The Microsporidian *Encephalitozoon Hellem* Secretes A Host Nucleus-Targeting Protein (Ehhntp1) to Upregulate Endoplasmic Reticulum-Associated Degradation and Promote Protein Ubiquitination

Yinze Han
Southwest University
Hailong Gao
Southwest University
Bing Han
Shandong University
Jinzhi Xu
Southwest University
Jian Luo
Southwest University
Bin Yu
Southwest University
Chunxia Wang
Southwest University
Boyan Pei
Southwest University
Jiajing Chen
Southwest University
Tian LI (lit@swu.edu.cn)
Southwest University https://orcid.org/0000-0003-4362-0130
Zeyang Zhou
Southwest University

Research

Keywords: Microsporidia, Encephalitozoon hellem, EhHNTP1, Secreted protein, ERAD

Posted Date: March 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-319485/v1

Abstract

Background: Microsporidia, a group of obligate intracellular parasites that can infect humans and nearly all animals, have lost the pathways for de novo amino acid, lipid and nucleotide synthesis and instead evolved strategies to manipulate host metabolism and immunity. The endoplasmic reticulum (ER) is a vital organelle for producing and processing proteins and lipids and is often hijacked by intracellular pathogens. However, little is known about how microsporidia modulate host ER pathways. Herein, we identified a secreted protein of *Encephalitozoon hellem*, EhHNTP1, and characterized its subcellular localization and functions in host cells.

Methods: A polyclonal antibody against EhHNTP1 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 EhHNTP1 fused with HA-EGFP, and the impacts on pathogen proliferation, protein subcellular localization and sequence functions were assessed. RNA sequencing of EhHNTP1-transfected cells was conducted to identify differentially expressed genes (DEGs) and pathway responses by bioinformatics analysis mainly with R packages. The DEGs in the transfected cells were experimentally confirmed with RT-qPCR and Western blotting. The regulatory effects of candidate DEGs were analyzed via RNA interference and cell transfection, and the effects were determined with RT-qPCR and Western blotting.

Results: EhHNTP1 is secreted into the host nucleus, and its translocation depends on a nuclear localization signal sequence (NLS) at the C-terminus from amino acids 239 to 250. Transfection and overexpression of EhHNTP1 in HEK293 cells significantly promoted pathogen proliferation. RNA-seq of the transfected cells showed that genes involved in 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 in 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 a vital ERAD component exploited by EhHNTP1. Moreover, EhHNTP1$^{\Delta HRD}$, a deletion mutant lacking the histidine-rich domain (HRD) in the C-terminus, predominantly suppressed the upregulation of ERAD genes, indicating that the HRD is essential for EhHNTP1 functions.

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.

1. Introduction

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 1400 species 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 with AIDS, organ transplant recipients, aged individuals and children [4, 5, 6, 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, 9, 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, 14, 15, 16, 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, 19, 20, 21], which generally includes the following three steps. First, proteolytic secretory and membrane proteins involved in the immune response, including MHC class 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, 27, 28, 29, 30]. For example, pathogen proteins secreted into the host nucleus play important roles in regulating immunity, proliferation and apoptosis [31, 32, 33]. Here, for the first time, we report that a secreted protein of *E. hellem*, EhHNTP1, is delivered into the host nucleus and disturbs the host ERAD pathway and protein degradation.

2. Materials And Methods

2.1. Cell cultures

RK13 (rabbit kidney) cells (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, FuDna 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.

2.2. Preparation of *E. hellem* spores

Confluent 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 1000 rpm for 10 min, and washed three times with sterile distilled water by centrifugation at 1000 rpm for 5 min. The purified spores were then counted and stored in sterile distilled water at 4°C.

2.3. Preparation of recombinant EhHNTP1 and antiserum

The coding sequence (CDS) of EhHNTP1 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-*EhHNTP1*-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. EhHNTP1 was then induced for 20 h at 16°C by the addition of 0.5 mM isopropyl-D-thiogalactoside to the culture medium. Subsequently, the bacterial cells were harvested by centrifugation for 15 min at 5000 rpm, resuspended in 20 mL of buffer A (100 mM sodium chloride, 10 mM Tris-HCl, 8 M urea, pH 8.0), lysed by sonication on ice and centrifuged at 1000 ×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 mM sodium chloride, 10 mM Tris-HCl, 8 M urea, 250 mM imidazole). Polyclonal antiserum against EhHNTP1 (anti-EhHNTP1) was prepared by intradermally inoculating mice with purified rEhHNTP1 (100 μg/mouse).

HFF cells infected with *E. hellem* for five days 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-EhHNTP1 diluted 1:150 in blocking buffer for 2 hours 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 ECL Western blot detection kit (Thermo Fisher Scientific) and imaged with an Azure Biosystems C300 imaging system.

2.4. EhHNTP1 transfection of HEK293 cells

The CDSs for EhHNTP1, EhHNTP1$^{\Delta 1-16}$, EhHNTP1$^{\Delta 239-278}$ and EhHNTP1$^{\Delta HRD}$ were amplified from *E. hellem* gDNA with specific primers (Supplementary 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 hours, the transfected cells were harvested to analyze protein expression and subcellular localization.

2.5. 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-EhHNTP1 (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 1:1000 in PBS) for 1 hour. 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.

2.6. RNA-seq analysis of EhHNTP1-transfected HEK293 cells

The transcriptomic responses of HEK293 cells transfected with HA-EGFP and EhHNTP1-HA-EGFP were investigated with RNA-seq, with three replicates of each group. RNA samples were extracted and assessed using a Nanodrop 2100 and Agilent 2000 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 [34] and assembled with StringTie [35]. Fragments per kilobase per million (FPKM) values were calculated and used to compare gene expression levels. Differentially expressed genes (DEGs) between the EhHNTP1-HA-EGFP- and HA-EGFP-transfected cells were analyzed using DEseq2 [36]. The DEGs were filtered with the thresholds of a P-value < 0.01 and a fold change ≥ 2, and DEGs found in at least in two replicates were considered credible. KEGG enrichment analysis of the DEGs was performed using clusterProfiler [37], which is an R package for comparing biological themes among gene clusters. Pathways enriched in at least two genes and a P-value < 0.05 were considered significant.

2.7. Real time quantitative PCR (RT-qPCR)

Total RNA was extracted from RK13 cells transfected with *EhHNTP1* 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 Supplementary Table 2. The transcription level was elevated by the $2^{-\Delta\Delta t}$ 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-value < 0.05 were considered significant.

To assess the growth of *E. hellem* in cell culture, quantitative PCR was performed with *β-tubulin* primers (F: TGAAGATGAGCAATCCAGGGTA, R: TAGCAATCAGGGGTGCAAAT). The gDNA of EhHNTP1- 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.

2.8. Western blot analysis of protein expression and ubiquitination

Proteins of HEK293 cells transfected with HA-EGFP and EhHNTP1-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.

3. Results

3.1. Sequence features of EhHNTP1

EhHNTP1, whose locus name is EHEL_071080 (GenBank accession: AFM98634.1), was annotated as a hypothetical protein and is composed of 278 amino acids. Predictions with SignalP 5.0 [38] and NLS Mapper [39] showed that EhHNTP1 contains a signal peptide (SP) from amino acids 1 to 16 and a nuclear localization signal sequence (NLS) from amino acids 239 to 250, respectively, indicating that EhHNTP1 is a secreted protein that probably targets the host nucleus (Fig. 1). In addition, the C-terminal region of EhHNTP1 encodes a histidine-rich domain (HRD) from amino acids 258 to 266, which was suggested to be functional in transcriptional regulation in the nucleus. Multiple sequence alignment analysis demonstrated that EhHNTP1 is highly conserved with homologs among *Encephalitozoon* species (Fig. 1), suggesting important functions of this protein in *Encephalitozoon*.

