Engineering Microbiology 4 (2024) 100149

Contents lists available at ScienceDirect

Engineering Microbiology

journal homepage: www.elsevier.com/locate/engmic

Original Research Article

Identification of host proteins that interact with African swine fever virus pE301R

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ARTICLE INFO

Keywords:
African swine fever virus
pE301R protein
Protein-protein interaction network
GO and KEGG analysis
Interferon

ABSTRACT

African swine fever virus (ASFV) infection poses enormous threats and challenges to the global pig industry; however, no effective vaccine is available against ASFV, attributing to the huge viral genome (approximately189 kb) and numerous encoding products (>150 genes) due to the limited understanding on the molecular mechanisms of viral pathogenesis. Elucidating the host-factor/viral-protein interaction network will reveal new targets for developing novel antiviral therapies. Using proteomic analysis, we identified 255 cellular proteins that interact with the ASFV-encoded pE301R protein when transiently expressed in HEK293T cells. Gene ontology (GO) annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) database enrichment, and protein-protein interaction (PPI) network analyses revealed that pE301R-interacting host proteins are potentially involved in various biological processes, including protein translation and folding, response to stimulation, and mitochondrial transmembrane transport. The interactions of two putative cellular proteins (apoptosis inducing factor mitochondria associated 1 (AIFM1) and vimentin (VIM)) with pE301R-apoptosis inducing factor have been verified by co-immunoprecipitation. Our study revealed the inhibitory role of pE301R in interferon (IFN) induction that involves VIM sequestration by pE301R, identified interactions between ASFV pE301R and cellular proteins, and predicted the potential function of pE301R and its associated biological processes, providing valuable information to enhance our understanding of viral protein function, pathogenesis, and potential candidates for the prevention and control of ASFV infection.

1. Introduction

Depending on its genotype, African swine fever virus (ASFV) can cause acute, subacute, and chronic disease types. ASFV is a fatal infectious disease affecting domestic and wild swine [1]. ASFV infection induces high virus titers in porcine blood, spleen, and lymph nodes, and as the disease progresses, it results in characteristic pathological changes associated with vasculitis [2]. The first documented case occurred in Africa, followed by a case in China in 2018. ASF has caused substantial economic losses to countries that have experienced outbreaks and is a crucial threat to the global food supply and its safety [3]. No effective vaccines or antiviral drugs are currently available to prevent or treat ASFV infection [4,5]. A rigorous prevention and control strategy executed by China that is generally internationally accepted involves early laboratory diagnosis and culling of infected animals or those potentially infected with ASFV [6,7].

African swine fever virus (ASFV) is a DNA arbovirus that is transmitted by only one known vector-soft ticks of the genus Ornithodoros

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https://doi.org/10.1016/j.engmic.2024.100149
Received 15 December 2023; Received in revised form 29 March 2024; Accepted 1 April 2024
Available online 5 April 2024

[3]. Viral particles are enveloped and display icosahedral structure, and belong to the nucleocytoplasmic large DNA virus (NCLDV) family [8]. The ASFV genome is a double-stranded (ds) DNA between 180 and 190 kb in length. At least 150 open reading frames (ORFs) are encoded within the viral genome. Till date, the number of verified viral proteins and understanding of their functions are limited [9]. Some ASFV proteins have been identified as viral particle structural proteins, whereas other viral proteins are associated with viral pathogenesis [8]. The identified viral structural proteins include pEP402R in the outer envelope, p72/pE120R/pB438L/H240R in the viral capsid, p17/pE183L/p12/pE248R/pH108R/pE199L in the inner envelope, p5/p14/p34/p37/p150/p15/p35/p8/pS273R in the core shell, and p10/pA104R in the nucleoid [10–12]. Many viral proteins modulate cell signaling pathways to evade host defense and facilitate efficient infection through various strategies [13]. ASFV multigene families (MGF), located at either variable end of the genome, play a critical role in ASFV pathogenesis in domestic and wild pigs. For instance, MGF360–12 L/MGF360–15R/MGF360–13 L/MGF360–9 L/MGF360–11 L/MGF360–14 L/MGF505–3R/MGF505–7R/CD2v-CSF2RA inhibits interferon (IFN) production by targeting signaling molecules including NF-κB, IRF3, STAT1/2/3, TBK1, JAK1/2, MAVS, and STING [14–16]. pI329L/pDP96R/pE120R/pI215L/pF334L/pI267L/pE301R/MGF300–2R inhibits IFN and ISG production by targeting TRIF/TBK1/IKK/IRF3/IRF9/RIG-I/P65/STING [17–19]. The protein pA238L/pF317L/pL83L/pI226L/pA151RpI226L/pA151R/pNP419/pQP383R/H240R inhibits inflammasome assembly and IL1-β secretion [20,21]. ASFV pA224L/pA179L/EP153R/pDP71L/P54/pE199L protein modulates programmed cell death, and DP71L/A238L/pE66L protein can affect cellular protein translation and cytokine transcription [22–26]. Identification of more ASFV proteins and their functions in ASFV pathogenesis will likely facilitate genetic engineering-attenuated vaccine development [27,28].

Host antiviral defenses are reprogrammed by the virus using various strategies to facilitate viral propagation. Understanding the underlying molecular mechanisms will contribute to development of disease control and treatment strategies [29]. High-throughput proteomic techniques have been developed to identify many host proteins that interact with viruses or viral proteins from viruses including PCV2, HPIV, and ASFV, furthering our understanding of pathogenesis mechanisms [30–33]. ASFV-encoded proteins, especially those without known functions and mechanisms, such as protein E301R (pE301R), require further study. pE301R contains 301 amino acid residues, and includes a proliferating cell nuclear antigen (PCNA) domain, which probably participates in viral DNA replication during ASFV infection [8]. pE301R has been predicted to act as a DNA clamp homolog or sliding clamp homolog [34] to improve processivity of the DNA replication complex containing dsDNA and the clamp loader complex [35]. Using structural approaches, a recent study identified pE301R as the first viral protein to act as a ring-shaped DNA clamp in viral DNA replication [36]. Knockdown of pE301R reduced viral DNA replication and highlighted the essential role of pE301R in the ASFV life cycle [37]. pE301R has also been identified as an IRF3 interactor that attenuates IFN-I [38]; however, this detailed molecular mechanism remains unclear. Collectively, these studies suggest that pE301R is a multifunctional viral protein involved in viral DNA replication, host antiviral immune evasion, and other biological processes.

To explore the host factor interaction network of pE301R and the potential functions of pE301R in ASFV pathogenesis, we performed co-immunoprecipitation-coupled mass spectrometry and identified 255 proteins that potentially interact with pE301R in HEK293T cells transiently expressing pE301R. Bioinformatics analysis of these pE301R-interacting proteins were used to assemble a protein-protein interaction (PPI) network. Host proteins that interact with ASFV pE301R implicate different cellular pathways, including protein translation and folding, myeloid leukocyte activation, mitochondrial transmembrane transport, and chromatin silencing. Several proteins associated with mitochondrial function and translational control were selected and verified to interact with ASFV pE301R in HEK293T cells transiently expressing both pE301R and its cellular interactors. Our study uncovers potential functions of pE301R and its associated biological processes, increasing our knowledge of viral proteins and ASFV pathogenesis.

