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Research article

Featured interactome of homocysteine-inducible endoplasmic reticulum protein uncovers novel binding partners in response to ER stress

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ABSTRACT

Homocysteine-inducible endoplasmic reticulum protein (HERP) is an endoplasmic reticulum (ER)-resident protein and important for the adaptation of cellular protein homeostasis by ER-associated degradation (ERAD) system. HERP interactors are critical for cellular viability and the reaction to ER stress. To explore the exact mechanisms by which HERP performed the biological functions, we conducted an interaction analysis of HERP protein in HeLa cells by co-immunoprecipitation (Co-IP) and liquid chromatography-mass spectrometer (LC-MS)/MS coupled with label-free quantification (LFQ). Among the interactome results, 123 proteins significantly interacted with HERP, which leads to numerous biological processes including protein import into nucleus, ubiquitin-dependent ERAD pathway, negative regulation of apoptotic process, and protein transport from ER, along with multiple pathways including several diseases, protein processing in ER, fatty acid metabolism, and steroid biosynthesis. Furthermore, we selected several prey proteins from the interactome data and confirmed that HERP interacted with ancient ubiquitous protein 1 (AUP1), Fas-associated factor family member 2 (FAF2), tripartite motif containing 47 (TRIM47), acyl-CoA synthetase long-chain family member 3 (ACSL3), sequestosome 1 (SQSTM1), and poly(rC) binding protein 2 (PCBP2) by Co-IP and confocal microscopy experiments, respectively. Moreover, the expression and location of several interacted proteins were obviously altered in response to ER stress induced by Thapsigargin stimulation and Enterovirus 71 infection. In conclusion, our findings revealed that the vital proteins interacted with HERP to mediate signaling transduction, thus providing novel clues for the mechanisms of HERP associated with ERAD and metabolism in response to ER stress under physiological and pathological conditions.

1. Introduction

The ER is a unique organelle responsible for protein folding and maturation, lipid metabolism, and Ca^{2+} homeostasis [1,2]. Because the processes of protein folding are complicated and prone to errors, various physiological and pathological conditions, including Ca^{2+} imbalances, glucose deprivation, viral infection, oxidative damage, damaged protein expression, and natural toxins can disturb the homeostasis of ER and lead to the load of misfolded proteins [3]. Ultimately, accumulated improperly folded proteins in ER lumen induce the occurrence of ER stress [3]. Cells detect and monitor such abnormal physiological functions by initiating a regulatory system called the unfolded protein response (UPR), which includes three signaling pathways, namely Protein kinase R-like endoplasmic reticulum kinase (PERK), Inositol requiring enzyme 1 (IRE1), and Activating transcription factor 6 (ATF6) [1]. The UPR processes the aberrant proteins by adjusting the protein folding capacity of ER and removing the misfolded proteins through directing them to ERAD [4]. ERAD is a highly controlled and sophisticated mechanism that restores protein homeostasis within the ER [5,6]. During ERAD, maturation-defective and surplus poly-peptides are

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recognized by an integral membrane protein quality-control system, ubiquitinated by the E3 ligase complexes, retro-translocated from ER, and ultimately degraded by the 26 S proteasome in the cytoplasm [7]. Impairment of ERAD may cause the formation and accumulation of protein aggregates, even cell death [6].

HERP, an ER-resident membrane protein with a molecular weight of 54 kDa, was originally identified as a homocysteine-induced gene in vascular endothelial cells [8]. Subsequently, HERP was found ubiquitously expressed in various tissues and organs, such as heart, liver, skeletal muscles, kidneys, and pancreas [8,9]. Structurally, HERP has an N-terminal ubiquitin-like (UBL) domain and a C-terminal transmembrane domains. The UBL domain mediates the interaction of HERP with ERAD system to gain a ubiquitylation function, thus expands the proper protein folding and secretory capacity of ER and acts on the recovery of ER homeostasis [2,10–12]. HERP is strongly upregulated by the unfolded protein response, which has been closely related to the pathogenesis of Parkinson's disease, type 2 diabetes, and neurodegeneration [13–15]. According to a previous report, HERP may play a role in nonalcoholic fatty liver disease (NAFLD) [16], which indicates that HERP could influence metabolism. Emerging data suggests that HERP mediates the regulation of liver cancer cell survival, inhibiting cell apoptosis and ferroptosis by different signaling pathways [17,18].

Different interactors of HERP are responsible for its diverse biological functions. HERP interacts with retro-translocation factor derlin-1, p97, and the E3 ubiquitin-protein ligase Hrd1 to constitute a high molecular-weight protein complex, which promotes the ubiquitination and subsequent relocation from ER to the cytosol of substrate protein [10,19,20]. In our previous study, we described that EV71-upregulated HERP interacts with TANK-binding kinase 1 (TBK1) to act as an integrator of mitochondrial antiviral signaling protein (MAVS)-mediated antiviral immunity pathway [11]. In order to investigate diverse regulatory mechanisms of HERP and understand its distinct biological functions, a global screening of HERP interacting proteins is essential.

In this study, we further assessed the function of HERP by conducting an LFQ-based proteomics analysis of HERP with Co-IP assays followed by LC-MS/MS approach. Ultimately, 123 proteins were identified as prey proteins. Moreover, we confirmed the association of HERP with AUP1, FAF2, TRIM47, ACSL3, SQSTM1, and PCBP2 using Co-IP experiment, implying the important relationship of HERP with ERAD and fatty acid metabolism. Consistently, we demonstrated the colocalization of overexpressed HERP and these proteins in ER by confocal microscope. Additionally, the endogenous expression and location of several interacted proteins were obviously altered in response to Thapsigargin (Tg) and Enterovirus 71 (EV71) induced ER stress. Therefore, these findings reveal highlighted insights into the regulatory manner underlying the ERAD and fatty acid metabolism, also shape the diverse functions of HERP in response to ER stress.

2. Materials and methods

2.1. Cell cultures

HeLa and HEK293T cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). RD cells were provided by China Center for Type Culture Collection (CCTCC) (Wuhan, China). During the experiment, all cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin sulfate at 37 ℃ in a 5% CO_2 incubator. Lipofectamine 2000 (Lipo2000) (Invitrogen; Carlsbad, CA, USA) was used to conduct transfection experiments according to the manufacturer's instructions.

2.2. Antibodies and reagents

ER-Tracker Red dye was purchased from Invitrogen. Mouse anti-FLAG and rabbit anti-HA antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-EV71 VP1 antibody was obtained from Abnova (Walnut, CA, USA). Mouse anti-HERP and anti-ERO1-La antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-GAPDH, rabbit anti-AUP1, anti-FAF2, anti-TRIM47, and anti-SQSTM1 were purchased from Proteintech Group (Wuhan, China). Rabbit anti-ATF6 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Tg, an ER stress inducer, was purchased from the MedChemExpress (Shanghai, China).

