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Evolutionary and functional studies on microsporidian ATP-binding cassettes: Insights into the adaptation of microsporidia to obligated intracellular parasitism

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ABSTRACT

ATP-binding cassette (ABC) transporters comprise the largest family of transmembrane proteins and are found in all domains of life. The ABCs are involved in a variety of biological processes and as exporters play important roles in multidrug resistance. However, the ABC transporters have not been addressed in microsporidia, which are a very large group of obligate intracellular parasites that can infect nearly all animals, including humans. Here, a total of 234 ABC transporters were identified from 18 microsporidian genomes and classified into five subfamilies, including 74 ABCBs, 2 ABCCs, 18 ABCEs, 15 ABCFs, 102 ABCGs and 23 uncategorized members. Two subfamilies, ABCA and ABCD, are found in most organisms, but lost in microsporidia. Phylogenetic analysis indicated that microsporidian ABCB and ABCG subfamilies expanded by recent gene duplications, which resulted in the two largest subfamilies in microsporidia. Functional analysis via qRT-PCR and Western blotting revealed that *NoboABCG1.1*, an ABCG member of *Nosema bombycis*, is expressed in mature spores and upregulated from 1 dpi to 6 dpi in infected silkworm midgut. IFA and IEM analysis showed that NoboABCG1.1 is localized on the plasma membrane of the sporoplasm, meront and mature spore. The propagation of *N. bombycis* was significantly inhibited after the RNAi of *NoboABCG1.1* expression, indicating that *NoboABCG1.1* is important to the pathogen proliferation. In conclusion, our study uncovered that the ABCs evolved during microsporidia adaption to intracellular parasitism and play important roles in the pathogen development.

1. Introduction

Microsporidia are a group of obligated intracellular parasites that infect a broad range of animal phyla including vertebrates including immunocompromised humans. Microsporidia are reported to be composed of at least 187 genera and 1500 species (Keeling, 2009; Vávra and Lukeš, 2013). Microsporidial genomes are extremely compact and are missing genes for many metabolic pathways (Corradi et al., 2010; Katinka et al., 2001; Nakjang et al., 2013; Pombert et al., 2012), including the biosynthesis of nucleotides and amino acids, the tricarboxylic acid cycle, beta-oxidation of fatty acids and the electron transport chain (Bakowski et al., 2014; Chen et al., 2013; Corradi et al., 2009; Corradi et al., 2010; Heinz et al., 2012; Katinka et al., 2001; Keeling et al., 2010; Pan et al., 2013; Pombert et al., 2012). In order to compensate for this lack of metabolites, microsporidia have transport systems to acquire nutrients from the host and export cytotoxic compounds across the plasma membrane (Cuomo et al., 2012; Heinz et al., 2014; Heinz et al., 2012). It has been suggested that the copying of the microsporidial genome may be a limiting step in the rate of reproduction and that the this may be the reason for genome compression, including the loss of genes in microsporidia (Xu et al., 2016).

The ATP-binding cassette (ABC) transporters comprise one of the largest superfamilies of transmembrane proteins found in all living organisms (Jungwirth and Kuchler, 2006). ABC transporters are divided into two major subtypes; importers and exporters, although the importers seem to be found primarily in prokaryotes and plants (Rice et al., 2014). Most of ABC transporters rely on the binding and hydrolysis of ATP to transport substrates, such as amino acids, lipids,

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sugars, peptides, ions, and drugs, from the cytosol to intracellular or extracellular regions (Dermauw and Van Leeuwen, 2014). Typical ABC full-length transporters contain two integral transmembrane domains (TMDs) and two nucleotide binding domains (NBDs), while half-length transporters are composed of one TMD and one NBD domain (Beis, 2015; Hollenstein et al., 2007; Schneider and Hunke, 1998). According to the homology of their NBDs, seven ABC subfamilies (ABCA - ABCG) have been identified in humans (Dean et al., 2001). An additional ABC transporter, ABCH, was first discovered in *Drosophila melanogaster*, and has been found in other arthropods and zebrafish but not in mammals, plants or fungi (Dermauw and Van Leeuwen, 2014; Popovic et al., 2010).

Previous studies of parasite ABC transporters have shown their important role in exporting antibiotic compounds resulting in drug resistance. At present, 20 predicted genes encoding ABC transporters have been identified in the genome of *Toxoplasma gondii* (Sauvage et al., 2006), 33 in *Cryptosporidium parvum* (Zapata et al., 2002), and 16 in *Plasmodium falciparum* (Koenderink et al., 2010). A recent study in *T. gondii* has show that five of ABCG half transporters facilitate the export of cholesterol and phosphatidylserine when expressed in mammalian COS cells (Ehrenman et al., 2010). However, another ABCG transporter, TgABC$_{107}$, localized in the parasite plasma membrane and parasitophorous vacuoles of *T. gondii* can import cholesterol from the host cell (Ehrenman et al., 2010). *P. falciparum* encodes a single ABCG transporter (PfABCG) which is localized in the parasite plasma membrane and may be play a role in lipid transport consistent with human ABCG1 function (Edaye and Georges, 2015; Ehrenman et al., 2010). Meanwhile, Specific ABC transporters have been implicated in drug resistance in protozoan parasites such as TcABCG1 are naturally resistant to Benznidazole through over-expressed in *Trypanosoma cruzi* (Klokouzas et al., 2003; Leprohon et al., 2006; Sauvage et al., 2009; Zingales et al., 2015). The first report of ABC transporters in microsporidia were EiABC1 and EiABC2 from *Encephalitozoon intestinalis* (Bonafonte et al., 2001). Subsequently thirteen ABC transporter genes were identified in the *Encephalitozoon cuniculi* and ten from *Trachipleistophora hominis* (Cornillot et al., 2002; Heinz et al., 2012).

Here, we analyzed the ABC transporters from 18 microsporidian genomes and characterized the subcellular localization and function of a G-subfamily member, NoboABCG1.1, in *Nosema bombycis*.

