The mitosome of the anaerobic parasitic protist *Entamoeba histolytica:* A peculiar and minimalist mitochondrion-related organelle

Herbert J. Santos | **Tomoyoshi Nozaki**

Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Correspondence
Tomoyoshi Nozaki, Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
Email: nozaki@m.u-tokyo.ac.jp

Abstract

The simplest class of mitochondrion-related organelles (MROs) is the mitosome, an organelle present in a few anaerobic protozoan parasites such as *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium parvum*. *E. histolytica* causes amoebiasis in humans, deemed as one of the important, yet neglected tropical infections in the world. Much of the enigma of the *E. histolytica* mitosome circles around the obvious lack of a majority of known mitochondrial components and functions exhibited in other organisms. The identification of enzymes responsible for sulfate activation (AS, IPP, and APSK) and a number of lineage-specific proteins such as the outer membrane beta-barrel protein (MBOMP30), and transmembrane domain-containing proteins that bind to various organellar proteins (ETMP1, ETMP30, EHI_170120, and EHI_099350) showcased the remarkable divergence of this organelle compared to the other MROs of anaerobic protozoa. Here, we summarize the findings regarding the biology of the mitosomes in *E. histolytica*, from their discovery up to the present understanding of its roles and interactions. We also include current advances and future perspectives on the biology, biochemistry, and evolution of the mitosomes of *E. histolytica*.

KEYWORDS
Entamoeba histolytica, membrane contact site, mitochondrion-related organelle, mitosome, mitosome fission, protein import, sulfate activation

MITOCHONDRIA are endosymbiotic organelles of α-proteobacterial origin, whose role includes ATP synthesis, iron–sulfur (Fe–S) cluster formation, tricarboxylic acid (TCA) cycle, heme biosynthesis, lipid and amino acid metabolism, calcium homeostasis, and programmed cell death (Lill & Kispal, 2000; Susin *et al.*, 1999). Organisms that inhabit oxygen-deficient environments have adapted to a life that does not require the roles and components of the canonical aerobic mitochondrion, with only one organism, the oxymonad *Monocercomonoides exilis*, having completely lost mitochondrion (Karnkowska *et al.*, 2016). In some eukaryotes whose respiratory and metabolic pathways are independent of molecular oxygen, the roles and components of the mitochondrion were differentially diminished and diversified. *Entamoeba histolytica* is one of several anaerobic protozoan parasites that lack canonical mitochondria. Instead, it has a remarkably divergent and

reduced mitochondrion-related organelle (MRO) called mitosome. Mitosomes of *E. histolytica*, and other anaerobic protozoan parasites such as *Giardia intestinalis*, and *Cryptosporidium parvum* are currently considered to be one of the most highly reduced classes of MROs, as evidenced by the absence of genomic DNA, cristae structure, electron transfer chain, and their inability to generate ATP (Makiuchi & Nozaki, 2014; Santos *et al.*, 2018; Stairs *et al.*, 2015). Interestingly, the mitosomes of these organisms also demonstrate divergent functionalities and features. In this review, we highlight the mitosomes of *E. histolytica*, the parasite that causes amoebiasis in humans.

DISCOVERY OF *ENTAMOEBA HISTOLYTICA* MITOSOMES

Like other "early branching" eukaryotes, *E. histolytica* was originally thought as amitochondriate. Ultrastructural analysis of *E. histolytica* trophozoites revealed the apparent lack of mitochondria, rough endoplasmic reticulum, Golgi apparatus, and microtubular cytoskeleton (Martinez-Palomo, 1986). The absence of enzymes involved in aerobic respiratory and glutathione metabolism pathways (Fahey *et al.*, 1984) further reinforced this notion. However, phylogenetic analysis based on ribosomal RNA sequences suggested that *E. histolytica* diverged much later than other eukaryotes that possess the aforementioned organelles, implying that this parasite may have lost its mitochondrion secondarily (Sogin *et al.*, 1989; Tovar *et al.*, 1999). This hypothesis was supported by the detection of nuclear genome-encoded orthologs of two mitochondrial proteins chaperonin 60 (Cpn60) and pyridine nucleotide transhydrogenase (PNT), which suggested that this parasite retained a previously unknown and reduced version of the mitochondrion (Clark & Roger, 1995). The discovery of this MRO called mitosome (also named Crypton in 1999 due to its cryptic nature and function) in *E. histolytica* was almost simultaneously reported upon the identification and expression of the Cpn60 ortholog that is targeted to this compartment (Mai *et al.*, 1999; Tovar *et al.*, 1999). Later on, characterization of the other protein, *E. histolytica* PNT, determined that its localization is on vesicular/vacuolar membranes, rather than on mitosomes (Yousuf *et al.*, 2010), despite PNT containing a predicted mitochondrial targeting presequence. Soon after, other genes encoding mitochondrial proteins were reported to be present in the *E. histolytica* genome such as the mitochondrial-type Hsp70 (Arisue *et al.*, 2002) and Cpn10 (van der Giezen *et al.*, 2005).

