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Research Article 研究报告

Microsporidian *Encephalitozoon hellem* secretes EhPTP4 to regulate host endoplasmic reticulum-associated degradation

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Abstract: [Objective] Microsporidia are a group of obligate intracellular parasites that can infect humans and nearly all animals. Here, we studied the polar tube protein 4 of *Encephalitozoon hellem* (EhPTP4) on its subcellular localization and functions as a potential secretory virulence factor in host cells. **[Methods**] A polyclonal antibody against EhPTP4 was produced to verify the protein subcellular localization in *E. hellem*-infected cells using indirect immunofluorescence assay (IFA) and Western blotting. HEK293 cells were transfected with wild-type or mutant EhPTP4 fused with HA-EGFP, and the impacts on pathogen proliferation, protein subcellular localization and sequence functions were assessed. RNA sequencing of EhPTP4-transfected cells was conducted to identify differentially expressed genes (DEGs) and pathway responses. The regulatory effects of candidate DEGs were analyzed via RNAi and cell transfection, and the effects were determined with RT-qPCR and Western blotting. **[Results]** EhPTP4 contains a signal peptide at the N-terminal, a nuclear localization sequence (NLS) and a histidine-rich domain (HRD) at the C-terminal. In the infected and transfected cells, EhPTP4 was secreted into the host nucleus. Transfection and overexpression of EhPTP4 in HEK293 cells significantly promoted pathogen proliferation. RNA-seq of the transfected cells showed that genes involved in endoplasmic reticulum (ER)-associated degradation (ERAD), a quality control mechanism that allows for the targeted degradation of proteins in the ER, were prominently upregulated. Upregulation of the ERAD genes *PDIA4*, *HERP*, *HSPA5* and *Derlin3* determined by RNA-seq data was verified using RT-qPCR and Western blotting. Protein ubiquitination of the transfected cells was then assayed and found to be markedly increased, confirming the activation of ERAD. PDIA4 knockdown with RNAi significantly suppressed the expression of HERP, indicating that PDIA4 is vital for the modulation by EhPTP4. Moreover, EhPTP4$^{\Delta HRD}$, a deletion mutant lacking the HRD, could not cause

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the upregulation of ERAD genes, indicating that the HRD is essential for the function of EhPTP4. **[Conclusion]** This study is the first report on a microsporidian secretory protein that targets the host nucleus to upregulate the ERAD pathway and subsequently promote protein ubiquitination. Our work provides new insights into microsporidia-host interactions.

Keywords: microsporidia; *Encephalitozoon hellem*; EhPTP4; secreted protein; ERAD; ubiquitination

海伦脑炎微孢子虫分泌蛋白 EhPTP4 对宿主内质网蛋白降解通路的调控作用

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摘　要：【目的】微孢子虫是一类专性细胞内寄生的真核病原微生物，能够感染人类和几乎所有的动物。本课题以海伦脑炎微孢子虫(*Encephalitozoon hellem*)为研究对象，探讨其极管蛋白 4 (EhPTP4)作为一个潜在的分泌性毒力因子在宿主细胞内的定位和功能。【方法】制备 EhPTP4 的鼠源多克隆抗体，利用间接免疫荧光分析和 Western blotting 确定 EhPTP4 在感染细胞中的亚细胞定位；基于序列特征，在 HEK293 细胞中转染野生型和突变体 EhPTP4，分析该蛋白的定位及其对病原增殖的作用；利用 RNA-seq 对转染 EhPTP4 的 HEK293 细胞进行转录组测序，分析 EhPTP4 引起的宿主基因表达和通路的变化; 进一步通过 RNAi 和细胞转染分析差异表达基因的调控作用，利用 RT-qPCR 和 Western blotting 验证调控效果。【结果】EhPTP4 的 N 端具有信号肽，C 端具有富含组氨酸的结构域(HRD)和核定位信号序列(NLS)。蛋白定位分析显示，在感染和转染细胞中，EhPTP4 均被分泌至宿主细胞核内。在 HEK293 细胞中过表达 EhPTP4 显著促进了病原的增殖。RNA-seq 和蛋白泛素化分析发现，EhPTP4 显著上调了宿主内质网蛋白降解通路(endoplasmic reticulum-associated degradation，ERAD)关键基因(*PDIA4*、*HERP*、*HSPA5* 和 *Derlin3*)的表达和宿主蛋白的泛素化修饰，表明 EhPTP4 具有调控宿主内质网蛋白降解的功能。在 HEK293 细胞中过

表达 PDIA4 发现，蛋白泛素化修饰明显上调，而 RNA 干扰 *PDIA4* 则导致 *HERP* 基因表达下调，表明 PDIA4 在病原调控宿主 ERAD 过程中发挥着关键作用。进而，对 EhPTP4 的 HRD 进行了缺失突变(EhPTP4$^{\Delta HRD}$)并转染细胞，发现此缺失突变体并未引起 PDIA4 和 HERP 的表达发生显著上调，表明 HRD 对 EhPTP4 的毒力功能起到关键作用。【结论】首次报道了微孢子虫利用分泌蛋白调控宿主的 ERAD 和蛋白泛素化，为深入解析该病原的致病机理提供了重要参考。

关键词：微孢子虫；海伦脑炎微孢子虫；极管蛋白 4；分泌蛋白；内质网蛋白降解；泛素化

Microsporidia are a group of obligate intracellular parasites with a broad range of hosts from invertebrates to vertebrates, including humans. More than 200 genera and 1 400 species of microsporidia have been identified[1]. *Encephalitozoon* is recognized as a common mammal-infecting species[1−2] that causes comprehensive immune responses[3]. Having emerged as important opportunistic pathogens in humans, microsporidia have been identified in patients infected by human immunodeficiency virus (HIV), organ transplant recipients, aged individuals and children[4−7]. Microsporidia have undergone extreme genomic compaction and reduction and lost canonical mitochondria and the genes for many metabolic pathways, such as the tricarboxylic acid cycle, and the *de novo* synthesis of nucleotides and amino acids[8−10]. Instead, microsporidia evolved strategies to manipulate pathways and rely on host nutrients, as well as escape host immunity[3,11].

The endoplasmic reticulum (ER) is a key organelle that functions in protein synthesis and processing, lipid synthesis, and calcium (Ca^{2+}) homeostasis[12]. Misfolded and misassembled proteins are degraded by the ER-associated degradation (ERAD) pathway, which includes a series of tightly coupled steps: substrate recognition, dislocation, and ubiquitin-dependent proteasomal destruction[13−17]. Thus, ERAD maintains protein quality by degrading proteins that fail to attain their native conformation due to mutations, errors in transcription or translation, or inefficient assembly into their native oligomeric complexes. ERAD has also been found to play important roles during pathogen infection. Certain types of pathogens, such as viruses, bacteria and protozoa, preferably hijack the host ERAD machinery to support their requirements[18−21], which generally includes the following three steps. First, proteolytic secretory and membrane proteins involved in the immune response, including major histocompatibility complex (MHC) class I and CD4, are exploited by pathogens to evade host immunity[22−23]. Second, ERAD is hijacked as transportation machinery from the ER to the cytosol for the invasion of pathogens[24]. Third, ERAD is utilized by pathogens to favor their nutritional requirements for virulence[25].

