The interactome of porcine epidemic diarrhea virus nucleocapsid protein

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Abstract

Background Many viral proteins specifically interact with cellular proteins to facilitate virus replication. Understanding these interactions can decipher the viral infection mechanism and provide potential targets for antiviral therapy. Porcine epidemic diarrhea virus (PEDV), the agent of PED, causes numerous economic losses for the swine industry each year. Till now, no effective vaccine or drugs are available to contain this disease. As a result, it is critical urgent to elucidate the PEDV interactome. The nucleocapsid (N) of PEDV plays an important role in viral replication. **Results** In this study, the N gene was cloned into pEGFP-C1 and transfected into 293T cells. The interactome of N was elucidated by label-free mass spectrometry. A total of 125 cellular proteins interacting with PEDV N protein were discovered, of which 4 cellular proteins, DHX9, NCL, KAP1, TCEA1, were confirmed by pull down, immunoprecipitation, and co-localization. **Conclusions** The interactome of N protein supplied a powerful tool to explore the role of N in PEDV infection and therapeutic targets.

Background

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease characterized by watery diarrhea, vomiting, dehydration, severe enteritis, and weight losses [1, 2]. Inactivated and live-attenuated CV777-based vaccines have been used as a major strategy to control PED for many years until the outbreak of PED in China in October 2010 [3-5]. This outbreak occurred on vaccinated and non-vaccinated pig farms and caused nearly 100% morbidity and mortality rates among suckling piglets leading to great economic losses to the swine industry. In April 2013, a PED outbreak emerged in the United States causing high mortality in piglets and also huge economic loss [6, 7]. These reemerging outbreaks indicated PED is a serious threat to the swine industry worldwide.

PED is caused by PED virus (PEDV), a large-enveloped RNA virus, which is belonging to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, and genus *Alphacoronavirus* [8-10]. Its genome is about 28 kb in length, with a 5' cap and a 3' polyadenylated tail and comprises a 5' untranslated region (UTR) and a 3' UTR, encoding two replicase polyproteins (pp1a and pp1ab), spike (S), envelope (E), membrane (M), and nucleocapsid (N) four structural proteins, and one hypothetical accessory protein [11]. N protein is a multifunctional viral protein and plays a key role in PEDV infection, such as the RNA-binding protein, viral RNA synthesis and modulating host cell processes [12-14]. It can subvert innate immunity by antagonizing beta and lamda interferon production [15, 16], prolonging the host cell S phase, inducing endoplasmic reticulum stress and up-regulating interleukin-8 expression [17].

As obligate intracellular parasites, the successful replication of viral pathogen in a host is a complex process involving many interactions to achieve viral invasion, replication, and packaging processes. Proteome study is a powerful tool to uncovering the cellular proteins taking part in the viral life cycle by interacting specific viral protein, and also by using which to find new therapeutics against virus infection [18-21]. In this study, to explore the biological function of PEDV N protein and the role of N protein in viral

replication, the interactome of N protein was uncovered, which supplied useful information on the further study the function of N proteins and also gave hints for antiviral drug targets.

Methods

Cells and viruses

Human Embryonic Kidney 293T (HEK293T) cells and Vero E6 cells were obtained from the cell bank of Shanghai Academy of Sciences and grown in Dulbecco's modified Eagle's medium (DMEM; sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; GemCell. the USA) at 37 °C with 5% CO₂. The PEDV LJX01/2014 strain was isolated and preserved by our laboratory.

Plasmids and transfection

The full length of the N gene was amplified from PEDV LJX01/2014 strain with the primers: 5'-AGGATCCATGGCTTCTGTCAGCTTTC-3' and 5'-GGCTCGAG TTAATTTCTGTATCGAAG-3'. The purified N gene was cloned into the pEGFP-C1 vector with the *Bam*H I and *Xho* I restriction enzymes to generate the pEGFP-N recombinant plasmid. The plasmids pEGFP-N and pEGFP-C1 were transfected into 293T cells with 50-70% confluency using the X-tremeGENE HP DNA Transfection Reagent (Roche) at 1:3 ratio according to the procedure. Monolayer Vero E6 cells were transfected with Lipofectamine 2000 (Life Technologies) according to the instruction.