2.3. Virus infection

EV71 (Xiangyang-Hubei-09 strain) was described as previously [21]. RD cells were used to propagate virus. For viral infection experiment, HeLa cells were added with EV71 at multiplicities of infection (MOI) of 1. Two hours later, the cells were washed with PBS twice, and maintained in DMEM containing 2% FBS for indicated time.

2.4. Co-immunoprecipitation and western blotting

For Co-IP experiment, 1×10^6 HEK293T cells were seeded in a 6-cm dish, then transiently incubated with a mixture of 4 μg plasmids and transfection reagents Lipo2000. After twenty-four hours, the cells were harvested and lysed in 1 ml RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, pH7.4) containing protease inhibitor mixture (Roche; Basel, Switzerland). Next, centrifuge the cell lysates at 12,000 rpm for 10 min at 4 ℃ and collect the supernatant after centrifugation. A part of the lysates was removed as input sample. The rest of the supernatant was incubated with specific primary antibodies on a rotator overnight at 4 ℃. Next, the Protein G agarose was added to the mixture for another two hours to collect the IP complex. The beads were washed 4 times with RIPA buffer, then resuspended and boiled with SDS-PAGE loading buffer. Subsequently, the prepared proteins were loaded onto 10% SDS-PAGE gel to separate and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore; Burlington, MA, USA). The membranes were blocked with 5% skim milk and then incubated with the specific primary antibodies overnight at 4 ℃. Then, the above membranes were rinsed three times with TBST (TBS with 0.1% Tween) and incubated with peroxidase-conjugated IgG for another 1 h at room temperature. After the last washing, a luminescent image analyzer was used to detect the protein bands (Bio-Rad; Hercules, CA, USA).

2.5. Protein separation and in-gel digestion

HeLa cells were grown in 10-cm dishes and transfected with Lipo2000 and 8 μg either Vector or FLAG-HERP plasmids. At 24 h post-transfection, the harvested cells were lysed for Co-IP experiment as described above. After separating on a 10% SDS-PAGE gel and staining with Coomassie blue dye, the gel lane of proteins was divided into about 1 mm^3 slices. The cubes were washed with double distilled water (ddH_2O) for 10 min. Next, the gel cubes were destained three times using 50% acetonitrile (ACN) solution containing 100 mM NH_4HCO_3 (pH 8.0) for 10 min shaking, and dehydrated with 100% ACN for additional 10 min shaking. The dehydrated gel pieces were disulfide reduced for 1 h at 56 °C in 10 mM DTT containing 50 mM NH_4HCO_3, alkylated for 10 min in 55 mM iodoacetamide containing 50 mM NH_4HCO_3 at room temperature avoiding the light, and dehydrated with 100% ACN for additional 20 min shaking. The gel pieces were digested with trypsin in 50 mM NH_4HCO_3 at 37 ℃ overnight. Subsequently, the supernatants were transferred into new Eppendorf tubes, and the gel pieces were sonicated twice with extraction buffer (60% ACN and 5% formic acid). Finally, the mixture of supernatant and peptide extraction were dried with a SpeedVac, and desalted with C18 columns for further MS analysis.

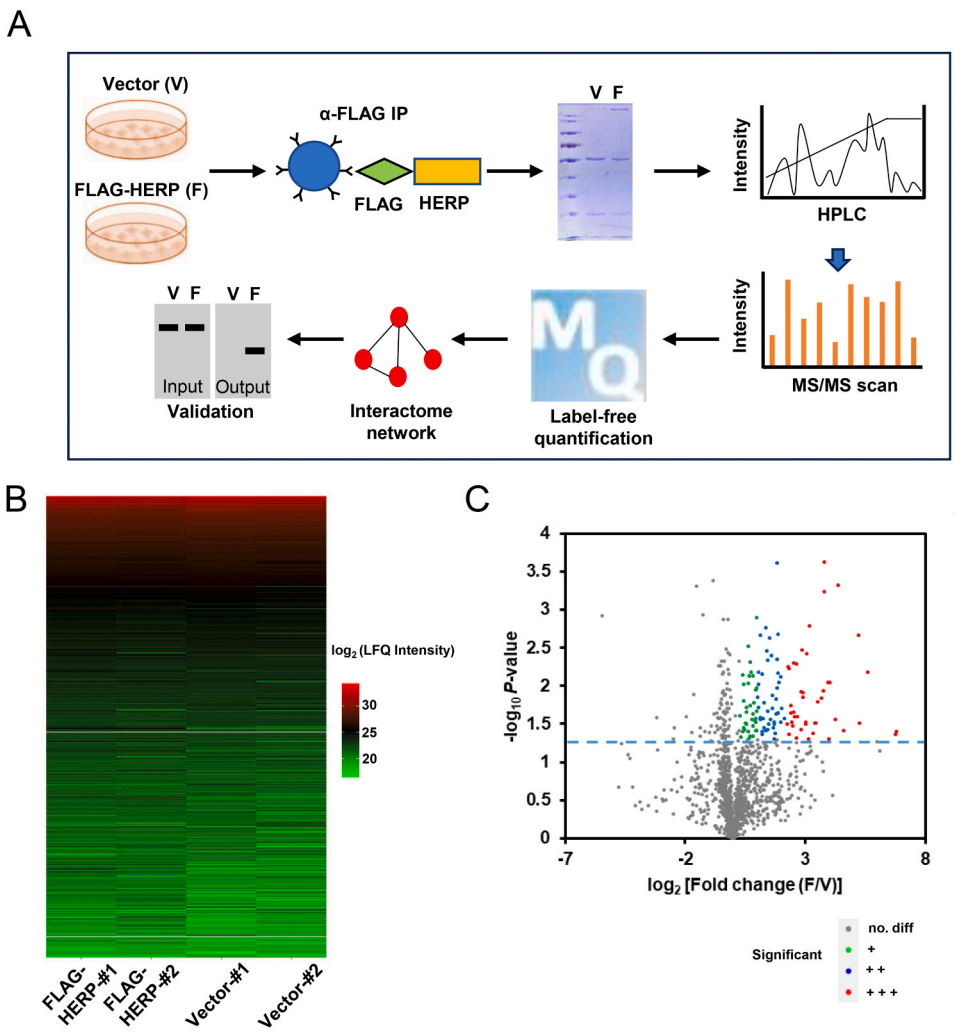

Fig. 1. Systematic identification of HERP binding proteins. (A) Overview of the experimental workflow in this study. Cell lysates were immunoprecipitated using anti-FLAG antibody and protein G beads. The samples eluted from Co-IP were separated by a 10% SDS-PAGE gel and processed with Coomassie staining. The entire gel lanes were excised from the gels, and in-gel digestion was performed for LC-MS/MS and LFQ analysis. MS data were processed, and a list of HERP interacting proteins was generated. Bioinformatics analyses were performed, and several critical HERP interacting proteins were selected for further validation. (B) Heatmap presents the $\log_2$ (LFQ intensity) of proteins in Vector and FLAG-HERP groups. (C) Volcano plot of *P* values ($-\log_{10}$) vs the Fold change ($\log_2$) of FLAG-HERP/Vector protein for all the quantified proteins. The significant standards of HERP interacting proteins were defined: +, $0.26 < \log_2$ (Fold change) < 1, $P < 0.05$; ++, $1 \leq \log_2$ (Fold change) < 2.2, $P < 0.05$; +++, $2.2 \leq \log_2$ (Fold change), $P < 0.05$.

