Review

Diversity and reductive evolution of mitochondria among microbial eukaryotes

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All extant eukaryotes are now considered to possess mitochondria in one form or another. Many parasites or anaerobic protists have highly reduced versions of mitochondria, which have generally lost their genome and the capacity to generate ATP through oxidative phosphorylation. These organelles have been called hydrogenosomes, when they make hydrogen, or remnant mitochondria or mitosomes when their functions were cryptic. More recently, organelles with features blurring the distinction between mitochondria, hydrogenosomes and mitosomes have been identified. These organelles have retained a mitochondrial genome and include the mitochondrial-like organelle of *Blastocystis* and the hydrogenosome of the anaerobic ciliate *Nyctotherus*. Studying eukaryotic diversity from the perspective of their mitochondrial variants has yielded important insights into eukaryote molecular cell biology and evolution. These investigations are contributing to understanding the essential functions of mitochondria, defined in the broadest sense, and the limits to which reductive evolution can proceed while maintaining a viable organelle.

Keywords: mitochondria; hydrogenosomes; mitosomes; mitochondrial-like organelles

1. INTRODUCTION

Some eukaryotes including members of the obligate intracellular parasitic Microsporidia (e.g. *Encephalitozoon*), and microaerophilic human parasites from the Amoebozoa (e.g. *Entamoeba*), Diplomonadida (e.g. *Giardia*) and Parabasalia (e.g. *Trichomonas*) (for taxonomy see table 1 and Adl *et al.* 2005), were once thought to lack mitochondria because they separated from other eukaryotes before the mitochondrial endosymbiosis. As such, they were viewed as important model systems from which to infer the early stages of eukaryotic evolution and, to signify their importance, they were grouped together in a paraphyletic taxon called the Archezoa (Cavalier-Smith 1983, 1987). Detailed molecular cell biology and phylogenetic investigations over the past 15 years have now shown that all of the best-studied archezoans contain a double-membrane-bounded organelle of mitochondrial ancestry (Embley & Martin 2006). The first of these to be discovered was the *Trichomonas* hydrogenosome (Lindmark & Müller 1973), so-called because it makes molecular hydrogen through the activity of iron-[Fe]-hydrogenase. It was initially thought that the *Trichomonas*

hydrogenosome might have originated from a separate endosymbiosis between the ancestor of *Trichomonas* and a *Clostridium* that contained this biochemical pathway (Müller 1980). The localization of proteins to *Trichomonas* hydrogenosomes that are typical of mitochondria, including chaperonins (Bui *et al.* 1996), the NADH-dehydrogenase module of complex I (Hrdy *et al.* 2004) and components of the mitochondrial iron-sulphur (Fe/S) cluster (ISC) biosynthesis pathway (Sutak *et al.* 2004), is now taken to support the simpler hypothesis that the hydrogenosomes of trichomonads are related to mitochondria (Embley & Martin 2006).

A similar approach, namely the immuno-localization of mitochondrial chaperonin 60 (Cpn60), was used to discover a tiny double-membrane-bounded organelle of cryptic function in *Entamoeba histolytica*, which was called a mitosome (Clark & Roger 1995; Tovar *et al.* 1999), or crypton (Mai *et al.* 1999), with the former name used most commonly. Mitosomes were subsequently discovered in the microsporidian *Trachipleistophora hominis* using the immuno-localization of mitochondrial Hsp70 (mtHsp70) (Williams *et al.* 2002) and in *Giardia* by immuno-localization of components of the mitochondrial ISC biosynthesis pathway (Tovar *et al.* 2003). Table 1 lists all of the experimentally localized proteins for the mitochondrial homologues discussed in this review and also incorporates current ideas (Adl *et al.* 2005) about the taxonomy of the diverse host organisms. The molecular cell biology investigations have been complemented by improved phylogenetic analyses to resolve the relationships of the former archezoans. These analyses have identified

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One contribution of 12 to a Theme Issue 'Evolution of organellar metabolism in unicellular eukaryotes'.

Table 1. Taxa with mitochondrial homologues for which there are protein or DNA localization data.

species ^a	higher taxonomic level ^a	mitochondrial homologue	protein or DNA localized to the organelle
<i>E. histolytica</i>	Amoebozoa, Entamoebida	mitosome	Cpn60 (Mai <i>et al.</i> 1999; Tovar <i>et al.</i> 1999), Cpn10 (van der Giezen <i>et al.</i> 2005), mtHsp70 (Tovar <i>et al.</i> 2007), MCF ADP/ATP transporter (Chan <i>et al.</i> 2005)
<i>M. balamuthi</i>	Amoebozoa, Mastigamoebidae	mitochondria-like organelle	Cpn60 (Gill <i>et al.</i> 2007)
<i>T. hominis</i>	Opisthokonta, Microsporidia	mitosome	mtHsp70 (Williams <i>et al.</i> 2002), Nfs (Goldberg <i>et al.</i> 2008)
<i>E. cuniculi</i>	Opisthokonta, Microsporidia	mitosome	mtHsp70, NTT3 (Tsaousis <i>et al.</i> 2008), Nfs, Isu, frataxin (Goldberg <i>et al.</i> 2008), ferredoxin (Williams <i>et al.</i> 2008a)
<i>Neocallimastix</i> sp.	Opisthokonta, Fungi	hydrogenosome	mtHsp70, Cpn60 (van der Giezen <i>et al.</i> 2003), [Fe]-hydrogenase (Voncken <i>et al.</i> 2002), MCF ADP/ATP transporters (van der Giezen <i>et al.</i> 2002; Voncken <i>et al.</i> 2002), pyruvate formate-lyase (Akmanova <i>et al.</i> 1999)
<i>Prionyces</i> sp.	Opisthokonta, Fungi	hydrogenosome	pyruvate formate-lyase (Akmanova <i>et al.</i> 1999)
<i>C. parvum</i>	Chromalveolata, Apicomplexa	mitosome	Cpn60 (Riordan <i>et al.</i> 2003), mtHsp70 (Slapeta & Keithly 2004)
<i>Blastocystis</i> sp.	Chromalveolata, Stramenopiles	mitochondria-like organelle	DNA, [Fe]-hydrogenase (Stechmann <i>et al.</i> 2008), DNA, NAD7, β -SCS, PFOR (Wawrzyniak <i>et al.</i> 2008), DNA (Perez-Brocal & Clark 2008)
<i>N. ovalis</i>	Chromalveolata, Ciliophora	hydrogenosome	DNA and hydrogenase activity (Boxma <i>et al.</i> 2005)
<i>T. vaginalis</i>	Excavata, Parabasalina	hydrogenosome	mtHsp70, mtHsp40, Cpn60, Cpn10, Pam18, (Bui <i>et al.</i> 1996; Dyall <i>et al.</i> 2003; Dolezal <i>et al.</i> 2005), α and β MPP (Brown <i>et al.</i> 2007; Šmíd <i>et al.</i> 2008), acetate:succinate CoA-transferases (van Grinsven <i>et al.</i> 2008), succinyl CoA synthetase α - and β -subunits (Lahti <i>et al.</i> 1994), adenylate kinase (Lange <i>et al.</i> 1994), malic enzyme (Hrdy & Müller 1995b; Dyall <i>et al.</i> 2004), ferredoxin, Nfs, Isu (Sutak <i>et al.</i> 2004; Dolezal <i>et al.</i> 2005, 2007), [Fe]-hydrogenase, [Fe]-hydrogenase maturases HydG, HydE, and HydF, PFOR (Hrdy & Müller 1995a; Bui & Johnson 1996; Pütz <i>et al.</i> 2006), Ndh51/NuoF, Ndh24/NuoE (Dyall <i>et al.</i> 2004; Hrdy <i>et al.</i> 2004), serine hydroxymethyl transferase, glycine cleavage H proteins H1 and H2, dihydrolipoamide dehydrogenase (Mukherjee <i>et al.</i> 2006a,b), SOD, rubrerythrin, thiol peroxidase (Pütz <i>et al.</i> 2005), MCF ADP/ATP transporter Hmp31 (Dyall <i>et al.</i> 2000), Hmp35 (Dyall <i>et al.</i> 2003), TrxR (Mentel <i>et al.</i> 2008), flavodiiron protein (Smutna <i>et al.</i> 2009)
<i>G. lamblia</i>	Excavata, Diplomonadida	mitosome	Nfs, Isu (Tovar <i>et al.</i> 2003), mtHsp70, Cpn60, ferredoxin (Regoes <i>et al.</i> 2005), β GPP (Šmíd <i>et al.</i> 2008), Tom40 (Dagley <i>et al.</i> in press)

