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RESEARCH ARTICLE

Identification of Canine Parvovirus NS1-Interacting Host Proteins and Functional Characterization of PCNA in Regulating Viral Replication

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ABSTRACT

Canine parvovirus (CPV) is capable of inducing hemorrhagic gastroenteritis and myocarditis in dogs, which imposes financial burdens on dog breeding and pet industries. To elucidate the complex interactions between the CPV non-structural protein NS1 and host proteins during CPV infection, this study employed co-immunoprecipitation (Co-IP) approach coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to screen 401 host proteins interacting with NS1 in F81 cells. Subsequent protein-protein interaction (PPI) network construction, Gene Ontology (GO) functional categorization, and Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping analysis revealed that the NS1-interacting proteins are involved in metabolism, gene expression, and the cell cycle. Based on these findings, Co-IP and GST pulldown assays were used to verify the interactions of seven proteins (PCNA, FHL2, SEH1L, DHRS7B, RFC4, POLB, and FRMD6) that specifically bind to CPV NS1. Further investigation of the interaction with PCNA revealed that overexpression of PCNA reduced CPV replication, whereas knockdown or inhibition of PCNA increased CPV replication. Additionally, knockdown of PCNA during CPV infection resulted in an increased proportion of cells in the S phase, thereby modulating the cell cycle. These results establish a basis for comprehending the molecular processes involved in CPV-host interactions, and they could assist in pinpointing possible antiviral targets.

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INTRODUCTION

Initially isolated and characterized in the late 1970s, CPV has undergone rapid evolution into various new strains, including CPV-2a, CPV-2b, and CPV-2 (Parrish, 1999; Fu *et al.*, 2022). This virus mainly infects actively dividing tissues, including lymphoid tissues, intestinal epithelium, and bone marrow, in addition to the hearts of neonatal puppies (Jiang, 2018; Capozza *et al.*, 2023). Following infection with CPV, dogs typically exhibit symptoms such as anorexia, depression, fever, vomiting, diarrhea, and dehydration (Decaro and Buonavoglia, 2012). CPV has a morbidity rate of up to 100%, with a mortality rate of up to 91% in unvaccinated puppies. This has resulted in significant economic losses to the dog breeding and pet industries (Alexis *et al.*, 2021; Temizkan and Sevinc Temizkan, 2023).

CPV is classified within the Parvoviridae family, specifically in the subfamily Parvovirinae, under the genus *Protoparvovirus*, and it is identified as the species Carnivore protoparvovirus 1 (Zaher *et al.*, 2020; Tuteja *et al.*, 2022). The viral capsid of CPV is a T = 1 icosahedron, measuring roughly 25 nm in diameter, housing a linear, single-stranded DNA genome of negative polarity that spans about 5 kb (Franzo *et al.*, 2023; Dema *et al.*, 2021). It comprises two open reading frames (ORFs) encoding a pair of non-structural proteins (denoted as NS1 and NS2) alongside two structural proteins (denoted as VP1 and VP2) (Hoelzer *et al.*, 2008; Zheng *et al.*, 2018; Li *et al.*, 2022). The non-structural protein NS1 consists of a DNA-binding region at the N-terminus, an ATP-dependent helicase module, and a transactivation segment at the C-terminus. It participates in multiple host cell activities, encompassing DNA repair signaling, type I interferon

response, apoptosis, and tumor suppression (Xie *et al.*, 2023). NS1 is vital for regulating viral promoters and facilitating viral replication.

During the infection of host cells, the interactions between viral proteins and host proteins are key processes for viral replication. In particular, non-structural proteins of the virus play a critical role in regulating viral replication and suppressing the host cell's antiviral responses (Hauswirth *et al.*, 2023; Xie *et al.*, 2023). As a member of the parvovirus family, CPV lacks its own DNA polymerase and therefore relies on the host cell's replication machinery for proliferation (Wang *et al.*, 2025). This process is typically mediated through interactions between viral and host proteins (Shah *et al.*, 2022). Earlier research has reported interactions between the NS1 proteins of minute virus of mice (MVM) and rodent parvovirus H1 (H1-PV) and host proteins (Adeyemi *et al.*, 2010; Hauswirth *et al.*, 2023). In this study, we employed Co-immunoprecipitation (Co-IP) in conjunction with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify host proteins that specifically associate with the CPV NS1 protein and elucidated the role of PCNA in regulating viral replication. This approach provides valuable insights into the interactions between the non-structural protein NS1 and host proteins, laying a theoretical foundation for mechanistic studies and the discovery of possible antiviral targets.

MATERIALS AND METHODS

Cells and Virus: Feline kidney (F81) cells were acquired from the Cell Bank of the Chinese Academy of Sciences. Human embryonic kidney (HEK 293T) cells were sourced from the American Type Culture Collection (ATCC). The CPV-2a strain SD6 was maintained in our laboratory (Zhou *et al.*, 2019), with all infection procedures executed under BSL-2 biocontainment standards.

Plasmid Construction and Cell Transfection: cDNAs of fca PCNA , fca FHL2, fca SEH1L, fca DHRS7B, fca RFC4, fca POLB, and fca FRMD6 were amplified with the use of specific primers (Table 1) and subsequently cloned into pCMV-Myc-C and/or pGEX-4T-1 vectors (YouBio, Changsha, Hunan, China). Prior to downstream assays, plasmids or siRNAs (GenePharma, Shanghai, China) (Table 2) were introduced using Lipofectamine™ 3000 or Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

Western blot: Cell lysates were separated by SDS-PAGE and were transferred onto PVDF membranes (Millipore, Burlington, MA, USA). Membranes underwent blocking with 5% milk-TBST (1 h, RT), incubated with primary antibodies overnight (4°C), washed (3 × TBST), then detected with secondary antibodies (1 h, 37°C). Bands were detected using SuperSignal™ West Pico Plus substrate (Thermo Scientific, Waltham, MA, USA).

Co-IP and Glutathione S-Transferase (GST) Pull-Down Experiments: For Co-IP, HEK 293T cells transfected with recombinant plasmids were lysed, and clarified lysates were incubated with Protein A/G beads (4°C, 2h). Immunoprecipitation was performed using anti-Flag/anti-c-Myc beads (MedChemExpress, Monmouth Junction, NJ, USA), followed by Western blot detection with anti-FLAG/anti-c-Myc antibodies (Sigma-Aldrich, St. Louis, MO, USA). In GST pull-down, GST-fused proteins (GST, GST-PCNA, GST-FHL2) bound to glutathione-conjugated beads were then mixed with 3xFlag-NS1 (4°C, 4h). Protein complexes were resolved through SDS-PAGE and detected with anti-GST/anti-FLAG monoclonal antibodies.