2. Materials and methods

2.1. Cells and cell culture

HEK293T cells (CRL-11,268; ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (FBS) (Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin.

2.2. Antibodies and reagents

Mouse monoclonal antibodies (mAbs) against β-actin (ab8226), horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (ab6721), and HRP-labeled goat anti-mouse IgG (ab6789) were purchased from Abcam (Boston, MA, USA). Anti-FLAG mAbs (F1804) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and anti-FLAG rabbit polyclonal Abs (pAbs) (A00170–40) were obtained from GenScript (Nanjing, China). Rabbit pAbs against Myc (R1208–1) were purchased from Huaan Biological Technology (Hangzhou, China). Anti-Myc mAb (60,003–2-Ig) was purchased from Proteintech (Wuhan, China). NP-40 cell lysis buffer was purchased from Beyotime (P0013F; Shanghai, China).

2.3. Plasmid construction and cell transfection

DNA encoding pE301R was synthesized using codon optimization and cloned into the pCMV-N-FLAG eukaryotic expression plasmid (Clontech, Palo Alto, CA, USA). Apoptosis-inducing factor mitochondria-associated 1 (AIFM1; accession no. NM_004208.4) and vimentin (accession no. NM_003380.5) were cloned from HEK293T cells and ligated into the pcDNA3.1/myc-His(-)A expression vector. The resulting protein-encoding plasmids were FLAG-pE301R, Myc-AIFM1, and Myc-VIM. The primers used were as follows: AIFM1-F, ATGTTCCGGTGTGGAGGCCTGGCGG; AIFM1-R, GTCTTCATGAATGTTGAATAGTTTG; VIM-F, ATGTCCACCAGGTCCGTGTCCT; and VIM-R, TTCAAGGTCATCGTGATGCTGAGA.

HEK293T cells were cultured in plates for DNA or RNA transfection. Attractene Transfection Reagent (301,005, Qiagen, Germany) was used for plasmid transfections. The targeting sequences for vimentin were as follows: #1, GCAGAAGAATGGTACAAAT; #2, CTGGTTGATACCCACTCAA; #3, GCATCACGATGACCTTGAA; and #4, CACCCTGCAATCTTTCAGA. Negative control RNAi sequences were UUCUCCGAACGUACGUTT (5′–3,' sense) and ACGUGACACGUUCGGAGAATT (5′–3,' antisense). Lipofectamine™ RNAiMAX Transfection Reagent (13,778,030, Invitrogen, Carlsbad, CA, USA) was used to transfect small interfering RNA.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Lysates were subjected to SDS-PAGE and blotted to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA) to analyze cell lysates after DNA transfection. Tris-buffered saline (TBS) with 5 % non-fat milk and 0.05 % Tween 20 was used to block membranes at 37 °C for 1 h. Membranes were then incubated with the indicated primary antibodies overnight at 4 °C, then washed and incubated with the appropriate secondary antibodies at room temperature (25 °C) for 2 h. Immunoreactive protein bands on the membranes were visualized by incubation with an enhanced chemiluminescence reagent (34,096, Thermo Scientific, Waltham, MA, USA).

2.5. Co-immunoprecipitation (Co-IP)

After transfection, HEK293T cells were lysed in NP-40 cell lysis buffer supplemented with a protease inhibitor mixture for 30 min at 4 °C. Supernatants were then clarified by centrifugation. The supernatants were incubated with protein A/G magnetic beads (HY-K0202; MedChemExpress) for 1 h at 4 °C to eliminate nonspecific binding. The corresponding antibodies were mixed with the aforementioned samples for 12 h at 4 °C and subsequently with protein A/G magnetic beads for another 12 h. Beads were washed four times with lysis buffer and analyzed using SDS-PAGE. Silver staining reagents (P0017S, Beyotime, China) were used to visualize multiple electrophoretic bands on the gel, which were cut for mass spectrometric analysis, excluding the IgG band.

2.6. Liquid chromatography coupled with mass spectrometry (LC-MS)

LC-MS analysis was performed by APTBio (Shanghai, China) to identify host proteins captured by Co-IP. An EASY column (2 cm × 100 mm 5 μm-C18; 75 μm × 100 mm 3 μm-C18; Thermo Finnigan) was used to concentrate and desalt peptides and an analytical RP column (0.18 150 mm BioBasic-18, Thermo Electron) was used to elute. Mascot 2.2 software was used to search for proteins. The following criteria were applied to identifiy proteins: (a) trypsin-digested peptides with 2 max missed cleavages allowed, (b) mascot, (c) >1 unique peptides, and (d) filter by score ≥ 20. Experimental (FLAG-pE301R-expressing) and negative control eluates were each subjected to two independent LC-MS analyses. Cellular proteins identified in the vector control group were considered to be non-specific interactions and eliminated from the dataset. Proteins represented by at least one unique peptide found in the FLAG-pE301R-expressing group in both replicates were considered for further analyses.

2.7. Construction and analysis of a protein-protein interaction network

The experimentally derived datasets were used to plot a pE301R-host protein interaction network using Cytoscape software (version 3.7.1). Interactions among host proteins were analyzed using the STRING database. Only interactions that were confirmed via direct physical binding were used to plot the protein-protein interaction map. The network analyzer tool in Cytoscape version 3.7.1 was used to calculate network topological parameters and central measures. The STRING database was used to analyze human protein-protein interactions. NCBI gene names were used to represent proteins throughout the study to reach a consensus on protein accessions.

2.8. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses

We annotated identified genes in terms of cellular component (CC), biological process (BP), and molecular function (MF) using the GO database.GO was analyzed using Cytoscape software (version 3.7.1) with the plugin GO clue (Supplementary Table S4). We predicted relevant pathways using the KEGG database, viaKEGG enrichment analysis. APTBio (Shanghai, China) performed GO and KEGG pathway analyses, and a corrected p-value < 0.05 in the GO and KEGG pathways was set as the criterion for significant enrichment.

2.9. Luciferase reporter assay

An IFN-β reporter plasmid and pRL-TK were used to assess RIG-I-N-mediated IFN-β promoter activation. HEK293T cells were cotransfected with the plasmids described above. A Dual-Luciferase Reporter Assay System (GeneCopoeia, GCP-LF001) was used to assess luciferase activity. Relative firefly luciferase activity was normalized to Renilla luciferase activity.

