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The role of NbTMP1, a surface protein of sporoplasm, in *Nosema bombycis* infection

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Abstract

Background: *Nosema bombycis* is a unicellular eukaryotic pathogen of the silkworm, *Bombyx mori*, and is an economic and occupational hazard in the silkworm industry. Because of its long incubation period and horizontal and vertical transmission, it is subject to quarantine measures in sericulture production. The microsporidian life-cycle includes a dormant extracellular phase and intracellular proliferation phase, with the proliferation period being the most active period. This latter period lacks spore wall protection and may be the most susceptible stage for control.

Methods: In order to find suitable target for the selective breeding of *N. bombycis*-resistant silkworm strains, we screen highly expressed membrane proteins from the transcriptome data of *N. bombycis*. The subcellular localization of the candidate protein was verified by Indirect immunofluorescence analysis (IFA) and immunoelectron microscopy (IEM), and its role in *N. bombycis* proliferation was verified by RNAi.

Results: The *N. bombycis* protein (NBO_76g0014) was identified as a transmembrane protein and named NbTMP1. It is homologous with hypothetical proteins NGRA_1734 from *Nosema granulosis*. NbTMP1 has a transmembrane region of 23 amino acids at the N-terminus. Indirect immunofluorescence analysis (IFA) results suggest that NbTMP1 is secreted on the plasma membrane as the spores develop. Western blot and qRT-PCR analysis showed that *NbTMP1* was expressed in all developmental stages of *N. bombycis* in infected cells and in the silkworm midgut. Downregulation of *NbTMP1* expression resulted in significant inhibition of *N. bombycis* proliferation.

Conclusions: We confirmed that NbTMP1 is a membrane protein of *N. bombycis*. Reduction of the transcription level of *NbTMP1* significantly inhibited *N. bombycis* proliferation, and this protein may be a target for the selective breeding of *N. bombycis*-resistant silkworm strains.

Keywords: *Nosema bombycis*, Membrane protein, Localization, RNAi, Monoclonal antibody

Introduction

Microsporidia are obligate intracellular parasitic eukaryotes with a wide range of hosts, infecting almost all vertebrates and invertebrates, including humans [1–3]. These spore-forming unicellular parasites have a unique ultrastructure and life-cycle [3]. They were

originally considered to be primitive organisms, such as protozoans or protists [4]. However, microsporidia are now considered to be related to fungi or perhaps to be a sister branch of fungi that has lost many genes and undergone genome compression, possibly due to their adaptation to intracellular parasitism [5–8]. The lack of mitochondria is taken as evidence that microsporidia are ancient eukaryotes [9]. As a result of the loss of many metabolic pathways, microsporidia must obtain nutrients from the host and export the cytotoxic compounds through the cell membrane [10–12].

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Microsporidia have a distinctive mechanism for infecting host cells. The polar tube is ejected when microsporidia are stimulated by suitable conditions, and the infective sporoplasm is transferred into the host cells through the polar tube prior to proliferation [13]. The sporoplasm can also adhere to host cells and enter the host cells by phagocytosis [14]. The membrane proteins of the sporoplasm then interact with the cytoplasmic content of host cell.

Nosema bombycis, the first microsporidian to be recognized, by Nageli in 1857, causes silkworm pébrine disease by vertical and horizontal transmission [15]. The prevention and control of *N. bombycis* is an important focus of sericulture research. In this study, we identified a transmembrane protein (NBO_76g0014) of *N. bombycis*, which we designated NbTMP1. We cloned and expressed recombinant protein NbTMP1 and prepared monoclonal antibodies. We also characterized the subcellular localization of NbTMP1 in *N. bombycis* and explored its function in *N. bombycis* proliferation.

Materials and methods

Preparation of *N. bombycis* and cell cultivation

Mature spores of *N. bombycis* CQ1, obtained from the China Veterinary Culture Collection Center (CVCC No. 102059), were isolated from infected silkworm pupae and purified by Percoll density gradient centrifugation (21,000 g, 40 min) [16]. Cells of ovarian cell line *Spodoptera frugiperda* (Sf9-III) in Sf-900 IIITM SFM were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and cultured in Sf-900TM III SFM medium at 28 °C.

NbTMP1 sequence analysis and amplification of the open reading frame

NbTMP1 (GenBank Accession No. EOB13409.1), which is a highly expressed protein in the early stages of infection, was screened from the transcriptome data of *Bombyx mori* infected with *N. bombycis*. The amino acid sequence of NbTMP1 was submitted to the SignalP 5.1 server (<http://www.cbs.dtu.dk/services/SignalP/>) and the TMHMM server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) for signal peptide and transmembrane domain predictions. The molecular weight and isoelectric point (pI) were predicted using the ExPASy server (http://web.expasy.org/compute_pi/), and the protein function domain was predicted using SMART (<http://smart.embl-heidelberg.de/>). BLAST (<https://blast.ncbi.nlm.nih.gov/>) was used to analyze the multiple sequence alignment. We cloned the extramembraneous region of *NbTMP1* using the forward primer