Table 1: List of primers used in this study.

Gene/Gene ID	Sequence 5' - 3' (restriction site)
MYC-PCNA	GCGTCGACATGTTCGAGGCGCGCC(*Sal*I)
101080704	GGGGTACCAGATCCTTCTTCATCCTCGATCTTG GG(*Kpn*I)
MYC-FHL2	CGGAATTCATGATGATGACGGAGCGCTTTGAC T(*Eco*RI)
101100121	CCGCTCGAGGGATGTCTTTCCCACAGTCCGGG (*Sal*I)
MYC-SEH1L	GCGTCGACATGTTTGTGGCGCGCAGTATC(*Sal*I)
101094793	CCCTCGAGGAACCCCTTCATTCTCAGGTAAGG GATTAA(*Xho*I)
MYC-DHRS7B	GCGTCGACATGGTCACTGAGGCGACCAG(*Sal*I)
101089810	CCCTCGAGGGGAGTTCTTGGATTTCCTCTCCTT TCTG(*Xho*I)
MYC-RFC4	GCGTCGACATGCAGGCTTTTCTAAAAGGCACA T(*Sal*I)
101098886	CCCTCGAGGGCAATTCTGAGTTAACTGCTGCAT CA(*Xho*I)
MYC-POLB	GCGTCGACATGAGCAAACGGAAGGCGC(*Sal*I)
101099797	GGGGTACCTTCACTTCGATCCTTGGGTTCTCG(*Kpn*I)
MYC-FRMD6	GCGTCGACATGAACAAATTGAATTTCCACAACA ACAGAGT(*Sal*I)
101097070	CCCTCGAGGTACAACGAACTCTGGAACTTCGT CATGAG(*Xho*I)
GST-PCNA	CGGGATCCATGTTCGAGGCGCGCC(*Bam*HI)
101080704	GCGTCGACAGATCCTTCTTCATCCTCGATCTTG GG(*Sal*I))
GST-FHL2	CGGAATTCATGATGATGACGGAGCGCTTTGAC T(*Eco*RI)
101100121	CCGCTCGAGTCAGATGTCTTTCCCACAGTCCG GG(*Sal*I)

Table 2: siRNA sequences used in this study.

siRNA	sense (5'-3')	antisense (5'-3')
PCNA-Felis catus-639	GCCGAGAUCUCAGU CAUAUTT	AUAUGACUGAGAUCU CGGCTT
PCNA-Felis catus-441	CUGGCAAUGAAGACA UCAUTT	AUGAUGUCUUCAUUG CCAGTT
PCNA-Felis catus-776	GCUGUUACCAUAGAA AUGATT	UCAUUUCUAUGGUAA CAGCTT

Liquid Chromatography–Mass Spectrometry (LC-MS/MS): Immunoprecipitated specimens underwent 45-minute LC-MS/MS on a timsTOF Pro instrument (Bruker) coupled to NanoElute liquid chromatography. APTBio (Shanghai, China) conducted analyses. Raw data were processed via MaxQuant for identification/quantification, excluding negative control-identified proteins to remove non-specific binding.

Protein–Protein Interaction Network Assembly and Evaluation: The STRING repository (https://cn.string-db.org/) and the Cytoscape v.3.10.2 software platform were utilized to integrate experimental datasets and construct an interaction network between the CPV NS1 viral protein and host proteins.

GO and KEGG Pathway Analyses: For the host proteins specifically interacting with CPV NS1, GO

analysis (http://geneontology.org) was conducted using the Cytoscape v.3.10.2 analysis platform. The enrichment of signaling pathways for the host proteins specifically interacting with CPV NS1 was also analyzed by using KEGG database (http://www.genome.jp/kegg). GO terms and KEGG pathways with a p-value < 0.05 were recognized as significantly enriched items.

RNA Extraction and qPCR: For PCNA expression analysis, F81 cells were harvested 48 h post-knockdown. Total RNA was extracted via RLT lysis, reverse-transcribed, and quantified by qPCR using SuperReal PreMix Plus (SYBR Green) (TIANGEN, Beijing, China) on a CFX Connect™ Real-Time PCR System (Bio-Rad, Hercules, CA, USA). β-actin was used as internal control, with relative expression calculated via $2^{-\Delta\Delta CT}$ method.

Viral copy numbers were quantified via qPCR 24 h post-CPV infection (MOI=0.1) in PCNA-modulated cells (knockdown, overexpression, or inhibitor-treated), using established method (Zhou *et al.*, 2019).

Flow cytometry: CPV-infected (MOI 0.1, 24 h) F81 cells with PCNA knockdown were processed as follows: cells were PBS-washed, digested with 0.05% trypsin-EDTA, quenched with 10% FBS medium. Fixed in 70% ethanol (-20°C overnight), cells were washed, resuspended in 500 µL PI staining solution. After 40 µm filtration, data were collected using a CytoFLEX S flow cytometer (Beckman Coulter).

Statistical Analysis: Data were processed in GraphPad Prism, with results shown as mean ± SD. Statistical significance was evaluated via Student *t*-test, with thresholds defined as *P <0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

RESULTS

The interaction between CPV NS1 protein and host proteins was identified through LC-MS/MS analysis: To investigate the interaction between CPV NS1 and host-derived proteins in F81 cells, the eukaryotic expression plasmids p3xFlag-NS1 and p3xFlag-CMV empty vector were transfected into F81 cells, respectively. After 24 hours of overexpression, samples were collected for Co-IP analysis. The samples obtained after immunoprecipitation were subjected to PAGE and visualized using silver staining (Fig. 1A). The remaining immunoprecipitated samples of each group were subjected to Coomassie Brilliant Blue staining, and based on the silver staining results, we removed clearly identical bands and collected the remaining gel, which was then pooled into a single sample for subsequent mass spectrometry analysis to explore the interactions between NS1 and the proteome of F81 cells. Raw MS files were subjected to database searching via MaxQuant for protein identification and quantification. Modified sites were filtered at a false discovery rate (FDR) of 0.01, only peptides containing at least seven amino acids were retained, enzymatic missed cleavages were capped at two, and variable modifications were defined as methionine oxidation and protein N-terminal acetylation. The analysis revealed a total of 1108 host proteins that associate with NS1 in cells transfected with Flag-NS1. Out of these, 707 host proteins were also detected in F81 cells harboring the p3xFlag-CMV vector control. Consequently, this study identified 401 host proteins that distinctly bind to CPV NS1 protein (Fig. 1B).