2.10. Transfection, RNA isolation, and quantitative PCR

HEK293T cells seeded in 12-well plates were transfected with siRNA for 24 h and co-transfected with the indicated plasmids, including myc-RIG-I-N, FLAG-pE301R, or empty vector as a negative control. At the indicated time points, total RNA was extracted using TRIzol Reagent (Invitrogen, 15,596,026). Next, a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, K1622) was used to prepare cDNA, using the following thermocycler program: 25 °C for 10 min followed by 37 °C for 60 min and then 75 °C for 15 min. Quantitative PCR (qPCR) was performed using the prepared cDNA with TB Green Premix Ex Taq II (Takara Bio). The primers used to amplify target genes were as follows: Vim-F: GACGCCATCAACACCGAGTT, Vim-R: CTTTGTCGTTGGTTAGCTGGT; Ifn-β-F: GACATCCCTGAGGAGATTAAG, Ifn-β-R: ATGTTCTGGAGCATCTCATAG; E301R-F: TCAACCTGACCAGCACCAT, pE301R-R: TCCTCGCACAGGATTCTAATT; Gapdh-F: CGGGAAGCTTGTGATCAATGG, Gapdh-R: GGCAGTGATGGCATGGACTG. qPCR data were normalized to GAPDH mRNA levels. Relative mRNA levels were calculated with the $2^{-\Delta\Delta CT}$ method.

2.11. Statistical analyses

Data are expressed as means ± standard deviation (SD) from three independent experiments with consistent results. One-way analysis of variance (ANOVA) was performed using GraphPad Prism software to determine the significance of the variability between groups. Asterisks indicate statistical significance: $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. Elucidation of pE301R-host protein interactions via liquid chromatography-mass spectrometry

To map host protein interactions with ASFV pE301R, we conducted Co-IP assays coupled with gel electrophoresis, silver staining, and LC-MS/MS using whole-cell lysates prepared from HEK293T cells transiently expressing the FLAG-tagged pE301R protein or, as a control, an empty vector (Fig. 1A). Specific host factors detected in both biological replicates, excluding nonspecifically bound proteins present in the mock group, were selected for further analysis, leading to the identification of 255 cellular proteins from the UniProt database as pE301R-binding cellular protein candidates (Fig. 1B).

3.2. Construction of a protein-protein interaction network

Verifying protein-protein interactions (PPIs) and establishing a network is critical for understanding the biofunction of identified proteins, the mechanisms of diverse cellular signaling events, and mechanisms of disease pathogenesis. An interaction network of pE301R-binding host proteins was first plotted with the STRING database, then used for network skeleton and functional analyses (Fig. 2 and Supplementary Table S1). To elucidate crosstalk between host factors, reported protein interactions identified by gene fusion, coexpression, and text mining were used to construct a network. Host proteins in the interaction network were predominantly cataloged into three large distinct clusters, framed by dashed circles: Cluster 1 proteins are mainly associated with translational initiation and protein folding chaperones. Cluster 2 proteins are mainly involved in mitochondrial transmembrane transport. Cluster 3 proteins play roles in chromatin silencing. The number of edges in the network (859) was notably higher than the expected value (302) for a constant number of nodes (210), suggesting a high prevalence of interactions. The results indicate that the main cellular activities associated with pE301R are translation and mitochondria-associated cellular activities.

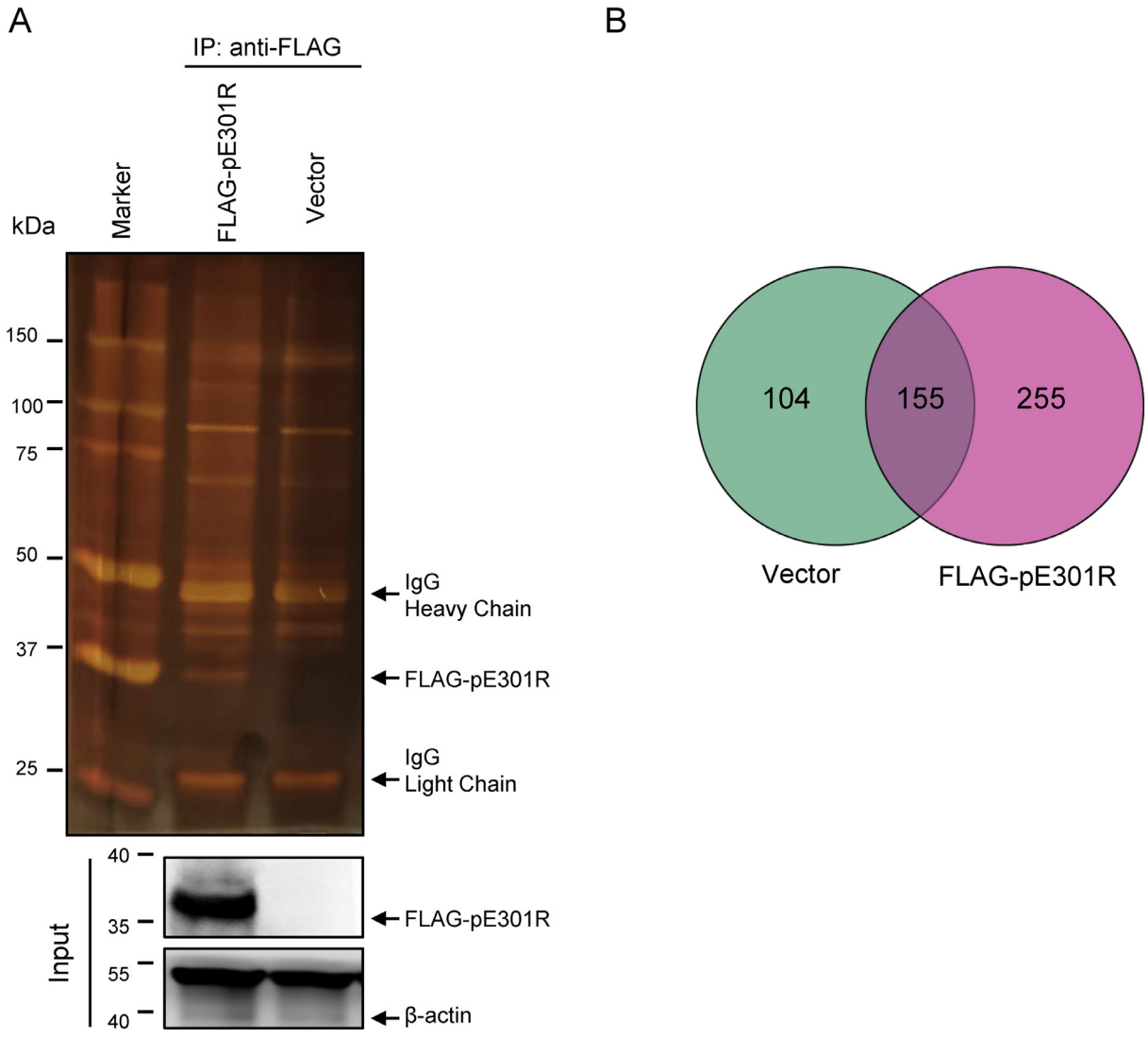

Fig. 1. Characterization of pE301R-interacting cellular proteins. (A) Empty vector or FLAG-pE301R-expressing plasmid was transfected into HEK293T cells, and whole cell lysates were harvested at 24 hpt. Co-immunoprecipitations were performed using anti-FLAG antibody. pE301R-interacting host proteins were eluted and separated via SDS-PAGE followed by silver staining. Lane 1, protein molecular weight (in kDa) size markers; lane 2, FLAG-pE301R-transfected; lane 3, empty vector-transfected. Expression of pE301R was verified by Western blotting with anti-FLAG antibody, with β-actin as the internal loading control. (B) Venn diagram of the pE301R-interacting protein candidates identified in two independently repeated IP/MS assays, and excluding host proteins identified in the vector control CO-IP. Common proteins within the datasets are indicated in the colored intersections. Proteins are labeled with their respective NCBI gene names.

