12. Mitochondrion-related organelles in free-living protists

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ABSTRACT

Mitochondrion-related organelles (MROs) are a set of functionally diverse organelles that have independently evolved from mitochondria in eukaryotes that live in low-oxygen conditions. These organelles are functionally diverse, possessing a range of ancestrally mitochondrial or horizontally-acquired biochemical pathways. Early studies of MROs focused mainly on parasitic organisms; however, the past decade has seen a growing body of work on the MROs of free-living eukaryotes based on comparative genomics, making it possible to tease apart adaptations to low-oxygen conditions from adaptations to parasitism. Here, we review current knowledge of MROs in free-living eukaryotes.

KEYWORDS

Mitochondria, mitochondrion-related organelles, MROs, free-living protists, free-living microbial eukaryotes, fornicata, excavata

INTRODUCTION

‘Mitochondrion-related organelles’ (MROs) is a term that encompasses the functionally diverse set of organelles that are derived from canonical mitochondria, and which are found in various distantly-related eukaryotes that are capable of surviving under low-oxygen conditions. Five classes of MROs have been proposed according to their role in ATP generation (Müller et al. 2012): 1) **aerobic mitochondria**, which produce ATP aerobically using the electron transport chain, with oxygen as the terminal electron acceptor; 2) **anaerobically functioning mitochondria**, which use alternative electron acceptors such as fumarate or succinate under low-oxygen conditions; 3) **hydrogen-producing mitochondria**, which possess both an electron transport chain and a [FeFe]-hydrogenase that, in combination with other enzymes, allows them to produce ATP under anaerobic conditions; 4) **hydrogenosomes**, which have lost the capacity for oxidative phosphorylation and instead couple ATP biosynthesis to hydrogen production using an [FeFe]-hydrogenase; and 5) **mitosomes**, which lack any role in ATP generation. Recent studies have blurred the distinctions between these categories, instead showing that the functional diversity of MROs falls along a continuum (Roger et al. 2017; Gawryluk et al. 2016).

Mitochondrion-related organelles were first described in parabasalid parasites (Lindmark and Müller 1973) (See **Chapter 1**), and most of the early work on MROs was carried out in a variety of other parasites or commensals ((Yarlett et al. 1981; Yarlett et al. 1986; Mai et al. 1999; Tovar et al. 1999); reviewed in (Santos et al. 2018)), including *Trichomonas vaginalis* (Lindmark et al. 1975) and *Giardia lamblia* (Tovar et al. 2003). As a result, uncertainty surrounded the adaptations that they displayed: these might have been primarily adaptations to a low-oxygen environment, but some might equally be attributed to a parasitic lifestyle. In the past ten years, work focusing on MROs in a range of different free-living eukaryotes has broadened our understanding of these organelles. However, almost all of these organisms exist in culture with dense and diverse populations of bacteria, which complicates and limits possible experiments; as a result, the majority of these organelles have so far only been studied using *in-silico* predictions of biochemical functions based on transcriptomic data. Therefore, any conclusions about the functions of the MROs of these organisms must be drawn with this limitation in mind. The picture that these studies paint is one of functional diversity amongst the MROs of free-living eukaryotes, with commonalities to the MROs of parasites at the level of ATP generation but with a larger complement of ancestrally mitochondrial pathways and proteins.

In the following, we first describe the range of processes inferred for the MROs of the free-living representatives of the Fornicata subclade of Metamonada, the most extensively studied group of anaerobes that includes a diversity of both free-living and endobiotic species. Then we survey free-living anaerobes from across the diversity other eukaryotes, most

of which are poorly understood, often with only one or two species examined per clade.

FORNICATA

Fornicata is a diverse group of flagellated eukaryotes that includes parasitic, commensal and free-living species. Ancestrally free-living representatives of Fornicata are crucial for understanding the evolution MROs in these anaerobes: Fornicata is a sister lineage to the hydrogenosome-bearing Parabasalia, and the free-living members form the deepest branches of the lineage that includes the mostly parasitic Diplomonadida, which have either hydrogenosomes or mitosomes (Kolísko et al. 2010; Takishita et al. 2012; Yubuki et al. 2016). Several free-living members of Fornicata have been studied for the presence and function of MROs - *Carpodiemonas membranifera*, *Ergobibamus cyprinoides*, *Aduncisulcus palustris*, *Chilomastix cuspidata*, *Kipferlia bialata* and *Dysnectes brevis* (Leger et al. 2017; Tanifuji et al. 2018). Transmission electron microscopy has shown that all of these lineages contain organelles that have a similar appearance to the hydrogenosomes of *Trichomonas vaginalis* (Park et al. 2009; Kolísko et al. 2010; Park et al. 2010; Yubuki et al. 2016). Overall, the predicted mitochondrial metabolism of free-living fornicates is similar to that of *Trichomonas* hydrogenosomes; however, there are a few additional pathways that appear to be related to the free-living lifestyle.