2.6. LC-MS/MS analysis

Peptides from each fraction were analyzed with the Ultimate 3000 RSLCnano system (Dionex; Sunnyvale, California, USA) coupled to the electrospray ion-trap mass spectrometer Q Exactive Plus (Thermo Fisher Scientific; New York, NY, USA). The MS samples were first dissolved in 0.1% formic acid with 2% ACN, then loaded on a C18 captured column (100 μm × 2 cm, 3 μm, 120 Å) and eluted on a C18 analytical column (750 μm × 15 cm, 2 μm, 120 Å). The peptides samples were eluted during a gradient (Buffer A, 3% DMSO, 0.1% formic acid and 97% H_2O; Buffer B, 3% DMSO, 0.1% formic acid and 97% ACN) over 120 min for reversed phase chromatograph separation, with a flow rate of 300 nL min^{-1}. Subsequently, the peptides were analyzed with a full MS scan from 350 to 1800 *m/z* at a resolution of 70,000. For each MS/MS scan, the top 15 intense precursors were submitted to higher energy collision dissociation (HCD) fragmentation at 200 *m/z* with a resolution of 17,500. AGCs for MS and MS/MS were set to 3×10^6 and 1×10^5, respectively. The intervals of maximum injection for MS and MS/MS were 20 and 100 ms, respectively. Selected ions were dynamically excluded for 35 s

2.7. Protein identification and quantification

The Data Analysis 4.0 (Bruker Compass Software) was used to process raw files, and the MaxQuant software (V1.6.2.10) was applied to compare the raw MS/MS data with Uniport/Proteome human database (May 10, 2020). Several parameters were set as follows: enzyme specificity, trypsin and with no proline restriction; maximum missed cleavages, two; variable modification, oxidation (Met) and acetylation (Protein N-terminal); carbamidomethylation of cysteine, fixed modification; precursor ion mass tolerance, 0.4 Da; MS/MS mass tolerance, 0.6 Da; LFQ, TRUE; LFQ min ratio count, 2; the false discovery rate (FDR), 0.01.

The MaxLFQ algorithm was used for LFQ quantification based on the intensity of the same protein peptide in different samples. Proteins that only identified by site, reverse hits, or contaminants were excluded from protein list generated by MaxQuant. The remaining proteins were used for relative quantification, and the Fold change and *P*-value between two groups were calculated. *P*-value was obtained by unpaired two-tailed Student's *t*-test. Only proteins with Fold change > 1.2 and $P < 0.05$ in the LFQ data were accepted as HERP interacting proteins and selected for further analysis. To screen the significant difference of these proteins, the normal distribution was fitted with $\log_2$ (Fold change) and the 95% confidence was calculated (mean ± 1.64 ×SD). The corresponding screening threshold was set as $P = 0.05$, $\log_2$ (Fold change) = 1 or mean + 1.64 ×SD. The significant standards were defined as follow: +, $0.26 < \log_2$ (Fold change) < 1, $P < 0.05$; ++, $1 \leq \log_2$ (Fold change) < 2.2, $P < 0.05$; +++, $2.2 \leq \log_2$ (Fold change), $P < 0.05$.

2.8. Bioinformatics analysis

The bioinformatics analysis was performed to investigate the biological relevance of HERP binding partners. HERP interacting proteins were classified into several categories by PANTHER system (http://

