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Transporter gene acquisition and innovation in the evolution of Microsporidia intracellular parasites

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The acquisition of genes by horizontal transfer can impart entirely new biological functions and provide an important route to major evolutionary innovation. Here we have used ancient gene reconstruction and functional assays to investigate the impact of a single horizontally transferred nucleotide transporter into the common ancestor of the Microsporidia, a major radiation of intracellular parasites of animals and humans. We show that this transporter provided early microsporidians with the ability to steal host ATP and to become energy parasites. Gene duplication enabled the diversification of nucleotide transporter function to transport new substrates, including GTP and NAD⁺, and to evolve the proton-energized net import of nucleotides for nucleic acid biosynthesis, growth and replication. These innovations have allowed the loss of pathways for mitochondrial and cytosolic energy generation and nucleotide biosynthesis that are otherwise essential for free-living eukaryotes, resulting in the highly unusual and reduced cells and genomes of contemporary Microsporidia.
Microsporidia are obligate intracellular parasites related to fungi that infect both immuno-competent and immuno-compromised humans and commercially important animals including fish, silkworms and honeybees. Microsporidia can only complete their life cycle inside a host cell and survive in the external environment as resistant thick-walled spores. The intracellular parasites grow rapidly and undergo several rounds of division before differentiating into new spores that are released into the environment to repeat the infection cycle. All microsporidian genomes have a highly reduced coding content comprising approximately 2000 to 3000 protein-coding genes and all species have lost the mitochondrial pathways for making ATP, retaining a minimal genome-lacking mitochondrion (called a mitosome) only for its essential role in Fe/S protein biogenesis. Although some species have retained genes for glycolysis, published data suggest that this pathway functions mainly in spores and is not used for making ATP in actively replicating parasites. Other species, including the major human pathogen Enterocytozoon bieneusi have lost glycolysis altogether and thus have no independent means of making their own ATP. This raises the question of how intracellular parasites acquire the enormous amounts of ATP and other nucleotides that they need to support their rapid growth and replication. It has been estimated that it takes at least \( 10^9 \) ATP molecules just to make one \( E. coli \) cell and ATP demand by the larger and more complex cells of Microsporidia is likely to be much higher. The loss of indigenous pathways for energy generation means that intracellular parasites must now obtain all of this ATP from the infected host cell.

Genome analyses suggest that reduction in metabolic capabilities is the predominant mode of microsporidian genome evolution and that this has been supported by expansion of transporter gene families to compensate for pathway loss. Given their predicted essential roles in supporting parasite growth and replication, surprisingly few of these transport proteins have been functionally characterised. Exceptions include the nucleotide transport (NTT) proteins that are expressed in the plasma membrane of Encephalitozoon cuniculi and Trachipleistophora hominis and which can transport ATP in heterologous transport assays using \( E. coli \). Phylogenetic analyses suggest that NTT transporters were acquired by horizontal gene transfer into the common ancestor of Microsporidia and Rozellia allomyces, a fungal endoparasite belonging to the paralytic group Rozellomyces that also contains Mitosporidium daphnia, and which is mainly known from environmental sequencing datasets.

Horizontal gene transfers into eukaryotes have been suggested to be important drivers of adaptive evolution but experimental data supporting this hypothesis is limited. To test the hypothesis that the horizontal acquisition of NTT transporters has played an important role in the adaptation of Microsporidia, we used phylogenetic methods and ancestral sequence reconstruction to infer the sequences of ancestral NTTs at two key points in their evolutionary history. Functional assays show that the reconstructed NTTs can transport ATP and hence would have provided early Microsporidia with the capacity to become intracellular energy parasites. To investigate how NTTs have evolved since their initial acquisition, we characterised the NTTs of three different contemporary Microsporidia species that can infect humans. The results show that NTT function has evolved to increase the range of purine nucleotides transported and to include a change in transport mechanism by individual NTTs to allow the net import of nucleotides for parasite growth and biosynthesis. The evolution of NTT function has enabled the loss of endogenous pathways for nucleotide biosynthesis and energy generation making Microsporidia dependent on NTT-mediated import for their survival. Our work demonstrates the fundamental importance of nucleotide transport proteins for the major group of medically and economically important obligate intracellular parasites infecting most animal groups.

