微生物学报
Acta Microbiologica Sinica
2024, 64(4): 1203–1218
http://journals.im.ac.cn/actamicrocn
DOI: 10.13343/j.cnki.wsxb.20230678

Research Article 研究报告

家蚕微粒子虫分泌蛋白己糖激酶 NbHK 对家蚕基因表达及相关通路的调控作用

文远[1,2]，邹子筠[1,2]，汪春霞[1,2]，于滨[1,2]，周泽扬[1,2,3]，李田[1,2*]

1 西南大学 资源昆虫高效养殖与利用全国重点实验室，重庆 400715
2 西南大学 微孢子虫感染与防控重庆市重点实验室，重庆 400715
3 重庆师范大学生命科学学院，重庆 400047

文远, 邹子筠, 汪春霞, 于滨, 周泽扬, 李田. 家蚕微粒子虫分泌蛋白己糖激酶 NbHK 对家蚕基因表达及相关通路的调控作用[J]. 微生物学报, 2024, 64(4): 1203-1218.
WEN Yuan, ZOU Ziyun, WANG Chunxia, YU Bin, ZHOU Zeyang, LI Tian. Hexokinase of *Nosema bombycis* modulates gene expression and related pathways in silkworm[J]. Acta Microbiologica Sinica, 2024, 64(4): 1203-1218.

摘　要：【目的】微孢子虫是一种营专性细胞内寄生的微生物，它可以感染几乎所有动物种类，包括人类和重要的经济动物。本研究对家蚕微粒子虫分泌蛋白己糖激酶(*Nosema bombycis* hexokinase, NbHK)在家蚕胚胎细胞中表达特征、亚细胞定位、调控作用和宿主互作蛋白质进行了系统分析，为阐明该蛋白在侵染中的作用与机理提供参考。【方法】利用原核表达蛋白免疫小鼠，制备 NbHK 的多克隆抗体，并利用 Western blotting 和间接免疫荧光法分析家蚕微粒子虫在感染的家蚕胚胎细胞(*Bombyx mori* embryo, BmE)中的表达和定位；通过过表达和 RNA 干扰实验，分析 NbHK 对病原增殖的作用；利用 RNA-seq 分析 NbHK 调控的家蚕基因表达和通路；利用生物素-链霉亲和素系统和质谱技术，从 NbHK::APEX2 转基因细胞中分离鉴定 NbHK 的互作蛋白。【结果】在感染家蚕微粒子虫的 BmE 中，NbHK 持续上调表达，主要被定位于宿主细胞核内。过表达 NbHK 显著促进了病原增殖，而敲低 NbHK 则明显抑制了病原增殖，说明在 NbHK 感染过程中发挥关键作用。利用 RNA-seq 分析鉴定了 94 个差异表达基因(differentially expressed genes, DEGs)，其中 58 个基因上调，36 个基因下调。DEGs 的富集分析显示，细胞寿命和内质网蛋白加工通路受到显著激活，而线粒体自噬途径受到明显抑制。互作蛋白鉴定分析发现，NbHK 可能与宿主细胞核内

资助项目：重庆市自然科学基金(cstc2019yszx-jcyj0006)；国家自然科学基金(32272944，31772678，31472151)；西南大学先导计划青年团队项目(SWU-XDJH202322)；重庆市现代农业产业技术体系(COMAITS202311)
This work was supported by the Natural Science Foundation of Chongqing (cstc2019yszx-jcyj0006), the National Natural Science Foundation of China (32272944, 31772678, 31472151), the Southwest University Pilot Program Youth Team Project (SWU-XDJH202322), and the Chongqing Modern Agricultural Industry Technology System (COMAITS202311).
*Corresponding author. E-mail: lit@swu.edu.cn
Received: 2023-11-04; Accepted: 2023-12-22; Published online: 2023-12-26

的核蛋白易位启动子区(nucleoprotein translocated promoter region, NTPR)等蛋白间存在相互作用。【结论】NbHK 主要被定位至家蚕细胞核中，调控家蚕细胞寿命等多个重要通路的基因表达，以利于病原增殖。本研究为深入解析 NbHK 在感染过程中的功能及其调控机理提供了新的参考。

关键词：微孢子虫；家蚕微粒子虫；己糖激酶；分泌蛋白；功能

Hexokinase of *Nosema bombycis* modulates gene expression and related pathways in silkworm

WEN Yuan[1,2], ZOU Ziyun[1,2], WANG Chunxia[1,2], YU Bin[1,2], ZHOU Zeyang[1,2,3], LI Tian[1,2*]

1 State Key Laboratory of Resource Insects, Southwest University, Chongqing 400715, China
2 Chongqing Key Laboratory of Microsporidia Infection and Control, Southwest University, Chongqing 400715, China
3 College of Life Sciences, Chongqing Normal University, Chongqing 400047, China

Abstract: [Objective] Microsporidia are obligate intracellular parasites capable of infecting a wide range of animal species, including both humans and animals of economic interests. We explored *Nosema bombycis* hexokinase (NbHK) in terms of the expression, subcellular localization, regulatory functions, and interacting proteins in *Bombyx mori* embryo cells, aiming to provide insights into the function and mechanism of this protein during infection. **[Methods]** We prepared a polyclonal antibody against NbHK to analyze the expression and localization of NbHK in *N. bombycis*-infected BmE cells by using Western blotting and the indirect immunofluorescent assay. Overexpression and RNA interference experiments were performed to assess the impact of NbHK on pathogen proliferation. RNA-seq was employed to analyze the transcriptional responses of the NbHK-transgenic BmE cells. A biotin-streptavidin system and mass spectrometry were employed to identify the interacting proteins of NbHK from NbHK::APEX2-transgenic BmE cells. **[Results]** NbHK was predominantly localized in the nucleus of infected cells, with consistently upregulated expression during infection. The overexpression of NbHK significantly increased the pathogen load, while the knock-down of NbHK suppressed pathogen proliferation, which indicated the crucial roles of NbHK during infection. RNA-seq analysis identified 94 differentially expressed genes (DEGs) responsive to infection, comprising 58 up-regulated genes and 36 down-regulated genes. The enrichment analysis of DEGs revealed significant activation of pathways related to cell lifespan regulation and protein processing in the endoplasmic reticulum while significant inhibition of the mitophagy pathway. Additionally, we identified host proteins including nucleoprotein translocated promoter region (NTPR) in the nucleus that potentially interacted with NbHK. **[Conclusion]** NbHK is secreted into silkworm nucleus to modulate the expression of genes involved in multiple pathways for promoting pathogen proliferation. Our study offers novel insights into the roles of NbHK in the infection of *N. bombycis*.

Keywords: microsporidia; *Nosema bombycis*; hexokinase; secretory protein; function

✉ actamicro@im.ac.cn, ☏ 010-64807516

Microsporidia constitute a vast assemblage of obligatory intracellular parasites that have the ability to infect a wide range of vertebrates and invertebrates, encompassing humans and economically significant animals[1]. Microsporidia do not harbor a canonical mitochondrion but retain a mitochondrial remnant, the mitosome[2]. Therefore, microsporidia greatly depend on host metabolism for nutrients.

Microsporidia have evolved genes encoding nucleotide transport proteins (NTTs) to steal ATP from its eukaryotic host[3]. In the early stage of human microsporidian *Encephalitozoon hellem* infection, the spore surface protein EhSSP1 interacted with the host mitochondria voltage-dependent channel (VDAC), which might be a key factor for the absorption of ATP[4]. Genomic and proteomic studies have identified a secretory hexokinase (HK) in microsporidia[5-6]. HK orthologs have been found in all examined microsporidia, but show amino acid variations among species[7].

HK is a multi-functional protein that not only acts as an innate immune receptor of bacterial peptidoglycan[8] but also inhibits apoptosis mediated by the death receptor[9]. In 2012, the secretion of microsporidian HK was firstly reported and characterized in *Nematocida parisii*, a natural pathogen of *Caenorhabditis nematocides*[10]. Subsequently, this secretory HK was confirmed in multiple microsporidian species[11-12]. In *Nosema bombycis*-infected *Spodoptera frugiperda* Sf9 cells, HK was secreted into the host cytosol and nucleus[13]. Besides, *Trachipleistophora hominis* HK localized on the surface of parasitic vesicles and was speculated to increase glycolytic capacity and ATP generation close to the parasite surface[14]. Proteomic analysis showed that HK increased after the spore germination of *N. bombycis*[15]. Moreover, the heterogeneous overexpression of HK from *N. bombycis* and *N. ceranae* showed ability to phosphorylate host glucose[16]. Furthermore, overexpression of antibody fragment against *N. ceranae* HK in Sf9 cell inhibited microsporidia proliferation[17]. Therefore, microsporidian HK likely plays vital roles during infection. However, the functions and regulatory mechanisms of microsporidian HK are yet to be elucidated.

