

A secretory hexokinase plays an active role in the proliferation of *Nosema bombycis*

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ABSTRACT

The microsporidian *Nosema bombycis* is an obligate intracellular parasite of *Bombyx mori*, that lost its intact tricarboxylic acid cycle and mitochondria during evolution but retained its intact glycolysis pathway. *N. bombycis* hexokinase (NbHK) is not only a rate-limiting enzyme of glycolysis but also a secretory protein. Indirect immunofluorescence assays and recombinant HK overexpressed in BmN cells showed that NbHK localized in the nucleus and cytoplasm of host cell during the meront stage. When *N. bombycis* matured, NbHK tended to concentrate at the nuclei of host cells. Furthermore, the transcriptional profile of *NbHK* implied it functioned during *N. bombycis*' proliferation stages. A knock-down of *NbHK* effectively suppressed the proliferation of *N. bombycis* indicating that NbHK is an important protein for parasite to control its host.

Subjects Microbiology, Molecular Biology, Parasitology

Keywords Microsporidia, Hexokinase, Secretory protein, RNAi

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INTRODUCTION

Microsporidia are unicellular eukaryotes and obligate intracellular parasites that have broad host range (Cali & Takvorian, 1999). Microsporidia can infect invertebrates and vertebrates including human beings (Cali & Takvorian, 1999; Franzen, 2004). The life cycle of a microsporidia can be divided into the dormant extracellular and intracellular proliferation stages (Bigliardi & Sacchi, 2001). As obligate parasites, most microsporidia no longer have an intact tricarboxylic acid cycle or mitochondria, which implies an acute dependence on host energy during the intracellular stage (Hacker et al., 2014). In *Encephalitozoon cuniculi* and *Trachipleistophora hominis*, a series of nucleotide transport proteins (NTTs) can steal ATP from hosts (Heinz et al., 2014; Tsaousis et al., 2008). Owing to the absence of oxidative phosphorylation in microsporidia, glucose metabolism by glycolysis releases 7% of full ATP potential (Berg, Tymoczko & Stryer, 2007). Even so, some microsporidia still maintain their intact glycolytic pathways, which may function during the extracellular stage (Wiredu et al., 2017). Although some microsporidia, such as

Enterocytozoon bieneusi, lack its intact glycolytic pathway, most microsporidia still possess a hexokinase (HK) (Wiredu et al., 2017), which implies that HK may be a necessary protein for the infection and proliferation of microsporidia.

Hexokinase is a multifunctional protein that can act in transcription regulation (Herrero et al., 1995; Niederacher & Entian, 1991) and apoptosis (Bryson et al., 2002; Gottlob et al., 2001), as well as with the cytokine neuroleukin (a nerve growth factor) (Chaput et al., 1988; Faik et al., 1988; Gurney et al., 1986). Furthermore, HK also participates in interaction between pathogen-host. For example, in macrophage, HK is an innate immune receptor for the detection of bacterial peptidoglycan (Wolf et al., 2016). In intracellular pathogen, such as *Plasmodium falciparum*, HK usually plays a basic role in the conversion of glucose to glucose 6-phosphate (Faik et al., 1988). A pathogen secreted HK was first reported in the microsporidia *Nematocida parisii* (Cuomo et al., 2012), which was also demonstrated using biochemical experiments (Reinke et al., 2017). This phenomenon was reported in *Paranosema (Antonospora) locustae* as well. The *P. locustae* HK was predicted to contain a signal peptide, and the indirect immunofluorescent assay (IFA) showed a nuclear localization in the host (Senderskiy et al., 2014; Timofeev et al., 2017). Then, special secreting hexokinase function which work as a regulator to increase ATP generation to parasite surface, was explored in *Trachipleistophora hominis* (Ferguson & Lucocq, 2018).

Nosema bombycis can infect Lepidoptera, including the economical insect silkworm, causing pebrine disease by vertical and horizontal transmission (Han & Watanabe, 1988). During infection, *N. bombycis* synthesizes many kinds of secretory proteins to control the host cells (Tian, 2013). Just like *P. locustae*, *N. bombycis* has an intact glycolysis pathway (Wiredu et al., 2017). Therefore, we hypothesized that NbHK can be secreted into host cells and has a significant impact on *N. bombycis*' proliferation.