GFP pull down

pEGFP-N and pEGFP-C1 were transfected respectively into 293T cells and harvested 24 h post-transfection. The cells were resuspended by lysis buffer (0.5% NP40; 10 mM Tris/Cl pH 7.4; 0.5 mM EDTA; 150 mM NaCl) supplemented with cOmplete™, EDTA-free protease inhibitor cocktail (Roche) and lysed on ice for 30 min. After centrifugation at 14,000 g for 10 min, the supernatant was collected and mixed with GFP-Trap (Chromotek) and incubated for 6 h in a shaker at 4 °C. The mixtures were centrifuged at 2,500g for 2 min and washed 2 times with wash buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA) supplemented with cOmplete™, EDTA-free protease inhibitor cocktail (Roche). For samples for mass spectrometry, after the removal of wash buffer, the cellular proteins were eluted with 50 µL of elution buffer (200 mM Glycine pH 2.5). The supernatants were separated by centrifugation and transferred to a new 1.5 mL centrifuge tube. This step was repeated 2 times to ensure the maximum elution to get 100 µL of eluted proteins, and then 10 µL of Tris-base buffer (pH 10.4) was added to neutralize the eluate. The eluted proteins were analyzed by label-free mass spectrometry. For samples for Western blot analysis, after the removal of wash buffer, the beads were resuspended with 2×SDS-Sample buffer.

Liquid Chromatography (LC) - Electrospray Ionization (ESI) Tandem MS (MS/MS) Analysis by Q Exactive.

The GFP pulldowns (250 µg for each sample) were purified with 200 µl UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) to remove the detergent, DTT and other low-molecular-weight components by repeated ultrafiltration (Microcon units, 10 kD). Then 100 µL UA buffer with 0.05 M iodoacetamide was added to block reduced cysteine residues and the samples were incubated for 20 min in darkness. Proteomic grade trypsin (Promega) was added (3 µg), and samples were incubated at 37 °C overnight.

Each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100µm*2cm, nanoViper C18) connected to the C18-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 µm inner diameter, 3µm resin) in buffer A (2% acetonitrile and 0.1% Formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% Formic acid) at a flow rate of 300 nl/min controlled by IntelliFlow technology. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) over 120 min. MS data were acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Determination of the target value is based on predictive Automatic Gain Control (pAGC). Dynamic exclusion duration was 25 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200. The normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled. MS experiments were performed triplicate for each sample.

Immunoprecipitation (IP)

293T cells were transfected with plasmids pEGFP-N and pEGFP-C1. The transfected cells were harvested at 24 h post transfection and washed three times with cold PBS (pH 7.4), and incubated with 200 µl lysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5%NP40) on ice, with PierceTM protease inhibitor tablets (Thermo Scientific) for 30 min, then centrifuged at 14,000 g for 10 min. The cellular proteins specific antibodies for ATP-dependent RNA helicase A (DHX9) (Proteintech, 17721-1-AP), transcription intermediary factor 1-beta (KAP1) (Abcam, ab10484), nucleolin (NCL) (Abcam, ab22758), transcription elongation factor A protein 1 (TCEA1) (Abcam, ab185947) (2 µg/ antibody) was added into the supernatant and incubated for 2 h on a rotator at 4 °C. Then the protein G resin (GenScript) were mixed overnight at 4 °C on a rotator. The immunoprecipitated samples were collected by centrifugation and washing, finally, eluted with 100 µL of the 2×SDS-sample buffer.

Western blot analysis

The protein samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). After blocking with 5% skim milk for 2 h at room temperature, the specific primary cellular antibodies or anti-N monoclonal antibody prepared by our laboratory [22] were added for 2 h at room temperature. After washing 3 times, horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse secondary antibodies (Kang Wei Century; 1:5000 dilution) were added and incubated for 1 h at room temperature. After another 3 times washing, the target bands were developed using a developing reagent Clarity™ Western ECL Substrate (Bio-Rad).

