A High Throughput Method to Analyze the Interaction Proteins With p22 Protein of African Swine Fever Virus in Vitro

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Research

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Abstract

Background: African Swine Fever Virus (ASFV) has been identified as the agent of ASF, which has resulting in a mortality rate of nearly 100% in domestic pigs worldwide. Protein p22 encoded by pKP177L was reported to be localized at the inner envelope of the virus, while the function of p22 remains unclear.

Methods: Protein p22 interacted proteins of the host were immune-precipitated and identified by Liquid Chromatography Mass Spectrometry, analyzed by Go terms and KEGG pathways.

Results: Numerous cellular proteins in 293-T that interacted with p22 protein were identified. These interacted proteins were related to the biological processes of binding, cell structure, signal transduction, cell adhesion, etc., and their interactions. At the same time, the interacted proteins participated in several KEGG pathways like ribosome, splicesome, etc. The key proteins in PPI network were closely related to actin filament organization and movement, resulting in affecting the process of phagocytosis and endocytosis.

Conclusions: The identified high number of proteins interacted with p22 and as a large database should be very useful to elucidate the function of p22 in the near future, and lay the foundation for elucidating the mechanism of ASFV.

Background

African swine fever (ASF) is caused by ASFV, a linear, large, double stranded DNA virus that is the only member of the *Asfarviridae* family [1]. ASFV is an enveloped DNA virus with genome length of 170–193 kbp [1]. The genome encoded 151–167 orfs. ASFV is an icosahedral symmetric virus that replicates in the cytoplasm of infected cells. Warthogs, Bush pigs and soft ticks are natural hosts of the virus, which can persistent to infect without any signs of disease [2]. Once introduced into domestic pigs, ASFV is highly lethal to the pigs, and could spread directly among pigs, resulting in a nearly 100% mortality [3]. Typical clinical symptoms include high fever, cyanosis, hemorrhagic lesions, anorexia and ataxia [4]. The lesion tissues displayed severe pathological vascular changes, such as renal ecchymosis, skin erythema, and diffuse hemorrhages in lymph nodes, kidneys, lungs and urinary bladder, pulmonary edema, disseminated intravascular coagulation and thrombocytopenia [5].

The disease has caused serious economic losses to the pig industry and has a severe impact on the world. Especially that in 2018, the break-out of ASF in China and spread quickly over the country, threatening the pig industry severely [6]. Recent studies have shown that some viral proteins are involved in the adhesion and entry of ASFV, some encoded structural proteins are involved in genome replication and virus infection [7]. It is reported that 15 Of the 26 virus-encoded proteins were detected in the virus proteome with predicted transmembrane domains [8]. However, some detected proteins remain uncharacterized. Among the detected proteins on the membrane, protein p22 (pKP177L) has been predicted to be externally located in the virion [9]. Some studies have reported that p22 was localized around the virus factories, rather than at the cell surface [10, 11]. It was tricky that Protein p22 was

weakly detected throughout the cytoplasm, including the virus factories, but could be detected at the periphery of assembling and mature icosahedral particles. As a result, protein p22 was localized at the inner envelope [12].

Other viral membranes proteins, like p17, pE183L, p12, and pE248R were also at the cell surface but localized at precursor viral membranes and intracellular icosahedral particles within the viral factories [13–16]. Some structural proteins have been reported to be involved in virus entry, like p12, pE248R and pE199L, some are required for the assembly process, like protein p17 and pE183 [17]. However, though these proteins are localized at the membrane of the virus, helping the entry or assembly process of the virus, the receptors of ASFV are still unclear.

In this study, we studied the interacted proteins with p22 of ASFV by proteomics analysis. Although a large number of structural protein studies have been performed, further research on the function and molecular mechanism is needed, will help to prevent and control the spread of the disease.

