Freibert, S-A., Goldberg, A. V., Hacker, C., Molik, S., Dean, P., Williams, T., Nakjang, S., Long, S., Sendra, K. M., Bill, E., Heinz, E., Hirt, R. P., Lucocq, J. M., Embley, T. M., & Lill, R. (2017). Evolutionary conservation and in vitro reconstitution of microsporidan iron–sulfur cluster biosynthesis. *Nature Communications, 8*, [13932]. https://doi.org/10.1038/ncomms13932

Publisher's PDF, also known as Version of record
License (if available): CC BY
Link to published version (if available): 10.1038/ncomms13932

Link to publication record in Explore Bristol Research
PDF-document

This is the final published version of the article (version of record). It first appeared online via Nature Publishing Group at doi:10.1038/ncomms13932. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research
General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available: http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/
Evolutionary conservation and in vitro reconstitution of microsporidian iron-sulfur cluster biosynthesis

Sven-A. Freibert1,*, Alina V. Goldberg2,*, Christian Hacker3,4, Sabine Molik1, Paul Dean2, Tom A. Williams2, Sirintra Nakjang2, Shaojun Long2, Kacper Sendra2, Eckhard Bill5, Eva Heinz2, Robert P. Hirt2, John M. Lucocq3, T. Martin Embley2 & Roland Lill1,6

Microsporidians are obligate intracellular parasites that have minimized their genome content and sub-cellular structures by reductive evolution. Here, we demonstrate that cristae-deficient mitochondria (mitosomes) of Trachipleistophora hominis are the functional site of iron-sulfur cluster (ISC) assembly, which we suggest is the essential task of these organelles. Cell fractionation, fluorescence imaging and immunoelectron microscopy demonstrate that mitosomes contain a complete pathway for [2Fe–2S] cluster biosynthesis that we biochemically reconstituted using purified mitosomal ISC proteins. The T. hominis cytosolic iron-sulfur protein assembly (CIA) pathway includes the essential Cfd1-Nbp35 scaffold complex that assembles a [4Fe–4S] cluster as shown by spectroscopic methods in vitro. Phylogenetic analyses reveal that the ISC and CIA pathways are predominantly bacterial, but their cytosolic and nuclear target Fe/S proteins are mainly archaeal. This mixed evolutionary history of Fe/S-related proteins and pathways, and their strong conservation among highly reduced parasites, provides compelling evidence for the ancient chimeric ancestry of eukaryotes.
Microsporidia are an enormously successful group of highly reduced obligate intracellular parasitic fungi that infect a wide range of eukaryotic hosts including immuno-compromised AIDS patients. They are also pathogenic for a number of economically significant animals including key pollinators and fish. Microsporidia have played a central role in hypotheses about early eukaryotic evolution and the importance of mitochondria in that process. These microorganisms show a remarkable reduction of their metabolic capacities as well as a striking simplification of core eukaryotic cellular structures including the mitochondrion. This organelle appears as a tiny, double membrane-bounded cell compartment commonly called a mitosome. The discovery of the microsporidian mitosome and similar organelles in, for example, Giardia was an important milestone in formulating the hypothesis that mitochondria are an ancestral feature of all eukaryotic cells, but the selective pressure(s) governing their retention in modern eukaryotes are generally unknown, particularly for non-model species. Since some homologues of the mitochondrial iron-sulfur cluster (ISC) assembly machinery have been found encoded in microsporidian genomes and localized to mitosomes, it was suggested that this biosynthetic process was connected to the retention of the organelle. However, the biological function the microsporidian ISC proteins has not been experimentally verified, and the exclusive localization of some ISC components to the mitosome has been controversial.

The maturation of iron–sulfur (Fe/S) proteins is the only known essential biosynthetic function of Saccharomyces cerevisiae mitochondria. The pathway is conserved in other eukaryotes including humans. In brief, the process consists of three steps. First, a [2Fe–2S] cluster is synthesized on the scaffold protein Isu1 requiring the cysteine desulfurase complex Nfs1–Isd11 as a sulfur donor, the ferredoxin Yah1 as an electron donor and frataxin Yfh1 (ref. 11). Second, the newly synthesized cluster is released from Isu1 by a dedicated Hsp70 chaperone system (Ssq1, Jac1) and transferred to the monothiol glutaredoxin Grx5 from where [2Fe–2S] proteins can be assembled. The final step is specific for [4Fe–4S] proteins such as respiratory complexes I and II, and requires a dedicated set of ISC proteins including Isa1–Isa2–Iba57 for [4Fe–4S] cluster synthesis, and Nfu1, Boll1, Bol3 and Ind1 for specific cluster insertion into apoproteins.

Surprisingly, the essentiality of mitochondrial Fe/S protein biogenesis in yeast and humans is not explained by the maturation of endogenous, mitochondrial Fe/S proteins, but by the indispensable role of the ISC assembly machinery in the maturation of cytosolic-nuclear Fe/S proteins involved in key pathways of life including ribosome assembly and function, DNA replication and repair, chromosome segregation, and telomere length regulation (for review see ref. 14). The ISC assembly machinery produces an unknown, sulfur-containing compound that is exported by the mitochondrial ABC transporter Atm1 for utilization by components of the cytosolic iron–sulfur protein assembly (CIA) machinery that catalyse the maturation of both cytosolic and nuclear Fe/S proteins.

We have also identified the components of the microsporidian CIA pathway, and have functionally characterized the putative microsporidian CIA scaffold complex. Finally, by using phylogenetic methods we examined the evolutionary origins of the microsporidian ISC and CIA machineries as well as their cytosolic-nuclear target Fe/S proteins. Our work provides strong evidence for the functional importance of the mitosome for these highly reduced intracellular parasites in Fe/S protein metabolism. Further, the work allows detailed insights into the complex evolutionary history of this ancient and essential biosynthetic pathway of eukaryotes.

Results
Sub-cellular localization of microsporidian ISC components.