MATERIALS & METHODS

Hexokinase sequence analysis and ORF amplification

The amino acid sequence of NbHK was submitted to SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) and NCBI (<https://www.ncbi.nlm.nih.gov/>) for signal peptide and domain predictions. The HK of *N. bombycis* (GenBank Accession No. EOB11276.1) was amplified from genomic DNA (gDNA) by PCR. The amplification reaction consisted of 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min using the forward primer 5'-CGCGGATCCATGATAATTTTCTATTGT-3', containing a *Bam* HI restriction site (GGATCC), and the reverse primer 5'-CCGCTCGAGATAAATAATTCGATGTAAAG-3' containing a *Xho* I restriction site (CTCGAG). PCR products were recovered (Omega, Norcross, GA, USA), integrated into pET-32 vector (TaKaRa, Kusatsu, Japan), then the recombinant vectors were transformed into *Escherichia coli* DH5 α competent cells. The identified pET-32a-NbHK vector was sequenced by Sangon (Shanghai, China).

Protein expression, purification and polyclonal antibody preparation

The pET-32a-NbHK vector was transformed into *E. coli* Rosetta for expression. After cultivation in 37 °C, the recombinant bacteria was induced for 20 h at 16 °C with 1 mM IPTG in LB medium. Nickel chelating affinity chromatography (Roche, Basel, Switzerland)

was used to purify the target protein fused with hexahistidine. BALB/c mice were used to generate antiserum by immunizing with 100 μ g recombinant HK protein homogenized with Freund's adjuvant (V/V=1:1, Sigma-Aldrich, St. Louis, MO, USA) four times. Complete Freund's adjuvant was used in the first injection, then the incomplete Freund's adjuvant was used in the following injections. All animal experiments were approved by Laboratory Animals Ethics Review Committee of Southwest University guidelines (Chongqing, China) (Permit Number: SYXK- 2017-0019).

Indirect immunofluorescent assay

Infected cells and healthy cells were fixed in PBS with 4% paraformaldehyde, then treated with 0.5% Triton X-100 for 10 min at room temperature, blocked with blocking reagent (0.5% (v/v) bovine serum albumin and 10% (w/v) goat serum) for 1 h, incubated with 1:500 dilutions of HK-antiserum (mouse) and Nb β -tubulin polyclonal antibody (rabbit) at 37 °C for 60 min. After washing with PBS three times (5 min each time), Alexa Fluor 488 and 594 (Thermo Fisher, Santa Clara, CA, USA) were used to detect the binding of primary antibodies at room temperature. The nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher, Santa Clara, CA, USA) for 10 min. Samples were observed and imaged with Olympus FV1200 (Olympus, Tokyo, Japan).

Protein preparation

Protein of infected cells, mature spores and healthy cells were prepared by the glass-bead breaking method as reported ([Geng et al., 2013](#)). The samples with 0.4 g glass beads (212–300 μ m), were lysed in RIPA Lysis Buffer (Beyotime, Shanghai, China) containing a protease inhibitor (phenylmethylsulfonyl fluoride), and then crushed for 5 min at 4 °C using a Bioprep-24 Homogenizer (ALLSHENG, Hangzhou, China). The treated samples were centrifuged at 12,000 rpm for 5 min and the supernatant were collected.

Immunoblot analysis

Proteins were separated by sodium dodecyl SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). After blocking in blocking buffer (5% (w/v) skim milk, 20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20), membrane incubated with an HK-antiserum or Nb β -tubulin-antiserum (diluted 1:3,000) ([Chen et al., 2017](#)), washed, and incubated with HRP-labeled goat anti-mouse IgG (diluted 1:6,000; Sigma, Saint Louis, MI, USA). The bound antibodies were detected by ECL Plus Western Blotting Detection Reagents (Bio-Rad, Richmond, CA, USA). The protein concentrations were detected with BCA Kit (Beyotime, Shanghai, China), and loading quantity of samples were normalized on the basis of Nb β -tubulin quantity.

Expression of recombinant HK fused with DsRed in BmN

NbHK was cloned using the forward primer 5'-GGGTACCATGATAATTTCTATTGTCTAC-3' or 5'-GGTACCTTAATTAAGACATTGGGAAATA-3' (to remove the signal peptide (Δ SG)), each containing a *KpnI* restriction site (GGTACC) and the reverse primer 5'-TGACCCTGAGCCTCCATAAATAATTCGATGTAAAG-3' containing a G₃S₂ linker sequence (GGAGGCTCAGGGTCA). The DsRed, a red fluorescent protein, was cloned