3.2. The expression profile and subcellular localization of EhHNTP1

To characterize the subcellular localization of EhHNTP1 in infected cells, recombinant EhHNTP1 was expressed in *E. coli* and purified for the immunization of mice and production of a mouse polyclonal antibody (anti-EhHNTP1) (Fig. 2a), which was then used to examine the localization of EhHNTP1-infected cells using an IFA. EhHNTP1 was found to translocate into the nuclei of infected cells (Fig. 2b), suggesting that EhHNTP1 is a secreted protein targeting the host nucleus. Therefore, we renamed it a host nucleus-targeting protein (EhHNTP1). Moreover, in parasitophorous vacuoles (PVs), EhHNTP1 was detected in only meronts but not spores, indicating that this protein is mainly synthesized and secreted by meronts and likely plays important roles during proliferation (Fig. 2b).

3.3. The NLS is required for the translocation of EhHNTP1 into the host nucleus

The NLS is an essential sequence for the targeting of some proteins to the nucleus [40, 41, 42]. The predicted NLS of EhHNTP1 is probably necessary for nuclear localization. Therefore, EhHNTP1 deletion mutants lacking the SP (EhHNTP1$^{\Delta 1-16}$-HA-EGFP) and NLS (EhHNTP1$^{\Delta 239-278}$-HA-EGFP) were constructed and expressed in HEK293 cells. As detected by IFA and Western blotting, EhHNTP1-HA-EGFP and EhHNTP1$^{\Delta 1-16}$-HA-EGFP were found in the nucleus (Fig. 3, Supplementary Fig. 2), while EhHNTP1$^{\Delta 239-278}$-HA-EGFP was present in only the cytoplasm (Fig. 3), suggesting that the NLS is indispensable for the nuclear targeting of EhHNTP1.

3.4. EhHNTP1 promotes *E. hellem* proliferation

The expression profile of EhHNTP1 was investigated in *E. hellem*-infected HEK293 cells using RT-qPCR. The results showed that EhHNTP1 was expressed from 12 hours postinfection (hpi) and highly expressed from 36 hpi (Fig. 4a), at which time the pathogens were at the meront stage. In HEK293 cells transfected with EhHNTP1-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 EhHNTP1 significantly promoted the proliferation of *E. hellem* (Fig. 4b). These results indicate that EhHNTP1 is vital to pathogen growth.

3.5. EhHNTP1 activates the host ERAD response

Being secreted into the host nucleus, EhHNTP1 probably regulates host gene expression. Thus, the gene expression levels of HEK293 cells transfected with EhHNTP1-HA-EGFP and HA-EGFP (control) were determined with RNA-seq (Supplementary Fig. 3). As shown by the results, a total of 82 DEGs, including 41 upregulated genes and 41 downregulated genes, were detected (Supplementary Table 3). KEGG enrichment analysis of the DEGs showed that the ERAD pathway was significantly enriched in the DEGs, including four upregulated genes, *PDIA4*, *HERP*, *HSPA5* and *DERL3*, which are vital components in ERAD (Fig. 5). The upregulation of these genes was verified by RT-qPCR and Western blotting (Fig. 6a and 6b). These results indicated that EhHNTP1 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 EhHNTP1-transfected HEK293 cells (Fig. 6c).

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 [43, 44]. PDIA4 is one of the largest members of the PDI family and acts as an inducer of the ER stress response by forming a chaperone complex with other proteins that binds

unfolded protein substrates [45]. In EhHNTP1-transfected HEK293 cells, both PDIA4 expression and protein ubiquitination were increased (Fig. 6d and 6e), suggesting that EhHNTP1 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 (Fig. 6e). Moreover, the knockdown of PDIA4 with RNAi suppressed HERP (Fig. 6f), 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 EhHNTP1.

3.6. HRD is essential for the regulatory function of EhHNTP1

The HRD is conserved among homologs of EhHNTP1 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 [46]. This suggests that the localization of EhHNTP1 in the host nucleus probably promotes host gene expression with an HRD-dependent mechanism. Therefore, we constructed EhHNTP1$^{\Delta HRD}$-HA-EGFP-transfected HEK293 cells and checked the localization of the mutant and ERAD responses. EhHNTP1$^{\Delta HRD}$-HA-EGFP showed markedly increased aggregation and formed significantly larger multimers in the nucleus than EhHNTP1-HA-EGFP (Fig. 7a). As determined by RT-qPCR, the expression levels of *PDIA4, HSPA5* and *HERP* were significantly decreased in EhHNTP1$^{\Delta HRD}$-HA-EGFP-transfected cells (Fig. 7b), indicating that the HRD is essential for EhHNTP1 in activating host ERAD.

4. 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, 47]. 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, 47]. 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 (Fig. 6c) 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 EhHNTP1. The UPR is a cytoprotective process that promotes ERAD [17, 44, 47, 48], suggesting that EhHNTP1 may promote the ERAD machinery by activating the UPR. Through

our RNA-seq data, however, we found no DEGs involved in the UPR (Supplementary Table 3), indicating that EhHNTP1 probably dysregulates ERAD through other pathways.

Our study revealed that EhHNTP1 is a secreted protein that targets the host nucleus. A previous study, however, showed that this protein 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 [49]. Therefore, EhHNTP1 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. Regarding the functions of EhHNTP1 in the host nucleus, it is necessary to determine how EhHNTP1 upregulates ERAD genes, what proteins it interacts with, what proteins are degraded, and what downstream pathways are regulated.

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 (Fig. 8). This work provides a new viewpoint for an in-depth understanding of the mechanisms with which microsporidia and hosts interact.

Declarations

Acknowledgments

The authors would like to thank all the authors who published the manuscripts included in this work.

Authors' contributions

TL, ZYZ and BH conceived and designed the experiments; YZH, BH, JZX, JL, BY, CXW, BYP and JJC contributed experimental reagents, materials and analytical tools; YZH and HLG performed the experiments; YZH and TL analyzed the data; TL and YZH developed the manuscript; and TL, BH, YZH and ZYZ reviewed the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the National Natural Science Foundation of China [31772678, 31770159 and 31472151] and the Natural Science Foundation of Chongqing, China (cstc2019yszx-jcyjX0010).

Availability of data and materials

Data are available in Additional file 1: Tables S1-S2 and Additional file 2: Table S3.