Iron-sulfur cluster assembly

The one mitochondrial pathway that appears almost universally present across eukaryote lineages (exceptions are discussed below) is the mitochondrial Iron-Sulfur Cluster (Fe-S) assembly system (ISC). This consists of several proteins, but the two signature proteins are IscU, which provides a scaffold for newly formed Fe-S clusters, and IscS, which functions as a cysteine desulfurase and transfers sulfur to a newly formed Fe-S cluster. Fe-S clusters are crucial parts of iron-sulfur proteins, which are essential for a variety of biological processes, including transcription, translation, and mitochondrial electron transport. In addition to its role in the biosynthesis of mitochondrial Fe-S enzymes, the mitochondrial ISC system is also essential for the cell because the mitochondrial ISC system provides the yet-unknown signal molecule that transmits the iron status of the mitochondria and is crucial for proper functioning of cytosolic Fe-S synthesis (the CIA machinery) (Lill et al. 2012). This molecule is transferred from the organelle to the cytosol through the *ATP-binding cassette transporter of the mitochondria* (ATM (Lill et al. 2012)). It is hypothesized that most highly reduced type of MRO known in Fornicata, the mitosomes of *Giardia*, still exists because the essential ISC pathway is localized to them (Tovar et al. 2003), even though the ATM has not yet been identified in the *Giardia* genome. All studied Fornicata, whether free-living, commensal or parasitic, appear to possess proteins involved in the ISC system (Leger et al. 2017).

Pyruvate metabolism and energy generation

All members of Fornicata lack the following: mitochondrial genomes; all components of the ETC, with the exception of the 24 kDa and 51 kDa subunits of Complex I (CI); and all components of the TCA cycle, with the exception of succinyl-CoA synthetase (SCS), which is capable of functioning without oxygen in these organisms (see below; **Figure 12.1**). Instead, most of the free-living Fornicata appear to possess typical anaerobic pyruvate fermentation and ATP generation pathways. Aerobic pyruvate decarboxylation in typical mitochondria is carried out by the pyruvate decarboxylation complex (PDC). In contrast, the pyruvate fermentation that is widespread in MROs involves the conversion of pyruvate to acetyl-CoA by one of three enzymes: a pyruvate:ferredoxin oxidoreductase (PFO), the related enzyme pyruvate:NADP oxidoreductase (PNO), or pyruvate:formate lyase (PFL) (reviewed in (Stairs et al. 2015)). Acetyl-CoA is subsequently metabolised to either acetate (while generating ATP) or to ethanol (without ATP generation). PFO enzymes are indeed found in free-living fornicates, but their localization is unclear, and therefore the conversion of pyruvate to acetyl-CoA may be localized to either the cytosol or MROs or both (Leger et al. 2017). There are two ways by which acetyl-CoA can be converted to acetate: 1) acetate:succinyl-CoA transferase (ASCT) may convert acetyl-CoA to acetate and succinate to succinyl-CoA, a reaction that is coupled to the generation of ATP through substrate level phosphorylation catalysed by SCS (Steinbuchel and Muller 1986; Müller et al. 2012); and 2) acetyl-CoA may be directly converted to acetate by acetyl-CoA-synthetase (ACS), with concomitant generation of ATP (Sanchez and Muller 1996). SCS and ASCT are both enzymes that localize exclusively to MROs, with SCS

being part of the TCA cycle in aerobic mitochondria (Tielens et al. 2010). The ACS enzyme most likely originated through a horizontal gene transfer (HGT) event from bacteria, and is usually localized to the cytosol (Sanchez and Muller 1996; Tielens et al. 2010). In deeper-branching free-living fornicates – *C. membranifera*, *Ergobibamus*, *Aduncisulcus*, and *Kipferlia* – genes encoding mitochondrially-targeted ASCT and SCS, and cytosolic ACS, have been identified, suggesting a dual location for the conversion of acetyl-CoA to acetate in both the cytosol and in mitochondria. In contrast, only ACS has been identified in *Chilomastix* and *Dysnectes*, suggesting a metabolism highly similar to that of *Giardia*, in which ATP is generated only in the cytosol. Based on the phylogeny of ACS, this enzyme appears to have been acquired within Fornicata several times by independent HGT events (Leger et al., 2017). *Kipferlia*, *Chilomastix* and *Dysnectes* contain ACS of the same evolutionary origin as *Giardia* (Leger et al. 2017), which has been experimentally shown to generate ATP while converting acetyl-CoA to acetate (Sanchez and Muller 1996). A second species of *Carpediemonas*, *C. frisia*, has been reported to possess MROs that lack any role in pyruvate metabolism or hydrogen production (Hamann et al. 2017), although this may have been based on incomplete sequence data.