Hidden Markov models (HMM, see ‘Methods’ section) were used to search the genomes of two phylogenetically distinct microsporidia, Encephalitozoon cuniculi and T. hominis, for homologues of yeast and human ISC proteins. The two microsporidia differ in genome size and genomic content. E. cuniculi has a small genome of 2.9 Mb with ~2,000 genes, whereas T. hominis is over 8.5 Mb with ~3,000 genes. In addition to the previously detected five of ten core ISC homologues (that is, the cysteine desulfurase complex Nfs1–Isd11, the scaffold protein Isu1, frataxin Yfh1, a putative glutaredoxin Grx5 (ref. 7) and mitochondrial Hsp70 (Ssc1)3,4), we identified the genes for ferredoxin Yah1, its reductase (Arh1) and the co-chaperone Jac1 in both genomes (Supplementary Table 1; Supplementary Fig. 1). Phylogenetic analyses recovered ThArh1 and EcArh1 in a monophyletic group with S. cerevisiae Arh1 (Supplementary Fig. 2), consistent with them being orthologues of the yeast mitochondrial enzyme. We did not detect a gene with similarity to the nucleotide exchange factor Mge1, which in yeast cooperates with Ssq1–Jac1 (ref. 18). Consistent with the absence of genes encoding mitochondrial [4Fe–4S] proteins such as aconitase and respiratory complexes I and II, we did not detect any ISC genes (for example, ISA1, ISA2 and IBA57) for this specific part of the ISC maturation pathway in either microsporidian suggesting that only the core ISC system for [2Fe–2S] protein assembly is present in microsporidia.

Previously, some microsporidian ISC proteins have been shown to localize to mitosomes in E. cuniculi and T. hominis using indirect immunofluorescence microscopy. By contrast, T. hominis ThIsu1 and ThYfh1 were predominantly detected in the cytosol. To reinvestigate the functional locations of these two T. hominis core ISC components, we made new antibodies to T. hominis ThIsu1 and ThYfh1 for immunoelectron microscopy. Glutaraldehyde-fixed, T. hominis-infected RK cells were cryo-sectioned and immunogold-labelled using these specific antisera. Both ThIsu1 and ThYfh1, like other core ISC proteins including controls, localized exclusively to mitosomes that were detectable as small, double membrane-bounded organelles with minor and major axes ranging between 47 to 119 nm and 78 to 267 nm, respectively (Fig. 1a,b). To precisely localize the ISC proteins, we compared the distribution of gold label for each individual protein with that of random points, placed over the corresponding mitosome profiles (Fig. 1c,d; Supplementary Fig. 3). The resulting frequency distribution demonstrated that the ISC proteins were located predominantly towards the matrix side of the mitosomal inner membranes (86.8% in a 20 nm band inside the mitosome matrix and 13.2% outside). For independent...
biochemical verification of the mitosomal localization, mitosomes were enriched by differential centrifugation of *T. hominis*-infected cells. All analysed core ISC proteins (ThNfs1, ThIsu1, ThYah1, ThYfh1, ThArh1) were accumulated in the 25,000 g (mitosome-enriched) pellet of infected RK-13 cells, which also contained the *T. hominis* homologue of mitochondrial ThIsu1.

![Figure 1](image1.png) **Figure 1 | Sub-cellular localization of *T. hominis* Fe/S-cluster assembly components.** Thawed cryo-sections of glutaraldehyde-fixed *T. hominis*-infected RK-13 cells were labelled with antibodies to *T. hominis* ISC components and protein-A gold. (a) Quantitative analysis. Labelling is expressed as density of gold labelling over compartment profile area (estimated by point counting; see ‘Methods’ section; 34–40 micrographs analysed per protein/experiment with following number of golds per experiment: ThIsu1, 64; ThNfs1, 67; ThYah1, 35; ThYfh1, 70; ThIsd11, 111). Error bars indicate the s.e.m. (N = 3). (b) Images illustrating the distribution of labelling for ISC components in mitosomes (these show examples of positive labelling only and therefore do not reflect densities quantified in a). Labelling appears to be located over the matrix of the double membrane-bounded organelle profiles (with mean minor and major axes of 80 and 127 nm, respectively; N = 50 mitosome profiles). The analysed profiles were morphologically undistinguishable from mitosomes that labelled positively for ThHsp70 as shown here and described previously. Labelling was expressed as gold particles per random point count and indicates the concentration of labelling at the IM/matrix interface. The numbers of gold and random counts were as follows: ThIsu1, 79; ThNfs1, 66; ThYah1, 37; ThYfh1, 63; ThIsd11, 50. Labelling for all tested ISC components was towards the mitosomal matrix with an enrichment at the IM. (c) Immunogold labelling distribution over mitosome matrix. Cumulative fraction plot shows pooled gold labelling data for all five ISC components (ISC labelling) compared with points located simple uniform random (random). ISC components show relative enrichment of labelling close to the IM (see Supplementary Fig. 3 for individual analyses).

![Figure 2](image2.png) **Figure 2 | Biochemical localization of the *T. hominis* ISC pathway components.** (a) Western blots using antibodies to *T. hominis* proteins for fractions obtained by differential centrifugation from RK cells or RK cells infected with *T. hominis* (Th). Centrifugation speeds of pellet fractions are given above each lane. Sup = final 100,000 g (100 K) supernatant. (b) Western blot of the 25,000g (25 K) pellet fraction of *T. hominis*-infected RK cells treated with or without proteinase K (PK) and Triton X-100 detergent as indicated.
Hsp70 (ThHsp70), a validated mitosomal marker protein\(^3\), and ThTom70, an orthologue of the yeast mitochondrial outer membrane protein import receptor Tom70 (Fig. 2a). The ISC proteins were protected against degradation by protease \(K\), unlike the cytosol-exposed ThTom70, but were degraded on membrane lysis using detergent (Fig. 2b). These independent datasets support the exclusive mitosomal matrix location of all core ISC proteins.

**Functional analysis of the mitosomal ISC proteins.** Since there are no well-established genetic tools for the manipulation of microsporidians, we employed heterologous complementation of yeast mitochondrial ISC depletion mutants to test the functionality of the microsporidian ISC genes\(^7\), although it is difficult to predict *a priori* which genes will complement successfully. For example, targeting of *T. hominis* ThIsu1 to yeast mitochondria rescued growth of a conditional yeast mutant and restored mitochondrial Fe/S-cluster biosynthesis, but the *E. cuniculi* orthologue (EcIsu1) did not complement\(^7\). In the present study, we tested complementation of yeast ISC mutants depleted for Isd11, Arh1, Yah1 and Yfh1 by the respective *T. hominis* homologues. Correct mitochondrial localization of the *T. hominis* ISC proteins was achieved by fusing a well-characterized and highly effective fungal mitochondrial presequence to the N terminus of each ISC protein\(^21\). Of the four *T. hominis* ISC proteins tested (Supplementary Table 2) only ThIsd11 rescued growth of the respective Gal–ISD11 mutant (Supplementary Fig. 4a). Co-expression of ThIsd11 together with the cysteine desulfurase ThNfs1 actually inhibited the growth of yeast wild-type cells suggesting a negative effect on endogenous cell function (Supplementary Fig. 4b). Complementation also failed when the electron transport chain partners ThArh1 and ThYah1 were co-expressed in the respective yeast mutants (Supplementary Table 2). The reasons why the tested microsporidian proteins did not complement yeast mutants, despite being unambiguous homologues of the respective yeast proteins, are not known. *T. hominis* ISC proteins are typically highly divergent in their primary sequence (see the long branches in the phylogenetic trees; Supplementary Fig. 2), and hence it is possible that they may not correctly interact with the residual endogenous yeast ISC partners\(^11\). However, the small number of residues known to be involved in yeast Nfs1–Isu1, Nfs1–Yfh1 and Isu1–Yfh1 interactions are conserved in *T. hominis* homologues suggesting that the mitosomal proteins still use similar interaction surfaces\(^22,23\).