used the forward primers 5'-GGAGGCTCAGGGTCAATGGTGCCTCCTCCAAGAAC-3' containing a G₃S₂ linker sequence (GGAGGCTCAGGGTCA) and the reverse primer 5'-CCTCGAGGCGGCCGCTACAGGAACAGG-3' containing a *Xho* I restriction site (CTCGAG). Then the *DsRed* was linked to the *rHK* and *rHK*Δ*SG* respectively through a G₃S₂ linker peptide by overlapping PCR. Then the overlapping PCR products were integrated into pCR-Blunt II-TOPO vector (Thermo Fisher, Santa Clara, CA, USA), after which the products of linkage were transform into *E. coli* DH5α. The recombinant pCR-Blunt II-TOPO vector contained the targets fragments, were extracted from *E. coli* DH5α. The above vectors and pIZ/V5-His (Thermo Fisher, Santa Clara, CA, USA) were digested by *Kpn*I and *Xho* I (Thermo Fisher, CA, USA). The digested *rHK* and *rHK*Δ*SG* fusing with *DsRed* were integrated into digested pIZ/V5-His, which was induced by T4 DNA ligase (New England Biolabs, MA, USA). Two μg constructed vectors were transiently transfected into BmN cells using X-tremeGENE™ HP DNA Transfection Reagent (Thermo Fisher, Santa Clara, CA, USA). Three days later, the transfected cells nuclei were labeled with Hoechst (Thermo Fisher, Santa Clara, CA, USA). Then the samples were examined by confocal microscopy (Olympus, Tokyo, Japan).

RNA interference (RNAi) fragments synthesis

The sequence of *NbHK* was submitted to BLOCK-iT™ RNAi Designer (<http://rnaidesigner.thermofisher.com/rnaexpress/design.do>). A 352-bp fragment that contain five potential interferential dsRNA fragments was amplified by the primers F-RI-Hexokinase-T7 5'-TAATACGACTCACTATAGGGAGAAGGAATATACTTGTCTGGGA-3' and R-RI-Hexokinase-T7 5'-TAATACGACTCACTATAGGGAGATTGACAGGTCTCTCAAATGC-3', each containing the T7 promoters (TAATACGACTCACTATAGGGAGA). The amplified product was used as template to synthesize dsRNA using a RiboMAX Large Scale System-T7 Kit (Promega, Madison, WI, USA). The dsRNA-*EGFP*, which was used as the mock group, was prepared with the same method by the primers F-RI-EGFP 5'-TAATACGACTCACTATAGGGAGAACGGCAAGCTGACCCTGAA-3' and R-RI-EGFP 5'-TAATACGACTCACTATAGGGAGATGTTGTAGTTGTACTCCAG-3', each containing the T7 promoters as well.

DNA and cDNA collection from infected samples

Spores were separated from hemolymph of severely infected silkworm pupae. The spores which were pretreated with 0.1 mol/L KOH, were added to the Sf9-III cells (cell: spores ratio, 1:5) (*Kawarabata & Ren, 1984*). Infected cells were collected at 1, 3, 5 days post infection (d. p. i) and stored in PBS or TRIzol (Invitrogen, Carlsbad, CA, USA). The newly molted 4th instar silkworm larvae were oral fed with 1×10^7 spores per. Then, the infected silkworms' midguts were collected and stored in TRIzol at -80°C immediately. The gDNA of infected Sf9-III cells was extracted using a DNA Extraction Kit (Omega, Norcross, GA, USA), while the cDNA of infected cells and midguts were prepared using Total RNA Kit II (Omega, Norcross, GA, USA) and RT-PCR Kit (Promega, Madison, WI, USA). Samples were taken from three separate experimental, mock and blank groups at each time point.

RNAi of *N. bombycis* in infected Sf9-III

The Sf9-III (Thermo Fisher, Santa Clara, CA, USA) cells were infected by using the previous method described above. After infection, two μg dsRNAs of *NbHK* or *EGFP* were transfected into Sf9-III respectively and then cultured in 6-well plates (Saleh *et al.*, 2016). The samples were collected at 1, 3 and 5 d. p. i.

Cell counting

The cell samples were suspended with one mL culture medium, then 10 μL cell suspensions were mixed with 10 μL trypan blue. Living counting were finished with Countess II FL (Thermo Fisher, Santa Clara, CA, USA).

Real-time quantitative PCR analysis

For transcription detections, one μg RNA was used to conduct reverse transcript PCR. Then, the products were diluted ten times as templates. Quantitative PCR was amplified using Hexokinase-qF 5'-CAAAATGTGATTATTATGGGAGATG-3' and Hexokinase-qR 5'-CGATGTAAAGTATAAAGGGCTGAT-3' primers, and reference gene primers SSU-qF 5'-CTGGGGATAGTATGATCGCAAGA-3' and SSU-qR 5'-CACAGCATCCATTGGAAA CG-3' (Huang *et al.*, 2018). Quantitative PCR was performed with following program: a pre-denaturation of 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 20 s (LightCycle 96, Roche, Switzerland). Expression of *SfHk* was detected using the same method with SfHK-qF 5'-TCACTTACATTCAAGATTTACCCAA-3' and SfHK-qR 5'-CTACGCCAGAACAAGAAAAGC-3' primers, and reference gene primers SfGAPDH-qF 5'-GGCTGGCGCTGAATACATCGTTGAGTCCAC-3' and SfGAPDH-qR 5'-TTAGCAACGGGAACACGGAAAGCCATAACCAG-3'.

