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Hydrogenosomes and Mitosomes: Conservation and Evolution of Functions[1]

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ABSTRACT. The field studying unusual mitochondria in microbial eukaryotes has come full circle. Some 10–15 years ago it had the evangelical task of informing the wider scientific community that not all eukaryotes had mitochondria. Advances in the field indicated that although some protists might not have mitochondria, the presence of genes of mitochondrial ancestry suggested their lineage once had. The subsequent discovery of mitochondrial compartments in all supposedly amitochondriate protists studied so far indicates that all eukaryotes do have mitochondria indeed. This assertion has fuelled novel eukaryotic origin theories and weakened others. But what do we know about these unusual mitochondria from anaerobic protists? Have they all converged onto similar roles? Iron–sulphur cluster assembly is often hailed as *the* unifying feature of these organelles. However, the iron–sulphur protein that is so important that a complete organelle is being maintained has not been identified. Is it to be expected that all unusual mitochondria perform the same physiological role? These organelles have been found in numerous protists occupying different ecological niches. Different selection pressures operate on different organisms so there is no reason to suspect that their mitochondria should all be the same.

Key Words. Anaerobic respiration, endosymbiosis, iron–sulphur proteins, mitochondrion, organelles.

THOUGHTS about the origin of eukaryotes have generated a multitude of models (reviewed in Martin et al. 2001). Arguably the most well-known is the serial endosymbiosis theory popularised by Margulis (then named Sagan (1967)) which stated that a heterotrophic anaerobe engulfed a bacterium to become the mitochondrion. Somewhat later, Doolittle published molecular data confirming the bacterial origin of mitochondria and embedded the endosymbiosis theory into modern evolutionary thinking (Doolittle 1980). All endosymbiosis theories contained a very important, but hypothetical organism: a ''primitive'' eukaryote devoid of classic eukaryotic features such as mitochondria (or perhaps even a nucleus). This amitochondriate host was formalised by Cavalier-Smith and these organisms were called the Archezoa (Cavalier-Smith 1983). The Archezoa contained several groups of extant organisms; the metamonads, microsporidia, parabasalia, and the archamoebae (Keeling 1998). All the organisms belonging to these groups were thought to have branched off the main line of eukaryotic evolution before the establishment of the mitochondrial endosymbiont.

The Archezoa hypothesis was based on various observations indicating the primitive nature of its constituents. Each group consisted of anaerobic microbial eukaryotes that lacked mitochondria, peroxisomes and often Golgi too (see, e.g. Friend 1966; Rosenbaum and Wittner 1970). In addition, their ribosomes were thought to resemble those of bacteria and not of eukaroytes. When molecular biological techniques were developed, rRNA sequencing confirmed the supposedly ancient nature of the Archezoa as they were represented by deep long branches in phylogenetic reconstructions (Sogin et al. 1989; Vossbrinck et al. 1987).

Therefore, organisms such as *Giardia*, *Entamoeba*, *Trichomonas* and microsporidia were thought to have arisen before the establishment of the mitochondrion in the eukaryotic lineage. Studying these organisms would therefore reveal a wealth of information about early eukaryotic evolution.

Interestingly, all these organisms are parasites of humans (microsporidia are even intracellular parasites) which might hint at completely different evolutionary forces at work. Additionally, trichomonads were known to harbour an energetically important organelle, the hydrogenosome (Cerkasovová et al. 1973; Lindmark and Müller 1973). These organelles were known to decarboxylate pyruvate and produce ATP akin to a process also taking place in classic mitochondria. In addition, the first rRNA analyses indicated that *Entamoeba histolytica* is not as basal as assumed and that it clusters with mitochondriate groups (Sogin 1989).

More serious trouble for the Archezoa started in 1995 with a publication from Graham Clark and Andrew Roger. Their discovery of genes encoding proteins (chaperonin 60 and pyridine nucleotide transhydrogenase [PNT]) that are normally localised in mitochondria of other organisms in *E. histolytica* was a prelude to the downfall of this popular theory (Clark and Roger 1995). Interestingly, the activity of PNT had already been detected before the formulation of the Archezoa hypothesis (Harlow, Weinbach, and Diamond 1976). Both genes encoding PNT and Cpn60 seemed to contain mitochondrial-like targeting signals. In addition, phylogenetic analysis of Cpn60 (PNT is too short for phylogenetic analysis) clearly showed its mitochondrial affinity as it clustered with mitochondrial homologues from other eukaryotes. In addition to *Entamoeba*, similar discoveries relating to the other Archezoa followed rapidly (Bui, Bradley, and Johnson 1996; Germot, Philippe, and Le Guyader 1996, 1997; Hirt et al. 1997; Horner et al. 1996; Roger, Clark, and Doolittle 1996; Roger et al. 1998).

So, in the late 1990s it seemed that the Archezoa concept was incorrect as all supposedly primitive amitochondriate eukaryotes contained evidence of a mitochondrial past in their genomes. Their amitochondriate status clearly was secondarily derived.

Apart from in trichomonads, hydrogenosomes were found in various ciliates, chytrid fungi and heterolobosean flagellates (reviewed in van der Giezen, Tovar, and Clark 2005). The disparate distribution of hydrogenosomes in the eukaryotic tree, combined with the fact that mitochondria and hydrogenosomes are mutually exclusive (van der Giezen and Tovar 2005) and their repeated appearance in ciliates in between mitochondrial clades (Embley et al. 1995) suggested that hydrogenosomes are modified mitochondria (van der Giezen and Müller 1998). High-resolution electron microscopy indicated the presence of two bounding membranes, the hallmark of organelles of endosymbiotic ancestry (Henze and Martin 2003), surrounding chytrid hydrogenosomes (Benchimol, Durand, and Almeida 1997; van der Giezen et al. 1997a). Mitochondrial targeting signals were discovered on hydrogenosomal proteins in chytrids (Brondijk et al. 1996; van der Giezen et al. 1997b) and *Psalteriomonas* (Brul et al. 1994). Around the same time, different laboratories independently