As a direct functionality test of the mitosomal ISC components we used a recently developed *in vitro* system that follows the *de novo* synthesis of a [2Fe–2S] cluster on the scaffold protein Isu1. Detection of this key reaction of mitochondrial Fe/S protein biogenesis makes use of a circular dichroism (CD) signal change at 431 nm that reflects Fe/S-cluster assembly on Isu1 (ref. 11). The *in vitro* reaction closely mimics the physiological situation, as it is kinetically fast, depends on all six ISC factors required in vivo, and occurs independently of the artificial reductant dithiothreitol. The *T. hominis* ISC proteins ThNfs1–ThIsd11, ThYfh1, ThYah1 and ThIsu1 were over-produced and purified from *E. coli* (Supplementary Fig. 5). Since we were unable to purify functional *T. hominis* ThArh1

**Figure 3** | *In vitro* reconstitution of Fe/S-cluster synthesis on *T. hominis* Isu1. (a) The *T. hominis* proteins ThIsu1, ThNfs–ThIsd11, ThYfh1, ThYah1 and human FdxR (the homologue of yeast Arh1) were mixed anaerobically in buffer R (standard reaction). Cysteine was added to start Fe/S-cluster synthesis, which was recorded by the CD signal change at 431 nm. Replicate reactions were performed, in which individual components of the *T. hominis* core ISC pathway or iron (Fe) were systematically omitted. (b) After 15 min full CD spectra were recorded for each reaction mixture, and compared with the spectrum of a standard reaction using ISC proteins from *C. thermophilum* (C.t.). (c) The initial rates of Fe/S-cluster synthesis on ThIsu1 for the different reactions were recorded. (d) The initial rates were estimated by linear regression and compared with the standard reaction using C.t. ISC components. N\(\geq 5\); Error bars = s.d.
for this assay, we used purified human FdxR. This protein has previously been shown to be functional for de novo Fe/S-cluster synthesis with yeast ISC proteins. The synthesis reactions were carried out anaerobically in the presence of reduced iron, cysteine and NADPH. When all purified T. hominis ISC proteins and human FdxR were mixed, a rapid and efficient generation of [2Fe–2S] clusters on ThIsu1 was observed (Fig. 3a,b). Omission of any of the six ISC factors greatly decreased the CD signal, as previously described in experiments using the yeast ISC proteins. The initial rates of ISC factor-lacking reactions were close to the background levels observed in the absence of iron (Fig. 3c,d; see also ref. 11). Both the initial rates and the efficiencies of [2Fe–2S] cluster formation by the mitosomal ISC proteins were similar to those
obtained with mitochondrial ISC proteins from either the thermophilic fungus Chaetomium thermophilum (Fig. 3b) or S. cerevisiae\(^1\). A comparison of the CD signal intensities of ISC-reconstituted and chemically reconstituted ThIsu1 (which binds one [2Fe–2S] cluster per dimer\(^1\)) revealed the association of about one [2Fe–2S] per ThIsu1 dimer, similar to yeast or Chaetomium Isu1. The ability of mitosomal ISC components to de novo assemble a [2Fe–2S] cluster on ThIsu1 at rates and efficiencies comparable to those of mitochondrial ISC proteins demonstrates the functional equivalence of both biosynthetic systems.

**Localization and function of ISC export and CIA components.**

We next investigated the components of the ISC export pathway\(^14\). The mitochondrial ABC transporter Atm1, together with glutathione (GSH) and Erv1, is crucial for yeast cytosolic and nuclear Fe/S protein biosynthesis, by exporting a sulfur-containing product that is essential for the function of the CIA machinery\(^24–27\). We first used an HMM-generated profile based on fungal Atm1 sequences to search for homologues in the predicted *T. hominis* proteome. Among 12 potential candidates, the three highest scoring *T. hominis* sequences (termed ThAtm1\(_1\), ThAtm1\(_2\) and ThAtm1\(_3\)) possessed structural features of the B sub-family of ‘half-size’ ABC transporters as expected for Atm1-like proteins (Supplementary Figs 1d and 6a)\(^28\). All three ThAtm1\(_k\) sequences also clustered strongly with the yeast and *Neurospora* Atm1 protein sequences in phylogenetic analysis (Supplementary Figs 2 and 6b), consistent with their common ancestry.

Western blots and proteinase K protection assays of cell fractions identified a protein of the correct size for ThAtm1\(_1\) in the mitosome-enriched fraction (Fig. 4), whereas no specific labelling was detected by immunostaining with antibodies against ThAtm1\(_2\) or ThAtm1\(_3\). Immunofluorescence microscopy using the antibody to ThAtm1\(_1\) showed the co-localization of the punctate signals for ThAtm1\(_1\) with those of the mitosomal marker ThHsp70 suggesting that ThAtm1\(_1\) is a credible candidate for a functional homologue of yeast Atm1 (Fig. 4). We also tried to localize the ThAtm1 proteins by immuno-EM using specific antibodies, but did not observe specific labelling of *T. hominis* mitosomes or any other intracellular compartment. Further searches in the *T. hominis* or *E. cuniculi* genomes identified the two glutathione biosynthesis genes GSH1 and GSH2 (coding for γ-glutamyl-cysteine synthase Gsh1 and glutathione synthase Gsh2), but no orthologue of yeast GSH1 and GSH2, or *E. cuniculi* GSH1 and GSH2, respectively. This species is not associated with any other intracellular compartment.