In this study, we have conducted a novel and comprehensive characterization of *N. bombycis* HK (NbHK) in its natural host, the silkworm *Bombyx mori*, including its expression profile, subcellular localization, impact on infection and regulatory functions. As a result, we found that NbHK was secreted into host nucleus to modulate expression of genes involved in multiple pathways like cell longevity regulation and protein processing in the endoplasmic reticulum. Our findings provide insights into the roles and mechanisms of NbHK during the infection.

1 Materials and Methods

1.1 Preparation of polyclonal antibody against NbHK

The coding sequence of NbHK (GenBank accession number KB910227.1) was downloaded from the SilkPathDB[18], and amplified from genomic DNA of *N. bombycis* by PCR. The amplification reaction consisted of 30 cycles at 98 ℃ for 15 s, 55 ℃ for 15 s, and 72 ℃ for 20 s using the forward primer 5′-CGCGGATCCATGATAATTTTCTATTGT-3′, containing a *Bam*H I restriction site (GGATCC), and the reverse primer 5′-CCGCTCGAGATAAATAATTCGATGTAAAG-3′, containing a *Xho* I restriction site (CTCGAG). The PCR product was recovered with Gel Extract Kit (Omega) and integrated into pET32a vector. Then, the recombinant vector was transformed into *Escherichia coli* Trans5α competent cells and verified by sequencing. The pET32a-NbHK-His vector was transformed into *E. coli* Rosetta competent cells for protein expression. After cultivation in 37 ℃, the bacteria were induced for 20 h at 16 ℃ with 0.1 mmol/L IPTG in LB medium. The recombinant protein was purified using nickel chelating affinity chromatography. KM mice were inoculated using the rNbHK-His, which was fixed with Freund's adjuvant (*V*/*V*=1:1,

Sigma-Aldrich) for four times. Complete Freund's adjuvant was used at first immunization and incomplete adjuvant was used other three times.

1.2 Protein preparation and Western blotting

Protein samples of infected, uninfected and transfected *B. mori* embryo (BmE) cells were prepared as described in our previous work[13].

Protein samples of mature spores were prepared *via* glass bead breaking in RIPA lysis buffer. The samples were shocked for 5 min using a homogenizer, lysed for 5 min at 4 ℃, and then centrifuged at 13 000 r/min for 5 min, and the supernatants were collected.

The protein samples were separated using 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (03010040001, Roche). After blocking for 1 h in blocking buffer (5% skimmed milk dissolved in TBST buffer), the membrane was incubated with NbHK antiserum diluted at 1:1 000 for 2 h. The membrane was then washed and incubated with HRP-goat anti-mouse IgG (Biosharp) diluted at 1:8 000 for 1 h. The protein was detected using ECL Plus Western blotting Detection Reagents (Bioground).

1.3 Indirect immunofluorescence assay (IFA)

BmE cells (approximate 1×10^5 cells in 1.5 mL medium) were inoculated with 1×10^5 *N. bombycis* spores. The uninfected and infected BmE cells (approximate 3×10^5 cells 48 h post transfection) were fixed on a glass plate with 4% paraformaldehyde (BL539A, Biosharp) for 10 min and treated with 0.5% Triton X-100 (1139ML100, BioFroxx) for 15 min at room temperature. The samples were washed, blocked with blocking buffer (5% bovine serum and 10% goat serum) for 1 h, and then incubated with NbHK antiserum diluted at 1:500 for 2 h at room temperature. After washing with PBST buffer three times, Alexa Fluor 488 (A-11001, ThermoFisher Scientific) diluted at 1:1 000 was incubated to detect the HK antibody for 1 h. The nucleus was stained with DAPI (D1306, ThermoFisher Scientific) for 30 min. The treated samples were observed and imaged with an Olympus FV1200 (Olympus).

1.4 Transfection of BmE cells with NbHK::EGFP

The coding sequence of NbHK was amplified using the forward primer 5′-TAAAGCTTGGTACCGAGCTCGGATCCATGATAATTTTCTATTGTCTACTC-3′ and the reverse primer 5′-CAGAGCCACCTCCGCCAGAGCCGCCTCCGCCATAAATAATTCGATGTAAAGT-3′. The EGFP, a green fluorescent protein, was amplified using the forward primer 5′-TGGCGGAGGTGGCTCTGGGGGCGGCGGATCGGTGAGCAAGGGCGAGGAGCTG-3′ and the reverse primer 5′-CCGCGGGCCCGCTCTAGAGCCTTGTACAGCTCGTCCATGC-3′. Then, NbHK and EGFP were linked with a $(G_4S_1)_3$ linker peptide using overlapping PCR. The overlapped product was integrated into pEHI/V5-His vectors which were digested using *Bam*H I with a homologous recombinant method. Before transfection, 1×10^5 BmE cells in 1.5 mL medium contained in a 12-well plate were inoculated with *N. bombycis* spores (spore:cell=1:30). After 24 h, 2 μg recombinant vector DNA and 3 μL X-tremeGENE^{TM} HP DNA Transfection Reagent (XTG360-RO, Roche) were transiently transfected into the BmE cells. After 48 h, the cell samples were observed with confocal microscopy (Olympus).

1.5 RNA interference (RNAi)

The coding sequence of NbHK was submitted to the BLOCK-IT RNAi Designer (https://rnaidesigner.thermofisher.com), and a 308 bp fragment containing five potential interfering fragments was designed. The entire fragment was amplified using the forward primer F-Ri-NbHK (5′-TAATACGACTCACTATAGGGAGAAGGAATATACTTGTCTGGGA-3′) and reverse primer R-Ri-NbHK (5′-TAATACGACTCACTATAGGGAGATTGACAGGTCTCTCAAATGC-3′). The amplified product served as a template for synthesizing dsRNA using the RiboMAX Large Scale System-T7 Kit (A54989, ThermoFisher Scientific). The dsRNA of EGFP was used as the control group and prepared with forward primer F-Ri-EGFP (5′-TAATACGACTCACTATAGGGA

GAACGGCAAGCTGACCCTGAA-3′) and reverse primer R-Ri-EGFP (5′-TAATACGACTCACTATAGGGAGATGTTGTAGTTGTACTCCAG-3′). Prior to dsRNA transfection, 5×10^5 BmE cells were inoculated with *N. bombycis* spores at a ratio of 1:30 in 2 mL medium contained in a 6-well plate. 24 h after infection, the BmE cells were transfected with dsRNA using X-tremeGENE HP DNA Transfection Reagent (XTG360-RO, Roche). The BmE cells were then collected 24 h post-transfection.

1.6 Extraction of RNA and cDNA from infected BmE cells

The *N. bombycis* spores were isolated from infected Sf9-III cells, treated with 0.1 mmol/L KOH, and added to the BmE cells at a ratio of 1:10 (cells:spores). Following infection, the BmE cells in a six-well plate were transfected separately with 2 μg of EGFP and NbHK dsRNA. The samples were collected at 1, 3, and 5 days post-infection and stored either in PBS buffer or TRIzol (Invitrogen) at −80 ℃. Genomic DNA was extracted from the infected cells using a DNA Extraction Kit (Omega), while total RNA was extracted using a Total RNA Kit (Omega) followed by cDNA synthesis using an cDNA Synthesis Kit (Yeasen).

1.7 Real-time quantitative PCR analysis of NbHK expression and infection

The detection of transcription was performed using RT-qPCR, with the template obtained from 1 μg of RNA. RT-qPCR amplification was conducted using the forward primer qF-NbHK (5′-CAAAATGTGATTATTATGGGAGATG-3′) and the reverse primer qR-NbHK (5′-CGATGTAAAGTATAAAGGGCTGAT-3′). The RT-qPCR program consisted of pre-denaturation at 95 ℃ for 5 min, followed by 40 cycles of denaturation at 95 ℃ for 10 s and annealing/extension at 60 ℃ for 30 s. *Nbβ-tubulin* was used as the reference gene of *N. bombycis*, and *GAPDH* was used as the reference gene of BmE cells.