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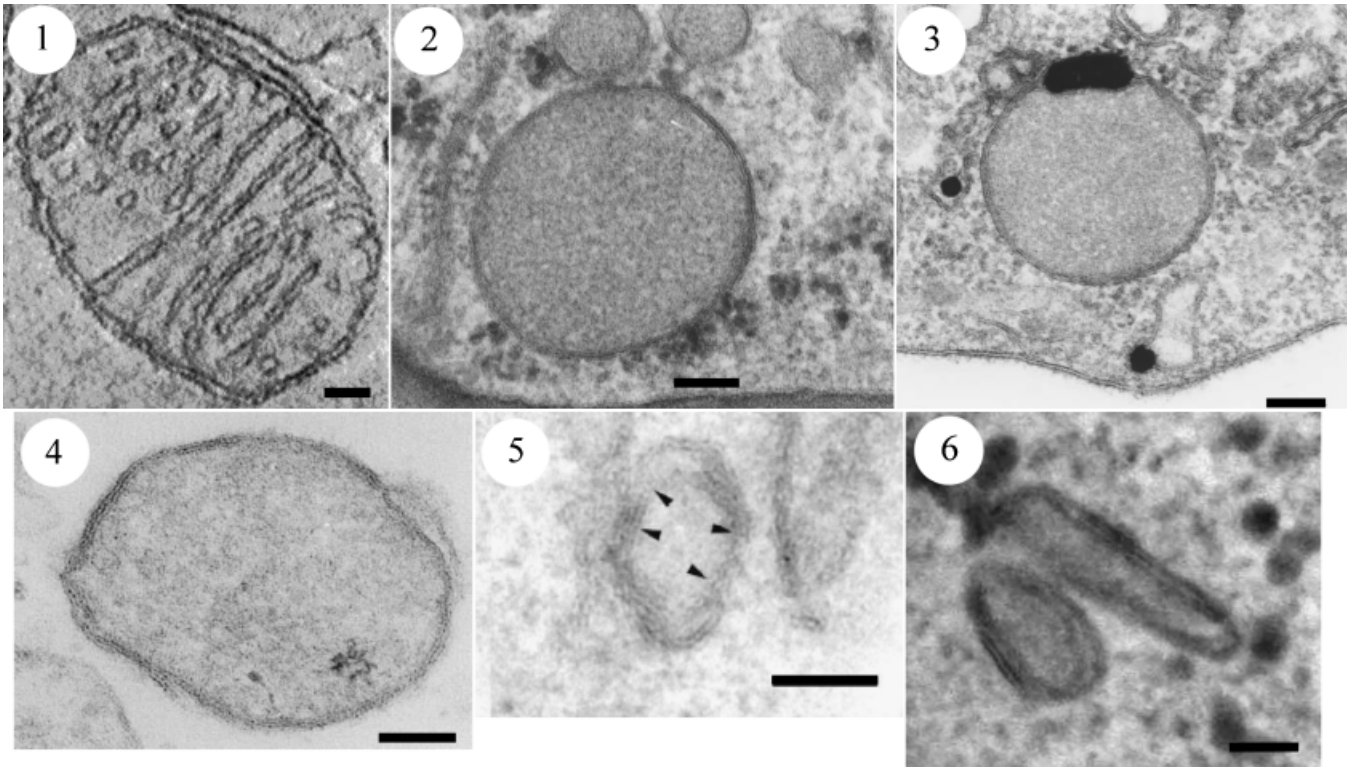

Fig. **1–6.** Electron micrographs of different mitochondrial manifestations. **1.** Mitochondrion from chicken cerebellum. **2, 3.** Hydrogenosomes from (2) the anaerobic fungus *Neocallimastix patriciarum* and (3) the cattle parasite *Tritrichomonas foetus*. **4–6.** Mitosomes from (**4**) the intestinal parasite *Entamoeba histolytica*, (5) the microsporidian *Trachipleistophora hominis* and (6) the diplomonad *Giardia intestinalis*. Scale bars: (1–4) 100 nm and (5, 6) 50 nm. Reproduced with permission from van der Giezen and Tovar (2005).

showed that hydrogenosomal targeting signals function as mitochondrial targeting signals in heterologous hosts (Bradley et al. 1997; Häusler et al. 1997; van der Giezen et al. 1998). So, the realisation that these hydrogenosomes were modified mitochondria was widely accepted in the late 1990s. Parsimony therefore dictates to assume the trichomonad organelle is mitochondrial too.

The Archezoa hypothesis was as good as rejected with having genes of mitochondrial ancestry in all Archezoan lineages and even a mitochondrial organelle in the trichomonads. The notion that Archezoan ribosomes were more similar to prokaryotic than to eukaryotic ribosomes had also been clearly refuted (Arisue et al. 2004; Shirakura et al. 2001). In addition, the presence of primitive Golgi-lacking lineages has also been dismissed and this organelle must have been present in the last common eukaryotic ancestor (Dacks et al. 2003). However, it was about to get worse as organelles of mitochondrial origin were subsequently discovered in all remaining Archezoan lineages. The first to be discovered was the mitosome (or crypton) in *E. histolytica* (Mai et al. 1999; Tovar, Fischer, and Clark 1999). Using antibodies generated against the earlier discovered Cpn60, both laboratories detected discrete locations in the cell indicative of the presence of an organelle. The Tovar et al. (1999) study demonstrated that removal of the mitochondrial-like targeting signal of Cpn60 resulted in accumulation of the protein in the cytosol. A phenomenon that could be reversed using a trypanosomal mitochondrial targeting signal from Hsp70 (Tovar et al. 1999). Subsequently, a mitochondrial relic was described in the microsporidian *Trachipleistophora hominis* (Williams et al. 2002) using antibodies raised against the mitochondrial Hsp70 protein (Hirt et al. 1997). The discovery of this organelle was pre-empted by the team that annotated the *Encephalitozoon cuniculi* genome (Katinka et al. 2001), they discovered several further genes normally encoding mitochondrial proteins. Finally, in 2003, the last Archezoan revealed its secrets (Tovar et al. 2003). *Giardia intestinalis* was shown to contain mitosomes as well. The *Giardia* organelles were shown to be involved in the essential mitochondrial process of iron–sulphur cluster assembly (Lill and Kispal 2000) (see Fig. 1–6 for images of typical mitochondria, hydrogenosomes, and mitosomes).

So, after some 10–15 yr, the field studying unusual mitochondria in microbial eukaryotes has come full circle. All eukaryotes proposed to be related to the original primitive eukaryote that engulfed a prokaryote that became the mitochondrion are derived from this actual event.

The Archezoa hypothesis is an appealing one and the discovery of a true Archezoan would be greatly welcome. However, it is currently not possible to entertain this hypothesis any longer in polite biological society.[2] Similarly, the endosymbiont theory in its most popular form needs some serious re-thinking too as its essential and hypothetical host is currently nothing more than a ghost.