**Results**

**Ancestral sequence reconstruction of NTT transporters.** The best fitting CAT + GTR model was used to infer a phylogeny for NTT sequences from Microsporidia, Rozellia allomyces, and outgroup Bacteria. CAT + GTR is particularly appropriate for analysis of NTTs because it explicitly models the site-specific biochemical properties of the amino acid alignment, such as the preference for hydrophobic residues in transmembrane domains. Both the monophyly of all Microsporidia NTTs, and a sister-group relationship between Microsporidia and Rozella, were recovered with maximal posterior support (PP = 1) in the consensus tree (Figs. 1a and 2a). We used the ancestral program in the PhyloBayes package to sample the most probable amino acid alignment, such as the preference for hydrophobic residues in transmembrane domains. Analyses of the inferred secondary structure of the two ancestral protein sequences containing residues known to be functionally important for NTTs (Supplementary Fig. 1 & 2) using HHpred analyses demonstrate that, like contemporary NTTs, they are members of the Major Facilitator Superfamily (MFS; Supplementary Table 2). Analyses of the inferred secondary structure of the two ancestral protein sequences containing residues known to be functionally important for NTTs (Supplementary Fig. 1 & 2) using HHpred analyses demonstrate that, like contemporary NTTs, they are members of the Major Facilitator Superfamily (MFS; Supplementary Table 2).

The ancestral sequences AncNTT \(_{Roz/Mic}\) and AncNTT \(_{Mic}\) were synthesised as codon-optimised genes for expression in \( E. coli \), a heterologous host that can be used for transport assays with radioactive \([\alpha^{32}\text{P}]\)-ATP. Consistent with their predicted secondary structure (Supplementary Fig. 3a), the expressed proteins were detected in the \( E. coli \) membrane fraction (Fig. 1c; Supplementary Fig. 4). Both proteins also transported \([\alpha^{32}\text{P}]\)-ATP in a time-dependent manner (Fig. 1d) and at high affinity as demonstrated by the low apparent \( K_m \) values (Fig. 1e; Supplementary Fig. 3b). Competition assays using a range of substrates to compete with \([\alpha^{32}\text{P}]\)-ATP indicated that the “ancestral” NTTs have relatively narrow substrate ranges, as only ATP and ADP consistently inhibited transport below 50% compared to the controls (Fig. 1f). Competition assays can only give an indication of whether a particular substrate might be transported because they do not actually measure transport of the cold substrate, so we tested transport of radio-labelled UTP, dTTP, GTP and NAD\(^+\) by AncNTT \(_{Roz/Mic}\) and AncNTT \(_{Mic}\). Both of the ancestral NTTs exhibited strong uptake of radio-labelled ATP; at least a tenfold increase compared to the empty vector controls (Fig. 1g) and only weak uptake of the other tested nucleotides. Some members of the MFS transporter family, including some NTTs used by intracellular bacteria, are symporters that can use proton gradients to drive net import. The protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) can be used to dissipate the proton gradient to identify symporters experimentally. We therefore tested transport of radio-labelled ATP by both ancestral transporters in the presence or absence of CCCP, and included PamNTT5 of Protothecomydia.
amoebophila, an intracellular bacterial symbiont of Acanthamoeba, as a known symporter and positive control. The addition of CCCP (Fig. 1h) did not inhibit transport indicating that AncNTT_Roz/Mic and AncNTT_Mic are ATP/ADP exchangers, a result that was supported by a counter-exchange assay for AncNTT_Roz/Mic (Fig. 1i). In these assays, [α-32P]-ATP-loaded bacteria were exposed to buffer or buffer containing unlabelled external ATP, and the residual radiolabel associated with harvested bacteria followed over time. AncNTT_Roz/Mic exhibited a higher rate of export of radionucleotide when incubated with ATP (Fig. 1i), as reported previously for the E. cuniculi exchanger EcNTT3. Together these data suggest that acquisition of an...
NTT gene by the common ancestor of Microsporidia and Rozella would have provided a tool to steal host ATP and thus to become an energy parasite.