To subvert and manipulate host pathways, pathogens usually secrete proteins as virulent factors into host organelles[26−30]. For example, pathogen proteins secreted into the host nucleus play important roles in regulating immunity, proliferation and apoptosis[31−33]. Here, for the first time, we report that the polar tube protein 4 of *E. hellem* (EhPTP4)[34] is a secretory protein targeting host nucleus to disturb the ERAD pathway and protein degradation.

1 Materials and Methods

1.1 Cell cultures

Rabbit kidney cells (RK13, ATCC CCL37, Shangcheng Beina Chuanglian Biotechnology Co. Ltd) were cultured in minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/mL penicillin, 100 μg/mL streptomycin) in an incubator with 5% CO_2. Human embryonic kidney cells (HEK293, ATCC CRL-1573, Fudan IBS Cell Center) and

human foreskin fibroblasts (HFF, ATCC CRL-2522, Shangcheng Beina Chuanglian Biotechnology Co. Ltd) were grown in 10% FBS-containing Dulbecco's modified MEM with penicillin-streptomycin in an incubator with 5% CO_2 at 37 °C.

1.2 Preparation of *E. hellem* spores

Monolayer RK13 cells were infected with *E. hellem* spores and maintained in MEM with penicillin-streptomycin supplemented with 10% FBS. Spores were collected from the culture medium, purified with 75% Percoll by centrifugation at 1 000 r/min for 10 min, and washed three times with sterile distilled water by centrifugation at 1 000 r/min for 5 min. The purified spores were then counted and stored in sterile distilled water at 4 °C.

1.3 Preparation of recombinant EhPTP4 and antiserum

The coding sequence (CDS) of EhPTP4 was amplified from *E. hellem* genomic DNA (gDNA) using PrimeSTAR Max Premix DNA polymerase with the forward primer 5′-CCATGGCTGATATCGGATCCGAATTCATGTCAACGTTTGTGGGTGC-3′ and the reverse primer 5′-GTGCTCGAGTGCGGCCGCAAGCTTTCTTTATAGACGGTAAGTGC-3′. The PCR products were inserted into the pET32a (+) vector, which contains a hexa-histidine tag (His), using homologous recombinase (Yeasen) according to the instruction manual. The recombinant plasmid pET32-*EhPTP4*-6×His was verified by sequencing. *Escherichia coli* Transetta (DE3) cells were transformed with the plasmid and cultured at 37 °C overnight in 5 mL of Luria-Bertani (LB) medium (10% tryptone, 5% yeast extract, 10% sodium chloride) with 100 μg/mL ampicillin, inoculated into 400 mL of LB medium and cultured to an OD_{600}=0.6. EhPTP4 was then induced for 20 h at 16 °C by the addition of 0.5 mmol/L isopropyl-D-thiogalactoside to the culture medium. Subsequently, the bacterial cells were harvested by centrifugation for 15 min at 5 000 r/min, resuspended in 20 mL of buffer A (100 mmol/L sodium chloride, 10 mmol/L Tris-HCl, 8 mol/L urea, pH 8.0), lysed by sonication on ice and centrifuged at 1 000 ×g and 4 °C for 1 hour to harvest the lysate. The supernatant was loaded onto a Ni-NTA Superflow cartridge (Qiagen) and eluted with buffer B (100 mmol/L sodium chloride, 10 mmol/L Tris-HCl, 8 mol/L urea, 250 mmol/L imidazole). Polyclonal antiserum against EhPTP4 (anti-EhPTP4) was prepared by intradermally inoculating mice with purified rEhPTP4 (100 μg/mouse).

HFF cells infected with *E. hellem* spores for 5 d were harvested to extract nuclear proteins using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). The extracted proteins were boiled at 100 °C upon the addition of protein loading buffer, separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with western blocking buffer [2 g of skim milk in 40 mL of Tris-buffered saline supplemented with 0.05% Tween 20 (TBST)] for 2 h at room temperature (RT). Then, the membranes were incubated with anti-EhPTP4 diluted 1:150 in blocking buffer for 2 h at RT and washed three times with TBST. The blots were then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) or goat anti-rabbit IgG conjugated to HRP. After three washes, the membranes were exposed with an enhanced chemiluminescence (ECL) Western blotting detection kit (Thermo Fisher Scientific) and imaged with an Azure Biosystems C300 imaging system.

1.4 EhPTP4 transfection of HEK293 cells

The CDSs for EhPTP4, EhPTP4$^{\Delta 1-16}$, EhPTP4$^{\Delta 239-278}$ and EhPTP4$^{\Delta HRD}$ were amplified from *E. hellem* gDNA with specific primers (Table 1). DNA fragments conjugated to an HA tag and EGFP were inserted into the pcDNA3.0 plasmid and transformed into *E. coli* DH5α for replication. The replicated plasmid DNA was extracted using an Endofree Minimal Plasmid Kit II (Tiangen) and transfected into HEK293 cells using a Lipofectamine 3000 kit (Invitrogen). After 48 h, the transfected cells were harvested to analyze protein expression and subcellular localization.

Table 1 Primers for amplifying *EhPTP4* and mutants

Genes	Forward primers	Reverse primers
EhPTP4	CACTATAGGGAGACCCATGGAACTGGGTTTGATCTTG	GAACATCGTATGGGTAGAAAACTATGGGCTCTCTTCC
$EhPTP4^{\Delta 1-16}$	ACTCACTATAGGGAGACCCATGAAGGACAGGGAGCTGG	ATCTGGAACATCGTATGGGTAGAAAACTATGGGCTCTC
$EhPTP4^{\Delta 239-278}$	CACTATAGGGAGACCCATGGAACTGGGTTTGATCTTG	ATCTGGAACATCGTATGGGTAAACCACCCTTCTTTCG
$EH7108^{\Delta HRD}$	CACTATAGGGAGACCCATGGAACTGGGTTTGATCTTG	ATCTGGAACATCGTATGGGTACTCAACGTAGGTTCTCCT

1.5 Indirect immunofluorescence assay (IFA)

Infected HEK293 cells were fixed with 4% paraformaldehyde for 10 min, washed three times with PBS, and permeabilized in 0.1% Triton X-100 for 30 min. The cells were then blocked in PBST containing 10% goat serum and 5% BSA for 1 h at 37 °C and washed three times with PBS. Samples were incubated with anti-EhPTP4 (diluted 1:150 in blocking buffer) or negative serum (diluted 1:150 in blocking buffer) for 1 h at RT, washed three times, and then incubated with secondary anti-mouse IgG conjugated to Alexa Fluor 488 (diluted 1:1 000 in PBS) for 1 h. Both host cells and pathogenic nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) for 30 min at RT and washed three times. The samples were observed and photographed using an Olympus FV1200 laser scanning confocal microscope.

1.6 RNA-seq analysis of EhPTP4-transfected HEK293 cells

The transcriptomic responses of HEK293 cells transfected with HA-EGFP and EhPTP4-HA-EGFP were investigated with RNA-seq, with three replicates of each group. RNA samples were extracted and assessed using a Nanodrop 2 100 and Agilent 2 000 system. Libraries were constructed and sequenced on the Illumina NovaSeq platform. Clean sequencing reads were mapped to the human genome (ftp://ftp.ensembl.org/pub/release-95/) using HISAT2[35] and assembled with StringTie[36]. Fragments per kilobase per million (FPKM) values were calculated and used to compare gene expression levels. Differentially expressed genes (DEGs) between the EhPTP4-HA-EGFP- and HA-EGFP-transfected cells were analyzed using DEseq2[37]. The DEGs were filtered with the thresholds of a $P<0.01$ and a fold change $\geqslant 2$, and DEGs found in at least in two replicates were considered credible. KEGG enrichment analysis of the DEGs was performed using clusterProfiler[38], which is an R package for comparing biological themes among gene clusters. Pathways enriched in at least two genes and a $P<0.05$ were considered significant.