Ethics approval and consent to participate

This study was conducted in the Laboratory Animal Facility under supervision and assessment by the Laboratory Animal Ethics Committee of Southwest University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Fayer R, Santin-Duran M. Epidemiology of Microsporidia in Human Infections. Microsporidia; 2014. p. 135-64.
2. Han B, Weiss LM. Microsporidia: Obligate Intracellular Pathogens Within the Fungal Kingdom. Microbiol Spectr. 2017;5 2; doi: 10.1128/microbiolspec.FUNK-0018-2016. https://www.ncbi.nlm.nih.gov/pubmed/28944750.
3. Han Y, Gao H, Xu J, Luo J, Han B, Bao J, et al. Innate and Adaptive Immune Responses Against Microsporidia Infection in Mammals. Front Microbiol. 2020;11:1468; doi: 10.3389/fmicb.2020.01468. https://www.ncbi.nlm.nih.gov/pubmed/32670257.
4. Wang Z-D, Liu Q, Liu H-H, Li S, Zhang L, Zhao Y-K, et al. Prevalence of Cryptosporidium, microsporidia and Isospora infection in HIV-infected people: a global systematic review and meta-analysis. Parasites & vectors. 2018;11 1:28-; doi: 10.1186/s13071-017-2558-x. https://pubmed.ncbi.nlm.nih.gov/29316950 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5759777/.
5. Ghoyounchi R, Mahami-Oskouei M, Rezamand A, Spotin A, Aminisani N, Nami S, et al. Molecular Phylodiagnosis of *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* in Children with Cancer: Microsporidia in Malignancies as an Emerging Opportunistic Infection. Acta Parasitol. 2019;64 1:103-11; doi: 10.2478/s11686-018-00012-w. https://www.ncbi.nlm.nih.gov/pubmed/30644064.
6. Abu-Akkada SS, El Kerdany ED, Mady RF, Diab RG, Khedr GA, Ashmawy KI, et al. *Encephalitozoon cuniculi* infection among immunocompromised and immunocompetent humans in Egypt. Iran J Parasitol. 2015;10 4:561-70. https://www.ncbi.nlm.nih.gov/pubmed/26811722.
7. Kicia M, Wesolowska M, Kopacz Z, Jakuszko K, Sak B, Kvetonova D, et al. Prevalence and molecular characteristics of urinary and intestinal microsporidia infections in renal transplant recipients. Clin Microbiol Infect. 2016;22 5:462 e5-9; doi: 10.1016/j.cmi.2016.01.014. https://www.ncbi.nlm.nih.gov/pubmed/26827672.
8. Corradi N, Pombert JF, Farinelli L, Didier ES, Keeling PJ. The complete sequence of the smallest known nuclear genome from the microsporidian *Encephalitozoon intestinalis*. Nat Commun. 2010;1:77; doi: 10.1038/ncomms1082. https://www.ncbi.nlm.nih.gov/pubmed/20865802.

9. Nakjang S, Williams TA, Heinz E, Watson AK, Foster PG, Sendra KM, et al. Reduction and expansion in microsporidian genome evolution: new insights from comparative genomics. Genome Biol Evol. 2013;5 12:2285-303; doi: 10.1093/gbe/evt184. https://www.ncbi.nlm.nih.gov/pubmed/24259309.
10. Heinz E, Williams TA, Nakjang S, Noel CJ, Swan DC, Goldberg AV, et al. The genome of the obligate intracellular parasite *Trachipleistophora hominis*: new insights into microsporidian genome dynamics and reductive evolution. PLoS Pathog. 2012;8 10:e1002979; doi: 10.1371/journal.ppat.1002979. https://www.ncbi.nlm.nih.gov/pubmed/23133373.
11. Bernal CE, Zorro MM, Sierra J, Gilchrist K, Botero JH, Baena A, et al. *Encephalitozoon intestinalis* Inhibits Dendritic Cell Differentiation through an IL-6-Dependent Mechanism. Front Cell Infect Microbiol. 2016;6:4; doi: 10.3389/fcimb.2016.00004. https://www.ncbi.nlm.nih.gov/pubmed/26870700.
12. Phillips MJ, Voeltz GK. Structure and function of ER membrane contact sites with other organelles. Nat Rev Mol Cell Biol. 2016;17 2:69-82; doi: 10.1038/nrm.2015.8. https://www.ncbi.nlm.nih.gov/pubmed/26627931.
13. Qi L, Tsai B, Arvan P. New Insights into the Physiological Role of Endoplasmic Reticulum-Associated Degradation. Trends Cell Biol. 2017;27 6:430-40; doi: 10.1016/j.tcb.2016.12.002. https://www.ncbi.nlm.nih.gov/pubmed/28131647.
14. Wu X, Rapoport TA. Mechanistic insights into ER-associated protein degradation. Curr Opin Cell Biol. 2018;53:22-8; doi: 10.1016/j.ceb.2018.04.004. https://www.ncbi.nlm.nih.gov/pubmed/29719269.
15. Stevenson J, Huang EY, Olzmann JA. Endoplasmic Reticulum-Associated Degradation and Lipid Homeostasis. Annu Rev Nutr. 2016;36:511-42; doi: 10.1146/annurev-nutr-071715-051030. https://www.ncbi.nlm.nih.gov/pubmed/27296502.
16. Olzmann JA, Kopito RR, Christianson JC. The mammalian endoplasmic reticulum-associated degradation system. Cold Spring Harb Perspect Biol. 2013;5 9; doi: 10.1101/cshperspect.a013185. https://www.ncbi.nlm.nih.gov/pubmed/23232094.
17. Hwang J, Qi L. Quality Control in the Endoplasmic Reticulum: Crosstalk between ERAD and UPR pathways. Trends Biochem Sci. 2018;43 8:593-605; doi: 10.1016/j.tibs.2018.06.005. https://www.ncbi.nlm.nih.gov/pubmed/30056836.
18. Morito D, Nagata K. Pathogenic Hijacking of ER-Associated Degradation: Is ERAD Flexible? Mol Cell. 2015;59 3:335-44; doi: 10.1016/j.molcel.2015.06.010. https://www.ncbi.nlm.nih.gov/pubmed/26253026.
19. Bennett SM, Jiang M, Imperiale MJ. Role of cell-type-specific endoplasmic reticulum-associated degradation in polyomavirus trafficking. J Virol. 2013;87 16:8843-52; doi: 10.1128/JVI.00664-13. https://www.ncbi.nlm.nih.gov/pubmed/23740996.
20. Dupzyk A, Tsai B. How Polyomaviruses Exploit the ERAD Machinery to Cause Infection. Viruses. 2016;8 9; doi: 10.3390/v8090242. https://www.ncbi.nlm.nih.gov/pubmed/27589785.
21. Tang Y, Zheng M, An R, Chen L, Gong L, Cai H, et al. Proteasomal degradation of T. gondii ROP18 requires Derlin2. Acta Trop. 2017;174:106-13; doi: 10.1016/j.actatropica.2017.06.027.

https://www.ncbi.nlm.nih.gov/pubmed/28669563.