Nbβ-tubulin copies were analyzed through qPCR, utilizing a reaction system containing 1 μL (about 150 ng/μL) of genomic DNA sample extracted from infected cells and the forward primer qF-Nbβ-tubulin (5′-AGAACCAGGAACAATGGACG-3′) along with the reverse primer qR-Nbβ-tubulin (5′-AGCCCAATTATTACCAGCACC-3′). The standard plasmid constructed by *Nbβ-tubulin* was diluted to 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10 copies/μL by gradient to establish a standard curve, to analyze the copy number of *N. bombycis*.

1.8 RNA-seq analysis of NbHK-transfected BmE cells

The transcriptional response of BmE cells transfected with EGFP and NbHK::EGFP was analyzed using RNA-seq. Three independent replicates were performed for each group, and RNA samples were extracted and sequenced on the Illumina NovaSeq platform. The clean reads were mapped to the silkworm genome (http://silkbase.ab.a.u-tokyo.ac.jp/pub/)[19] using HISAT2[20]. Fragments per kilobase per million (FPKM) values were calculated using FeatureCounts software to compare gene transcriptomic levels. differentially expressed genes (DEGs) between EGFP- and NbHK::EGFP-transfected BmE cells were analyzed using DESeq2[21], filtered by a standard *P* value<0.05 and fold change≥1. KEGG enrichment analysis[22] of DEGs was performed using Fisher's exact test, considering pathways enriched in at least two genes with $P<0.05$ as significant.

1.9 Isolation and identification of NbHK interacting proteins

NbHK::APEX2 transgenic BmE cells were cultured with Grace Insect Medium (ThermoFisher Scientific), which contained biotin-phenol (Iris BIOTECH) and incubated 30 min at 28 ℃. Then, 150 μL of 100 mmol/L H_2O_2 was added into the medium for 1 min, the liquid in the cell flask was discarded, and the reaction termination buffer was added and incubated for 5 min. The cell samples were collected, and the total proteins were extracted with RIPA lysis buffer and centrifuged at 13 000 r/min for 5 min, and the supernatant was collected. Furthermore, using 500 μL of streptavidin magnetic beads (ThermoFisher Scientific) per 4 mg total protein, we extracted an

appropriate amount of total protein and incubated it with the magnetic beads at room temperature for 2 h. After incubation, the magnetic beads were washed with RIPA, KCl, Na_2CO_3, and urea buffer, respectively. Finally, the proteins combined with the magnetic beads were eluted with 80 μL SDT lysis buffer and incubated in boiling water for 10 min. The supernatant was collected and stored at −80 ℃.

The identification of NbHK interacting proteins was performed using mass spectrometry, with three independent replicates for each group. The protein samples were separated and analyzed using a Q-Exactive Mass Spectrometry device. The raw data were mapped to the UniProt (https://www.uniprot.org) database[23] and quantitatively analyzed using MaxQuant software (v1.5.3.17)[24]. The candidate interacting proteins were filtered with strict standards including unique peptide number≥2 and peptide only determined in the NbHK::APEX-transfected cells. KEGG and GO enrichment analyses[22] of the candidates were performed using Fisher's exact test with a standard P value<0.05.

2 Results and analyses

2.1 The expression and subcellular localization of NbHK

The NbHK gene was successfully cloned (Figure 1A), followed by expression and purification of the protein (Figure 1B). Subsequently, a polyclonal antibody against NbHK (anti-NbHK) was generated and its specificity confirmed through Western blotting analysis (Figure 1C). Western blotting analysis of the temporal expression profile revealed a consistent increase in NbHK levels from 12 to 96 h post-infection (Figure 2A), indicating its crucial role during infection. Furthermore, IFA performed on *N. bombycis*-infected BmE cells demonstrated that NbHK is secreted into host cells and predominantly localizes within the nucleus of infected cells (Figure 2B).

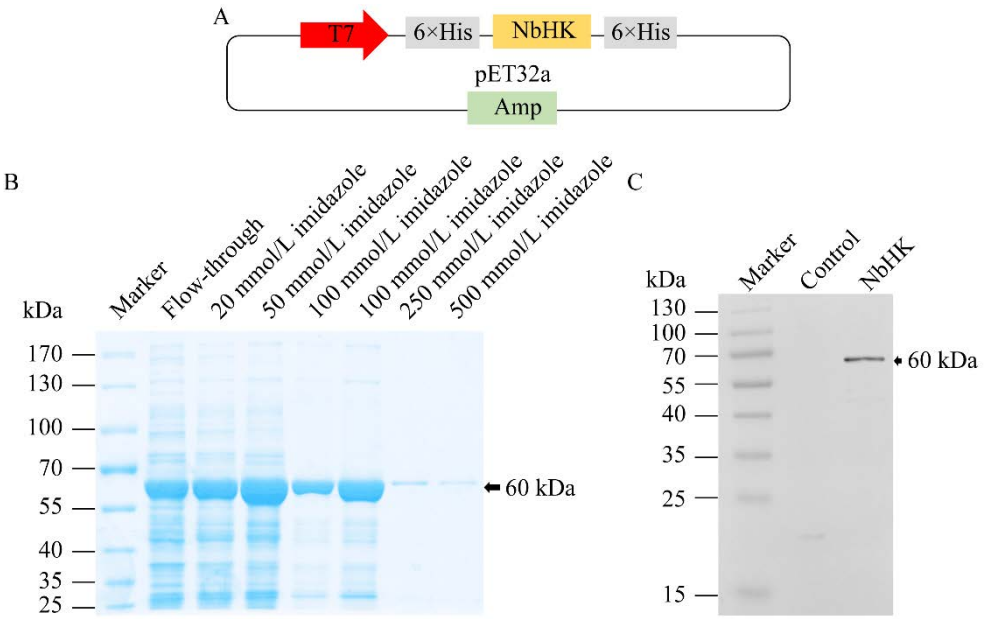

Figure 1 Preparation of polyclonal antibody using recombinant NbHK (rNbHK). A: Diagram of the pET32a-NbHK-His expression vector. The pET32a contains a T7 promoter and double 6×His tags. B: Purification of rNbHK expressed in *Escherichia coli*. The rNbHK was eluted with elution buffer containing a different concentration of imidazole and analyzed *via* SDS-PAGE. The arrow indicates the size of NbHK::His. C: Western blotting analysis of the polyclonal antibody against NbHK (anti-NbHK). Proteins were extracted and purified from the transformed and untransformed (control) *E. coli*.

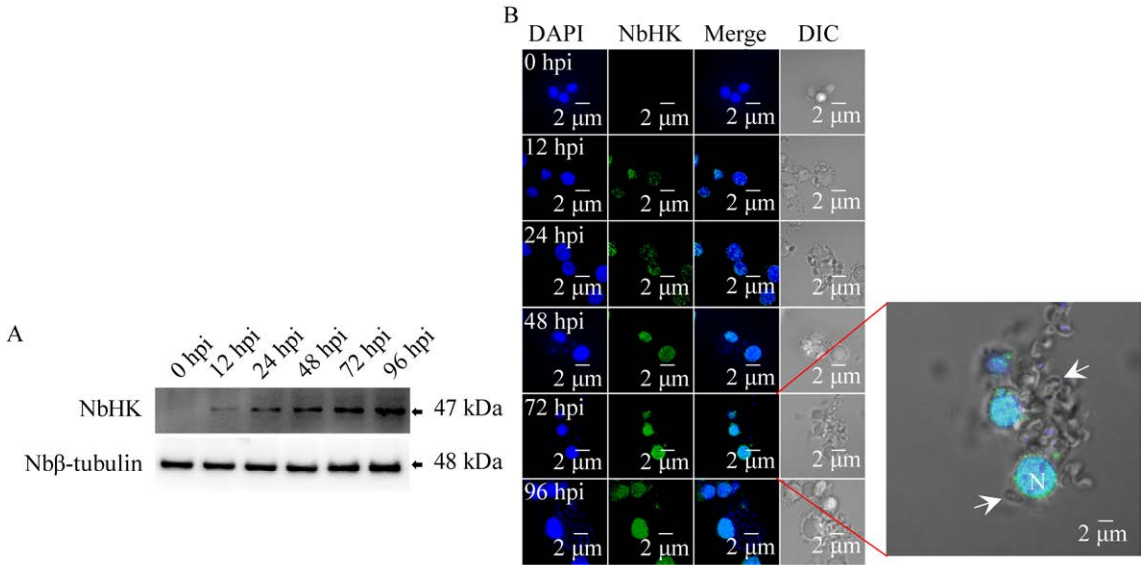

Figure 2 Expression profile and subcellular localization of NbHK in *Nosema bombycis*-infected BmE cells. A: Western blotting analysis of NbHK expression. Protein contents were homogenized referring to the Nbβ-tubulin (internal reference). The NbHK and Nbβ-tubulin were determined with relative antibodies, respectively. B: IFA analysis of the subcellular localization of NbHK in infected BmE cells. Arrowhead indicates the *N. bombycis* spore. Green fluorescence shows the localizations of NbHK labeled with the anti-NbHK. The nucleus of the host cells and *N. bombycis* were labeled with DAPI (blue). N: Nucleus.