Protein import

The protein import machinery of MROs in free-living Fornicata is highly reduced to a ‘bare-bones’ version of the canonical mitochondrial import system. Most free-living Fornicata possess the key components of

each of the major complexes of the protein import machinery (Dudek et al. 2013; Leger et al. 2017): these include Tom40 and Sam50, which facilitate import across and into the outer membrane, as well as a set of Tim proteins facilitating import across and into the inner membrane (Dudek et al. 2013). Interestingly, all free-living Fornicates possess a single Tim17/22/23 pore-forming protein, like that recently discovered in *Giardia* (Pyrihova et al. 2018); meanwhile, most other eukaryotes have all three of this family of paralogs (Mokranjac and Neupert 2010). Accessory proteins for the import machinery have also been identified, including Pam18, Pam16, Tim44, and a single small Tim8/9/10/13 protein (Leger et al. 2017). TIM23 and mitochondrial HSP70, which is the molecular motor that pulls proteins across the inner membrane (Schneider et al. 1994) through the TIM23 complex. Similarly, both subunits of mitochondrial processing peptidase (MPP) are present in free-living members of Fornicata (Leger et al. 2017). This is an interesting contrast to the diplomonads, in which the alpha subunit of MPP has been secondarily lost (Šmid et al. 2008).

Glycine Cleavage System

The glycine cleavage system (GCS), a component of amino acid metabolism, is essential for the degradation of glycine. It is a set of four enzymes – T-protein, P-protein, L-protein, and H-protein – and is exclusively localized to mitochondria in aerobes (Kikuchi 1973). The predicted mitochondrial targeting signals found in many of the GCS sequences of free-living fornicates suggests that they are likewise localized to MROs (Leger et

al. 2017). The presence and absence of GCS within Fornicata shows an interesting evolutionary pattern (**Figure 12.1**): all free-living representatives most likely have a fully functioning GCS, with all four enzymes being present, suggesting that the presence of GCS is somehow essential for free-living species (though see *Trepomonas*, below). By contrast, the host-associated species appear to have lost the GCS completely or partially (Leger et al. 2017; Tanifuji et al. 2018). Concerning ‘partial losses’: some host-associated species contain the L-protein; however, this is commonly involved in other metabolic processes (Nývltová et al. 2016) and therefore its presence cannot be taken as evidence for a functioning GCS.

Hydrogen production coupled with GCS

[FeFe]-hydrogenase, its maturases (HydE, HydF and HydG) (Putz et al. 2006) and ferredoxin have been identified in all free-living fornicates (**Figure 12.1**). The phylogeny of [FeFe]-hydrogenase weakly suggests multiple independent origins within free-living Fornicata, while maturases show strong monophyly with other eukaryotic homologs (Leger et al. 2017). The 24 kDa and 51 kDa subunits of complex I (CI), which are often the only CI subunits present in MROs (Müller et al. 2012; Stairs et al. 2015; Hrdý et al. 2004; Dyall et al. 2004), have been identified in *C. membranifera*, *Ergobibamus*, *Chilomastix*, *Kipferlia*, and *Dysnectes* (Leger et al. 2017; Tanifuji et al. 2018). These subunits form an electron bifurcating trimeric complex with [FeFe]-hydrogenase to reoxidize NADH to NAD⁺ and ferredoxin, ultimately generating hydrogen (Schut and Adams 2009). The

presence of these NADH-oxidizing enzymes suggests an interesting metabolic link with the GCS, as their presence in the MROs will help to maintain the NAD⁺/NADH required for GCS function (Hampson et al. 1983). Altogether, this suggests that MROs of all primarily free-living Fornicata are capable of the following: NADH production (via the GCS), H₂ production (via [FeFe]-hydrogenase), NADH oxidation (24 kDa and 51 kDa subunits of CI), and electron transport through ferredoxin (Leger et al. 2017).

Overall characteristics and evolution of MROs in ancestrally free-living Fornicata

Recent comparative transcriptomic studies of metamonad MROs have shed light on the evolutionary trajectory of primarily free-living lineages of Fornicata. Ancestrally, Fornicata possessed an organelle similar to the hydrogenosomes of parabasalids (**Figure 12.2**), with several additional metabolic functions, but with an already highly reduced protein import system. The ancestral lineages were capable of ATP generation by converting acetyl-CoA to acetate, in both MROs via coupled SCS and ASCT enzymes and in the cytosol via the ACS enzyme; they possessed a functioning GCS; and they were capable of hydrogen production. In addition, they likely possessed additional amino acid metabolism enzymes and cardiolipin synthase.