We therefore employed an in vitro functional test for the ability of ThCfd1 and ThNbp35 to assemble a [4Fe–4S] cluster\(^34\). Both *E. cuniculi* and *T. hominis* Cfd1–Nbp35 contain the conserved cysteine residues shown to coordinate the Fe/S clusters of this complex\(^35\) (Supplementary Figs 1e and 8a). ThCfd1 and His-tagged ThNbp35 were co-overexpressed in *E. coli* and co-purified indicating hetero-complex formation (Fig. 5b, inset right). The brownish colour of the protein solution indicated the presence of Fe/S clusters, but at under-stoichiometric amounts. We therefore chemically reconstituted ThCfd1–HisThNbp35 under anaerobic conditions in the presence of ferric ammonium citrate and Li2S to yield a dark-brown protein complex with 9.5 Fe and 8.6 S bound per heterodimer (Fig. 5b, inset left)\(^34\). This amount is close to the expected stoichiometry of 8 Fe and 8 S for binding of the N terminal and bridging [4Fe–4S] clusters. Both ultraviolet–vis and electron paramagnetic resonance (EPR) spectroscopy showed spectral features (absorption peak at 420 nm; EPR g values of 1.89, 1.92 and 2.05) consistent with the presence of [4Fe–4S] clusters (Fig. 5b,c). When ThCfd1 was purified and reconstituted

We tested all three *T. hominis* Atm1 candidates for complementation of a yeast ATM1 mutant using high and low level expression vectors with the addition of a fungal mitochondrial precursor\(^21\) at the N terminus of the *T. hominis* sequences, but none of them showed any detectable rescue of the growth defect of an Atm1-deficient yeast mutant (Supplementary Fig. 7; Supplementary Table 2). Using this approach, we were thus unable to functionally confirm that ThAtm1\(_1\) is a mitosomal homologue of yeast Atm1. However, in a recently published crystal structure of yeast Atm1 that contained a bound GSH ligand in a positively charged binding pocket\(^32\), several residues were identified that are generally conserved in eukaryotes. These include the disease-relevant position D398 (corresponding to residue E433 in human ABCB7) in the case of X-linked sideroblastic anaemia and cerebellar ataxia (XLSA/A)\(^33\), and yeast residues R280, R284 and N343. Intriguingly, in the putative *E. cuniculi* and *T. hominis* Atm1 sequences the latter residues are also conserved suggesting a functional similarity (Supplementary Fig. 1d).

![Figure 4 | Localization of the T. hominis homologue of mitochondrial Atm1.](image-url)
without ThNbp35, only small amounts of Fe/S cluster were bound, even after reconstitution (Supplementary Fig. 8b) showing that this protein only binds clusters in a labile fashion. All these findings are strikingly similar to those made previously for yeast Cfd1–Nbp35 (ref. 35). Taken together, the results demonstrate the binding of two [4Fe–4S] clusters to ThCfd1–ThNbp35, and suggest that it can potentially serve as the microsporidian CIA scaffold complex.

Eukaryotes contain multiple monothiol glutaredoxins36 that function in different cellular compartments, with mitochondrial yeast Grx5 and human GLRX5 playing a role in cellular Fe/S protein biogenesis37–39 and cytosolic yeast Grx3–Grx4 and human GLRX3 (PICOT) being involved in intracellular iron distribution and cytosolic-nuclear Fe/S protein biogenesis40–42. We previously showed the functional complementation of a Grx5-depleted yeast cell by an E. cuniculi monothiol glutaredoxin (previously annotated as EcGrx5 (ref. 7)) on its targeting to mitochondria with a foreign presequence. Here, we tested the T. hominis Grx homologue (annotated here as ThGrx3) for yeast Grx5 mutant complementation, but unlike EcGrx5, ThGrx3 did not rescue the growth of the Grx5-depleted yeast mutant on targeting to mitochondria43, and this could explain why we had observed complementation by the E. cuniculi EcGrx5 (ref. 7). Because cytosolic monothiol glutaredoxins contain an additional N-terminal thioredoxin-like (Trx) domain36, we searched for its presence in the microsporidian glutaredoxins. We found a divergent, N terminally-truncated Trx domain in EcGrx5 using standard bioinformatics tools and a similar (40% identity) stretch of sequence in ThGrx3 that was nevertheless not recognized as a Trx domain in the same analyses (Supplementary Figs 1c and 9c). Taken together, our data suggest a predominant cytosolic localization of ThGrx3 and, consistent with that location, some structural similarity of both microsporidian proteins to yeast Grx3/Grx4, rather than Grx5 (Supplementary Table 1).
Fe/S proteins play a significant role in eukaryotic physiology. Microsporidians, in general, have a monophyletic origin, suggesting that there is strong negative selection against gene replacement due to the important roles that these proteins play. In contrast, important nuclear and cytosolic Fe/S proteins do appear to have an archaeal origin. Monophyly of eukaryotic sequences, including those from microsporidia, is generally considered to descend from an Alphaproteobacterium vertically inherited from the mitochondrial endosymbiont, which is not archaeal as might be expected based on evidence for an archaeal origin of the host for the mitochondrial endosymbiosis. By contrast, important nuclear and cytosolic Fe/S proteins do appear to have an archaeal origin. Monophyly of eukaryotic sequences, including those from microsporidia, is generally observed, suggesting that there is strong negative selection against gene replacement due to the important roles that Fe/S proteins play in eukaryotic physiology.

**Evolutionary origin of microsporidian ISC and CIA components.** We inferred phylogenies for the mitochondrial ISC and CIA pathways in the genomes of *E. cuniculi* and *T. hominis*. Individual components are coloured according to their inferred evolutionary origins (Supplementary Fig. 2). Components of the mitosomal ISC pathway appear to have originated from the mitochondrial endosymbiont. The CIA pathway is largely bacterial in character, and not archaeal as might be expected based on evidence for an archaeal origin of the host for the mitochondrial endosymbiosis. By contrast, important nuclear and cytosolic Fe/S proteins do appear to have an archaeal origin. Monophyly of eukaryotic sequences, including those from microsporidia, is generally observed, suggesting that there is strong negative selection against gene replacement due to the important roles that Fe/S proteins play in eukaryotic physiology.

**Figure 6 | Evolutionary origins of microsporidian Fe/S-related proteins.** The cartoon shows the components of the eukaryotic ISC and CIA machineries conserved on the genomes of *E. cuniculi* and *T. hominis*. Individual components are coloured according to their inferred evolutionary origins (Supplementary Fig. 2). Components of the mitosomal ISC pathway appear to have originated from the mitochondrial endosymbiont. The CIA pathway is largely bacterial in character, and not archaeal as might be expected based on evidence for an archaeal origin of the host for the mitochondrial endosymbiosis. By contrast, important nuclear and cytosolic Fe/S proteins do appear to have an archaeal origin. Monophyly of eukaryotic sequences, including those from microsporidia, is generally observed, suggesting that there is strong negative selection against gene replacement due to the important roles that Fe/S proteins play in eukaryotic physiology.
Dre2 sequences are shorter than other eukaryotic Dre2 sequences and lack the N-terminal region, which has been shown to be dispensable for yeast viability. Microsporidia have retained the conserved Dre2 C-terminal domain that interacts with Tah1 and carries both [2Fe–2S] and [4Fe–4S] clusters. Recent analyses of Dre2 from other eukaryotes suggested that it was present in the LECA, but did not resolve its specific prokaryotic origin. Taken together, these data suggest that the CIA pathway is of mainly bacterial heritage and was already in place in the LECA.