However, the downfall of the Archezoa hypothesis has sparked some other models of eukaryotic evolution (Martin and Müller 1998; Moreira and López-García 1998; Vellai, Takacs, and Vida

[2]''To many, no doubt, such speculations may appear too fantastic for present mention in polite biological society'', this was the damning comment of E.B. Wilson (1928) to Wallin's hypothesis of the endosymbiotic origin of mitochondria (1927). See Martin and Kowalik (1999).

1998). Moreira and López-García (1998) are often cited as describing the origin of the mitochondrion in their synthrophy hypothesis. However, in their scenario, an archaebacterial endosymbiont becomes the nucleus in a eubacterial host. In an updated hypothesis (López-García and Moreira 1999), a second, α-proteobacterial endosymbiont is introduced that becomes the mitochondrion. Parsimony would suggest that the less-endosymbiotic intense scenarios from Martin and Müller and from Vellai and colleagues seem more likely.

In contrast to previous evolutionary models, all these models are based on biochemical arguments. The difference between Martin and Müller (1998) and Vellai et al. (1998) is that the latter (as in the original endosymbiont theories) suggests the endosymbiont to have been an aerobe. Martin and Müller suggest it has been a facultative anaerobe. This would make explaining the various anaerobic enzymes found in the former Archezoa easier and enable the inclusion of hydrogenosomes and mitosomes (the latter had not been discovered then but were predicted) in a unifying theory explaining the origin of all eukaryotes.

Mitochondria, both aerobic and anaerobic, are highly relevant to the general topic of hydrogenosomes and mitosomes, but they will not be considered here. The reader is referred to other texts (Scheffler 2008; Tielens et al. 2002; van Hellemond et al. 2003). This reviews aims to place current findings relating to hydrogenosomes and mitosomes in a broader (historical) context and summarises relevant findings from large scale genome-type analysis. In order to avoid confusing vocabulary regarding mitochondrial relicts, mitochondrial-like organelles, etc. the reader should consult Table 1.

ORGANELLAR TARGETING

A consequence of the integration of a bacterial endosymbiont into the host was the transfer of most of its genome to the hosts' nucleus (Timmis et al. 2004). Gene loss and transfer must have been massive assuming the original endosymbiont had a genome similar in size to modern α-proteobacteria. α-proteobacterial genomes range from about 1 Mb for the intracellular parasite *Rickettsia prowazekii* to about 9 Mb for *Bradyrhizobium japonicum*. Mitochondrial genomes on the other hand vary between 6 kb for *Plasmodium falciparum* to 2400 kb for cucurbit (van der Giezen and Tovar 2005). This enormous reduction in genome size was accompanied by a similar reduction in coding capacity as modern mitochondrial genomes contain five (*P. falciparum*) to 97 (*Reclinomonas americana*) genes. In contrast, mitochondria contain anything between 400 and 1,200 proteins (Truscott, Brandner, and Pfanner 2003). Most of these proteins are not encoded by the mitochondrial genome but originate from the nucleus. To this end, nuclear-encoded proteins destined for the mitochondrion need to be imported (see Neupert and Herrmann (2007) for extensive information gleaned from studies on yeast).

The first characterised gene encoding a hydrogenosomal protein (*Trichomonas vaginalis* ferredoxin) contained a short presequence that was absent from the mature protein (Johnson et al. 1990). Subsequent work revealed similar short presequences on many *T. vaginalis* hydrogenosomal proteins (Hrdý and Müller 1995a, b; Lahti, Bradley, and Johnson 1994; Lahti, D'Oliveira, and Johnson 1992; Länge, Rozario, and Müller 1994; Mentel et al. 2008; Pütz et al. 2005, 2006; Richards and van der Giezen 2006; Smutná et al. 2008; van Grinsven et al. 2008). Interestingly, molecular characterisation of the *Psalteromonas lanternae* ferredoxin indicated the presence of a similar presequence on this hydrogenosomal protein from this free-living amoeboflagellate (Brul et al. 1994). In contrast, the reported presence of peroxisomal targeting signals on hydrogenosomal hydrogenase from an anaerobic chytrid (Marvin-Sikkema et al. 1993a) has not been confirmed by sequencing of the corresponding gene (Davidson et al. 2002; Voncken et al. 2002b). Similarly, the *inter alia* report

Table **1.** Distribution of hydrogenosomes and mitosomes throughout the eukaryotes (see note below).

Organisms	Supergroups[a]	Organelle-types[b]	Metabolic types[c]	Reference
Tritrichomonas foetus	Excavata	Hydrogenosome	Type II	Lindmark and Müller (1973), Cerkasovová et al. (1973)
Trichomonas vaginalis	Excavata	Hydrogenosome	Type II	Lindmark et al. (1975)
Giardia intestinalis	Excavata	mitosome	Type I	Tovar et al. (2003)
Trimastix pyriformis	Excavata	Hydrogenosome	Type II	Hampl et al. (2008)
Neocallimastix sp. (representing anaerobic chytrids)	Opisthokonta	Hydrogenosome	Type II	Yarlett et al. (1986)
Trachipleistophora hominis (representing microsporidia)	Opisthokonta	Mitosome	Type I	Williams et al. (2002)
Nyctotherus ovalis	Chromalveolata	Hydrogenosome	Type II	Akhmanova et al. (1998)
Cryptosporidium parvum	Chromalveolata	Mitosome	Type I	Riordan et al. (1999)
Blastocystis	Chromalveolata	Hydrogenosome	Type II	Stechmann et al. (2008)
Dasytricha ruminantium (representing rumen ciliates)	Chromalveolata	Hydrogenosome	Type II	Yarlett et al. (1981)
Trimyema compressum (representing free-living ciliates)	Chromalveolata	Hydrogenosome	Type II	Zwart et al. (1988)
Entamoeba histolytica	Amoebozoa	Mitosome	Type I	Tovar et al. (1999), Mai et al. (1999)
Psalteriomonas lanternae	Amoebozoa	Hydrogenosome	Type II	Broers et al. (1990)
Mastigamoeba balamuthi	Amoebozoa	Hydrogenosome	Type II	Gill et al. (2007)
Breviata anathema	Amoebozoa	Hydrogenosome	Type II	Walker (2007), Minge et al. (2008)
Chlamydomonas reinhardtii	Plantae	See text for this peculiar case.	Mitochondriate	Atteia et al. (2006), Mus et al. (2007)

[a]See Simpson and Roger (2004) for an overview of the six eukaryotic supergroups.