Major changes occurred within the lineage leading to *Dysnectes* and diplomonads (**Figure 12.2**), in which the MRO lost its capacity for ATP generation, but retained a functioning GCS as well as NADH dehydrogenase

and [FeFe]-hydrogenase. This resulted in the so-far unique MRO of *Dysnectes*, which is predicted to be capable of hydrogen production, but which is unable to produce ATP (in contrast to the canonical hydrogenosomes of *Trichomonas*) (Leger et al. 2017). Further reduction of the organelle occurred in diplomonads, which are ancestrally host-associated and have completely lost the GCS. The pattern of MRO reduction continued in the lineage leading to *Giardia*, which resulted in the existence of mitosomes (Tovar et al. 2003; Jedelský et al. 2011). In contrast, *Spironucleus salmonicida*, another parasitic diplomonad, regained ATP generation capacity in its MRO by localizing the ACS enzyme, acquired through an independent HGT event, to the organelle (Jerlström-Hultqvist et al. 2013).

Trepomonas sp.

Trepomonas represents an interesting lineage within diplomonads, as it is likely a secondarily free-living species (Xu et al. 2016). Unfortunately, there are very few data available and therefore it is hard to draw strong conclusions. However, *Trepomonas* possesses cytosol-localized ACS of the same evolutionary origin as that of *Giardia*, suggesting that the MROs of both organisms share characteristic features (Leger et al. 2017). It does not possess the GCS, which is intriguing as all other free-living taxa do. It is very likely that the GCS was lost from the genome of the *Trepomonas* lineage during its evolutionary history as parasites and has never been regained. To date, it remains unclear whether GCS-like functions in glycine

decarboxylation and 5,10-methylenetetrahydrofolate production are carried out in *Trepomonas*.

OTHER TAXA

Jakobida

Stygiella incarcerata (formerly *Jakoba incarcerata* or *Andalucia incarcerata* (Pánek et al. 2015)) is a member of Jakobida, a group of free-living flagellates which together with Euglenozoa, Heterolobosea and *Tsukubamonas* forms the clade known as Discoba (Hampl et al. 2009; Yabuki et al. 2011). Most jakobids are aerobes with canonical mitochondria, yet members of the family Stygiellidae (Pánek et al. 2015) appear to have secondarily adapted to an obligatory anaerobic lifestyle, possessing uniformly-staining mitochondrion-related organelles that lack cristae (Simpson and Patterson 2001; Pánek et al. 2015).

As with the free-living fornicates, predictions of the biochemical features of these organelles are based on a transcriptomic survey from cells in culture with diverse prokaryotes (Leger et al. 2016). Based on this, *Stygiella* appears to lack all components of the ETC save the two CI subunits described above, and all components of the TCA cycle. Searches for orthologs of known jakobid mitochondrial gene transcripts, PCR screens using mitochondrial 16S rRNA gene probes, and pulsed-field gel electrophoresis failed to uncover any evidence for a mitochondrial genome (Leger et al. 2016).

Stygiella MROs possess pyruvate metabolism similar to that of *Trichomonas* hydrogenosomes. [FeFe]-hydrogenase, all three [FeFe]-hydrogenase maturases, PFO, SCS, and ASCT are present (although the transferase present in *Stygiella*, ASCT1B, is a different subfamily (Tielens et al. 2010) from the ASCT1C found in *Trichomonas*), and immunoelectron microscopy showed that [FeFe]-hydrogenase was primarily located in the MROs, suggesting that they are the site of pyruvate metabolism and ATP production. IscS, the cysteine desulfurase component of the ISC system, was similarly localized to the MROs. Overall, *Stygiella* appears to have retained a wider complement of mitochondrial amino acid metabolism genes than *Trichomonas*, including a complete branched-chain amino acid degradation pathway and cardiolipin synthase (Leger et al. 2016) (**Figure 12.1**).