3.3. Gene ontology annotation

To identify the cellular pathways in the pE301R-host protein interaction network, we performed gene ontology annotation. The three categories of biological functions annotated by gene ontology are biological processes, molecular functions, and cellular components. Interacting proteins are enriched in many biological processes, including the generation of precursor metabolites and energy, RNA localization, protein stability, translation, and viral transcription. Briefly, unfolded protein binding, electron transfer activity, and ribonucleoprotein complex binding were enriched within the molecular function category, whereas the organelle envelope, mitochondrial membrane protein complex, and ribosomal subunit were enriched under the cellular component category (Fig. 3A, B, and Supplementary Tables S2–S4). Overall, GO annotation of common proteins indicated that pE301R may modulate biological processes implicated in translation, mitochondrial activity, and RNA localization.

3.4. KEGG pathway enrichment

To further analyze pE301R-interacting host proteins involved in host signal transduction pathways, we conducted KEGG pathway enrichment analysis and identified the top 20 enriched pathways with the highest representation of each term. Most candidate pE301R-interacting cellular proteins are involved in ribosome function, neutrophil extracellular trap information, or prion disease. KEGG enrichment analysis indicated that pathways involving neurodegenerative diseases and diabetic cardiomyopathy were preferentially enriched (Fig. 4A, B, and Supplementary Table S5). In addition, KEGG enrichment analysis showed that these proteins may play critical roles in regulating distinct pathways, such as those involved in neurodegeneration.

3.5. Validation of pE301R/host protein interactions

To verify protein interactions identified by mass spectrometry, we performed in vitro co-IP and immunoblotting assays. Two host proteins

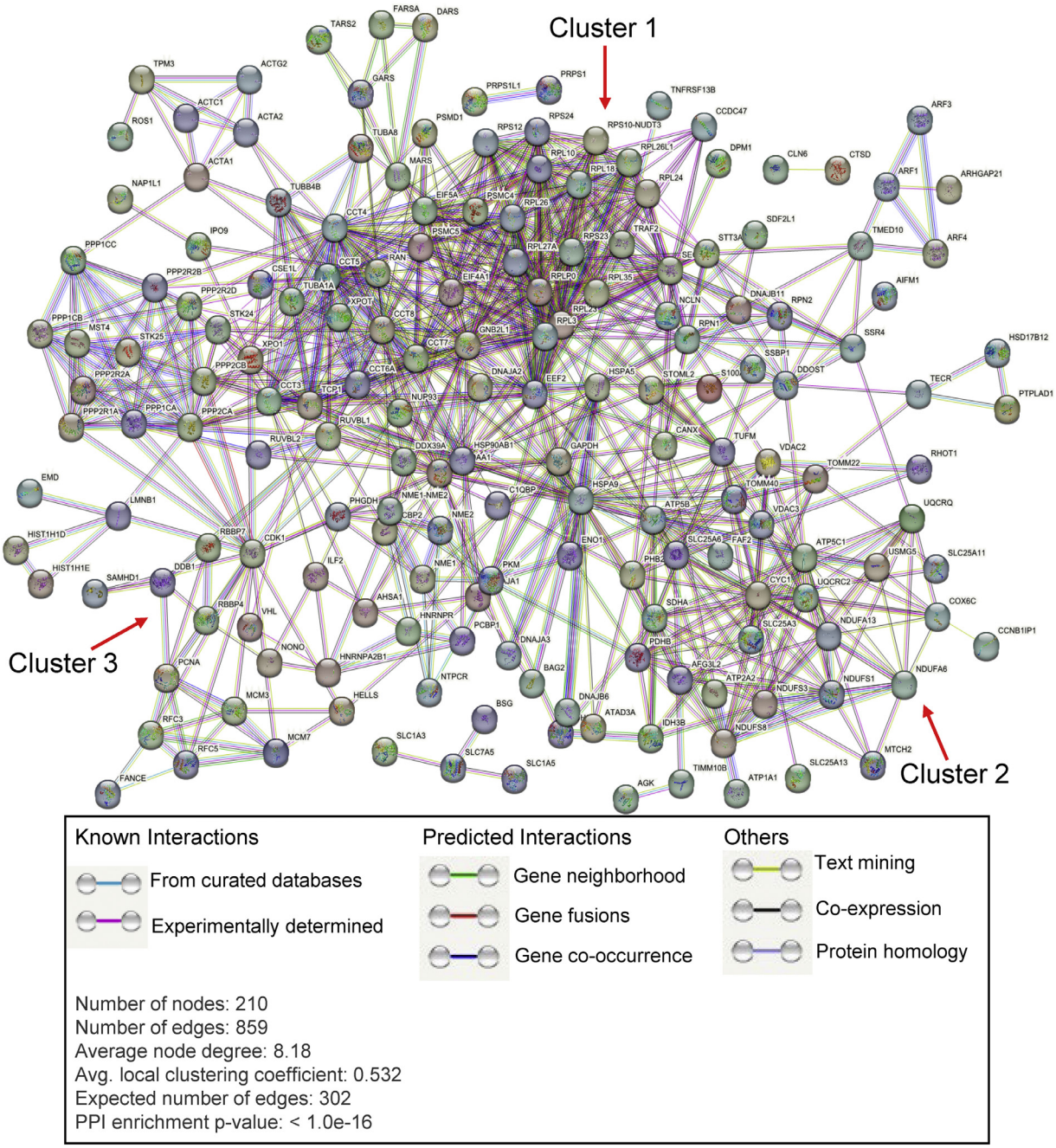

Fig. 2. Establishment of a protein-protein interaction network using the STRING database. Each edge color represents a different method of protein-protein interaction identification, as indicated below the figure. A map depicting interactions between 255 host proteins identified as pE301R interactors was plotted using the network analyzer tool in Cytoscape, version 3.7.1. The corresponding symbols indicate different protein classes. Protein names are consistent with their respective NCBI database names (Supplementary Table S1).

identified in pE301R-transfected samples were selected for further analysis: the programmed cell death associated protein AIFM1, and the cytoskeletal protein, VIM. HEK293T cells were co-transfected with FLAG-pE301R or empty vector together with Myc-AIFM1 or Myc-VIM expression plasmids. Whole cell lysates were subjected to immunoprecipitation with the specified antibody and protein A/G beads. The results showed that ASFV pE301R bound specifically to AIFM1 and VIM. No signals were detected when using empty vector (Fig. 5A, B). These results validate the IP/LC-MS/MS data, showing that AIFM1 and VIM interact with pE301R.