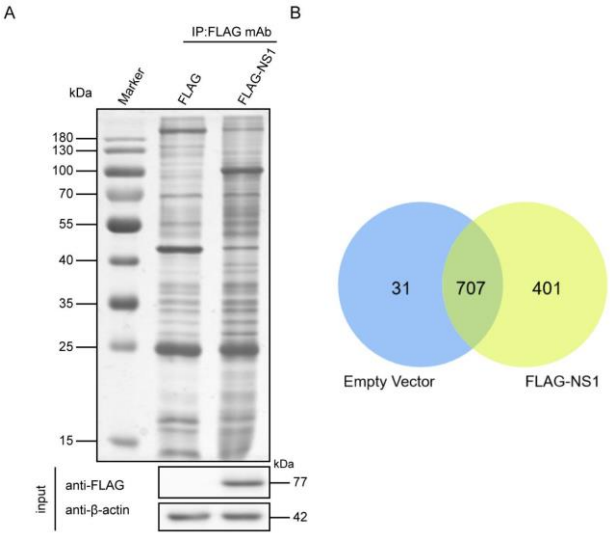

Fig.1: Detection of host proteins that interact with the CPV NS1 protein. (A) F81 cells were transfected with either the p3xFlag-CMV vector or p3xFlag-NS1. An immunoprecipitation assay was conducted 24 hours post-transfection, with subsequent analysis via SDS-PAGE and silver staining. Lane 1 shows the protein molecular weight ladder; lane 2 corresponds to the p3xFlag-CMV control; and lane 3 represents the transfection of p3xFlag-NS1. (B) Venn diagrams depicting proteins associated with CPV NS1 derived from the empty p3xFlag-CMV control and p3xFlag-NS1.

Protein–Protein Interaction (PPI) Network Construction: Protein-protein interactions play a critical role in molecular biology research, as they are essential for numerous biological processes and provide the foundation for understanding the complex networks within cells. In this study, an interaction network was assembled through the STRING database and Cytoscape v.3.10.2 software, followed by functional analysis. PPI analysis revealed that the observed node connectivity (523) markedly exceeded the predicted value (285), indicating a higher-than-expected frequency of interactions. In the PPI network, proteins associated with ribosome biogenesis, such as WDR36, WDR43, and UTP4, were identified; proteins related to the cell cycle, including PCNA and CDK1; and proteins involved in DNA replication, such as PCNA, POLA1, and PRIM2 (Fig. 2).

GO Analysis: Gene Ontology (GO) enrichment analysis serves as a robust strategy for investigating the biological roles of genes exhibiting altered expression patterns. The GO annotation system is structured into three distinct categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), and it is applicable across a wide range of species. In this study, GO annotation was performed on host proteins that specifically expressed and interacted with CPV NS1 protein. The results indicated that CPV NS1 primarily influences biological activities including cellular macromolecule metabolic processes, gene expression, cellular component biogenesis, and the cell cycle. Furthermore, within the Cellular Component category, the GO terms significantly enriched included membrane-

bounded organelles, the nucleus, and intracellular non-membrane-bounded organelles. In the Molecular Function category, enrichment was observed for GO terms related to organic cyclic compound binding, nucleic acid binding, RNA binding, and purine ribonucleotide binding (Fig. 3).

KEGG Analysis: To further elucidate the cellular pathways through which CPV NS1 targets host protein metabolism and signal transduction, we performed KEGG pathway enrichment profiling and extracted the top20 statistically significant pathways. The enrichment assessment demonstrated that the target proteins were predominantly enriched in pathways such as the spliceosome, RNA transport, ribosome biogenesis in eukaryotes, and the cell cycle. In addition, several proteins were enriched in pathways including DNA replication, RNA degradation, mismatch repair, homologous recombination, the Hedgehog signaling pathway, and the Hippo signaling pathway (Fig. 4).

Validate the interaction between CPV NS1 non-structural protein and host proteins: F81 cells are highly susceptible to CPV and efficiently support CPV replication, making them suitable for virus-host interaction studies in this research. For downstream validation, HEK 293T cells were employed on account of their superior transfection efficiency and robust protein expression capacity and were subjected to co-immunoprecipitation (Co-IP) and GST pull-down assays. To validate the protein-protein interactions identified by LC-MS/MS, this study employed *in vitro* Co-IP experiments to verify the interaction between CPV NS1 and seven selected host proteins. The p3xFlag-NS1 recombinant plasmid or an empty vector control was co-transfected into HEK 293T cells with expression plasmids encoding MYC-tagged PCNA, MYC-FHL2, MYC-SEH1L, MYC-DHRS7B, MYC-RFC4, MYC-POLB, and MYC-FRMD6. Immunoprecipitation was carried out using anti-FLAG and anti-MYC magnetic beads. The results demonstrated that CPV NS1 exhibited specific interactions with PCNA, FHL2, SEH1L, DHRS7B, RFC4, POLB, and FRMD6, with varying binding intensities. No binding signals were detected in the empty vector control group (Fig. 5A, 5B).