Functional analysis of NTTs from contemporary Microsporidia. To investigate the role(s) that NTT transporters play in contemporary parasite biology, we characterised the NTTs of three phylogenetically distinct Microsporidia (Fig. 2) that can infect humans as well as animals. The presence of NTT genes in all microsporidan genomes and the expansion of NTT gene copy number by independent lineage- and clade-specific gene duplication events (Fig. 2), coupled with the central roles of nucleotides in cellular metabolism, suggest that NTT-mediated transport plays an important role in the biology of contemporary Microsporidia. In classical theory, gene duplication is thought to provide an important pathway to evolutionary innovation because it can free individual gene copies to evolve new functions. Expansion of transporter functional repertoire must have occurred during Microsporidia evolution because, in addition to the absence of pathways for ATP production, the early ATP-expensive steps needed for the de novo biosynthesis of the nucleotides needed to make DNA, RNA and nicotinamide cofactors are also missing from sequenced genomes. To test the hypothesis that NTT transporter function has evolved after duplication, and thus enabled pathway loss, we investigated the functional biology of NTTs from Encephalitozoon cuniculi and Trachipleistophora hominis which cause opportunistic infections of humans and are among the few model species that can be grown in co-culture with mammalian cells. The NTTs from Enterocytozoon bieneusi, the most common cause of human infection and which cannot yet be grown in the laboratory, were also characterised as was the single NTT of Rozella allomycis, the endoparasitic fungal sister lineage to Microsporidia. Rozella has less reduced gene content than Microsporidia including components of a mitochondrial electron transport chain for making ATP, and a lifestyle that alternates between a free-living motile zoospore and a naked endoparasitic stage.

The draft genome sequence of Enterocytozoon bieneusi was used to guide synthesis of its four NTTs (EbNTT1–4) as codon-optimised genes for expression in E. coli. The four NTT genes from E. cuniculi (EcNTT1–4) and from T. hominis (ThNTT1–4) as well as the single NTT gene from R. allomycis were all cloned and expressed in E. coli. All of the NTTs transported [α-32P]-ATP (Fig. 2b) in a time-dependent manner (Fig. 2c). To calculate an apparent Km and Vmax for the T. hominis, E. bieneusi, and R. allomycis NTTs, [α-32P]-ATP uptake was performed in the presence of increasing concentrations of non-radio-labelled ATP (Fig. 2d). The low Km values for all of these transporters are similar to published values for E. cuniculi NTTs (Fig. 2d; Supplementary Fig. 3b) and suggest that they have high affinity for ATP. Moreover, the Km values for the Microsporidia and Rozella NTTs are well below the estimated ATP concentration for the cytoplasm of eukaryotes (~3 mM), suggesting that transport of ATP from infected host cells would not be limiting during the parasite life cycle.

Analysis of the E. bieneusi, E. cuniculi, T. hominis, and Rozella allomycis genomes demonstrate that they lack genes for the enzymes needed to make purine and pyrimidine nucleotides de novo (Supplementary Fig. 5 and 6). To investigate if the Microsporidia NTTs can transport other nucleotides to fill these gaps, we carried out competition assays for NTT-mediated 32P-ATP uptake in the presence of an excess of a variety of individual cold purine and pyrimidine nucleotides, nucleosides, and nicotinamide derivatives. The results of the competition experiments and published data for E. cuniculi suggest that the NTTs of all three Microsporidia are purine nucleotide transporters with a preference for adenosine and guanosine triphosphates and diphosphates (Fig. 3a). By contrast, transport by R. allomycis RaNTT1 was strongly reduced only by cold ATP and ADP (Fig. 3a). The addition of cold ITP-reduced and IDP-reduced ATP transport below 50% by some Microsporidia NTTs (Fig. 3a), but these are unlikely to be substrates for nucleotide biosynthesis since the genomes of all three species lack the enzymes for incorporating ITP or IDP into cellular metabolism (Supplementary Fig. 6). ATP transport by some T. hominis, E. cuniculi and E. bieneusi NTTs was also inhibited to varying degrees by nicotinamide derivatives (mainly NAD+, NADH and NADP+) suggesting that these might be transported (Fig. 3a), as demonstrated previously for one of the NTTs (PamNTT4) of Protochlamydia amoebophila. NADH and NADPH are predicted to be essential co-factors in core metabolic reactions in Microsporidia but genome analyses suggest that unlike Rozella allomycis, the Microsporidia species E. cuniculi, T. hominis and E. bieneusi cannot make NAD+ derivatives de novo (Supplementary Fig. 7). Retention of the NAD+ kinase and phosphohydrolase enzymes needed to interconvert between NAD+ and NADP+ (Supplementary Fig. 7) does suggest, however, that if NAD+ can be transported, then all three species could make NADH, NADP+ and NADPH.