3.6. Inhibition of pE301R in IFN response is vimentin (VIM)-dependent

We performed a reporter gene assay in HEK293T cells to identify the novel role of ASFV pE301R in the IFN response. An IFN-β promoter-driven luciferase (luc) reporter was transfected into HEK293T cells together with the N terminus of RIG-I (RIG-I-N) to activate the IFN-β promoter with or without ectopic pE301R expression. We found that IFN-β-luc activity was stimulated by RIG-I-N, and that this activation effect was significantly weakened by pE301R ($p < 0.01$) in a dose-dependent manner (Fig. 6A). Recent studies have reported that VIM

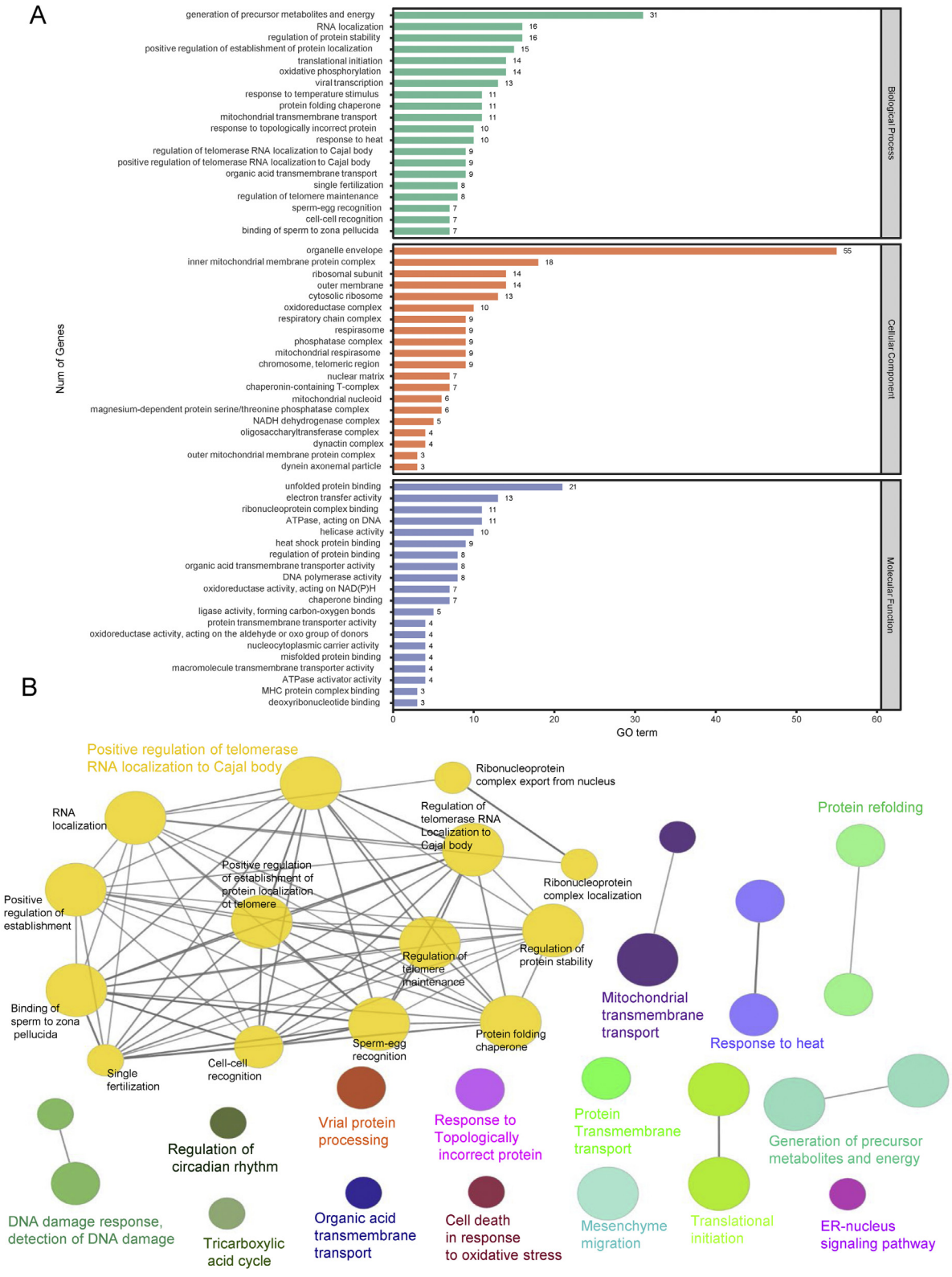

Fig. 3. Gene ontology analysis of the identified pE301R-host interactome. (A, B) Representative overrepresented GO terms of protein clusters and GO distribution were analyzed with the GO clue plugin in Cytoscape, version 3.7.1. All proteins selectively pulled down in pE301R-transfected cells versus empty vector-transfected cells were classified into three categories. The significantly enriched biological process (BP), molecular function (MF), and cellular component (CC) terms with p-values < 0.05 are shown. Roman numerals represent the detailed GO terms, as shown in Supplementary Tables S2, S3, and S4. The size of the spots represents the amount of protein involved in each biological process.