For *Nb β -tubulin* copy number detection, one μL (~ 200 ng/ μL) of the each gDNA samples extracted from the above infected cells was analyzed by qPCR. The 10 μL reaction systems were conducted using the Nb β -tubulin-qF 5'-AGAACCAGGAACAATGGACG-3' and Nb β -tubulin-qR 5'-AGCCCAATTATTACCAGCACC-3' primers. Real-time PCRs were performed with the above program (LightCycle 96; Roche, Basel, Switzerland). The standard template had been constructed in previous research (Huang *et al.*, 2018). The standard curve covered four orders of magnitude for the starting quantity (1.3×10^3 – 10^6). The multiple T tests were conducted with GraphPad.Prism.v6.01.

RESULTS

Recombinant hexokinase purification and immunoblot analysis

The amino acid sequence analysis showed that NbHK contained a signal peptide, which implied it was a secretory protein (Fig. 1A). The ORF of *HK* was cloned by PCR using specific primers based on the gDNA of *N. bombycis*. The target gene were successfully integrated into pET-32a vector, which was validated by PCR and restriction enzyme digestion (Fig. 1B). The sequencing results showed a 1,287-bp fragment encoding 428 amino acids, which was consistent with the data from the genomic sequence of *N. bombycis* in SilkPathDB (<https://silkpathdb.swu.edu.cn/>) (Fig. S1).

SDS-PAGE analysis showed that rHK was expressed at a molecular mass of ~ 60 kDa, which was consistent with the predicted size (Fig. 1C). The purified protein was used to

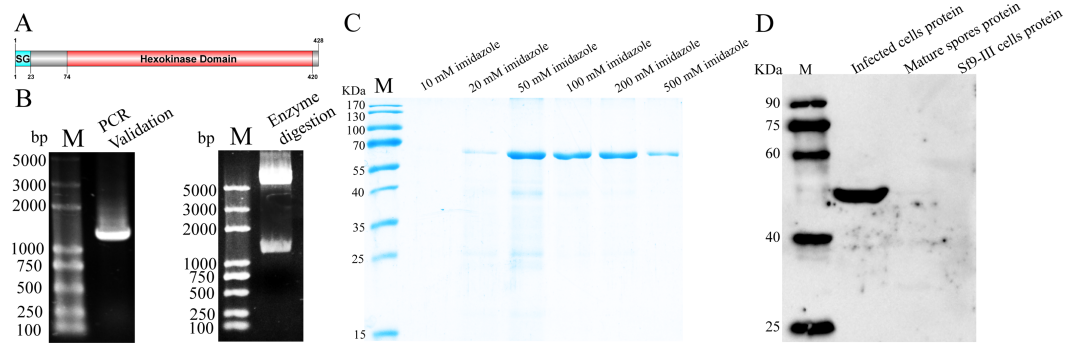


Figure 1 Expression and analysis of NbHK. (A) Protein structure of NbHK. There is a signal peptide contacting 23 amino acids in the N terminus. (B) Validation of the pET-32-NbHK vector by PCR (left) and *Bam* HI/*Xho* I enzyme digestion (right). Products 1,300 bp were amplified by PCR or cleaved from the constructed vector. M: 2K Plus (TaKaRa, Kusatsu, Japan). (C) Purification of recombinant HK. Recombinant HK was eluted by elution buffer containing different concentration of imidazole and analyzed by SDS-PAGE. (D) Specificity of the HK antiserum. Proteins extracted from infected Sf9-III, mature spores and healthy Sf9-III were subjected to western blot using polyclonal antibody against HK. M, Protein maker (Transgene, Shanghai, China).

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prepare the antibody. The titer of the HK antiserum was determined by ELISA (Fig. S2). Western blot indicated that the HK antiserum can recognize a 50 kDa protein specifically in the infected cells proteins, but the signal was not demonstrated in mature spores and healthy Sf9-III cells. (Fig. 1D). The molecular mass difference between rHK and native HK, which was consist in infected cells, was due to tag proteins.