1.7 Real time quantitative PCR (RT-qPCR)

Total RNA was extracted from RK13 cells transfected with *EhPTP4* using the E.Z.N.A.® Total RNA Kit II (OMEGA, China) according to the manufacturer's instructions. cDNA was synthesized with 1 μg of total RNA using the Hifair® III 1st Strand cDNA Synthesis Kit (gDNA digester plus) (Yeasen). RT-qPCR was performed using the primers for candidate genes shown in Table 2. The transcription level was elevated by the $2^{-\Delta\Delta C_t}$ values with three replicates. All statistical *t*-tests were conducted using GraphPad Prism 6.0 for two-tailed comparison tests, the results of which with a $P<0.05$ were considered significant.

To assess the growth of *E. hellem* in cell culture, quantitative PCR was performed with *β-tubulin* primers (F: TGAAGATGAGCAATC CAGGGTA, R: TAGCAATCAGGGGTGCAAAT). The gDNA of EhPTP4- and EGFP-transfected HEK293 cells infected with *E. hellem* was extracted using an E.Z.N.A.™ Tissue DNA kit (OMEGA, China) according to the manufacturer's instructions.

Table 2 Primers used for cloning and RT-qPCR

Genes	Forward primers	Reverse primers
EhPTP4	TTACAAAGGGACCTGATGAAACC	CTTCTTCATAGGAGGAGCATTCA
Eh-βtubulin	TGAAGATGAGCAATCCAGGGTA	TAGCAATCAGGGGTGCAAAT
GADPH	GTATCGTGGAAGGACTCATGAC	ACCACCTTCTTGATGTCATCAT
PDIA4	CGTTGATTACATGATCGAGCAG	ATTTCTCAGGCTGCATTACAAC
HSPA5	GCTCTCTGGTGATCAAGATACA	GTTCCTTGGAATCAGTTTGGTC
HERP	TTGGTTGGATTGGACCTATTCA	GTCTAAATGGAAACCACCCAAC

1.8 Western blotting analysis of protein expression and ubiquitination

Proteins of HEK293 cells transfected with HA-EGFP and EhPTP4-HA-EGFP for 48 h were extracted using cell lysis buffer for Western blotting and IP (Beyotime, China, P0013) containing protease inhibitor cocktail (MedChemExpress, China, HY-K0010) and DUB inhibitor (Beyotime, China, SG0020). Western blotting was performed with mouse monoclonal antibody against actin (Beyotime, China, AA128), rabbit monoclonal antibody against HERP (Abcam, UK, ab150424), rabbit monoclonal antibody against PDIA4 (Abcam, UK, ab190348), mouse monoclonal antibody against EGFP (Roche, Switzerland, Cat. No. 11814460001), rabbit polyclonal antibody against HSPA5 (Beyotime, China, AF0171), mouse monoclonal antibody against FLAG (Sigma-Aldrich, USA, F3165) and anti-ubiquitin rabbit pAb (NT) (PTM Bio, China, PTM1106) to analyze protein expression and ubiquitination, respectively.

2 Results

2.1 Sequence features of EhPTP4

EhPTP4, which locus name is EHEL_071080 (GenBank accession: XP_003887615), was annotated as a hypothetical protein and is composed of 278 amino acids. Predictions with SignalP 5.0[39] and NLS Mapper[40] showed that EhPTP4 contains a signal peptide (SP) from amino acids 1 to 17 and a nuclear localization signal sequence (NLS) from amino acids 239 to 249, respectively, indicating that EhPTP4 is a secreted protein that probably targets the host nucleus (Figure 1). In addition, the C-terminal region of EhPTP4 encodes a histidine-rich domain (HRD) from the 258th to 265th amino acids, which was suggested to be functional in transcriptional regulation in the nucleus. Multiple sequence alignment analysis demonstrated that EhPTP4 is highly conserved with homologs among *Encephalitozoon* species (*E. hellem*, *E. romaleae*, *E. cuniculi* and *E. intestinalis*), suggesting important functions of this protein in *Encephalitozoon*.

2.2 The expression profile and subcellular localization of EhPTP4

To characterize the subcellular localization of EhPTP4 in infected cells, recombinant EhPTP4 was expressed in *E. coli* and purified for the immunization of mice and production of a mouse polyclonal antibody (anti-EhPTP4) (Figure 2, Figure 3A), which was then used to examine the localization of EhPTP4-infected cells using IFA. EhPTP4 was found to translocate into the nuclei of infected cells (Figure 3B), suggesting that EhPTP4 is a secreted protein targeting the host nucleus. Moreover, EhPTP4 was detected in meronts inside parasitophorous vacuoles (PVs), indicating that this protein is mainly synthesized and secreted by meronts and likely plays important roles during proliferation (Figure 3B).