Confocal imaging

The plasmids pEGFP-N and pEGFP-C1 were transfected in 293T and Vero E6 cells. After 24 h, cells were fixed with 4% paraformaldehyde for 30 minutes, washed 3 times. The 0.1% (v / v) Triton X-100 was used to permeabilize cells for 15 min. After washing 3 times, specific antibodies were added, incubated for 2 h at room temperature, and washed 3 times with PBS. Then PE-labeled goat anti-rabbit IgG (Southern Biotech) secondary antibody was added and incubated for 1 h at room temperature. Cell nucleus was stained with DAPI (Vectorlabs; H-1200) for 10 min at room temperature then observed under a laser confocal microscope (Leica; Germany).

Results

Expression of N protein

The N gene was amplified directed by the template of PEDV LJX01/2014 strain and cloned into the pEGFP-C1 vector to make recombinant plasmid pEGFP-N. The pEGFP-N and blank vector were transfected into 293T cells. The expression of the N gene was confirmed by observing under fluorescence microscopy (Fig. 1A) and Western blot analysis (Fig. 1B).

Identification of the potential cellular interacting partners of N proteins

In order to obtain the interactome of N protein, the plasmids pEGFP-N and pEGFP-C1 were transfected in 293T and the cellular binding partners were pulled down using the GFP-trap (Fig. 2A). These proteins were identified by label-free MS. The results showed that approximately 1200 cellular proteins were initially identified and quantified, which represented both specific and nonspecific interactions. Under the criteria of more than 2 unique peptides, a false discovery rate (FDR) $\leq 1\%$ and a p value < 0.05 for the t-test analysis, 125 cellular potential proteins were identified (Table 1). After analyzed by the biological database STRING (<https://string-db.org/>), 79 proteins were in the nucleus, 4 proteins were in cytosolic part and 17 proteins exist in both parts. Four KEGG pathways were significantly enriched: ribosome,

spliceosome, RNA transport, and non-homologous end-joining. Biological function GO-terms significantly enriched with RNA processing including mRNA metabolic process, translational initiation, translation, RNA catabolic process, mRNA catabolic process, gene expression and so on. Molecular functions of RNA binding, structural constituent of ribosome, heterocyclic compound binding, and structural molecule activity was enriched.

Validation of cellular proteins interacting with N proteins

Four cellular proteins including transcription elongation factor A protein 1 (TCEA1), ATP-dependent RNA helicase A (DHX9), nucleolin (NCL) and transcription intermediary factor 1-beta (KAP1) were selected to validate the cellular proteins potentially interact with N proteins. Independent GFP pull down were carried out on 293T cells, the pulldowns were analyzed by Western blot. The results showed that DHX9, NCL, KAP1, and TCEA1 were all in pull downs which pulled down by N protein (Fig. 2B). The same procedure was applied onto Vero E6 cell, PEDV susceptible cell line, to further confirm their interaction. The results showed that in the N pulldowns, DHX9, NCL, KAP1, and TCEA1 were all detected (Fig. 2C). These results showed that N could pull down all these four cellular proteins.

IP assay was employed to confirm the interaction of the cellular proteins with N protein in both 293T cells and Vero E6 cells. The IPs were immunoprecipitated by anti-DHX9, NCL, KAP1, and TCEA1 antibodies, respectively. The Western blot results showed that N protein was in the immunoprecipitated products of DHX9, NCL, KAP1, and TCEA1 (Fig. 3A, 3B).

Above results showed that all four cellular proteins of DHX9, NCL, KAP1, and TCEA1 were pulled down by N protein and all four proteins could immunoprecipitated N proteins, indicating the interaction of the four cellular proteins with N protein.

Co-localization of pEGFP-N with cellular proteins

The plasmids pEGFP-N and pEGFP-C1 were transfected in 293T and Vero E6 cells. At 24h post-transfection, the cells were fixed and probed by anti-DHX9, NCL, KAP1, and TCEA1. The co-localization of pEGFP-N with cell proteins was observed by laser confocal technique. Results showed that N protein co-localized with NCL in both 293T and Vero E6 cells. While no colocalization between DHX9, KAP1, TCEA1, and N protein was observed (Fig. 4A, 4B).

Discussion

Viral proteins often interact with cellular proteins to facilitate finishing the viral life cycle or creating a favorable environment for viral replication. Studying these interactions will help for the analysis of viral pathogenesis and the function of viral proteins to reveal the viral infection mechanism and provides more

options for antiviral targets [23, 24]. In the present study, the interactome of PEDV N protein was discovered, which would supply a great platform for studying the role of N protein in PEDV infection and the selection of anti-PEDV therapies.