2.2 NbHK played important roles in *Nosema bombycis* infection

To investigate the functions of NbHK during infection, we generated pEHI-NbHK::EGFP vectors for transfection into BmE cells (Figure 3A). As depicted in Figure 3B, NbHK::EGFP was successfully expressed in the transfected cells and localized within both the cytosol and nucleus (Figure 3B). Subsequently, these transfected cells were infected with *N. bombycis*, and the pathogen load was determined by quantifying the copy number of *Nbβ-tubulin* using quantitative PCR. The results demonstrated a significant increase in pathogen load within the NbHK::EGFP- transfected cells compared to those transfected with EGFP alone (Figure 3C).

Moreover, the expression of NbHK was downregulated using RNAi, and this was confirmed by both RT-qPCR and Western blotting analysis. Consequently, there was a significant reduction in both the transcriptional level (Figure 4A) and protein abundance (Figure 4B) of NbHK on the third day after infection. Subsequently, pathogen load was assessed by qPCR, revealing substantial decreases on the third- and fifth-days post-infection, respectively (Figure 4C).

2.3 Transcriptomic responses of BmE cells to NbHK

The secretion of NbHK into the host nucleus implies its potential role in regulating host gene expression. Therefore, NbHK-transgenic BmE cells were generated (Figure 5A) to investigate their transcriptomic responses using RNA-seq. The transfected cells were verified using Western blotting (Figure 5B) and counted for an over 50% transfection-positive rate (Figure 5C). Differentially expressed genes (DEGs) were identified based on the following criteria: (1) Only one difference between the control and experimental groups in three biological replicates; and (2) A P value<0.05 and absolute log fold change>1; (3) FPKM>1. The analysis revealed a total of 94 DEGs in the RNA-seq data, comprising of 58 upregulated genes and 36 downregulated genes (Figure 6A, supplementary file 1). KEGG enrichment analysis demonstrated significant regulation of multiple pathways including longevity

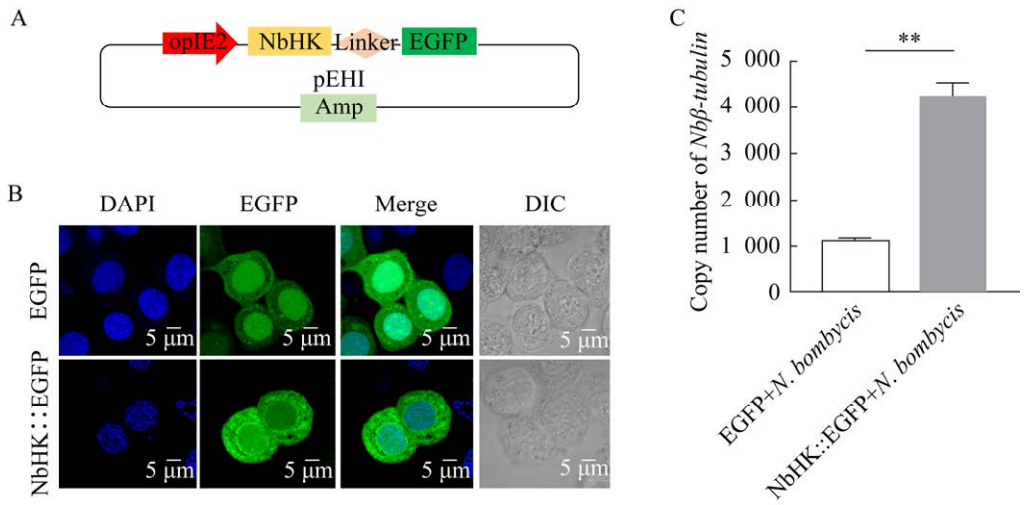

Figure 3 Effect of NbHK overexpression on *Nosema bombycis* infection. A: Diagram of pEHI-NbHK::EGFP expression vector. The vector contained an opIE2 promoter, a $(G_4S_1)_3$ linker, and a EGFP tag. B: Subcellular localization of NbHK::EGFP in transfected BmE cells. The nucleus was labeled with DAPI (the blue signal). The green fluorescence of NbHK::EGFP was directly observed in the cells. C: Comparison of pathogen load in the BmE cells infected by *N. bombycis* after RNAi NbHK. 1×10^5 BmE cells were firstly infected (spore: cell=1:30) for 24 h, and then transfected for 24 h. Total genomic DNA of the infected and transfected BmE cells was extracted to determine the copy number of *Nbβ-tubulin* (copies/μL) by qPCR, which indicated the change of pathogen load. Statistically significant differences are represented with asterisks (**: P<0.01). Bars represent the standard deviations of 3 independent replicates.

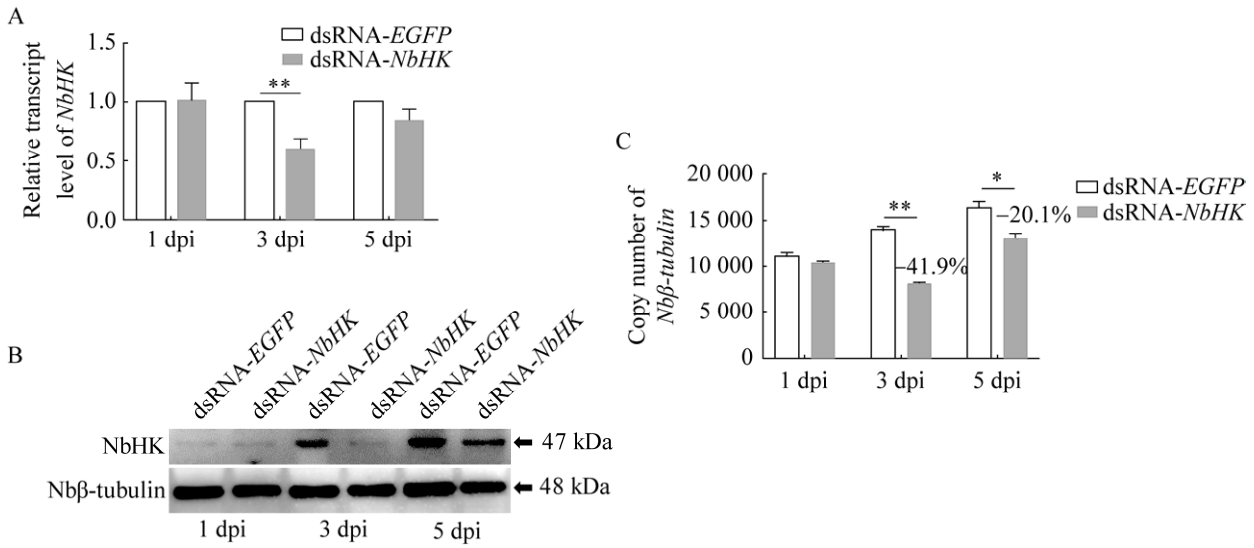

Figure 4 The effect of NbHK on *Nosema bombycis* infection. A: Suppression of NbHK expression by RNAi. Transcripts of NbHK were determined using RT-qPCR after BmE cells were transfected with dsRNA-*EGFP* and dsRNA-*NbHK*. B: Western blotting analysis of the protein content of NbHK after RNAi. The sample loads were homogenized referring to the content of Nbβ-tubulin. The NbHK contents were then determined using anti-NbHK. C: Detection of the pathogen load after RNAi of NbHK. 5×10^5 BmE cells were firstly infected (spore:cell=1:30) for 24 h, and then transfected with dsRNA. Total genomic DNA of the infected and transfected BmE cells was extracted to determine the copy number of *Nbβ-tubulin* (copies/μL) by qPCR, which indicated the change of pathogen load. *: P<0.05, **: P<0.01.