One point of controversy about the *E. histolytica* mitosome was the contradicting findings on the presence or absence of organellar DNA in this MRO. Cpn60-containing mitosomes do not contain DNA based on Hoechst and propidium iodide staining (Mai *et al.*, 1999;

Tovar *et al.*, 1999). However, DNA signal in cryptons/mitosomes stained with anti-Hsp60 (synonymous with Cpn60) antibody was detected using SYTOX green, acridine orange, propidium iodide, and a mouse monoclonal antibody to double-stranded DNA, respectively (Ghosh *et al.*, 2000). Nevertheless, the absence of organellar DNA in Cpn60-containing mitosomes has been reaffirmed by confocal microscopy and in situ nick translation coupled to immunofluorescence microscopy in fixed cells and in partially purified organellar fractions (Léon-Avila & Tovar, 2004). Another controversy is the absence of iron–sulfur (Fe–S) cluster biosynthesis machinery in *E. histolytica* mitosomes, which is one of the common features and functions shared among mitochondria and MROs. *E. histolytica* and other amoebozoans such as the free-living *Mastigamoeba balamuthi* (Nývltová *et al.*, 2015) and the archamoeba *Pelomyxa schiedti* (Záhonová *et al.*, 2022) contain orthologs of the nitrogen fixation (NIF) enzyme system for Fe–S cluster formation (Nývltová *et al.*, 2013). In *M. balamuthi*, the NifS and NifU subunits were demonstrated to localize in both cytosol and its MRO, the hydrogenosome, (Nývltová *et al.*, 2013). In the case of *P. schiedti*, NifU (two out of three homologs), but not NifS, was demonstrated to be targeted to yeast mitochondria by heterologous localization experiments (Záhonová *et al.*, 2022). The localization of *Entamoeba* NifS and NifU remains unclarified as results of imaging and biochemical analyses (Maralikova *et al.*, 2010) were not corroborated by organellar proteomic analysis (Mi-ichi *et al.*, 2009).

ENTAMOEBA HISTOLYTICA MITOSOMES AND SULFATE ACTIVATION

Clues on the role of the mitosome in *E. histolytica* emerged from a proteomic survey and biochemical analyses conducted in 2009 (Mi-ichi *et al.*, 2009). It was reported that *E. histolytica* mitosomes produce 3′-phosphoadenosine-5′-phosphosulfate (PAPS) from cytosolic sulfate, resulting from the compartmentalized activity of the following three enzymes: ATP sulfurylase (AS), adenosine-5′-phosphosulfokinase (APSK), and inorganic pyrophosphatase (Mi-ichi *et al.*, 2009). Such finding established sulfate activation as potentially the sole metabolic process that exists in this organelle, although sulfate activation conventionally occurs in the cytosol/plastids of eukaryotic cells (Patron *et al.*, 2008). However, it had also been shown that a unicellular flagellate *Euglena gracilis* performs sulfate activation in its aerobic mitochondrion (Saidha *et al.*, 1985, 1988). Interestingly, other amoebozoans such as *M. balamuthi* (Nývltová *et al.*, 2015) and *P. schiedti* (Záhonová *et al.*, 2022) were also reported to carry out sulfate activation pathway in their respective MROs. However, the genomes of other parasites that similarly thrive