We used the combination of EGFP-trap with Label-free LC–MS/MS approach to elucidate the N protein interactomes which have been successfully used on other viral proteins, including human respiratory syncytial virus, infectious bronchitis virus, porcine reproductive and respiratory syndrome virus and Ebola virus VP24 [18, 20, 21, 23, 24]. The specific interaction partners of N would be selectively enriched in the pEGFP-N samples. To provide a statistically robust data set, pull downs with both the pEGFP control and pEGFP-N were conducted independently in triplicate. Selected under stringent criteria, 125 cellular proteins were listed (Table 1). The interaction of these proteins was analyzed using the STRING algorithm and found that most of these proteins were in the nucleus. The function of these proteins was mostly related with RNA including mRNA metabolic process, translation, RNA binding and so on, which was coincident with the characteristic of nucleocytoplasmic trafficking of N protein the reported function of RNA-binding protein, viral RNA synthesis [12-14]. This interactome also gives us hints for the novel role of N protein and cellular proteins, for example, analyzed by UniProt database showed that 107 among 125 cellular proteins were related to acetylation. This showed us a new way about the epigenetic changes during the expression of N protein and PEDV infection.

Because nearly 80% cellular proteins of N partners were in the nucleus part, nuclear proteins of NCL, DHX9, KAP1, and TCEA1 with different fold changes were selected to validate the MS results. Firstly, the interaction was confirmed by pull down and IP in 293T and Vero E6 cells. N protein was found to interact with all four cellular proteins by pull down and all four proteins interacted with N protein by IP in both cell lines. These results supply strong proof of their interaction. NCL is a multifunctional DNA/RNA-binding protein widely conserved among eukaryotes. It is involved in RNA metabolism, in particular in rRNA maturation [25]. It also plays multiple and important roles during virus infection by helping the formation of infectious virus particles, virus replication, virus internalization, trafficking, immune evasion and so on, such as [Epstein-Barr virus](#) (EBV), dengue virus, feline calicivirus, influenza A virus, herpes simplex virus 1 [26-30]. The most important thing is that the N protein of Avian infectious bronchitis virus within the same family *Coronaviridae* with PEDV interacted with NCL. DHX9 is a multifunctional ATP-dependent nucleic acid helicase which unwinds DNA and RNA and that plays important roles in DNA or RNA processes [31]. It also takes part in the virus life cycle to [regulate viral RNA synthesis by interacting with N protein of porcine reproductive and respiratory syndrome virus](#) [32]. DHX9 is a component of virus replication complexes of chikungunya virus (KSHV) and also EBV and hepatitis B virus [33-35]. KAP1 is a ubiquitously expressed protein involved in many critical functions which are dependent upon post-translational modifications, such as phosphorylation or sumoylation [36]. Its function also can be hijacked by the virus to mediate viral gene expression and play a role during viral latency, such as KSHV, Murine Leukemia Virus [37, 38]. TCEA1 is a transcription elongation factor S-II which stimulates mRNA chain elongation catalyzed by RNA polymerase II [39]. Little information is available on its function and virus infection. Based on the role of these proteins on other viruses, we believe that these four cellular proteins had a role in PEDV life cycle. However, these proteins may form a complex with other cellular

proteins which are highly structured and dynamic nuclear organelle which may a reason for no colocalization of DHX9, KAP1, and TCEA1 with N proteins. The real role of cellular proteins in N expression or PEDV infection needs more study. Other cellular proteins which were not selected for confirmation also take parts in the virus life cycle, such as LARP1, G3BP1, SERBP1, SRP14, IGF2BP1, YBX1, HMGB2. All these indicated that the interactome of N proteins was reliable.

In the present research, the interactome of PEDV N protein was elucidated and confirmed by pull down and IP. These results would supply a powerful tool to study the role of N in PEDV infection and therapeutic targets.