5'-GGATCCATGTTCAAGTCTAGTGATGA-3' containing a *Bam*HI restriction site (underlined bases) and the reverse primer 5'-GTCGACCTTATCATTTCATTATTTCCC-3' containing a *Sal*I restriction site (underlined bases). Complementary DNA (cDNA) was obtained by reverse transcription of RNA from the Sf9-III cells (Thermo Fisher Scientific) infected with *N. bombycis* and used as the PCR template. The PCR products were purified with the Gel Extraction Kit of Omega Bio-tek, Inc. (Norcross, GA, USA) and integrated into the pET-28 vector, following which the vectors were transformed into competent cells of *Escherichia coli* strain DH5 α . The positive pET-28-*NbTMP1* recombinant vectors were sequenced by Sangon (Shanghai, China).

Recombinant protein expression, monoclonal antibody preparation and immunoblotting

The identified pET-28-*NbTMP1* vector was transformed into *E. coli* strain Rosetta for expression. The recombinant bacteria were induced for 4 h at 37 °C with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) in LB medium. The target protein was purified with nickel-chelating affinity chromatography (Roche Applied Science, Penzberg, Germany). All animal experiments were approved by the Laboratory Animals Ethics Review Committee of Southwest University (Chongqing, China) under Permit Number AERC-SWU2017-7. The mice were maintained according to the recommendations of the committee, and food and water were provided ad libitum. Female BALB/c mice (6- to 8 weeks-old) were injected subcutaneously four times with rNbTMP1 (100 μ g/mouse) mixed with Freund's complete/incomplete adjuvant (1:1; Sigma-Aldrich, St. Louis, MO, USA). The injection interval was 7 days. Monoclonal antibodies (mAbs) were produced and screened as previously described [17]. The mAb immunoglobulin G (IgG) subtypes were detected using the Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science) according to manufacturer's recommendations.

For immunoblotting, the proteins of mature spores and infected cells were prepared by the glass bead breaking method [18], isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PDVF membranes (Roche Applied Science). After blocking in 5% skim milk diluted in TBST (150 mM NaCl, 20 mM Tris-HCl, 0.05% Tween-20), the membrane was incubated for 1 h in anti-NbTMP1 ascites (1:1000). After washing three times, the membrane was incubated with

goat anti-mouse IgG (1:5000; Sigma-Aldrich) conjugated with horseradish peroxidase (HRP) label. Finally, the protein bands were visualized with ECL Plus Western Blotting Detection Reagents (Bio-Rad, Hercules, CA, USA).

Silkworm midgut paraffin section

Newly molted fifth instar silkworm larvae were starved for 1 day and then fed mulberry leaves coated with spores (1×10^7 spores per larva). At 4 days post-infection (dpi), the infected silkworms were killed, and the midguts were collected and stored at -80°C . For embedding, the midguts were first washed three times, 15 min each time, in phosphate buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 and 2.7 mM KCl), following which the midguts were fixed in 4% paraformaldehyde for 24 h. The sections were prepared as previously described [19]. After deparaffination, the sections were boiled in sodium citrate buffer solution for 10 min and then used in the immunolocalization studies.

Immunofluorescence analysis

For the immunofluorescence analysis (IFA), the samples were fixed in 4% paraformaldehyde and then washed three times in PBS. The samples were then permeabilized with 0.5% Triton X-100 for 30 min and then blocked with PBS-bovine serum albumin for 1 h at room temperature. Next, the samples were incubated with the primary antibodies, including anti-NbTMP1 ascites (mouse), or with negative serum and Nb β -tubulin (rabbit) antibody which was used to label meronts of *N. bombycis* [20]. After washing three times with PBS, Alexa 488 conjugate Goat anti-Mouse IgG and Alexa 568 conjugate Goat anti-Rabbit IgG (Thermo Fisher Scientific) were used to detect the bound primary antibodies, and 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) was used to label the nucleus. The results were observed under a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Immunoelectron microscopy study

Infected silkworm midguts were used for the immunoelectron microscopy (IEM) study. Ultrathin sections (70 nm) were prepared as previously described [19] and placed on nickel grids. After blocking with PBS-bovine serum albumin, the grids were incubated with anti-NbTMP1 ascites diluted 1:30 or with negative serum at room temperature for 1 h. The grids were then incubated with gold-conjugated anti-mouse IgG (Aldrich-Sigma) diluted to 1:100. After staining with uranium and lead, the sections were examined and photographed with a JEM-1400 Plus TEM transmission electron microscope (JEOL Co. Ltd., Tokyo, Japan).