Subcellular localization of NbHK

The subcellular localization of NbHK was analyzed by IFA. Nb β -tubulin was used as a marker protein to distinguish the meront stage from mature spores. Meronts can be labeled using the Nb β -tubulin antibody, while the mature spores can be observed visibly in differential-interference microscopy (DIC) (Chen et al., 2017; Huang et al., 2018). The IFA demonstrated the NbHK can be secreted into host cells (Fig. 2). In the proliferation stage, NbHK was located in the nucleus and cytoplasm of host cell (Figs. 2A–2E). When *N. bombycis* matured, the NbHK tended to be concentrated in the nuclei of host cells (Figs. 2F–2J). The change in localization implied that NbHK's functions might be different, depending on the growth stage of *N. bombycis*. The antibody of NbHK cannot bind to host cell in uninfected cells (Fig. S3).

The vectors that encode fusion proteins HK::DsRed and HK Δ SG::DsRed were independently transfected into BmN cells (Fig. 3A). The fluorescence signal showed that rHK was localized in the cytoplasm of healthy cells, independent of presence of its signal peptide (Figs. 3B, 3C). When the BmN cells' nuclei became diffuse, indicating a trend toward apoptosis, the HK::DsRed co-localized with the nuclei (Fig. 3B, white arrow). The similar results were demonstrated in transfected Sf9-III cells (Fig. S4).

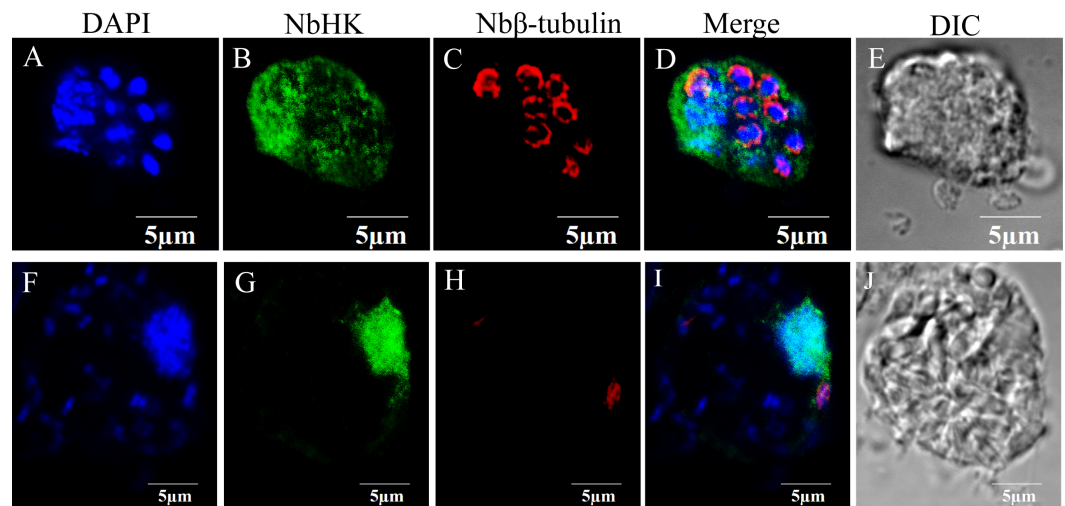


Figure 2 Subcellular localization of NbHK in infected Sf9-III. Localization of NbHK was imaged with confocal microscopy at different stage of infection. Green and red fluorescence were observed in the samples incubated with the HK antibody (mouse) and rabbit Nb β -tubulin antiserum, respectively. The nuclei of host cells and *N. bombycis* were labeled with DAPI (blue fluorescent signal) (Thermo Fisher, Santa Clara, CA, USA). (A–E) Localization of NbHK in the meront stage of *N. bombycis*. The parasites were bound by Nb β -tubulin antibody, which showed that the meronts lacked a chitin layer. The NbHK dispersed in the whole host cell. (F–J) Localization of NbHK in the mature spore stage of *N. bombycis*. The chitin layer of the mature spore blocked the binding of the Nb β -tubulin antibody, and the mature spore could be observed by DIC. NbHK was concentrated in host cell nuclei at this stage.

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Transcriptional profile of *NbHK* in infected cells and midguts

In infected Sf9-III cells, *NbHK* was expressed at the early stage of infection and showed a highly significant increase from 8 to 48 h post infection (h. p. i). Then, transcription levels were relatively stayed stable from 48 to 72 h.p.i and were slightly down-regulated from 72 to 96 h.p.i (Fig. 4A). In infected midgut of *B. mori*, the *NbHK* expression pattern was similar to that of infected cells. *NbHK* was gradually up-regulated, except for a slight down-regulation at 24 h. p. i (Fig. 4B). The transcript profile of *NbHK* implied it functioned during all proliferation stages of *N. bombycis*.