By contrast to the predominantly bacterial origin of both the ISC and CIA pathways, important nuclear and cytosolic Fe/S proteins from eukaryotes including microsporidia appear to have been inherited from the archaeal host (Fig. 6; Supplementary Fig. 2). Phylogenies for Elp3 and the paralogous genes Rad3, Chl1 and RETL1 are consistent with a closer relationship of eukaryotic sequences to Archaea rather than Bacteria, but the low support values at this depth from trees of single proteins prevented inference of a particular founding prokaryote. Homologues of the ABC protein Ril1 (full-length version including both the Fe/S and ABC domains) and the primase Pri2 were only found among eukaryotes and Archaea. On the basis of phylogeny and the close structural similarities between the B-family of replicative DNA polymerases of eukaryotes and Archaea, it has been suggested that eukaryotic Fe/S proteins are derived from archael versions, with eukaryotic DNA polymerases of the epsilon type having a chimeric prokaryotic ancestry. Our analyses for the four Fe/S-cluster-containing paralogues (α, δ, ε, ζ) of replicative B family DNA polymerases from eukaryotes including microsporidia are consistent with that hypothesis, but the support values were generally low. Trees for Ppat, Ntg1 and Dna2 sequences are consistent with the early common origin of most eukaryotic Fe/S proteins, but were insufficiently resolved to infer their prokaryotic affinities (Fig. 6; Supplementary Fig. 2).

**Discussion**

In microsporidia and other eukaryotes, a bacterial pathway for making Fe/S clusters now serves to support the maturation of ‘archaeal-like’ nuclear and cytosolic proteins. The ISC pathway components that function inside the mitochondrion/mitosome provide evidence for their origin from the alphaproteobacterial mitochondrial endosymbiont. The cytosolic pathway is also largely bacterial in character, but the proteins show no particular link to the Alphaproteobacteria in our trees. Strictly speaking, our analyses cannot exclude the possibility of the co-origin of both pathways, because the genomes of modern Alphaproteobacteria are chimeric due to lateral gene transfer events between prokaryotes, and there is no reason to believe that ancient Alphaproteobacteria were any different. However, it is interesting that the Fe/S protein biogenesis pathway inside the organelle retains strong evidence of alphaproteobacterial ancestry but the CIA components do not. An origin of both the ISC and CIA pathway ‘en bloc’ would have facilitated the replacement of the original archael host pathways for Fe/S protein biosynthesis with an already co-functioning pathway of bacterial character.

Our data strongly suggest that the function of the T. hominis mitosome is to support the maturation of essential cytosolic and nuclear Fe/S proteins by a cytosolic pathway linked to the organanelle-hosted ISC system by its dependence on an exported sulfur-containing substrate of unknown identity. Interestingly, the only mitosomal Fe/S-cluster-containing protein identified in the genomes of *E. cuniculi* and *T. hominis* is the [2Fe–2S] protein ferredoxin (Yah1), which is itself an essential component of the mitochondrial core ISC pathway representing a ‘chicken and egg’ situation. Studies in yeast and human cells have already shown that depletion of the mitochondrial membrane potential or defective mitochondrial ISC or cytosolic CIA pathways cause genome instability and lead to induction of the DNA damage pathway. This data demonstrates that there is strong negative selection against any perturbation of these pathways explaining why a role in Fe/S protein biogenesis is so strongly conserved — more so than ATP production for example — among diverse mitochondrial homologues. The ISC and CIA pathways and target Fe/S proteins are retained in all available microsporidian genomes, and there are no other mitochondrial biosynthetic pathways similarly conserved. A role in Fe/S protein biosynthesis therefore appears to be the essential function and remaining selective pressure for retention, of this most minimal version of the eukaryotic mitochondrion.

**Methods**

**Identification of microsporidian Fe/S-related proteins.** Clusters of orthologous groups (KOGs) containing human or yeast proteins involved in mitochondrial or cytosolic Fe/S protein biosynthesis or nuclear or cytosolic Fe/S-cluster-containing proteins were used as the starting material for our analyses. Each KOG was supplemented with the orthologues from *T. hominis*, *Dictyostelium discoideum*, *Entamoeba histolytica* HM-1:IMSS, *Giardia lamblia* ATCC 8080, *Trichomonas vaginalis*, *Cryptosporidium parvum* Iowa type II and *Plasmodium falciparum*. These were identified using the COGNITOR method, which assigns a protein sequence to a KOG if the top two BLASTP hits (E-value cutoff of 0.001) from different species are members of the same KOG. In cases where a putative orthologue was not detected for one of the parasites using this approach, we also searched using HMMER to apply more sensitive HMM profile (Hidden Markov model)-based methods. To identify any protein-coding genes that might have been omitted from published proteomes by mis-annotation during automated analyses we used TBlastN to search the primary genome data.

**Evolutionary origin of microsporidian Fe/S-related proteins.** To investigate the evolutionary origins of the microsporidian Fe/S protein biogenesis machinery and nuclear and cytosolic Fe/S-cluster-containing proteins, we constructed sets of homologous sequences for each gene from eukaryotes, Bacteria and Archaea using a variety of eukaryotic and prokaryotic reference sequences as starting seeds for database searches against the NCBI non-redundant database using BLAST. It has already been suggested that some genes of the mitochondrial Fe/S protein biogenesis pathway originated with the mitochondrial endosymbiont, which is thought to have been a member of the Alphaproteobacteria. We therefore included a representative sample of Alphaproteobacteria in our analyses as well as a broad sample of bacterial and archael diversity. To ensure that we included the most similar prokaryotic homologues of eukaryotic proteins, we complemented our analyses with the ten best prokaryotic BLASTP hits to eukaryotic sequences.