22. Magadan JG, Perez-Victoria FJ, Sougrat R, Ye Y, Strebel K, Bonifacino JS. Multilayered mechanism of CD4 downregulation by HIV-1 Vpu involving distinct ER retention and ERAD targeting steps. PLoS Pathog. 2010;6 4:e1000869; doi: 10.1371/journal.ppat.1000869. https://www.ncbi.nlm.nih.gov/pubmed/20442859.
23. van den Boomen DJ, Lehner PJ. Identifying the ERAD ubiquitin E3 ligases for viral and cellular targeting of MHC class I. Mol Immunol. 2015;68 2 Pt A:106-11; doi: 10.1016/j.molimm.2015.07.005. https://www.ncbi.nlm.nih.gov/pubmed/26210183.
24. Geiger R, Andritschke D, Friebe S, Herzog F, Luisoni S, Heger T, et al. BAP31 and BiP are essential for dislocation of SV40 from the endoplasmic reticulum to the cytosol. Nat Cell Biol. 2011;13 11:1305-14; doi: 10.1038/ncb2339. https://www.ncbi.nlm.nih.gov/pubmed/21947079.
25. Rodino KG, VieBrock L, Evans SM, Ge H, Richards AL, Carlyon JA. Orientia tsutsugamushi Modulates Endoplasmic Reticulum-Associated Degradation To Benefit Its Growth. Infect Immun. 2018;86 1; doi: 10.1128/IAI.00596-17. https://www.ncbi.nlm.nih.gov/pubmed/29109174.
26. Asrat S, de Jesus DA, Hempstead AD, Ramabhadran V, Isberg RR. Bacterial pathogen manipulation of host membrane trafficking. Annu Rev Cell Dev Biol. 2014;30:79-109; doi: 10.1146/annurev-cellbio-100913-013439. https://www.ncbi.nlm.nih.gov/pubmed/25103867.
27. Senderskiy IV, Timofeev SA, Seliverstova EV, Pavlova OA, Dolgikh VV. Secretion of *Antonospora* (*Paranosema*) *locustae* proteins into infected cells suggests an active role of microsporidia in the control of host programs and metabolic processes. PLoS One. 2014;9 4:e93585; doi: 10.1371/journal.pone.0093585. https://www.ncbi.nlm.nih.gov/pubmed/24705470.
28. Escoll P, Mondino S, Rolando M, Buchrieser C. Targeting of host organelles by pathogenic bacteria: a sophisticated subversion strategy. Nat Rev Microbiol. 2016;14 1:5-19; doi: 10.1038/nrmicro.2015.1. https://www.ncbi.nlm.nih.gov/pubmed/26594043.
29. Von Dwingelo J, Chung IYW, Price CT, Li L, Jones S, Cygler M, et al. Interaction of the Ankyrin H Core Effector of Legionella with the Host LARP7 Component of the 7SK snRNP Complex. mBio. 2019;10 4; doi: 10.1128/mBio.01942-19. https://www.ncbi.nlm.nih.gov/pubmed/31455655.
30. Bao J, Liu L, An Y, Ran M, Ni W, Chen J, et al. *Nosema bombycis* suppresses host hemolymph melanization through secreted serpin 6 inhibiting the prophenoloxidase activation cascade. J Invertebr Pathol. 2019;168:107260; doi: 10.1016/j.jip.2019.107260. https://www.ncbi.nlm.nih.gov/pubmed/31622597.
31. Panas MW, Naor A, Cygan AM, Boothroyd JC. Toxoplasma Controls Host Cyclin E Expression through the Use of a Novel MYR1-Dependent Effector Protein, HCE1. MBio. 2019;10 2; doi: 10.1128/mBio.00674-19. https://www.ncbi.nlm.nih.gov/pubmed/31040242.
32. Wang W, Chen J, Liao B, Xia L, Hou S, Wang Z, et al. Identification and functional characterization of Histone-like DNA-binding protein in *Nocardia seriolae* (NsHLP) involved in cell apoptosis. J Fish Dis. 2019;42 5:657-66; doi: 10.1111/jfd.12962. https://www.ncbi.nlm.nih.gov/pubmed/30854666.

33. Gay G, Braun L, Brenier-Pinchart MP, Vollaire J, Josserand V, Bertini RL, et al. *Toxoplasma gondii* TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-gamma-mediated host defenses. J Exp Med. 2016;213 9:1779-98; doi: 10.1084/jem.20160340. https://www.ncbi.nlm.nih.gov/pubmed/27503074.
34. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015;12 4:357-60; doi: 10.1038/nmeth.3317. https://www.ncbi.nlm.nih.gov/pubmed/25751142.
35. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol. 2015;33 3:290-5; doi: 10.1038/nbt.3122. https://www.ncbi.nlm.nih.gov/pubmed/25690850.
36. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15 12:550; doi: 10.1186/s13059-014-0550-8. https://www.ncbi.nlm.nih.gov/pubmed/25516281.
37. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16 5:284-7; doi: 10.1089/omi.2011.0118. https://www.ncbi.nlm.nih.gov/pubmed/22455463.
38. Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol. 2019;37 4:420-3; doi: 10.1038/s41587-019-0036-z.
39. Kosugi S, Hasebe M, Tomita M, Yanagawa H. Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. Proc Natl Acad Sci U S A. 2009;106 25:10171-6; doi: 10.1073/pnas.0900604106. https://www.ncbi.nlm.nih.gov/pubmed/19520826.
40. Lobingier BT, Huttenhain R, Eichel K, Miller KB, Ting AY, von Zastrow M, et al. An Approach to Spatiotemporally Resolve Protein Interaction Networks in Living Cells. Cell. 2017;169 2:350-60 e12; doi: 10.1016/j.cell.2017.03.022. https://www.ncbi.nlm.nih.gov/pubmed/28388416.
41. Pemberton LF, Paschal BM. Mechanisms of Receptor-Mediated Nuclear Import and Nuclear Export. Traffic. 2005;6 3:187-98; doi: 10.1111/j.1600-0854.2005.00270.x. https://doi.org/10.1111/j.1600-0854.2005.00270.x.
42. Wu WW, Sun YH, Pante N. Nuclear import of influenza A viral ribonucleoprotein complexes is mediated by two nuclear localization sequences on viral nucleoprotein. Virol J. 2007;4:49; doi: 10.1186/1743-422X-4-49. https://www.ncbi.nlm.nih.gov/pubmed/17547769.
43. Wang Z, Zhang H, Cheng Q. PDIA4: The basic characteristics, functions and its potential connection with cancer. Biomed Pharmacother. 2020;122:109688; doi: 10.1016/j.biopha.2019.109688. https://www.ncbi.nlm.nih.gov/pubmed/31794946.
44. Perri ER, Thomas CJ, Parakh S, Spencer DM, Atkin JD. The Unfolded Protein Response and the Role of Protein Disulfide Isomerase in Neurodegeneration. Front Cell Dev Biol. 2015;3:80; doi: 10.3389/fcell.2015.00080. https://www.ncbi.nlm.nih.gov/pubmed/26779479.

45. Meunier L, Usherwood YK, Chung KT, Hendershot LM. A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. Mol Biol Cell. 2002;13 12:4456-69; doi: 10.1091/mbc.e02-05-0311. https://www.ncbi.nlm.nih.gov/pubmed/12475965.

46. Lu H, Yu D, Hansen AS, Ganguly S, Liu R, Heckert A, et al. Phase-separation mechanism for C-terminal hyperphosphorylation of RNA polymerase II. Nature. 2018;558 7709:318-23; doi: 10.1038/s41586-018-0174-3. https://www.ncbi.nlm.nih.gov/pubmed/29849146.

47. Merulla J, Fasana E, Solda T, Molinari M. Specificity and regulation of the endoplasmic reticulum-associated degradation machinery. Traffic. 2013;14 7:767-77; doi: 10.1111/tra.12068. https://www.ncbi.nlm.nih.gov/pubmed/23521725.

48. Celli J, Tsolis RM. Bacteria, the endoplasmic reticulum and the unfolded protein response: friends or foes? Nat Rev Microbiol. 2015;13 2:71-82; doi: 10.1038/nrmicro3393. https://www.ncbi.nlm.nih.gov/pubmed/25534809.

49. Han B, Polonais V, Sugi T, Yakubu R, Takvorian PM, Cali A, et al. The role of microsporidian polar tube protein 4 (PTP4) in host cell infection. PLoS Pathog. 2017;13 4:e1006341; doi: 10.1371/journal.ppat.1006341. https://www.ncbi.nlm.nih.gov/pubmed/28426751.