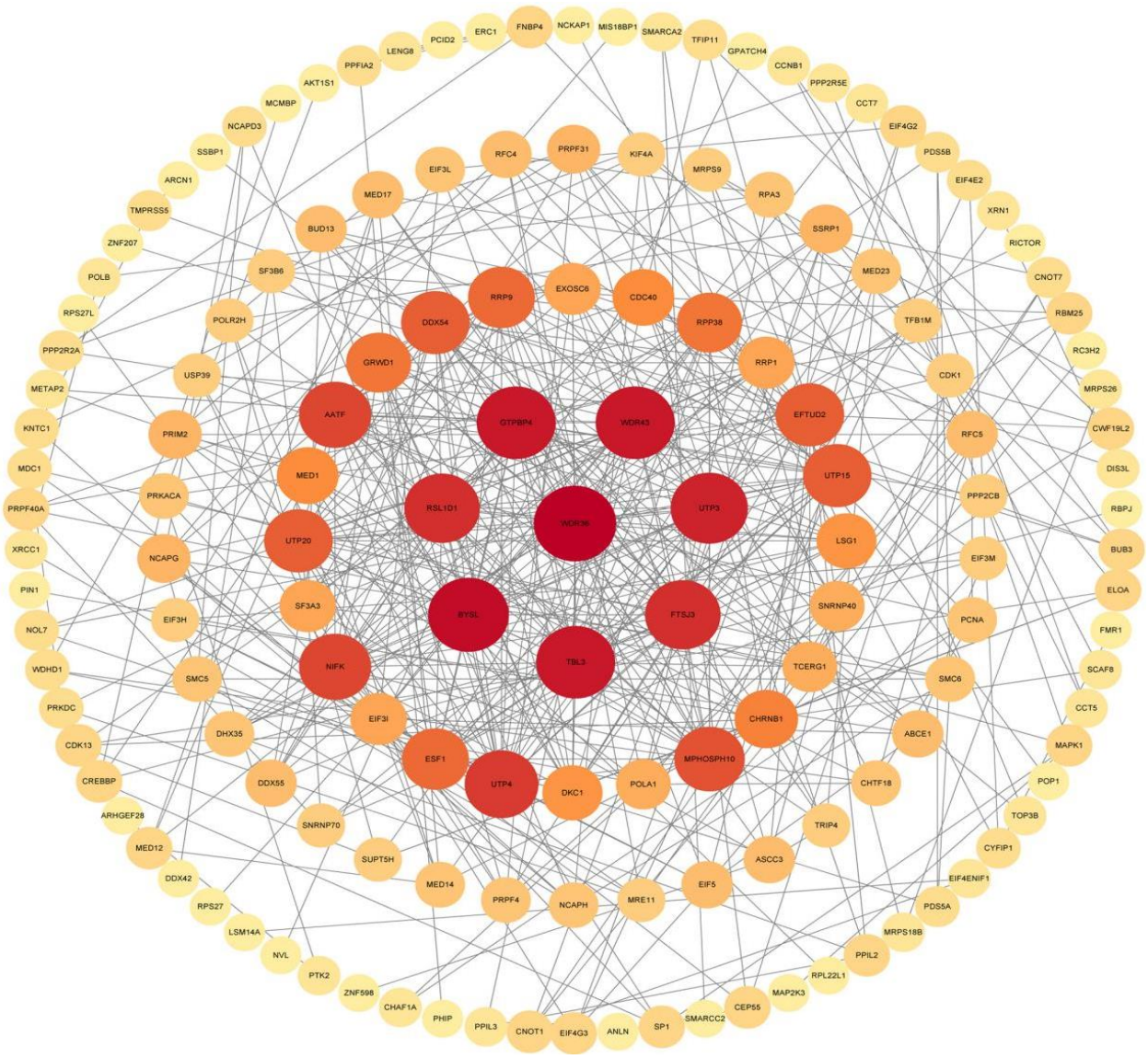

Fig.2: Development and examination of the PPI network. The interaction network diagram of host proteins associated with CPV NS1 was created with the STRING database and visualized using Cytoscape v.3.10.2. Each node symbolizes a host protein, while edges indicate interactions among these nodes. A larger node signifies a higher degree of interaction, and darker colors represent greater significance. The respective NCBI gene names were used for the proteins.

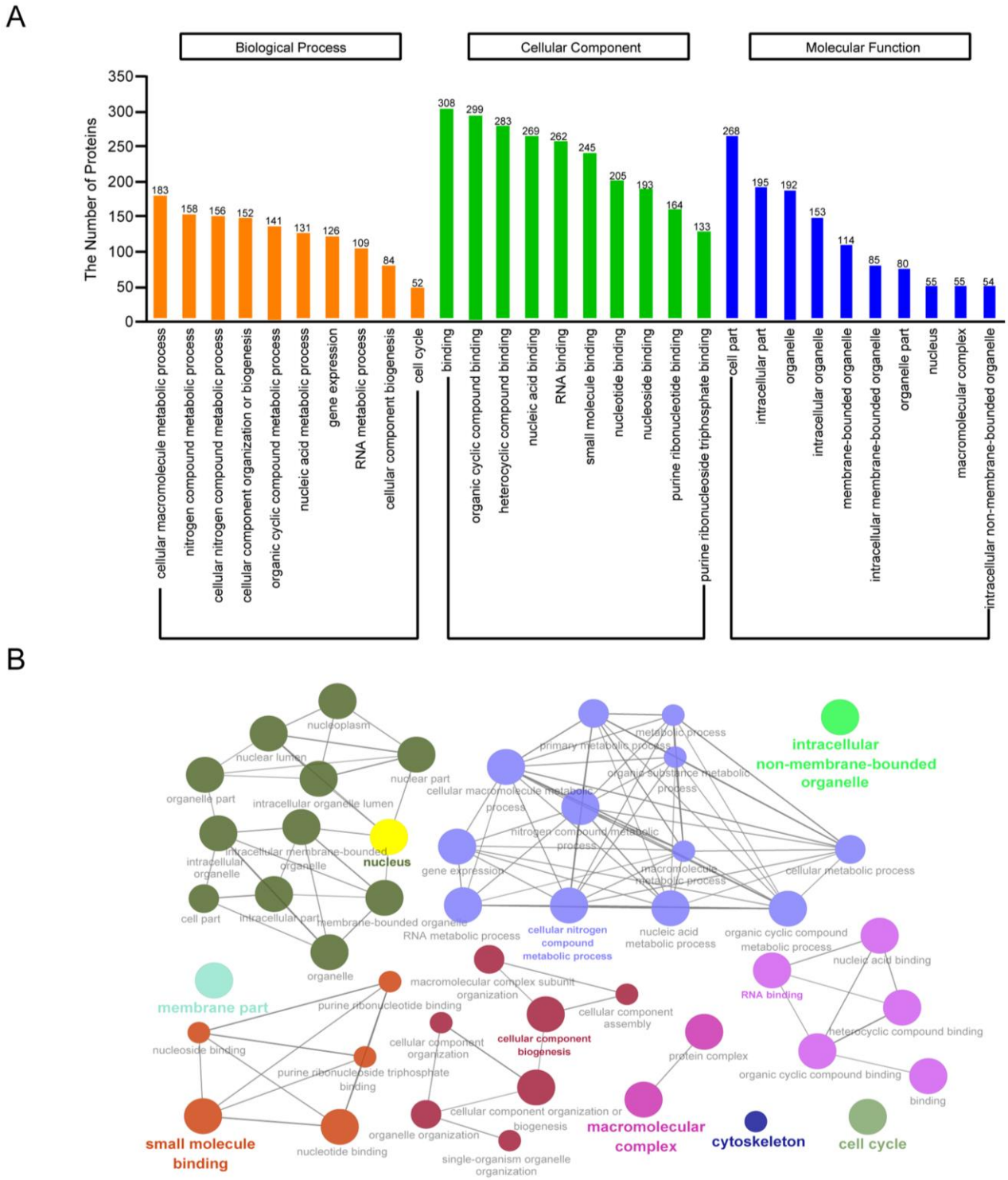

Fig.3: GO assessment based on the interaction between cellular proteins and CPV NS1. The Gene Ontology Resource website facilitated the GO analysis. The distributions of proteins in the GO were classified into three categories: BP, MF, and CC.