To test the hypotheses of substrate transport generated by the competition data, we investigated the transport of radio-labelled UTP, dTTP, GTP and NAD+ by the individual NTTs when expressed in E. coli. The results of these experiments demonstrate that GTP is transported at high levels for all of the Microsporidia.

Fig. 1 Ancestral reconstruction and functional characterisation of nucleotide transporters. a Schematic representation showing the position of the nodes in the NTT phylogenetic tree for which ancestral sequences (AncNTT<sub>Roz/Mic</sub> and AncNTT<sub>Mic</sub>) were inferred and functionally characterised. We obtained point estimates of the ancestral NTT protein sequences by selecting the amino acid with the highest posterior probability at each site in the alignment (Supplementary Data 1 and 2). b Inferred secondary structure and membrane topology of one contemporary (ThNTT4) and the two ancestral sequences, predicted using HHMTOPIER. c Truncation western blot of fractionated E. coli expressing different NTTs detected using an anti-His antibody. Ancestral gene = AncNTT<sub>Roz/Mic</sub>, E. cuniculi = Ec, R. allomycis = Ra. Total = sonicated bacteria, Inclusions = 20,000 g pellet, Membranes = 150,000 g pellet. Cytosol = supernatant after 150,000 g spin. Complete blots are shown in Supplementary Figure 4. d Kinetics of [32P]-ATP uptake by ancestral NTTs expressed in E. coli: pET16b = empty vector control. e Substrate saturation curve for the uptake of [32P]-ATP in the presence of increasing concentrations of unlabelled ATP. Data is fitted to a Michaelis–Menten equation to determine Km (µM) and Vmax (pmol/min/mg) by iteration. f Competitive substrate inhibition against [32P]-ATP uptake. Competitors were at 50,000× excess over the radio-labelled ATP. Data points represent residual radioactivity within the bacteria after subtraction of the empty vector control. g Nucleotide uptake of [32P]-labelled pyridine (dTTP and UTP) or purine (ATP and GTP) nucleotides or NAD+ by the ancestral NTTs. h Effect of the prokaryotic CCCP on [32P]-nucleotide uptake by the two ancestral proteins and PamNTT5 of Protochlamydia. Significant difference (∗) to the control was only seen for PamNTTs (p < 0.05; one-way ANOVA). i Back-exchange assay whereby [32P]-ATP-loaded E. coli expressing AncNTT<sub>Roz/Mic</sub> were incubated in the presence or absence (=Buffer) of unlabelled ATP. Data shows residual intracellular label in harvested E. coli cells. All data points represent means ± SD of at least three independent experiments.
NTTs (Fig. 3b). This suggests that the two main classes of purine nucleotides are transported by Microsporidia NTTs and this is consistent with genome analyses that suggest that both types would need to be imported (Figs. 2b and 3). By contrast, we found no evidence for GTP transport above background by the single NTT of *R. allomycis* (RaNTT1), and thus it appears that transport by RaNTT1 is restricted to ATP and ADP among the substrates tested (Figs. 2b and 3). Radio-labelled \(^{32}\text{P}-\text{NAD}^+\) uptake assays in *E. coli* revealed that all of the NTTs from *T. hominis* and *E. bieneusi* could transport \(^{32}\text{P}-\text{NAD}^+\) (Fig. 3b). The weak \(^{32}\text{P}-\text{NAD}^+\) transport detected for two (EcNTT2 and EcNTT3) of the four *E. cuniculi* NTTs (Fig. 3b) was only slightly higher than, and not
significantly different \((p > 0.05)\) to, the empty vector control. The observation that the *R. allomycis* RaNTT1 did not transport NAD\(^+\) \((\text{Fig. 3b})\) is consistent with genome analysis that suggests that, unlike Microsporidia, *R. allomycis* can make this important cofactor (Supplementary Fig. 7).