Figures

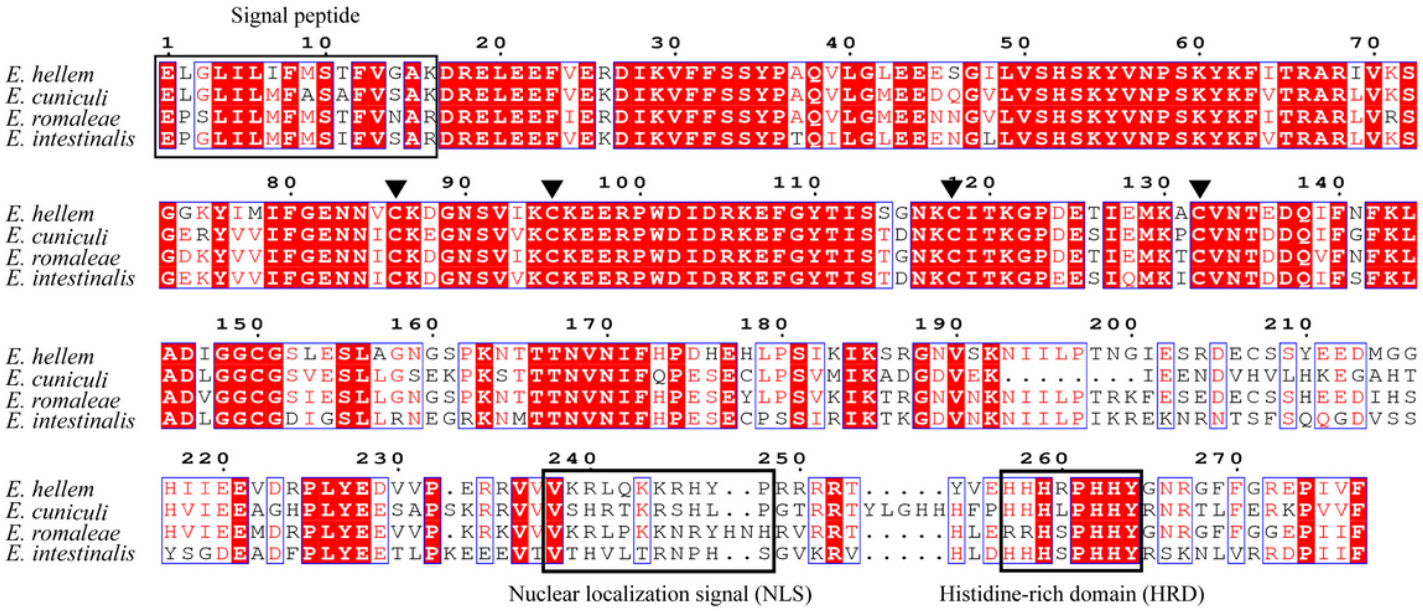

Figure 1

Sequence features and multiple alignment of EhHNTP1. EhHNTP1 encodes 278 amino acids containing a signal peptide from amino acids 1 to 16, a nuclear localization signal sequence (NLS) from amino acids 239 to 250, and a histidine-rich domain (HRD) from amino acids 258 to 266.

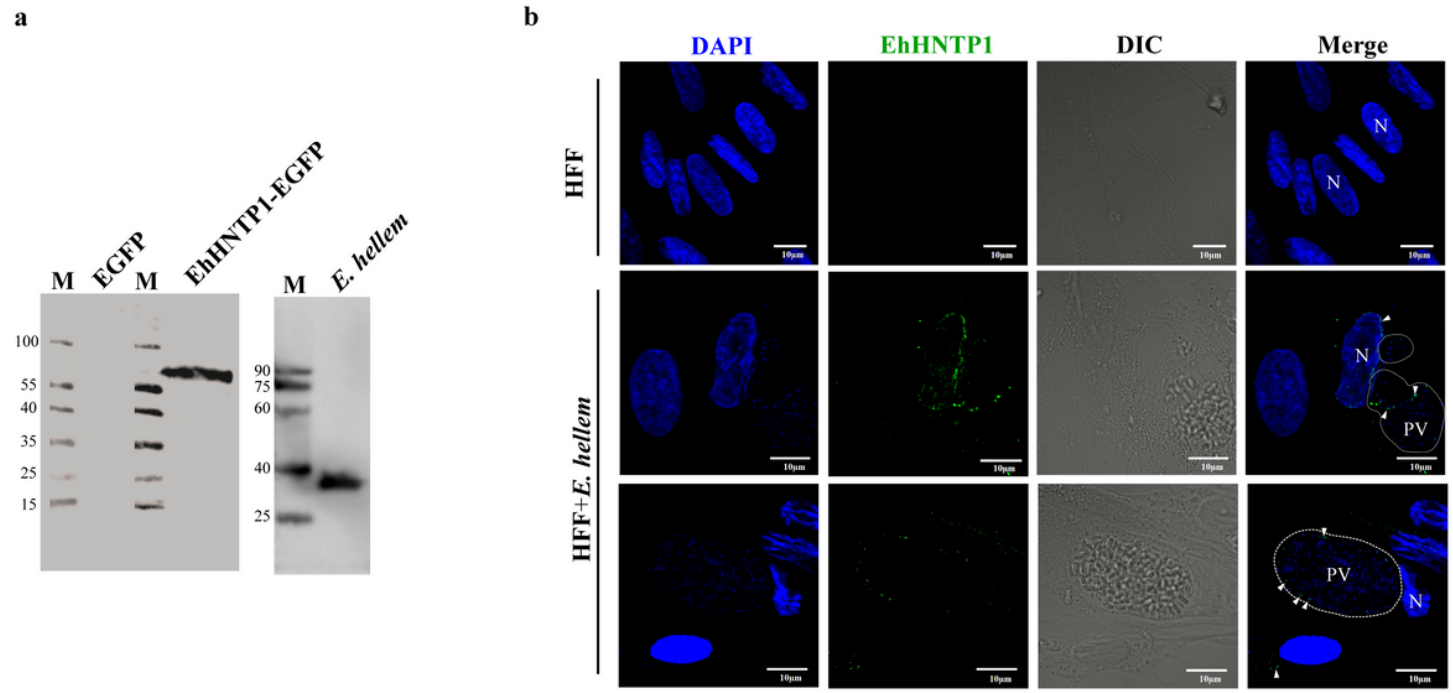

Figure 2

EhHNTP1 was secreted from E. hellem-infected cells and translocated to the host nucleus. (a) Western blot analysis of EhHNTP1 in the total proteins of EhHNTP1-HA-EGFP-transfected cells and E. hellem spores. (b) Subcellular localization analysis of EhHNTP1 in HFF cells infected with E. hellem at 48 hpi with anti-EhHNTP1 (green). Nuclei were stained with DAPI (blue). N, nucleus. PV, parasitophorous vacuole. Arrowhead, E. hellem meront. Scale bar, 10 μM.