To further validate the direct interaction between CPV NS1 and host proteins, GST pull-down assays were performed using PCNA and FHL2. Recombinant plasmids encoding p3xFlag-NS1 were incubated with the lysates of GST-PCNA and GST-FHL2 fusion proteins, followed by GST affinity purification. Protein-protein interactions were subsequently assessed by immunoblotting. As shown in the figure, CPV NS1 directly interacted with both PCNA and FHL2 (Fig. 5C). Collectively, the results from the Co-IP and GST pull-down experiments confirmed the protein-protein interactions identified through LC-MS/MS-based proteomic analysis.

The role of PCNA on CPV viral infection: Given that NS1 is linked to cell cycle arrest and PCNA is one of the molecules involved in regulating the cell cycle, we chose to focus our further studies on PCNA. To examine the role of PCNA on CPV viral infection, siRNA was employed to decrease the expression of PCNA in F81 cells.

Quantitative PCR analysis demonstrated that PCNA transcript levels were substantially reduced after interference treatment (Fig. 6A). The transfected cultures were subsequently challenged with CPV at an MOI of 0.1, and after 48 hours, viral VP2 protein expression was assessed by qPCR and WB. The results revealed that, compared to the negative control group, the VP2 protein expression in the PCNA knockdown group was significantly increased (Fig. 6B-6D). To further verify the regulatory role of PCNA in viral replication, PCNA was overexpressed in F81 cells. After confirming the overexpression efficiency by WB, the transfected cultures were challenged with 0.1 MOI CPV for 48 hours. The VP2 protein levels in the PCNA overexpression group were considerably reduced compared with the control counterpart (Fig. 6E-6G). Additionally, F81 cells were treated with the PCNA inhibitor T2AA for 1 hour prior to infection with CPV at a MOI of 0.1. After 24 hours of infection, samples were collected for qPCR and WB analysis. The findings demonstrated that T2AA-treated cells exhibited elevated VP2 protein levels relative to the DMSO-treated control (Fig. 6H-6J). Collectively, these data suggest that PCNA negatively regulates CPV replication.

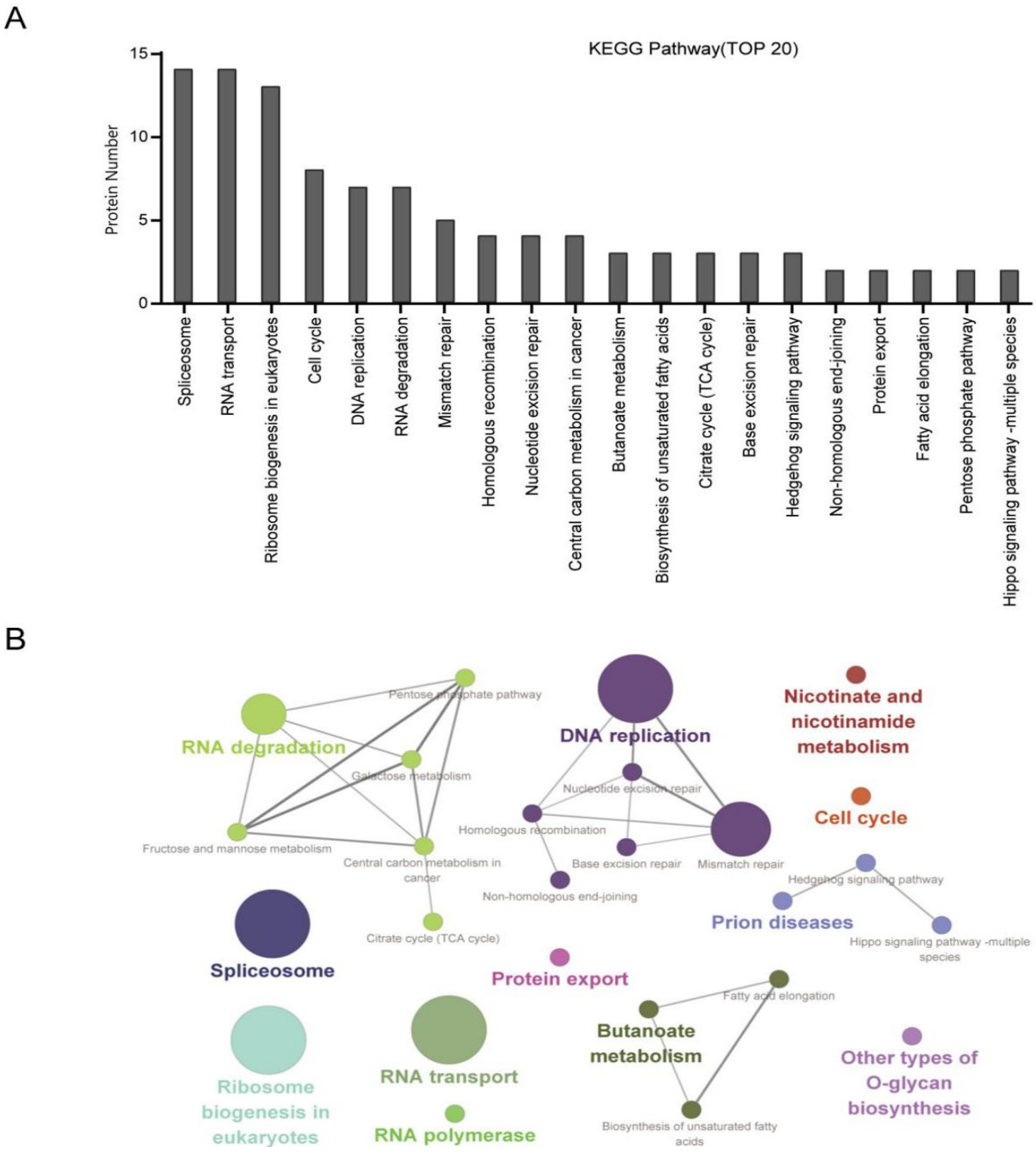

Fig. 4: KEGG pathway enrichment evaluation. (A) Pathway enrichment analysis was conducted on proteins interacting with Canine Parvovirus (CPV) NS1, utilizing the KEGG pathway database. (B) Analysis of proteins that interact with CPV NS1 through KEGG functional annotation was performed using the GOclue plug-in in Cytoscape software, with $P<0.05$.