Abbreviations

Porcine epidemic diarrhea: PED; Porcine epidemic diarrhea virus: PEDV; Nucleocapsid: N; Human Embryonic Kidney 293T: HEK293T; Dulbecco's modified Eagle's medium: DMEM; Fetal bovine serum: FBS; Immunoprecipitation: IP; Transcription elongation factor A protein 1: TCEA1; ATP-dependent RNA helicase A: DHX9; Nucleolin: NCL; Transcription intermediary factor 1-beta: KAP1; Chikungunya virus: KSHV.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MT and GD conducted the research and interpreted the results. MT, GD, XC, SC, FC, JL, LL, YZ, SL, GL and YX participated in data collection. MT, GL and YX contributed to data analysis and helped draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Not applicable.

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Tables

Table 1. Cellular proteins showing more than 2-fold changes in abundance compared GFP-N with GFP

Protein IDs	Protein Names	Proteins	Unique peptides	Sequence coverage [%]	Fold change	Mol. weight [kDa]	T test
N	N	PEDV nucleocapsid protein	28	45.9	994.4	49.5	0.016
Q6PKG0	LARP1	La ribonucleoprotein domain family, member 1 (1019 aa)	30	37.2	391.2	123.5	0.048
P62263	RPS14	40S ribosomal protein S14	10	43.0	221.4	16.3	0.009
O60506	SYNCRIP	Heterogeneous nuclear ribonucleoprotein Q	10	30.3	183.8	69.6	0.015
P62249	RPS16	ribosomal protein S16 (146 aa)	17	69.9	142.7	16.4	0.006
Q13151	HNRNPA0	heterogeneous nuclear ribonucleoprotein A0	13	37.7	142.1	30.8	0.008
Q13283	G3BP1	GTPase activating protein (SH3 domain) binding protein 1	14	46.1	124.5	52.2	0.008
B4DLR3	HNRPU	Heterogeneous nuclear ribonucleoprotein U	3	44.5	95.8	86.9	0.014
E7EVA0	MAP4	microtubule-associated protein 4	45	21.8	82.3	245.4	0.030
P19338	NCL	nucleolin	57	52.0	75.1	76.6	0.018
P62081	RPS7	40S ribosomal protein S7	13	53.1	74.4	22.1	0.001
Q12905	ILF2	interleukin enhancer binding factor 2	14	43.3	70.2	43.1	0.002
P38159	RBMX	RNA-binding motif protein, X chromosome	20	44.0	70.1	42.3	0.024
D3DQ69	SERBP1	SERPINE1 mRNA binding protein 1	2	64.4	68.6	49.1	0.003
P27695	APEX1	DNA-(apurinic or apyrimidinic site) lyase	13	41.2	66.5	35.6	0.010
P84103	SRSF3	Serine/arginine-rich splicing factor 3	6	36.0	63.5	19.3	0.013
P13010	XRCC5	X-ray repair cross-complementing protein 5	25	29.8	63.0	82.7	0.011
Q14157	UBAP2L	Ubiquitin-associated protein 2-like	20	28.9	62.4	114.5	0.012
P15880	RPS2	40S ribosomal protein S2	7	37.9	59.5	31.3	0.017
O75534	CSDE1	Cold shock domain-containing protein E1	23	25.9	52.9	88.9	0.028
Q13442	PDAP1	Heat- and acid-stable phosphoprotein	22	66.3	51.7	20.6	0.007
P37108	SRP14	Signal recognition particle 14 kDa protein	4	30.9	49.6	14.6	0.031
P62269	RPS18	40S ribosomal protein S18	13	53.3	49.3	17.7	0.032
Q9NZI8	IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1	27	49.9	47.6	63.5	0.012
P67809	YBX1	Nuclease-sensitive element-binding protein 1	17	73.5	47.4	35.9	0.012