The sequence sets resulting from this protocol were aligned with Muscle, Mafft, ProbCons, Kalign and FastA, a consensus alignment was generated using M-Coffee, and poorly-aligning regions were detected and removed using the ‘automated’ mode in trimAl (ref. 69), which chooses from among several editing modes of varying strictness depending on the overall level of alignment conservation. We built initial 100-bootstrap maximum likelihood trees using the LG + F model in RaxML to verify that our homology searches had identified orthologues of the eukaryotic genes. Bayesian phylogenetic trees were made using the C20 model implemented in PhyloBayes, which is an empirical variant of the flexible CAT model, which contains a mixture of twenty site-specific frequency profiles estimated from a large database of alignments. Like CAT, it performs well on alignments of highly divergent sequences that are typical of
overnight. After washing the cells were used to inoculate 2 l of TB medium and

Yeast mutant complementation by microsporidian genes. The T. hominis genes

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13932 | www.nature.com/naturecommunications

ascorbate, 0.3 mM FeCl2). The reaction was transferred to a CD cuvette, sealed

In vitro reconstitution of mitosomal Fe/S-cluster synthesis. The assay followed a procedure described earlier11. In brief, recombinant T. hominis proteins were expressed in E. coli with a His-tag and purified by Ni-NTA affinity chromatography followed by gel filtration (Akta Purifier System 10, Column 16/60 Superdex 2000 pg, GE Healthcare). Samples for the in vitro Fe/S-cluster synthesis assay were prepared in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA). Protein solutions and reagents were incubated under anaerobic conditions overnight at 10 °C before the experiments. The 300 μl standard reaction assay contained 2.5 μM ThNf1–ThId11, 3 μM ThYfh1, 3 μM ThYah1, 0.3 μM human FdsR and 100 μM of either ThIsul1 or Ctsul5 in buffer R (35 mM Tris–HCl, pH 8, 150 mM NaCl, 0.2 mM MgCl2, 0.2 μM FeCl3, 20 μM PLP, 0.5 mM NADPH, 0.5 mM ascorbate, 0.3 mM FeCl3). The reaction was transferred to a CD cuvette, sealed tightly and placed at 30 °C in a CD spectrophotometer equipped with an automatic stirring device (J-815, Jasco). After 2 min of temperature equilibration the Fe/S-cluster synthesis reaction was initiated by anaerobic addition of 0.5 mM cysteine. The CD signal change at 415 nm was recorded. Subsequently, full spectra were recorded from 300 to 650 nm. Initial rates were estimated by a linear fit to the initial 4.5 min of the reaction. Evaluation of the data were carried out using Origin 8 G software.

Purification of the T. hominis Cfd1–Nbp35 complex. The pETDuet-1 plasmid (Novagen) was used for heterologous co-expression of N terminally His-tagged ThNbp35 and untagged ThCfd1 in E. coli strain BL21 (DE3). Cells were incubated in 50 ml LB medium with ampicillin (100 mg l−1) final concentration) at 37 °C overnight. After washing the cells were used to inoculate 21 of TB medium and grow at 37 °C. Protein expression was induced at OD600 of 0.8 by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside in the presence of 50 μM FeCl3 followed by incubation at 28 °C overnight. Cells were collected by centrifugation and shock-frozen.

Cells were resuspended in 50 ml of lysis buffer (35 mM Tris–HCl pH 7.4, 300 mM NaCl, 5% (v/v) glycerol) and disrupted by sonication (20 min on ice with 1 s intervals). After centrifugation for 90 min at 60,000g the lysate was passed over a HisTrap HP column (1 ml; GE Healthcare) and bound ThCfd1–HisThNbp35 complex eluted using an Akta Purifier System 10 (GE Healthcare) applying an isotropic imidazole gradient from 50 mM to 1 M. The eluate was subjected to size exclusion chromatography (16/60 200 pg, GE Healthcare), and ThCfd1–HisThNbp35 was purified to apparent homogeneity in storage buffer (35 mM Tris–HCl pH 7.4, 120 mM NaCl). Purified protein was aliquoted, shock-frozen and stored at −80 °C until use.

Chemical reconstitution of Fe/S clusters on ThCfd1–HisThNbp35. For chemical Fe/S-cluster reconstitution ferric ammonium citrate and L-β-S (each 10 mM in storage buffer) were freshly prepared. ThCfd1–HisThNbp35 (80 μM) was reduced with 720 μM DTT for 1 h at 25 °C in an anaerobic chamber, and subsequently diluted with storage buffer to 20 μM final concentration. Reconstitution was started by stepwise addition of ferric ammonium citrate to a final concentration of 200 μM. Subsequent additions of iron were added to a final concentration of 200 μM. After incubation for 15 min reconstituted proteins were desalted using a PD-10 column equilibrated with storage buffer and concentrated to a final concentration of 40 μM.

EPR spectroscopy of ThCfd1–HisThNbp35. For EPR spectroscopy, chemically reconstituted ThCfd1–HisThNbp35 complex (40 μM) was anaerobically reduced with sodium dithionite (200 μM). Samples were shock-frozen after incubation for 3 min. X-band EPR derivative spectra were recorded on a Bruker ELEXSYS E580 spectrometer equipped with a Bruker dual mode cavity (ER11EMD) and a helium flow cryostat (Oxford Instruments ESR 900). The microwave bridge was a high-sensitivity Super-X bridge (Bruker ER-049X) with integrated microwave frequency counter. The magnetic field controller (ER032T) was calibrated with a Bruker NMR field probe (ER035M). EPR simulations were performed with the self-made routine eismght (by E.B.).

Protein expression and generation of antibodies. Full-length ThGRX5/3 and ThARH1 genes were cloned using the Champion pET100 Directional TOPO Expression kit, a TOM70 gene fragment encoding the cytoplasmic domain was cloned into the expression vector pET16b and all the proteins were expressed in BL21 (DE3) E. coli cells as recombinant histidine-tagged proteins. Expressed proteins were purified by gel electrophoresis for the commercial (Agrisera, Sweden) generation of rabbit antibodies. Antibodies for the third candidate T. hominis Atm1 homologues were generated commercially (BioGenes, GmbH) against two peptides corresponding to most variable domain of each candidate Atm1. The sequences of the peptides were: Atm1_1: DYNIYKNSFIEIKK and KNEKSTKNAESQD; Atm1_2: RGEHEETNDRGNNS and QDDTRYQNATEDGST and Atm1_3: DTIKKLERSPHMSK and DEGQEPAAKRYLET.