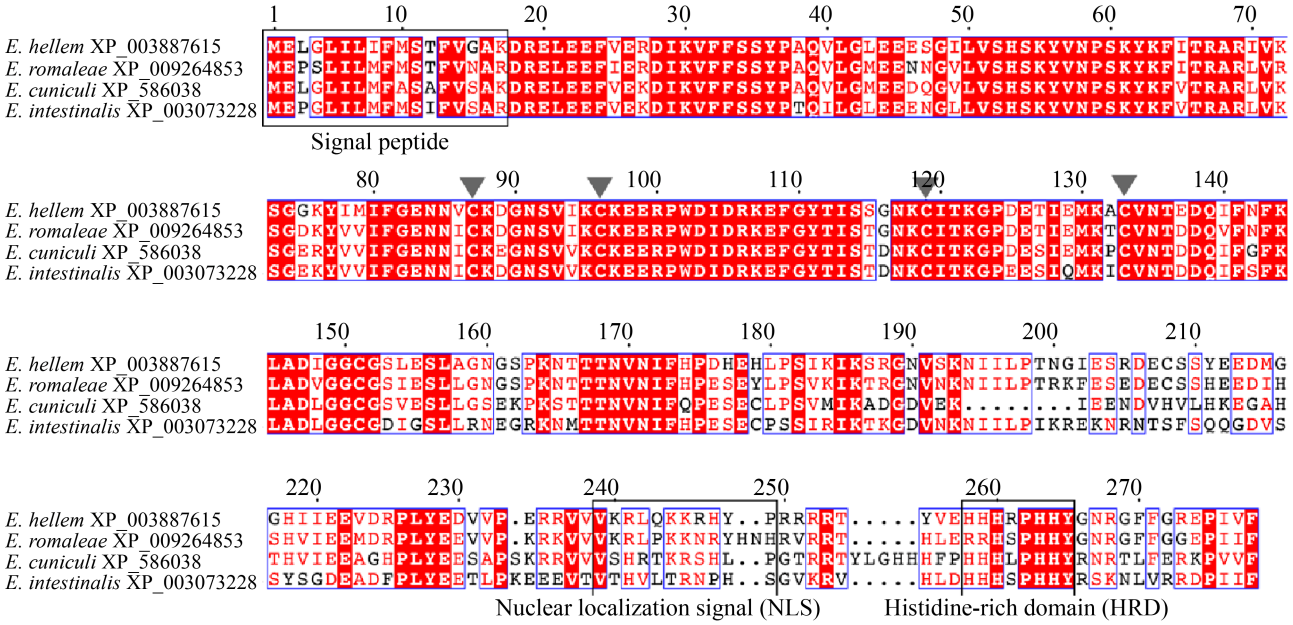

Figure 1 Sequence features and multiple alignment of EhPTP4. Protein sequences of EhPTP4 (XP_003887615) and homologs in *E. romaleae* (XP_009264853), *E. cuniculi* (NP_586038) and *E. intestinalis* (XP_003073228) were aligned using MAFFT. EhPTP4 encodes 278 amino acids containing a signal peptide from 1 to 17, a nuclear localization signal sequence (NLS) from 239 to 249, and a histidine-rich domain (HRD) from 258 to 265.

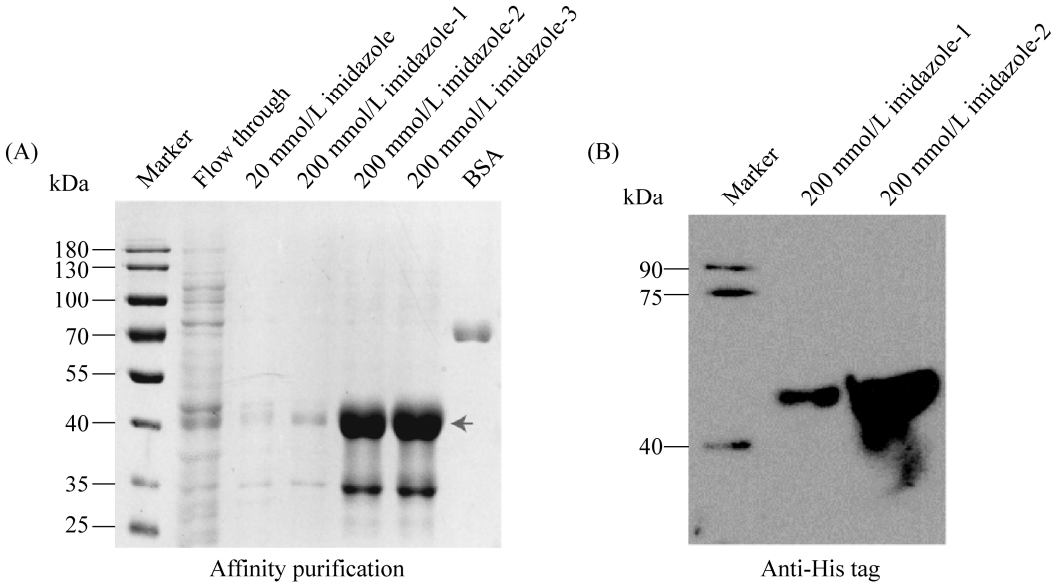

Figure 2 Purification and examination of recombinant EhPTP4. A: affinity purification of rEhPTP4-His (arrowhead) with Ni-NTA columns; B: the purified rEhPTP4-His was detected using Western blotting with antibody against His-tag (anti-His tag).

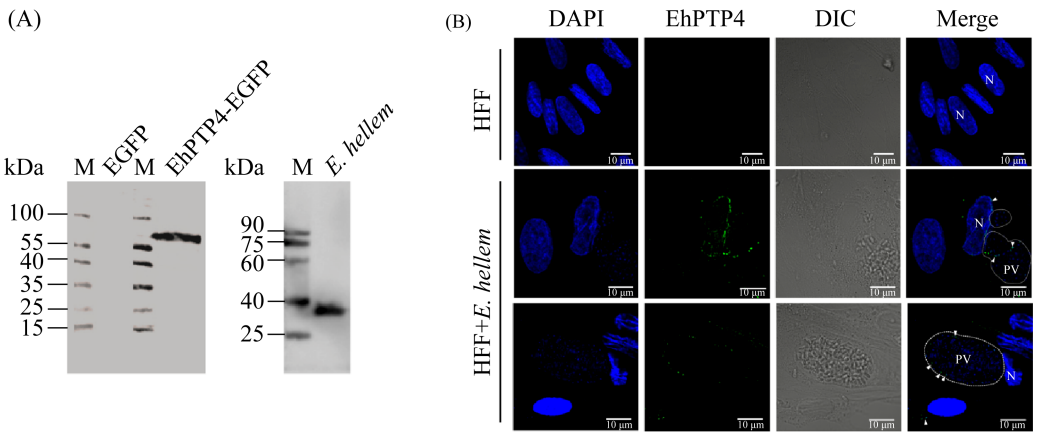

Figure 3 EhPTP4 was secreted from *E. hellem*-infected cells and translocated to the host nucleus. A: Western blotting analysis of EhPTP4 in the total proteins of EhPTP4-HA-EGFP-transfected cells and *E. hellem* spores; B: subcellular localization analysis of EhPTP4 in HFF cells infected with *E. hellem* at 48 hpi with anti-EhPTP4 (green). Nuclei were stained with DAPI (blue). N: nucleus; PV: parasitophorous vacuole; arrowhead: *E. hellem* meront. Scale bar, 10 μm.

2.3 The NLS is required for the translocation of EhPTP4 into the host nucleus

The NLS is an essential sequence for the targeting of some proteins to the nucleus[41–43]. The predicted NLS of EhPTP4 is probably necessary for nuclear localization. Therefore, EhPTP4 deletion mutants lacking the SP (EhPTP4$^{\Delta 1-16}$-HA-EGFP) and NLS (EhPTP4$^{\Delta 239-278}$-HA-EGFP) were constructed and expressed in HEK293 cells. As detected by IFA and Western blotting, EhPTP4-HA-EGFP and EhPTP4$^{\Delta 1-16}$-HA-EGFP were found in the nucleus (Figure 4, Figure 5), while EhPTP4$^{\Delta 239-278}$-HA-EGFP was present in only the cytoplasm (Figure 4), suggesting that the NLS is indispensable for the nuclear targeting of EhPTP4.