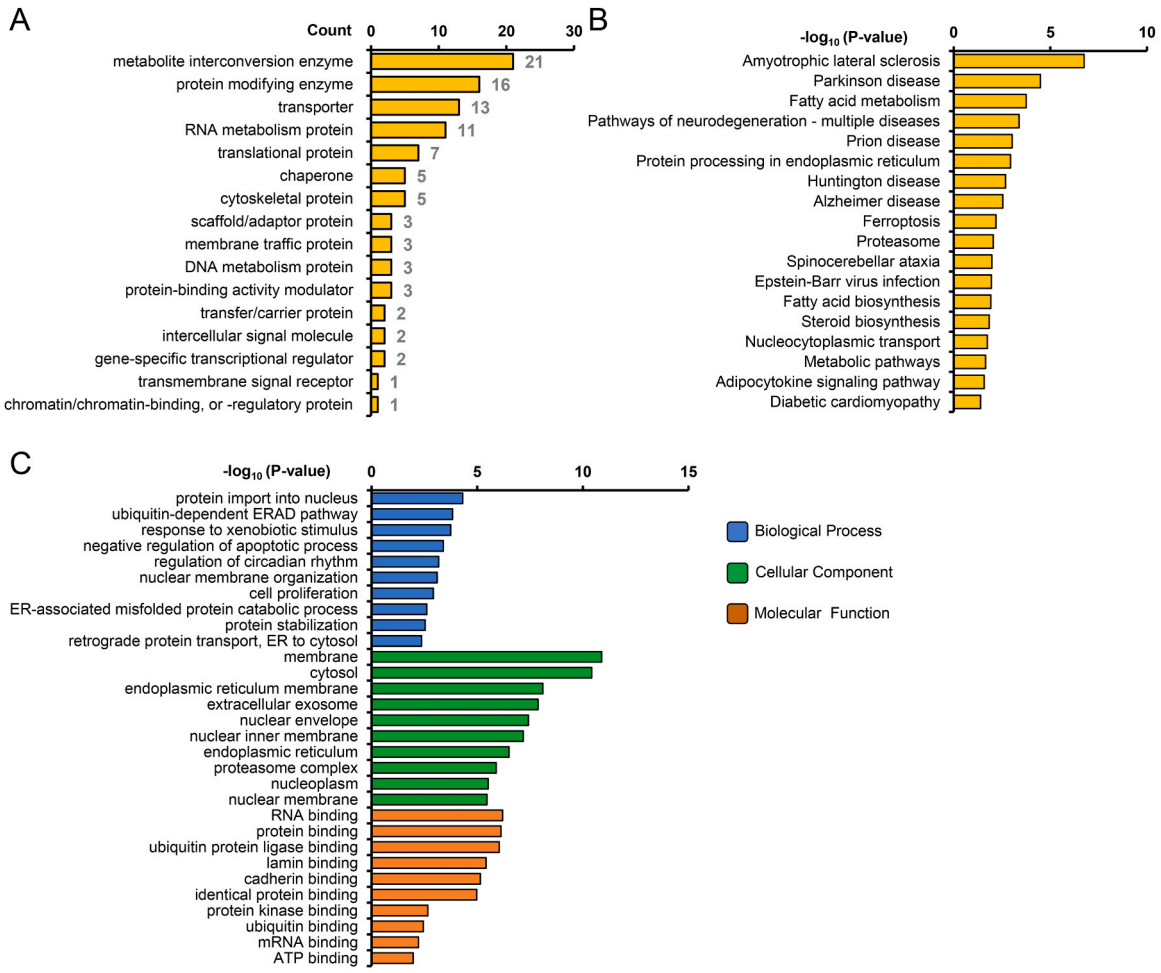

Fig. 2. Functional enrichment analysis of HERP interactome. (A) Protein class analysis of identified HERP binding proteins. (B) Bar chart of KEGG pathway enrichment of identified HERP interacting proteins. (C) Bar chart of the GO enrichment of identified HERP binding proteins using Cytoscape. The top ten GO terms enriched in the HERP interacting proteins are shown.

www.pantherdb.org), a useful protein class tool [22]. Based on DAVID Bioinformatics Resources 6.8 (https://david-d.ncifcrf.gov/), Gene ontology (GO) classification and enrichment were carried out to understand the biological functions of HERP interactome [23]. The identified HERP binding proteins are classified into several subfamilies with common functions based on biological process (BP), molecular function (MF), and cellular component (CC), as well as the most related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. To understand the relation among identified HERP interacting proteins globally, the protein-protein interaction (PPI) networks were constructed using the search tool for the retrieval of interacting genes/proteins (STRING) database v10.0 [24], while visualizing the interaction network with Cytoscape v3.2.1 (http://www.cytoscape.org) [25].

2.9. Confocal microscopy

According to the previous report [26], the immunofluorescence experiment was performed. In brief, 1×10^5 HeLa cells were seeded in 24-well plate with coverslips, then the cells were transfected with specific plasmids. After 30 min fixation with 4% formaldehyde, the samples were added with saponin buffer (0.1% saponin and 1% BSA in PBS) for 30 min at room temperature. After three washes with PBS, specific primary antibodies were used to probe the samples at 4 °C overnight, followed by the incubation with luciferin-conjugated goat anti-rabbit IgG or anti-mouse IgG (Proteintech Group) for 1 h at room temperature, and DAPI (Roche) solution staining for nuclei for 10 min. After the last wash, the cell images were observed by a Leica TCS SP8 confocal microscopy (Leica Microsystem; Wetzlar, Germany).

3. Results

3.1. Identification of HERP interacting proteins by Co-IP and LC-MS/MS approaches

HERP is an important protein in ER, and the expression levels of HERP increased strongly in response to ER stress [8]. In this study, an LFQ-based proteomics analysis was conducted to systematically explore the potential proteins interacting with HERP. After transfected with FLAG-HERP or Vector plasmids, the HeLa cells from two biological duplicates were lysed and immunoprecipitated with anti-FLAG antibody, then the IP samples were separated on SDS-PAGE gel, followed by in-gel digestion and LC/MS-MS analysis (Fig. 1A). Consequently, a total of 1279 proteins were identified and quantified (Table S1A), and the heatmap indicated the relative LFQ intensity of quantifiable proteins in FLAG-HERP and Vector groups (Fig. 1B). Our analysis identified 123 binding proteins of HERP based on Fold change and *P*-value, among which the significant difference of 43, 44, and 36 proteins presented as "+ + + ", "+ +", and "+ " respectively (Fig. 1C and Table S1B). Of the interacting proteins, ubiquitin specific peptidase 7 (USP7) has been reported to be a HERP binding protein in Yeast Two-Hybrid (Y2H) screens [27], which suggests that our LC-MS/MS data are authentic.

