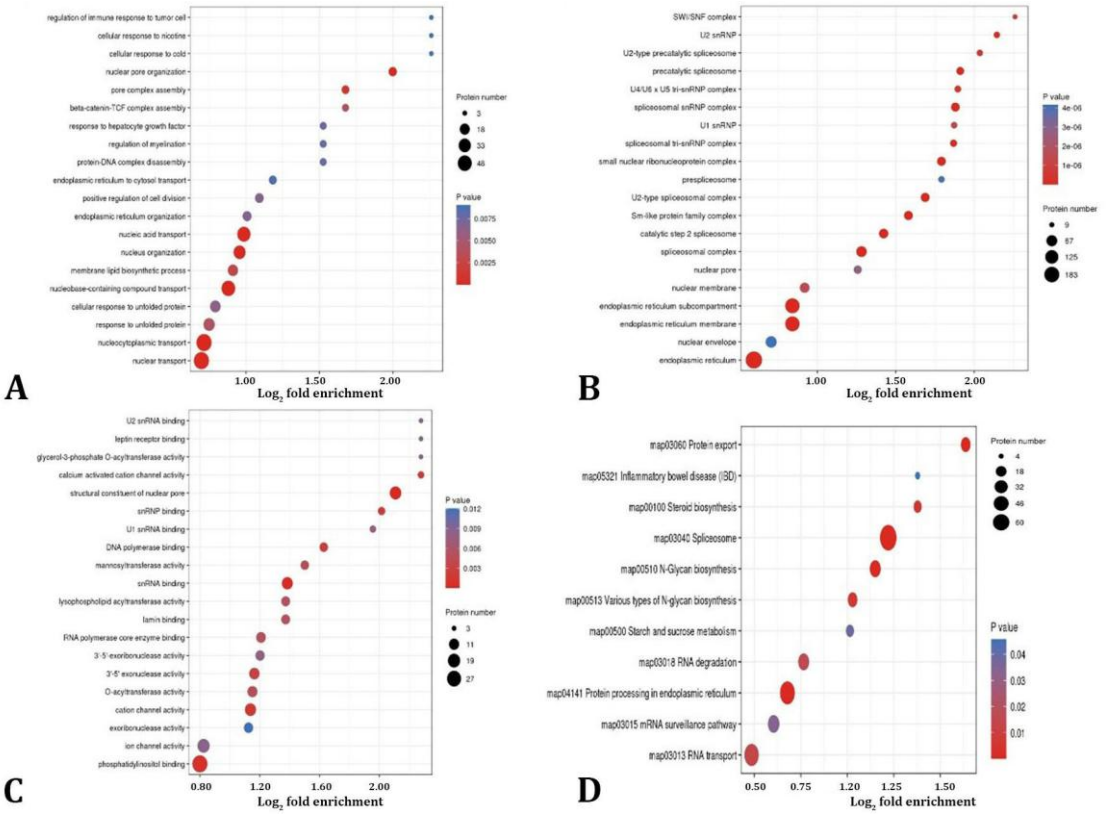

Fig. 4. Analysis of the enriched pathways. **A)** Biological process, **B)** Cellular component, **C)** Molecular function, and **D)** Proteins' Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment.

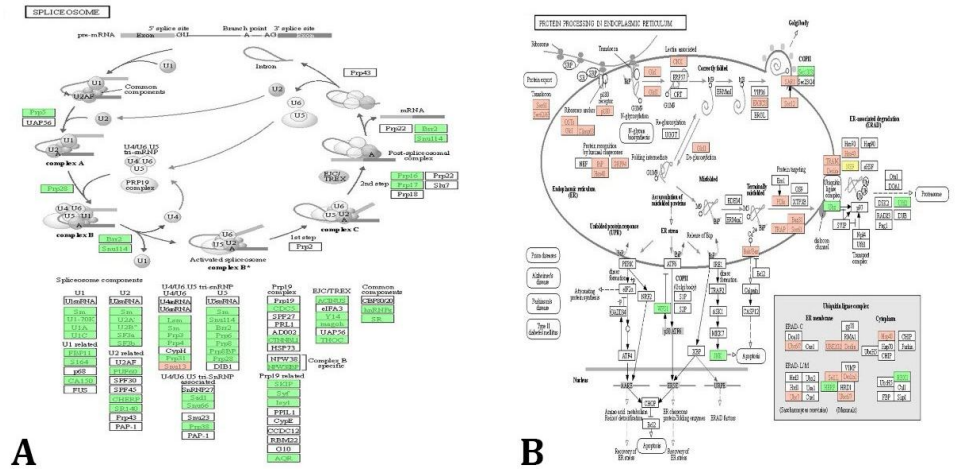

Fig. 5. The network in spliceosome and endoplasmic reticulum. **A)** The composition of spliceosome, **B)** Protein processing in endoplasmic reticulum. The proteins in green were reduced, while the proteins in red were increased.