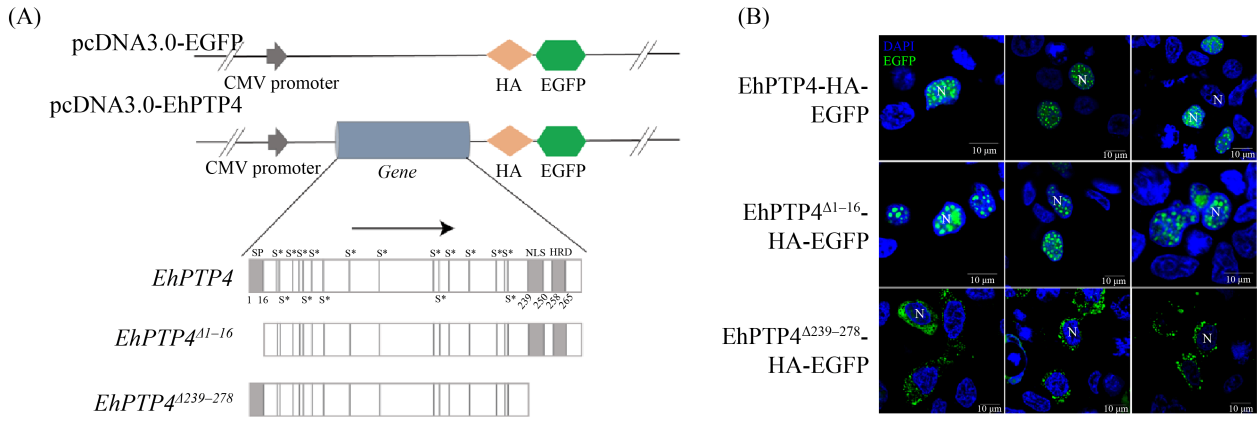

Figure 4 EhPTP4 translocation to the host nucleus depends on its nuclear localization signal sequence (NLS). A: constructions of EhPTP4 deletion mutants lacking the signal peptide (EhPTP4$^{\Delta 1-16}$-HA-EGFP) and NLS (EhPTP4$^{\Delta 239-278}$-HA-EGFP); B: the subcellular localization of EhPTP4-HA-EGFP, EhPTP4$^{\Delta 1-16}$-HA-EGFP and EhPTP4$^{\Delta 239-278}$-HA-EGFP (shown in green) in transfected HEK293 cells was observed using a laser scanning confocal microscope. Nuclei were stained with DAPI (blue). N: nucleus.

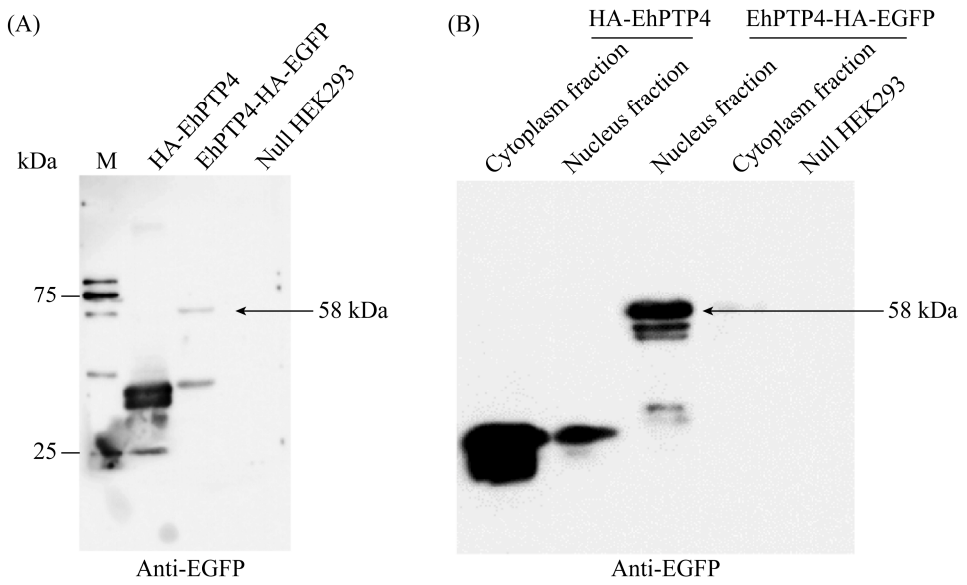

Figure 5 Western blotting analysis of the subcellular localization of EhPTP4 in transfected cells. A: the expression of EhPTP4-HA-EGFP and HA-EGFP in HEK293 were detected using anti-EGFP; B: the cytoplasm and nucleus of HEK293 cells transfected with EhPTP4-HA-EGFP and HA-EGFP were extracted, respectively. Each fraction was then detected for EhPTP4 using Western blotting with antibody against EGFP (anti-EGFP), respectively.

2.4 EhPTP4 promotes *E. hellem* proliferation

The expression profile of EhPTP4 was investigated in *E. hellem*-infected HEK293 cells using RT-qPCR. The results showed that EhPTP4 was expressed from 12 h post infection (hpi) and highly expressed from 36 hpi (Figure 6A), at which time the pathogens were at the meront stage. In HEK293 cells transfected with EhPTP4-HA-EGFP and HA-EGFP (control), the pathogen load was determined and indicated by the copy number of *E. hellem* *β*-tubulin. The overexpression of EhPTP4 significantly promoted the proliferation of *E. hellem* (Figure 6B). These results indicate that EhPTP4 is vital to pathogen growth.

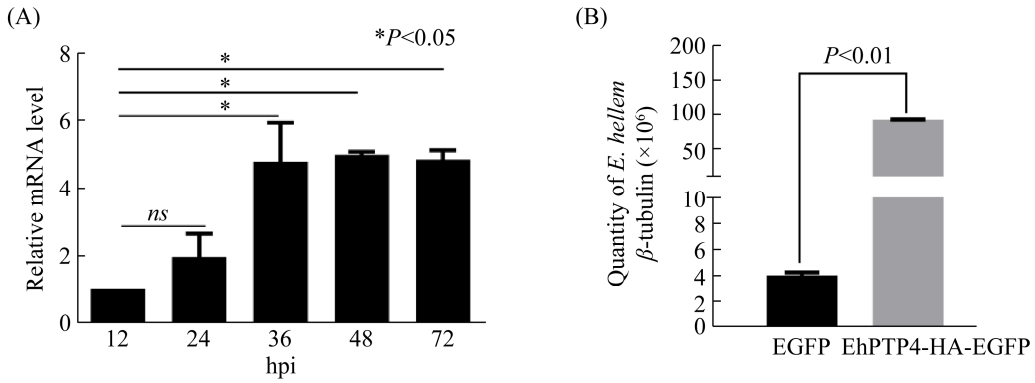

Figure 6 EhPTP4 overexpression promoted *E. hellem* proliferation. A: RT-qPCR analysis of EhPTP4 expression in *E. hellem*-infected RK13 cells from 12 to 72 hpi; B: qPCR analysis of *E. hellem* proliferation in EhPTP4-HA-EGFP- and HA-EGFP-transfected HEK293 cells. The transfected cells were infected with 1×10^6 *E. hellem* spores, and genomic DNA was then extracted. Pathogen load was determined as indicated by quantification of the *E. hellem* *β*-tubulin gene.

2.5 EhPTP4 activates the host ERAD response

Being secreted into the host nucleus, EhPTP4 probably regulates host gene expression. Thus, the gene expression levels of HEK293 cells transfected with EhPTP4-HA-EGFP and HA-EGFP (control) were determined with RNA-seq (Figure 7). As shown by the results, a total of 82 DEGs, including 41 upregulated genes and 41 downregulated genes, were detected (Table 1). KEGG enrichment analysis of the DEGs showed that the ERAD pathway was significantly enriched in the DEGs, including 4 upregulated genes encoding the protein disulfide isomerase family A member 4 (PDIA4), homocysteine-induced endoplasmic reticulum protein (HERP), heat shock protein family A (Hsp70) member 5 (HSPA5) and degradation in endoplasmic reticulum protein 3 (DERL3), which are vital components in ERAD (Figure 8). The upregulation of these genes was verified by RT-qPCR and Western blotting (Figure 9A, B). These results indicated that EhPTP4 is secreted into the host nucleus and dysregulates the ERAD pathway. ERAD is vital for protein homeostasis due to the degradation of misfolded and unfolded proteins in the ER. In this process, substrate proteins undergo recognition, dislocation, ubiquitination, translocation from the ER, and then degradation by the proteasome. Hence, we checked protein ubiquitination using Western blotting with anti-ubiquitin and found it to be increased in EhPTP4-transfected HEK293 cells (Figure 9C).