[b]This table should be taken as a ''best guest'' approach as in not all cases has the true identity of an organelle been established. In addition, as mentioned in the main text, strictly speaking molecular hydrogen production needs to be detected in order to be able to label an organelle as a hydrogenosome Müller (1993).

[c]Metabolic types are based on the classification by Martin and Müller (1998) where Type I means no core ATP production in organelles and ATP production is cytosolic, Type II means fermentative core ATP production in organelles (producing hydrogen) and mitochondriate means respiratory/chemiosmotic ATP production in the organelle.

of a peroxisomal targeting signals on chytrid adenylate kinase (Akhmanova et al. 1998a) has never been confirmed either. Work from others (Brondijk et al. 1996; Dacks et al. 2006; Davidson et al. 2002; van der Giezen et al. 1997b, 2003) and the same laboratory (Akhmanova et al. 1999; Voncken et al. 2002b) have clearly demonstrated the presence of amino terminal presequences with mitochondrial characteristics on chytrid hydrogenosomal proteins. The presence of amino terminal presequences on putative hydrogenosomal and mitosomal proteins seems more or less universal. The report describing the discovery of a putative hydrogenosomal genome in the cockroach hindgut ciliate *Nyctotheris ovalis* also reported a possible mitochondrial targeting signal on the discovered hydrogenase (Akhmanova et al. 1998b). More recent genomic analysis indicated the presence of amino terminal presequences on putative organellar proteins from *Mastigamoeba balamuthi* (Gill et al. 2007), *Blastocystis* (Stechmann et al. 2008) and *Trimastix pyriformis* (Hampl et al. 2008).

Although mitosomes were discovered later than hydrogenosomes, proteins with mitochondrial-like presequences were discovered more or less at the same time as hydrogenosomal targeting signals were discovered (i.e. before the actual organelle had been identified). The first to be discovered were putative presequences on PNT and Cpn60 from *Entamoeba histolytica* (Clark and Roger 1995). This was followed by similar findings in microsporidia (Germot et al. 1997; Hirt et al. 1997), *G. intestinalis* (Nixon et al. 2002 [although not mentioned]) and *Cryptosporidium parvum* (LaGier et al. 2003).

Even though several groups demonstrated processing of these presequences, their actual role in targeting proteins to hydrogenosomes or mitosomes had not been demonstrated. Bradley et al. (1997) developed an in vitro import system for *T. vaginalis* hydrogenosomes. They convincingly demonstrated that, similar to mitochondrial protein import, the ferredoxin presequence was essential for proper import and was dependent on temperature, ATP but affected by a protonophore (Bradley et al. 1997). In the same year, the sufficiency of trichomonad hydrogenosomal targeting signals was shown by Häusler et al. (1997). They demonstrated that the putative presequences of *T. vaginalis* ferredoxin, β-succinyl-CoA synthetase (SCS) and pyruvate: ferredoxin oxidoreductase (PFO) were capable of targeting dihydrofolate reductase into yeast and *Trypanosoma brucei* mitochondria in vitro. The ferredoxin presequence was functional in vivo in yeast as well. Import of SCS was less efficient than that of ferredoxin and PFO though (Häusler et al. 1997). Imported SCS and ferredoxin were both efficiently processed in contrast to PFO. Similar heterologous import studies using anaerobic chytrid hydrogenosomal malic enzyme demonstrated the requirement of the amino terminal presequence for mitochondrial targeting as well (van der Giezen et al. 1998, 2003). These studies using hydrogenosomal proteins clearly demonstrated the mitochondrial nature of the hydrogenosomal protein import machinery.

Amino terminal presequences are used to target proteins to the organellar matrix. However, proteins destined for the mitochondrial membranes use a different import pathway (see Dolezal et al. 2006 for an overview of the different mitochondrial protein import pathways) and have internal, cryptic, targeting signals. Heterologous targeting experiments using the *T. vaginalis* (Dyall et al. 2001) and chytrid (van der Giezen et al. 2002) hydrogenosomal ADP/ATP carrier, an inner membrane protein, clearly indicated that this import pathway had been conserved between hydrogenosomes and mitochondria as well.

Import studies using mitosomal proteins also indicated the requirement of amino terminal targeting signals for some proteins. Of the five mitosomal proteins known for *G. intestinalis* (Cpn60, Hsp70, IscS, IscU, and ferredoxin), only the import of the latter two is reliant on an amino terminal targeting signal (Dolezal et al. 2005; Regoes et al. 2005). The other three proteins have no recognisable (amino terminal) targeting signals but are nonetheless present inside the mitosomes and are processed upon import (Dolezal et al. 2005; Regoes et al. 2005). Perhaps more surprisingly, the ''leader-less'' *Giardia* IscS was imported into *T. vaginalis* hydrogenosomes (Dolezal et al. 2005) indicating that, despite the absence of a clear-cut targeting signal, the hydrogenosomal import machinery recognised another (cryptic) signal and properly imported the protein. Similar confusing results were obtained in a study using microsporidian mitosomes. Bioinformatics analyses predicted the presence of amino terminal targeting signals on some but not all mitosomal proteins. Targeting experiment in yeast using GFP-fusion constructs indicated that there is no correlation between these predictions and the actual location where these fusions ended up (Burri et al. 2006). Although mistargeting could be due to the taxonomic distance of the two organisms, leader-less mitosomal proteins were imported as well similar to the situation with *Giardia* (Burri et al. 2006). It has been suggested that the advanced state of reductive evolution of mitosomes has affected organellar protein import as well (Burri et al. 2006; Regoes et al. 2005).

More recent work indicates that hydrogenosomal protein import is not so straightforward anymore either. Studies using the hydrogenosomal thioredoxin reductase (ThxR) from *T. vaginalis* indicated that there must be multiple signals present that guide protein import (Mentel et al. 2008). A (naturally) leader-less ThxR (TrxRh2) was imported properly while a ThxR (TrxRh1) with an obvious amino terminal signal was not upon removal of that signal. However, the leaders of the α subunits of SCS and PFO (known hydrogenosomal proteins) were not capable of restoring hydrogenosomal targeting of TrxRh1 when the original leader was removed (Mentel et al. 2008). This suggests the TrxRh1 leader contains an incomplete signal that needs another (internal) incomplete signal to function. However, the leaders of SCS and PFO apparently interfere with and/or are rendered inactive due to the internal incomplete signal. More confusingly, when the leader of the α subunit of SCS was removed, SCS was still imported into *T. vaginalis* hydrogenosomes (Mentel et al. 2008). Similar to mitosomal protein import, it seems internal cryptic signals might be needed to import certain proteins into hydrogenosomes too. As SCS functions as a dimer, the possibility that the α subunit piggybacks (Glover, Andrews, and Rachubinski 1994) on the β subunit for import cannot be excluded. However, this kind of import has not been observed for mitochondria.