Fractionation of infected rabbit kidney cells. Rabbit kidney cells (RK-13) were routinely cultured in 175 cm2 flasks, infected with T. hominis spores and grown at 37 °C in Dulbecco’s Modified Eagle Medium (DMEM), containing kanamycin 100 μg ml−1, penicillin 100 μg ml−1, streptomycin 100 μg ml−1 and fungizone 1 μg ml−1 (refs 3,7,5). Infected cells were harvested by trypsinisation for 5 min at 37 °C and washed by one wash in complete medium and two washes with PBS. A final wash was done in HSDP buffer (0.25 M sucrose, 10 mM HEPEs pH 7.2) containing 1 × protease inhibitor cocktail (Sigma). All subsequent manipulations were performed on ice. Cells were resuspended in cold HSDP buffer containing DNase (1 U ml−1) and RNase (10 mg/ml) and homogenized by 30 strokes in a Dounce homogenizer or by sonication. Cell lysis was verified by trypan blue exclusion. The RK-13 cell lysate was left at room temperature for 20 min and subjected to differential ultracentrifugation in a fixed-angle Ti 70.1 rotor (Beckman) using the following parameters: 1,000g for 10 min, 10,000g for 30 min, 25,000g and 100,000g for 1 h. The final 100,000g supernatant represented the cytosolic fraction. After each centrifugation step pellets were resuspended in PBS and protein concentration was determined using standard BCA assay. All samples were diluted in loading buffer and were boiled for 5 min except samples that were to be used for Atm1 protein visualization, which were heated at 37 °C for 10 min. Twenty micrograms of protein for each fraction was loaded per lane for SDS–PAGE and Western blot analysis using HRP-conjugated secondary antibodies (Jackson Laboratories). Proteins were visualized by chemiluminescence using a ChemiDoc XRS + imager system (Biorad).

To affinity-purify the anti-Atm1_1 antibody, the original antiserum was diluted 1:10 in TBS-Tween 1% milk and incubated overnight with a nitrocellulose membrane blot with recombinant Atm1_1 run on an SDS–PAGE gel. After washing the membrane three times with TBS-T 1% milk solution, the antibody was diluted 1/250 with TBS-Tween 1% milk and incubated overnight with a nitrocellulose membrane blot with recombinant Atm1_1 run on an SDS–PAGE gel. After washing the membrane three times with TBS-T 1% milk solution, the antibody was eluted with 0.1 M glycine, pH 2.5 and 0.15 M NaCl. The concentration of IgG was quantified using a BCA Protein Assay Kit (Pierce) and stored with 10% BSA at −20 °C before use for IFA and WB. As an alternative approach to reduce host background, the antigenic activity of the anti-Atm1_1 antiserum, it was diluted 1/250 with TBS-T 1% milk solution and incubated for 1 h (twice) with nitrocellulose membranes blotted with RK-13 total protein extract separated by SDS–PAGE. The purified antisera were then used directly for IFA and/or WB.

Protease protection assay for mitosomal-enriched fractions. Proteins K protection assays were performed on the 25,000g (mitosomal-enriched) pellet of infected RK-13 cells obtained as described above. The 25,000g pellet was washed twice with HSDP buffer without protease inhibitors and incubated with 50 μg ml−1 proteinase K (Roche) for 20 min, or 0.2% (v/v) Triton X-100 for 10 min at room temperature followed by incubation with 50 μg ml−1 – proteinase K (Roche) for 20 min. The membrane fractions were then incubated with trichloracetic acid at 20% (v/v) final concentration for 30 min on ice. Precipitated proteins were sedimented by centrifugation and solubilized in loading buffer. The mitosomal proteins were subsequently analysed by western blot.

Immunofluorescence assay localization of T. hominis proteins. Immunofluorescence assay (IFA) was done as described previously7. Briefly, RK-13 cells infected with T. hominis were grown on coverslips until confluent and then fixed in acetone/methanol 50:50 v/v at −20 °C for 2 h. After blocking with 5% milk in PBS, slides were incubated for 1 h with a 1% (v/v) milk/PBS solution containing the relevant antigen, washed in PBS and then incubated for 1 h with the appropriate secondary antibodies: Rat anti-ThHsp70 was used as a mitosomal marker, the remaining antibodies against T. hominis proteins were raised in rabbit. Goat anti-rabbit antibodies (Invitrogen) conjugated to Alexa 594 (red) or 488 (green) fluorophores were used as secondary antibodies. Cells were incubated with the secondary antibody and permeabilized for DNA. All cells were visualized using either a Leica TCS SP2 UV confocal microscope or a Zeiss Axioimager II epifluorescence microscope with a X63 objective lens.
Small fragments of cell pellets were mounted onto specimen carriers, plunge-frozen and freeze-dried. For cryo-electron microscopy, the cell pellets were fixed in 50% acetone at −20°C for 20 min, then in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4) for 2 h, and post-fixed in 1% osmium tetroxide buffered with 0.1 M cacodylate (pH 7.4) for 1 h. After dehydration in a series of increasing concentrations of ethanol and acetone, the samples were infiltrated with and embedded in Araldite 502 resin (TAAB Laboratories). Ultrathin sections were then picked up onto 200-mesh copper grids (Agar, £). Ultrathin sections were stained with 2% w/v uranyl acetate in 50% ethanol (2 × 15 min) and 0.5% lead citrate (10 min) before examination in a Tecnai Spirit T12 electron microscope (FEI, Hillsboro, USA). All images were acquired at a nominal 80-kV accelerating voltage. The images were contrast enhanced and exported using Gatan Digital Micrograph software (Gatan). The gold label density was analyzed using ImageJ software (available at：https://imagej.nih.gov/ij/). The total number of gold particles and the distribution of gold label per mitosome were quantified for each sample. The gold label density was analyzed using ImageJ software (available at：https://imagej.nih.gov/ij/). The total number of gold particles and the distribution of gold label per mitosome were quantified for each sample. The gold label density was analyzed using ImageJ software (available at：https://imagej.nih.gov/ij/) and two independent observers blinded to the sample conditions. The statistical significance of the gold label density was determined using the Mann-Whitney U-test.
39. Ye, H. et al. Glutaredoxin 5 deficiency causes sideroblastic anemia by specifically impairing heme biosynthesis and depleting cytosolic iron in human erythroid progenitors. Cell Metab. 12, 373–385 (2010).

40. Haunhorst, P. et al. Crucial function of vertebrate glutaredoxin 3 (PICOT) in iron homeostasis and hemoglobin maturation. Mol. Biol. Cell 24, 1895–1903 (2013).

41. Mühlenhoff, U. et al. Cytosolic monothiol glutaredoxin function in intracellular iron sensing and trafficking via their bound iron–sulfur cluster. Cell Metab. 12, 373–385 (2010).

42. Zhang, Y. et al. Conserved electron donor complex Drx2-Tah18 is required for ribonucleotide reductase metallocofactor assembly and DNA synthesis. Proc. Natl Acad. Sci. USA 111, E1695–E1704 (2014).

43. Molina, M. M., Belli, G., de la Torre, M. A., Rodriguez-Manzaneque, M. T. & Herrero, E. Nuclear monothiol glutaredoxins of Saccharomyces cerevisiae can function as mitochondrial glutaredoxins. J. Biol. Chem. 279, 51923–51930 (2004).

44. Tachery, J., Sanchez, L. B. & Muller, M. Mitochondrial type iron–sulfur cluster assembly in the amitochondriate eukaryotes Trichomonas vaginalis and Giardia intestinalis, as indicated by the phylogeny of Isc. Mol. Biol. Evol. 18, 1341–1344 (1999).