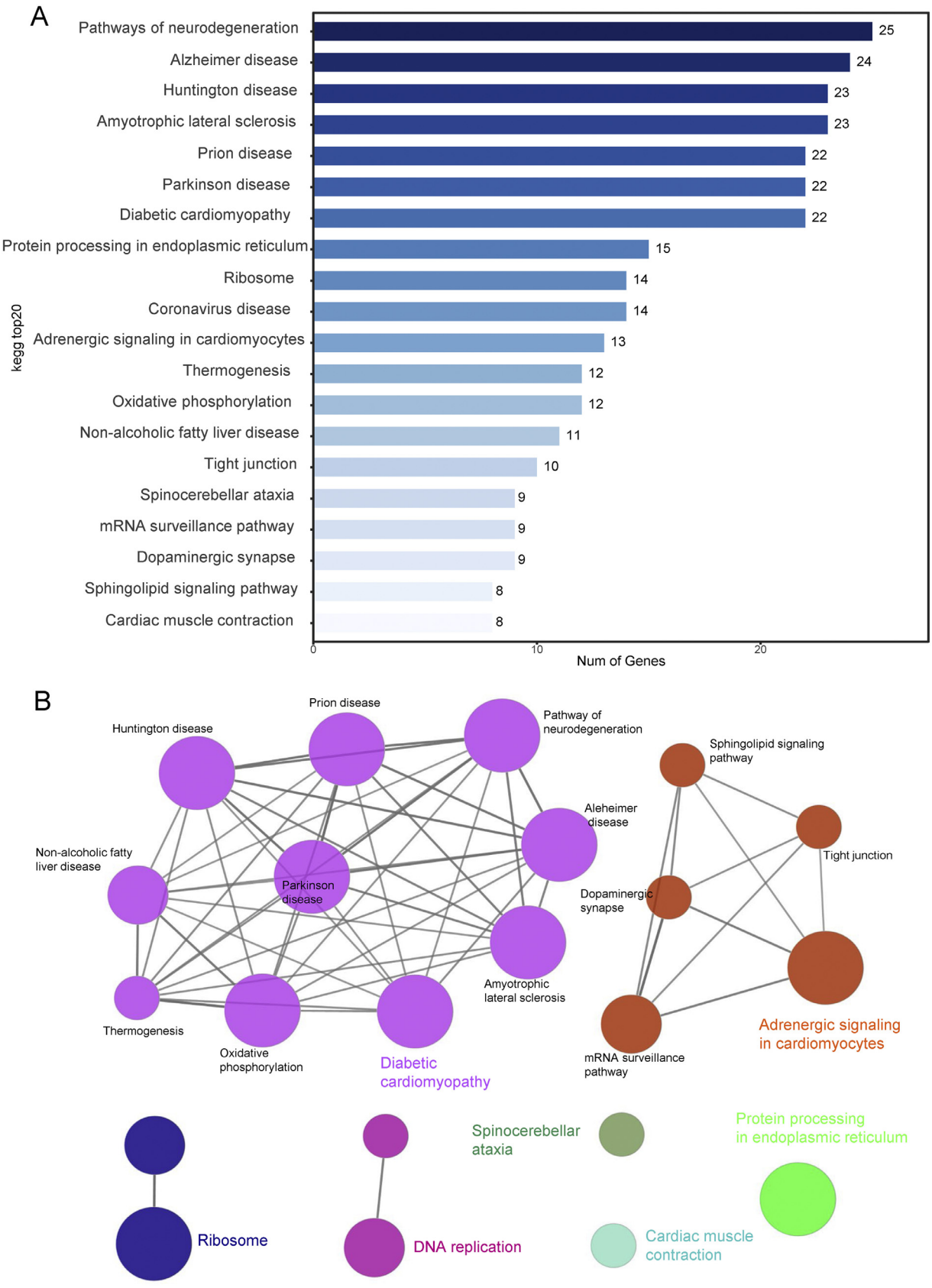

Fig. 4. KEGG pathway enrichment. (A, B) Pathways enriched in pE301R-interacting proteins are presented, as analyzed via KEGG functional annotation (Supplementary Table S5) using the GO clue plugin in Cytoscape, version 3.7.1. The size of the spots represents the amount of protein involved in each pathway.

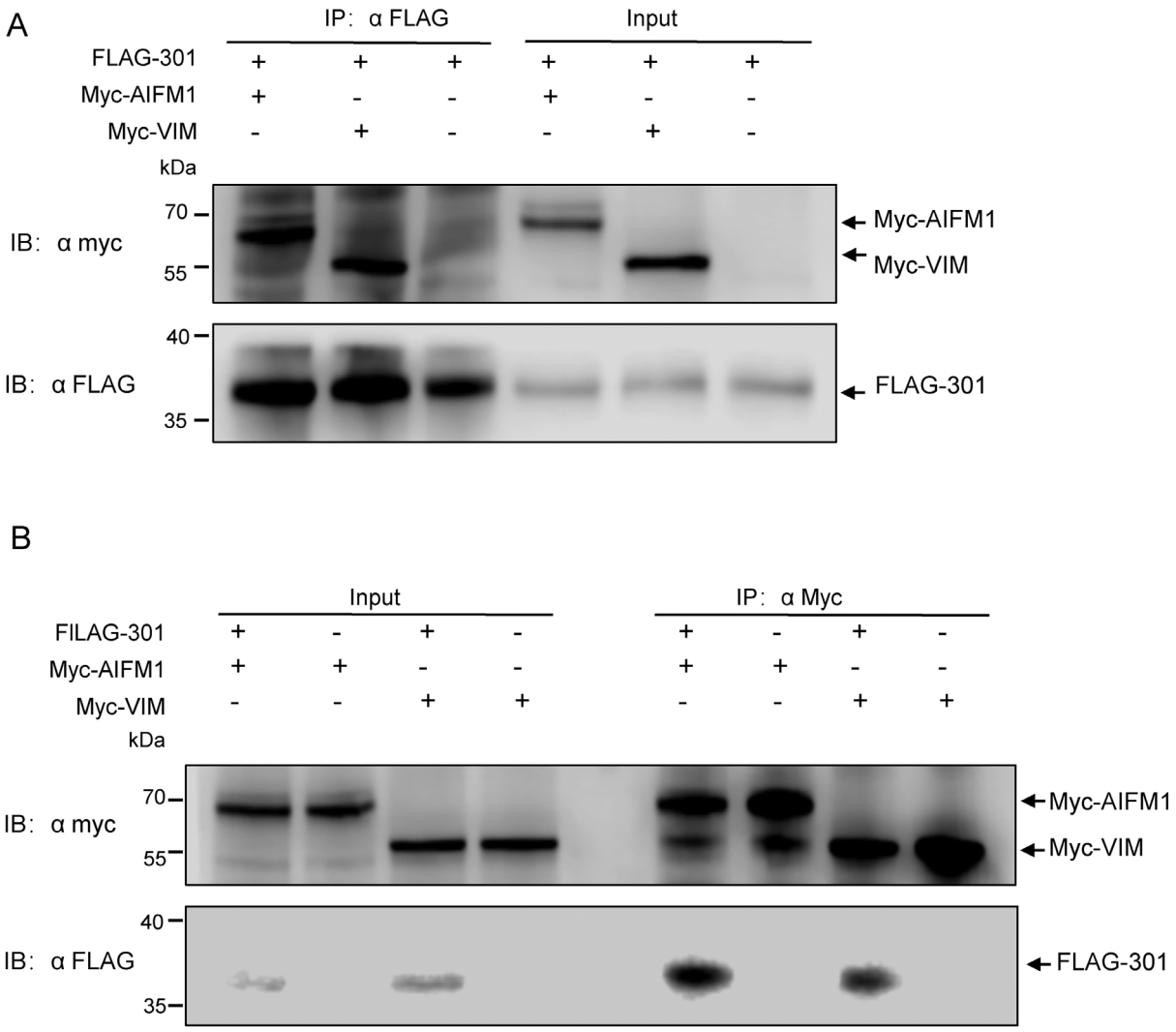

Fig. 5. Validation of Cap-host protein interactions. (A, B) HEK293T cells were co-transfected with a plasmid expressing FLAG-pE301R, together with plasmids expressing Myc-AIFM1 and Myc-VIM, respectively. Among them, FLAG-pE301R co-transfected with an empty vector served as the negative control. Cell lysates were immunoprecipitated with FLAG pAbs, or Myc pAbs and protein A/G beads, separated via SDS-PAGE, and then subjected to immunoblotting with the corresponding primary and secondary antibodies.