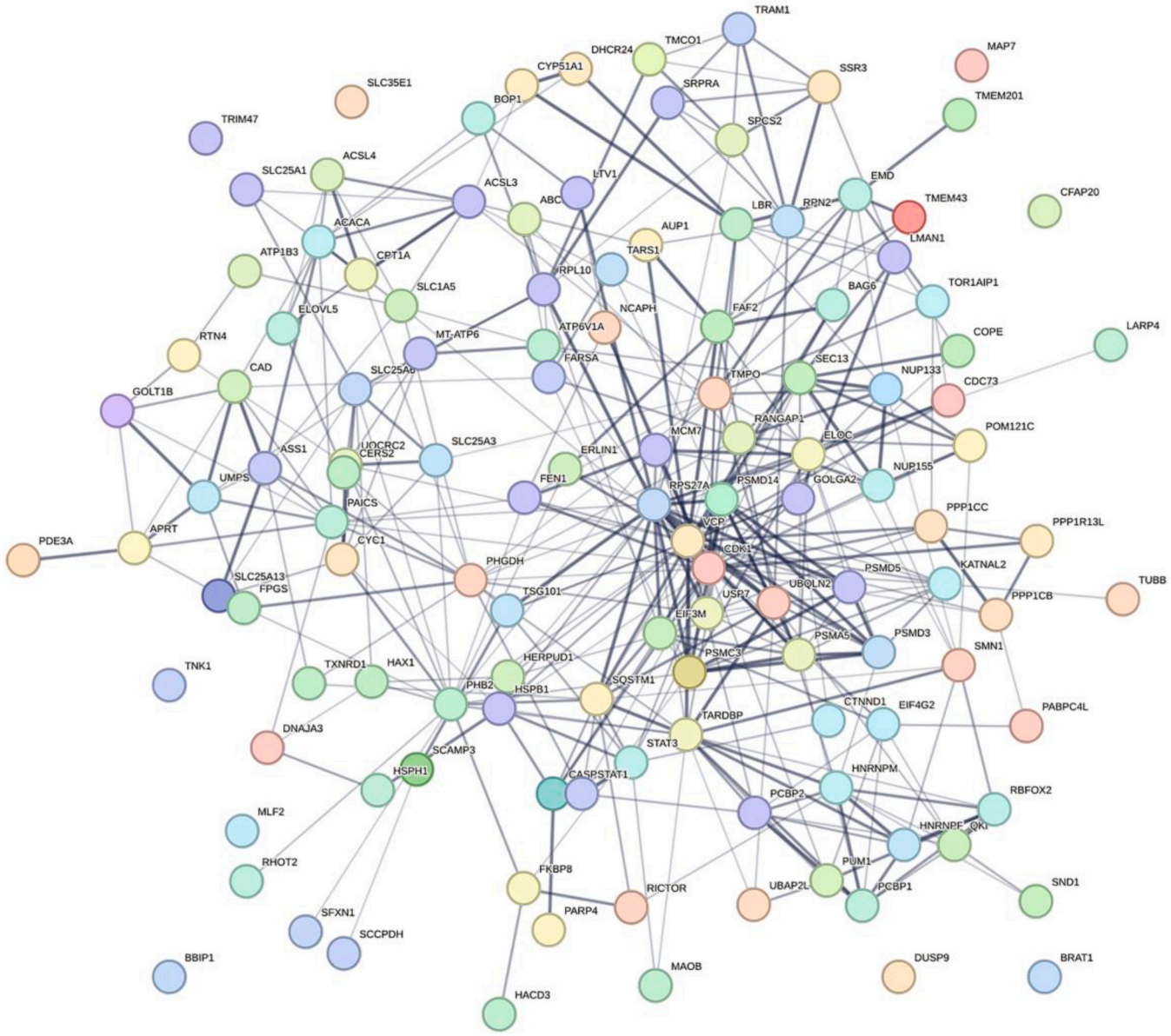

Fig. 3. PPI analysis for the identified HERP interacting proteins. PPI analysis for the identified HERP interacting proteins. The PPI network of HERP interacting proteins was constructed using the STRING database. Of the 114 HERP interacting proteins were connected in this network.

3.2. Bioinformatics analysis of HERP binding proteins

To investigate the biological roles of the HERP interactome adequately, these identified proteins were classified with PANTHER classification systems. Using the method, 98/123 proteins were divided into 16 protein classes (Fig. 2A and Table S2). The top five classes were metabolite interconversion enzyme (21 proteins), protein modifying enzyme (16 proteins), transporter (13 proteins), RNA metabolism protein (11 proteins) and translational protein (7 proteins). Furthermore, DAVID Bioinformatics Resources was used to perform KEGG pathway and GO enrichment analysis. In detail, the KEGG pathway enrichment analysis displayed that the HERP interacting partners were mostly enriched in the development of some diseases, including amyotrophic lateral sclerosis, Parkinson disease, neurodegeneration, prion disease, Huntington disease, Alzheimer disease, and spinocerebellar ataxias (Fig. 2B and Table S3). Importantly, we observed that the interactors were involved in protein process in ER, and proteasome (Fig. 2B and Table S3), which may be an important mechanism of HERP as an ER-stress induced protein associated with ERAD signaling [19,28]. Besides, fatty acid metabolism, steroid biosynthesis, and adipocytokine signaling pathway were also enriched, indicating that HERP may play a vital role in metabolism (Fig. 2B and Table S3). The GO enrichment analysis was carried out based on three parts, namely biological processes, molecular functions, and cellular components. Our data demonstrated that most identified proteins were categorized into protein metabolism and protein transport, such as ubiquitin-dependent ERAD pathway, ER-associated misfolded protein catabolic process, and retrograde protein transport from ER to cytosol (Fig. 2C and Table S4). Consistently, the most HERP interacting partners were markedly localized in membrane, cytosol, and ER membrane (Fig. 2C and Table S4. With the molecular function category, we showed that the identified proteins participated in RNA binding, protein binding, and ubiquitin protein binding (Fig. 2C and Table S4). Overall, these results indicated that HERP has diverse functions in important biological pathways and processes.

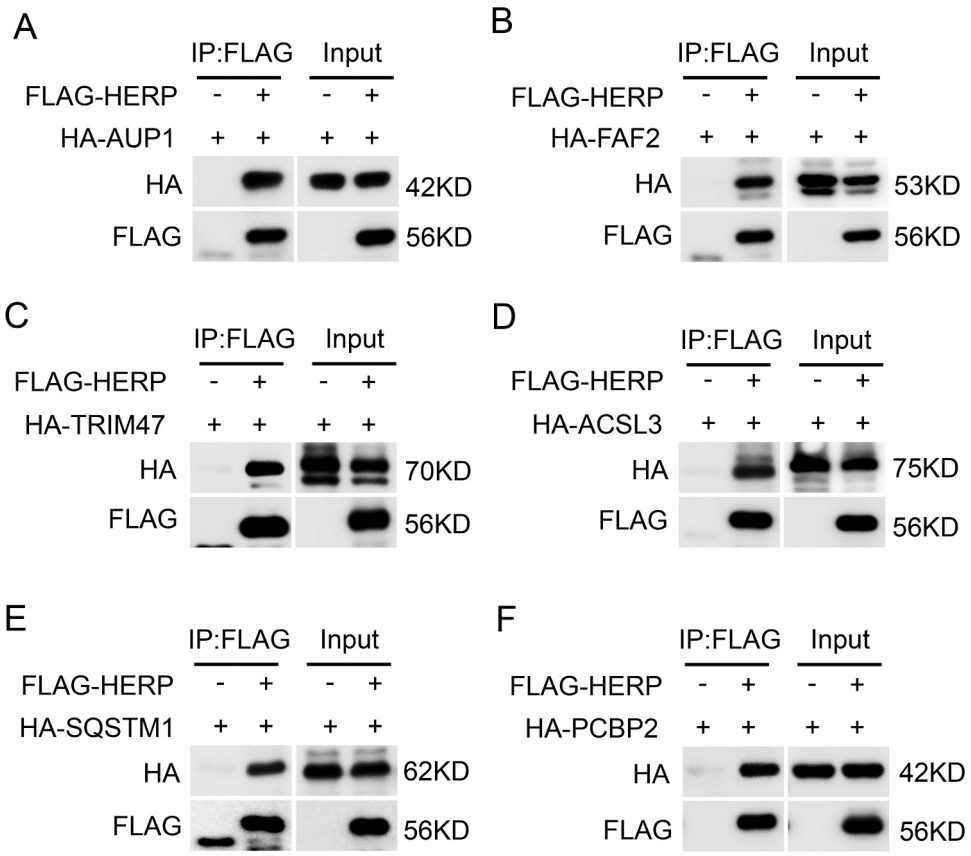

Fig. 4. Validation of HERP interacting proteins by Co-IP assay. HEK293T cells were plated in 6-well plates and co-transfected with FLAG-HERP and HA-AUP1 (A), HA-FAF2 (B), HA-TRIM47 (C), HA-ACSL3 (D), HA-SQSTM1 (E), or HA-PCBP2 (F) plasmids. Cells were lysed in RIPA lysis buffer. The immunoprecipitations and whole-cell lysates were analyzed by Western blot with HA and FLAG antibodies.