2. Materials and methods

2.1. Genome sequence data

All protein sequences were downloaded from GenBank database, these include: *Encephalitozoon cuniculi* GB-M1 (GCA_000091225.1) (Katinka et al., 2001); *Encephalitozoon intestinalis* ATCC 50506 (GCA_000146465.1) (Corradi et al., 2010); *Encephalitozoon hellem* ATCC 50504 (GCA_000277815.3) (Pombert et al., 2012); *Encephalitozoon romaleae* SJ-2008 (GCA_000280035.2) (Pombert et al., 2012); *Ordospora colligata* OC4 (GCA_000803265.1) (Pombert et al., 2015); *Nosema bombycis* CQ1(GCA_000383075.1) (Pan et al., 2013); *Nosema antheraeae* (SilkPathDB, http://silkpathdb.swu.edu.cn) (He et al., 2015); *Nosema apis* BRL 01 (GCA_000447185.1) (Chen et al., 2013); *Nosema ceranae* (GCA_000988165.1) (Pelin et al., 2015); *Anncaliia algerae* PRA339 (GCA_000385875.2); *Pseudoloma neurophilia* (GCA_001432165.1) (Ndikumana et al., 2017); *Trachipleistophora hominis* (GCA_000316135.1) (Heinz et al., 2012); *Vavraia culicis* subsp. floridensis (GCA_000192795.1) (Desjardins et al., 2015); *Vittaforma corneae* ATCC 50505 (GCA_000231115.1); *Nematocida parisii* ERTm1 (GCA_000250985.1) (Cuomo et al., 2012); *Nematocida parisii* ERTm3 (GCA_000190615.1) (Cuomo et al., 2012); *Nematocida* sp. 1 ERTm6 (GCA_000738915.1) (Bakowski et al., 2014); *Edhazardia aedis* USNM 41457 (GCA_000230595.3) (Desjardins et al., 2015).

2.2. Identification of putative ABC transporter proteins

To identify ABC transporter proteins in Microsporidia, all protein sequences were searched against the TransportDB and Transporter Classification Databases (TCDB) based on sequence similarity using the default parameters (Ren et al., 2007; Saier et al., 2016). All candidate ABC transporters were then searched against PFAM database to predict the nucleotide binding domain (NBD) using the Hidden Markov Model (HMM) and confirmed using the NCBI CD-Search tool to predict conserved domains. The transmembrane helices were predicted using the TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) web portal. Graphical representations of transmembrane topology were created with TMRPres2D (http://bioinformatics.biol.uoa.gr/TMRPres2D).

2.3. Phylogenetic analysis

Protein sequences were aligned using MUSCLE v3.8.31 and regions of uncertain alignment were eliminated with TrimAl using the default parameters (Capella-Gutiérrez et al., 2009; Edgar, 2004). The substitution model prediction option on MEGA (v6.06) (Tamura et al., 2011) was used to identify the best substitution models for the individual protein homologue sets. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method in MEGA (Tamura et al., 2011). The best substitution model was used and bootstrap support was evaluated based on 100 replicates. The phylogenetic trees were visualized using Evolview (He et al., 2016).

2.4. Preparation of Nosema bombycis and cell cultivation

Mature spores of *N. bombycis* isolate CQ1, conserved in the China Veterinary Culture Collection Center (CVCC No. 102059) in Chongqing, China, were propagated in and isolated from silkworm larvae as previously described (Wu et al., 2008). *Spodoptera frugiperda* ovary cell (Sf9) were purchased from Thermo Fisher Scientific and cultured in Sf-900™ III SFM medium at 28 °C.

2.5. Expression of fusion proteins, antiserum production, and Western blotting

DNA was extracted as previously described (Pan et al., 2013). The outer membrane region of NoboABCG1.1 (GenBank: EOB15202.1) was predicted by TMHMM 2.0 and amplified from spore genomic DNA using primers (F: 5`-cgcggatccATGTCACAGAAAGTTTTTGAATT-3` and R: 5`aaatatgcggccgcAAAGAATCTCTCAGTTCTGTATACG-3`). The resulting 1002 bp DNA fragment encoding 334 amino acids was cloned into prokaryotic expression vectors pET32a, then transformed into *Escherichia coli* Rosetta cells. A single positive colony was induced for 16 h at 16 °C with 0.1 mM IPTG. The fused expression proteins were purified using the Ni-NTA Superflow Cartridge (QIAGEN). Four mice were intradermally immunized with recombinant NbABCG1.1 (80 μg/mouse) homogenized with Freund's adjuvant (1: 1; Sigma). One week after the fourth injection, antisera were collected and stored at −20 °C.

For immunoblotting, the total protein of *N. bombycis* was extracted as previously described (Li et al., 2012), separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Roche). The membrane was blocked with 5% (*w/v*) skim milk for 1 h at 37 °C, and incubated with the anti-NoboABCG1.1 sera or the negative control sera diluted 1:1000 with skim milk for 1 h at 37 °C. Following several washes, the membrane was then incubated with horseradish peroxidase-labeled goat anti-mouse IgG antiserum (Sigma Aldrich) at 1:1000 dilution. After three washes, the membrane were developed with the ECL Western blot detection kit (Thermo Fisher Scientific), and imaged using an Azure Biosystems C300 imaging system.

2.6. Indirect immunofluorescence assay (IFA)

Infected cells (meronts) were fixed with 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS and permeabilized in 0.1% Triton X-100 for 1 h. The cells were then blocked in PBST containing 10% goat serum and 5% BSA for 1 h at 37 °C. After three washes with PBS, samples were incubated for 1 h at room temperature with NoboABCG1.1 mouse antibody and Nb-β-Tubulin rabbit antiserum (Chen et al., 2017) (diluted 1:100 in blocking solution) or a negative serum (diluted 1:100 in blocking solution), washed, and then incubated further for 1 h with a 1:1000 dilution of Alexa Fluor 488 conjugate Goat anti-Mouse IgG and a 1:1000 dilution of Alexa Fluor 594 conjugate Goat anti-Rabbit IgG (Thermo Fisher) in a dark moist chamber at 37 °C. Nuclear material was stained with DAPI (4,6-diamidino-2-phenylindole) at room temperature for 15 min. The samples were then observed and photographed using an Olympus FV1200 laser scanning confocal microscope.