^aSpecies are grouped according to super-groups and first rank or second rank names as defined in Adl *et al.* (2005). SOD, superoxide dismutase. Other abbreviations of protein names are defined in the text.

mitochondria-containing sister groups for *Entamoeba* and Microsporidia, in *Dictyostelium* and fungi, respectively, and have suggested that the deep branching positions of *Giardia* and *Trichomonas* are incorrect, or at best poorly supported (Embley & Martin 2006).

In summary, the prevailing view is that the Archezoa hypothesis has been falsified for the key taxa for which it was originally proposed. The discovery that all eukaryotes appear to contain an organelle related to mitochondria suggests that mitochondrial homologues are vital and defining features of eukaryotes. In that sense, the former Archezoa have played a central role in recasting hypotheses for eukaryogenesis (Embley & Martin 2006), and they have provided seminal examples of the diversity of mitochondrial form and function under different lifestyles. It is also now apparent that similarly diverse mitochondrial homologues can be found in other free-living and parasitic protists and fungi that were never grouped with archezoans.

2. MITOCHONDRIAL HOMOLOGUES: DIVERSE IN FORM AND FUNCTION

Hydrogenosomes, defined minimally as double-membrane-bounded organelles that make hydrogen, have also been described in diverse anaerobic ciliates and in anaerobic chytrid fungi isolated from the guts of herbivores such as *Piromyces* sp. and *Neocallimastix* sp. (Embley et al. 2003; Hackstein et al. 2008a). Among ciliates, anaerobic species with hydrogenosomes interleave among aerobes with mitochondria, suggesting that for this group at least, the transition from mitochondria to hydrogenosome is an easy one to make (Embley et al. 1995). Anaerobic ciliates with hydrogenosomes have been isolated from the cockroach gut (*Nyctotherus ovalis*), the gut of herbivores (*Dasytricha ruminantium*) and marine (*Plagiopyla frontata*, *Metopus contortus*) and fresh water sediments (*Trimyema compressum*, *Trimyema* sp.) (Fenchel & Finlay 1995; Boxma et al. 2005). In these habitats, they often harbour intracellular methanogenic archaeobacteria that use the hydrogen produced by the ciliate host hydrogenosomes to reduce CO₂ and to make ATP (Embley & Finlay 1994; Fenchel & Finlay 1995).

The case of the anaerobic ciliate *N. ovalis* is of particular interest, as its hydrogenosomes still retain a mitochondrial genome which has been partially sequenced (Boxma et al. 2005), providing a direct link between the two types of organelle. The partial hydrogenosomal genome encodes some of the genes for complex I, as well as ribosomal (r)RNA, tRNA and ribosomal proteins: consistent with the retention of a capacity for organelle protein synthesis (Boxma et al. 2005). Other components of mitochondrial complex I and complex II are encoded by the nuclear genome, suggesting that the hydrogenosomes of *Nyctotherus* may have retained a partial electron transport chain (ETC), consistent with the detected membrane potential (Boxma et al. 2005). The [Fe]-hydrogenase of *Nyctotherus* is probably nuclear-encoded since it has a mitochondrial-like targeting signal. The gene itself was initially described as a fusion protein comprising an [Fe]-hydrogenase linked to the 24 and 51 kD subunits of mitochondrial

complex I (Hackstein et al. 1999), but subsequent phylogenetic analyses provided no support for this hypothesis (Horner et al. 2000). In most analyses, the *Nyctotherus* 51 kD subunit clustered with the 51 kDa-like component of the NAD(H)-dependent [Ni-Fe] hydrogenases from bacteria rather than complex I-associated sequences (Horner et al. 2000). Phylogenetic analyses do provide weak evidence that the *Nyctotherus* [Fe]-hydrogenase component of the fusion protein might have a separate origin to other eukaryotic [Fe]-hydrogenases (Horner et al. 2000; Embley et al. 2003; Hampl et al. 2008). Interestingly, when the [Fe]-hydrogenase of the fresh water anaerobic ciliate *Trimyema* sp. was analysed, it was found to form a monophyletic group with the *Nyctotherus* sequence (Embley et al. 2003). This suggests that a gene for [Fe]-hydrogenase was already present in the common ancestor of these disparate ciliate lineages, helping to explain the apparent facility whereby different anaerobic ciliates have been able to make hydrogenosomes from mitochondria (Embley et al. 1995).

Mitosomes are double-membrane-bounded organelles that have so far been found in parasitic members of the lineages Amoebozoa, Microsporidia, Diplomonads and Apicomplexa (Tovar et al. 1999; Williams et al. 2002; Tovar et al. 2003; Keithly et al. 2005). Based upon the published biochemical studies over many years (Müller 1988, 2003) and more recent genomic studies (Katinka et al. 2001; Abrahamsen et al. 2004; Loftus et al. 2005; Morrison et al. 2007), it appears unlikely that mitosomes have a role in cellular ATP generation. Indeed, mitosomes are largely defined by what they lack; they are remnant mitochondria that lack ATP-generating pathways and do not make hydrogen. Mitosomes are morphologically simple and lack cristae and are typically smaller than mitochondria, varying in size from approximately 50 nm to approximately 500 nm in diameter depending on the species (Scheffler 2008; Tachezy & Smid 2008). Their small size and simple morphology along with the absence of mitochondrial marker enzymes undoubtedly contributed to past failures to recognize mitosomes as mitochondrial homologues. Interestingly, however, there are references to mitochondrial-like organelles lacking cristae for Microsporidia and for *Giardia lamblia*, in the historical literature (Cheissin 1965; Vávra 1976).

The number of mitosomes within each cell also varies between species. *Cryptosporidium parvum* contains a single mitosome (Keithly et al. 2005) but *E. histolytica* (Leon-Avila & Tovar 2004) and *Giardia* (Regoes et al. 2005) may contain between 25 to 150 mitosomes per cell (Tachezy & Smid 2008). An average cell of the microsporidian *T. hominis* contains around 28 mitosomes (Williams et al. 2002) but its smaller relative *Encephalitozoon cuniculi*, typically contains less than 10 mitosomes per cell (Tsaousis et al. 2008). Very little is known about how these organelles are inherited during host cell division (Regoes et al. 2005; Tsaousis et al. 2008), but, given the small number of mitosomes in species such as *C. parvum* and *E. cuniculi*, a mechanism must exist to ensure the reliable segregation of mitosomes between daughter cells.