Heterolobosea

Heterolobosea is a diverse group of amoebae, flagellates and amoeboflagellates that includes a large number of anaerobic species, almost all of which belong to one clade, Psalteriomonadidae (Pánek et al. 2017). ***Psalteriomonas lanterna*** and ***Sawyeria marylandensis***, both free-living members of Psalteriomonadidae, possess uniformly-staining MROs. In *Psalteriomonas*, these are elongated ('sausage- and dumb-bell-shaped' (de Graaf et al. 2009)) and form clusters; in *Sawyeria*, they are cup-shaped (Barberà et al. 2010). The transcriptome data available for both organisms were derived from Sanger-sequenced expressed sequence tag clones, and as a result are relatively sparse. Nevertheless, the transcriptomes of both

organisms encode [FeFe]-hydrogenase and PFO with mitochondrial targeting signals as well as the 51 kDa subunit of CI, suggestive of the hydrogenosomal pyruvate fermentation pathway (de Graaf et al. 2009; Barberà et al. 2010). Both also encode ISC system enzymes. The larger *Sawyeria* transcriptome additionally encodes SCS, the 24 kDa subunit of CI, elements of the branched-chain amino acid degradation pathway, proline, serine and ornithine metabolism pathways, and the GCS (Barberà et al. 2010) (**Figure 12.1**).

Stramenopila

MROs with cristae have been described in four taxa of anaerobic stramenopile: the commensal flagellate *Proteromonas lacertae* (Perez-Brocal et al. 2010), the commensalic/parasitic *Blastocystis* (Zierdt et al. 1988; Zierdt 1991), and the free-living flagellate *Rictus lutensis* (Yubuki et al. 2010) and ***Cantina marsupialis*** (Yubuki et al. 2015). Despite their very different appearance, *Proteromonas* and *Blastocystis* are closely related and likely descend from a common ancestor that was anaerobic and endobiotic (Kostka 2017). The MROs of *Blastocystis* spp. and of *Cantina* have been studied at the molecular level (Lantsman et al. 2008; Stechmann et al. 2008; Denoeud et al. 2011; Tsaousis et al. 2011; Noguchi et al. 2015; Noguchi et al. 2016; Gentekaki et al. 2017). *Blastocystis* MROs retain a mitochondrial genome and CI as well as complex II (CII) of the ETC (Lantsman et al. 2008; Stechmann et al. 2008; Denoeud et al. 2011; Gentekaki et al. 2017) (see **Chapter 11**). By contrast, *Cantina* MROs appear to lack both the genome

and all ETC complexes other than CII (Noguchi et al. 2015). The MROs of both organisms appear to possess anaerobic ATP generation enzymes – [FeFe]-hydrogenase and its associated maturases, PFO, ASCT1B, ASCT1C, and ACS. *Blastocystis* also possesses RQUA (Stairs et al. 2014), an enzyme that catalyzes the biosynthesis of rhodoquinone, which is essential for fumarate reduction by CII in some anaerobes (Van Hellemond et al. 1995; Lonjers et al. 2012; Stairs et al. 2018); however, RQUA was not identified in *Cantina* (Noguchi et al. 2015; Stairs et al. 2018). Both also possess the ISC system, a complete GCS, and the branched-chain amino acid degradation pathway (Noguchi et al. 2015; Gentekaki et al. 2017) (**Figure 12.1**). Interestingly, while both organisms possess an MRO-targeted cardiolipin synthase, the specific type of enzyme is not the same, due to differential loss in the two lineages: the *Cantina* enzyme contains two phospholipase D domains and synthesizes cardiolipin from two phosphatidylglycerol molecules, while the *Blastocystis* enzyme contains an CDP-alcohol phosphatidyltransferase domain, and synthesizes cardiolipin from one phosphatidylglycerol molecule and one cytidine diphosphate diacylglycerol molecule (Noguchi et al. 2016).

Rhizaria

As in stramenopiles, MROs have only been studied in two rhizarians that are not closely related to each other; the first, *Mikrocytos mackini*, is an obligate parasite with highly reduced MROs that have only retained the ISC system and elements of the mitochondrial protein import machinery. In

stark contrast, the organelle of the free-living *Brevimastigomonas motovehiculus* appears to represent an early step in mitochondrial adaptation to low-oxygen conditions. *Brevimastigomonas* MROs retain a partial electron transport chain consisting of CI, CII and Complex V (CV); at least seven subunits of these complexes are encoded in the mitochondrial genome, as are the highly divergent and possibly non-functional *cob* and *cox1* genes. Also retained are PDH and at least seven, and possibly all, enzymes of the TCA cycle. The TCA cycle enzymes malate dehydrogenase, fumarase and succinate dehydrogenase (CII) may also be capable of acting reductively under anaerobic conditions, as they are predicted to do in *Blastocystis*. Supporting this possibility, the *Brevimastigomonas* genome also encodes RQUA (Gawryluk et al. 2016). *Brevimastigomonas* MROs are also predicted to contain [FeFe]-hydrogenase and its maturases, PFO, PNO, ASCT1B, and SCS (Gawryluk et al. 2016). Consistent with this co-occurrence of mitochondrial and hydrogenosomal-like elements of pyruvate metabolism, *Brevimastigomonas* grows well under low-oxygen conditions, but also tolerates aerobic conditions (Gawryluk et al. 2016). In addition, the ISC system, branched-chain amino acid degradation pathway, cardiolipin biosynthesis pathway, and GCS were identified as putatively present in the MROs (**Figure 12.1**).