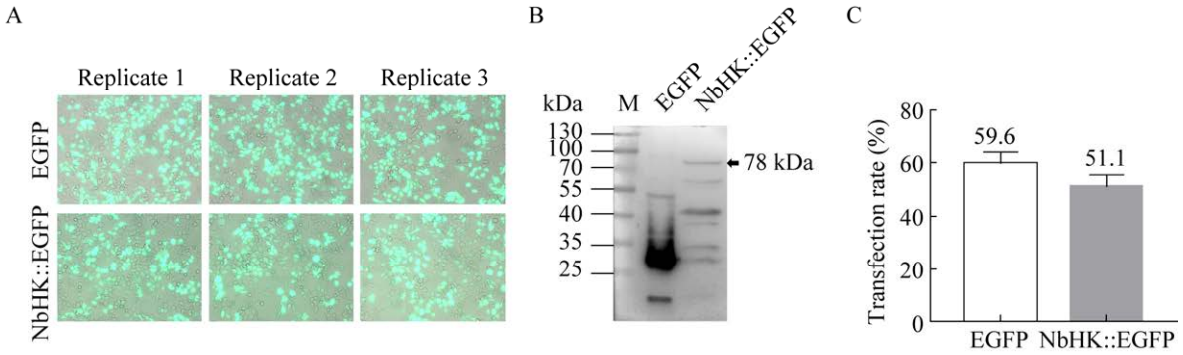

Figure 5 Preparation of cell samples for RNA-seq. A: Observation of RNA-seq samples. Transfected BmE cells were observed using a fluorescence microscope. The mock (EGFP) and experimental (NbHK::EGFP) groups included three independent replicates, respectively. B: Detection of NbHK::EGFP in RNA-seq samples *via* Western blotting. NbHK::EGFP was detected using a mouse monoclonal antibody against the EGFP. The arrow indicates the theoretical size of NbHK::EGFP. C: Transfection rate of BmE cells for the RNA-seq analysis.

regulation, protein processing in the endoplasmic reticulum (ER), and mitophagy (Figure 6B). Moreover, three upregulated genes associated with longevity and protein processing in the ER were identified: heat shock protein 19.9 (HSP19.9), heat shock protein family A member 1 (HSPA1), and small heat shock protein 19.3 (sHSP19.3) (Figure 6C), which were validated by RT-qPCR (Figure 6D).

2.4 Interacting proteins of NbHK

In order to further analyze how NbHK upregulated gene expressions involved in cell longevity and protein processing in the ER, a pEHI-NbHK::APEX2::HA expression vector was constructed (Figure 7A), and the potential interacting proteins of NbHK were isolated and identified using a biotin streptavidin system and mass spectrometry. The catalytic activity of APEX2 was detected using IFA, which showed that the NbHK::APEX2::HA (green fluorescence) was located in the cytoplasm and nucleus and co-localized with biotin labeled proteins (red fluorescence) (Figure 7B). In addition, the protein biotinylation was confirmed using Western blotting (Figure 7C). Finally, the isolation and enrichment of the biotinylated proteins were detected using Western blotting. As a result, abundant biotin-labeled proteins were obtained after enrichment (Figure 7D).

The mass spectrometry data showed that a total of 49 proteins were specifically identified in the NbHK::APEX-transfected BmE cells (supplementary file 2). These proteins were mainly isolated from the nucleus and cytoplasm (Figure 7E). Considering the predominant localization of NbHK in the host nucleus, we firstly focused on the 8 nucleus-localized and well annotated proteins, which UniProt accession numbers are Q1HQ86, Q2F5X7, H9JQ89, H9J002, Q0ZAL8, Q2F5K2, H9JJY3, and H9J682. Among these candidates, we found that only H9JQ89 (nucleoprotein TPR, NTPR) was reported to regulate HSP expression[25]. The NTPR is a component of the nuclear pore complex, which is mainly involved in transport between the nucleus and the cytoplasm and can also regulate gene expression as a transcription factor[26-28]. Therefore, we speculated that NbHK might interact with NTPR to regulate the expression of HSP19.9, HSPA1, and sHSP19.3, as identified *via* RNA-seq, thus regulating protein processing in the endoplasmic reticulum and cell longevity.

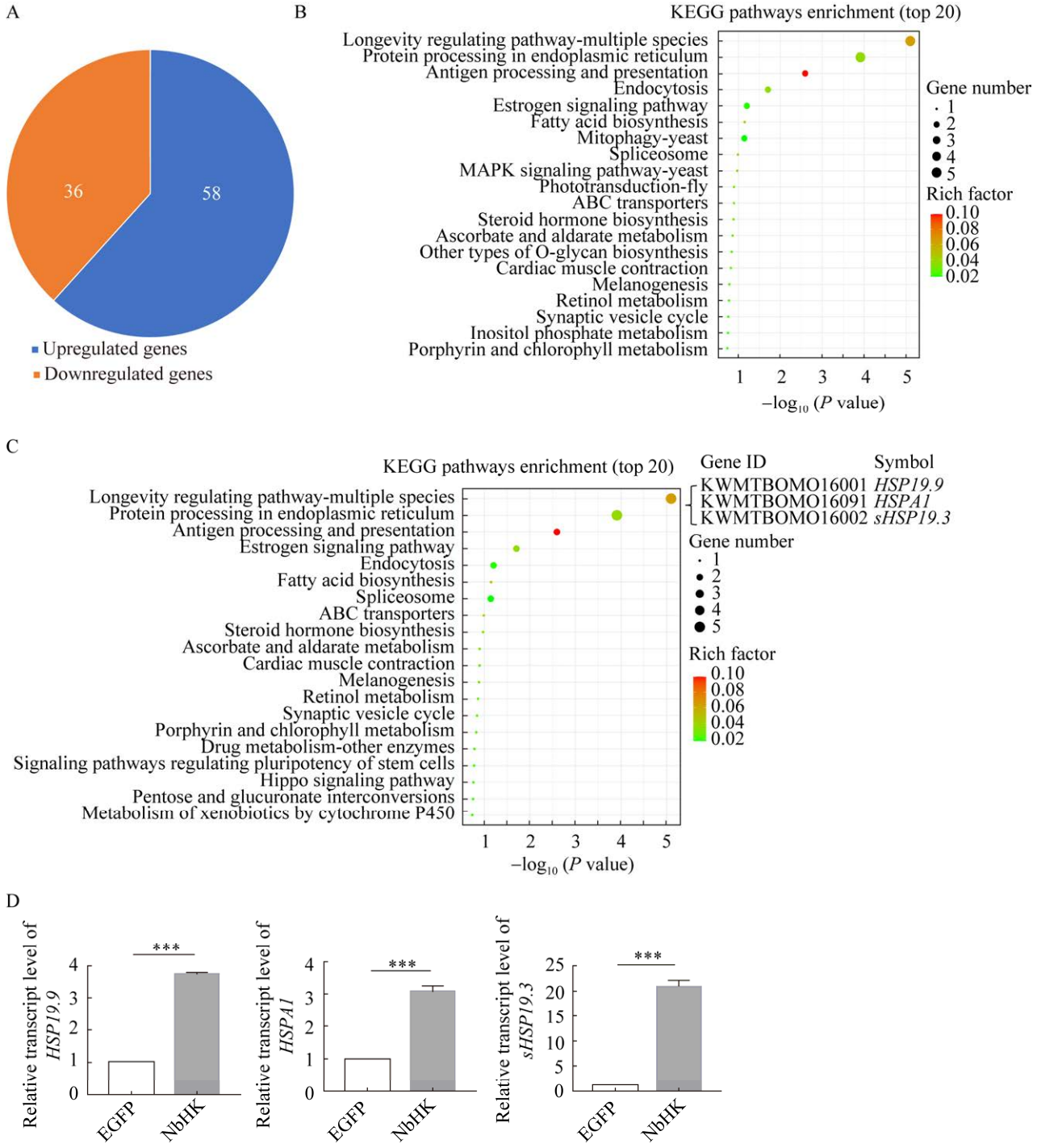

Figure 6 RNA-seq analysis of the BmE cells transfected with NbHK::EGFP and EGFP. A: DEGs of BmE cells transfected with NbHK::EGFP, including 58 upregulated and 36 downregulated genes. B: KEGG enrichment of the DEGs. C: KEGG enrichment analysis of the upregulated genes. D: Verification of the significantly upregulated genes *via* RT-qPCR. ***: $P<0.001$. Bars represent the standard deviations of 3 independent replicates.