RNA interference of NbTMP1

A 331-bp interferential fragment was selected by BLOCK-iTTM RNAi Designer (<http://rnaidesigner.thermofisher.com/rnaiexpress/design.do>). The fragment was amplified by the F-RI-NbTMP1-T7 (5'-TAATACGAC TCACTATAGGGAGATGTCTCTAGGGCAGATGC-3') and R-RI-NbTMP1-T7 (5'-TAATACGACTCACTATAG GGAGATCCAGTACGTGTTGCCTG-3') primers and used as the template for synthesizing double-stranded RNA (dsRNA). The RiboMAXTM Large Scale RNA Production System-T7 (Promega, Madison, WI, USA) was used to obtain the dsRNA. The dsRNA of enhanced green fluorescent protein (EGFP) was used as a negative control and was synthesized as described above [17]. After the Sf9-III cells were cultured in 12-well plates overnight, 2 μg dsRNA of *NbTMP1* or *EGFP* was transfected into the cells. After 4 h, the spores were germinated with 0.1 M KOH and added to the Sf9-III cells (spores:cell, 5:1). Samples of infected cells were collected at 1, 3, and 5 dpi and immediately stored in PBS or TRIzol (Invitrogen, Carlsbad, CA, USA) at -80°C .

Real-time quantitative PCR analysis

A DNA Extraction Kit (Omega Bio-tek, Inc.) was used to extract the genomic DNA (gDNA) of the infected cells. Total RNA Kit II (Omega Bio-tek, Inc.) and EvoScript Universal cDNA Master (Roche Applied Science) were used to prepare the cDNA. Real-time quantitative PCR (qPCR) was amplified by the F-q-Nb TMP1 (5'-CCT ATCTCTAAAGACGGT-3') and R-q-Nb TMP1 (5'-CTT TTTCTATTTTGGCAGCA-3') primers, and reference gene primers F-q-SSU (5'-CTGGGGATAGTATGATCG CAAGA-3') and R-q-SSU (5'-CACAGCATCCATTGG AAACG-3'). The transcription levels were calculated by the $2^{-\Delta\Delta t}$ values method with three replicates. GraphPad Prism v6.01 (GraphPad Software, San Diego, CA, USA) was used to conduct the multiple *t* tests.

The Nb β -tubulin copy number was used to count *N. bombycis*. The gDNA of infected cells samples was analyzed by qPCR. The reaction systems were conducted using the primers: Nb β -tubulin-qF (5'-AGAACCAGG AACAAATGGACG-3') and Nb β -tubulin-qR (5'-AGC CCAATTATTACCAGCACC-3'). The standard template used was described in previous research [17]. The standard curve covered six orders of magnitude (1.3×10^2 – 10^7).

Results

Sequence characteristics and immunoblot analysis of NbTMP1

Due to the loss of many metabolic pathways, microsporidia must obtain nutrients and energy from the host through the cell membrane. In order to facilitate

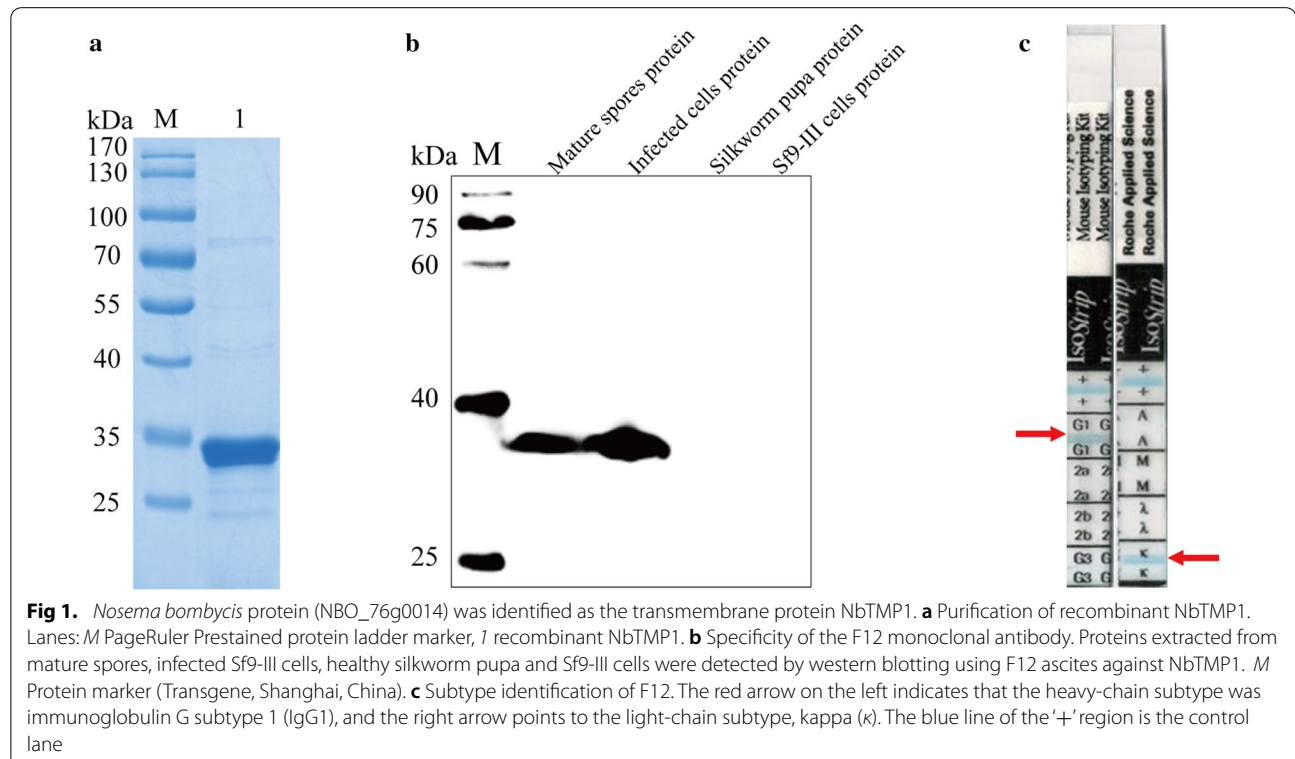
the control of *N. bombycis* in the cell, we attempted to screen highly expressed membrane proteins from the transcriptome data of *N. bombycis*. NbTMP1, a hypothetical membrane protein, was highly expressed in the transcriptome data of *Bombyx mori* infected with *N. bombycis*, suggesting that it may be involved in the proliferation of *N. bombycis*. Sequence analysis showed that NbTMP1 is 43% homologous to the hypothetical protein NGRA_1734 (GenBank Accession No. KAF9762805.1) in *Nosema granulosis* [21]. NbTMP1 contained a complete open reading frame (ORF) that was 732 bp in length and encoded a polypeptide of 243 amino acids with a transmembrane domain. The protein has a calculated molecular weight of 27.59 kDa and a theoretical pI value of 9.92. It does not have a signal peptide and typical functional domains. The recombinant NbTMP1 protein was expressed as a soluble protein of about 30 kDa (Fig. 1a) and was purified to prepare the monoclonal antibody F12.