The competition data provided no evidence that ATP transport by Microsporidia NTTs was significantly inhibited by pyrimidine substrates (Fig. 3a), despite evidence from genome analysis (Supplementary Fig. 5) that Microsporidia lack the enzymes needed to make pyrimidines de novo. Genome analyses do suggest that, if UTP were to be transported, then Microsporidia have the enzymes to make the other pyrimidine nucleotides needed for nucleic acid biosynthesis (Supplementary Fig. 5).\(^{15,29}\)

We therefore investigated if radio-labelled UTP or dTTP were transported in our assays, but detected no convincing evidence for transport of either substrate by the Microsporidia or *Rozella* NTTs (Fig. 3). It has previously been suggested\(^{15}\) that a conserved family of Microsporidia putative transporters related to the *E. coli* NupG nucleoside transporter\(^{14}\) might be used by the parasites to import host cell nucleosides, potentially providing starting substrates for pyrimidine nucleotide biosynthesis. However, in addition to the absence of any published experimental data to support nucleoside transport by these parasite proteins, the genome of *E. cuniculi*\(^{15}\) and other Microsporidia\(^{29}\) appear to lack the kinases needed to use nucleosides for pyrimidine nucleotide biosynthesis.\(^{29}\) This suggests that nucleoside import would not solve the pyrimidine deficit for these species.

Our data suggest that NTT substrate range has evolved over time facilitated by gene duplication, allowing the loss of parasite pathways for ATP and nucleotide biosynthesis. However, the inferred ancestral NTT phenotype of nucleotide exchange cannot provide the net import of nucleotides needed for nucleic acid synthesis and the increase in parasite biomass observed during intracellular infection (Fig. 4a,b). We therefore tested transport of radio-labelled ATP by all of the NTTs in the presence or absence of CCCP\(^{27}\). The results suggest that whereas the *Rozella* NTT and most of the Microsporidia NTTs have retained the ancestral exchange phenotype (Fig. 3c), *T. hominis* ThNTT4, and *E. bieneusi* EbNTT1 and EbNTT2 have independently evolved into symporters capable of carrying out net nucleotide import (Figs. 3c and 5a). To investigate further, we carried out counter-exchange assays\(^{14,27}\) for a representative sample of both types of NTT (Fig. 3d). ThNTT4 and PamNTT5 exhibited no increased rate of export of radio-labelled nucleotide consistent with them being symporters\(^{14,27}\), while EcNTT3\(^{14}\), ThNTT2 and RaNTT1 exhibited a higher rate of export when incubated with ATP (Fig. 3d), suggesting that they are exchangers. Taken together, our data suggest that, in addition to using NTTs for net import of NAD\(^+\) and purine nucleotides.

**Fig. 2** Phylogeny and ATP transport by Microsporidia and *Rozella* NTTs. a NTT phylogeny for the Microsporidia/R. allomycis clade of endoparasitic fungi inferred under the CAT+GTR model in PhyloBayes. The tree is the posterior consensus tree inferred under the CAT+GTR model, in which all relationships with posterior support <0.5 were collapsed. Branch lengths are proportional to the expected number of substitutions per site. Scale bar = 0.5 changes per site. A single-ancestral acquisition of a bacterial NTT gene is inferred in the common ancestor of Microsporidia and *Rozella* followed by independent gene duplications and family expansion during the radiation of Microsporidia. The NTTs that were functionally characterised in this study are indicated, including the two ancestral NTTs shown at the base of the tree. Support values are Bayesian posterior probabilities. b Uptake of \([32P]\)-ATP by *E. coli* cells expressing NTTs for 30 min. All NTT gene and species names are given. ATP uptakes for all NTTs were significantly different \((p < 0.05, \text{one-way ANOVA})\) to the pET16b control. c Kinetics of \([32P]\)-ATP uptake in *E. coli* expressing NTTs from *E. bieneusi* (EbNTT1–4) or *R. allomycis* (RaNTT1). d Substrate saturation curves for \([32P]\)-ATP to determine \(K_m\) and \(V_{max}\) for NTTs from *T. hominis* (ThNTT1–4), *E. bieneusi* (EbNTT1–4), and *R. allomycis* (RaNTT1). Curves were fitted to the Michaelis–Menten equation and the \(K_m\) and \(V_{max}\) were calculated by iteration. All data points represent means ± SD of at least three independent experiments.
**Fig. 3** Substrate competition assays and nucleotide uptake assays for Microsporidia and Rozella NTTs. 