participates in regulating IFN-I production [39]. To determine whether VIM is involved in pE301R-downregulation of the IFN-β response, we performed VIM knockdown in HEK293T cells with four siRNAs (#1, #2, #3, #4) targeting different regions of VIM. Compared to negative control siRNA (siNC), VIM-mediated reduction efficacy ranged from 12 % to 42 % (Fig. 6B and C). The effect of VIM knockdown on RIG-I-N-stimulated IFN-β signaling was tested with a reporter system with or without pE301R. RIG-I-N-stimulated IFN-β-luc reporter activity inhibition by pE301R was partially relieved by VIM knockdown (Fig. 6D) with 63 % inhibition in the siNC group decreased to 47–50 % inhibition in the siVIM group ($p < 0.01$, compare siNC bar 3 vs. siVIM#3/#4 bar 3). The effects of VIM knockdown and pE301R expression were verified at the transcription level. Suppression of the RIG-I-N-stimulated increase in endogenous IFN-β mRNA by pE301R was partially attenuated by VIM knockdown, with 70 % inhibition in the siNC group decreased to 60 % inhibition in the siVIM group ($p < 0.05$, compare siNC bar 3 vs. siVIM#3/#4 bar 3) (Fig. 6E). Collectively, these data show that the interaction between VIM and pE301R is associated with IFN-β signaling suppression.

4. Discussion

ASFV is an acute, highly contagious, and fatal infectious disease that underlies substantial economic losses. As no approved vaccine is available, the only way to control viral spread is to cull animals infected or potentially infected with ASFV. Recent studies have explored safe ASFV infection systems with specific isolates or smaller animal models; the current study is limited to animal biosafety level 3 using a pig model [40]. The ASFV genome is a large double-stranded DNA (dsDNA) encoding a complex collection of products. Despite efforts to identify viral proteins and their functions, many unidentified ASFV-encoded products and host factors involved in viral pathogenesis require clarification.

This study focused on pE301R, as previous reports have suggested that pE301R is a multifunctional viral protein involved in viral genome replication [36] and IFN-I suppression [38]. Using IP/LC-MS/MS proteomic tools, we identified host proteins that interact with pE301R and analyzed the potential biological functions of their interactions with pE301R. In total, 255 cellular protein candidates with potential roles in ASFV pathogenesis interacted with ASFV pE301R. GO and KEGG path-