Methods

Sample preparation

Gene pKP177L (p22) of the ASFV and tagged by HA at C- terminal were synthesized into the plasmid pCDNA-3.1(+) by the Genescript cooperation (ShangHai,China), and sequenced correct. The plasmids were transfected into 293-T cells by lipo3000 according the instruction of manuscript (Thermo scientific, USA), and were proven successfully expressed in 293-T cells. PAMs were prepared and pCDNA-3.1-p22 and mock plasmid were transfected into PAMs, respectively. At 24h post transfection, cells were lysed by lysis buffer (Beyotime, China) with 1% protease inhibitor cocktail, Cell lysates were centrifuged (5000 × g, 5 min), and the supernatants were incubated overnight at 4°C with 1 μg of anti-HA, and then pre-coupled to 40 μl of A/G Plus agarose beads for 4 h at 4°C. The immune complexes were precipitated, washed, and subjected to Western blotting analysis, triplicate sample of each group were prepared as above and sent to high throughput method. Liquid Chromatography Mass Spectrometry (LC-MS/MS) was performed by Shanghai Applied Protein Technology Company on Q Exactive (Thermo Scientific).

Western blot. 293-T cells cells were transfected with 1 μg of pcDNA3.1-p22-HA or mock plasmid for 24 h. At 24 h post transfection, 293-T cells were lysed by lysis buffer containing 1% protein inhibitor. The cell lysate proteins were subjected to SDS-PAGE and transferred onto 0.22-μm nitrocellulose membranes (Pall, Port Washington, NY, USA). Then, the membranes were incubated with 5% defatted milk at room temperature for 2 h, washed by phosphate-buffered saline (PBS) containing 0.05% Tween 20 for three times, followed by anti-HA rabbit polyclonal antibody incubation at 4°C overnight, washed by PBST for three times then incubated with horseradish peroxidase-conjugated goat anti- rabbit IgG secondary antibody at room temperature for 1 h. After three times wash by PBST, detection was performed using ECL Kit (Thermo Fisher Scientific).

Indirect immunofluorescence assay. 293-T cells cells were transfected with 1 μg of pcDNA3.1-p22-HA or mock plasmid for 24 h. At 24 h post transfection, cells were fixed in 4% paraformaldehyde at 4°C for 30 min, and then the cell membranes were permeabilized by PBS containing 0.2% Triton X-100 for 5 min. The cells were incubated with 1:200 diluted anti-HA antibody at 37°C for 1 h. Then, the cells were incubated with Alexa Fluor 555-conjugated goat anti-rabbit IgG at 1:400 dilution at 37°C for 1 h and washed by PBS for three times before examination.

GO Enrichment and KEGG Pathway Analysis of the interacted proteins

Gene ontology (GO) is the concept of a combination of gene-gene functions, is designed to detect cell biological functions via a systematically dynamic and computational interpretation of genes, RNA and proteins. It covers three main areas and clustered by directed acyclic graph (DAG) [18], including cellular components, molecular function, and biological processes. Kyoto Encyclopedia of Gene and Genomes (KEGG) database aims to systematic analyze gene and their related gene functions with an interacting network of molecules in the cells in a hierarchical order [19]. GO enrichment and KEGG pathway analysis of the interacted proteins were conducted. DAVID (http://david.abcc.ncifcrf.gov/) used in this study is short for Database for Annotation, Visualization and Integrated Discovery.

PPI Network construction

Protein-protein interaction (PPI) plays an extremely important role in understanding cellular or systemic processes of cell growth, reproduction and metabolism [20], provides a platform for the annotation of functional, structural, and evolutionary properties of proteins. To further investigate the molecular mechanism of p22 of ASFV, PPI network of the interacted proteins was constructed through STRING database (http://www.string-db.org/). STRING is an online database which includes experimental as well as predicted interaction information and comprises >1,100 completely sequenced organisms. To select core genes from PPI network, we analyzed the top ological structure of the network and obtained the proteins that directly interact with the target protein in the network. We selected the protein-protein interactions especially functioned in the process of endocytosis to construct the PPI network for visualization.