**a** Substrate competition assays whereby [32P]-ATP uptake by NTT-expressing *E. coli* was performed in the presence of 50,000 × excess unlabelled substrate. Th *T. hominis*, Eb *E. bieneusi*, Ra *R. allomycis*. 

**b** [32P]-nucleotide uptake assays with *E. coli* cells expressing NTT genes. Uptake of NAD⁺ by *E. cuniculi* NTTs was not significantly different to empty vector controls (p > 0.05, one-way ANOVA). Coloured bars show transport data for the indicated pyrimidine nucleotides (dTTP or UTP), purine nucleotides (ATP or GTP) or NAD⁺. pET empty vector control. 

**c** [32P]-nucleotide uptake by NTT-expressing *E. coli* in the absence (set to 100% after control was subtracted) or presence of carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Significant difference at p < 0.05 (one-way ANOVA) is shown with *. 

**d** Back-exchange assay with [32P]-ATP-loaded *E. coli* incubated in the presence or absence of 100 µM unlabelled ATP. Data shows the residual radioactivity following washing of the bacteria. PamNTT5 is a positive control symporter from the bacterium *Protochlamydia amoebophila*. All data (mean ± SD) is representative of at least three independent experiments.
**Fig. 4** Protein localisation and transcript abundance for *T. hominis* NTT transporters over a time course of *T. hominis* infection. 

**a** Immunofluorescence time course (3–96 h) of rabbit kidney (RK-13) cells infected with *T. hominis* spores using published individual rabbit anti-ThNTT antibodies (red). Rat anti-HSP70 (green) was used to label the mitosomes of intracellular parasites (meronts). The first time point at 3 h is shortly after injection of the *T. hominis* sporoplasm into the host cell when labelling by antisera to ThNTT1 and ThNTT4 is already apparent. The top DIC image shows the spore bags (arrows) at 96 h with superimposed labelling (red) by antisera to ThNTT1 and ThNTT3 but not by antisera to ThNTT4. Scale bar is 1 µm.

**b** Increase in parasite biomass during *T. hominis* infection time course measured using cell diameter and cell volume. Diameter of parasite cells (minimum 100 counted) was taken at their widest point, and cell volumes were calculated using Axiovision software. Error bars are standard deviation. *N* = 3.

**c** RNAseq analysis showing transcript abundance (log_{10} FPKM (Fragments per kilobase per million mapped reads)) for the four ThNTTs, spore protein (PTP2), a glycolytic enzyme (PGK1) and ribosomal protein L37 throughout the infection time course.
Fig. 5 Gene duplication and evolution of Microsporidia NTTs. a Phylogeny of Microsporidia and Rozella NTTs and their transported substrates. ATP transport by the predicted ancestral NTTs AncNTT_{Roz/Mic} and AncNTT_{Mic} are shown at the respective nodes. The tree topology suggests that the common ancestor of Microsporidia clade III and IV could already transport both purine nucleotides and potentially NAD⁺ as indicated by the cross-hatched boxes. b A model for T. hominis NTT-mediated acquisition of energy and nucleotides from infected cells. NTTs are located at the parasite plasma membrane and can act as exchangers (ThNTT1-3) or symporters (ThNTT4) enabling energy parasitism or net nucleotide uptake, respectively. The transporters and/or pathways used by T. hominis to acquire pyrimidine nucleotides are currently unknown.
exchanger to symporter has been coupled with increased gene expression, providing critical complementary adaptations that can support rapid parasite growth.

**Discussion**

Ancestral sequence recreation (ASR) brings with it a number of uncertainties associated with the reliability of ancient phylogenetic reconstruction, but it nevertheless represents a powerful tool to test hypotheses about the function of genes in the deep past. Here, we provide experimental evidence that the inferred ancestral NTT sequences at two early and critical points in Microsporidia evolution (Fig. 1a), encode proteins that can insert into biological membranes and undergo the necessary conformational changes required to transport ATP and ADP. Moreover, the transport kinetics (apparent $K_m$ and $V_{max}$) of the ancestral proteins fall within the variation reported for contemporary NTTs, and they are consistent with the observed in susceptible eukaryotic populations. Their success as intracellular parasites is the major reason for the reduced host fitness and increased mortality imposed by the translocation of large volumes of ATP and ADP, which requires a huge metabolic drain on host resources imposed by the translocation of nucleotides.