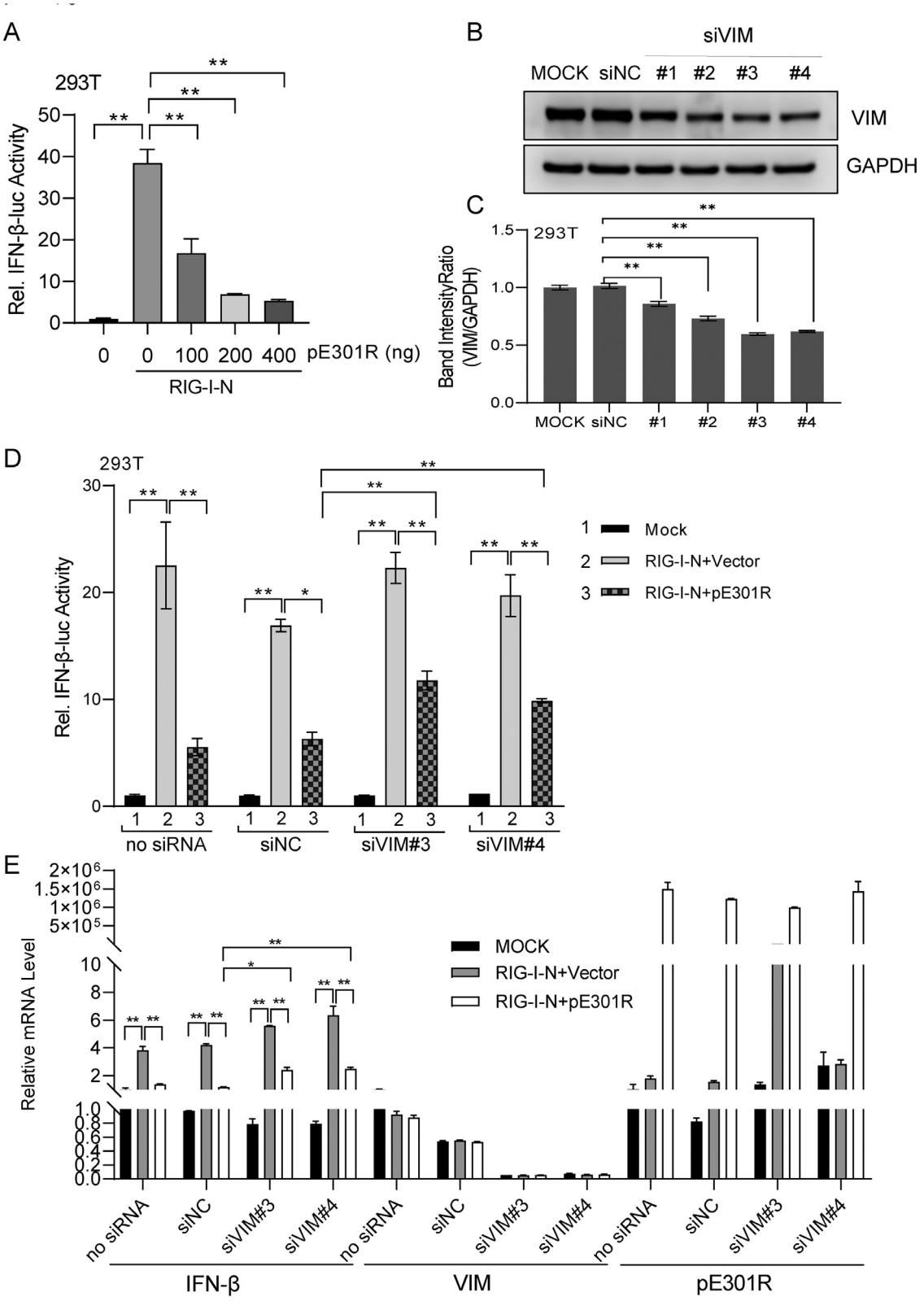

Fig. 6. An antagonistic role and mechanism of pE301R on IFN induction. (A) HEK293T cells were transfected with the following DNA, including pIFN-β-Luc reporter plasmid (0.2 μg), pRL-TK plasmid (0.02 μg), RIG-I-N plasmid (0.2 μg), and a plasmid (0.1, 0.2 or 0.4 μg) expressing pE301R protein. Luciferase activity was tested 24 h post transfection (hpt). (B) HEK293T cells were transfected with human siRNA targeting vimentin siVIM-#1,#2, -#3, -#4 or siNC, and 24 h later immunoblotting was perfomed with cell lysates. (C) The band intensity in (B) was measured. (D) HEK293T cells were transfected with siVIM-#3/#4 or siNC, and 24 h later cells were transfected with the mix DNA, including a pIFN-β Luc reporter plasmid (0.2 μg), the pRL-TK plasmid (0.02 μg), a plasmid expressing RIG-I-N, FLAG-pE301R as indicated, with vector alone as control. Luciferase activity was quantified at 24 hpt, and the level of luciferase activity, normalized with the corresponding RIG-I-N group, was indicated in percentage. (E) HEK293T cells were transfected with siVIM or siNC, and 24 h later cells were transfected with a plasmid expressing RIG-I-N, FLAG-pE301R as indicated, with vector as control. At 24 hpt, mRNA level of target genes was measured and quantified.

way analyses showed that the pE301R-binding host proteins participate in diverse biological processes, including RNA localization, translation, cellular metabolic processes, and nucleic acid binding (Figs. 3 and 4). Our data also indicate that specific host factors might be recruited by pE301R to control viral DNA replication in the cell nucleus and modulate translation and mitochondrial function.

Many viruses have evolved strategies to evade host surveillance and promote their replication, including alteration of host ubiquitination pathways, stress granule formation, intracellular trafficking, and protein quality control, among others [41–44]. The current study showed that in early stage infection, the viral protein pE301R modulates ribosome function by binding to the translation initiation complex. Previous studies have also found that ASFV stimulates cap-dependent translation by activating the eIF4F complex to initiate viral mRNA translation [45]. Both ASFV pE66L and MGF110–7 L suppress host translation through the PKR/eIF2α pathway [25,46]. Overall, the interactions between ASFV proteins and host translation components play a critical role in the viral infection cycle.

Our proteomic data also showed that pE301R could potentially regulate mitochondrial function (Fig. 3), for instance via a verified interaction with AIFM1 (Fig. 5). Evidence supports that viruses can regulate host immune responses by interacting with mitochondrial proteins. For example, mitochondrial Tu translation elongation factor (TUFM) may be recruited by human parainfluenza virus type 3 to induce mitophagy and suppress IFN responses [32]. In addition, Kaposi's sarcoma-associated herpesvirus (KSHV) may interact with the mitophagy receptors NIX and TUFM to induce mitophagy, thereby inhibiting antiviral innate immune responses [47]. An interaction between the mitophagy receptor SQSTM1/p62 and translocase of outer mitochondrial membrane 70 (TOMM70) is facilitated by ASFV p17 to promote mitophagy and regulate innate immunity [48]. Several studies have shown that AIFM1 can be recruited by viral proteins to modulate cellular processes. SARS-CoV-2 upregulates apoptosis-inducing genes and triggers mitochondrial pathways, thus exacerbating COVID-19 pneumonia [49]. In addition, herpes simplex virus can induce cell death via mitochondrial apoptosis-inducing factor (AIF) translocation or block programmed cell death by regulating AIF translocation to the nuclei of human embryonic lung fibroblasts [50,51]. Influenza virus infection induces AIF translocation in A549 cells to regulate apoptosis and viral propagation [52]. Porcine epidemic diarrhea virus induces caspase-independent apoptosis by activating AIFM [53].

The host cytoskeletal protein vimentin was also identified as an ASFV pE301R partner (Fig. 5). Previous studies have shown that vimentin is involved in various viral infection strategies [54,55]. For example, the SARS-CoV-2 spike protein binds to vimentin for viral attachment [56]. The classical swine fever virus replication complex formation is facilitated by the viral non-structural protein NS5A via its interaction with vimentin [57]. Vimentin also plays a critical role in maintaining the integrity of Zika virus replication complexes and RNA-binding regulatory hubs to improve Zika viral replication [58]. Cellular vimentin is modulated by foot-and-mouth disease virus for viral survival [59] via the Nonstructural Protein 3A-vimentin interaction [60]. Cellular vimentin targets the HN protein and regulates Newcastle disease virus [61] infectivity. Vimentin was also identified as a negative regulator of type I interferon production upon RNA- and DNA-virus infection [39], as vimentin binds to TBK1 and IKKε to inhibit TBK1-IRF3 and IKKε-IRF3 interactions, suppressing IRF3 phosphorylation and nuclear translocation. ASFV pE301R negatively regulates IFN induction by inhibiting nuclear translocation of IRF3 [38]. Using proteomics to identify partner proteins associated with pE301R, we demonstrated that pE301R interacts with vimentin to inhibit IFN-β production (Figs. 5 and 6), thereby verifying that pE301R targets IRF3, independent of receptors. However, the detailed molecular mechanisms and biofunctions of the pE301R vimentin interaction need to be clarified using a viral infection model. The crystal structure of pE301R revealed a ring-shaped trimeric DNA sliding clamp [36]. Further studies should analyze the role of pE301R in virus replication. The subcellular localization and dynamics of pE301R expression and activity need to be clarified, as identifying the host factors recruited by pE301R will help us understand its function in ASFV pathogenesis.

In summary, the current study reveals potential functions of pE301R. Deep bioinformatic analysis of available datasets and systematic future studies will help in understanding the roles of pE301R in regulating host responses and viral infections.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Given their roles as Editor-in-Chief and Associate Editor, respectively, Dr. Youming Zhang and Dr. Cheng-Ming Chiang had no involvement in the peer-review of this article, and had no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Wenyuan Han.

CRediT Authorship Contribution Statement

Menghan Shi: Software, Methodology, Formal analysis, Data curation. **Niu Zhou:** Writing – review & editing, Software, Methodology. **Mengchen Xiu:** Methodology, Investigation, Data curation. **Xiangzhi Li:** Writing – review & editing, Visualization, Formal analysis. **Fen Shan:** Writing – review & editing, Software. **Wu Chen:** Writing – review & editing. **Wanping Li:** Software, Methodology. **Cheng-Ming Chiang:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Xiaodong Wu:** Resources, Project administration, Conceptualization. **Youming Zhang:** Supervision, Project administration, Funding acquisition. **Aiying Li:** Writing – review & editing, Visualization, Validation, Methodology. **Jingjing Cao:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Acknowledgments

This work was supported by the National Key R&D Program of China (2019YFA0905700, 2018YFA0900400), Natural Science Foundation of China (31900147, 32170038, 32270088, M-0348 and 32161133013), the 111 Project (B16030), and a Sino-German Helmholtz International Lab grant. C.M.C's research was supported by US National Institutes of Health grant 1R01CA251698–01 and CPRIT grants RP180349 and RP190077.

Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2024.100149.

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