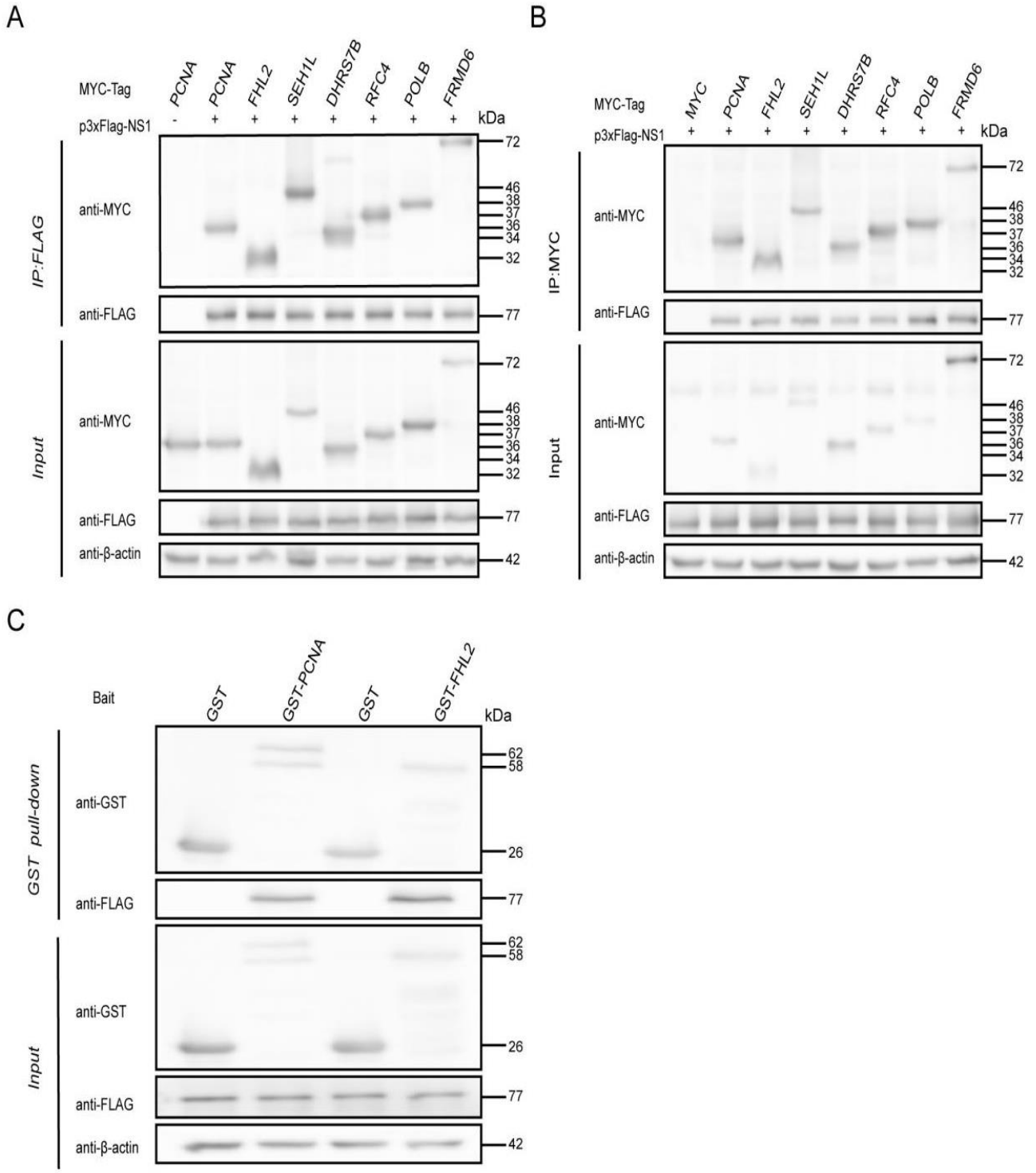

Fig. 5: Validation of interactions between CPV NS1 and host proteins. (A, B) HEK 293T cells underwent co-transfection with plasmids that express MYC-PCNA, MYC-FHL2, MYC-SEH1L, MYC-DHRS7B, MYC-RFC4, MYC-POLB, MYC-FRMD6, alongside a plasmid for p3xFlag-NS1. In these experiments, p3xFlag-NS1 or MYC-PCNA was also co-transfected with an empty vector used as a negative control. The resulting cell lysates were subjected to immunoprecipitation using anti-Flag or anti-MYC antibodies, followed by separation using SDS-PAGE. The proteins were subsequently transferred onto a membrane and detected utilizing the relevant antibodies, with β-actin serving as an internal standard. The calculated molecular weights for the host proteins that interacted are approximately: PCNA (36 kDa), FHL2 (32 kDa), SEH1L (46 kDa), DHRS7B (34 kDa), RFC4 (37 kDa), POLB (38 kDa), and FRMD6 (72 kDa). (C) Full-cell lysates of p3xFlag-NS1 were incubated with GST, GST-PCNA, and GST-FHL2 to conduct the GST pull-down assay; the immunoblotting was then executed with antibodies directed against GST, and β-actin was employed as an internal standard.

PCNA is associated with the regulation of cell cycle after CPV infection: To explore the mechanistic basis of PCNA function during CPV infection, flow cytometry was used to analyze the cell cycle in the si-776 knockdown group and the control si-NC group following infection (Fig. 7A, 7B). Compared to the si-NC infection group, the si-776 infection group (si-776 vs si-NC) showed an increased proportion of cells in the S phase (38.1±0.1% vs. 25.17±1.44%), a decreased proportion in the G2/M phase (15.37±0.55% vs. 32.53±2.34%), and no significant change in the proportion of cells in the G0/G1 phase (43.4±1.04% vs. 41.3±2.01%) (Fig. 7C).