**Nucleotide uptake assays.** Prior to uptake experiments, freshly transformed colonies were screened for NTT expression by western blot (2–3 days prior) and with the highest NTT expression levels used in subsequent experiments. For all uptake assays, bacteria were resuspended to an OD$_{600}$ of 5.0 in PBS. Single endpoint experiments were performed for 30 min for all radio-labelled nucleotides. An empty pET16b vector was used as a control in all experiments. To determine the initial rate of transport, $[^{32}P]$-ATP uptake activity was recorded in the presence of increasing concentrations of non-radio-labelled substrates as described previously. Data was fitted with the Michaelis–Menten equation to determine the apparent $K_m$ and $V_{max}$ of transport. Back-exchange assays were carried out as described previously, whereby E. coli cells were loaded with $[^{32}P]$-ATP and incubated in PBS containing 100 $\mu$M cold ATP and the residual radioactivity was detected by filtering the bacteria through a 0.45 $\mu$m cellulosic nitrocellulose membrane filter at different times post-washing. Carboxyl cyanide m-chlorophenyl hydroxylamine (5 min; 250 $\mu$M CCHP; Sigma Aldrich) was used to dissipate the proton gradient prior to uptake assays. All radiochemicals were obtained from Hartmann except $[^{32}P]$-nicotinamide adenine dinucleotide (NAD$^+$), which was obtained from Perkin Elmer. Unlabelled nucleotides that were used in competition and uptake assays were obtained from Sigma Aldrich and prepared according to the manufacturer’s instructions. All uptake experiments were performed as independent triplicates and where indicated, significant difference was assessed using a one-way ANOVA performed using the statistical program SPSS.

**Methods**

**Host cell culture and parasite propagation.** The Microsporidia *Trachipleistophora hominis* (ATCC—PRA-404) was initially isolated from a human HIV/AIDS patient and is now routinely sub-cultured within the rabbit kidney cell line RK-13 (ATCC—CCL-37) at 33 °C in Dulbecco’s Modified Eagle Medium (DMEM), containing 10% FCS, penicillin (100 $\mu$g/ml) and streptomycin (100 $\mu$g/ml). Spores used in time course experiments were harvested from infected RK-13 cells grown in 20 175 cm$^2$ tissue culture flasks. Cells were washed and scraped into phosphate buffered saline (PBS, pH 7.4) and then lysed by sonication. The released spores were purified on a Percoll gradient prior to adding them to uninfected RK-13 cells.

**Immunofluorescence microscopy of NTT transporters.** Samples of infected RK-13 cells used in the RNASeq analysis were grown in parallel on 13 mm coverslips for immunofluorescence. At selected times post-infection, cells were washed twice in PBS prior to being fixed at 20°C for 1–2 h with methanol/acetic acid (50:50). Fixed cells were washed in PBS, blocked in 1% milk in PBS and then labelled using published rabbit antiserum (1:100) against *T. hominis* NTT transporters. Anti-rat anti-sera (1:100) to *T. hominis* mitosomal Hsp70 (THHSP70) were previously published. Cells were also stained with DAPI (Molecular Probes) and mounted in Vectashield Hard Set (VectorLabs). Microscopy was performed with a Zeiss Axioslager II epifluorescence microscope using a x63 objective lens and images were processed using Axiosvision software.

**Cloning and gene synthesis of nucleotide transporters.** Primers for the amplification of NTT transporter genes are given in Supplementary Table 1. *Entocytozoon bieneusi* genomic DNA was obtained from purified spores (kindly provided by Dr. Elizabeth Didier, Tulane University, USA) and NTT genes for sequence checking were PCR amplified and named EbNTT1–4. The synthetic NTT genes used for transport assays were codon-optimised for expression in E. coli and synthesised by GeneArt. The single NTT gene from *Roseella* sp. was PCR-amplified from *R. allomyces* genomic DNA (kindly provided by Dr. Timothy James, University of Michigan, USA) and named RaNTT1. All transporter genes were cloned into pET16b (Novagen) in frame an N-terminal dual-astatin tag and confirmed by sequencing. pET16b plasmids encoding *T. hominis* or *E. cuniculi* NTT transporters were prepared previously.