Alveolata

The majority of MROs in alveolates have been described in ciliates that are commensals of mammals (Yarlett et al. 1981; Yarlett et al. 1983; Yarlett et

al. 1984; Paul et al. 1990), however hydrogenosomes have also been described in free-living members of at least six orders of **ciliates** (reviewed in (Fenchel and Finlay 1991)). These hydrogenosomes contain cristae, and are generally associated with endosymbiotic methanogenic archaea, which consume the hydrogen that is produced as a metabolic byproduct ((van Bruggen et al. 1983); reviewed in (Fenchel and Finlay 1991; Hackstein 2010)). Apart from the presence of [FeFe]-hydrogenase, little is known about the metabolic potential of the MROs of free-living ciliates (but see also **Chapter 5**).

MROs have also been described in two free-living chromerids, ***Chromera velia*** and ***Vitrella brassicaformis***. Both are photosynthetic organisms that are found associated with corals; both belong to the outgroup to the parasitic apicomplexan clade (which includes *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*). The MROs of both organisms are capable of oxidative phosphorylation, but lack CI; *Chromera* additionally lacks complex III (CIII) (Flegontov et al. 2015; Oborník and Lukes 2015). Instead, their ETCs include an alternative NADH dehydrogenase and an alternative oxidase, features that are shared with apicomplexan parasites. The ETC is continuous in *Vitrella*, but is uniquely split in *Chromera*, with Complex IV (CIV) and CV acting independently of the alternate NADH dehydrogenase, the alternative oxidase and CII (Flegontov et al. 2015). The genomes of both organisms encode MRO-targeted [FeFe]-hydrogenase and its maturases, PNO, and SCS (Flegontov et al. 2015; Oborník and Lukes 2015); both genomes also encode ACS, but only that of *Vitrella* possesses a mitochondrial targeting peptide, while *Chromera* also possesses mitochondrially-targeted ASCT1B

(Flegontov et al. 2015; Oborník and Lukes 2015; Stairs et al. 2015) (**Figure 12.1**).

Breviatea

Pygsuia biforma is a member of Breviatea, a group of free-living flagellates that, together with apusomonads, are the closest relatives to Opisthokonta (Brown et al. 2013). Superficially similar MROs are present in *Pygsuia*, *Breviatea anathema* and *Subulatomonas tetraspora*, although only partial transcriptome data are available for the latter two organisms (Stairs et al. 2014; Grant et al. 2012). The single MRO of *Pygsuia* is uniformly-staining and elongated (Brown et al. 2013; Stairs et al. 2014). It appears to lack a mitochondrial genome, and most components of the electron transport chain: only CII and the 24 kDa and 51 kDa subunits of CI are encoded in its transcriptome (Stairs et al. 2014). Of the TCA cycle, only fumarase and succinate dehydrogenase are present. Consistent with these enzymes acting reductively, *Pygsuia* also possesses RQUA for rhodoquinone (RQ) biosynthesis, like *Blastocystis* and *Brevimastigomonas*; recently, RQ was detected in *Pygsuia* lipid extracts (Stairs et al. 2018 REF). [FeFe]-hydrogenase and its maturases, PFO, PNO, ASCT1B, ASCT1C, and SCS are also predicted to be targeted to the MROs. The GCS, branched-chain amino acid degradation pathway, and cardiolipin biosynthesis pathway are also present (Stairs et al. 2014) (**Figure 12.1**).

Interestingly, *Pygсуia* lacks all components of the ISC system. Instead, it possesses two genes encoding an enzyme, SufCB, that appears to be a fusion of the SufC and SufB components of the sulfur mobilization (SUF) system of iron sulfur cluster biosynthesis (Stairs et al. 2014). SufCB is also encoded in the transcriptomes of *Blastocystis* (Tsaousis et al. 2012) and *Stygiella* (Leger et al. 2016), where it is likely located in the cytosol; in *Pygсуia*, however, one of the SufCB proteins possesses a mitochondrial targeting signal and has been localized to the MRO (Stairs et al. 2014). Phylogenies suggest that SufC and SufB were acquired from an archaeon, possibly as part of an operon, and became fused and then transferred in a handful of eukaryotes. An independent transfer event, this time from bacteria, appears to have led to the acquisition of cytosolic Suf genes in *Preaxostyla* ((Karnkowska et al. 2016; Vacek et al. 2018); see **Chapter 13**).