Western blot analysis with F12 ascites revealed a unique positive band in mature spores and in the proteins of infected cells (Fig. 1b). The band is about 35 kDa, which is more than the molecular weight of NbTMP1, possibly due to post-translational modification, such as phosphorylation and glycosylation, of

the native NbTMP1. Bioinformatics analysis showed that there are two glycosylation sites and 20 phosphorylation sites. Since the recombinant protein lacks a transmembrane domain, the molecular mass of the recombinant NbTMP1 and the native protein differed. The Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science) was used to identify the subtypes of monoclonal antibody F12, and the test strip demonstrated that the subtypes of F12 were IgG1-κ (Fig. 1c).

Subcellular localization of NbTMP1

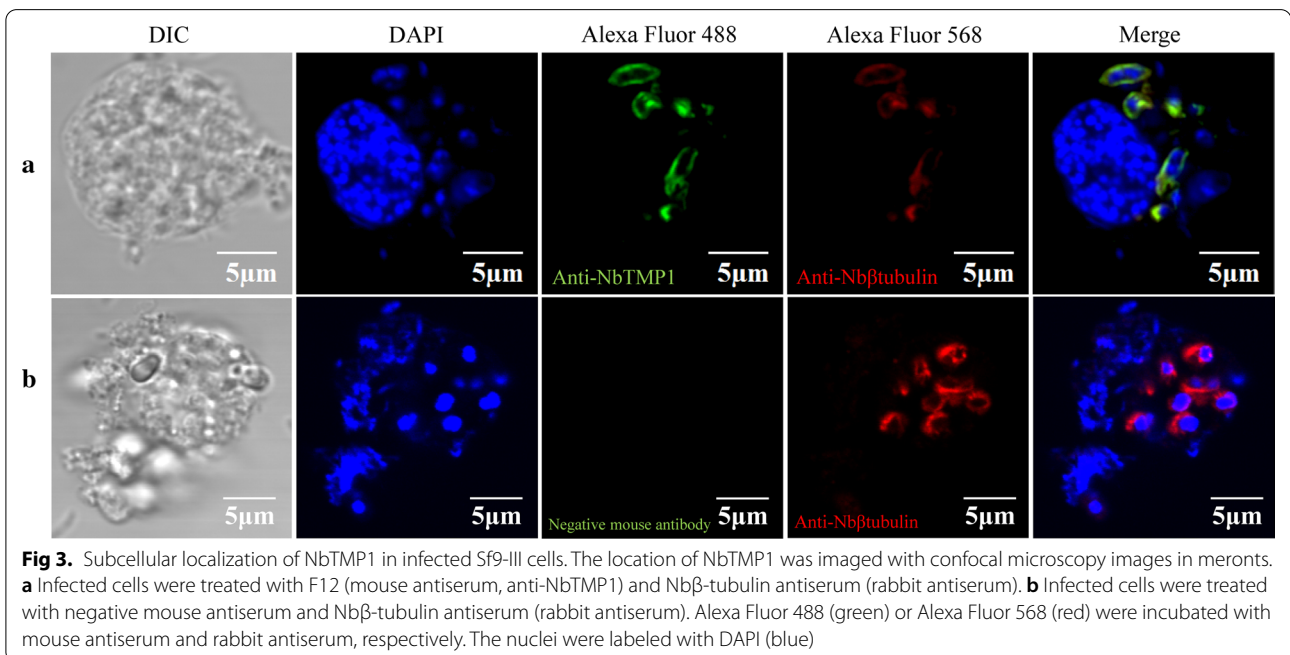
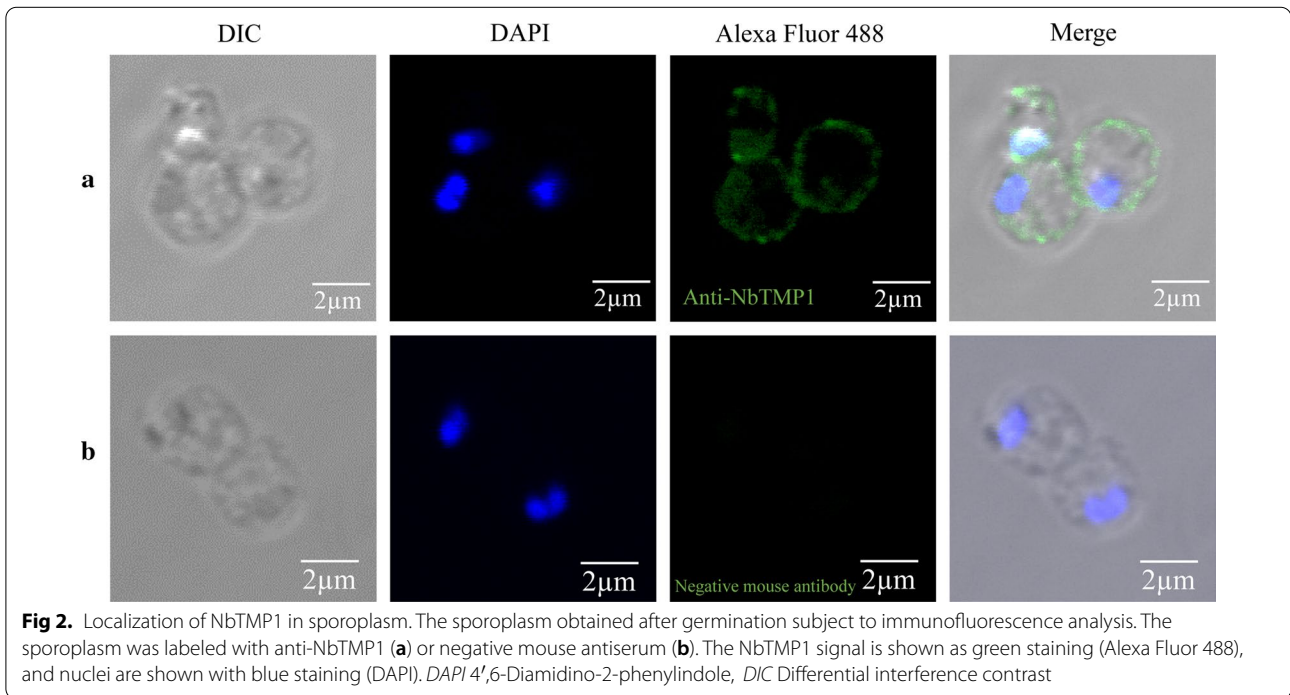
To study the subcellular localization of NbTMP1, we performed IFA with the F12 ascites. Sporoplasm was obtained by allowing mature spores to germinate in 0.1 M KOH. The IFA indicated that NbTMP1 is localized on the plasma membrane of the sporoplasm (Fig. 2). We used the Nbβ-tubulin antibody to label meronts and noted that NbTMP1 co-located with Nbβ-tubulin in the proliferation stage (Fig. 3). The subcellular localization of NbTMP1 was verified by examining paraffin sections of infected tissues. The fluorescence signal of NbTMP1 was consistent with the contour of mature spores (Fig. 4a), but there was no fluorescence signal in mature spores that had not been sectioned (Fig. 4b). As antibodies cannot penetrate the spore wall of *N. bombycis*, this