45. Emelyanov, V. V. Phylogenetic affinity of a Giardia lamblia cysteine desulphurase conforms to canonical pattern of mitochondrial ancestry. FEBS Microbiol. Lett. 226, 257–266 (2003).

46. Richards, T. A. & van der Giezen, M. Evolution of the Isd11–IscS complex Fe–S containing Dre2 C-terminus is essential for yeast viability. Mol. Microbiol. 82, 54–67 (2011).

47. Zhang, Y. et al. Drx2, a conserved eukaryotic Fe/S cluster protein, functions in cytosolic Fe/S protein biogenesis. Mol. Cell. Biol. 28, 5569–5582 (2008).

48. Netz, D. J. et al. The conserved protein Dre2 uses essential [2Fe–2S] and [4Fe–4S] clusters for its function in cytosolic iron–sulfur protein assembly. Biochem. J. 473, 2073–2085 (2016).

49. Tsaouis, A. D., Gentekaki, E., Eme, L., Gastro, D. & Roger, A. J. Evolution of the cytosolic iron–sulfur cluster assembly machinery in Blastocystis species and other microbial eukaryotes. Eukaryot. Cell 13, 163–153 (2014).

50. Tahirov, T. R., Makarova, K. S., Rogozin, I. B., Pavlov, Y. I. & Koonin, E. V. Evolution of DNA polymerases: an inactivated polymerase-exonuclease module in Pol epsilon and a chimeric origin of eukaryotic polymerases from two classes of archaeal ancestors. Biol. Direct. 4, 11 (2009).

51. Ku, C. et al. Endosymbiotic gene transfer from prokaryotic pan-genomes: inherited chimerism in eukaryotes. Proc. Natl Acad. Sci. USA 112, 10319–10146 (2015).

52. Tatusov, R. L. et al. The conserved protein Dre2 uses essential [2Fe–2S] and [4Fe–4S] clusters for its function in cytosolic iron–sulfur protein assembly. Biochem. J. 473, 2073–2085 (2016).

53. Tsaouis, A. D., Gentekaki, E., Eme, L., Gastro, D. & Roger, A. J. Evolution of the cytosolic iron–sulfur cluster assembly machinery in Blastocystis species and other microbial eukaryotes. Eukaryot. Cell 13, 163–153 (2014).

54. Tahirov, T. H., Pavlov, Y. I. & Koonin, E. V. Evolution of DNA polymerases: an inactivated polymerase-exonuclease module in Pol epsilon and a chimeric origin of eukaryotic polymerases from two classes of archaeal ancestors. Biol. Direct. 4, 11 (2009).

55. Ku, C. et al. Endosymbiotic gene transfer from prokaryotic pan-genomes: inherited chimerism in eukaryotes. Proc. Natl Acad. Sci. USA 112, 10319–10146 (2015).

56. Veatch, J. R., McMurray, M. A., Nelson, Z. W. & Gottschling, D. E. Mitochondrial dysfunction leads to nuclear genome instability via an iron–sulfur cluster defect. Cell 137, 1247–1258 (2009).

57. Gari, K. et al. MMS19 links cytoplasmic iron–sulfur cluster assembly to DNA metabolism. Science 337, 243–245 (2012).

58. Stehling, O. et al. MMS19 assembles iron–sulfur proteins required for DNA metabolism and genomic integrity. Science 337, 195–199 (2012).

59. Tatusov, R. L. et al. The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4, 41 (2003).

60. Tatusov, R. L., Koonin, E. V. & Lipman, D. J. A genomic perspective on protein families. Science 278, 631–637 (1997).

61. Eddy, S. R. Profile hidden Markov models. Bioinformatics 14, 755–763 (1998).

62. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 33, 1792–1797 (2004).

63. Katoh, K., Kuma, K., Toh, H. & Miyata, T. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 38, 1122–1128 (2010).

64. Do, C. B., Mahbabahshyam, M. S., Brudno, M. & Batzoglou, S. ProbCons: probabilistic consistency-based multiple sequence alignment. Genome Res. 15, 330–340 (2005).

65. Lassmann, T. & Sonnhammer, E. L. Kalign—an accurate and fast multiple sequence alignment algorithm. BMC Bioinformatics 6, 298 (2005).

66. Bradley, R. K. et al. Fast statistical alignment. PLoS Comput. Biol. 5, e1000392 (2009).

67. Lartillot, N., Lepage, T. & Blanquart, S. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. Bioinformatics 25, 2268–2269 (2009).

68. Capella-Gutierrez, S., Silla-Martinez, J. M. & Gabaldon, T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25, 1972–1973 (2009).

69. Tatusov, R. L. A. RAxML VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690 (2006).

70. Lartillot, N. & Peel, A. Matches between the B. n. n. sp. after in vitro isolation from an AIDS patient and inoculation into athymic mice. Parasitology 112(1 Pt 1): 143–154 (1996).

71. Wallrichs, G., McDowell, A., Back, R. & Dubochet, J. On the preparation of cryosections for immunocytochemistry. J. Ultrastruct. Res. 89, 65–78 (1984).

72. Lucoq, J. M. Can data provenance go the full monty? Trends Cell Biol. 22, 220–230 (2012).

Acknowledgements
We gratefully acknowledge the contribution of the Core Facility Protein Spectroscopy and Protein-Biochemistry of Philippus-Universitat Marburg. Technical support from Ekaterina Kozhevnikova is gratefully acknowledged. This work was supported by Marie Curie Postdoctoral Fellowships to T.A.W., E.H. and S.L., a European Research Council Advanced Investigator Grant (ERC-2010-AdG-268701) to T.M.E., and a Wellcome Trust Programme Grant (Number 045404) to T.M.E. and J.M.L. R.L. acknowledges generous financial support from Deutsche Forschungsgemeinschaft (SFB 593, SFB 987, GRK 1216, LI 415/5), LOEWE program of state Hessen, Max-Planck Gesellschaft, von Behring Röntgen Stiftung.

 Authors contributions
J.M.L., T.M.E and R.L. designed the study. S.-A.F., A.V.G., C.H., S.M., P.D., T.A.W.-S., N.I., K.S., E.B., E.H. and R.P.H. performed the experiments. All authors analysed data. T.M.E. and R.L. wrote the manuscript with contributions from S.-A.F., A.V.G. and J.M.L. All authors edited and approved the final manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Freibert, S.-A. et al. Evolutionary conservation and in vitro reconstitution of microsporidian iron–sulfur cluster biosynthesis. Nat. Commun. 8, 13932 doi: 10.1038/ncomms13932 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.