Blastocystis is an anaerobic parasite commonly found in the human gut that belongs to a diverse eukaryotic group called the stramenopiles or heterokonts, which also includes the brown algae, diatoms and oomycetes (Perez-Brocal & Clark 2008). The mitochondrion-like organelles (MLO) of *Blastocystis* spp. resemble the hydrogenosomes of the anaerobic ciliate *Nyctotherus* hydrogenosome, in that they have retained an organelle genome (Perez-Brocal & Clark 2008; Stechmann *et al.* 2008; Wawrzyniak *et al.* 2008). The characterized circular-mapping MLO genomes (Perez-Brocal & Clark 2008; Wawrzyniak *et al.* 2008) are DNA molecules of between 27–29 kbp that are A + T rich (approx. 80%) and gene dense. Each contains 45 genes of which 27 are open reading frames, including genes for ribosomal proteins and some subunits of mitochondrial complex I. The rest of the genes encode structural ribosomal RNAs and tRNAs (Perez-Brocal & Clark 2008; Wawrzyniak *et al.* 2008). These data, in combination with EST data (Stechmann *et al.* 2008) have been interpreted (Perez-Brocal & Clark 2008) to suggest that a proton-pumping complex I may function in the *Blastocystis* MLO: this would be consistent with the previously detected organelle membrane potential (Zierdt *et al.* 1988; Hamblin *et al.* 2008). By contrast, and unlike the mitochondrial genomes of related stramenopiles, the MLO genomes lack genes for cytochromes and ATPase subunits, suggesting that *Blastocystis* lacks mitochondrial complexes III and IV (Zierdt *et al.* 1988; Perez-Brocal & Clark 2008).

Blastocystis also has an iron-[Fe]-hydrogenase of the same type as used by *Trichomonas* hydrogenosomes to make hydrogen (Stechmann *et al.* 2008). Antiserum to the *Blastocystis* [Fe]-hydrogenase was used to localize the protein to the *Blastocystis* MLO (Stechmann *et al.* 2008), suggesting that it resembles the *Nyctotherus* hydrogenosome in this feature too. However, in a recent biochemical study of another strain of *Blastocystis* (Lantsman *et al.* 2008), no hydrogenase activity could be detected in fractions enriched for the MLO or in cell-free extracts. The reasons for this discrepancy are unknown: it could be down to strain differences, to differences in growth conditions or simply that detecting hydrogen production by *Blastocystis* will need a more sensitive assay (Lantsman *et al.* 2008). For example, mass spectrometry was needed to detect *Giardia* cytosolic hydrogen production (Lloyd *et al.* 2002), which is typically 10-fold less than that produced by *Trichomonas* under the same conditions. As a consequence of the failure to detect hydrogen production, the *Blastocystis* organelle is generally referred to as an MLO rather than as a hydrogenosome (Lantsman *et al.* 2008).

The examples discussed above illustrate that reductive evolution of mitochondria to MLO, hydrogenosome or mitosome is common across the eukaryotic tree. Some of this reductive evolution is convergent, the occurrence of multiple anaerobic ciliate lineages with hydrogenosomes interleaved among aerobic lineages with mitochondria (Embley *et al.* 1995), and the similarities between the *Nyctotherus* (Boxma *et al.* 2005) and *Blastocystis* organelles (Perez-Brocal & Clark 2008; Stechmann *et al.* 2008)

provide particularly striking examples of this trend. The organelles described so far are probably just the tip of a very large iceberg of mitochondrial homologue diversity that remains to be discovered. Intracellular methanogens have been described in a variety of otherwise poorly characterized anaerobic protists, suggesting that hydrogenosomes may be relatively common in nature (Fenchel & Finlay 1995). The protozoological and microbial ecology literature is also littered with descriptions of protists living in low-oxygen environments, and which contain uncharacterized double-membrane-bounded organelles (Fenchel & Finlay 1995; Hampl & Simpson 2008).

3. ALL MITOCHONDRIAL HOMOLOGUES NEED TO MAKE OR IMPORT ATP

Canonical mitochondria can be classified into two different types: aerobic mitochondria, which use oxygen as the final acceptor during aerobic respiration, and anaerobic mitochondria, present in facultative anaerobes, which can use organic or inorganic compounds, such as nitrate or fumarate, as final acceptors during anaerobic respiration (Tielens *et al.* 2002). Both pathways make ATP for the organelle to sustain its own metabolism, to import proteins and to import and export solutes. The excess of ATP produced by mitochondria is exported to the cytosol using members of the mitochondrial carrier family (MCF) (Kunji 2004). Some ATP is generated by the *Trichomonas* hydrogenosome by substrate level phosphorylation involving acetyl CoA released by decarboxylation of pyruvate by the enzyme pyruvate:ferredoxin oxidoreductase (PFOR) (Hrdy *et al.* 2008). The MLO of *Blastocystis* probably makes ATP in the same way, but using the PFOR homologue pyruvate: NADP(+) oxidoreductase, to decarboxylate pyruvate (Hamblin *et al.* 2008; Lantsman *et al.* 2008). Based upon biochemical studies (Reeves 1984; Müller 1988, 2003) and genomic sequences (Katinka *et al.* 2001; Abrahamsen *et al.* 2004; Loftus *et al.* 2005; Morrison *et al.* 2007), it appears unlikely that the mitosomes of Microsporidia, *Giardia*, *Entamoeba* and *Cryptosporidium* can make ATP. However, at least according to the best studied mitochondrial models, mitosomes must require ATP to import nuclear encoded proteins and to support the activities of their Cpn60 and mtHsp70 ATPases (Tovar *et al.* 1999; Williams *et al.* 2002; Riordan *et al.* 2003; Regoes *et al.* 2005; Tovar *et al.* 2007; Tsaousis *et al.* 2008). So how do these organelles acquire the ATP they need?

(a) Mitochondrial ATP transport proteins

The genomes of eukaryotes with canonical mitochondria typically contain between 35 and 55 members of the MCF family of transport proteins (Kunji 2004). For example, the human genome codes for about 48 MCF proteins, about half of which have been functionally characterized. MCF proteins support mitochondrial metabolic energy generation, DNA replication and amino acid metabolism by linking biochemical pathways in the mitochondrial matrix with those in the cytosol (Kunji 2004). The number and

diversity of MCF proteins thus closely mirrors important facets of mitochondrial metabolic diversity, and the functional characterization of hydrogenosomal and mitosomal MCF proteins is an important part of investigating how these organelles function. A specialized member of the MCF, the mitochondrial ADP/ATP carrier (AAC), located in the inner mitochondrial membrane, exchanges mitochondrial-generated ATP with cytosolic ADP on an equimolar basis—so the adenine nucleotide pool of the mitochondrial matrix does not change (Kunji 2004). It is also thought to be an MCF protein, possibly an ATP-Mg/Pi carrier, that replenishes adenine nucleotide pools during mitochondrial division (Kunji 2004). In the classical endosymbiosis theory, insertion of a host-nuclear-encoded MCF ATP/ADP transporter into the protomitochondrion is viewed as a key step that allowed the host cell to harvest ATP from the enslaved endosymbiont (John & Whatley 1975).