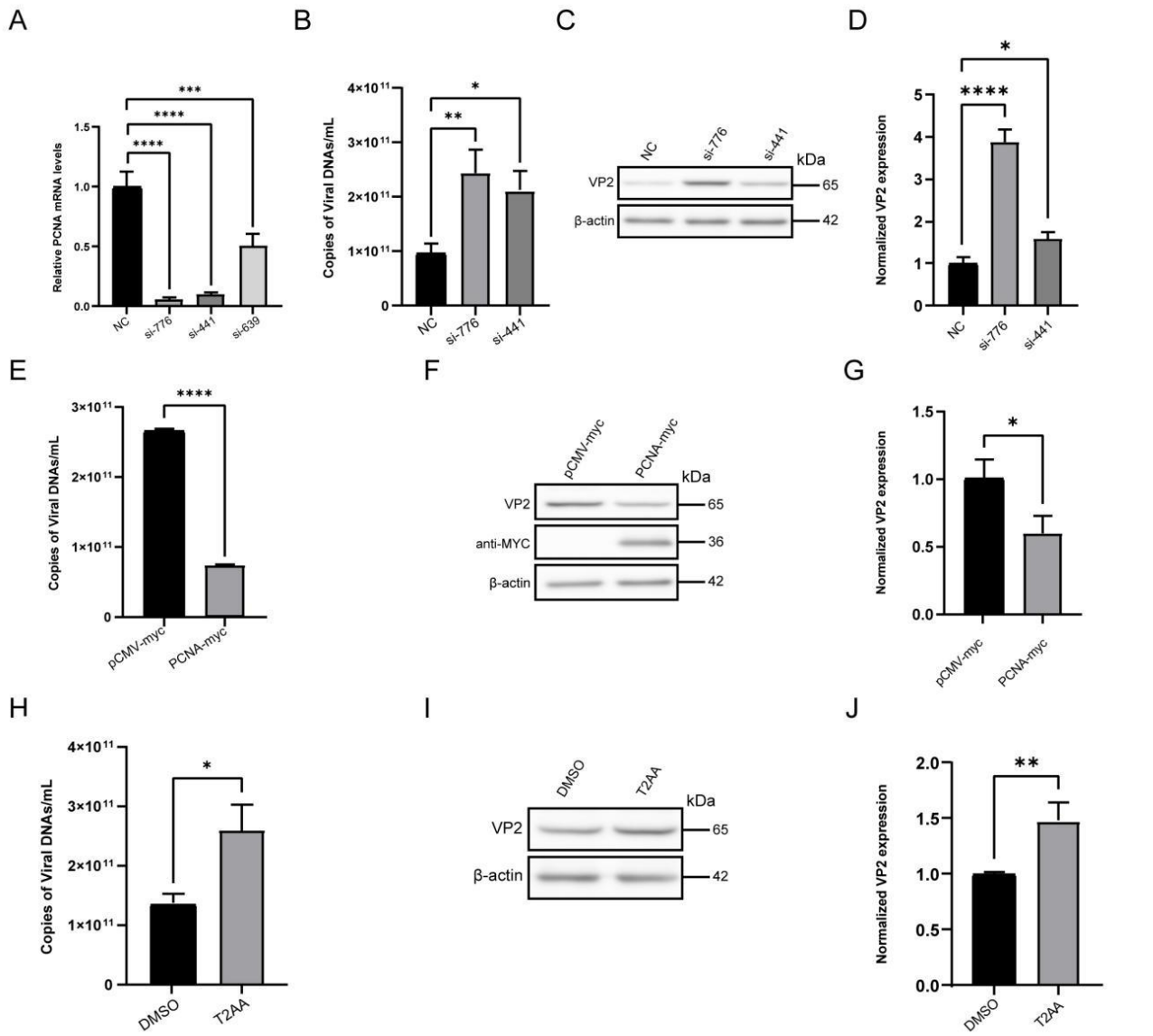

Fig. 6: Influence of PCNA on CPV replication. (A) Assessment of siRNA interference efficiency through RT-qPCR analysis. (B) qPCR analysis evaluating the expression levels of the CPV VP2 gene. (C, D) Evaluation of alterations in CPV protein levels and associated grayscale quantification post-PCNA knockdown. (E) qPCR analysis measuring the expression levels of the CPV VP2 gene. (F, G) Evaluation of variations in CPV protein levels and associated grayscale quantification after PCNA overexpression. (H) qPCR analysis assessing CPV VP2 gene expression levels. (I, J) Assessments of changes in CPV protein levels and corresponding grayscale quantification following T2AA treatment in cells. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

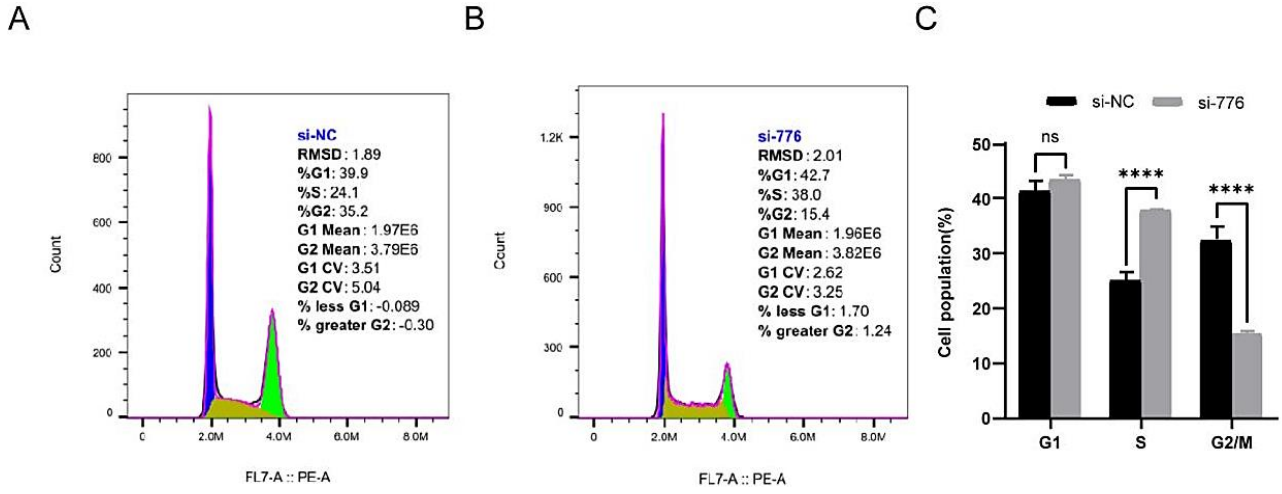

Fig.7: Flow cytometry evaluation of the impact of PCNA knockdown on the cell cycle. (A, B) Percentages of cells in distinct phases of the cell cycle for the si-NC and si-776 groups. (C) Statistical analysis comparing the distribution changes of cells across different phases of the cell cycle between the si-NC and si-776 groups. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