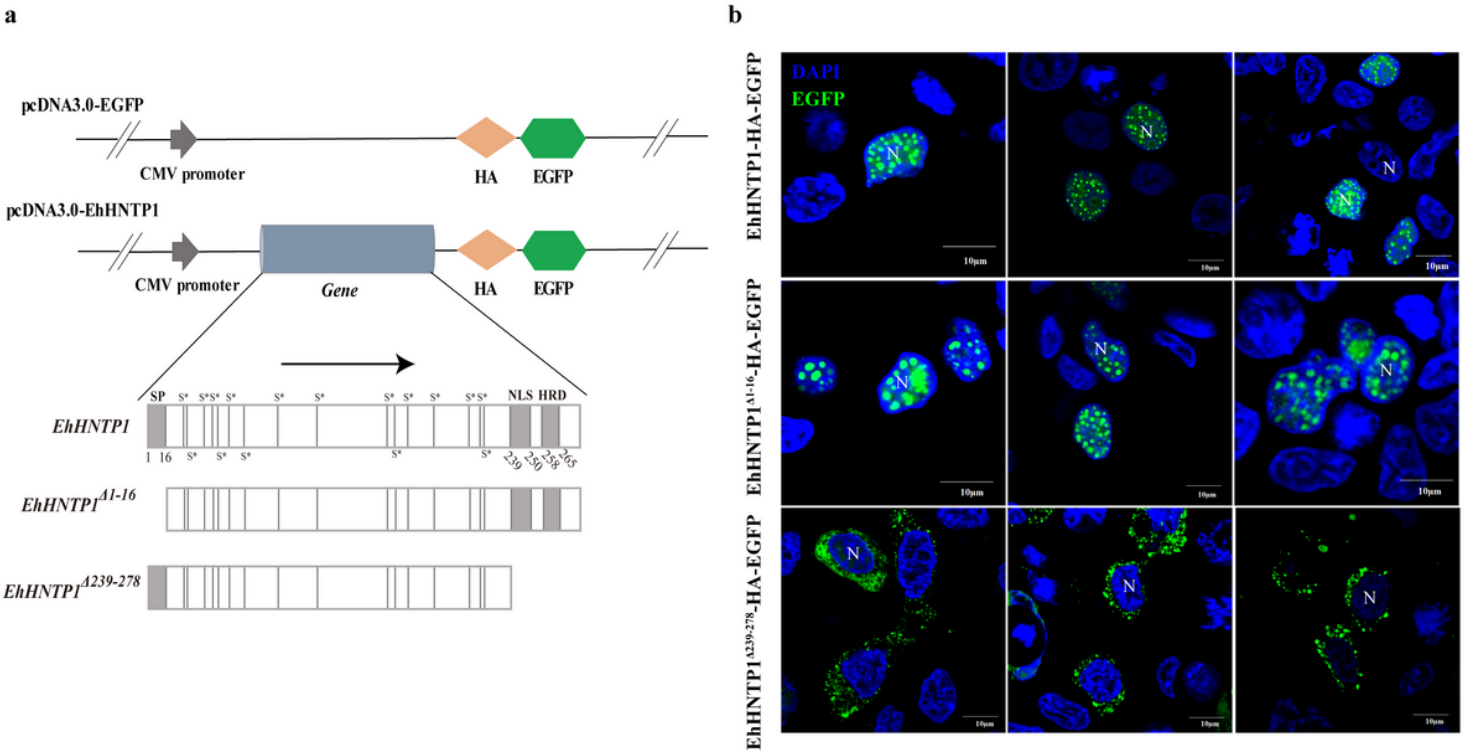

Figure 3

EhHNTP1 translocation to the host nucleus depends on its nuclear localization signal sequence (NLS). (a) Constructions of EhHNTP1 deletion mutants lacking the signal peptide (EhHNTP1Δ1-16-HA-EGFP) and NLS (EhHNTP1Δ239-278-HA-EGFP). (b) The subcellular localization of EhHNTP1-HA-EGFP, EhHNTP1Δ1-16-HA-EGFP and EhHNTP1Δ239-278-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.

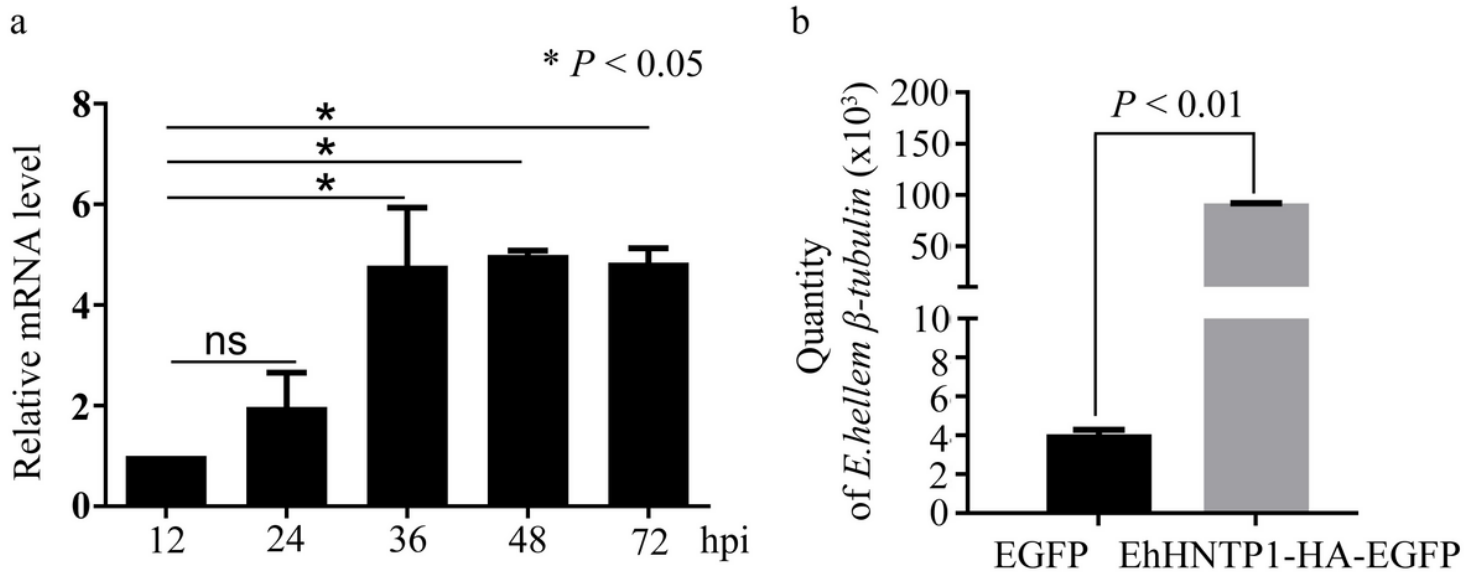

Figure 4

EhHNTP1 overexpression promoted E. hellem proliferation. (a) RT-qPCR analysis of EhHNTP1 expression in E. hellem-infected RK13 cells from 12 to 72 hpi. (b) qPCR analysis of E. hellem proliferation in EhHNTP1-HA-EGFP- and HA-EGFP-transfected HEK293 cells. The transfected cells were infected with 1×106 E. hellem spores, and genomic DNA was then extracted. Pathogen load was determined as indicated by quantification of the E. hellem β-tubulin gene.