The hydrogenosomes of the anaerobic ciliate *N. ovalis* (Hackstein et al. 2008b) and the anaerobic chytrid fungus *Neocallimastix* (van der Giezen et al. 2002; Voncken et al. 2002) contain functionally characterized mitochondrial-type AAC. These proteins share strong structural and functional similarities to mitochondrial AAC and cluster with AAC in phylogenetic trees (van der Giezen et al. 2002; Hackstein et al. 2008b). The *Neocallimastix* AAC was able to functionally complement a yeast mitochondrial mutant showing conservation of function and targeting signals. Moreover, when hydrogenosomal membranes were fused with liposomes, ADP/ATP transport was inhibited by bongkreikic acid, a known inhibitor of mitochondrial ADP/ATP transporters (van der Giezen et al. 2002). The *Trichomonas vaginalis* genome contains five genes annotated as coding for MCF proteins, none of which cluster with the classic AAC (Carlton et al. 2007). One of these genes codes for an abundant protein, named Hmp31 (for hydrogenosomal membrane protein), in the hydrogenosome-enriched membrane fraction of *T. vaginalis* (Dyall et al. 2000). Hmp31 was targeted to the inner membrane of *Saccharomyces cerevisiae* mitochondria as measured using radiolabelled Hmp31 in isolated mitochondria (Dyall et al. 2000). An Hmp31 homologue from *Trichomonas gallinae* was also shown to localize to the hydrogenosomal membrane fraction (Tjaden et al. 2004). The *T. gallinae* Hmp31 protein showed substrate specificity and comparable kinetic properties to mitochondrial-type AAC from mitochondria and fungal hydrogenosomes (Tjaden et al. 2004). However, unlike the classic AAC, the *T. gallinae* Hmp31 protein was not sensitive to bongkreikic acid (Tjaden et al. 2004). Several ESTs from a *Blastocystis* cDNA library appear to code for putative MCF proteins, but none of these have been localized or functionally characterized (Stechmann et al. 2008).

The hydrogenosomes of *Trichomonas* (Müller 2003) and the *Blastocystis* MLO (Hamblin et al. 2008; Lantsman et al. 2008) can generate ATP by substrate level phosphorylation, but mitosomes appear to depend on the cytosol for the ATP and other solutes that they need to function. Relatively few proteins

have been experimentally localized to mitosomes so far, but all mitosomes appear to contain at least one protein, typically either Cpn60 or Hsp70 or both, that requires ATP for its activity (Embley & Martin 2006). In principle, an MCF AAC could operate in reverse along a concentration gradient to exchange mitosomal ADP for cytosolic ATP (Kunji 2004). The genome of *C. parvum* contains genes for MCF proteins annotated as ADP/ATP transporters (Abrahamsen et al. 2004), but these have not been functionally characterized or localized to the organelle. The *E. histolytica* mitosome contains a single type of MCF protein that, in principle, could be used to fuel its mitosome. The *Entamoeba* MCF protein is resistant to bongkreikic acid and it transports ATP and ADP using a novel mechanism that does not appear to require a membrane potential (Chan et al. 2005). Phylogenetic analyses confirmed that the *Entamoeba* AAC is distinct from archetypal mitochondrial AACs (Chan et al. 2005).

A gene for an MCF protein was found in an EST library for the microsporidian *Antonospora locustae* (Williams et al. 2008b). The *Antonospora* MCF was targeted to the mitochondria of *Saccharomyces*, suggesting conservation of targeting signals, and it showed high specificity for ATP and ADP when expressed in *Escherichia coli* (Williams et al. 2008b). Although the *Antonospora* ATP/ADP transporter was not shown to actually localize to a mitosome in *Antonospora*, the data are consistent with at least one microsporidian retaining an MCF. By contrast, the genome of the microsporidian *E. cuniculi* lacks any genes for MCF proteins (Katinka et al. 2001). It does, however, contain four genes for bacterial-type nucleotide transporters (named EcNTT1-4) similar to those used by intracellular pathogens, including *Rickettsia* and *Chlamydia*, to steal ATP from their host cells (Winkler & Neuhaus 1999). As proliferating microsporidia recruit host mitochondria near their plasma membrane, it has previously been proposed that microsporidians use host-derived ATP to supplement their energy budget (Weidner et al. 1999). The presence in *E. cuniculi* of NTT homologues provides a potential means of achieving this goal (Katinka et al. 2001). Consistent with this hypothesis, three of the EcNTT were expressed on the surface of the parasite when living inside rabbit kidney cells (Tsaousis et al. 2008). The fourth transporter (EcNTT3) colocalized with the mitosomal marker protein mtHsp70, consistent with it being targeted to *E. cuniculi* mitosomes (Tsaousis et al. 2008) (figure 1). Thus, the *E. cuniculi* mitosome appears to be using the same strategy as the parasite itself to acquire ATP. Heterologous expression in *E. coli* demonstrated that all four EcNTT were able to transport ADP and ATP in contrast to bacterial pathogens where typically only one paralogue will mediate ATP uptake (Audia & Winkler 2006; Haferkamp et al. 2006). Adenine nucleotide efflux (figure 1) was observed for EcNTT3 when the external substrate was removed, indicating that it is able to equilibrate nucleotide pools across a concentration gradient. Efflux was stimulated by the addition of external cold ATP or ADP, confirming that EcNTT3 is an

Figure 1. Detection of *E. cuniculi* mitochondria using electron and light microscopy and functional evidence that mitochondria use a novel route for ATP acquisition. Transmission electron micrographs of fixed rabbit kidney cell (RK-13) cultures infected with *E. cuniculi* showing double-membrane-bounded organelles inside the parasite. (a) One parasite shows a group of three double-membrane-bounded organelles (red arrows) situated close to the parasite nucleus (N). The inner and outer membranes of the parasite nuclear envelope are indicated with blue arrowheads. The double-membrane-bounded structures are associated with a rather homogeneous zone, which has a higher electron density than the surrounding cytoplasm (asterisk), and which in turn is applied closely to the nuclear envelope. The inset shows another field where putative microtubules (blue arrowheads) radiate from this zone (asterisk), and associated mitochondria are indicated by red arrows. (b) Higher magnification of the three mitochondria displayed in (a). The outer and inner membranes of one mitochondrion are indicated with red arrows. Scale bars, (a) 200 nm and (b) 100 nm. Localization and expression of *E. cuniculi* mitochondrial Hsp70 and a novel nucleotide transport protein (*EcNTT3*). Mitochondrial Hsp70 shares structural features with characterized mtHsp70 proteins that require ATP for their activities (Williams *et al.* 2002; Tsaousis *et al.* 2008). (c) Western blot analyses using published antisera (Tsaousis *et al.* 2008) demonstrate that the *EcNTT3* protein is present in *E. cuniculi* spores (lane 1) and RK-13 cells infected with *E. cuniculi* (lane 2), but it is not detected in control non-infected RK-13 cells (lane 3). The corresponding signal for the mitochondrial *E. cuniculi* Hsp70 is shown for comparison. The reasons for the different signal intensities for the two proteins in the different preparations (lanes 1 and 2) are unknown. (d–f) Three *E. cuniculi* cells isolated from infected RK-13 cells were colabelled with the anti-Hsp70 sera (red signal) and anti-*EcNTT3* sera (green signal). The signal for *EcNTT3* clearly colocalizes with the signal for the mitochondrial marker Hsp70. The transport properties of the NTT3 protein were investigated in *E. coli* cells expressing the complete NTT3 protein fused to a His-tag. (g) Back-exchange properties of *E. coli* cells pre-loaded with [α^{32} P]-ATP and expressing recombinant NTT3 protein. The *E. coli* cells took up [α^{32} P]-ATP in an NTT3-dependent manner (data not shown; Tsaousis *et al.* 2008). Efflux of radio-labelled adenine nucleotides was observed when the external substrate was removed, indicating that NTT3 can equilibrate nucleotide pools across a gradient. Addition of cold ADP or ATP further stimulated efflux, confirming that NTT3 is an exchanger of adenine nucleotides. Data points represent the mean and s.d. of three independent experiments. Open circles, none; filled triangles, ADP; filled squares, ATP. Adapted from Tsaousis *et al.* (2008). Copyright © Macmillan Publishers Ltd.