Protein disulfide isomerase (PDI), most abundant ER protein, is responsible for the formation, breakage and rearrangement of protein disulfide bonds and also helps with the binding of misfolded proteins for subsequent degradation[44–45]. PDIA4 is one of the largest members of the PDI

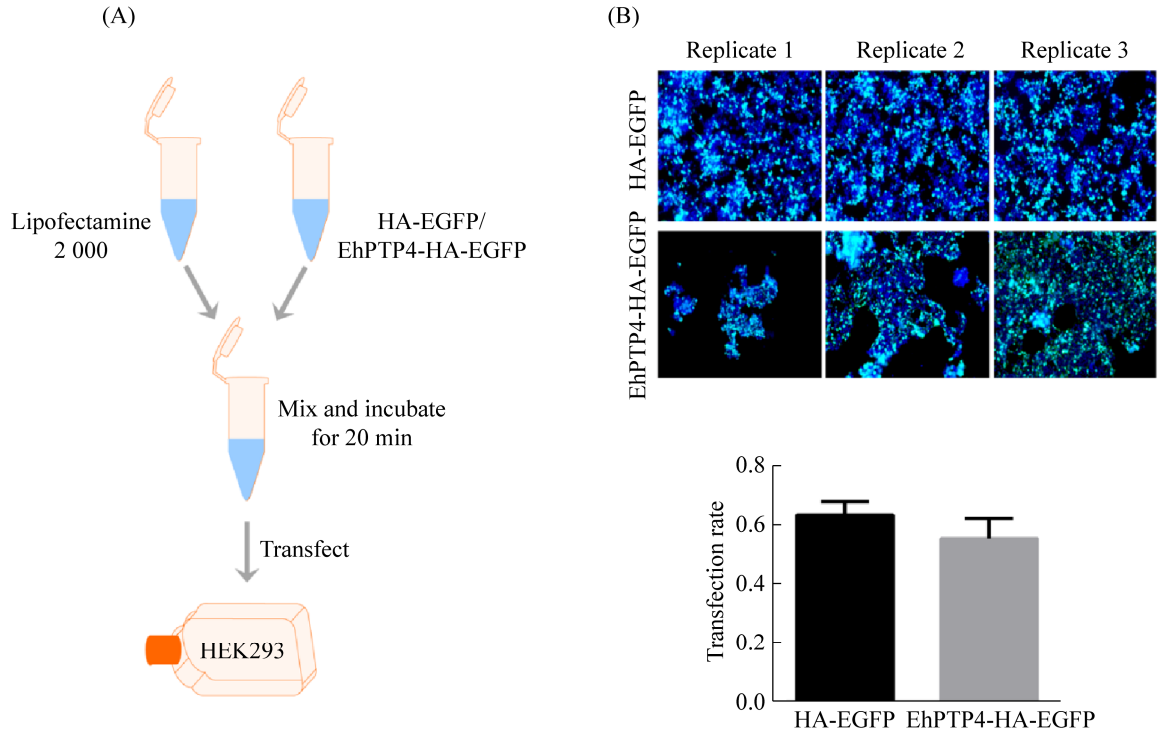

Figure 7 Preparation of RNA-seq samples. A: basic work flow for preparing of RNA-seq samples; B: HEK293 cells transfected with EhPTP4-HA-EGFP and HA-EGFP were observed using fluorescence microscope. The nucleus was stained with DAPI (blue): C: the transfection rates of HEK293 cells were statistically counted.

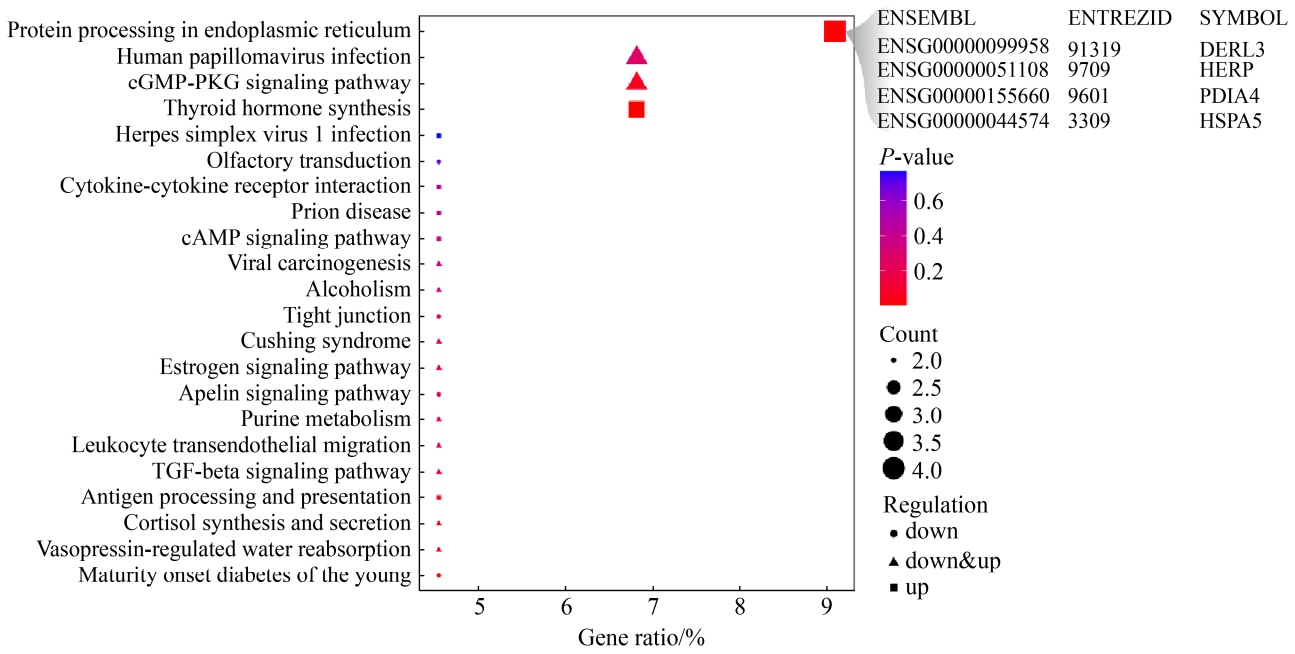

Figure 8 KEGG enrichment analysis of DEGs between HEK293 cells transfected with EhPTP4-HA-EGFP and HA-EGFP (control). DEGs were statistically determined from RNA-seq data from the transfected cells, and enrichment was assessed by mapping to the KEGG database. The results showed that genes involved in ERAD, including *PDIA4*, *HERP*, *HSPA5* and *DERL3*, were significantly enriched and upregulated.

family and acts as an inducer of the ER stress response by forming a chaperone complex with other proteins that binds unfolded protein substrates[46]. In EhPTP4-transfected HEK293 cells, both PDIA4 expression and protein ubiquitination were increased (Figure 9D and 9E), suggesting that EhPTP4 induces host ERAD via PDIA4. For further validation, HEK293 cells were transfected with PDIA4-FLAG, and protein ubiquitination was found to be increased upon the overexpression of PDIA4 (Figure 9E). Moreover, the knockdown of PDIA4 with RNAi suppressed HERP (Figure 9F), which is also an important component of the ERAD pathway, revealing that PDIA4 is a key factor hijacked by microsporidia to modulate host ERAD via EhPTP4.