Hydrogenosomal and mitosomal targeting signals from *T. vaginalis* and *G. intestinalis* are generally short and do not contain as many positive residues when compared with well-studied yeast signals. Some longer, more classic, signals do exist for *T. vaginalis* but the reason for this difference has been unclear for some time. Recently, it has been demonstrated that *Giardia* contains a unique monomeric mitochondrial processing peptidase (MPP) that is capable of processing those short presequences (Šmíd et al. 2008). It seems that the longer classic presequences are held in place by the α subunit of MPP in order for it to be processed by the β subunit. *Giardia* does not contain the α subunit any more and its β subunit only MPP (monomeric) is capable of processing the shorter *Giardia* presequences but not long ones from yeast (Šmíd et al. 2008).

Many authors (Burri et al. 2006; Regoes et al. 2005; Šmíd et al. 2008) suggest reductive evolution at work due to the parasitic nature of the organisms studied. Although this could well be the case, the fact that all our knowledge with respect to mitochondrial protein import mainly comes from the Opisthokonta severely blurs our overall vision of the evolution of this process. The universal distribution of mitochondria in all eukaryotic supergroups indicates that the origin of this organelle must have been a very

early event in eukaryotic evolution, if not *the* event that resulted in the eukaryotic lineage. As bacteria do not have the protein import machineries that are present in mitochondria, these machineries must have evolved very early in mitochondrial evolution as well. Comparative genomics studies suggest that major components of mitochondrial protein import complexes are present in all major lineages (Dolezal et al. 2006). The mitochondrial outer membrane complex SAM is involved in the assembly of β-barrel proteins into the mitochondrial outer membrane. This complex is related to the bacterial Omp85 protein and has been identified on the genomes of hydrogenosomal and mitosomal lineages such as *T. vaginalis* and *E. cuniculi* (Dolezal et al. 2006). Components of the translocase of the inner mitochondrial membrane (TIM) have been identified in *T. vaginalis* and *G. intestinalis* (Dolezal et al. 2005) as well. Although most information on mitochondrial protein import comes from yeast, more comparative work now suggests that mitochondrial import is build around a modular design with only a few core components (Dolezal et al. 2006). Further work using ''unusual'' model organisms will undoubtedly result in more exceptions to the ''rule''.

ORGANELLAR PHYSIOLOGY

Trichomonad hydrogenosomes were first described in the early 1970s (Cerkasovová et al. 1973; Lindmark and Müller 1973; Müller 1973). The organelles were described by means of the distribution of the activities of malate dehydrogenases, glycerol-3-phosphate dehydrogenase (called α-glycerophosphate dehydrogenase), hydrogenase and pyruvate synthase (PFO) (Lindmark and Müller 1973; Müller 1973). This was done in *Tritrichomonas foetus*, not *T. vaginalis* although this followed suit (Lindmark, Müller, and Shio 1975). During the 1970s, the basic biochemistry of ATP synthesis was unravelled and a metabolic map for comparative work on hydrogenosomes in other organisms was laid out (Brugerolle and Metenier 1973; Cerkasòv et al. 1978; Cerkasovová et al. 1973; Lindmark and Müller 1973, 1974; Lindmark et al. 1975; Müller 1973; Müller and Lindmark 1978). In principle, the metabolic map produced by Müller and Lindmark 1978 still shows the core carbon pathway of most hydrogenosomes (see Fig. 7). There is some controversy regarding whether chytrid hydrogenosomes use PFO or pyruvate formate lyase (PFL) to decarboxylate pyruvate. Marvin-Sikkema et al. (1993b) detected high activities of a cytosolic PFL and a hydrogenosomal PFO in *Neocallimastix* sp. L2. In contrast, Akhmanova and colleagues failed to isolate genes encoding PFO by PCR but identified six PFL encoding cDNAs (1999). They measured a specific activity of PFL that was hundred times lower than the value obtained by Marvin-Sikkema et al. (1993b). These data indicate that chytrids use, similarly to trichomonads, a hydrogenosomal PFO to decarboxylate pyruvate. The reported PFL sequences by Ahkmanova et al. (1999) most likely encode the cytosolic protein detected by Marvin-Sikkema et al. (1993b); these sequences do not contain typical chytrid hydrogenosomal targeting signals either. Further work is needed to clarify the complete metabolism of cythrid hydrogenosomes.

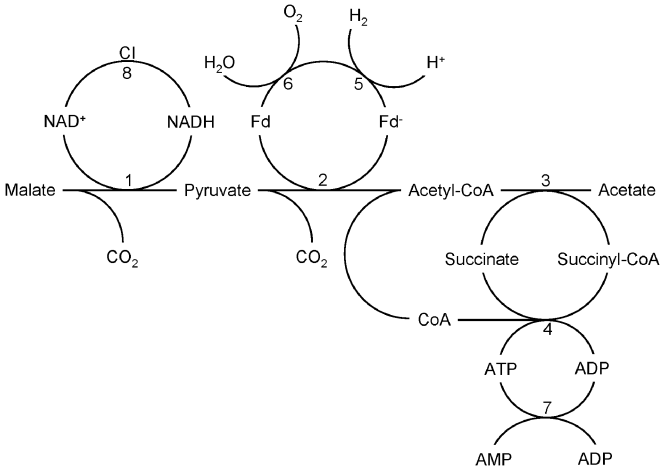

Fig. **7.** The basic carbon metabolic pathway of hydrogenosomes as originally described for *Tritrichomonas foetus* hydrogenosomes by Müller and Lindmark (1978). This core pathway seems present in most studied hydrogenosomes. The original scheme has been updated where new data became available. 1, Malic enzyme (decarboxylating); 2, pyruvate: ferredoxin oxidoreductase (PFO); 3, acetate: succinate CoA-transferase (ASCT); 4, succinyl-CoA synthetase (SCS); 5, hydrogenase; 6, flavodiiron protein (Smutná et al. 2008); 7, adenylate kinase; 8, Complex I components (Dyall et al. 2004; Hrdý et al. 2004; Smutná et al. 2008); Fd, ferredoxin. Reproduced with permission.