exchanger of adenine nucleotides and thus potentially capable of importing cytosolic ATP, in exchange for mitochondrial ADP, into the *E. cuniculi* mitochondrion (Tsaousis *et al.* 2008).

The genome of the diplomonad parasite *Giardia* (Morrison *et al.* 2007) appears to lack any genes for MCF proteins or NTT-like transporters, so how its mitochondria acquire the ATP needed for ISC assembly

(Tovar *et al.* 2003) and protein import (Dolezal *et al.* 2005; Regoes *et al.* 2005; Šmíd *et al.* 2008) is unknown.

4. PROTEIN TARGETTING AND PROTEIN IMPORT

(a) Presence of mitochondrial-like targeting signals

Even in species that have a mitochondrion with an associated organelle genome, the vast majority of organelle proteins are encoded by the host nuclear genome and must be targeted to the mitochondrion and its individual compartments using specific targeting signals (see also Lithgow & Schneider 2009). Many of the nuclear-encoded mitochondrial matrix proteins are synthesized with N-terminal cleavable leader sequences that are recognized by receptors on the mitochondrial surface (Neupert & Herrmann 2007). Typical pre-sequences contain 10 to 80 amino acid residues, many of which are positively charged, hydrophobic and hydroxylated (Dyall & Dolezal 2008). The N-terminus may often form an amphiphilic α -helix (Pfanner & Geissler 2001). Many mitochondrial pre-sequences contain an arginine residue at the -2 position from the cleavage site, and this is also a common feature of the relatively few, mostly for *Trichomonas* but also for *Entamoeba*, *Giardia* and *Neocallimastix*, experimentally verified N-terminal cleaved pre-sequences on hydrogenosomal or mitochondrial proteins (but see Smutna *et al.* 2009). The targeting sequences of hydrogenosomal or mitochondrial proteins are generally shorter than the mitochondrial versions: most predicted *Giardia* mitochondrial pre-sequences and a majority of *Trichomonas* hydrogenosomal pre-sequences have a length of between four and 21 amino acids (Šmíd *et al.* 2008). A full list of experimentally verified, or predicted, hydrogenosomal or mitochondrial targeting sequences is given by Dyall & Dolezal (2008) and Šmíd *et al.* (2008). The positive charges on mitochondrial pre-sequences are drawn through the TIM23 inner membrane channel aided by the membrane potential (Dyall & Dolezal 2008), after which the pre-sequence is normally cleaved by a mitochondrial-processing peptidase (MPP) (Šmíd *et al.* 2008). After cleavage, the protein is refolded into its native structure aided by the Cpn60/Cpn10 complex (Dyall & Dolezal 2008).

The 'classic' MPP typically consists of two subunits: an α -subunit implicated in substrate binding and release and a β -subunit that possesses catalytic activity (Šmíd *et al.* 2008). The processing peptidases of *Giardia* and *Trichomonas* have recently been functionally characterized (Brown *et al.* 2007; Šmíd *et al.* 2008). The *Giardia* MPP is so far unique in that it lacks the α -subunit and functions as a monomeric β -subunit (β GPP) (Šmíd *et al.* 2008). The recombinant *Giardia* β GPP was able to process *in vitro* the N-terminal extensions of *Giardia* mitochondrial ferredoxin and of two scaffold proteins (IscU and IscA) involved in mitochondrial ISC biosynthesis (Šmíd *et al.* 2008). The MPP of *T. vaginalis* was also initially thought to function as a single β -subunit MPP (Brown *et al.* 2007), but subsequent work has demonstrated that it functions most efficiently as a

heterodimer comprising a divergent α -subunit and a β -subunit (Šmíd *et al.* 2008). The *T. vaginalis* MPP processed the N-terminal extensions of *Trichomonas* hydrogenosomal ferredoxin, adenylate kinase, Hsp70 and IscU.

Targeting signals for other types of mitochondrial proteins, for example for members of the MCF targeted to the inner mitochondrial membrane, are less well characterized. These do not use an N-terminal cleaved leader sequence but instead rely on internal targeting signals to guide them to their final location (Pfanner & Geissler 2001). Intriguingly, the *T. vaginalis* MCF protein Hmp31 does have a cleavable 12 amino acid N-terminal extension, but this is not necessary for targeting Hmp31 to the hydrogenosomal membrane (Dyall *et al.* 2000). *Trichomonas* Hmp31 was efficiently targeted to the inner membrane of yeast mitochondria, and, in the reciprocal experiment, hydrogenosomes were able to import yeast AAC (Dyall *et al.* 2000). These data strongly suggest that elements of the inner membrane protein-targeting machinery are conserved between yeast mitochondria and *Trichomonas* hydrogenosomes. Mentel *et al.* (2008) recently demonstrated that other hydrogenosomal proteins possess internal targeting signals. One of the two hydrogenosomal thioredoxin reductase (TrxR) isoforms, TrxRh1, carried an N-terminal extension resembling known hydrogenosomal targeting signals and for this protein the N-terminal extension was necessary to import the protein into hydrogenosomes (Mentel *et al.* 2008). The second hydrogenosomal TrxR isoform, TrxRh2, had no N-terminal targeting signal but was nonetheless also efficiently targeted to hydrogenosomes. Moreover, the α -subunit of succinyl CoA synthetase, lacking its normal N-terminal extension, was still efficiently imported into hydrogenosomes, indicating that this extension is not required for import of this major hydrogenosomal protein. These data indicate the presence of additional targeting signals within the mature subunits of several hydrogenosome-localized proteins (Mentel *et al.* 2008).