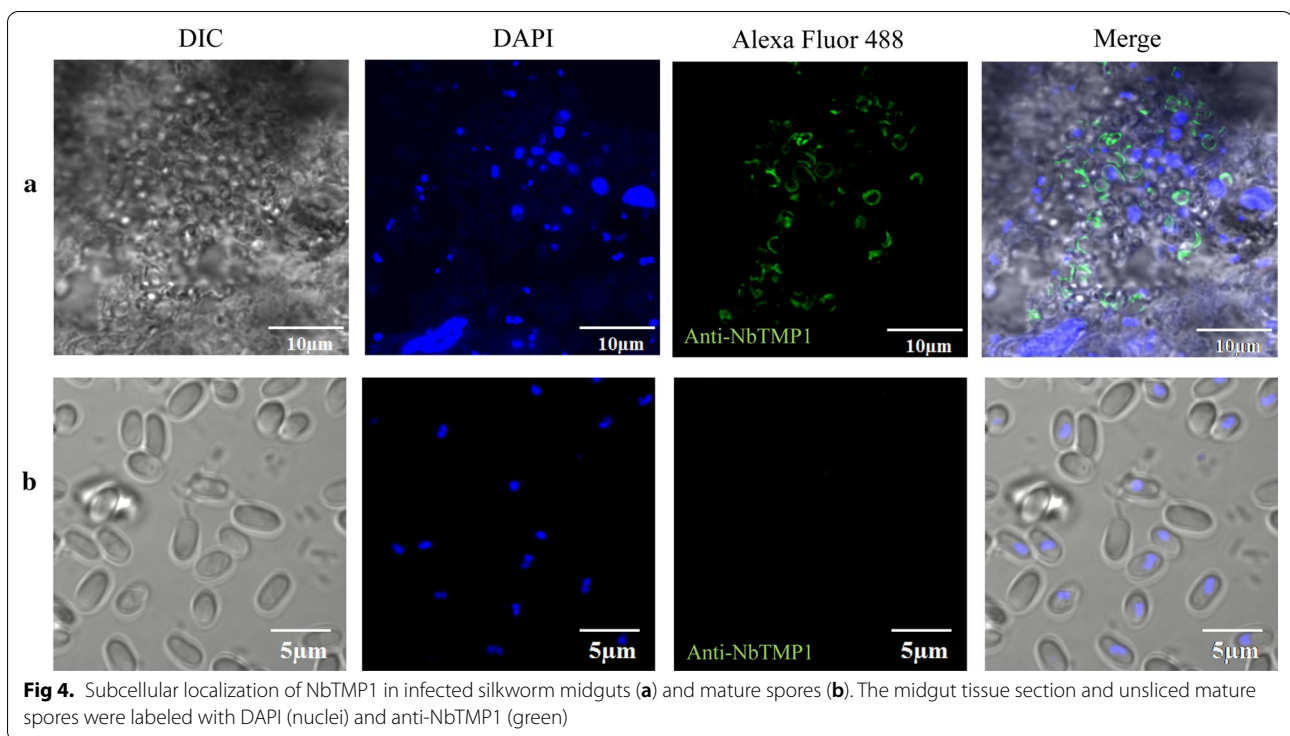


observation also implies that NbTMP1 is located on the plasma membrane. IEM analysis further demonstrated that NbTMP1 is located on the membrane of *N. bombycis* (Fig. 5). This result indicates that NbTMP1 may be secreted to the plasma membrane during the development of *N. bombycis*.

Transcriptional profile of *NbTMP1* in infected cells and midguts

After *N. bombycis* infected the Sf9-III cells, the expression of *NbTMP1* increased from the 1 dpi to 6 dpi. The drop in *NbTMP1* expression on 4 dpi may be due to the formation of mature spores (Fig. 6a). In the midguts





of infected silkworms, *NbTMP1* showed low expression level in the pre-infection phase, with expression increasing continuously after 4 dpi (Fig. 6b). On 4 dpi, *N. bombycis* began a new round of host infection, and the transcript profile of *NbTMP1* suggested that it may play a role in spore infection.

RNA interference of *NbTMP1* inhibited *N. bombycis*' proliferation

The effects of RNA interference (RNAi) were analyzed using qPCR. Following the addition of the dsRNA, the transcription level of *NbTMP1* was significantly down-regulated in the experimental groups (*NbTMP1*-dsRNA) (Fig. 7a). Since *Nbβ-tubulin* is a housekeeping and conserved gene of *N. bombycis*, its copy number was used to reflect the number of *N. bombycis* in the two groups [17]. The qPCR results showed that *N. bombycis* began to proliferate from 1 to 5 dpi in the mock groups (EGFP-dsRNA). However, the pathogen load was remarkably lower in the experimental groups (Fig. 7b). These results suggest that *NbTMP1* plays an important role in the proliferation of *N. bombycis*.

Discussion

Membrane protein is rarely studied in microsporidia. In *Trachipleistophora hominis*, plasma membrane-located purine nucleotide transport proteins (NTTs) are key components of the process involved in stealing ATP from

hosts [11]. EhSSP1 was identified as *Encephalitozoon hellem* sporoplasm surface protein 1; this protein binds to human foreskin fibroblasts and is associated with the final step of invasion on the invasion synapse [22]. *NbTMP1* was predicted to have a transmembrane domain, suggesting that it may be located on the membrane of the spore. The IFA and IEM results proved that *NbTMP1* actually did localize on the membrane of sporoplasm and spores. The expression profiles *in vitro* and *in vivo* and the western blot results showed that *NbTMP1* is present in all stages of the *N. bombycis* life-cycle. Although we showed that downregulation of *NbTMP1* expression can inhibit the proliferation of *N. bombycis*, the specific mechanism remains unknown.