in anoxic/hypoxic environments and possess MROs, namely *G. intestinalis* and *C. parvum* (mitosome) as well as *Trichomonas vaginalis* (hydrogenosome), completely lack the essential genes to carry out sulfate activation (Mi-ichi *et al*., 2009). Phylogenetic analyses indicate that AS of both *E. histolytica* and *M. balamuthi* branched as a sister clade to α-proteobacterial AS with high support (Mi-ichi *et al*., 2009; Nývltová *et al*., 2015) suggesting lateral gene transfer (LGT) as the means of acquisition (Mi-ichi *et al*., 2009; Nývltová *et al*., 2015). *Eh*IPP and one of three IPP paralogs in *M. balamuthi*, *Mb*IPP-1 form a distinct eukaryotic clade with other IPP homologs from Amoebozoa and other eukaryotes, suggesting that this IPP is ancestral and common to all eukaryotes, whereas *Mb*IPP-2 and *Mb*IPP-3 were grouped with bacterial sequences suggesting that they are independently acquired by LGT from an unidentified prokaryote (Nývltová *et al*., 2015). For APSK, the resolution of phylogenetic analysis is poor. Results showed that all four amoebozoan lineages including acanthamoeba and dictyostelids did not cluster together but formed a well-supported clade with other Eukaryota as well as bacteria except α-proteobacterial APSK. This suggests that APSK may exist in the common amoebozoan ancestor, but its exact evolutionary history remains ambiguous (Nývltová *et al*., 2015). Overall, these findings suggest that the acquisition of sulfate activation genes via LGT is unlikely ubiquitous among Amoebozoa but selectively occurred to some extent in both the *Entamoeba* and *Mastigamoeba* genera (Mi-ichi *et al*., 2011).

The sulfate activation pathway begins with the activation of inorganic sulfate to adenosine-5′-phosphosulfate (APS), via the reaction catalyzed by AS (Figure 1). The resulting pyrophosphate produced simultaneously in this reaction is degraded by IPP into phosphates. Then, APS is phosphorylated by APSK to form 3′-phosphoadenosine-5′-phosphosulfate (PAPS), which is subsequently released to the cytosol. Sulfate activation is essential to *Entamoeba* proliferation, as silencing of genes encoding AS, APSK, and IPP led to decreased growth rate as well as diminished sulfolipid production (Mi-ichi *et al*., 2011). The various sulfotransferases in the cytosol catalyze the transfer of the sulfuryl moiety of PAPS to corresponding acceptors to form sulfurylated metabolites, which include mucopolysaccharides, sulfoproteins, and sulfolipids (Mi-ichi *et al*., 2009). On the other hand, both APS and PAPS may undergo reduction and assimilation into sulfur-containing biomolecules such as cysteine, methionine, Fe-S, thiamine, and coenzyme A (Mi-ichi & Yoshida, 2019). However, the potential APS reductase, PAPS reductase, and sulfite reductase appear to be missing in the genomes of *Entamoeba* and *Mastigamoeba* (Kawano-Sugaya *et al*., 2020; Mi-ichi & Yoshida, 2019; Žárský *et al*., 2021), respectively. In sulfate-reducing bacteria, activated sulfate is reduced to sulfide, which is utilized as a terminal electron acceptor via anaerobic respiration (Mi-ichi *et al*., 2011).

One of the many resultant cytosolic sulfolipids that are synthesized due to the sulfate activation pathway in mitosomes is cholesteryl sulfate, formed by a reaction catalyzed by sulfotransferase-6 (SULT6) (Mi-ichi *et al*., 2015a, Mi-Ichi *et al*., 2015b). Cholesteryl sulfate was shown to be linked to increased cyst formation when supplemented to a culture medium in *Entamoeba invadens*. Such a feature seems to be unique to *Entamoeba*, since it was shown that *Mastigamoeba*, though capable of sulfate activation, does not have genes for SULTs that could process PAPS into sulfolipids (Mi-ichi *et al*., 2015a, Mi-Ichi *et al*., 2015b; Žárský *et al*., 2021). The role of PAPS in *M. balamuthi*, which lacks SULTs, remains elusive. Other sulfolipids linked to trophozoite proliferation are similarly synthesized via this pathway including fatty alcohol disulfates, formed by the activity of SULT1, SULT3-5, and SULT7-9, and the structurally undetermined SL-II, SL-III, and SL-IV produced by SULT1-5, and SULT7-9 (Mi-ichi *et al*., 2017; Mi-ichi & Yoshida, 2019). SL-VIII is synthesized by SULT10, but its structure and role in the parasite are still unknown (Mi-ichi *et al*., 2017; Mi-ichi & Yoshida, 2019). Finally, *E. histolytica* possesses five sulfatases (SFs) that were demonstrated to degrade SL-II, SL-III, and SL-IV (Mi-ichi *et al*., 2017; Mi-ichi & Yoshida, 2019). Like SULTs, *Mastigamoeba* lacks SFs in its genome, suggesting that LGT of this gene family uniquely occurred in *Entamoeba* (Mi-ichi *et al*., 2015a, Mi-Ichi *et al*., 2015b, Mi-ichi *et al*., 2017; Nývltová *et al*., 2015). Taken together, the mitosome and its compartmentalized sulfate activation, and the resultant metabolism of sulfolipids in the cytosol, influence not only proliferation but also, and equally important, the parasitic and pathogenic nature of *E. histolytica* (Mi-ichi *et al*., 2017; Mi-ichi & Yoshida, 2019; Santos *et al*., 2018).