DISCUSSION

Parvoviruses regulate host cell interactions through multiple mechanisms (Kailasan *et al.*, 2015; Gallinella, 2019; Haseeb *et al.*, 2025). Due to the limited genomic capacity of parvoviruses, both their structural and non-structural proteins exhibit multifunctionality during the infection process (Kailasan *et al.*, 2015; Haseeb *et al.*, 2025).The non-structural protein NS1 is a multifunctional phosphorylated protein with various enzymatic activities, including DNA helicase, endonuclease, and ATPase activities (Vargas-Ruiz *et al.*, 2025). It plays a crucial role in viral replication, transcriptional control, host apoptotic pathway modulation, and cell cycle checkpoint enforcement (Chen *et al.*, 2024). Parvoviruses are capable of infecting a wide range of species (Hsi *et al.*, 2025). Certain parvoviruses infecting companion animals, exemplified by CPV, exhibit extensive adaptability, rapid evolution, and frequent mutations (Lamm and Rezabek, 2008). Currently, vaccination is still the most efficient strategy to prevent CPV infection (Li *et al.*, 2025). However, the emergence of new CPV variants has led to numerous changes in antigenicity, immunogenicity, and virulence, which pose significant challenges to the efficacy of existing vaccines (Zhou *et al.*, 2024). Therefore, elucidating the intricate interactions between the NS1 protein and host proteins during CPV infection will advance our comprehension of the molecular underpinnings governing CPV. This knowledge may help discover potential antiviral targets.

LC-MS/MS is a critical tool in the field of protein and peptide bioanalysis (Kang *et al.*, 2020).In proteomics research, LC-MS/MS is widely employed for protein identification, characterization, and biomarker discovery, significantly advancing the ongoing development of proteomics studies (Henry and Meleady, 2017).We utilized LC-MS/MS technology to identify proteins that specifically bind to CPV NS1 and integrated these findings with existing PPI data, thereby constructing a comprehensive PPI network. This network not only reveals direct interactions between proteins but also expands our understanding of the functional networks of these proteins through known protein interaction data. Further, we conducted functional enrichment profiling using GO and KEGG databases for the identified proteins. GO enrichment analysis indicated significant enrichment of these host proteins in BP, MF, and CC. The enriched GO terms primarily reflect biological processes such as metabolic processes, gene regulation, and signal transduction. KEGG enrichment analysis further highlighted the involvement of these proteins in multiple signaling pathways, encompassing cell cycle control, Hedgehog signal transduction, and Hippo signaling pathway. These analyses suggest that CPV NS1 may assist the virus in infecting or replicating within host cells by interfering with cellular metabolism, regulating gene expression, and hijacking the cell cycle. These observations furnish a conceptual framework for subsequent mechanistic studies.

In this study, based on a comprehensive consideration of the number of unique peptide segments, molecular weight, and protein abundance, we have screened seven host proteins, including PCNA, FHL2, SEH1L, DHRS7B, RFC4, POLB, and FRMD6, and validated them through Co-IP experiments. The results showed their interaction with CPV NS1 protein. Furthermore, GST pull-down assays confirmed the direct interactions between PCNA, FHL2, and CPV NS1. The host proteins identified as interacting with NS1 participate in diverse intracellular activities, such as cell cycle control (PCNA, SEH1L), energy metabolism (DHRS7B), DNA repair (POLB), innate immunity (FHL2), Hippo signaling (FRMD6), and Notch1 signaling (RFC4).

Among these proteins, we preliminarily investigated the function of PCNA in the process of viral replication. PCNA belongs to the DNA sliding clamp (β-clamp) family and is primarily responsible for regulating DNA replication and repair, serving as a platform for DNA polymerase δ/ε and other replication factors (Maga and Hubscher, 2003; Strzalka and Ziemienowicz, 2011; Chen *et al*., 2024; Kwan *et al*., 2024). Our observations indicated that overexpression of PCNA in F81 cells inhibits viral replication, whereas knockdown of PCNA or treatment with the PCNA inhibitor T2AA enhances viral replication. Earlier reports have demonstrated that T2AA disrupts the interaction between PCNA and DNA polymerase δ in the cell chromatin (Drosopoulos *et al.*, 2020). Inhibition of DNA synthesis by T2AA results in cell cycle arrest at the S phase (Punchihewa *et al.*, 2012). Moreover, during parvovirus replication, the NS1 protein co-localizes with the nuclear foci of the "autonomous parvovirus-associated replication" (APAR) bodies, which are known to recruit PCNA and DNA polymerase δ (Adeyemi *et al.*, 2010). We hypothesize that the binding of NS1 to PCNA may interfere with the interaction between PCNA and DNA polymerase δ, preventing PCNA from effectively participating in cellular DNA replication and repair, thereby prolonging the S phase. This prolonged S phase provides ample DNA and protein resources, facilitating rapid CPV replication within the host cell (Dai *et al.*, 2020). Our findings parallel prior observations documenting the co-accumulation of MVM NS1 and PCNA in APAR, as well as the interaction between H1-PV NS1 and DNA replication factors (Adeyemi *et al.*, 2010; Hauswirth *et al.*, 2023). Nevertheless, additional experimental validation is required to confirm this hypothesis.

It should be noted that this study has certain limitations, and the following questions remain to be resolved in subsequent studies. First, the functional contributions of the NS1-associated host factors reported herein, especially those additional interactors confirmed by Co-IP, to CPV replication remain to be fully defined. Second, whether and how NS1 influences the PCNA–DNA polymerase δ association and subcellular distribution remains to be directly tested. Third, the physiological relevance of PCNA-dependent CPV replication control needs to be substantiated in animal models.

In conclusion, we have captured host-derived proteins that interact with NS1 in F81 cells. Through our bioinformatic assessment, we demonstrated that these proteins are primarily associated with cellular metabolic networks, DNA replication and repair, the cell cycle, and various signaling pathways. Following that, we chose seven host proteins for Co-IP and/or GST pull-down assays, and the results aligned with those derived from

mass spectrometry. Additionally, our further investigations revealed that altering the expression of the PCNA gene can affect viral replication, a process that involves the regulation of the cell cycle. Our findings establish a basis for comprehending the molecular processes involved in CPV-host interactions and contribute to identifying potential antiviral targets.

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Data availability statement: All data underpin the findings of this study are included within the main text. Any additional information can be requested from the corresponding author under reasonable conditions.

Authors contribution: HZ, XS, QL, SL and BY conceived and designed the experiments. HZ, XS, QL, KC, HC, JZ and QQ performed the experiments. HZ, XS, QL, KC, FX, BX, HC, SL and BY analyzed the data. HZ, XS, QL, KC, FX, SL and BY wrote the paper.

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