There are two main hypotheses on how microsporidia infect host cells. One is that the microsporidia eject a polar tube, penetrate the membrane of new host cells and then deliver the contents into the cytoplasm of the host cell; the other is that the microsporidia gain access to the host cell by phagocytosis, and then the polar tube is used to escape the phagosome and to infect the host cytoplasm [13]. Sporoplasm is the earliest stage of microsporidia infection of host cells. The mechanism used by microsporidia to transmit genetic information after infecting a host cell is unclear. However, surface proteins, such as NbAQP, NbSWP9 and NbSWP7, can play important roles in the process of spore germination and infection [23, 24]. *NbTMP1* is located on the

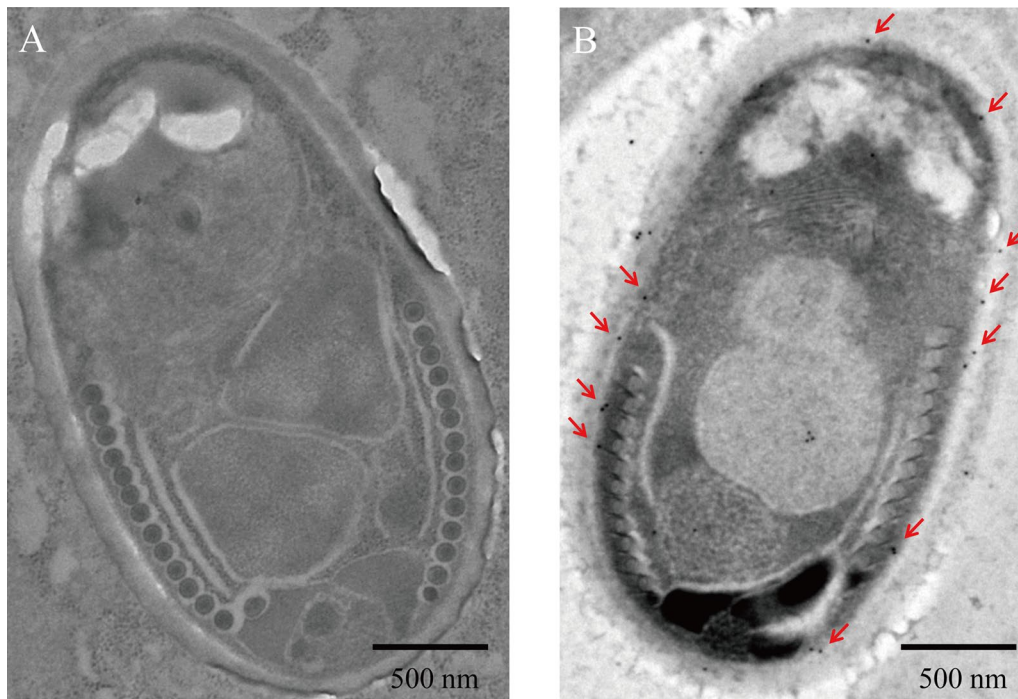


Fig 5. Immunoelectron microscopy of NbTMP1 in spores of infected midgut. **a** Negative control, **b** spore showing the presence of gold labeled anti-NbTMP1 on the plasma membrane. The red arrow indicates the gold label. Scale bars 500 nm

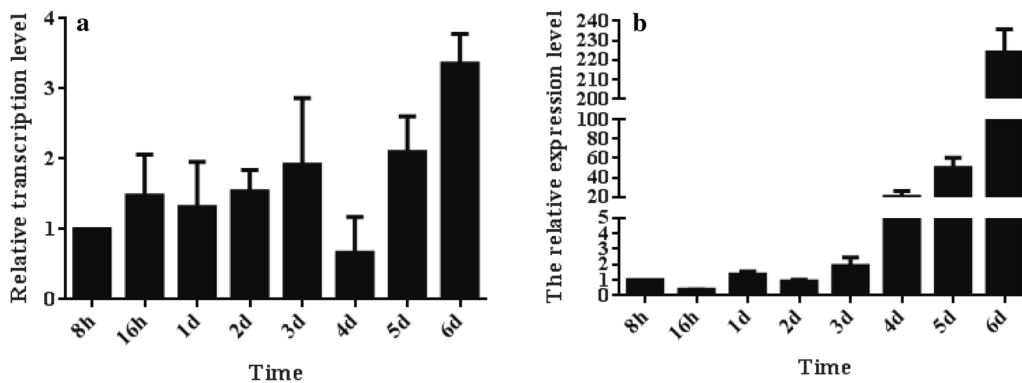
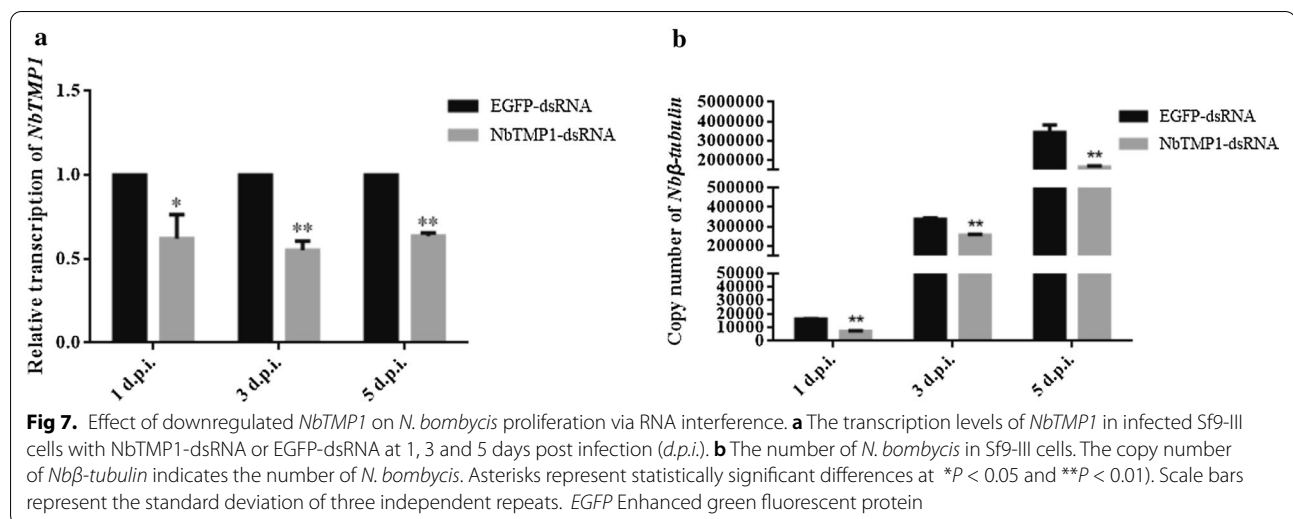