Analysis of an EST library for *Blastocystis* also revealed a large number of putative proteins with N-terminal extensions, indicative of mitochondrial-like targeting pre-sequences (Stechmann *et al.* 2008). Two of these proteins: [Fe]-hydrogenase (Stechmann *et al.* 2008) and the α -subunit of succinyl CoA synthetase (Hamblin *et al.* 2008) localized to the MLO of *Blastocystis*. Based upon the EST library, *Blastocystis* also has a putative MPP (Stechmann *et al.* 2008). An EST library for the anaerobic ciliate *N. ovalis* contained sequences with putative N-terminal pre-sequences for proteins that typically function in mitochondria, such as pyruvate dehydrogenase (Boxma *et al.* 2005). The N-terminal sequences of *C. parvum* mtHsp70, IscU and IscS can direct green fluorescent protein to yeast mitochondria (LaGier *et al.* 2003; Slapeta & Keithly 2004), and mtHsp70 and Cpn60 are known to localize to the *Cryptosporidium* mitosome (Riordan *et al.* 2003). The genome of *Cryptosporidium* (Abrahamsen *et al.* 2004) contains putative α - and β -subunits of an MPP, but these have not been functionally characterized.

Mitochondrial-like pre-sequences have also been detected on *E. histolytica* pyridine nucleotide transhydrogenase, Cpn60 and mtHsp70 (Tovar *et al.* 1999; Bakatselou *et al.* 2003). The targeting signal of Cpn60 was shown to be required for targeting the protein into the *Entamoeba* mitosome. Deletion of the first 15 amino acids of Cpn60 led to an accumulation of the truncated protein in the cytoplasm of *Entamoeba*. This mutant phenotype was reversed by the replacement of the deleted amino acids with a mitochondrial targeting signal from *Trypanosoma cruzi* Hsp70 (Tovar *et al.* 1999). Consistent with the cleavage of the leader sequence from Cpn60, there is evidence for a putative MPP on the *Entamoeba* genome sequence (Loftus *et al.* 2005). When the *Entamoeba* MCF was expressed in yeast, it was targeted to the inner membrane of mitochondria, suggesting that it retains targeting signals recognized by the yeast inner mitochondrial membrane import pathway (Chan *et al.* 2005).

The first protein shown to be targeted to a microsporidian mitosome, *T. hominis* mtHsp70, lacks any detectable N-terminal targeting sequence, and it was not targeted to mammalian mitochondria (Williams *et al.* 2002). Other proteins, experimentally verified to localize to *T. hominis* or *E. cuniculi* mitosomes, namely *T. hominis* cysteine desulphurase (abbreviated Nfs or IscS), and *E. cuniculi* Hsp70, IscS, IscU and frataxin, also lack detectable N-terminal targeting signals (Goldberg *et al.* 2008; Tsaousis *et al.* 2008). Consistent with the absence of cleaved leader sequences on *E. cuniculi* mitosomal proteins, its genome does not contain genes for an MPP (Katinka *et al.* 2001). It appears from these data that *E. cuniculi* has dispensed with cleaved N-terminal leader sequence-based targeting for mitosomal matrix proteins, and relies on internal signals to guide these proteins to their destination.

In yeast, some proteins, for example mitochondrial glycerol-3-phosphate dehydrogenase (G3PDH), destined for the inter-membrane space carry an N-terminal leader sequence that is cleaved by another type of protease: the inner membrane peptidase (IMP) (Burri *et al.* 2006). This peptidase consists of two catalytic subunits (Imp1 and Imp2) and a regulator (Som1). *Antonospora* protein extracts appear to contain two sizes of G3PDH, consistent with the processing of a 40 amino acid N-terminal extension (Burri *et al.* 2006). By contrast, *E. cuniculi* G3PDH does not appear to be processed. Consistent with this processing, there is a gene (found as an EST) for an IMP (IMP2) on the *A. locustae* (Burri *et al.* 2006), but not the *E. cuniculi* (Katinka *et al.* 2001), genome. Although the location of Imp2 inside *A. locustae* is currently unknown, it was shown to localize to yeast mitochondria in heterologous targeting experiments (Burri *et al.* 2006).

(b) Conservation of mitochondrial import pathways

The machinery used by yeast mitochondria to import or insert proteins into the outer membrane, inter-membrane space, inner membrane or mitochondrial

matrix is based on a number of major import complexes (Pfanner & Geissler 2001; Dyall & Dolezal 2008). The translocase of the outer mitochondrial membrane (TOM) complex provides the general import pore through the outer mitochondrial membrane. The sorting and assembly machinery (SAM) complex is involved in the correct insertion of β -barrel proteins such as Tom40 and porin into the outer membrane; Sam50 and Sam35 are essential components of the yeast SAM pathway (Dyall & Dolezal 2008). Two translocase of the inner mitochondrial membrane (TIM) complexes, namely TIM22 and TIM23, are responsible for protein translocation across, or insertion into, the inner mitochondrial membrane (Neupert & Herrmann 2007). There are additional complexes: the mitochondrial inter-membrane space import (MIA) complex, which inserts proteins into the inter-membrane space, and the OXA complex, which is mainly used for proteins encoded by mitochondrial genomes. Each of the import complexes comprises multiple components, many of which are conserved in fungi, animals and plants, suggesting that the last common ancestor of these species already had a sophisticated protein import machinery. The results of genomic sequence analyses and the small number of functional studies already suggest that species with hydrogenosomes and mitosomes have dramatically reduced the complexity of their organelle protein import machineries (Dyall & Dolezal 2008), raising intriguing questions as to how protein import functions and what are the driving forces behind its reductive evolution. In the following discussion, we focus mainly on the major outer (TOM) and inner membrane (TIM22, TIM23) import pathways, and on proteins for which there are at least some experimental data that they function in hydrogenosomes or mitosomes.

The TOM complex forms the general import pore through which proteins can cross the outer mitochondrial membrane. In yeast, it comprises the Tom40 pore and a variety of accessory proteins including a Tom20 receptor that recognizes N-terminal pre-sequences, and a Tom70 receptor that recognizes internal signal sequences. Homologues of the main Tom40 pore can be found on the fully sequenced genomes of *C. parvum* (Abrahamsen *et al.* 2004), *E. cuniculi* (Katinka *et al.* 2001), *E. histolytica* (Loftus *et al.* 2005), *G. lamblia* (Morrison *et al.* 2007) and *T. vaginalis* (Carlton *et al.* 2007), although some are not annotated as such. Tom40 shares a porin-3 motif with porin (also called the voltage-dependent anion channel), suggesting that they are ancient paralogs from the same broad 'mitochondrial porin' protein family (Pusnik *et al.* 2009). As the sequences from parasites are often very difficult to identify as specific types of 'mitochondrial porin', all we can say at the moment is that it seems possible (if not likely) that each species has a mitochondrial porin in the outer membrane of its mitochondrial homologue. However, experimental data will be needed to confirm this inferred cellular localization and to determine whether any of these proteins actually function as general import pores (Dagley *et al.* in press).

The localized *E. cuniculi* mitochondrial proteins all lack an N-terminal leader sequence (Goldberg *et al.* 2008; Tsaousis *et al.* 2008), consistent with the absence of an MPP (Katinka *et al.* 2001), and suggesting that they are imported using internal targeting signals. In yeast, the Tom70 protein is part of the outer membrane machinery that recognizes proteins with internal targeting signals (Pfanner & Geissler 2001). The genome of *E. cuniculi* contains a gene for a putative Tom70 (Katinka *et al.* 2001) that can be imported into the mitochondria of *S. cerevisiae* (Waller *et al.* 2009). The EcTom70 inserted with the correct topology into the outer membrane of yeast mitochondria but was unable to complement the growth defects of Tom70-deficient yeast.