Results

Samples identification

Protein p22 was expressed in the 293-T cells by western blotting analysis, the band was shown as the predicted size of 25 kDa (Fig. 1A). IFA analysis also proved the p22 protein expressed in 293-T cells (Fig. 1B).

Enriched GO Terms analysis

In this study, the cells expressed p22 protein were collected and sent to GO and KEGG analysis. There were thousands of proteins interacted with p22 compared with control samples. From the Go map,

thousands of enriched GO terms were obtained and their corresponding protein numbers were shown in Table.S1 and Table.S3. Go terms mainly covered three parts which the proteins were involved in biological process, molecular function and cellular component. 359 sequences were related to biological process (Fig. 2A). The top two enriched GO terms of biological process were cellular process and metabolic process, biological regulation, cellular component organization or biogenesis, etc.

463 proteins were related to molecular function (Fig. 2B). Major enriched GO terms of molecular function were binding (as high as 378 ptroteins were included), catalytic activity, structural molecule activity, etc, indicating that p22 may be an important role in virus entry.

For the cellular component, 374 sequences were related (Fig. 2C). The GO terms analysis of the interacted proteins mainly included cell part, organelle, protein-containing complex, membrane-enclose lumen membrane, might participate in cell structure maintence.

KEGG Pathways analysis

165 KEGG pathways were screened out and their corresponding protein number were shown in Table.S2 and Table.S3. Top 20 enriched KEGG pathways were listed in (Fig. 3A). According to the result, the KEGG pathways that p22-related proteins involved mainly were Ribosomes(Fig. 3B), splicesome (Fig. 3C), which the protein number as high as 31 and 23, respectivly. Pathogenic Escherichia coli infection, Tight junction, Necroptosis, Ribosome biogenesis in eukaryotes, RNA transport, Regulation of actin cytoskeleton, Cardiac muscle contraction, Adrenergic signaling in cardiomyocytes, Alzheimer disease, Huntington disease, etc were also screened out. It is noteworthy that KEGG pathways analysis showed that 7 related proteins participated in endocytosis (Fig. 3D), 6 proteins were involved in Cyclic GMP-dependent protein kinase (cGMP-PKG) signaling pathway and Focal adhesion. Minor proteins (4 proteins) participated in cAMP signaling pathway and AMP-activated protein kinase (AMPK) signaling pathway.

PPI network

The p22-interacted proteins were placed in the STRING database for PPI analysis and visualization in Cytoscape software. The selected proteins interacted with p22 protein in endocytosis process were connected as a network. The proteins including Myosin-9 (MYH9), Actin-related protein 2/3 complex subunit 2 (ARPC2), Actin-related protein 2, Actin-related protein 2/3 complex subunit 1B, ADP-ribosylation factor 6 (ARF6), Beta-actin-like protein 2 (ACTBL2), Alpha-actinin-4 (ACTN4), Clathrin heavy chain A (CLTC) and Ras-related protein-10 (RAB10). The PPI network contained 8 nodes and 20 edges. All the hub proteins were at key positions in the interaction network. The nodes represented the interacted proteins and the edges represented the interactions between these proteins (Fig. 4). The selected proteins in the PPI network might relate to p22 more closely in the process of endocytosis.

Discussion

As the membrane protein of ASFV, the function of p22 was rarely known. In attempt to get acquired of the p22 function, p22 interacted protein of the host were identified by a high throughput method and analyzed by Go terms and KEGG pathways, numerous cellular proteins in 293-T that interacted with p22 proteins were identified. This study provides a large database and useful tool. It will help to figure out the function of the p22.

In this study, Go terms mainly covered three parts: biological process, molecular function and cellular component. The top two enriched GO terms of biological process were cellular process and metabolic process, implying that p22 might utilize the host proteins directly or indirectly to affect the cell growth, function and stability. Main enriched GO terms of molecular function were binding, catalytic activity. GO analyses revealed that the most significant ontology categories of molecular function is binding, as the protein at inner envelop, we wish to dig the possible role of p22, like, the entry process of the virus, the high number of proteins that interacted with p22 protein were related to binding process, suggesting a role of p22 in virus binding and entry into the cell. The interesting result would inspire us to dig out the real function of p22 in virus entry.