2.7. Immunoelectron microscopy (IEM) analysis

Infected ovaries were fixed in 2% glutaraldehyde for 2 h and washed four times (15 min each) in 0.1 M PBS buffer (pH 7.2), then post-fixed for 2 h in 1% osmium tetroxide, and washed four additional times in 0.1 M PBS. The samples were then dehydrated using an ascending ethanol series followed by emersion in 100% acetone twice for ten minutes. The samples were then sequentially exchanged into Epon812 (SPI, USA) resin, embedded and polymerised at 70 °C for 48 h. Ultrathin sections were cut using a LEICA EM UC7 ultramicrotome and placed on nickel grids. After being blocked with a standard blocking solution, the grids were incubated with anti-NoboABCG1.1 serum diluted1:30 or anti-NoboABCG1.1 negative serum for 1 h at room temperature. Followed by incubation with gold conjugated anti-mouse IgG (Sigma) diluted 1:100, grids were rinsed with sterile water, dried, examined and photographed with a JEM-1400 Plus TEM transmission electron microscope at an accelerating voltage of 80 kV.

2.8. RNAi of NoboABCG1.1

To determine whether NoboABCG1.1 plays a role in the proliferation of *N. bombycis* in host cells, RNAi was used to knockdown the *NoboABCG1.1* expression (Paldi et al., 2010; Pan et al., 2017). A 328 bp fragment was selected: NoboABCG1.1-F: 5`(T7)TAATACGACTCACTATAGGGAGA TATACAACGGCAAAGAGCG-3`; NoboABCG1.1-R: 5`- (T7)TAATACGACTCACTATAGGGAGA GCAGAGTGTGAATCAAGTCCA-3`. The fragments were amplified and purified as the templates for synthesizing dsRNA. The double-stranded RNA fragments were obtained using RiboMAX™ Large Scale RNA Prouction System-T7 (Promega, USA). An enhanced green fluorescent protein (EGFP)-derived dsRNA was synthesized as described above and used as a negative control (Huang et al., 2018). The Sf9 cells were seed into 6-well plates at 1×10^6 cells per well where *dsNoboABCG1.1* and *dsEGFP* transfection was performed according to the instructions of X-tremeGENE HP DNA transfection reagents (Roche). The Sf-900™ III SFM medium were replaced with fresh Sf-900™ III SFM medium and *N. bombycis* spores were added to achieve a final ratio of 10:1 spores/cell after 5 h. Samples of infected Sf9 cells were collected at 1 and 6 days post-infection and immediately stored in TRIzol (Invitrogen) at −80 °C.

2.9. Real-time quantitative PCR (qRT-PCR) analysis

Total RNA of infected Sf9 and midgut cells was extracted using the E.Z.N.A.™ total RNA kit II (OMEGA, China) according to the manufacturer's instructions. The cDNA was synthesized with 1 μg total RNA using the GoScript™ Reverse Transcription System Kit (Promega) after DNA digestion with DNase I. The Real-time quantitative PCR was conducted using *NoboABCG1.1* primers (F: TGGACTTGATTCACACTCTGCTT; R: ACCGTCATAAAAAACACCACCTT) and reference gene primers (F: CTGGGGATAGTATGATCGCAAGA; R: CACAGCATCCATTGGAAACG). Transcription levels were calculated by the $2^{-\Delta\Delta t}$ values method using three replicates. All statistical *t*-tests were performed with GraphPad Prism version 6.0 by two-tailed comparison tests and any difference with a *P* value < .05 was considered significant.

To assess the growth of *N. bombycis* in cell culture Real-time quantitative PCR was performed for *Nb-β-tubulin* primers (F:AGAACCAGGAACAATGGACG; R: AGCCCAATTATTACCAGCACC) using standard curves. Genomic DNA (gDNA) of infected Sf9 cells was extracted using the E.Z.N.A.™ The Tissue DNA kit (OMEGA, China) was used to extract the DNA according to the manufacturer's instructions.

3. Result

3.1. Identification of ABC transporters in microsporidia

The search against the TCDB database containing 18 microsporidian genomes reveals a total of 234 ABC transporters classified into five subfamilies, comprising 74 ABCBs, 2 ABCCs, 18 ABCEs, 15 ABCFs, 102 ABCGs and 23 ABC transporters of undefined status (Table 1, Supplementary Table S1). Genes coding members of the ABCA and ABCD

Table 1
Comparatively analysis of ABC transporters in the microsporidian.

Species	ABCB (HMT)	ABCC (MRP)	ABCE (RLI)	ABCF (GCN)	ABCG (WHI)	Other	Total
Nosema bombycis	3	0	1	1	4	5	14
Nosema antheraeae	10	0	1	1	5	3	20
Nosema apis	4	0	1	1	2	2	10
Nosema ceranae	7	0	1	1	4	0	13
Encephalitozoon cuniculi	6	0	1	1	5	0	13
Encephalitozoon hellem	6	0	1	1	6	0	14
Encephalitozoon intestinalis	6	0	1	1	6	0	14
Encephalitozoon romaleae	5	0	1	1	5	1	13
Ordospora colligata	5	0	1	1	5	0	12
Anncaliia algerae	3	0	1	1	7	1	13
Pseudoloma neurophilia	3	0	1	1	8	2	15
Trachipleistophora hominis	2	1	1	1	7	0	12
Vavraia culicis subsp. floridensis	2	1	1	1	7	0	12
Vittaforma corneae	7	0	1	1	2	8	19
Nematocida parisii ERTm1	1	0	1	0	5	0	7
Nematocida parisii ERTm3	1	0	1	0	4	0	6
Nematocida sp. 1 ERTm6	1	0	1	0	6	0	8
Edhazardia aedis	2	0	1	1	14	1	19
Total	74	2	18	15	102	23	234

Table 2
Number of ABC genes in intracellular parasites and other eukaryotes.