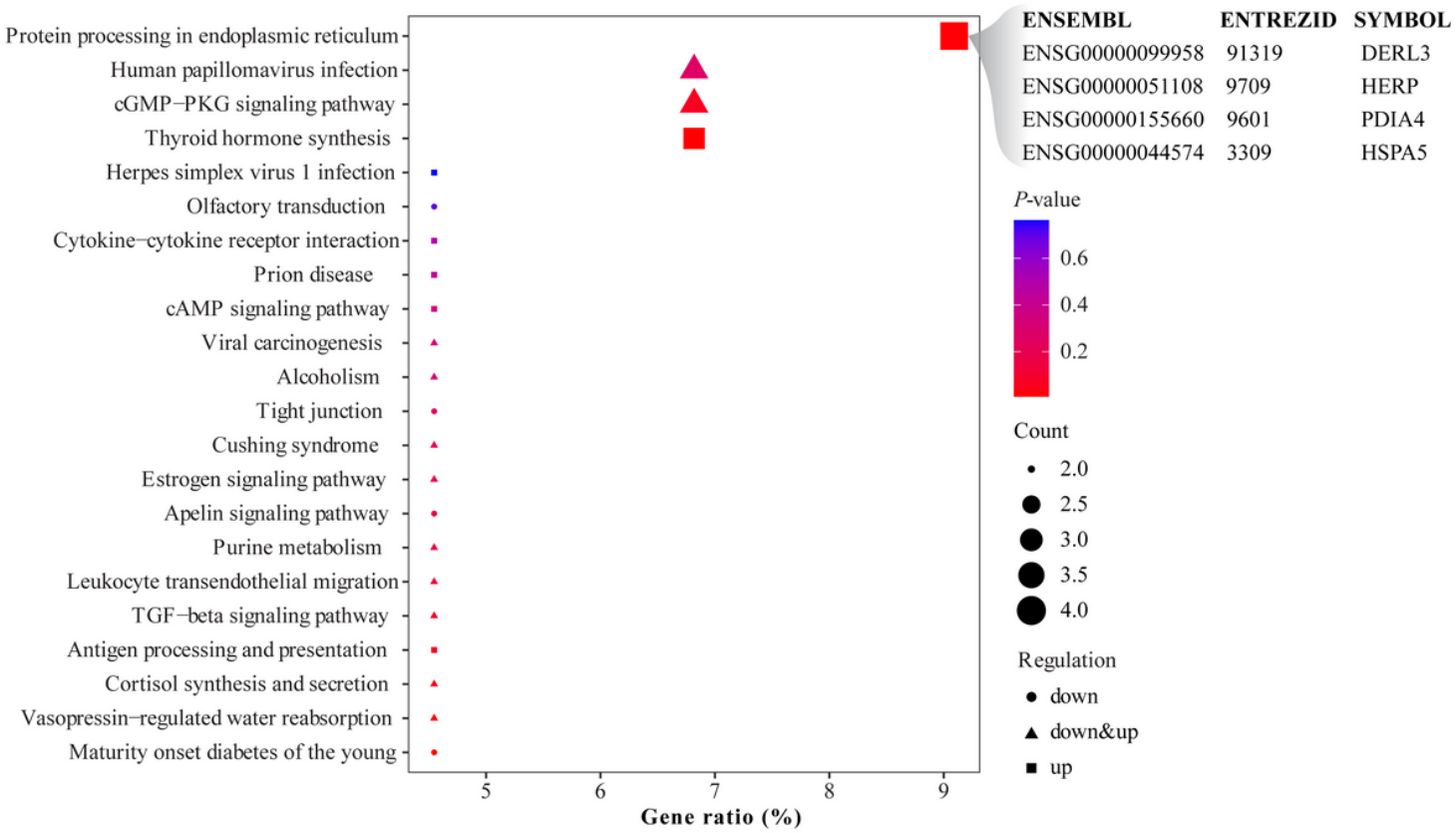

Figure 5

KEGG enrichment analysis of DEGs between HEK293 cells transfected with EhHNTP1-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.

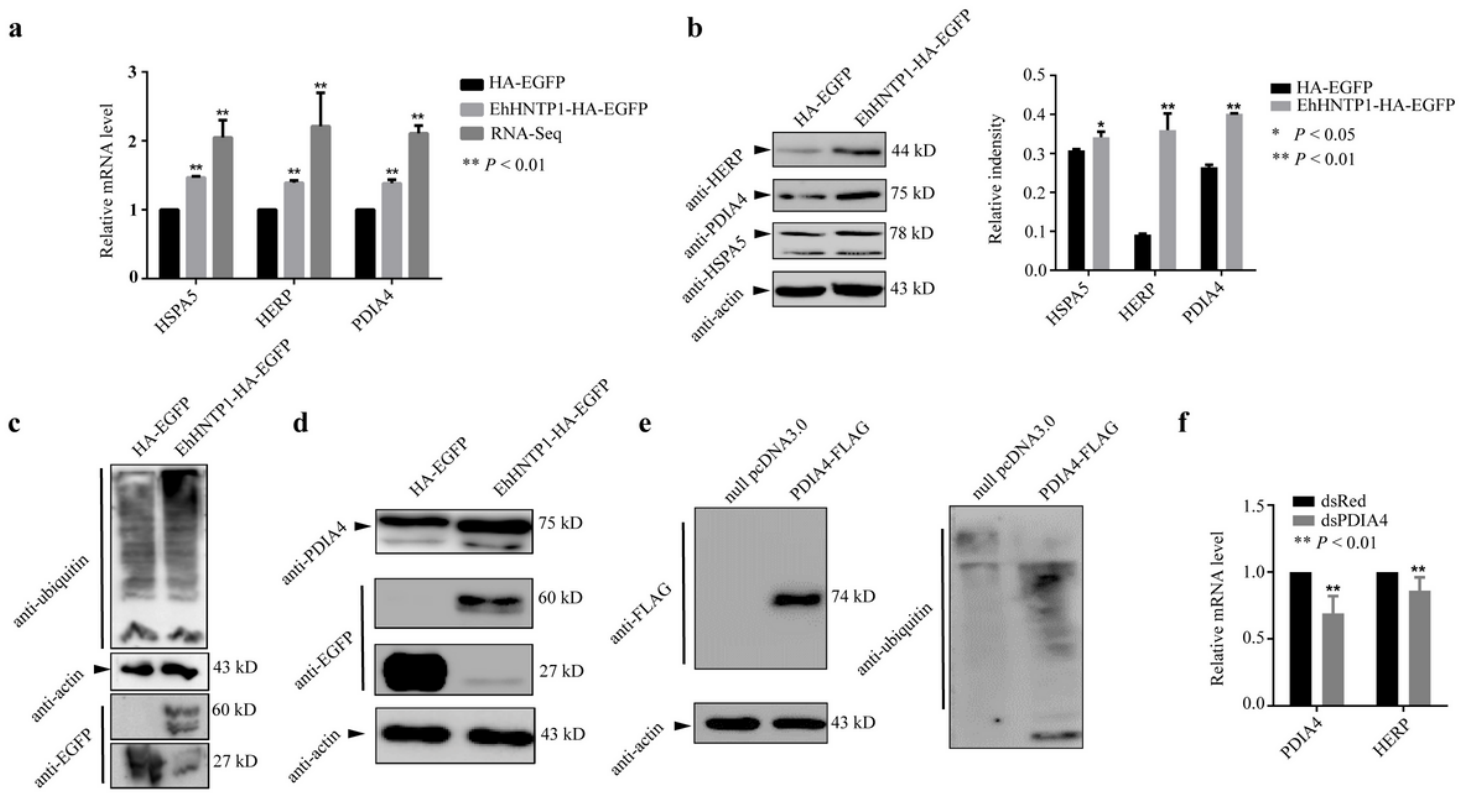

Figure 6

Verification of ERAD genes in HEK293 cells transfected with EhHNTP1-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.