The GO terms analysis of the interacted proteins in cell component mainly included cell part, organelle, protein-containing complex, membrane-enclose lumen, membrane, the results further verified the conclusion that p22 was located in the membrane of the viron and might participate in virus structure maintence and contact with the host membrane via the binding and endocytosis process. Of course the suspect needs further to be proven.

For KEGG pathways analysis, a large number of KEGG pathways were screened out as high as 165, the KEGG pathways that related proteins participated in mainly were Ribosomes, splicesome. Ribosomes are nanomachines essential for protein production and protein synthesis. The initial steps of ribosome biogenesis take place in the cell compartment. Spliceosome executes eukaryotic precursor messenger RNA (pre-mRNA) splicing to remove noncoding introns. It depends on the interaction of RNA-RNA, RNA-protein and protein-protein. It is composed of several nucleoproteins and has the function of recognizing 5 'splicing site, 3' splicing site and branching point of mRNA precursor. It indicated that p22 interacted proteins mainly participated in the process of gene expression in the host cells, gave us a hint that p22 affected the gene and protein expression of cell host, directly or indirectly affected the function of the biological process.

Morever, KEGG pathways that less number of p22 interacted proteins were involved in included pathogenic Escherichia coli infection, tight junction, necroptosis, ribosome biogenesis in eukaryotes, RNA transport, regulation of actin cytoskeleton, cardiac muscle contraction, adrenergic signaling in cardiomyocytes, Alzheimer disease, Huntington disease, etc. The widely affected pathways reflected the wide range of the functions that p22 or its related proteins.

It is noteworthy that KEGG pathways analysis showed that 7 p22-interacted proteins participated in endocytosis. As the results in GO analysis, a large number of p22 interacted proteins participated in binding. Above all, p22 was predicted to be involved in the entry process at the envelop of the virus.

At last, it was possible that other pathways had an important influence on the progression of ASFV entry via some biological process, such as cGMP-PKG signaling pathway, cAMP signaling pathway and AMPK signaling pathway which were screened out by KEGG analysis. cGMP is the intracellular second messenger that mediates the action of nitric oxide (NO) and natriuretic peptides (NPs), affecting a wide range of physiologic processes [21]. cAMP is also one of the most common and universal second messengers, cAMP regulates pivotal physiologic processes including metabolism, secretion, calcium homeostasis, muscle contraction, cell fate, and gene transcription [22]. AMPK is a central regulator of cellular energy homeostasis, regulating growth and reprogramming metabolism as well as in cellular processes including autophagy and cell polarity [23].

In those hub proteins connected in PPI network, the ADP-ribosylation factor (Arf) protein family is part of the large Ras superfamily which encompasses small GTPases [24]. Among this family, ARF6 stimulates actin polymerization, drives phagocytosis through multiple mechanisms, and also assists autophagy as well [25]. Other than Arf6, RAB10 also influenced the GTPase activity [24]. Rab10 localizes on both Golgi and early endosomal/recycling compartments, plays an important roles in lysosome exocytosis and plasma membrane repair [26]. Alpha actinins belong to the spectrin gene superfamily which represents a diverse group of cytoskeletal proteins. Alpha actinin is an actin-binding protein. In nonmuscle cells, it is involved in binding actin to the membrane. In skeletal, cardiac, and smooth muscle isoforms, it localized to the Z-disc and analogous dense bodies, participates in anchoring the myofibrillar actin filaments. ACTN4 encodes a nonmuscle, alpha actinin isoform which is concentrated in the cytoplasm and involved in metastatic processes [27]. MYH9 is involved in several important functions, including cytokinesis, cell motility and maintenance of cell shape [28]. ARPC2, Actin-related protein 2/3 complex subunit 2, containing 7 subunits, of which Arp2 and Arp3 belong to actin-related proteins [29]. The activation of Arp2/3 complex could promote the synthesis of F-actin in the suitable condition [30]. The Arp2/3 complex is involved in the rearrangement of the macrophage cytoskeleton and affected the phagocytosis of macrophages [31]. Knockout of the Arp2/3 complex APC2 gene in mouse macrophages results in decreased F-actin polymerization, followed by decreased phagocytic ability [32]. In summary, the key proteins mentioned above and other hub proteins in PPI network were closely related to actin filament organization and movement, resulting in affecting the process of phagocytosis and endocytosis. Additional studies on the role of p22 in the process of endocytosis should be conducted.