Subfamily	Microsporidian	Lema	Trbr	Plfa	Togo	Crpa	Sace	Human
ABCA	0	10	2	0	0	0	0	12
ABCB	1–10	4	2	7	6	2	4	11
ABCC	1	8	3	2	2	14	6	12
ABCD	0	3	3	0	0	1	2	4
ABCE	1	1	1	1	1	1	1	1
ABCF	1	3	3	1	1	1	5	3
ABCG	2–14	6	4	1	5	2	10	5
ABCH	0	3	1	2	4	0	0	0
Other	1–4	4	3	1	1	0	2	0
Total	6–20	42	22	16	20	21	32	48

Note: Lema: *Leishmania major*; Trbr: *Trypanosoma brucei*; Plfa: *Plasmodium falciparum*; Togo: *Toxoplasma gondii*; Crpa: *Cryptosporidium parvum*; Cael: *Caenorhabditis elegans*; Sace: *Saccharomyces cerevisiae*; Drme: *Drosophila melanogaster*.

transporters were not identified in the microsporidia examined in this study. The lack of ABCA and ABCD transporters is also the case for the apicomplexan parasites *P. falciparum* and *T. gondi* (Table 2). Microsporidian ABC transporters are half-sized transporters arranged in forward TMD–NBD (ABCB, ABCC) and reverse NBD–TMD (ABCG) configurations (Fig. 1). The TMD domain of most microsporidial ABC transporters has six α-helices but some only contain one α-helix (Supplementary Table S1). The ABCE and ABCF subfamilies only contain the NBD domain (Fig. 1).

3.1.1. ABCB subfamily

The ABCB is found in all 18 microsporidian genomes and shares approximately 30% amino acid sequence similarity with the yeast ATM1 protein involved in Fe—S cluster export (Kispal et al., 1997). There are 74 half-sized ABCBs, which belong to the Heavy Metal Transporter (HMT) family and are located in the mitochondria of other eukaryotic organisms. There are 10 ABCBs identified in *N. antheraeae* and only one found in each of the *Nematocida* isolates. The microsporidian ABCBs can be divided into two groups, in which Group I is found in all microsporidial genomes in this study (Fig. 2A).

3.1.2. ABCC subfamily

The only ABCC transportors found in microsporidial species examined were those of *T. hominis* and *V. culicis* each containing one. ABCC transporters belong to the Drug Conjugate Transporter (DCT) family. The ABCC transporters of most organisms have a $(TMD–NBD)_2$ organization whereas the microsporidial ABCC transporter contains only one TMD and NBD. Compared to other species, microsporidian ABCC subfamily is significantly reduced in number so that some microsporidia even lost this subfamily (Table 2).

3.1.3. ABCE and ABCF subfamily

Two putative homologues of the ABCE (RLI) and ABCF (GCN) families were identified in microsporidia. Unlike other ABC members two families do not contain transmembrane domain and are comprised of two NBDs, which are fused together. The ABCE and ABCF subfamily are very conserved and single-copied in eukaryotes (Table 2). All microsporidian ABCEs contain a RNase L inhibitor (RLI) and ferredoxin 4 Fe/4S (Fer4) motif (Supplementary Fig. S1). But the two members of ABCE in *N. bombycis* and *V. corneae* only have one NBD domain. The ABCF can not be found in *Nematocida*.

3.1.4. ABCG subfamily

We identified 102 members of the ABCG subfamily in the microsporidia. Sequence analysis show that microsporidian ABCG belongs to WHI family. The ABCGs can be phylogenetically classified into three groups (Fig. 2B). Group I and Group II are conserved in most microsporidian species, but Group III only exists in *A. algerae*, *E. aedis* and *Nematocida*. *P. neurophilia*, *E. aedis* and *A. algerae* have more ABCG members, indicating gene duplications happened in these microsporidian (Fig. 2B).

3.2. Characterization of NoboABCG1.1

Two of ABCG proteins in *N. bombycis*, NoboABCG1.1 and NoboABCG1.2, share a high sequence similarity as shown in Supplementary Fig. S2A. However, transmembrane topological analysis indicates that the NBD domain of NoboABCG1.1 is an extracellular transporter while NoboABCG1.2 transports into the cytoplasm (Supplementary Fig. S2B, C).

Measurement of expression of *NoboABCG1.1* from infected midgut tissue of fourth instar silkworms showed an increase from day 1 to day 6 post infection. There was a drop in expression on day 5 presumably during a period of spore formation (Fig. 3A).

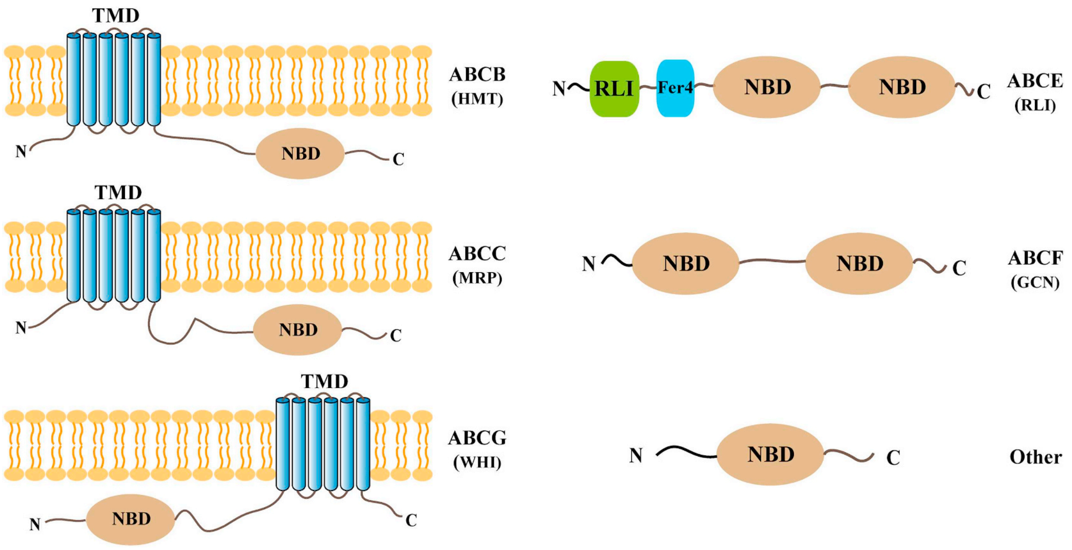

Fig. 1. Conserved domains of the ABC transporter families in microsporidia.
Conserved domains of various microsporidian ABC transporter subfamilies relative to the cell membrane. NBD indicates a nucleotide binding domain and TMD indicates a transmembrane domain.