P35637	FUS	RNA-binding protein FUS	9	25.5	45.6	53.4	0.032
P26599	PTBP1	Polypyrimidine tract-binding protein 1	13	27.7	44.6	57.2	0.010
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	26	64.2	43.3	43.8	0.003
Q15637	SF1	Splicing factor 1	9	13.1	42.0	68.3	0.017
P39748	FEN1	Flap endonuclease 1	9	24.7	41.6	42.6	0.009
Q14152	EIF3A	Eukaryotic translation initiation factor 3 subunit A	27	20.6	41.2	166.6	0.039
Q00839	HNRPU	Heterogeneous nuclear ribonucleoprotein U	3	42.9	41.0	90.6	0.014
P62701	RPS4X	40S ribosomal protein S4, X isoform	18	58.2	38.7	29.6	0.016
E7EUU4	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	43	29.0	37.5	171.6	0.016
P30050	RPL12	60S ribosomal protein L12	11	69.1	36.1	17.8	0.022
P62241	RPS8	40S ribosomal protein S8	10	49.0	33.5	24.2	0.039
P62280	RPS11	40S ribosomal protein S11	12	54.4	33.3	18.4	0.010
Q7Z417	NUFIP2	Nuclear fragile X mental retardation-interacting protein 2	19	35.3	33.3	76.1	0.044
P18077	RPL35A	60S ribosomal protein L35a	9	55.5	31.9	12.5	0.029
O75821	EIF3G	Eukaryotic translation initiation factor 3 subunit G	13	46.2	30.1	35.6	0.047
Q9Y3F4	STARP	Serine-threonine kinase receptor-associated protein	20	66.3	29.2	38.4	0.017
P52815	MRPL12	39S ribosomal protein L12, mitochondrial	6	23.7	28.4	21.3	0.017
P23193	TCEA1	Transcription elongation factor A protein 1	13	47.5	27.6	34.0	0.008
O15371	EIF3D	Eukaryotic translation initiation factor 3 subunit D	7	15.1	27.6	64.0	0.007
B4DZF2	EIF4G2	Eukaryotic translation initiation factor 4gamma 2	14	16.0	27.6	110.3	0.043
P11940	PABPC1	Polyadenylate-binding protein 1	24	43.7	26.4	70.7	0.020
P12956	XRCC	X-ray repair cross-complementing protein 6	28	44.2	25.6	69.8	0.010
P62753	RPS6	40S ribosomal protein S6	11	34.9	24.9	28.7	0.014
O60869	EDF1	Endothelial differentiation-related factor 1	17	72.3	24.8	16.4	0.033
P60866	RPS20	40S ribosomal protein S20	7	38.7	23.5	13.4	0.024
Q92945	KHSRP	Far upstream element-binding protein 2	32	55.1	22.9	73.1	0.011
A0A024RDF4	HNRPD	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1	6	54.6	22.2	32.8	0.004

Q07666	KHDRBS1	KH domain-containing, RNA-binding, signal transduction-associated protein 1	9	19.2	21.8	48.2	0.009
Q3MHD2	LSM12	Protein LSM12 homolog	7	35.4	21.6	21.7	0.029
Q08211	DHX9	ATP-dependent RNA helicase A	31	27.8	21.5	141.0	0.027
Q14011	CIRBP	Cold-inducible RNA-binding protein	6	36.6	21.3	18.6	0.023
Q13247	SRSF6	Serine/arginine-rich splicing factor 6	3	20.3	20.6	39.6	0.035
P26583	HMGB2	High mobility group protein B2	13	46.9	20.6	24.0	0.012
P84098	RPL19	60S ribosomal protein L19	9	31.1	20.1	23.5	0.000
Q9Y2S6	TMA7	Translation machinery-associated protein 7	6	40.6	19.5	7.1	0.001
P23396	RPS3	40S ribosomal protein S3	17	70.8	18.8	26.7	0.007
P62888	RPL30	60S ribosomal protein L30	8	67.0	18.4	12.8	0.010
Q01844	EWSR1	RNA-binding protein EWS	8	15.9	18.1	68.5	0.003
Q9Y265	RUVBL1	RuvB-like 1	17	50.0	17.5	50.2	0.017
Q9BQ61	C19orf43	Uncharacterized protein C19orf43	6	44.3	17.3	18.4	0.028
Q00688	FKBP3	Peptidyl-prolyl cis-trans isomerase FKBP3	16	46.4	17.2	25.2	0.002
P15311	EZR	Ezrin	12	28.5	16.9	69.4	0.049
P62633	CNBP	Cellular nucleic acid-binding protein	7	41.8	16.6	19.5	0.007
P62304	SNRPE	Small nuclear ribonucleoprotein E	2	40.2	16.4	10.8	0.039
P23246	SFPQ	Splicing factor, proline- and glutamine-rich	28	38.9	16.3	76.1	0.006
Q9Y5L4	TIMM13	Mitochondrial import inner membrane translocase subunit Tim13	6	60.0	16.3	10.5	0.032
P36873	PPP1CC	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	2	39.6	16.1	37.0	0.045
P62316	SNRPD2	Small nuclear ribonucleoprotein Sm D2	8	59.3	15.9	13.5	0.024
P05455	SSB	Lupus La protein	17	31.9	15.0	46.8	0.010
P62857	RPS28	40S ribosomal protein S28	5	56.5	13.8	7.8	0.005
P26373	RPL13	60S ribosomal protein L13	8	46.0	13.7	24.3	0.038
Q12849	GRSF1	G-rich sequence factor 1	9	19.6	13.5	53.1	0.006
Q9Y224	C14orf166	UPF0568 protein C14orf166	12	51.6	13.4	28.1	0.004
B3KSH1	EIF3F	Eukaryotic translation initiation factor 3 subunit F	6	17.2	12.9	39.1	0.006
B4DDB6	HNRPA3	Heterogeneous nuclear ribonucleoprotein A3,	3	35.1	12.7	37.0	0.018