Conclusion

Although several studies have been reported to elucidate the pathogenesis of ASFV, the viral protein function remains unclear. In this research, the proteins in the host cells interacted with p22 and the signaling pathways they might participate in were screened out by a high throughput method, laying the foundation to elucidate the function of p22. For the pig industry, it would also be advantageous to study the pathogenesis of the disease, monitor and predict outcome, to control the disease in the near future.

Abbreviations

ASFV: African Swine Fever Virus; LCMS: Liquid Chromatography Mass Spectrometry; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Gene and Genomes; PPI: Protein-protein interaction; MYH9: Myosin-9; ARPC2: Actin-related protein 2/3 complex subunit 2; ARF6: ADP-ribosylation factor 6; ACTBL2: Beta-actin-like protein 2; ACTN4: Alpha-actinin-4; CLTC: Clathrin heavy chain A; RAB10: Ras-related protein-10; pre-mRNA: precursor messenger RNA; cGMP: Cyclic GMP; PKG: cGMP-dependent protein kinase; NO: nitric oxide; AMPK: AMP-activated protein kinase.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and material

All the data have been included in this article and supplementary tables.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, FBC; Methodology, FBC, ZJM; Investigation, WDD; Writing—Original Draft, ZXJ; Writing—ZXJ, LB; Funding Acquisition, LB, FHY; Resources, FBC, ZJM; Supervision, LB, FHY.

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Figures

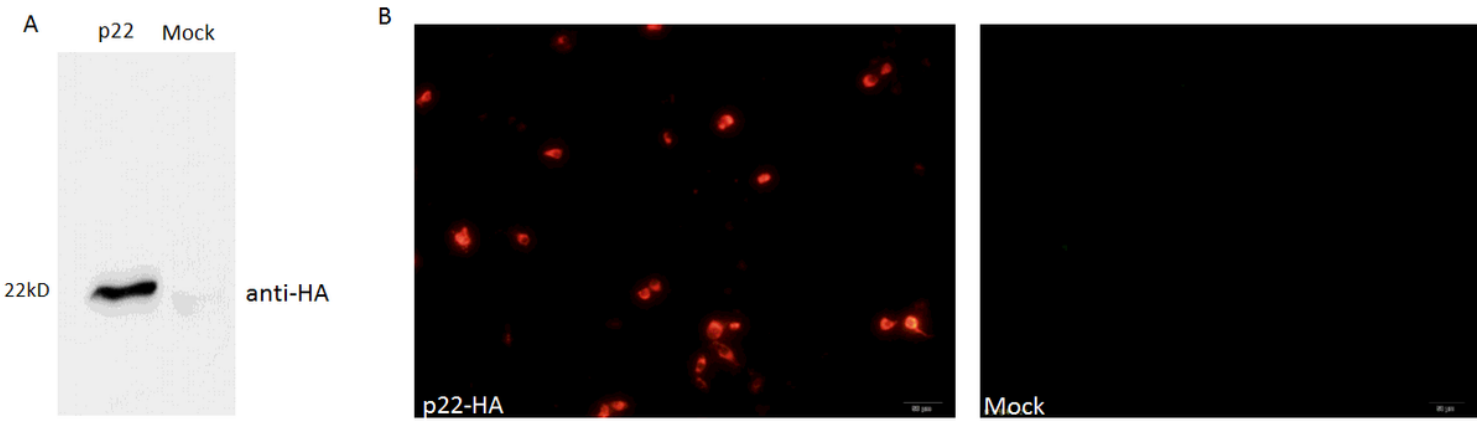

Figure 1

p22 expression identification. pCDNA-3.1-p22-HA and its mock were transfected in the 293-T cells and at 24h post transfection, cells were lysed and subjected to (A) western blotting analysis. The band of p22 of ASFV was shown as the predicted size. (B) IFA identification. The red fluorescence represented p22 protein.