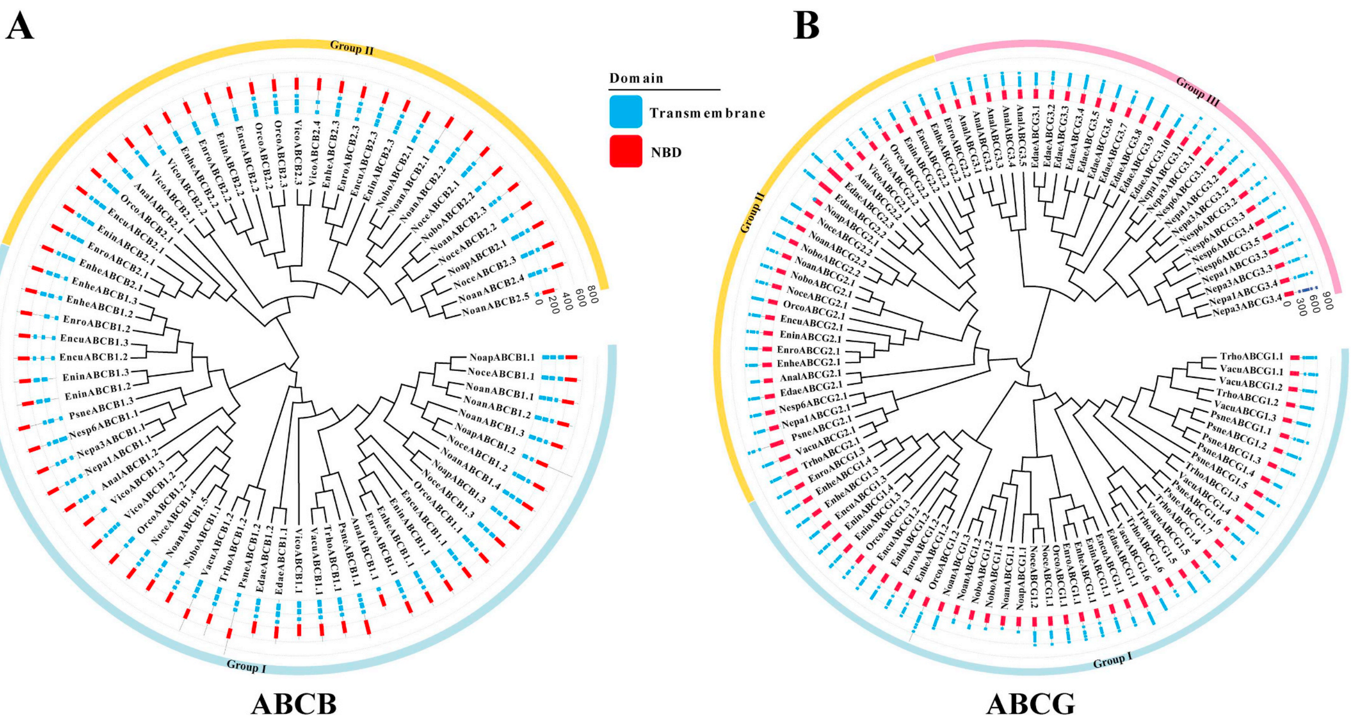

Fig. 2. Maximum-likelihood-based phylogenetic tree of two (A = ABCB and B = ABCG) transporter family sequences from the genomes of 18 microsporidian species. The blue and red rectangles represent the transmembrane and NBD domains. The microsporidial species are indicated with the following abbreviations: Nobo, *Nosema bombycis*; Noan, *Nosema antheraeae*; Noap, *Nosema apis*; Noce, *Nosema ceranae*; Encu, *Encephalitozoon cuniculi*; Enhe, *Encephalitozoon hellem*; Enin, *Encephalitozoon intestinalis*; Enro, *Encephalitozoon romaleae*; Orco, *Ordospora colligate*; Anal, *Anncaliia algerae*; Psne, *Pseudoloma neurophilia*; Trho, *Trachipleistophora hominis*; Vacu, *Vavraia culicis* subsp. floridensis; Vico, *Vittaforma corneae*; Nepa1, *Nematocida parisii* ERTm1; Nepa3, *Nematocida parisii* ERTm3; Nesp6, *Nematocida* sp. 1 ERTm6; Edae, *Edhazardia aedis*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Subcellular localization of NoboABCG1.1

A His-tagged recombinant protein of NoboABCG1.1 composed of the first 334 N-terminal amino acid residues and containing the NBD motif (His-NoboABCG$^{1-334}$) was expressed and purified, and used to make antiserum by inoculating mouse (Supplementary Fig. S3). The specificity of anti-NoboABCG1 was checked with total proteins of mature spore and identified the ~43 kDa recombinant protein (Fig. 3B).

To investigate the subcellular location of NoboABCG1.1, immunofluorescent assay (IFA) was performed with the anti-NoboABCG1.1, results of which indicated that the NoboABCG1.1 localizes on the plasma membrane of sporoplasm (Fig. 4). Besides, NoboABCG1.1 was also found on the plasma membrane of *N. bombycis* meronts (Fig. 5). Furthermore, IEM analysis demonstrated similar localization of NoboABCG1.1(Fig. 6). These results revealed that NoboABCG1.1 is located on the plasma membrane of *N. bombycis* mature spore and meront.