Down-regulation of *NbHK* suppressed *N. bombycis*' proliferation

The effects of RNAi were assessed using qPCR and western blot. Transcriptional levels revealed that the expression of *NbHK* was significantly down-regulated by dsRNA in the experimental groups (dsRNA-*HK*) compared with the mock groups (dsRNA-*EGFP*) at three and five d.p.i (Fig. 5A). Besides, the western blot also showed NbHK down-regulated at three d.p.i, which was basically consistent with transcriptional detection result (Fig. 5B).

The copy number of *Nbβ-tubulin* which lacks homology in the host was used to the reflect the infection level of the two groups (Huang et al., 2018). The infection of level the two groups kept similar at 1 d.p.i, because the effect of RNAi was not obvious at this time. In the mock groups, *N. bombycis* began to proliferate from one to five d.p.i while the proliferation of *N. bombycis* was remarkably inhibited in the experimental groups at three d.p.i (Fig. 5C).

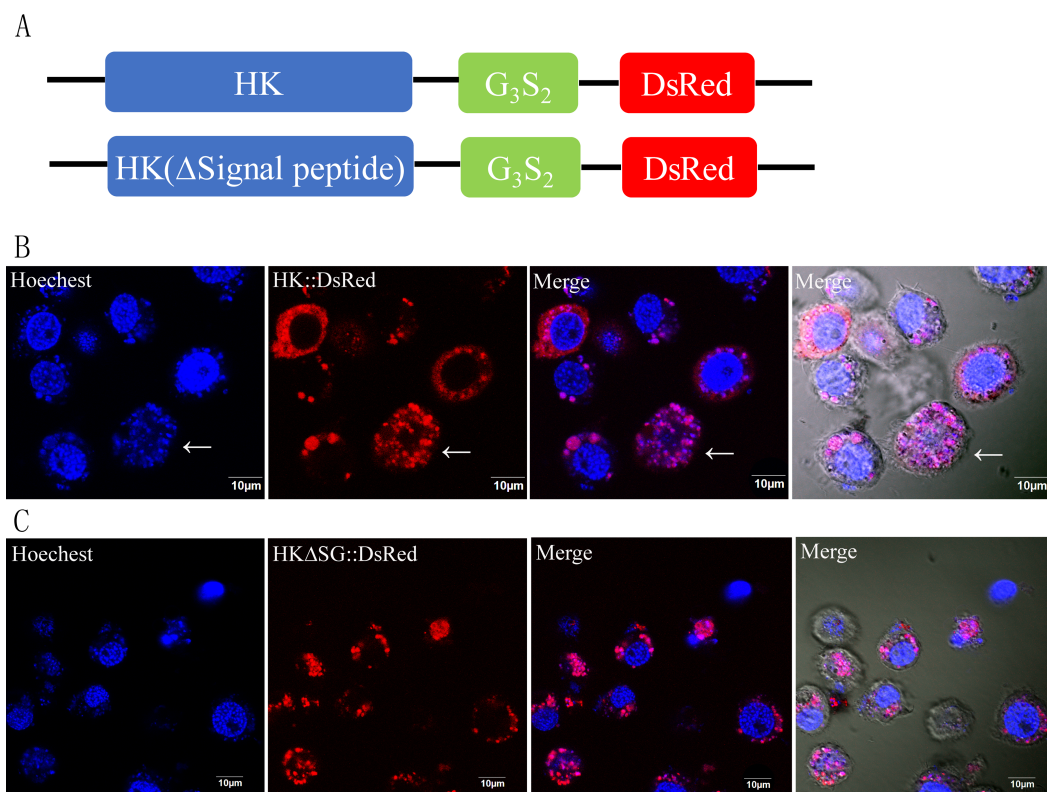


Figure 3 Subcellular localization of HK::DsRed and HK Δ SG::DsRed in BmN. (A) HK, with or without its signal peptide, was fused with DsRed. The two expression cassettes were integrated into pIZ/v5-His, and then the constructed vectors were transfected into BmN. (B) Subcellular localization of HK::DsRed in BmN. The cell nucleus was labeled with Hoechst. The white arrow indicates an apoptotic cell. (C) Subcellular localization of HK Δ SG::DsRed in BmN.

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The dsRNA-EGFP, dsRNA-HK and transfection have no effect on proliferation of host cells and expression of SfHK (Figs, S5A, SB). Besides, the RNAi constructs have no impact on proliferation of *N. bombycis* (Fig. S6).