The TIM22 and TIM23 complexes provide the main inner mitochondrial membrane translocases in yeast. The TIM22 complex is used to insert inner mitochondrial membrane proteins such as the MCF AAC (Neupert & Herrmann 2007). In yeast, the passage of the substrate through the twin pores formed by Tim22 is dependent on the membrane potential (Rehling *et al.* 2003). Putative Tim22 orthologues have been annotated for *E. cuniculi* (Katinka *et al.* 2001) and *A. locustae* (Williams & Keeling 2005) and potentially could be used to insert the experimentally localized *E. cuniculi* mitochondrial ADP/ATP transport proteins (Tsaousis *et al.* 2008) and the putative *A. locustae* mitochondrial MCF ADP/ATP transporter (Williams *et al.* 2008b). MCF proteins have been identified and functionally characterized for *Entamoeba* (Chan *et al.* 2005) and *Trichomonas* (Dyall *et al.* 2000), including their heterologous import into yeast mitochondria, presumably via the TIM22 complex. The failure to identify Tim22 orthologues on the genomes of *Entamoeba* (Loftus *et al.* 2005) or *Trichomonas* (Carlton *et al.* 2007) is thus particularly surprising.

In yeast, the TIM23 complex is the main inner membrane import pathway for matrix or inner membrane proteins carrying N-terminal leader sequences. It comprises the paralogues Tim23 and Tim17, which are also homologous with Tim22, and a number of accessory proteins including Tim50, which regulates the opening of the Tim23/Tim17 channel, and Tim21, which regulates module docking (Dyall & Dolezal 2008). In yeast, the translocation of proteins through the TIM23 complex is ATP dependent and requires a membrane potential. *Cryptosporidium parvum* (Abrahamsen *et al.* 2004) and *T. vaginalis* (Carlton *et al.* 2007) have genes for putative Tim17 and Tim23, so an inner membrane translocation pore could potentially be present for the organelles of these species. However, genes for Tim17 or Tim23 were not identified for *Entamoeba* (Loftus *et al.* 2005) or *Giardia* (Morrison *et al.* 2007), and this lack, together with the apparent absence of genes for Tim22, makes it difficult to understand how proteins are imported across the inner membrane of the mitosomes of these species.

Protein import into the matrix is driven by the PAM complex (pre-sequence translocase-associated motor), which in yeast comprises Pam16, Tim17/Pam17, Tim18/Pam18, Tim44/Pam44 and Mge1. The mtHsp70 also plays a part in this process (Pfanner &

Geissler 2001). The Cpn60/Cpn10 complex is thought to be involved in protein folding following their translocation into the matrix (Dyall & Dolezal 2008). Putative Pam18 homologues have been localized to the mitosome of *Giardia* and to the hydrogenosome of *Trichomonas* (Dolezal *et al.* 2005), and a putative Pam16 has been annotated for *E. cuniculi* (Waller *et al.* 2009). An mtHsp70 has been localized to the hydrogenosomes of *Trichomonas* (Bui *et al.* 1996) and to the mitosomes of *C. parvum* (Slapeta & Keithly 2004), *E. cuniculi* (Tsaousis *et al.* 2008), *T. hominis* (Williams *et al.* 2002) and *E. histolytica* (Tovar *et al.* 2007). Cpn60 and Cpn10 have been localized to the mitosomes of *E. histolytica* (Tovar *et al.* 1999, 2007), Cpn60 to *Giardia* (Regoes *et al.* 2005) and *C. parvum* (Riordan *et al.* 2003) and Cpn60 and Cpn10 to the hydrogenosomes of *T. vaginalis* (Bui *et al.* 1996).

5. IRON–SULPHUR CLUSTER BIOSYNTHESIS: AN ESSENTIAL COMMON FUNCTION OF MITOCHONDRIAL HOMOLOGUES?

The biogenesis of ISCs is the only essential biosynthetic function of yeast mitochondria (Lill & Muhlenhoff 2008) and is required for the maturation of mitochondrial and cytosolic Fe/S proteins of diverse functions. In humans, defects in Fe/S protein biogenesis cause haematological and neurological disorders (Lill & Muhlenhoff 2008) such as Friedreich's ataxia. In yeast, the synthesis of an ISC occurs inside mitochondria on the scaffold proteins Isu1/Isu2 (yeast nomenclature) (Muhlenhoff *et al.* 2003). This step requires a cysteine desulphurase (Nfs, also abbreviated IscS) complex (Nfs1–Isd11) (Zheng *et al.* 1993; Wiedemann *et al.* 2006) and frataxin (Yfh1) (Gerber *et al.* 2003), as the sulphur and iron donors, respectively. The electrons needed for the reaction are supplied by ferredoxin (Yah1) (Muhlenhoff *et al.* 2003). ISC transfer from Isu1/Isu2 to target mitochondrial apoproteins is thought to be facilitated by a dedicated chaperonin system comprising mtHsp70 (Ssq1) and the DnaJ-like cochaperone Jac1 (Lill 2009). This mitochondrial pathway for making ISCs is collectively called the ISC assembly system. Core components of the ISC machinery used to make ISCs inside mitochondria appear to have been inherited from the mitochondrial endosymbiont (Tachezy *et al.* 2001; Emelyanov 2003), making it potentially one of its aboriginal functions. Recent work suggests that ISC assembly may be an important function of diverse mitochondrial homologues too.

Trichomonas hydrogenosomes contain core components of the ISC machinery, including Hsp70, ferredoxin, Nfs, Isu and frataxin (Hrdy *et al.* 2008), and isolated hydrogenosomes catalyze the enzymatic assembly and insertion of ISCs into apo-ferredoxin (Sutak *et al.* 2004). When expressed in the mitochondria of a frataxin-deficient *S. cerevisiae* strain, *T. vaginalis* frataxin also partially restored defects in heme and ISC biosynthesis (Dolezal *et al.* 2007). The localization of Isu and Nfs to tiny double-membrane-bounded organelles provided the first evidence for a mitosome in *Giardia* (Tovar *et al.*

2003). High-speed fractions obtained using differential centrifugation and enriched in *Giardia* mitosomes were also able to add ISCs to apo-ferredoxin, suggesting a complete pathway for ISC assembly is present in the organelle (Tovar *et al.* 2003). Interestingly, however, no frataxin gene was detected on the *Giardia* genome (Morrison *et al.* 2007).

There is also published evidence that the mitosomes of two species of microsporidia, *E. cuniculi* and *T. hominis*, contain key components of the ISC machinery. Using indirect immuno-fluorescence analyses (IFA) of parasite-infected rabbit kidney cells, and antisera raised against recombinant *E. cuniculi* or *T. hominis* proteins, the mitosomes of *E. cuniculi* were shown to contain frataxin, Nfs, Isu and mtHsp70 (Goldberg *et al.* 2008; Tsaousis *et al.* 2008) and potentially ferredoxin (Williams *et al.* 2008a). By contrast, using the same IFA approach, the mitosomes of *T. hominis* appear to contain mtHsp70 and Nfs but the main pools of Isu and frataxin were apparently cytosolic (Goldberg *et al.* 2008). The IFA data cannot exclude the possibility that there is some minor pool of Isu and frataxin in the *T. hominis* mitosome or of Nfs in the cytosol, but the apparent disjunct distribution of major pools raises intriguing questions as to how the separated *T. hominis* ISC components can perform their usually tightly coordinated function (Goldberg *et al.* 2008). Functional data from the same study already demonstrate that *ThIsu* accepts sulphur from *Saccharomyces* mitochondrial Nfs1, because it can complement a yeast Isu1/2 mutant. How the persulphide moiety generated by the cysteine desulphurase activity of mitochondrial *ThNfs* might be delivered to a cytosolic *ThIsu* scaffold is also problematic. However, even in well-studied model systems like yeast (Lill & Muhlenhoff 2008), there are still uncertainties about what essential product of mitochondrial Fe/S biosynthesis is actually exported from mitochondria to support cytosolic Fe/S biosynthesis (Netz *et al.* 2007; Lill 2009).