3.3. Protein-protein interaction network analysis for the HERP interacting proteins

To decipher the interaction relationship among these HERP interacting proteins, PPI network was generated and visualized by STRING database and Cytoscape, respectively. The finding of a single dominant connected component implied that 114/123 prey proteins and bait protein HERP (also called HERPUD1) were involved in interaction network (Fig. 3 and Table S5), demonstrating that most of identified HERP binding proteins are functionally linked.

3.4. Validation of the selected HERP binding proteins by Co-IP assay

Based on the bioinformatic analysis, significant difference, and antibody availability of identified proteins, six HERP binding partners (AUP1, FAF2, TRIM47, ACSL3, SQSTM1, and PCBP2) from top ranked proteins according to fold change of LFQ were selected for further validation. We transfected with Vector or FLAG-HERP and HA-AUP1 (Fig. 4A), HA-FAF2 (Fig. 4B), HA-TRIM47 (Fig. 4C), HA-ACSL3 (Fig. 4D), HA-SQSTM1 (Fig. 4E), or HA-PCBP2 (Fig. 4F) plasmids into HEK293T cells and implemented Co-IP assays. After immunoprecipitated with FLAG antibody, all the above six proteins were detectable in the HERP-IP complex rather than in the Vector-IP complex (Fig. 4). Therefore, HERP can interact with AUP1, FAF2, TRIM47, ACSL3, SQSTM1, and PCBP2, which provides new clues to explore the functions of HERP.

3.5. Validation of the colocalization of selected HERP binding proteins with HERP in ER

Since HERP is an ER-resident membrane protein [8], the interaction and localization of HERP with these six proteins were investigated. Initially, we transfected FLAG-HERP and HA-AUP1, HA-FAF2, HA-TRIM47, HA-ACSL3, HA-SQSTM1, or HA-PCBP2 plasmids in HeLa cells, and stained with specific primary antibodies. To confirm the location, the cells were stained with ER tracker. Immunofluorescence (IF) observations revealed that HERP companied with AUP1 (Fig. 5A), FAF2 (Fig. 5B), TRIM47 (Fig. 5C), ACSL3 (Fig. 5D), SQSTM1 (Fig. 5E), or PCBP2 (Fig. 5F) were expressed and co-localized in ER.

3.6. Expression and colocalization of HERP and its binding protein upon ER stress activation

HERP is known to be upregulated reacting to ER stress, such as Tg stimulation or virus infection [8,11]. The role of ER stress induction on the function of HERP and its binding proteins was explored. Initial data showed that in response to Tg and EV71, the upregulated expression of HERP, endoplasmic reticulum oxidoreductin-1 (ERO1)-La, and ATF6 was observed (Fig. 6A), indicating the trigger of ER stress [3]. The expression of EV71 VP1 was also detected, confirming a robust viral replication in HeLa cells (Fig. 6A). Moreover, we detected that the expression levels of AUP1, FAF2, and TRIM47 increased when treated with Tg and EV71 (Fig. 6A), whereas the expression levels of SQSTM1 protein declined, which is identical with a previous report that the activation of ER stress triggers autophagy [29]. In the following IF experiments, our results demonstrated that EV71 and Tg induced the expression of AUP1 (Fig. 6B), FAF2 (Fig. 6C), TRIM47 (Fig. 6D) and