Hydrogenosomes are organelles that, under anoxic conditions, decarboxylate pyruvate into acetate, CO_2, and H_2 with the concomitant production of energy in the form of ATP (Müller 1993). Although clearly described, more recent genomics-based analyses of various organisms fall foul of this definition as hydrogenosomes cannot be defined based on their protein complement alone (Boxma et al. 2005; Gill et al. 2007; Hampl et al. 2008; Stechmann et al. 2008) and actual determination of hydrogen production needs to be performed. This is not an exclusive problem for the hydrogenosomal field though but a serious problem for the post-genomic era as a whole. A welcome exception was the recent study by Lantsman et al. (2008) who, similar to the early days of amitochondriate research, isolated the actual organelle and assayed for the presence of various enzymes. However, in contrast to the EST-based study from Stechmann et al. (2008), Lantsman et al. (2008) were not able to detect an active hydrogenase in *Blastocystis* extracts using the standard methyl viologen assay (Lindmark and Müller 1973). Nevertheless, this might be due to the use of a different *Blastocystis* subtype than the one used in the Stechmann et al. study that did localise a hydrogenase to the organelle (2008). Similarly, the *G. instinalis* hydrogenase was not shown to produce hydrogen until a novel detection method was used that clearly demonstrated the production of small amounts of molecular hydrogen (Lloyd, Ralphs, and Harris 2002). This hydrogen production was sensitive to oxygen in agreement with earlier work showing that low concentrations of oxygen fundamentally alter the metabolism of *Giardia* (Paget et al. 1993). Alternatively, many eukaryotic hydrogenases contain accessory domains of uncertain function at their carboxy termini. Perhaps these hydrogenases perform other functions in the cell. Related to hydrogenases is the discovery of components of complex I in *T. vaginalis* hydrogenosomes (Dyall et al. 2004; Hrdý et al. 2004). The identified components (NuoF and NuoE) are subunits from the NADH dehydrogenase part of complex I (Brandt 2006) and are thought to be involved in regenerating NAD^+ (see Fig. 7). Recent EST analyses identified further complex I components in *Blastocystis* (Stechmann et al. 2008). These components are normally involved with electron transfer from NADH to quinones and with proton pumping thereby generating a proton gradient across the hydrogenosomal membrane.

The basic route of carbon through trichomonad, and later chytrid (Marvin-Sikkema et al. 1993b; O'Fallon, Wright, and Calza 1991; Yarlett et al. 1986), hydrogenosomes seemed to confirm the notion that hydrogenosomes are remnant mitochondria and biochemically in demise. However, slowly, additional functions were uncovered that indicated hydrogenosomes do not only generate a little more ATP from glucose by dumping electrons on protons.

With a completed nuclear genome (Carlton et al. 2007) and over 30 yr of research, most is still known about the *T. vaginalis* hydrogenosomes. Most definitely when it comes to biochemically confirmed presences of activities. As oxygen-tolerant anaerobes, *T. vaginalis* has to deal with varying levels of oxygen. Unlike other eukaryotes, its defence mechanism is not relying on peroxisomes and glutathione but relies on thioredoxin-linked peroxiredoxin (Coombs et al. 2004) and a bacterial rubrerythrin (Pütz et al. 2005). These systems do not seem to have arisen via vertical evolution but have apparently been acquired laterally (Coombs et al. 2004; Pütz et al. 2005).

The glycine cleavage complex (GCV) is exclusively mitochondrial and normally catalyzes the oxidative decarboxylation and deamination of glycine. Together with serine hydroxymethyltransferase (SHMT) it is responsible for one-carbon transfers needed for the biosynthesis of folate cofactors (Salcedo, Sims, and Hyde 2005). These cofactors are essential for the production of purines and pyrimidines.

Serine hydroxymethyltransferase and two of the four GCV components have been identified in *T. vaginalis* hydrogenosomes (Mukherjee et al. 2006a, b). Despite extensive searches, these authors were unable to detect glycine decarboxylase (component P) or amino methyl transferase (component T) on the *T. vaginalis* genome. Large-scale EST analyses identified all components of GCV and SHMT in the free-living amoeba *Mastigamoeba balamuthi* (Gill et al. 2007) and in the intestinal parasite *Blastocystis* (Stechmann et al. 2008). In *Mastigamoeba* and *Blastocystis*, not all components contained amino terminal presequences (especially SHMT) so their actual localisation has not been tested. As SHMT can be found in both the cytosol and mitochondria, this would require further testing indeed. Analysis of ESTs from the preaxostylid flagellate *T. pyriformis* indicated the presence of all components of the GCV as well and most components contained amino terminal extensions (Hampl et al. 2008). As the L component of GCV can also be a component of the α-ketoacid dehydrogenase complexes (Carothers, Pons, and Patel 1989), and components P and T could not be identified on the *T. vaginalis* genome, some caution is needed in the interpretation of this data. In addition, it is known that trichomonads do not synthesise purines and pyrimidines de novo (Berens, Krug, and Marr 1995). Therefore, the role of GCV and SHTM with respect to production of purines and pyrimidines in *T. vaginalis* is circumspect.

In contrast to aerobic mitochondria, *T. vaginalis* hydrogenosomes play no role in arginine biosynthesis (or urea cycle). However, *T. vaginalis* (Yarlett et al. 1994), *G. intestinalis* (Schofield et al. 1992) and *T. foetus* (Yarlett et al. 1994) use the (cytosolic) arginine dihydrolase pathway that generates ATP via substrate level phosphorylation. The hydrogenosomes of the anaerobic fungus *Neocallimastix frontalis* contain the typical mitochondrial carbamoyl-phosphate synthase and ornithine transcarbamoylase (Gelius-Dietrich, Ter Braak, and Henze 2007). These two enzymes also partake in the arginine dihydrolase pathway. Similarly, *Blastocystis* seems to contain both enzymes as well, carbamoylphosphate synthase contains a mitochondrial-like targeting signal (Stechmann et al. 2008). Although the arginine dihydrolase pathway is cytosolic in *T. vaginalis*, *T. foetus* and *G. intestinalis*, there would be no reason why it could not be organellar in *N. frontalis* or *Blastocystis*. However, the possibility exists that the identified enzymes in the latter two species are part of the urea cycle. Argininosuccinate synthase, another urea cycle enzyme, has indeed been identified in a *Neocallimastix patriciarum* small-scale EST survey (van der Giezen and Embley, unpubl. observ.).