MITOSOMAL PROTEIN AND METABOLITE TRANSPORT MACHINERIES

The double-membrane barrier of the mitochondria and MROs is lined with various proteins that allow the transport of substances between the cytosol and the organelle. The outer membrane possesses channel proteins, acting as a nonselective passageway of solutes to and from the organelle. On the other hand, the inner membrane is practically impermeable to polar and large molecules, with the presence of a number of selective channels that enable a regulated transit of metabolites. These membrane proteins are assisted by accessory carriers and chaperones in the cytosol, intermembrane space, and matrix. To carry out sulfate activation, mitosomes require mechanisms for transport and translocation across their double membrane barrier. In *E. histolytica*, homologs of various members of transport machineries are either gone or substituted by noncanonical, genus-specific proteins. What is left are basic components that highlight
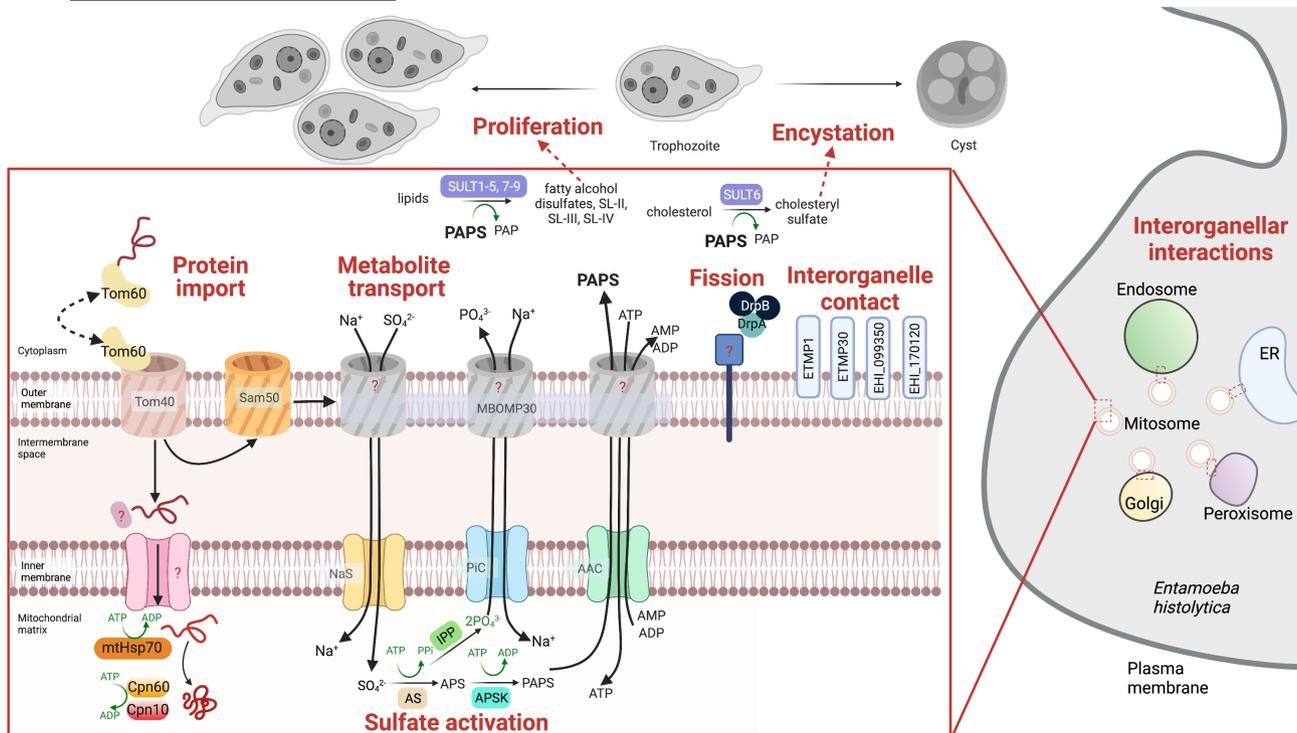
FIGURE 1 Mechanisms and components are currently known in the mitosome of *Entamoeba histolytica*. Protein import begins with the binding of mitosome-targeted proteins to the cytosolic receptor of the translocase of the outer membrane (Tom), Tom60, which docks to the Tom40 channel. Proteins pass through Tom40, to the intermembrane space where unknown proteins sort them for assembly to the outer membrane via Sam50 (for beta-barrel proteins) or transmit them to the also unidentified translocase of the inner membrane for delivery to the matrix. Soluble proteins are folded via heat-shock protein (mtHsp70) and chaperonins (Cpn60 and Cpn10) in the matrix. Metabolite transport in the outer membrane likely occurs through the novel beta-barrel protein MBOMP30, or the Tom40. Three inner membrane channels are identified, namely the sodium sulfate transporter (NaS), phosphate carrier (PiC), and ATP-ADP carrier (AAC). In the matrix, three enzymes perform activation of sulfate by three enzyme-catalyzed reactions. ATP sulfurylase (AS) catalyzes the formation of adenosine-5′-phosphosulfate (APS) using ATP and inorganic sulfate. Inorganic pyrophosphatase (IPP) degrades pyrophosphates into phosphates. APS kinase (APSK) phosphorylates APS to form 3′-phosphoadenosine-5′-phosphosulfate (PAPS). The product PAPS is exported to the cytosol, reacts with cholesterol via sulfotransferase 6 (SULT6) to produce cholesteryl sulfate, which is secreted outside the cell, and is known to induce trophozoite to cyst formation (encystation). Other secreted sulfolipids (SL-II, SL-III, SL-IV, and SL-V) synthesized in the cytosol by SULT1-5 and SULT7-9, were demonstrated to be essential to trophozoite proliferation. Mitosome fission occurs via the heterooligomer complex of DrpA and DrpB. The binding of the cytosolic Drps to the outer membrane of mitosomes is still unclear as the Drp receptor is not yet identified. Several *Entamoeba*-specific transmembrane domain-containing proteins (ETMP1, ETMP30, EHI_099350, and EHI_170120) are likely involved in establishing membrane contact sites with other organelles specifically endosomes, the Golgi apparatus, ER, and peroxisomes, respectively. Created with BioRender.com