DISCUSSION

NbHK was predicted to be a secretory protein with a signal peptide, suggesting its potential localization inside host cells. The location of NbHK during the proliferation stage of *N. bombycis* infection implied it may play a role in regulating and controlling the host's energy metabolism (Ferguson & Lucocq, 2018). HK, with or without its signal peptide, and fused with DsRed was expressed in BmN cells, which showed a similar subcellular localization. The localization of eukaryotic expressed rHK revealed that the signal peptide could not lead the rHK to be secreted or to enter into nuclei of healthy BmN or Sf9-III. The localization results were different from those of *P. locustae* HK which is mainly concentrated on hosts' nuclei (Senderskiy et al., 2014; Timofeev et al., 2017). Expression profiles *in vivo* and *in vitro* also indicated NbHK continuous high transcriptional level during proliferation stages of *N. bombycis* infection and the western blot showed NbHK

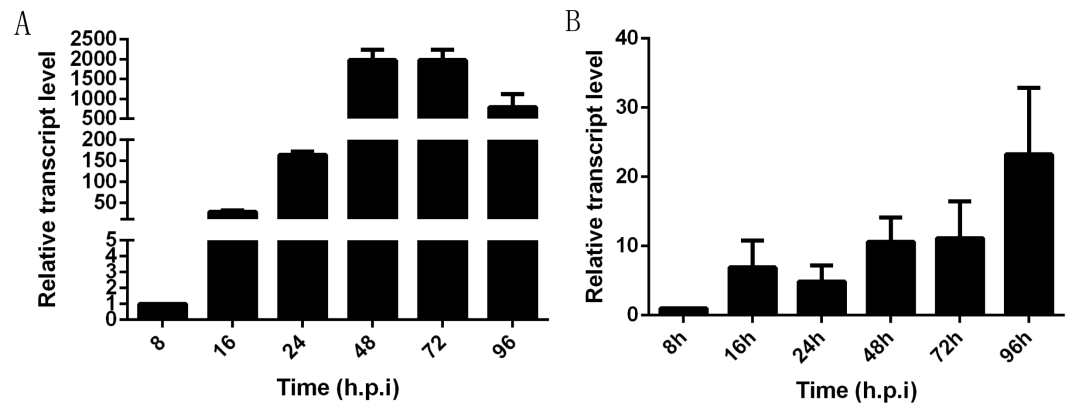


Figure 4 Transcriptional profile of *NbHK* in infected Sf9-III cells and midguts. The relative expression level of *NbHK* in infected Sf9-III cells and midguts at different time points. They are presented relative to the 8 h.p.i. *N. bombycis* SSU rRNA was the reference gene for the normalization of expression levels. (A) Transcript level of *NbHK* in infected Sf9-III cells. (B) Transcript level of *NbHK* in infected midgut of *B. mori*. Vertical bars show the mean \pm SEs ($n = 3$).

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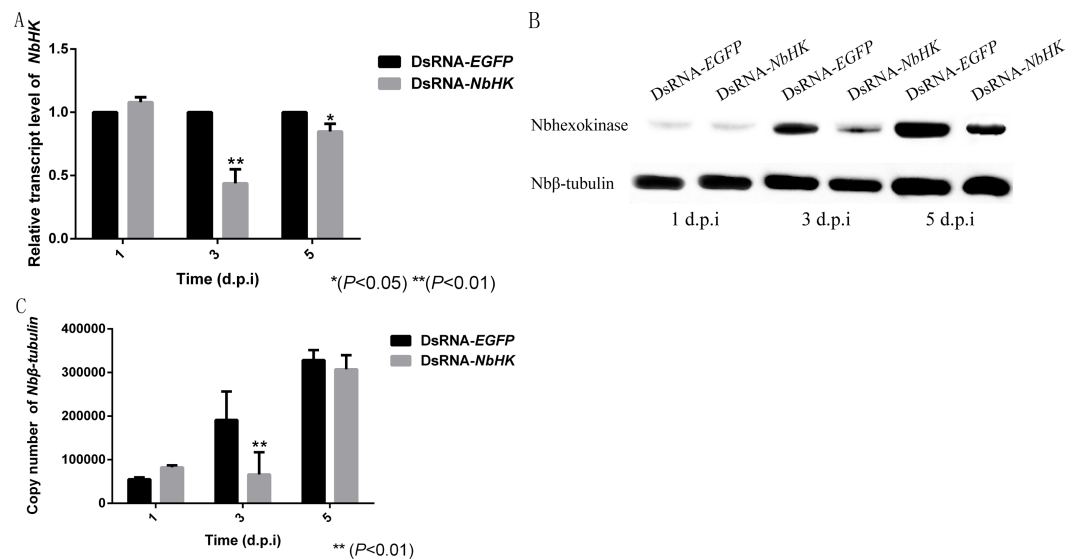