As the general perceived function of mitochondria is the provision of ATP to the rest of the cell, hydrogenosomal and mitosomal transporters involved in the transfer of ATP from the organellar matrix to the cytosol received considerable attention. The first to be discovered was the *T. vaginalis* Hmp31 carrier but unfortunately, the carrier was not characterised at the time and incorrectly labelled as being a classic mitochondrial AAC (Dyall et al. 2001). Later, work by others correctly phylogenetically classified this carrier as part of an unusual carrier family with unknown substrate specificity (Tjaden et al. 2004). Despite its unusual inhibitor profile and distinct phylogenetic placement this carrier was, however, capable of transporting ATP and ADP (Tjaden et al. 2004). A similar carrier has been identified in the *E. histolytica* mitosomes (Chan et al. 2005). Strikingly, although members of the mitochondrial carrier family are abundant in model eukaryotes, *E. histolytica* seems to have retained only one member of this extended carrier family. Although the carrier does transport ATP it is surprising that both *T. vaginalis* and *E. histolytica* do not utilise the classic ADP/ATP carrier. *Dictyostelium discoideum*, a social amoeba that is related to *Entamoeba*, does have the same unusual carrier although it has not been characterised yet (Chan et al. 2005). Classic ADP/ATP carriers have been molecularly characterised from chytrid hydrogenosomes (van der Giezen et al. 2002; Voncken et al. 2002a). Anaerobic ciliates known to contain hydrogenosomes (*N. ovalis* and *Euplotus*) seem to contain classic ADP/ATP carriers as well (Boxma et al. 2005; Voncken et al. 2002a). In addition, EST analysis of *Blastocystis* revealed a classic AAC transporter (Stechmann et al. 2008). More recently, a study of the microsporidian *E. cuniculi* revealed the presence of unusual ''reverse'' ADP/ATP carrier (Tsaousis et al. 2008) more commonly found in parasitic bacteria such as *Rickettsia* and *Chlamydia* (Winkler and Neuhaus 1999). Apparently, this microsporidian lost all members of the classic mitochondrial carrier family and replaced it with the ''ATP-stealing'' types from intracellular parasitic bacteria (Tsaousis et al. 2008). This work suggests that, as commonly believed, mitosomes are not involved in ATP generation but nonetheless harbour ATP requiring processes.

Mitochondria have been discovered to play an essential role in the assembly of iron sulphur (FeS) clusters as co-factors for various enzymes (Lill et al. 1999). Because of the simple nature of FeS clusters and their widespread distribution in many essential cellular processes, they have been assigned essential roles in the evolution of life (Hall, Cammack, and Rao 1971; Martin and Russell 2003; Russell and Hall 1997). The essential cysteine desulphurase (IscS) was indeed discovered on the *T. vaginalis* and *G. intestinalis* genomes (Tachezy, Sánchez, and Müller 2001). In the case of *Trichomonas*, a hydrogenosomal targeting signal was present on the N-terminus of the protein and the protein was indeed found to be localised in hydrogenosomes (Sutak et al. 2004). The *Giardia* IscS did not contain a presequence (Tachezy et al. 2001) but was nonetheless targeted to mitosomes (Dolezal et al. 2005; Regoes et al. 2005; Tovar et al. 2003) and shown to be, along with its partner protein IscU, involved in FeS cluster (ISC) assembly (Tovar et al. 2003). Similarly, both proteins are present and are targeted to the mitosome in the apicomplexan parasite *C. parvum* (LaGier et al. 2003). More recently, EST analyses identified components of the the FeS cluster assembly machinery for *M. balamuthi* (Gill et al. 2007) and *Blastocystis* (Stechmann et al. 2008) as well. Surprisingly, analyses of the *E. histolytica* genome resulted in the discovery of the non-homologous NIF system that is involved in FeS cluster assembly but generally not in mitochondria (Ali et al. 2004; van der Giezen, Cox, and Tovar 2004). It seemed the amoebae replaced the mitochondrial ISC system that originated from the mitochondrial endosymbiont (Emelyanov 2003; Tachezy et al. 2001; Tovar et al. 2003; van der Giezen et al. 2004) with the simpler NIF system (Huynen et al. 2001). Whether the Nif proteins identified in *E. histolytica* are

localised to the mitosomes awaits confirmation. The *nif* genes were also identified in *Mastigamoeba* but not in *D. discoideum* indicating that the replacement of the ISC system with NIF took place deep in the Archamoebae branch (Gill et al. 2007). The deep-branching Breviates (Minge et al. 2008) do contain the classic ISC system (Walker 2007). Perhaps more interestingly, the *Acanthamoeba castellanii* EST project at TBestDB (O'Brien et al. 2007) has annotated both *isc* and *nif* genes but these need further characterisation.

Although it has long been known that trichomonads contain very active malate dehydrogenases (Ryley 1955) it seemed that a complete and functional TCA cycle was absent in *T. foetus* (Danforth 1967). As the *T. vaginalis* genome has been sequenced (Carlton et al. 2007) no additional TCA cycle enzymes have been identified apart from the well-known SCS (Jenkins et al. 1991; Lahti et al. 1992, 1994; Steinbüchel and Müller 1986). Succinyl-CoA synthetase has been identified in chytrid hydrogenosomes as well (Brondijk et al. 1996; Dacks et al. 2006; Marvin-Sikkema et al. 1993b; Yarlett et al. 1986). Strangely enough, the hydrogenosome-like organelles from *M. balmuthi* seem to contain most of the TCA cycle enzymes but SCS was not identified among nearly 20,000 ESTs (Gill et al. 2007). The *Blastocystis* organelles only contain the latter half of the TCA cycle (Stechmann et al. 2008) which might run ''back-wards'' from oxaloacetate to succinate (or succinyl-CoA). This pathway has also been found to operate in the *N. ovalis* organelles (Boxma et al. 2005). However, and this needs some serious attention, the enzymatic analysis performed by Lantsman et al. (2008) on isolated organelles is in complete contrast to the above mentioned EST-based analyses by Stechmann et al. (2008). The latter identified the latter half of the TCA cycle while, in essence, Lantsman and colleagues found the first half of the TCA cycle. Both studies only agree on the absence of citrate synthase and the presence of SCS. In addition, some detected activities (isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) were reported to be absent by Zierdt et al. (1988). Although the differences could be due to strain differences that are known to be substantial in *Blastocystis* (Stensvold et al. 2007), it is truly remarkable and underscores the importance of combining genomic and biochemical analysis.