their essentiality in maintaining the operations of the organelle. Presently, a few membrane proteins have been identified in this organelle. On the outer membrane, there are three beta-barrel proteins: the respective core channels of the translocase of the outer membrane (TOM) complex, Tom40, and the sorting and assembly machinery (SAM) complex, Sam50 (Dolezal et al., 2010), and the lineage-specific mitosomal beta-barrel outer membrane protein of 30 kDa (MBOMP30) (Santos et al., 2015) (Figure 1). Both Tom40 and MBOMP30 are essential to proliferation, as indicated by growth defects or the total inability of parasites to survive transcriptional silencing of respective genes (Makiuchi et al., 2013; Santos et al., 2015). They also comprise the central gateway that enables protein trafficking and integration, as well as metabolite import/export. Tom40 is the core pore component of the TOM complex required for the import of mitochondrial precursor proteins into the mitochondria

(Baker et al., 1990; Hill et al., 1998). The protein import machinery lacks most of the members of the TOM and SAM complexes, as well as that of the translocase of the inner membrane (TIM) complex. Interestingly, a component so far only identified in the *E. histolytica* (Makiuchi et al., 2013) and recently in the *P. schiedti* (Záhonová et al., 2022) TOM complex, Tom60 was discovered. *E. histolytica* Tom60 is an essential protein, which contains tetratricopeptide repeat and acts as a cytoplasmic carrier of both soluble and membrane-bound mitosomal proteins (Makiuchi et al., 2013). Although MBOMP30, a unique beta-barrel present only in the genus *Entamoeba* (Santos et al., 2015), has not been functionally characterized, it is interesting to note that *Entamoeba* mitosomes lack a putative homolog of the voltage-dependent anion channel (VDAC). VDACs primarily serve as a nonspecific diffusion pore for small molecules entering or leaving the mitochondria (Colombini, 2004). The absence

of VDAC in *Entamoeba* may indicate that MBOMP30 could act as a substrate channel for the transport of ions (e.g. phosphate, sulfate, and sodium), nucleotides (AMP, ADP, and ATP), and PAPS. Metabolite transport in *E. histolytica* mitosomes may also be carried out by Tom40, as it had been shown that yeast Tom40 transports superoxide anion and NADH, in the absence of functional VDAC (Budzinska *et al*., 2009; Kmita & Budzinska, 2000). Even though these substrates transported by yeast Tom40 are unrelated to the sulfate activation pathway in *E. histolytica* mitosomes, it is plausible that the *E. histolytica* Tom40 may also be utilized as an alternative channel for metabolites in the absence of a functional VDAC.