Genome, transcriptome and proteome data have also been generated for a second free-living breviate, *Lenisia limosa* (Hamann et al. 2016). Unlike *Pygсуia*, *Lenisia* cells contain multiple, cristate MROs. Much like *Pygсуia* and other protists, *Lenisia* encodes MRO-targeted PFO, [FeFe]-hydrogenase and its associated maturases, the 24 kDa and 51 kDa subunits of CI, SCS, and ASCT. However, this protist uses the typical mitochondrial ISC system for Fe-S cluster biosynthesis, rather than the SUF system discussed above (Stairs, *pers. comm.*). Furthermore, no evidence of RQUA was found in the genome (Stairs et al. 2018). Like anaerobic ciliates (see above), *Lenisia* forms a symbiotic association with prokaryotic symbionts – in this case, epibionts of the bacterial genus *Arcobacter* – which consume the hydrogen gas produced by its MROs (Hamann et al. 2016).

Amoebozoa

The first free-living organism shown to contain a MRO was the amoebozoan ***Mastigamoeba balamuthi*** (Gill et al. 2007), a close relative of the human pathogen *Entamoeba histolytica*. Both of these archaeamoebae have replaced the ISC system for the biosynthesis of Fe-S clusters with a horizontally acquired nitrogen fixation (NIF) system of iron sulfur cluster assembly (Ali et al. 2004; Nývltová et al. 2013). While the pathway is exclusively cytoplasmic in *Entamoeba*, *Mastigamoeba* encodes two copies of each component, targeted to the cytoplasm and MRO respectively (Nývltová et al. 2013). Extensive biochemical and molecular biological analyses have revealed that the *Mastigamoeba* MROs oxidize pyruvate and generate hydrogen via the so-called ‘extended glycolysis’ pathway (PFO, [FeFe]-hydrogenase and ACS), malate dehydrogenase (MDH) and an NAD⁺-dependent D-lactate dehydrogenase (D-LDH) (Nývltová et al. 2015) (**Figure 12.1**). Here, the authors propose that malate is converted to oxaloacetate which in turn is used by an unidentified dicarboxylic acid/lactate antiporter to import D-lactate into the MROs. This D-lactate serves as the substrate for D-LDH to generate the pyruvate necessary for extended glycolysis. Therefore, unlike other MROs, the *Mastigamoeba* MROs derive pyruvate from lactate, and not oxaloacetate or via the pyruvate transporter (Nývltová et al. 2015). Like *Pygmaia bifurcata*, *Mastigamoeba* encodes MRO-targeted quinone-dependent metabolism (e.g., CII, RQUA, electron transferring flavoprotein system) despite lacking the remainder of the respiratory chain (i.e., no components of CI, CIII, CIV, or CV have been identified); however, the exact functions of these proteins have not been elucidated. Both *Entamoeba* and *Mastigamoeba* have MRO-localized sulfate activation (Mi-ichi et al. 2009; Nývltová

et al. 2015). In *Entamoeba*, this pathway is essential for the generation of sulfated lipids necessary for cell proliferation and encystation (Mi-ichi et al. 2009; Mi-ichi et al. 2011; Mi-ichi et al. 2015); however, its role in *Mastigamoeba balamuthi* MROs remains to be investigated.

CONCLUSIONS

Both the categories of ‘mitochondrion-related organelles’ and ‘free-living eukaryotes’ are artificial and lie on a spectrum. Several eukaryotes possess mitochondrially-targeted fermentation enzymes in addition to a complete mitochondrial electron transport chain (ETC) and tricarboxylic acid (TCA) cycle, resulting in mitochondria that may be capable of functioning anaerobically (Clarke et al. 2013; Fritz-Laylin et al. 2010; Hug et al. 2010; Leger et al. 2013; Ebenezer et al. 2017). Some of these are opportunistic pathogens that inhabit a variety of freshwater, marine or soil environments in addition to host tissues (Clarke et al. 2013; Leger et al. 2013); organisms with this type of lifestyle may reveal a wider diversity of mitochondrial functions than previously described in most MROs of parasites or commensals. Other eukaryotes have lost CI of the ETC, while retaining the capacity for oxidative phosphorylation and other mitochondrial functions (Gabaldón et al. 2005; Maclean et al. 2018; Senkler et al. 2018).