Figure 5 Effect of *NbHK* down-regulation on *N. bombycis* proliferation. (A) The transcription levels of *NbHK*. Complementary DNAs of the experimental (dsRNA-*NbHK*) and mock (dsRNA-*EGFP*) groups were analyzed by qPCR. The $\Delta\Delta C_t$ method was conducted to process the data. (B) *NbHK* expression levels of *NbHK*. Total proteins of experimental (dsRNA-*NbHK*) and mock (dsRNA-*EGFP*) groups were analyzed by western blot. Loading quantity of samples were normalized by Nbβ-tubulin, and *NbHK* quantities were detected with its antiserum. (C) Infection levels of Sf9-III. Genomic DNA was extracted from experimental (dsRNA-*NbHK*) and mock (dsRNA-*EGFP*) groups at 1, 3 and 5 d.p.i. Copy numbers of *Nbβ-tubulin* indicated the infection levels. Statistically significant differences are represented with asterisks (* $P < 0.05$; ** $P < 0.01$). Bars represent the standard deviations of three independent replicates.

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barely exist in mature spores, which implied the NbHK just functioned in intracellular stage. But how microsporidia kept basic energy metabolism was still a secret. It is uncommon for an energy metabolism-related enzyme to be secreted into host cells and acted as a potential moderator. Although we showed that the down-regulation of *NbHK* expression can inhibit the proliferation of *N. bombycis*, the specific mechanisms involved need further study.

In microsporidia, there is only a tiny mitochondrial remnant called a mitosome (Goldberg *et al.*, 2008; Williams *et al.*, 2002), which implied that they are less prone to produce energy by themselves. The unique NTTs of microsporidia allow for a simplified energy metabolism pathway (Dean *et al.*, 2018; Tsaousis *et al.*, 2008). Although there is no ATP generation during the conversion of glucose to glucose 6-phosphate, it indirectly promotes glycolysis. The high transcription of *NbHK* and its importance function implied the glycolysis also play a key role during its intracellular stages, even microsporidia could steal ATP from host. HK is a control valve of glycolysis. The secretory HK of microsporidia turn up the valve of glycolysis and the tricarboxylic acid cycle, which is a clever strategy to steal energy from the host (Ferguson & Lucocq, 2018).

The lack of a stable and reliable gene manipulation method has impeded research on *N. bombycis* protein functions. In 2016, an EGFP was expressed in *N. bombycis* through a non-transposon vector (Rui *et al.*, 2016). This was the first step toward the generation of successful transgenic microsporidia, but there were no follow-up reports regarding its application in protein function research. RNAi has been widely applied in other fungi including *Nosema ceranae* and *Heterosporis saurida* (Paladi *et al.*, 2010; Saleh *et al.*, 2016), to study protein functions. *N. bombycis* has all of the genes required for RNA silencing and it functions *in vivo*, but the RNA interference efficiency was unstable in different experimental groups (Pan *et al.*, 2017; Wang *et al.*, 2015). In our research, a stable RNAi strategy was established through an *in vitro* transcription system and lipofection in infected Sf9-III, which allowed high-throughput targets screening. Furthermore, RNAi in *N. bombycis* is not only a research method, but also an important strategy for breeding *N. bombycis*-resistant *B. mori*, which have been successful in BmNPV resistance (Kanginakudru *et al.*, 2007).

CONCLUSION

The *N. bombycis* hexokinase is a secretory protein which localized in host cells' cytoplasm and nuclei. The *NbHK* expressed during proliferation stage of infection *in vivo* and *in vitro*. The down-regulation of *NbHK* could inhibit the proliferation of this parasite. The results implied the NbHK was involved in controlling of host's energy metabolism.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Yukang Huang conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Shiyi Zheng performed the experiments.
- Xionge Mei, Bin Sun and Boning Li conceived and designed the experiments.
- Bin Yu contributed reagents/materials/analysis tools.
- Junhong Wei, Guoqing Pan and Zeyang Zhou contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Jie Chen and Chunfeng Li conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Tian Li conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All animal experiments were conducted in accordance with Laboratory Animals Ethics Review Committee of Southwest University guidelines (Chongqing, China) (Permit Number: SYXK-2017-0019).

Data Availability

The following information was supplied regarding data availability:

The raw data are provided in the [Supplemental Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.5658#supplemental-information>.

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