**NTT expression, western blotting and bacterial fractionation.** For all NTTs, expression was performed from a pET16b vector freshly transformed into E. coli Rosetta2 DE3 pLysS (EMDMillipore). Luria Broth was inoculated with individual colonies and shaken overnight at 37 °C prior to the inoculation of Terrific Broth (TB; Sigma). At OD$_{600}$ 0.4–0.6, cells were chilled to 18 °C prior to the addition of 1 ml of TB medium to induce gene expression for 16–18 h. Bacteria were centrifuged on ice, sedimented at 6000 × g for 5 min and washed twice with cold PBS. Standard western blot analysis was performed on 20 µg total bacterial lysate using an anti-polyHis antibody (H1029, Sigma) and the colonies expressing the highest levels of NTTs were used for subsequent studies. To assess membrane localisation, bacteria were sonicated followed by differential ultra-centrifugation to isolate a high-speed membrane fraction (20,000 × g × 10 min and 150,000 × g × 2 h) for western blot analysis.

**Synchronising infection of RK-13 cells by *T. hominis* and RNASeq analysis.** To synchronise the infection of *T. hominis* healthy RK-13 cells grown in 150 cm$^2$ round tissue culture dishes, were incubated with freshly prepared spores as previously described and then subjected to extensive washing after 2 h. Two dishes of cells were used for RNA purification at 3, 14, 22, 40, 70 and 96 h post-inoculation. Sequencing, including library preparation, was carried out using the Illumina stranded mRNA sample preparation kit, with sequencing carried out on two lanes of an Illumina HiSeq 2500, producing a total of 27.5 M paired-end reads representing *T. hominis* transcripts. Sequencing reads were trimmed using Cufflinks and CuffDiff as previously described.

**Phylogenetic analysis and inference of ancestral NTT sequences.** We augmented the sequence sampling of a published NTT phylogeny with the NTT protein sequences from recently sequenced Microsporidia genomes using BLASTP searches at NCBI, using *T. hominis* ThNTT4 as the query. Sequences were aligned using MUSCLE and the alignment was manually edited to remove insertions found in single sequences and the very C-terminal end of the alignment, where there was no recognisable homology between the bacterial and eukaryotic sequences. The alignment can be downloaded from Figshare (https://doi.org/10.6084/m9.figshare.5170729.v1) and is also included as a high-resolution pdf for on-screen visualisation (Supplementary Fig. 2). We tested the fit of the single-matrix LG model, and the site-heterogeneous CAT$^{+}$ Poisson and CAT$^{+}$ GTR$^{44}$ models to the alignment using PhyloBayes 3.3$^{33}$, in all cases using a discretised gamma distribution (four categories) to model among-site rate variation. Posterior
predictive simulations indicated that only the CAT + GTR model provided an adequate fit to the alignment with respect to site-specific sequence composition, which is known to be an important factor in accurate phylogenetic inference.  

We therefore used the CAT + GTR model both to infer the tree and to reconstruct ancestral sequences, using the ancestral program that is part of the PhyloBayes package. Figure 2 depicts the posterior consensus tree inferred under the CAT + GTR model, in which all relationships with posterior support <0.5 were collapsed. Branch lengths are proportional to the expected number of substitutions per site. We obtained point estimates of the ancestral NTT protein sequences in the Microsporidia common ancestor and in the common ancestor of Microsporidia and Rozella by selecting the amino acid with the highest posterior probability at each site. The JGI 15 genome was used to be an intranuclear parasite within Enterocytozoonidae microsporidia. 

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Author contributions

P.D., T.A.W., K.M.S., A.K.W., R.P.H., E.R.S.K. and T.M.E. conceived and designed experiments. P.D., K.M.S., A.K.W., A.V.G., E.K. and P.M. performed the experiments. P.D., S.N., K.M.S., A.K.W. and T.A.W. analysed the data. P.D., K.M.S., T.A.W., R.P.H. and T.M.E. wrote the paper in consultation with all authors.

Additional information

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