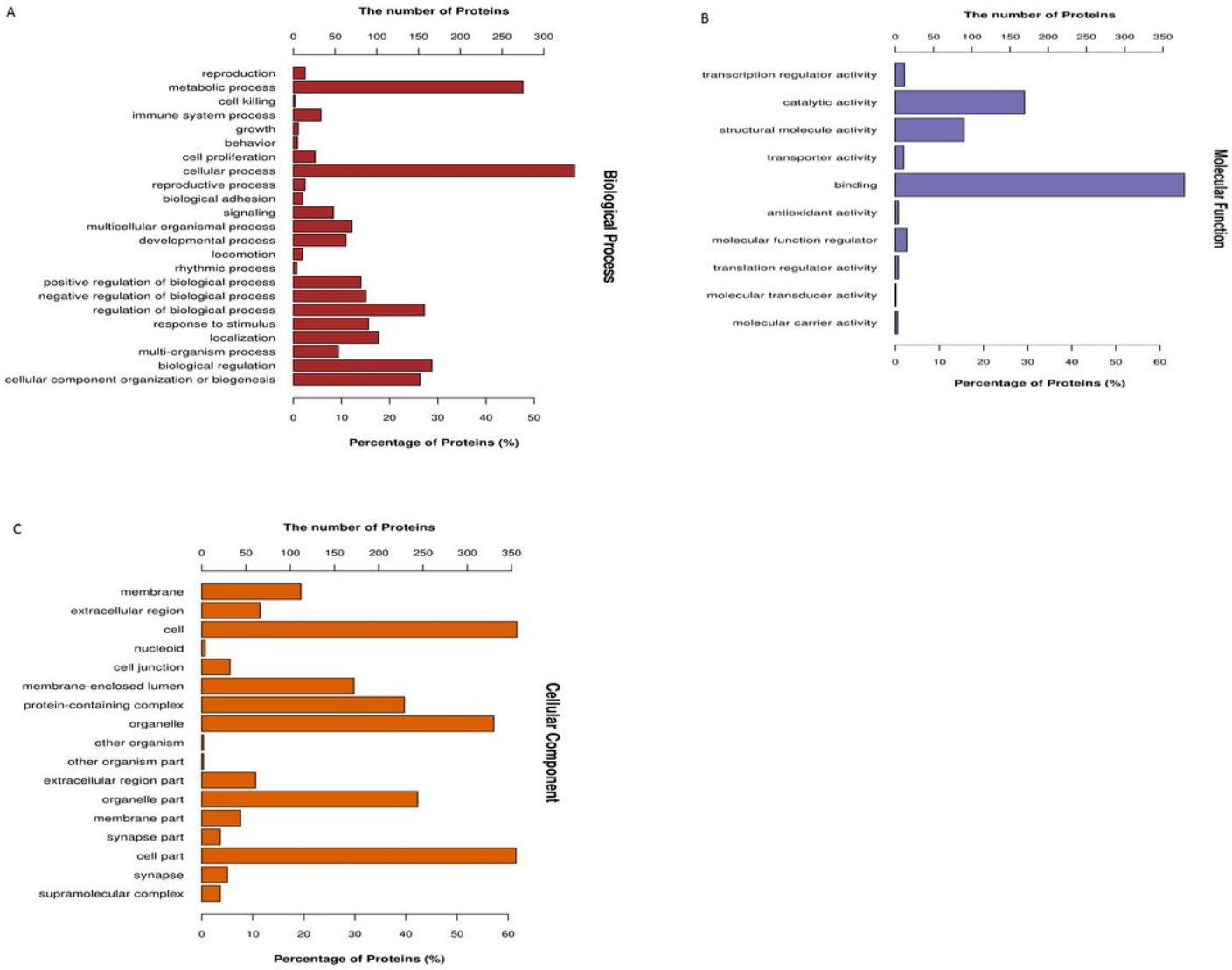

Figure 2

Distribution of GO terms on three groups: (A) biological process, (B) molecular function and (C) cellular component. Up coordinate represents the number of the proteins; right vertical coordinate represents the percentage of proteins.