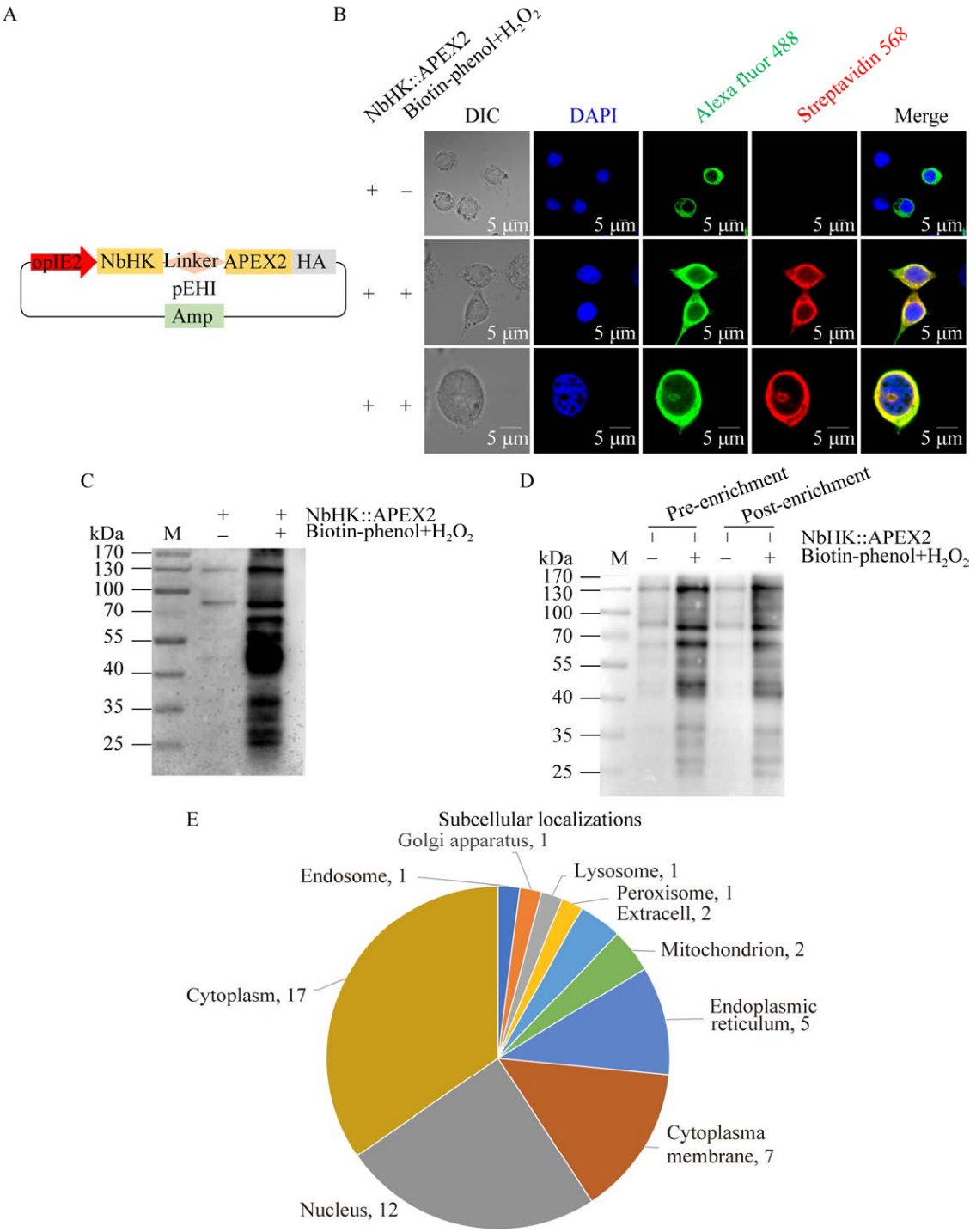

Figure 7 Isolation and identification of NbHK interacting proteins. A: Diagram of NbHK::APEX2 expression vector. The reconstructed pEHI vector contains an opIE2 promoter, a NbHK, a linker (G4S1)3, an APEX2 and a HA tag. B: Subcellular localization of biotin-labeled proteins. The nucleus was labeled with DAPI (blue). The green fluorescence represents NbHK::APEX2 labeled with the antibody against HA. The red fluorescence indicates the biotin-labeled proteins labeled with streptavidin 568 (ThermoFisher Scientific). C: Detection of the catalytic activity of APEX2. Protein biotinylation was detected *via* Western blotting using streptavidin-HRP. D: Detection on the enrichment of biotinylated proteins *via* Western blotting using streptavidin-HRP. E: Predicted subcellular localizations of the 49 proteins specifically identified in the NbHK::APEX2-transfected BmE cells.

3 Discussion and conclusion

Generally, HK serves as the initial and rate-limiting enzyme in glycolysis[29] or modulates glucose metabolism through the regulation of GAPDH[30]. However, extensive research has demonstrated that HK possesses multifunctional properties. Apart from its metabolic role as a glucose kinase, hexokinase 2 (ScHxk2) in *Saccharomyces cerevisiae* is also implicated in regulatory functions[31]. Under high-glucose conditions, ScHxk2 translocated from the cytoplasm to the nucleus to exert transcriptional control over glucose-repressible genes[32]. Similarly, *Candida albicans* hexokinase 2 (CaHxk2) localizes within the nucleus during glucose presence and contributes to signaling pathways involved in glucose repression while playing a crucial role in *C. albicans* virulence by promoting metabolic flexibility, stress response, and morphogenesis[33]. Therefore, the subcellular localization of NbHK varies in different host cells and infection stages[13], indicating its diverse functions during the infection process. NbHK is likely involved in promoting glucose metabolism in the host cytosol[16], while also potentially modulating gene expressions related to multiple pathways within the host nucleus. Thereby, it is intriguing to investigate the factors that determine the sorting of NbHK within host cells. In comparison to secreted NbHK in infected BmE cells, transfected NbHK::EGFP localizes both in the nucleus and cytosol, rather than predominantly aggregating in the nucleus. This suggests that translocation of NbHK may depend on other co-factors from either the host or parasite. However, these co-factors are likely absent without an infection present. Additionally, it is possible that fusion with EGFP peptides disrupts interactions between NbHK and translocating factors.

HSPs are pivotal molecular chaperones that play crucial roles in various cellular pathways. For instance, HSP27 and HSP70 directly safeguard cells against damage-induced cell death pathways[34]. Activation of heat shock factor 1 (HSF1) impedes cellular aging[35]. RNAi HSP83 expression of *Acyrthosiphon pisum* resulted in reduced longevity and fecundity, indicating the evolutionarily conserved function of this chaperone in insects[36]. Examination of longevity in two ecotypes of *Daphnia* revealed that *D. pulicaria*, the long-lived ecotype, exhibits robust induction of HSP70 compared to the short-lived ecotype, *D. pulex*[37]. The small heat shock protein subfamily members (sHSP12s), with a size of 12 kDa, appear to exert critical physiological roles in *Caenorhabditis elegans* by suppressing dauer formation while promoting both longevity and reproduction[38]. The four cytosolic yeast HSP70s, namely Ssa1−4, are considered functional; however, the absence of Ssa1 and Ssa2 leads to a severe reduction in cellular reproduction and accelerates replicative aging[39]. The findings of this study demonstrate that NbHK can activate host HSPs, suggesting that *N. bombycis* may regulate the lifespan of host cells through this protein. Additionally, previous research has indicated that microsporidia can induce alterations in host protein processing within the endoplasmic reticulum (ER) *via* secreted proteins[40]. Therefore, it is plausible to suggest that NbHK might also play a role in modulating host protein processing in the ER. However, further investigations are required to validate the protein contents and functions attributed to these HSPs during *N. bombycis* infection.