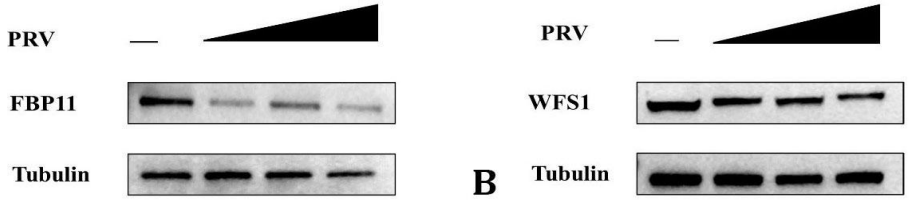

Fig. 6. A) PK-15 cells were infected with pseudorabies virus (PRV; multiplicity of infection = 0.50, 1.00, and 2.00) or mock-infected with Dulbecco's modified Eagle's medium for 40 hr. Cells were lysed and probed for the levels of formin binding protein 11 (FBP11) and tubulin. **B)** The levels of wolfram syndrome 1 (WFS1) and tubulin were examined from the same samples by western blotting.

Discussion

Scientists have always been interested in the host variables that dictate the course of the virus because they play a role in the complex process of host-virus interaction and could serve as possible targets for antiviral therapy. In this study, we found 1,354 differentially expressed proteins in response to PRV infection. KEGG analysis showed that these proteins exert their effects probably through spliceosome, RNA transport, protein transport and ER. This is completely different from previous reports. For example, Yang *et al.* reported that the DEPs induced by PRV infection were mainly involved in NIK/NF-κB signaling pathway and DNA damage response in Vero cells.[10] While another report indicated PRV-induced DEPs were more likely linked to the digestive system and the phosphoinositide-3-kinase-protein kinase B pathway.[18] One of the possible reasons why they are different is the MS technique. They used traditional Tandem Mass Tag (TMT)-based proteomic analysis, while we used 4D label free method. Nevertheless, we still cannot exclude the difference brought by various virus strains and cell lines. Among the regulated proteins associated with spliceosome and ER, the FBP11 and WFS1 were selected and validated via western blotting.

Spliceosome, composed of small nuclear RNA and protein subunits, serves to delete introns from a transcribed pre-mRNA segment. Among the subunits, FBP11 is a component of spliceosome. It has been reported that FBP11 modulates nuclear localization of Neural Wiskott-Aldrich Syndrome Protein (N-WASP) and suppresses N-WASP-dependent micro spike formation.[19] In addition, it plays a role in the proline-rich ligand recognition.[20] However, it has not been reported whether FBP11 involves viral infections. The WFS1, as an ER membrane-embedded protein, serves to play physiological roles in membrane trafficking, secretion.[21] In this study, for the first time we reported that PRV infection reduced the levels of FBP11 and WFS1. We speculated that disruption of FBP11 or WFS1 might impair spliceosome or ER function, thus, affecting cell cycles and reducing PRV replication. is worthy of exploration whether FBP11 or WFS1 indeed affects PRV replication.

The ER is an organelle that serves to help protein synthesis and lipid metabolism. Thus, disrupted ER would impair its ability in biological functions and affect cell cycle. In this study, we observed that many ER-related proteins were upregulated. We speculated that these upregulated proteins might affect ER function. Heat shock protein 40 (HSP40), also known as DnaJ heat shock protein family (HSP40) member B1 (DNAJB1), as an element of ER-associated degradation, mediates the proteasome pathway and ER stress.[17] Of note, HSP40 has been shown to modulate viral infections.[22] For example, it was involved in the replication of murine hepatitis virus .[23] Moreover, it could enhance influenza virus replication via aiding in nuclear import of viral ribonucleoproteins.[24] In this study, we found that HSP40 level was elevated by PRV infection. Nevertheless, whether this increased HSP40 modulates viral PRV replication and underlying mechanisms need to be further addressed.

In conclusion, we found that PRV infection could markedly disrupt the levels of proteins in PK-15 cells. Further analysis indicated that these regulated proteins were primarily enriched in spliceosome, ER, RNA transport, and protein transport. Additionally, we highlighted the alteration of proteins in spliceosome and ER and verified the levels of their components, FBP11 and WFS1.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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