P09651	HNRNPA1	isoform CRA_a Heterogeneous nuclear ribonucleoprotein A1	9	49.7	12.2	38.7	0.015
P63244	GNB2L1	Guanine nucleotide-binding protein subunit beta-2-like 1	15	46.1	11.9	35.1	0.028
P05204	HMGN2	Non-histone chromosomal protein HMG-17	3	54.4	11.9	9.4	0.006
O14979	HNRNPDL	Heterogeneous nuclear ribonucleoprotein D-like	10	23.8	11.8	46.4	0.026
Q9UMS4	PRPF19	Pre-mRNA-processing factor 19	9	18.7	11.7	55.2	0.014
P62913	RPL11	60S ribosomal protein L11	2	38.2	11.6	20.3	0.011
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	13	59.9	11.4	31.6	0.002
P23284	PPIB	Peptidyl-prolyl cis-trans isomerase B	13	50.9	11.3	23.7	0.011
Q16186	ADRM1	Proteasomal ubiquitin receptor ADRM1	5	15.0	11.1	42.2	0.048
P07910	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	13	31.4	10.6	33.7	0.001
Q14974	KPNB1	Importin subunit beta-1	15	20.5	10.4	97.2	0.020
P50402	EMD	Emerin	4	17.3	10.3	29.0	0.028
P19388	POLR2E	DNA-directed RNA polymerases I, II, and III subunit RPABC1	4	20.5	10.2	24.6	0.018
Q13347	EIF3I	Eukaryotic translation initiation factor 3 subunit I	10	33.2	10.2	36.5	0.024
Q04837	SSBP1	Single-stranded DNA-binding protein, mitochondrial	5	39.2	10.1	17.3	0.012
P05387	RPLP2	60S acidic ribosomal protein P2	9	79.1	10.0	11.7	0.011
P06748	NPM1	Nucleophosmin	31	88.4	10.0	32.6	0.002
P09661	SNRPA1	U2 small nuclear ribonucleoprotein A	6	25.1	9.8	28.4	0.036
G8JLB6	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H	6	35.8	9.6	51.2	0.016
P46782	RPS5	40S ribosomal protein S5	16	58.8	9.5	22.9	0.018
Q13263	KAP1	Transcription intermediary factor 1-beta	21	33.4	9.5	88.5	0.027
P55884	EIF3B	Eukaryotic translation initiation factor 3 subunit B	19	24.9	9.3	92.5	0.007
P98179	RBM3	RNA-binding protein 3	3	12.1	8.6	17.2	0.022
P52272	HNRNPM	Heterogeneous nuclear ribonucleoprotein M	18	46.8	8.3	77.5	0.005
P49006	MARCKSL1	MARCKS-related protein	6	65.1	7.4	19.5	0.004
P22626	HNRNP1	Heterogeneous nuclear ribonucleoproteins A2/B1	19	53.8	7.2	37.4	0.036
P05388	RPLP0	60S acidic ribosomal protein	11	46.1	7.1	34.3	0.019