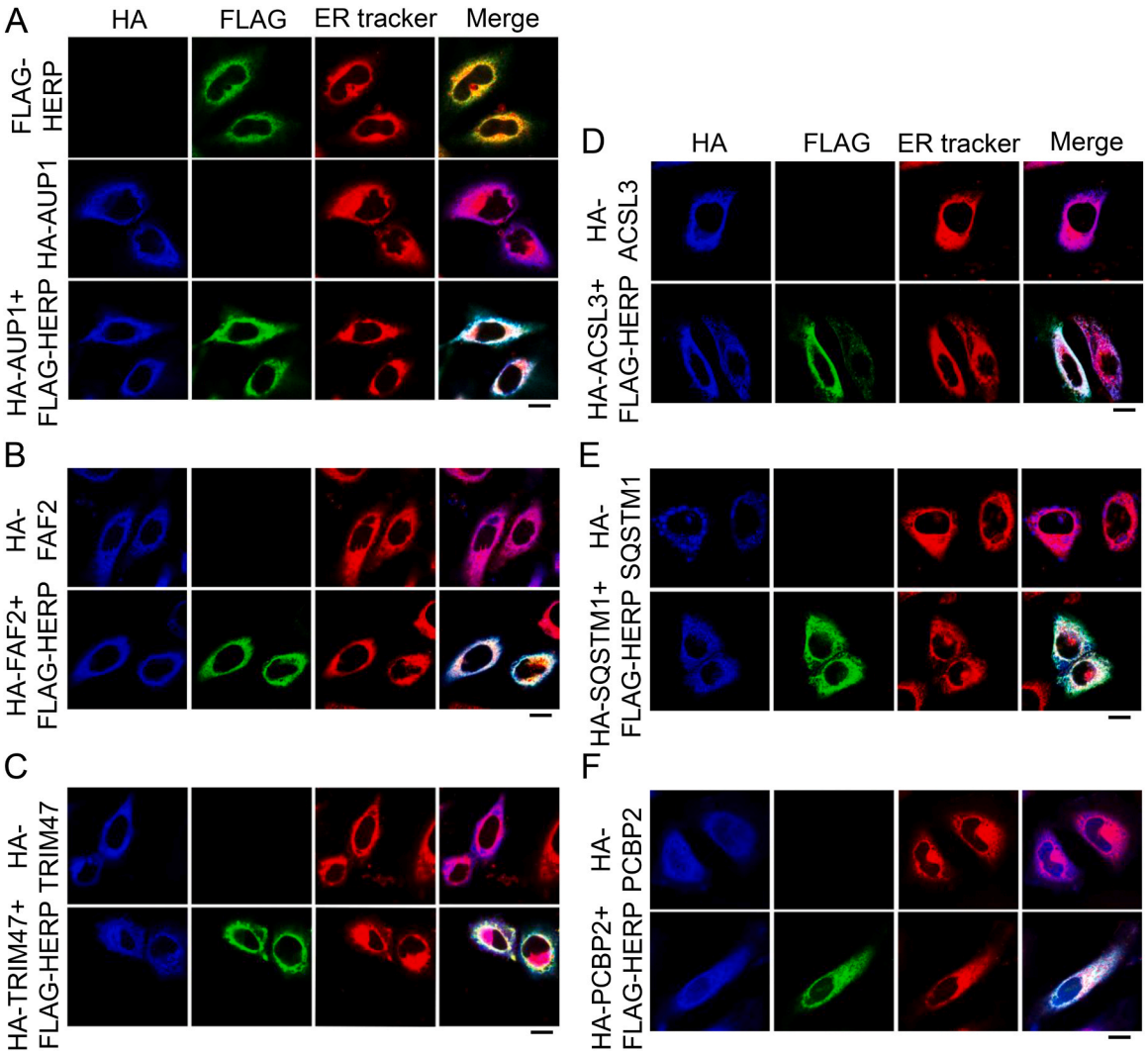

Fig. 5. Validation of the selected HERP binding proteins colocalization in ER. HeLa cells were plated in confocal dishes and transfected with FLAG-HERP and HA-AUP1 (A), HA-FAF2 (B), HA-TRIM47 (C), HA-ACSL3 (D), HA-SQSTM1 (E), or HA-PCBP2 (F). Cells were stained with HA and FLAG antibodies, and ER tracker for ER. Immunofluorescence analyses were performed: HERP (blue), AUP1 (green), FAF2 (green), TRIM47 (green), ACSL3 (green), SQSTM1 (green), PCBP2 (green), and ER tracker (red). ER: endoplasmic reticulum. Bar = 20 μm.

declined the expression of SQSTM1 (Fig. 6E), and these proteins were co-localized with upregulated HERP in ER significantly (Fig. 6B-E). Altogether, our results identified the interaction of HERP and several vital proteins with diverse functions in ER stress reaction.

4. Discussion

Numerous studies have shown that HERP is implicated in diverse cellular processes including ERAD [12,19,28], metabolic regulation [16], Ca^{2+} homeostasis [14], cell proliferation [17,30], cell apoptosis [17], and ferroptosis [18]. However, further functions and the exact mechanisms by which HERP carries out its biological functions are still unknown. The different functions of HERP depend on its specific interaction with other proteins. In this study, we used a proteomic approach to investigate the proteins interacting with HERP.

A total of 123 potential HERP binding proteins were identified. The bioinformatic analyses revealed that these proteins participate in protein import into nucleus, ubiquitin-dependent ERAD pathway, ER-associated misfolded protein catabolic process and ubiquitin protein ligase binding as well as multiply pathways including the development of several diseases, protein processing in ER, fatty acid metabolism, steroid biosynthesis, and ferroptosis. It is noteworthy that HERP has been reported to act a critical function in Parkinson's disease, diabetes, and neurodegeneration [13–15]. These findings imply that HERP is closely related to the occurrence of a variety of diseases and there is a potential connection of different pathological processes. In addition, the enrichment of HERP interacting proteins in proteasome and proteins processing in ER are in accord with the functions of HERP in directing misfolded proteins for ubiquitination and degradation by 26 S proteasome [19,28]. To evaluate the results from LC-MS/MS, we selected six identified proteins from several functional groups to validate these interactions, including AUP1, FAF2, TRIM47, ACSL3, SQSTM1, and PCBP2. Among them, AUP1 [31], FAF2 [32], ACSL3 [33], and SQSTM1 [34] are located mainly or partly in ER. As for TRIM47 and PCBP2, they have been reported to be located partly in the cytosol [35,36], which indicates the possible interaction and co-localization of these proteins with HERP in ER. Our results demonstrated that HERP interacted with these six proteins using Co-IP and confocal microscopy experiments, respectively. Furthermore, we attempted to search the motifs of the selected proteins and several other identified HERP interacting proteins using the MEME Suite (https://meme-suite.org). Unfortunately, we failed to verify the common motifs in these proteins, maybe further analysis or advanced approaches is required to be performed.