3.4. RNAi of NoboABCG1 inhibited pathogen proliferation

Previous studies have proved that dsRNA works to knock down gene expression in *Nosema* (Paldi et al., 2010; Pan et al., 2017). By adding dsRNA against *NoboABCG1.1* to the infected Sf9 cell, the *NoboABCG1.1* mRNA level decreased by about 40% and 60% after first day and sixth

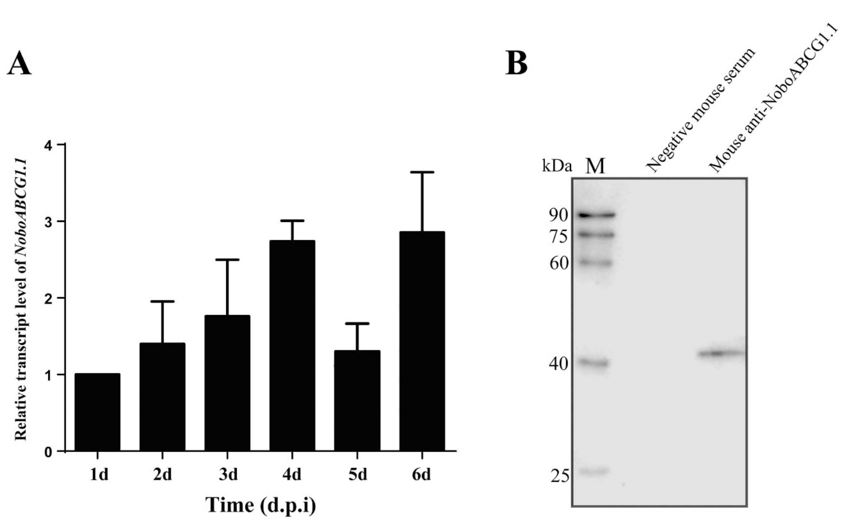

Fig. 3. Transcription and western analysis of *NoboABCG1.1*. (A) Transcription levels of *NoboABCG1.1* from infected silkworm guts. The relative expression level of *NoboABCG1.1* of 3 larval gut samples taken daily over a period of six days and measured by qRT-PCR. *N. bombycis* SSU rRNA levels were measured as a reference for normalization of expression level. The sample taken at day 1 was arbitrarily assigned a value of 1. The y-axis indicates the relative expression level of *NoboABCG1.1*, and the x-axis indicates the time post infection. Vertical bars represent the mean ± SE ($n = 3$). (B) Total protein of *N. bombycis* was used to detect specificity of NoboABCG1.1 antiserum by western blotting. M: protein marker.

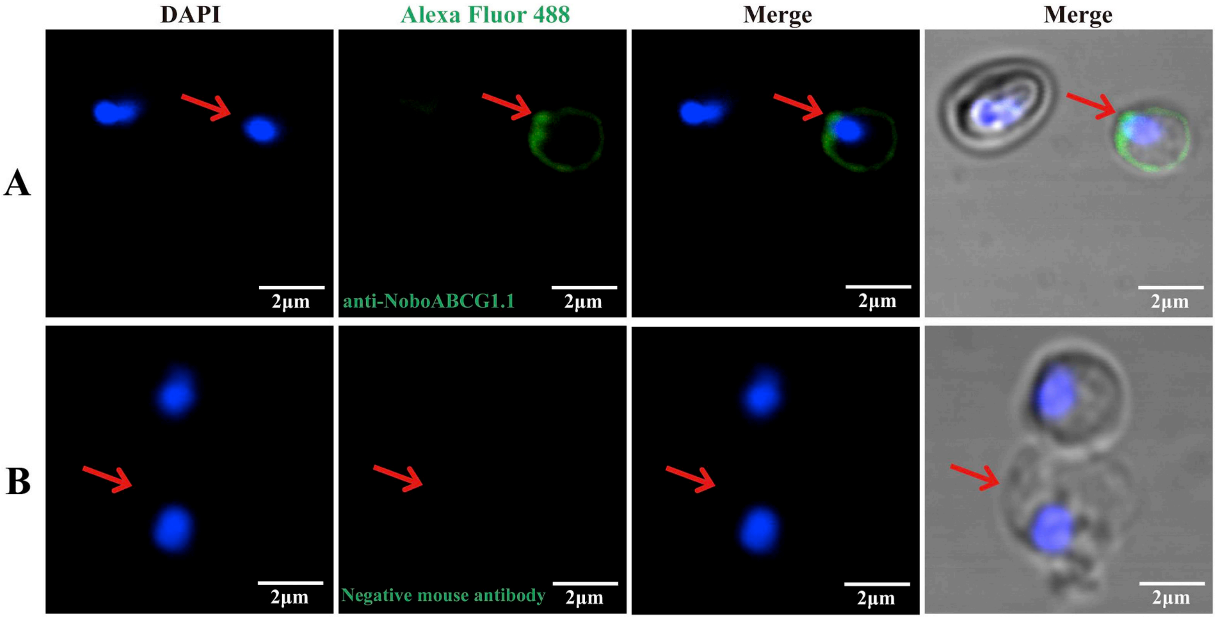

Fig. 4. Localization of NoboABCG1.1 in sporoplasm. Germinated spores stained with DAPI (nuclei) or Anti-NoboABCG1.1 (green) or both. The red arrows represent the sporoplasm from *N. bombycis*. The blue fluorescent signal represents the nuclear DNA stained with DAPI. (B) Negative control. (Bars = 2 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

day post-infection, respectively (Fig. 7A). The qRT-PCR results showed that there was no significant proliferation difference between the *dsEGFP* and *dsNoboABCG1.1* transfected cells on 1st d.p.i. Pathogens began to proliferate in *dsEGFP* transfected cells on 6th d.p.i. However, the pathogen load was significantly lower in *dsNoboABCG1.1* transfected cells on 6th dpi (Fig. 7B). Furthermore, Western blot using anti-NoboABCG1.1 was performed and confirmed the results of the qRT-PCR (Fig. 7C). Above results suggested that the NoboABCG1.1 plays important role during microsporidian proliferation as a substrate transporter.