Microbial eukaryotes, particularly free-living ones, remain relatively poorly studied. Given the recent advent of low-cost sequencing technologies, and

the discoveries of new major groups of eukaryotes that these have enabled, it is likely that much progress is still to be made in our understanding of eukaryote diversity and MROs. Nevertheless, the work summarized above has greatly expanded our understanding of these organelles and has enabled some themes to emerge. MROs in free-living eukaryotes may still retain the capacity for aerobic ATP generation or may have lost it entirely; most of MROs of free-living eukaryotes described to date are capable of pyruvate fermentation under low-oxygen conditions using any of several characteristic enzymes, although in some cases the capacity for ATP production may be absent under any conditions. Many have retained specific mitochondrial pathways not commonly found in the MROs of parasites or commensals: the glycine cleavage system, the branched-chain amino acid degradation pathway, and cardiolipin biosynthesis enzymes. However, evidence for other key mitochondrial enzymes, such as those involved in heme biosynthesis or the urea cycle, has not been described in any of these MROs. Comparative genomics based on high-quality, high-coverage sequence data has been instrumental in extending our predictions of the biology of MROs beyond a small number of key enzymes. Proteomic and biochemical analyses that focus on mitochondria and MROs in a wider range of eukaryotes may uncover new functions in these organelles, not previously identified in model eukaryotes. The interactions between MROs and the prokaryotic endosymbionts of their hosts, as in ciliates or *Leishmania*, are a particularly exciting avenue of future study.

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Figure 12.1. Selected proteins predicted to localize to mitochondria and related organelles in free-living eukaryotes and selected parasites and commensals.

Green, opisthokonts; yellow, amoebozoa; red, stramenopiles, alveolates and rhizaria (SAR clade); purple, discobids; blue, fornicates. Lighter colours indicate the taxa discussed in detail in this chapter. Squares indicate

presence in the genome or transcriptome and predicted localization to the organelle; small squares indicate that only a subset of the expected proteins or subunits are present and predicted to be present in the organelle.

Blue circles, free-living; purple circles, commensals; pink circles, parasites.

*, alternative NADH dehydrogenases. Ψ , pseudogenised. ?, insufficient literature. Grey, transcriptome only.

AOX, alternative oxidase; PDH, pyruvate dehydrogenase; PFO, pyruvate:ferredoxin oxidoreductase; PNO, pyruvate:NADP⁺ oxidoreductase; PFL, pyruvate formate lyase; RQUA, rhodoquinone biosynthesis methyltransferase-like protein RQUA; ASCT, acetate:succinyl-CoA transferase; ACS, acetyl-CoA synthetase; Hyd. matur., [FeFe]-hydrogenase maturases; ISC, ISC iron sulfur cluster assembly proteins; SUF, sulfur mobilization system of iron sulfur cluster assembly proteins; NIF, nitrogen fixation system of iron sulfur cluster assembly proteins; GCS+SHMT, glycine cleavage system and serine hydroxymethyltransferase; Mito.Br.ch.a.a.a.t., mitochondrial branched-chain amino acid aminotransferase; CLS_cap, cytidine diphosphate-alcohol phosphatidyltransferase domain-containing cardiolipin synthase; CLS_pld, phospholipase D domain-containing cardiolipin synthase; MPP, mitochondrial processing peptidase, SAM50, sorting and assembly machinery component 50; TOM40, translocase of the outer membrane component 40; TIM17/22/23, translocase of the inner membrane components 17, 22 and 23; PAM18, presequence translocase-associated motor component 18. Data from (Henriquez et al. 2005; Doležal et al. 2006; de Graaf et al. 2009; Barberà et al. 2010; Doležal et al. 2010; Fritz-Laylin et al. 2010; de Graaf et al. 2011; Jedelský et al. 2011; Opperdoes et al. 2011;

Stairs et al. 2011; Schneider et al. 2011; Tian et al. 2012; Burki et al. 2013; Jerlström-Hultqvist et al. 2013; Leger et al. 2013; Zubáková et al. 2013; Maguire and Richards 2014; Makiuchi and Nozaki 2014; Stairs et al. 2014; Flegontov et al. 2015; Noguchi et al. 2015; Nývltová et al. 2015; Oborník and Lukes 2015; Stairs et al. 2015; Gawryluk et al. 2016; Karnkowska et al. 2016; Leger et al. 2016; Noguchi et al. 2016; Gentekaki et al. 2017; Leger et al. 2017; Santos et al. 2018; Stairs et al. 2018)

Figure 12.2. Phylogenetic tree of Fornicata and the reconstruction of the putative metabolism of their MROs (adapted from (Leger et al. 2017). Capacity for ATP synthesis in the organelle is lost on the branch leading to *Dysnectes* and diplomonads and is subsequently regained in *Spironucleus salmonicida*. The organelle of *Dysnectes* represents a rare organelle capable of hydrogen production but incapable of ATP synthesis.

Free-living taxa are shown in black; parasitic or commensal taxa are shown in red.