Fig 6. Transcription analysis of NbTMP1. *N. bombycis* small subunit ribosomal ribonucleic acid was used as an internal control. The relative expression level of NbTMP1 is presented relative to expression at 8 h post-infection. **a** Transcription level of NbTMP1 in infected Sf9-III cells, **b** transcription level of NbTMP1 in infected silkworm midguts. Vertical bars show the mean \pm standard error ($n = 3$)

plasma membrane of the sporoplasm, suggesting that it may be involved in the proliferation of spores in cells.

Understanding the protein functions of *N. bombycis* has been hampered by the lack of stable and reliable gene manipulation methods. RNAi has been used in fungi, including *Heterosporis saurida* and *Nosema ceranae*, to study protein functions [25, 26]. *Nosema bombycis* possesses all of the genes required for RNA

silencing and *in vivo* function [27]. A stable RNAi strategy was previously established utilizing an *in vitro* transcription system and lipofection in Sf9-III infection [28]. RNAi has been successfully used to develop *Bombyx mori* nucleopolyhedrovirus (anti-BmNPV)-resistant silkworms [29]. RNAi can also be used as a strategy for breeding *N. bombycis*-resistant silkworms, and NbTMP1 is a potential target of RNAi. Single-chain



antibodies are also a strategy for breeding *N. bombycis*-resistant silkworms. In *Anopheles*, transposon-mediated transformation was used to generate m1C3, m4B7 and m2A10 single-chain antibodies (scFvs), and the transgenic mosquitoes expressing the scFv gene had significantly lower infection levels of *Plasmodium falciparum* [30, 31]. The transgenic Sf9-III cells, which express single-chain antibodies of SWP12 of *N. bombycis*, effectively inhibit the proliferation of *N. bombycis* in cells [17]. In this study, we obtained the monoclonal antibody (F12) of NbTMP1, and in future investigations we plan to evaluate the performance of this single-chain antibody for breeding of *N. bombycis*-resistant silkworms. In follow-up studies, we will try to express dsRNA of *NbTMP1* and scFv-F12 in silkworm individuals by transgenic technology to verify the function of NbTMP1 and evaluate the resistance of transgenic silkworms to *N. bombycis*.

Conclusions

We identified a novel membrane protein in the microsporidium *N. bombycis*. NbTMP1 was localized on the plasma membrane of the sporoplasm, meronts and spores, and its proliferation was significantly inhibited by RNAi. This membrane localized protein is important for pathogen development, and may be a potential target for construction of *N. bombycis*-resistant silkworms.

Abbreviations

IFA: Indirect immunofluorescence analysis; IEM: Immunoelectron microscopy; Sf9-III cells: Ovarian cell line of *Spodoptera frugiperda*; RNAi: RNA interference; qPCR: Real-time quantitative PCR.

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Authors' contributions

SZ, YH and HH performed the experiments and analyzed the data. SZ and CL drafted the manuscript and performed manuscript preparation. SZ, HH, BY and NZ supervised and performed the collection of material and all other laboratory experiments. SZ, JW, GP, CL and ZZ conceived the idea and coordinated the project. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article. All data are fully available without restriction upon reasonable request.

Ethics approval and consent to participate

All animal procedures were approved by Laboratory Animals Ethics Review Committee of Southwest University (Chongqing, China) under Permit Number: AERCSWU2017-7.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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