4. Discussion

A total of 234 ABC transporter genes were identified from 18 microsporidian genomes, demonstrating that ABC transporters are often duplicated in microsporidian genomes. A remarkable feature shared by

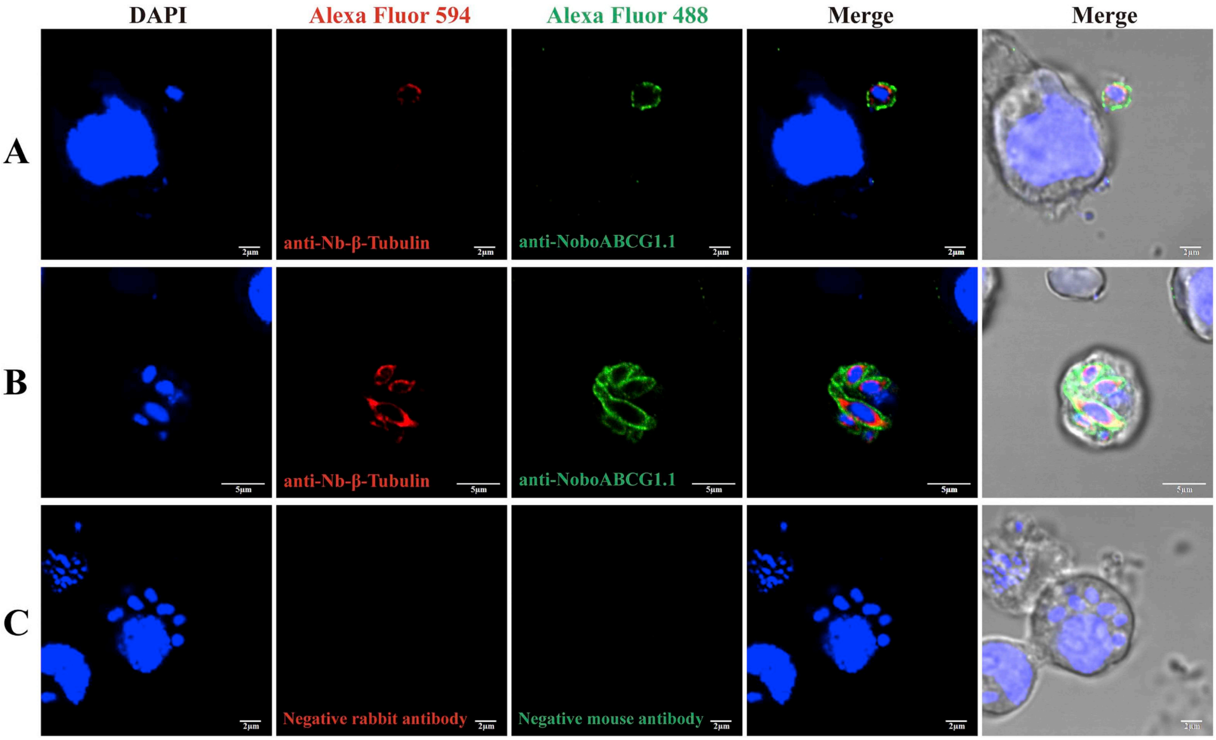

Fig. 5. Confocal micrograph showing subcellular localization of NoboABCG1.1 in SF9 cells infected with *N. bombycis*. Localization of NoboABCG1.1 (green) and tubulin (red) in meronts (A) and sporonts (B). (C) Negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

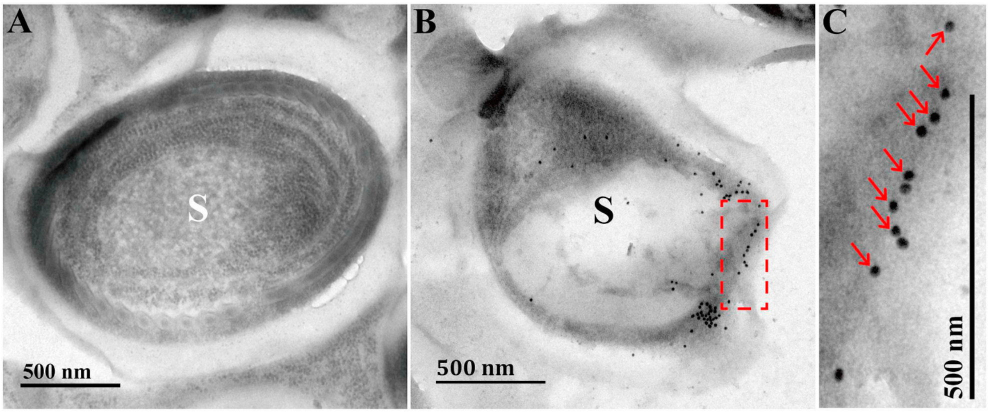

Fig. 6. Immunoelectron microscopy of NoboABCG1.1 in spores of infected ovaries. (A) Negative control. (B) Spore showing the presence of gold labeled anti-NoboABCG1.1 on the plasma membrane. (C) Enlargement of the red rectangle in (B) highlighting immuno-gold particles (red arrows). S = mature spores. (Bars = 500 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microsporidia is a dramatic genomic reduction coupled with losing the ability to synthesize key nutrients de novo during the transition from free-living to an obligate intracellular lifestyle.

ATP-Binding cassette transporters are one of the largest families of transporters. They are present in every species of microsporidia thus far examined. Fig. 2 A and B are phylogenetic representations of the ontogenies the ABCB and ABCG subfamilies and illustrates an active duplication and probable loss of these genes over time. The placement of copies on the genome as seen of Table 1 is further evidence of gene duplication.