To maintain redox balance in the absence of oxidative phosphorylation, several organisms utilise two pathways to catabolise malate (see Tielens et al. 2002). The reductive branch of this malate dismutation pathway reduces malate to succinate by reversing part of the TCA cycle (oxaloacetate to malate to fumarate to succinate). Importantly, in order to do so, a divergent complex II is needed. Organisms utilising malate dismutation need a fumarate reductase (FRD) instead of a succinate dehydrogenase (SDH). However, at sequence level, both complexes seem indistinguishable. Experiments using isotopically labelled carbon suggest that *N. ovalis* does indeed employ a FRD (Boxma et al. 2005). Because of the difference in midpoint potential between FRD and SDH, different electron carriers are needed. Aerobic mitochondria use ubiquinones as electron carriers. However, the midpoint potential of ubiquinone (UQ; $E^{0\prime} = +70\,\mathrm{mV}$) is too high to reduce fumarate to succinate and a more reduced carrier is needed. Rhodoquinone (RQ; $E^{0\prime} = -63\,\mathrm{mV}$) has indeed been detected in *N. ovalis* extracts suggesting that its complex II is indeed a FRD (Boxma et al. 2005). Whether the capacity to make RQ is ancestral for anaerobic eukaryotes has interesting implications for mitochondrial evolution. There is one bacterial group that can make both UQ and RQ, the facultative anaerobic α-proteobacterial *Rhodospirillum rubrum* (Parson and Rudney 1965). Physiologically, this bacterium might fit well with the hydrogen hypothesis (Martin and Müller 1998).

The oxidative branch of malate dismutation involves the subsequent decarboxylations from malate to acetyl-CoA (Tielens et al. 2002). Energy is conserved by transfer of the CoA-moiety to succinate using acetate: succinate CoA-transferase (ASCT). The activity of this enzyme had been discovered in the early days of hydrogenosome research (Müller and Lindmark 1978). However, isolating the associated gene prooved to be more difficult (van Grinsven et al. 2008). ASCT has also been discovered in ''normal'' mitochondria and this was the first link between mitochondria and hydrogenosomes coming from ''the other side'' (van Hellemond, Opperdoes, and Tielens 1998). An ASCT has been discovered in the *Blastocystis* ESTs as well as agreeing well with a postulated malate dismutation pathway linked to a FRD (Stechmann et al. 2008).

FUTURE DIRECTIONS?

The recent explosion of organisms with hydrogenosomes and mitosomes has made it clear that these organelles are not rare oddities but that they play important roles in eukaryotic cellular evolution. They have now been found in most eukaryotic supergroups (Simpson and Roger 2004) and it seems reasonable to predict that these organelles will be found in the Rhizaria too. However, the apparent absence of hydrogenosomes and mitosomes in the Plantae is striking. Perhaps this lineage is too oxygenic to allow for these organelles with their oxygen-sensitive enzymes? However, all enzymes normally present in hydrogenosomes are present in the single-celled photosynthetic alga *Chlamydomonas reinhardtii*. This facultative anaerobic alga contains a hydrogen-producing hydrogenase and PFO (in addition to pyruvate dehydrogenase and pyruvate formate lyase) (Atteia et al. 2006; Happe, Mosler, and Naber 1994; Mus et al. 2007). However, the hydrogenosome and PFO are distributed between the chloroplast and mitochondria. As such, no real hydrogenosome seems to be present in *C. reinhardtii* unless we allow for an even more flexible use of the term hydrogenosome. On the other hand, hydrogenosomes and mitosomes are absent in multicellular organisms in general. Most likely, the increased energy demands of multicellular organisms cannot be met by hydrogenosomes and mitosomes (Lane 2005).

It is clear that the hydrogenosomal and mitosomal protein import systems share many features with the well-studied import system from *Saccharomyces cerevisiae*. However, there are sufficient differences to indicate that the yeast model is not generally applicable for all other eukaryotes. On the other hand, perhaps the missing components are simply too divergent to be recognised and better models are needed to detect them (Likic et al. 2005). Would other complex organellar machineries such as organellar division be similarly different?

Some hydrogenosomes contain an organellar genome (Akhmanova et al. 1998b; Pérez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008) and their presence correlates with the presence of a partial electron transport chain. This observation is more in line with the CoRR hypothesis (Allen 1993, 2003) than the hydrophobicity hypothesis (von Heijne 1986) that both try to explain why organellar genomes are retained. CoRR proposes that the reason behind organellar genomes is that genes are close to, and in the same compartment as, their gene products. This colocation allows for rapid and direct regulatory coupling. It is reasonable to assume that even organelles with a smaller complement of electron transport chain components such as hydrogenosomes want to place these under genetic control. Therefore, as predicted by CoRR, a mitochondrial sensor kinase might control the crosstalk between the nucleus and the organellar genome, similarly to the recently discovered chloroplast sensor kinase (Puthiyaveetil et al. 2008).

Although more and more functions are discovered in hydrogenosomes it is clear that not all hydrogenosomes in all lineages perform the same functions. This would actually be highly sur-

prising as hydrogenosomes have repeatedly evolved throughout the eukaryotic tree. Some selection pressures must be more or less equal to enable the appearance of the hydrogenosomal phenotype which seems to be some sort of evolutionary stable state. However, the individual lineages that commit to this evolutionary path have distinct gene sets and the ultimate organelle will not be the same. Just as the standard mitochondrion does not exist, a standard hydrogenosome is non-existent. A standard mitosome seems even harder to define as we barely know what the currently studied ones actually do. Analyses of the genomes of *G. intestinalis* and *E. histolytica* have not revealed any additional information not already known with respect to their mitosomes. Full proteomic analysis of their mitosomes seems the way forward.

So, despite the fields initial vantage point, all eukaryotes do have mitochondria, be it not the standard text book ones. The discovery of hydrogenosomes and mitosomes has shown to be enormously important in understanding global eukaryotic cellular evolution and they will undoubtedly continue to serve this role for some years to come.

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