Our study revealed that NbHK potentially interacts with NTPR within the host nucleus. NTPR is a crucial component of the nuclear pore complex (NPC), which plays a vital role in genome organization and gene expression[41]. It has been reported that NPC indirectly regulates gene expression by facilitating the nuclear import of gene regulators through the nuclear envelope[42]. Furthermore, NTPR can associate with transcription factors to enhance HSP mRNA production[25]. These findings suggest that NbHK may modulate HSP expression through its interaction with NTPR. Actually, the infection

caused by *N. bombycis* significantly induces host gene expressions involved in pathways such as immune response, biosynthesis, mRNA and protein processing[43], wherein the pathogen secreted proteins as the NbHK likely play crucial roles.

In summary, our work revealed new possible functions of NbHK, providing an underlying model (Figure 8) for further investigation of its microsporidian pathogenesis, such as the control of host protein processing in the ER, mitophagy, and cell death.

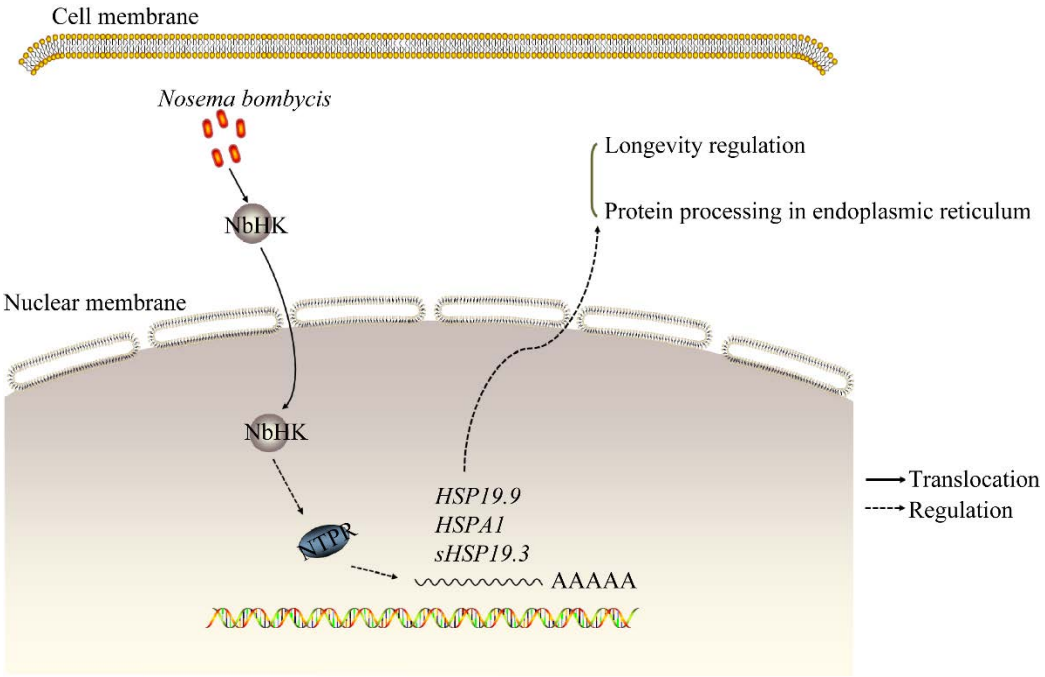

Figure 8 A model of *Nosema bombycis* secretion of NbHK to modulate host gene expression. *N. bombycis* secretes NbHK into the host nucleus, probably to interact with the nuclear pore complex component NTPR to regulate the expressions of *HSP19.9*, *HSPA1*, and *sHSP19.3*, thus modulating host cell longevity and promoting pathogen proliferation.

Availability of data and materials

The raw data of our RNA-seq has been deposited in the Genome Sequence Archive (GSA: CRA011225) that is publicly accessible at https://ngdc.cncb.ac.cn/gsa. The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) *via* the iProX partner repository with the dataset identifier PXD042970.

Competing interests

The authors declare that they have no competing interests.

Supplementary Materials

The supplementary materials have been deposited in National Microbiology Data Center with submission number SUB1702709887194.

Supplementary File 1. Differentially expressed genes in the NbHK::EGFP-transfected BmE cells identified *via* RNA-seq.

Supplementary File 2. Significantly changed proteins isolated from NbHK::APEX2::HA-transfected BmE cells using a biotin-streptavidin system and identified using LC-MS/MS spectra.

参考文献

[1] HAN B, WEISS LM. Microsporidia: obligate intracellular pathogens within the fungal kingdom[J]. Microbiology Spectrum, 2017, 5(2): 1-26.

[2] WILLIAMS B, KEELING P. Cryptic organelles in parasitic protists and fungi[M]//Advances in Parasitology Volume: 54. Amsterdam: Elsevier, 2003: 9-68.

[3] TSAOUSIS AD, KUNJI ERS, GOLDBERG AV, LUCOCQ JM, HIRT RP, EMBLEY TM. A novel route for ATP acquisition by the remnant mitochondria of *Encephalitozoon cuniculi*[J]. Nature, 2008, 453(7194): 553-556.

[4] HEINZ E, HACKER C, DEAN P, MIFSUD J, GOLDBERG AV, WILLIAMS TA, NAKJANG S, GREGORY A, HIRT RP, LUCOCQ JM, KUNJI ERS, EMBLEY TM. Plasma membrane-located purine nucleotide transport proteins are key components for host exploitation by microsporidian intracellular parasites[J]. PLoS Pathogens, 2014, 10(12): e1004547.

[5] HAN B, MA YF, TU V, TOMITA T, MAYORAL J, WILLIAMS T, HORTA A, HUANG H, WEISS LM. Microsporidia interact with host cell mitochondria *via* voltage-dependent anion channels using sporoplasm surface protein 1[J]. mBio, 2019, 10(4): e01944-e01919.

[6] REINKE AW, BALLA KM, BENNETT EJ, TROEMEL ER. Identification of microsporidia host-exposed proteins reveals a repertoire of rapidly evolving proteins[J]. Nature Communications, 2017, 8: 14023.

[7] TOKAREV YS, TIMOFEEV SA, MALYSH JM, TSAREV AA, IGNATIEVA AN, TOMILOVA OG, DOLGIKH VV. Hexokinase as a versatile molecular genetic marker for microsporidia[J]. Parasitology, 2019, 146(4): 472-478.

[8] WOLF AJ, REYES CN, LIANG WB, BECKER C, SHIMADA K, WHEELER ML, CHO HC, POPESCU NI, COGGESHALL KM, ARDITI M, UNDERHILL DM. Hexokinase is an innate immune receptor for the detection of bacterial peptidoglycan[J]. Cell, 2016, 166(3): 624-636.

[9] LAUTERWASSER J, FIMM-TODT F, OELGEKLAUS A, SCHREINER A, FUNK K, FALQUEZ-MEDINA H, KLESSE R, JAHREIS G, ZERBES RM, O'NEILL K, van der LAAN M, LUO X, EDLICH F. Hexokinases inhibit death receptor-dependent apoptosis on the mitochondria[J]. Proceedings of the National Academy of Sciences of the United States of America, 2021, 118(33): e2021175118.

[10] CUOMO CA, DESJARDINS CA, BAKOWSKI MA, GOLDBERG J, MA AT, BECNEL JJ, DIDIER ES, FAN L, HEIMAN DI, LEVIN JZ, YOUNG S, ZENG QD, TROEMEL ER. Microsporidian genome analysis reveals evolutionary strategies for obligate intracellular growth[J]. Genome Research, 2012, 22(12): 2478-2488.

[11] 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[J]. PLoS One, 2014, 9(4): e93585.

[12] TIMOFEEV SA, SENDERSKIY IV, TSAREV AA, TOKAREV YS, DOLGIKH VV. Heterologous expression of *Paranosema (Antonospora*) locustae hexokinase in lepidopteran, Sf9, cells is followed by accumulation of the microsporidian protein in insect cell nuclei[J]. Journal of Invertebrate Pathology, 2017, 143: 104-107.

[13] HUANG YK, ZHENG SY, MEI XE, YU B, SUN B, LI BN, WEI JH, CHEN J, LI T, PAN GQ, ZHOU ZY, LI CF. A secretory hexokinase plays an active role in the proliferation of *Nosema bombycis*[J]. PeerJ, 2018, 6: e5658.

[14] FERGUSON S, LUCOCQ J. The invasive cell coat at the microsporidian *Trachipleistophora hominis*-host cell interface contains secreted hexokinases[J]. Microbiology Open, 2019, 8(4): e00696.