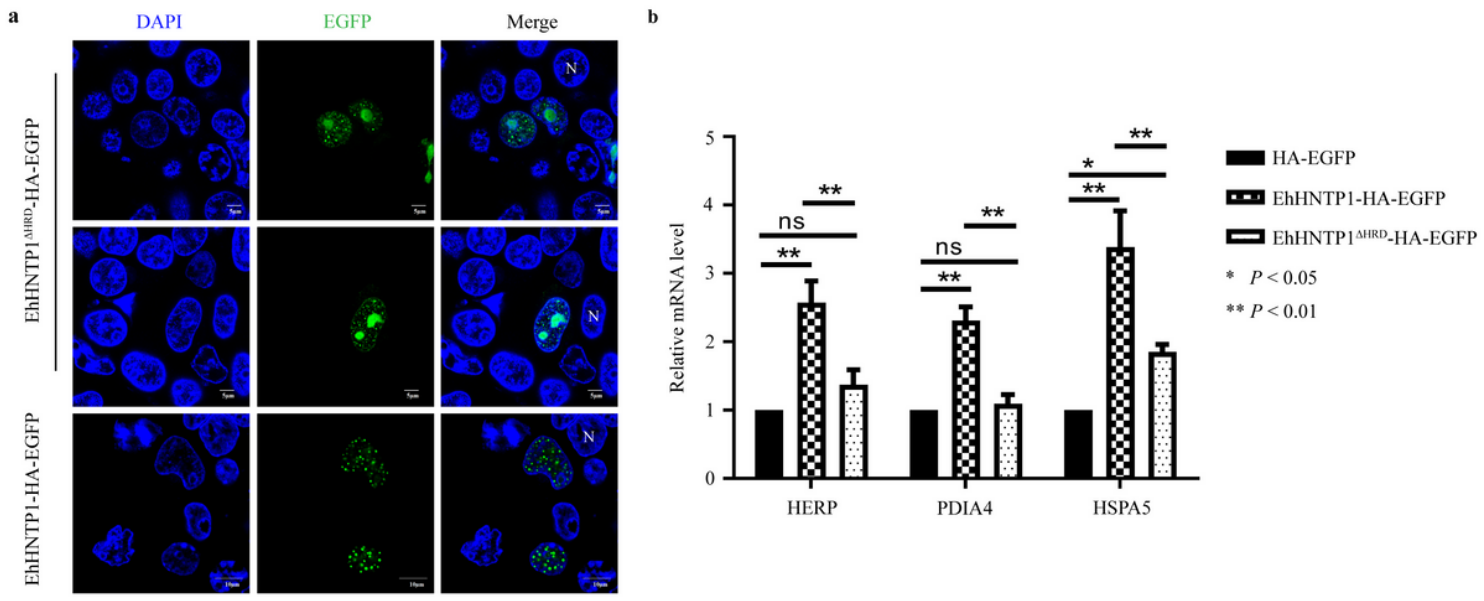

Figure 7

The HRD is essential for the regulatory function of EhHNTP1. (a) Deletion of the HRD changed the localization of EhHNTP1 in the nucleus. EhHNTP1ΔHRD-HA-EGFP aggregated and formed a large multimer, whereas wild-type EhHNTP1 was evenly distributed in the nucleus. (b) The expression of PDIA4, HERP and HSPA5 was dramatically decreased in EhHNTP1ΔHRD-HA-EGFP-transfected HEK293 cells compared with cells expressing EhHNTP1-HA-EGFP.

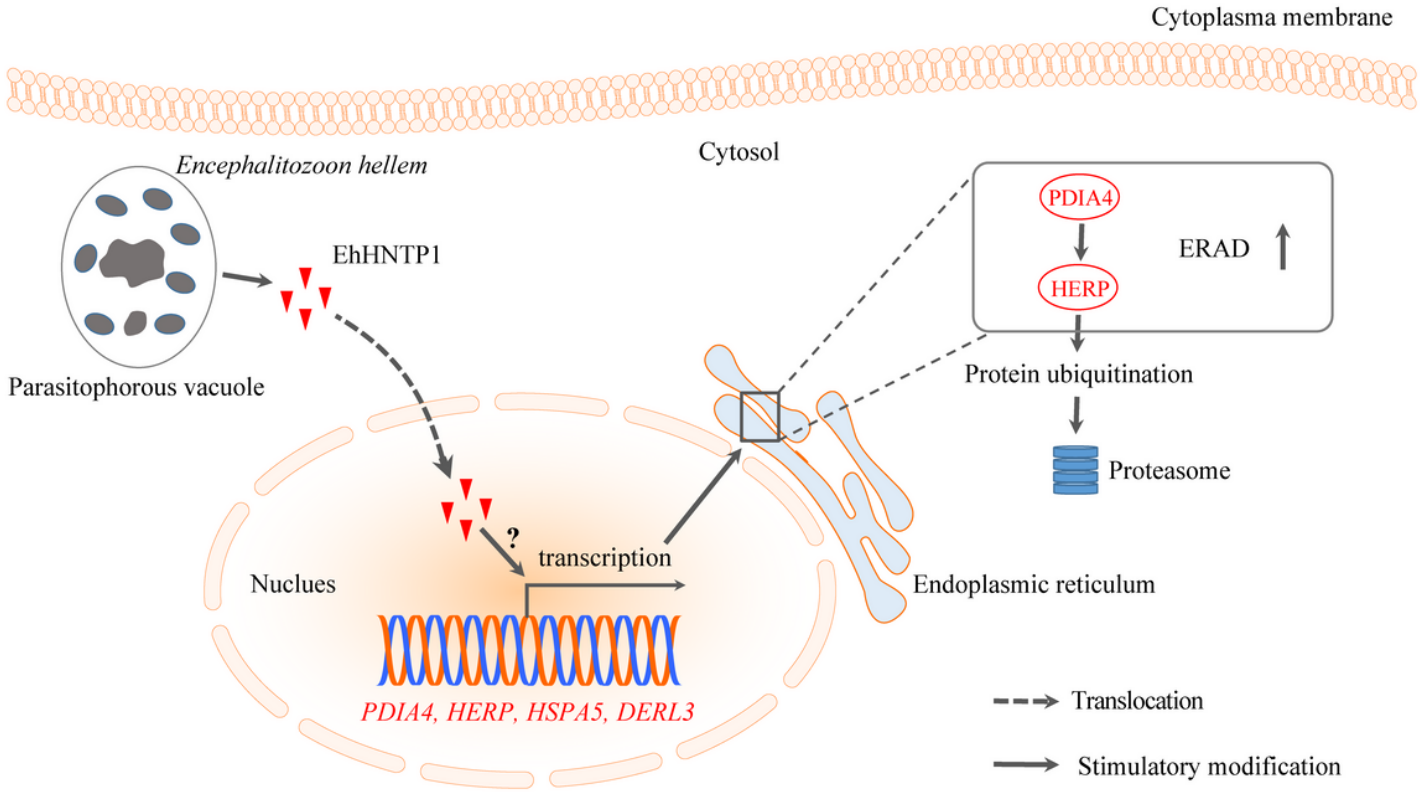

Figure 8

A schematic showing the model by which E. hellem secretes EhHNTP1 to dysregulate host ERAD and protein ubiquitination. During E. hellem proliferation, EhHNTP1 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.

Supplementary Files

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

- Additionalfile1.docx
- Addtioanlfile2.xlsx
- graphicalabstract.jpg