In the interactome, 21 proteins were assigned to the metabolite

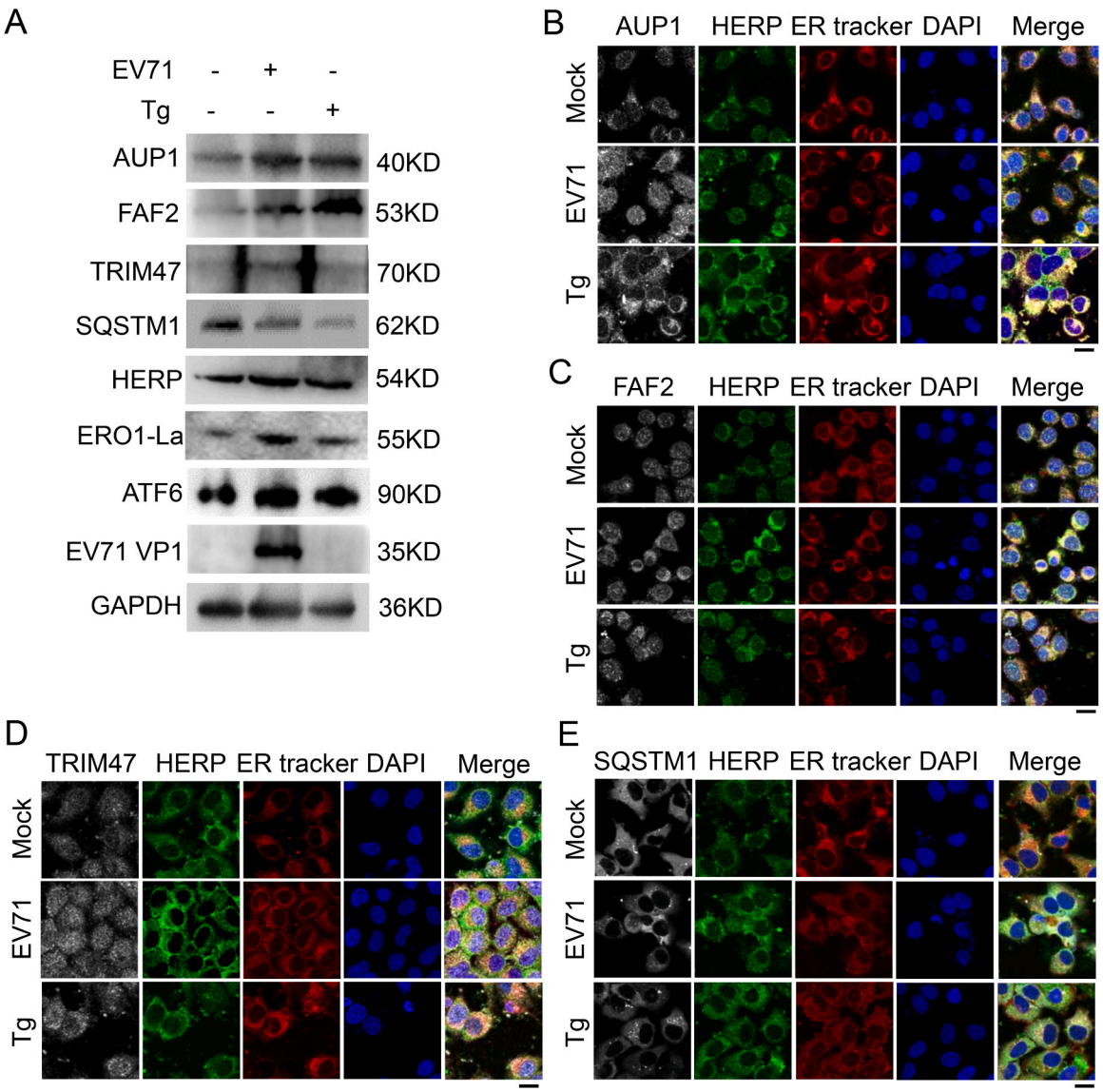

Fig. 6. Expression and colocalization of HERP and its binding protein in response to ER stress. (A) HeLa cells were treated with EV71 (MOI = 1) for 12 h or Tg (10 μM) for 6 h. The cells were lysed and indicated proteins were detected by Western blot analyses. (B-E) HeLa cells were plated in confocal dishes, then treated with EV71 for 12 h or Tg for 6 h. The localization of HERP (green) and AUP1 (B), FAF2 (C), TRIM47 (D), or SQSTM1 (E) (white), were analyzed by confocal microscopy. The ER was stained with ER tracker (red), and the nucleus was stained with DAPI (blue). Bar = 20 μm.

interconversion enzyme, which represents the largest class of interactors. Fatty acid metabolism was also enriched in KEGG pathway analysis, suggesting an essential role of HERP in cellular metabolize regulation. Among them, ACSL3 and ACSL4 are membranes of the long-chain fatty-chain-coenzyme A ligase family, exerting its critical functions in lipid biosynthesis and fatty acid degradation, thereby regulating several kinds of the progress of cancer [37,38]. Thus, we speculate that the interaction of HERP and ACSL3 or ACSL4 may promote fatty acid metabolism and eventually lead to the turnover of fatty acid.

The scaffold/adaptor protein plays an integral role in medicating signaling transduction. HERP interacted with several proteins enriched in protein binding and ubiquitin protein ligase binding including AUP1, FAF2 and SQSTM1, which regulates a series of biological functions. AUP1 and FAF2 contribute the functions of ERAD and cellular lipid droplet homeostasis [31,32,39,40], indicating a possible association between the quality control of ER protein and lipid droplets. A previous study has presented that flavivirus exploits AUP1 to trigger lipophagy and promotes its replication [41]. In addition, FAF2 enhances the ubiquitination and dimerization of stimulator of interferon genes (STING), and interacts with STING and TRIM56, thus upregulating STING-mediated antiviral immune response [42]. On this account, the binding of HERP and these proteins may participate in a range of functions. SQSTM1 is an important selective autophagy receptor which acts as a ubiquitin sensor and a signal transducing adapter with multiple functions in different situations. Recent studies revealed that ER stress induces SQSTM1-medicated selective autophagy, which is essential for the removing of unfolded protein and impairing the replication of alphaherpesvirus [3,29]. Therefore, we conclude that HERP may influence the autophagy pathway through directly regulating SQSTM1.

Protein modifying enzyme also occupied an important part in HERP binding proteins, including 16 proteins. The posttranslational modifications (PTMs) is pivotal in the functional proteome by affecting the activity, localization, and interaction with other cellular molecules of proteins [43]. TRIM47 is an oncogene and involved in promoting cancer cell proliferation by ubiquitination of substrates, including p53, fructose-1,6-bisphosphatase 1 (FBP1), forkhead box O1 (FOXO1) and SMAD4 [44–47]. In addition, TRIM47 also plays a critical function in innate immunity through the increase of the K48-linked ubiquitination

of NF90 and K63-linked ubiquitination of TNF receptor-associated factor 2 (TRAF2) [35,48]. Notably, HERP is required for the ubiquitination of ERAD substrates [2,12,20]. Thus, we validated the binding of TRIM47 and HERP that may contribute to regulating the functions of HERP in ERAD.

In addition, we found that HERP associated with RNA metabolism protein, 11 proteins were enriched in this classification. These results demonstrated a potential role of HERP in regulation of gene expression. PCBP2 from these molecular function categories was further validated as a HERP binding protein. The protein includes a linker region and three K-homologous (KH) domains, which has been linked to the regulation of poliovirus replication by binding to its 5′-terminal cloverleaf structure [49]. The function of PCBP2 in antiviral innate immunity by mediating the degradation of MAVS and the decrease of cGAS activity was reported recently [50,51]. In current study, the complexes of HERP and PCBP2 may regulate antiviral signaling or bind to virus' DNA/RNA directly, thus influencing the replication of virus.

Indeed, there were some limitations in our study and several issues should be considered. Firstly, overexpression system was selected to implement Co-IP experiment for LC-MS/MS with some disadvantages, including the dysregulation of endogenous protein expression and the production of non-specific interactions. Secondly, we used HeLa cells to study HERP interactome, which may produce uncertainty due to the change of expression profile in tumor cells. Thirdly, the complexity of the interactome should increase, such as adding more conditions to explore the changes of HERP interactome after Tg or EV71 stimulation.

5. Conclusions

In sum, we identified 123 potential HERP binding candidates. The bioinformatics analysis indicated that these prey proteins are linked to a variety of cellular processes and signaling pathways. Subsequently, six featured proteins were selected from distinct functional groups and confirmed as HERP interacting proteins. In general, this study illustrates that our interactome of HERP in HeLa cells offers a compatible model to investigate the functions of HERP and involved signaling pathways. It also presents a global view of the mechanism of HERP-associated functional response to ER stress under physiological and pathological conditions.

CRediT authorship contribution statement

Conceptualization, R. S., P. W., and Z. L.; Data Curation, R. S., J. Y., P. W., and Z. L.; Formal Analysis, J. Y., X. R., and Y. C.; Funding Acquisition, R. S., P. W., and Z. L.; Investigation, R. S., J. Y., and Z. L.; Methodology: R. S., J. Y., X. R., and Y. C.; Resources: R. S., J. Y., X. R., Y. C., and P. W.; Supervision, P. W., and Z. L.; Validation, P. W., and Z. L.; Writing, R. S.; Writing-Review & Editing: Z. L.

Declaration of Competing Interest

We declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.09.006.

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