2.6 HRD is essential for the regulatory function of EhPTP4

The HRD is conserved among homologs of EhPTP4 in *Encephalitozoon* species, indicating its important function for the protein. The HRD identified in vertebrate cyclin T1 was proven to markedly enhance the binding of positive transcription elongation factors to the C-terminal domain (CTD) of the RPB1 subunit of human RNA polymerase II, leading to the hyperphosphorylation of the CTD, which is essential for transcriptional elongation and mRNA processing[47]. This suggests that the localization of EhPTP4 in the host nucleus probably promotes host gene expression with an HRD-dependent mechanism. Therefore, we constructed EhPTP4$^{\Delta HRD}$-HA-EGFP-transfected HEK293 cells and checked the localization of the mutant and ERAD responses. EhPTP4$^{\Delta HRD}$-HA-EGFP showed markedly increased aggregation and formed significantly larger multimers in the nucleus than EhPTP4-HA-EGFP (Figure 10A). As determined by RT-qPCR, the expression levels of *PDIA4*, *HSPA5* and *HERP* were not significantly increased in EhPTP4$^{\Delta HRD}$-HA-EGFP-transfected cells (Figure 10B), indicating that the HRD is essential for EhPTP4 in activating host ERAD.

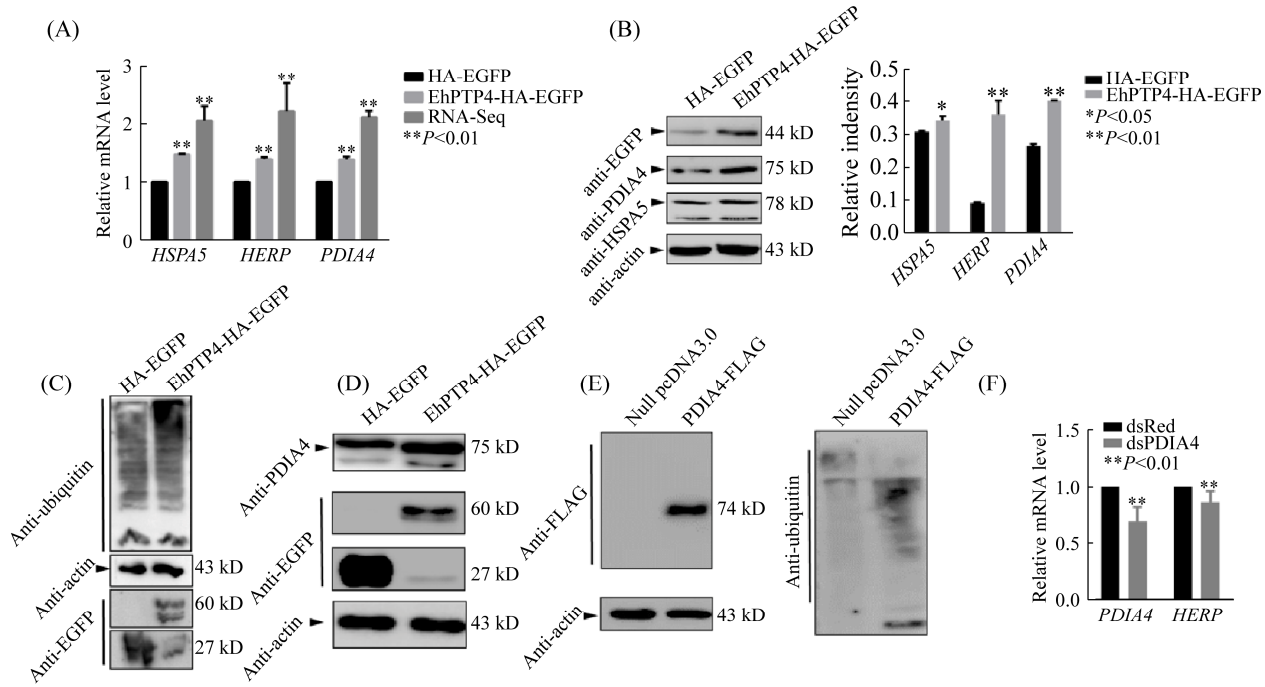

Figure 9 Verification of ERAD genes in HEK293 cells transfected with EhPTP4-HA-EGFP and HA-EGFP (control). A: the expression levels of *PDIA4*, *HERP* and *HSPA5*, which were increased in the RNA-seq data, were verified in the transfected cells using RT-qPCR; B: the expression levels of *PDIA4*, *HERP* and *HSPA5* in the transfected cells were verified using Western blotting with antibodies against each respective protein. Relative intensity was calculated with ImageJ software; C: protein ubiquitination was promoted in the transfected cells, as detected by Western blotting with an antibody against ubiquitin; D: the expression of *PDIA4* was detected in the transfected cells with antibodies against PDIA4, EGFP and actin (control); E: protein ubiquitination was promoted in PDIA4-FLAG-transfected HEK293 cells, as detected with antibodies against FLAG, ubiquitin and actin (control); F: the knockdown of *PDIA4* expression with RNAi suppressed the expression of *HERP*, which is a crucial component of the ERAD pathway.

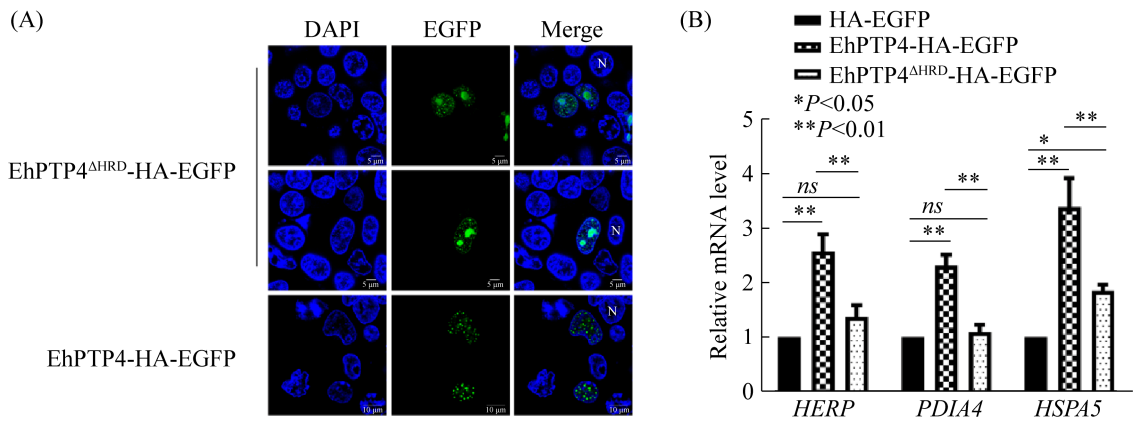

Figure 10 The HRD is essential for the regulatory function of EhPTP4. A: deletion of the HRD changed the localization of EhPTP4 in the nucleus. EhPTP4$^{\Delta HRD}$-HA-EGFP aggregated and formed a large multimer, whereas wild-type EhPTP4 was evenly distributed in the nucleus; B: the expression of *PDIA4*, *HERP* and *HSPA5* was dramatically decreased in EhPTP4$^{\Delta HRD}$-HA-EGFP-transfected HEK293 cells compared with cells expressing EhPTP4-HA-EGFP.