		P0					
A0A024R814	RPL7	Ribosomal protein L7, isoform CRA_a	11	27.0	6.9	30.4	0.035
P62906	RPL10A	60S ribosomal protein L10a	12	42.4	6.7	24.8	0.024
Q96CT7	CCDC124	Coiled-coil domain- containing protein 124	17	60.5	6.7	25.8	0.032
Q4VCS5	AMOT	Angiomotin	23	23.2	6.4	118.1	0.019
Q99832	CCT7	T-complex protein 1 subunit eta	14	30.9	6.4	59.4	0.047
B4DUQ1	HNRPK	Heterogeneous nuclear ribonucleoprotein K	24	49.2	6.0	48.5	0.039
P05198	EIF2S1	Eukaryotic translation initiation factor 2 subunit 1	11	40.6	5.9	36.1	0.006
P40939	HADHA	Trifunctional enzyme subunit alpha, mitochondrial	10	18.0	4.7	83.0	0.028
O00410	IPO5	Importin-5	12	17.9	4.6	123.6	0.046
O00264	PGRMC1	Membrane-associated progesterone receptor component 1	4	45.1	4.5	21.7	0.033
Q92552	MRPS27	28S ribosomal protein S27, mitochondrial	5	11.8	4.5	47.6	0.034
P11177	PDHB	pyruvate dehydrogenase E1 component subunit beta, mitochondrial	8	28.1	4.1	39.2	0.045
P30153	PPP2R1A	Serine/threonine-protein phosphatase 2A	9	17.3	3.8	65.3	0.029
H0Y7A7	CALM2	Calmodulin (Fragment)	12	48.1	3.7	20.8	0.009
P60842	EIF4A1	Eukaryotic initiation factor 4A-I	10	44.1	3.6	46.2	0.044
Q9Y3B4	SF3B6	Splicing factor 3B subunit 6	3	27.2	3.2	14.6	0.005
P26641	EEF1G	Elongation factor 1-gamma	22	42.3	2.9	50.1	0.041
A8MXP9	MATR3	Matrin-3	11	13.1	2.7	100.0	0.042
P00390	GSR	Glutathione reductase, mitochondrial	6	15.9	2.4	56.3	0.028
O43324	EEF1E1	Eukaryotic translation elongation factor 1 epsilon-1	6	32.8	2.3	19.8	0.049

Figures

Figure 1

Identification of the pEGFP-N expression in 293T cells by fluorescence microscopy (A) and Western blot (B). The predicted molecular size was 27, 81kDa for EGFP and EGFP-N.

Figure 2

Validation of the MS samples or results by pull down Western blot analysis of the GFP-trap pull down products. The pEGFP-C1 and pEGFP-N transfected 293T cells lysates were pull down by GFP-trap. The pull downs were analyzed by Western blot analysis using a specific antibody against GFP. B Validation the MS results by pull down in 293T cells. The pEGFP-C1 and pEGFP-N transfected 293T cells lysates were pull down by GFP-trap. The presence of the protein DHX9, KAP1, NCL, and TCEA1 were confirmed using the specific antibodies by Western blot. C Validation the MS results by pull down in Vero E6 cells. The procedure was the same as in (B).

Figure 3

Validation of the partners of N protein by IP Western blot analysis of the partners of N protein by IP in 293T cells. The partners of N proteins were immunoprecipitated from the pEGFP-C1 and pEGFP-N transfected 293T cells lysates using anti-DHX9, KAP1, NCL, and TCEA1 specific antibodies. The IPs were analyzed by Western blot analysis with anti-DHX9, KAP1, NCL, and TCEA1 specific antibodies or anti-GFP antibody. B The same procedure of IP was applied onto Vero E6 cell.

Figure 4

Validation of the partners of N protein by co-localization A Co-localization of cellular proteins with N in 293T cells. 293T cells were transfected with pEGFP-N or pEGFP-C1. The cellular proteins were probed by anti-DHX9, KAP1, NCL, and TCEA1 antibodies and visualized by PE-labeled goat anti-rabbit IgG (Red). Nuclei were stained with DAPI (blue). The colocalization was determined by the yellow signal in the merged images. B The same procedure of confocal technique was applied on Vero E6 cells.