Previous studies have shown that nucleotide transport proteins (NTT) located in the parasite's plasma membrane that were obtained from bacteria via horizontal gene transfer (HGT) where they can be used to import ATP, GTP and NAD$^+$ from the host cytoplasm (Corradi, 2015; Dean et al., 2018; Heinz et al., 2014; Tsaousis et al., 2008). ABCs was found in all living organisms, but our result show that no definitive evidence for obtaining ABC transporter via HGT in microsporidia based on these phylograms. And we found that ABCA, ABCC and ABCD subfamilies seem to be lost in most or all microsporidia. As observed in Table 2, the absence of genes encoding ABCA proteins seems to be a common feature in apicomplexans (Sauvage et al., 2006). The involvement of protozoan ABCA transporters in regulation of lipid transport and vesicular trafficking has been described for the *T. cruzi* and *Leishmania* species (Cristina et al., 2010; Sauvage et al., 2009). The function of ABCG subfamily is similar with ABCA which may be lost during adapt their parasitic life. ABCD members are involved in the import of very long chain fatty acids and/or the enzymes responsible of their oxydation into peroxisomes (Sauvage et al., 2006). The lack of peroxisomes or similar organelles in microsporidia, and more generally in Apicomplexa, could explain the apparent absence of ABCD-like proteins in these parasites. Transcriptome analysis has shown that ABC transporter genes identified in *E. cuniculi* are also expressed, indicating ABC transporter plays importer role in the basic biology of microsporidia (Grisdale et al., 2013). In addition, microsporidia seem to decreasing gene size through loss of domains, such as signal peptides (Xu et al., 2016). Our result also proved that the transmembrane helices numbers of most microsporidian ABCs were also reduced compared to the typical ABC (Hollenstein et al., 2007). These results agree with the idea of genome size reduction through the shortening of gene products including those that play key roles in cellular metabolism (Nakjang et al., 2013; Xu et al., 2016).

Microsporidian ABCs are half-size transporters thought to undergo homo or heterodimerization for their physiological activity (Beis, 2015; Velamakanni et al., 2007). The *N. bombycis* NoboABCG1.1 and NoboABCG1.2 are quite similar in amino acid sequence but different in transmembrane topology and in the locations of NBD domain. The NoboABCG1.1 protein faces the host cytoplasm while NoboABCG1.2 faces toward the pathogen's plasma membrane (Supplementary Fig. S2A, B, C). This suggests that the NBD domain toward host cytoplasm probably binds and hydrolyzes host ATP to transport substrates. The NoboABCG1.1 is expressed during all infectious stages, also suggesting its important role in substrate transportation. The PfABCG, which

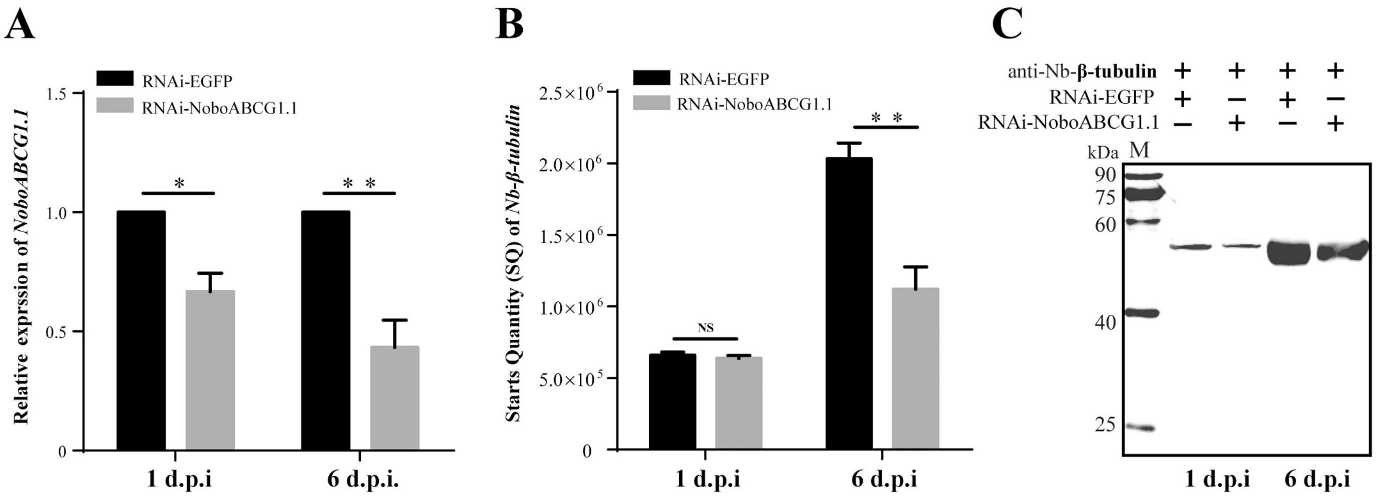

Fig. 7. Inhibition of spore proliferation after depression of *NoboABCG1.1* via RNAi. (A) The expression of *NoboABCG1.1* in infected Sf9 cell line with NoboABCG1.1-dsRNA and EGFP-dsRNA at 1 and 6 days post infection (dpi). Bars represent standard deviation of three independent replicates. Statistically significant differences are indicated with asterisks (* $P < .05$, ** $P < .01$). (B) Comparisons the number of *N. bombycis* spore between the two treatment group by qRT-PCR. (C) Quantitative detection the amounts of pathogen by western blot with anti-Nb-β-tubulin.

localized on the plasma membrane of *P. falciparum*, was reported to be involved in lipid transport (Edaye and Georges, 2015; Tran et al., 2014). In *Leishmania,* ABCG4 and ABCG6 were demonstrated to confer resistance to drugs across the plasma membrane (Bosedasgupta et al., 2008; Castanys-Muñoz et al., 2007). Here, we proved that the propagation of *N. bombycis* was significantly inhibited by knocking down the expression of *NoboABCG1.1*. These studies support our speculation that microsporidia ABCG plays important roles in substrate transportation.

This study shows the position of the ABCG transporter on the cell surface of the parasite and gives us a broader view on the microsporidian ABC transporters, and role that the ABCG subfamily plays for growth and reproduction of the microsporidia. These results provide references for further illuminating and understanding microsporidian substrates transportation, drug resistance and metabolism.

Ethics statement

The approval for animal experiments was obtained from The Laboratory Animal Welfare and Ethics Committee of The Third Medical University, Chongqing, China. The agreement number is SYXK-PLA-20120031.

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