Bacteria possess three distinct systems to generate Fe/S proteins. The ISC assembly system is used for 'house-keeping' proteins, the sulphur-mobilization (SUF) machinery probably repairs Fe/S proteins and the nitrogen-fixation (NIF) machinery is used primarily to form specific ISCs in the nitrogenase of nitrogen-fixing bacteria (Tachezy & Smid 2008). *Entamoeba histolytica* is the only eukaryote known to have lost key components of the ISC machinery (Loftus *et al.* 2005) and to have replaced it by lateral gene transfer of a NifS cysteine desulphurase and NifU scaffold protein, from bacteria related to *Campylobacter* and *Helicobacter* (Ali *et al.* 2004; van der Giezen *et al.* 2004). Heterologous expression of *EhNifS* and *EhNifU* successfully complemented, under anaerobic but not aerobic conditions, the growth defect of an *E. coli* strain, in which both the ISC and SUF operons were deleted. These data suggest that *EhNifS* and *EhNifU* are necessary and sufficient for ISCs of non-nitrogenase Fe/S proteins to form under anaerobic conditions (Ali *et al.* 2004). The cellular localization of *EhNifS* and *EhNifU* in *Entamoeba* is currently unknown, but is crucial to testing the hypothesis that all mitochondrial homologues make ISCs.

Genes for NifS and NifU were also identified in a cDNA library for the related anaerobic amoeba *Mastigamoeba balamuthi*, suggesting that gene transfer predates the diversification of *Entamoeba* and *Mastigamoeba* (Gill *et al.* 2007). By contrast, *Dictyostelium discoideum*, an aerobic relative of *Entamoeba*, contains genes for the mitochondrial ISC system, suggesting that transfer post-dates the last common ancestor of these two species. Intriguingly, *Acanthamoeba castellanii*, another relative of *Dictyostelium* and *Entamoeba*, is reported to contain genes for both ISC and NIF components, but the proteins have so far not been localized or functionally characterized (van der Giezen 2009).

6. SUMMARY AND FUTURE PROSPECTS

Over the past 15 years, we have gone from a perspective whereby some eukaryotes were held to be primitively without mitochondria to one where it seems reasonable to suggest that organelles related to mitochondria are essential components of all eukaryotic cells. Some of these mitochondrial homologues, the mitosomes of parasites, no longer make ATP and have undergone dramatic reductive evolution at every level. For example, it is difficult to understand how *Entamoeba* and *Giardia* mitosomes can import proteins through their inner membrane because they appear to have lost key components of the complexes used for this purpose in model systems. Studying protein import into mitosomes directly is needed to better understand how they achieve this and to reveal the limits to which reductive evolution can proceed while maintaining a viable organelle. Interesting progress has already been made in this area, for example the discovery that the mitochondrial peptidase of *Giardia* functions, uniquely among eukaryotes, as a monomer (Šmíd *et al.* 2008).

Some of the work needed will be based upon proteomics investigations of isolated organelles. Identifying parasite orthologues of functionally characterized proteins can be difficult because the parasite versions are often highly divergent. Using computers trained on targeting sequences of model organisms to try and predict the cellular locations of parasite proteins often suffers from the same limitations. High-throughput proteomics using mass spectrometry has proved invaluable for investigating the structures of model mitochondria and their functions in health and disease (Mathy & Sluse 2008). New highly sensitive mass-spectrometry methods that use differential isotope tagging to identify protein location (Lilley & Dunkley 2008), and which do not require large amounts of highly purified organelles—which can be difficult to isolate even from yeast, never mind obligate intracellular parasites like Microsporidia—are now available to facilitate proteomics studies of mitosomes and hydrogenosomes. The potential value of this work is illustrated by proteomics studies on *Trichomonas* hydrogenosomes that have already found novel hydrogenosomal proteins, including peroxidases and [Fe]-hydrogenase maturases (Henze 2008). So far, there have been no proteomics studies of mitosomes from *Entamoeba*, *Giardia* or Microsporidia, or of any MLO or non-*Trichomonas* hydrogenosomes. Such work, together with detailed

bioinformatics, will provide clues to the structure and functions of different organelles including lineage-specific differences and innovations.

The discovery of organelles such as the *Blastocystis* MLO and the *Nyctotherus* hydrogenosome provided important missing links between classic mitochondria, hydrogenosomes and mitosomes and examples of intermediate stages of reductive evolution. The key ATP-generating pathways in classic mitochondria are the TCA cycle and the ETC, both of which are inhibited in the absence of oxygen. Although the data for *Blastocystis* and *Nyctotherus* are only partial (Boxma et al. 2005; Perez-Brocal & Clark 2008; Stechmann et al. 2008), it already suggests that complexes of the ETC components have been lost, presumably because of the decreased selection pressure to retain genes for aerobic respiration in low-oxygen environments. The constraints of living under low-oxygen conditions have probably also been major drivers for the reductive evolution of the mitosomes of *Cryptosporidium*, *Entamoeba*, *Giardia* and the hydrogenosomes of *Trichomonas*. By contrast, microsporidia live inside eukaryotic cells that have perfectly good aerobically functioning mitochondria. It is not an anaerobic lifestyle that is driving the reductive evolution of microsporidia and their mitosomes, but obligate intracellular parasitism.

At present, the only common metabolic function identified for a diverse sample of mitochondria, hydrogenosomes and mitosomes is ISC assembly.

The biogenesis of ISCs is an essential biosynthetic function of yeast mitochondria, and defects in human Fe/S protein biogenesis cause severe haematological and neurological disorders (Lill & Muhlenhoff 2008). Quite why ISC biosynthesis might need to be contained within the organelle is not certain (Lill 2009). For organelles containing Fe/S proteins such as complex I or ferredoxin, it might simply be easier to insert ISCs into apo-proteins *in situ*. Another reason could be that cellular compartmentalization limits the toxicity of ferrous iron and sulphide, which are needed for Fe/S assembly (Tachezy & Smid 2008). In yeast and humans, the products of mitochondrial ISC biogenesis are also needed to make essential non-mitochondrial Fe/S proteins by a cytosolic iron-sulphur protein assembly system (Netz et al. 2007). These Fe/S proteins include Rli1, which is essential for a functional translation apparatus (Kispaal et al. 2005). The component exported from yeast mitochondria (by an ISC export machinery) has not been fully characterized; it appears to contain sulphur but not iron (Lill & Muhlenhoff 2008). It will be interesting to investigate whether hydrogenosomes and mitosomes are vital for the species that contain them because they also carry out this function.

Work done in our laboratory on this topic was supported by European Union Marie Curie Training Fellowships to K.H. and A.V.G., and by a Wolfson Research Merit Award from the Royal Society to T.M.E.

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