[15] LIU H, CHEN BS, HU SR, LIANG XL, LU XM, SHAO YQ. Quantitative proteomic analysis of germination of *Nosema bombycis* spores under extremely alkaline conditions[J]. Frontiers in Microbiology, 2016, 7: 1459.

[16] DOLGIKH VV, TSAREV AA, TIMOFEEV SA, ZHURAVLYOV VS. Heterologous overexpression of active hexokinases from microsporidia *Nosema bombycis* and *Nosema ceranae* confirms their ability to phosphorylate host glucose[J]. Parasitology Research, 2019, 118(5): 1511-1518.

[17] DOLGIKH VV, ZHURAVLYOV VS, SENDERSKIY IV, IGNATIEVA AN, TIMOFEEV SA, SELIVERSTOVA EV. Heterologous expression of scFv fragment against *Vairimorpha* (*Nosema*) ceranae

hexokinase in Sf9 cell culture inhibits microsporidia intracellular growth[J]. Journal of Invertebrate Pathology, 2022, 191: 107755.

[18] LI T, PAN GQ, VOSSBRINCK CR, XU JS, LI CF, CHEN J, LONG MX, YANG M, XU XF, XU C, DEBRUNNER-VOSSBRINCK BA, ZHOU ZY. SilkPathDB: a comprehensive resource for the study of silkworm pathogens[J]. Database, 2017, 2017: bax001.

[19] KAWAMOTO M, KIUCHI T, KATSUMA S. SilkBase: an integrated transcriptomic and genomic database for *Bombyx mori* and related species[J]. Database, 2022, 2022: baac040.

[20] KIM D, PAGGI JM, PARK C, BENNETT C, SALZBERG SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype[J]. Nature Biotechnology, 2019, 37(8): 907-915.

[21] LOVE MI, HUBER W, ANDERS S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2[J]. Genome Biology, 2014, 15(12): 1-21.

[22] WU TZ, HU EQ, XU SB, CHEN MJ, GUO PF, DAI ZH, FENG TZ, ZHOU L, TANG WL, ZHAN L, FU XC, LIU SS, BO XC, YU GC. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data[J]. Innovation (Cambridge (Mass)), 2021, 2(3): 100141.

[23] UNIPROT CONSORTIUM T. UniProt: the universal protein knowledgebase[J]. Nucleic Acids Research, 2018, 46(5): 2699.

[24] TYANOVA S, TEMU T, COX J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics[J]. Nature Protocols, 2016, 11(12): 2301-2319.

[25] SKAGGS HS, XING HY, WILKERSON DC, MURPHY LA, HONG YL, MAYHEW CN, SARGE KD. HSF_1-TPR interaction facilitates export of stress-induced HSP70 mRNA[J]. The Journal of Biological Chemistry, 2007, 282(47): 33902-33907.

[26] FROSST P, GUAN TL, SUBAUSTE C, HAHN K, GERACE L. Tpr is localized within the nuclear basket of the pore complex and has a role in nuclear protein export[J]. The Journal of Cell Biology, 2002, 156(4): 617-630.

[27] KRULL S, THYBERG J, BJÖRKROTH B, RACKWITZ HR, CORDES VC. Nucleoporins as components of the nuclear pore complex core structure and Tpr as the architectural element of the nuclear basket[J]. Molecular Biology of the Cell, 2004, 15(9): 4261-4277.

[28] MITCHELL PJ, COOPER CS. Nucleotide sequence analysis of human *tpr* cDNA clones[J]. Oncogene, 1992, 7(2): 383-388.

[29] HU YL, CAO KL, WANG F, WU WY, MAI WH, QIU LY, LUO YX, GE WP, SUN BG, SHI LG, ZHU JM, ZHANG JM, WU ZY, XIE YC, DUAN SM, GAO ZH. Dual roles of hexokinase 2 in shaping microglial function by gating glycolytic flux and mitochondrial activity[J]. Nature Metabolism, 2022, 4(12): 1756-1774.

[30] de JESUS A, KEYHANI-NEJAD F, PUSEC CM, GOODMAN L, GEIER JA, STOOLMAN JS, STANCZYK PJ, NGUYEN T, XU K, SURESH KV, CHEN YH, RODRIGUEZ AE, SHAPIRO JS, CHANG HC, CHEN CL, SHAH KP, BEN-SAHRA I, LAYDEN BT, CHANDEL NS, WEINBERG SE, et al. Hexokinase 1 cellular localization regulates the metabolic fate of glucose[J]. Molecular Cell, 2022, 82(7): 1261-1277.e9.

[31] LAURIAN R, RAVENT J, DEMENTHON K, LEMAIRE M, SOULARD A, COTTON P. *Candida albicans* hexokinase 2 challenges the *Saccharomyces cerevisiae* moonlight protein model[J]. Microorganisms, 2021, 9(4): 848.

[32] AHUATZI D, HERRERO P, de la CERA T, MORENO F. The glucose-regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent[J]. Journal of Biological Chemistry, 2004, 279(14): 14440-14446.

[33] LAURIAN R, DEMENTHON K, DOUMÈCHE B, SOULARD A, NOEL T, LEMAIRE M, COTTON P. Hexokinase and glucokinases are essential for fitness and virulence in the pathogenic yeast *Candida albicans*[J]. Frontiers in Microbiology, 2019, 10: 327.

[34] STEINKRAUS KA, SMITH ED, DAVIS C, CARR D, PENDERGRASS WR, SUTPHIN GL, KENNEDY BK, KAEBERLEIN M. Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in *Caenorhabditis elegans*[J]. Aging Cell, 2008, 7(3): 394-404.

[35] CALDERWOOD SK, MURSHID A, PRINCE T. The shock of aging: molecular chaperones and the heat shock response in longevity and aging: a mini-review[J]. Gerontology, 2009, 55(5): 550-558.

[36] WILL T, SCHMIDTBERG H, SKALJAC M, VILCINSKAS A. Heat shock protein 83 plays pleiotropic roles in embryogenesis, longevity, and fecundity of the pea aphid *Acyrthosiphon pisum*[J]. Development Genes and Evolution, 2017, 227(1): 1-9.

[37] SCHUMPERT C, HANDY I, DUDYCHA JL, PATEL RC. Relationship between heat shock protein 70 expression and life span in *Daphnia*[J]. Mechanisms of Ageing and Development, 2014, 139: 1-10.

[38] FU XM, EZEMADUKA AN, LU XP, CHANG ZY. The *Caenorhabditis elegans* 12 kDa small heat shock proteins with little *in vitro* chaperone activity play crucial roles for its dauer formation, longevity, and reproduction[J]. Protein Science, 2021, 30(10): 2170-2182.

[39] ANDERSSON R, EISELE-BÜRGER AM, HANZÉN S, VIELFORT K, ÖLING D, EISELE F, JOHANSSON G, GUSTAFSSON T, KVINT K, NYSTRÖM T. Differential role of cytosolic Hsp70s in longevity assurance and protein quality control[J]. PLoS Genetics, 2021, 17(1): e1008951.

[40] HAN Y, GAO H, XU J, LUO J, CHEN J, HAN B, LI T, ZHOU Z. Microsporidian *Encephalitozoon hellem* secretes EhPTP4 to regulate host endoplasmic reticulum-associated degradation[J]. Acta Microbiologica Sinica, 2022, 62(1): 357-373.

[41] AKSENOVA V, SMITH A, LEE H, BHAT P, ESNAULT C, CHEN SE, IBEN J, KAUFHOLD R, YAU KC, ECHEVERRIA C, FONTOURA B, ARNAOUTOV A, DASSO M. Nucleoporin TPR is an integral component of the TREX-2 mRNA export pathway[J]. Nature Communications, 2020, 11: 4577.

[42] TAMURA K. Nuclear pore complex-mediated gene expression in *Arabidopsis thaliana*[J]. Journal of Plant Research, 2020, 133(4): 449-455.

[43] 梁喜丽, 鲁兴萌, 邵勇奇. 鳞翅目昆虫病原微孢子虫研究进展[J]. 微生物学报, 2018, 58(6): 1064-1076.
LIANG XL, LU XM, SHAO YQ. Advances in studies on *Lepidoptera microsporidium* pathogen[J]. Acta Microbiologica Sinica, 2018, 58(6): 1064-1076 (in Chinese).