3 Discussion

ERAD is a principal quality control mechanism responsible for targeting native, misfolded and unfolded proteins for dislocation across the ER membrane and proteasomal degradation and plays vital roles in multiple cellular processes and functions[13,48]. Thus, by modulating ERAD, pathogens broadly affect host cell physiology. Studies have revealed that some intracellular pathogens, including viruses and bacteria, may dysregulate ERAD to enhance their chances of survival in the host[20,25,48]. For example, *Orientia tsutsugamushi*, an obligate intracellular bacterial pathogen that is auxotrophic for aromatic amino acids and histidine, can induce the host unfolded protein response (UPR) and promote ERAD to seize amino acids during early-stage growth[25]. This implies that microsporidia may obtain host amino acids with the same strategy because these pathogens lost the genes for the *de novo* synthesis of amino acids. On the other hand, hijacking ERAD for proteolytic suppression of immune proteins is also a strategy for certain pathogens[18]. The observed increases in protein ubiquitination and degradation (Figure 9C) suggest that the dysregulation of ERAD may help with the immune evasion of microsporidia. Therefore, determining the ubiquitylated proteins in transfected cells would help further understanding of the functions and regulation of EhPTP4. The UPR is a cytoprotective process that promotes ERAD[17,45,48–49], suggesting that EhPTP4 may promote the ERAD machinery by activating the UPR. Through our RNA-seq data, however, we found no DEGs involved in the UPR (Table 1), indicating that EhPTP4 probably dysregulates ERAD through other pathways.

Our study revealed that EhPTP4 is a secreted protein that targets the host nucleus. The EhPTP4 homolog of *E. cuniculi* was identified in late sporogonial stages by Brosson et al[50]. Moreover, Han et al revealed that EhPTP4 localizes on the tip of the polar tube and interacts with host transferrin receptor 1 (TfR1) on the membrane, demonstrating its important roles during infection[34]. Therefore, EhPTP4 is likely a multifunctional protein that plays roles in mediating invasion of the polar tube and targeting the host nucleus to modulate host gene expression. Both studies provide insights into the new functions of this secreted protein conserved in *Encephalitozoon* whose functions, however, require in-depth study. It is well known that intracellular pathogens can secretes effectors into host cytosol to mimic the activities of host proteins involved in ubiquitin pathway, for example, the E3 ubiquitin-protein ligase[51–52]. Localizing in host nucleus and without sequence features homologous to host ubiquitin system components, however, the EhPTP4 is interesting but poorly understood how to modulate host ubiquitinylation. Regarding the functions of EhPTP4 in the host nucleus, it is necessary to determine how EhPTP4 upregulates ERAD genes, what proteins it interacts with, what proteins are degraded, and what downstream pathways are regulated.

E. hellem was originally isolated from Madin-Darby canine kidney cells inoculated with corneal biopsies and conjunctival scrapings of three AIDS patients with keratoconjunctivitis[53]. *E. hellem* is mostly reported in birds, but, like many other *Encephalitozoon* species, can also infect human, monkeys, carnivore and rodents[54]. In research, the most common cell lines used for culturing *Encephalitozoon* are derived from epithelium or fibroblast tissues from rabbits, mice, monkeys, or humans, for example, the RK13 is often use to multiply these parasites, and the most successful cell modes to study the *Encephalitozoon* infections are HFF and HEK293[55]. Nonetheless, more cell types need to be investigated and utilized to better understand the infections of these parasites.

In summary, we first characterized a microsporidian protein targeting the host nucleus that upregulates the expression of genes involved in ERAD and subsequently increases protein ubiquitination (Figure 11). This work provides a

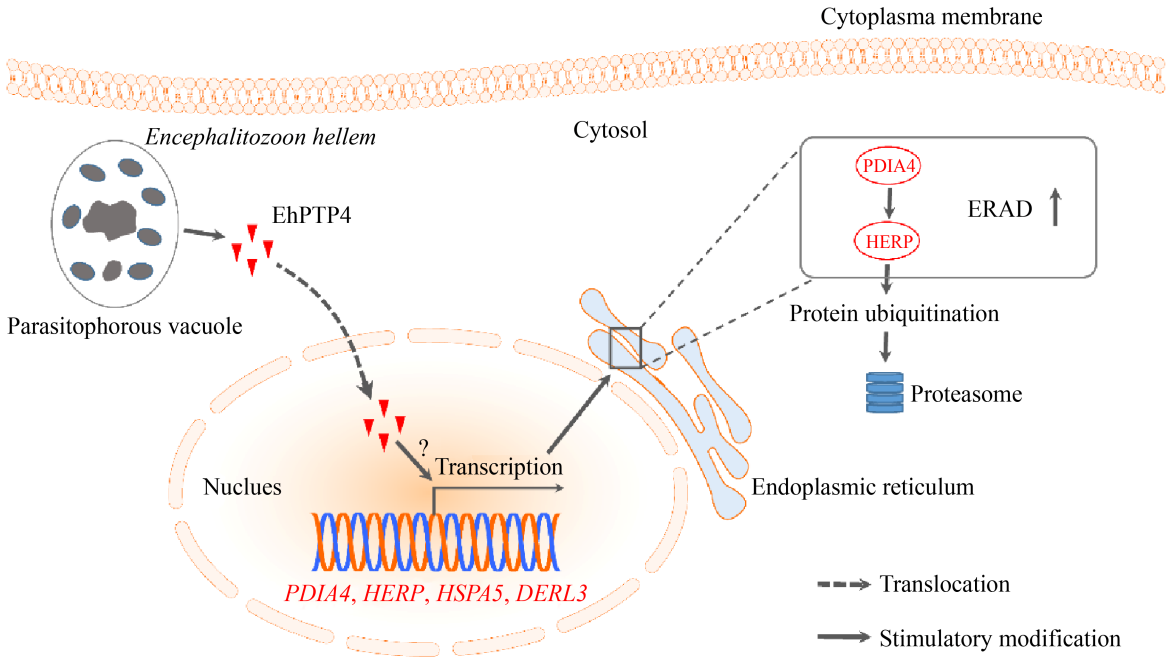

Figure 11 A schematic showing the model by which *E. hellem* secretes EhPTP4 to dysregulate host ERAD and protein ubiquitination. During *E. hellem* proliferation, EhPTP4 is secreted and translocated into the host nucleus. ERAD genes, including *PDIA4*, *HERP*, *HSPA5* and *DERL3*, are upregulated, resulting in the degradation of some proteins and facilitating *E. hellem* proliferation.

new viewpoint for an in-depth understanding of the mechanisms with which microsporidia and hosts interact.

Availability of data and materials

The raw data of our RNA-seq is deposited in NCBI SRA with accession number SAMN18995148 under BioProject PRJNA726947.

Competing interests: the authors declare that they have no competing interests.

Additional file: Table S1. Differentially expressed genes in HEK293 cells upon EhPTP4 transfection.

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