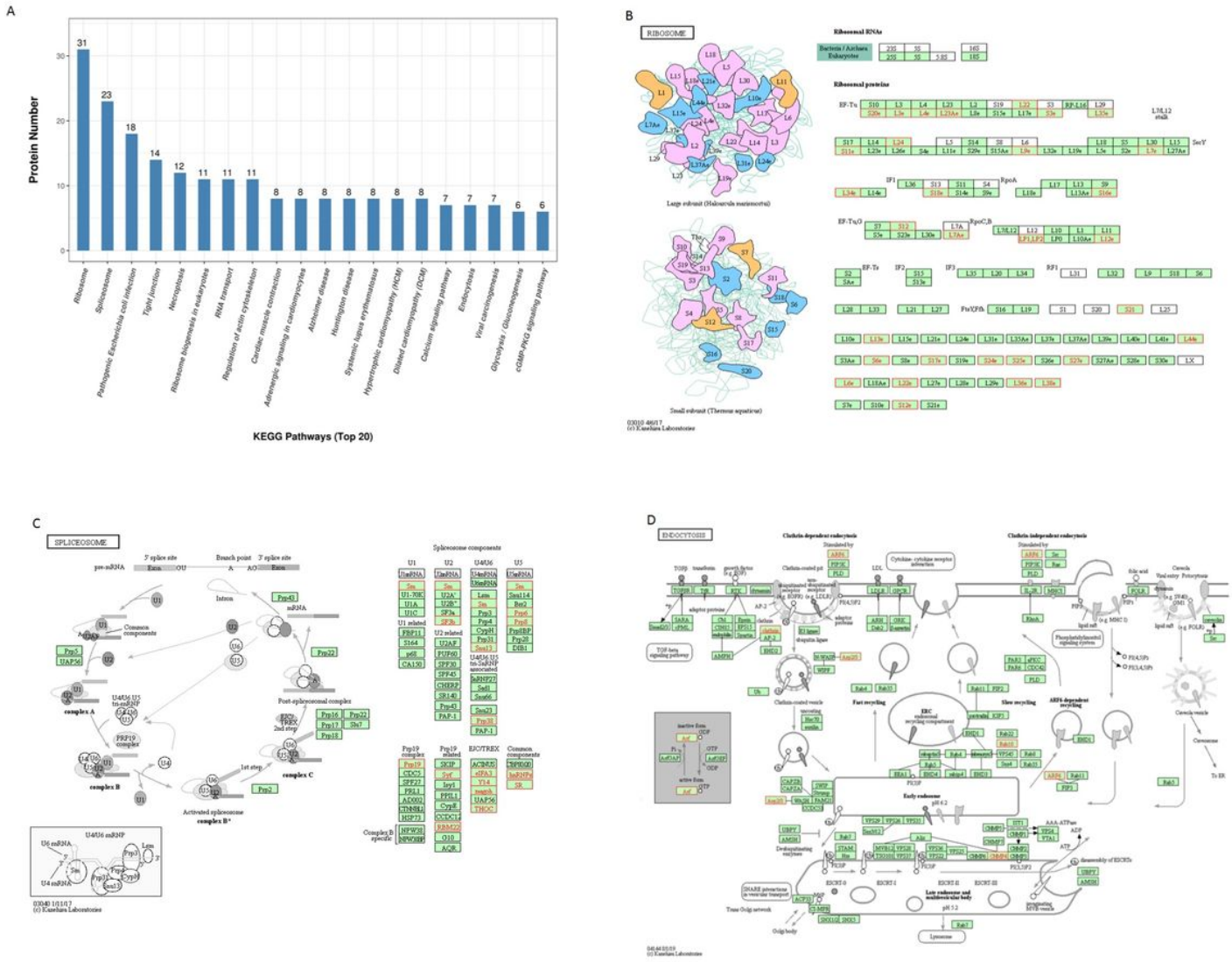

Figure 3

KEGG pathways analysis of interacted proteins with p22. (A)Top 20 enriched KEGG pathways distribution. KEGG pathways that p22-related proteins involved mainly were (B) ribosomes, (C) splicesome, (D) endocytosis.

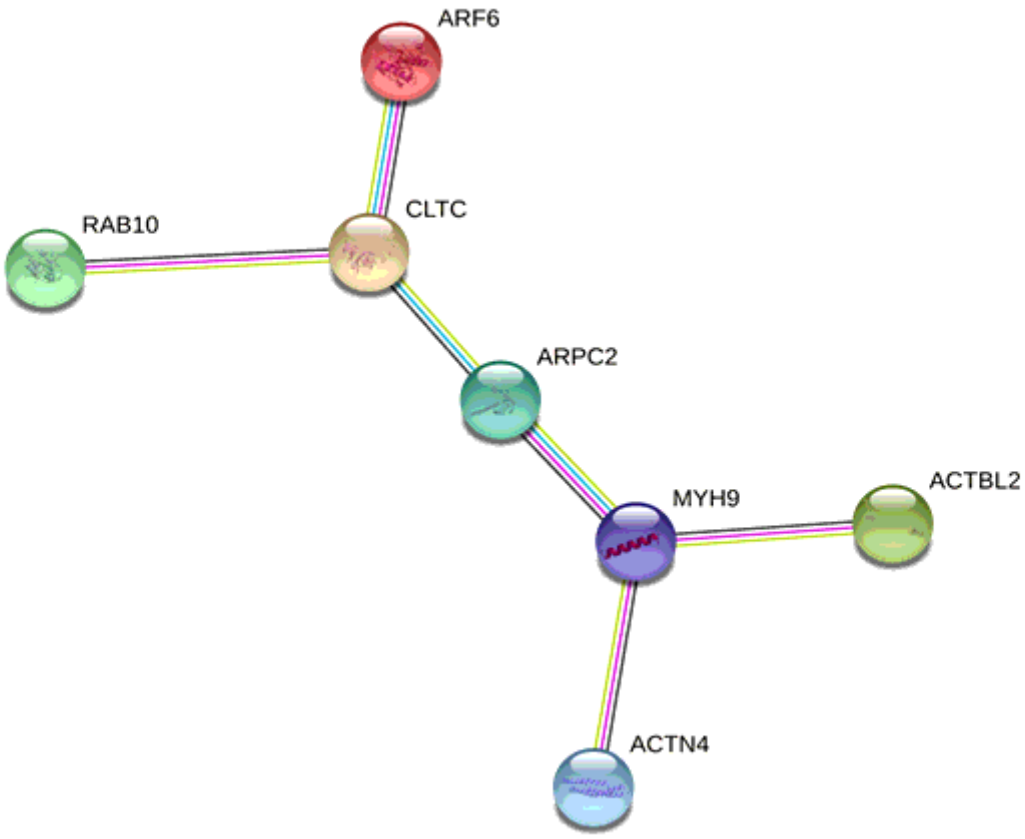

Figure 4

PPI network of key proteins that interacted with p22 in endocytosis process. The size of each node in the protein-protein interaction networks presents the connect degree of each gene. Those nodes that were not connected to any node were omitted in the network. The selected proteins interacted with p22 protein in endocytosis process were connected as a network. The network was generated in the STRING database and then visually edited in Cytoscape software.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- tableS1GO.xlsx
- tableS2kegg.xlsx
- tableS3proteins.xlsx