The transport of essential metabolic substrates across the inner membrane occurs via mitochondrial carrier family (MCF) proteins which are important for establishing cellular homeostasis by maintaining redox and phosphate potentials. So far, only three inner membrane channel proteins have been identified and confirmed to be localized to *E. histolytica* mitosomes: the ATP/ADP carrier (AAC), Na/sulfate transporter (Mi-ichi *et al*., 2009), and phosphate carrier (Jedelsky *et al*., 2011). AAC is a channel for ADP/ATP exchange via a novel membrane potential-independent mechanism (Chan *et al*., 2005). Interestingly, in addition to ATP and ADP, PAPS (Mi-ichi *et al*., 2015a; Mi-Ichi *et al*., 2015b), the terminal product of the sulfate activation pathway, was demonstrated to be transported by the AAC of *E. histolytica*.

Due to a lack of detectable homologs, we are still unable to identify even a single member of the TIM complex. Given the fact that soluble proteins are targeted to the matrix (Mi-ichi *et al*., 2009; Santos *et al*., 2020), we posit that a highly divergent protein import machinery likely exists as exemplified by the discovery of Tom60 (Makiuchi *et al*., 2013). Proteins destined to be in the matrix of mitosomes are recognized through the presence of either or both an amino-terminus located presequence and/or a single or multiple internal targeting sequence (ITS). A presequence in the amino-terminus is not predicted in most of the *E. histolytica* mitosomal matrix proteins identified in the proteome (Mi-ichi *et al*., 2009). One example is AS whose sequence is 60% identical to that of the sulfate-reducing δ-proteobacterium *Desulfovibrio vulgaris* (Santos *et al*., 2020). By swapping low homology sequences of AS in *E. histolytica* and *D. vulgaris*, respectively, two ITSs were found to enable traffic of AS into the mitosomal matrix (Santos *et al*., 2020). It is of note that the presence of two ITS segments in *Eh*AS is a feature that is different from the AS homolog of *M. balamuthi*, wherein the protein targeting the hydrogenosome matrix occurs likely through its predicted presequence (Santos *et al*., 2020).

Several factors are presumed to contribute to the selective pressure against a presequence-based target recognition in MROs. The partial or complete loss of the electron transport chain led to a minimal or total abolition of the membrane potential. As a result, the electrophoretic force required to direct protein import across the inner membrane is lost, making the possession of these positively charged residues of the presequence superfluous (Chacinska *et al*., 2009; Garg & Gould, 2016). Also, the loss of the membrane potential-dependent inner membrane complexes Tim23 and Tim22 (Chacinska *et al*., 2009) may have triggered the restructuring of the TOM complex, specifically the presequence receptor Tom22, which interacts with Tim23 (Bajaj *et al*., 2014; Bykov *et al*., 2020). In *Entamoeba*, the genes encoding the targeting sequence receptors Tom22, Tom20, and Tom70 are absent in the genome. Instead, a noncanonical component Tom60 acts as a receptor for the mitosomal import of both soluble and membrane proteins (Makiuchi *et al*., 2013). Furthermore, *Entamoeba* only has a single mitochondrial presequence peptidase (MPP), specifically the catalytic MPPβ subunit which cleaves presequences after protein translocation. However, *Eh*MPPβ is cytosolic and not localized in the mitosomes (Makiuchi & Nozaki, 2014), which further suggests that *Entamoeba* is less reliant on a presequence-mediated mitosome protein import machinery (Santos *et al*., 2020).

MITOSOME FISSION VIA A DRP HETERODIMER COMPLEX

Canonical mitochondria require a coordinated mechanism of organellar fission and fusion to maintain their number and quality. Mitochondrial fusion is a compensatory mechanism to prevent the loss of mitochondrial membrane potential (Chen *et al*., 2003) by balancing the contents of normal and damaged mitochondria (Elgass *et al*., 2013; Hoppins, 2014). Like mitochondria, the quality of mitosomes must be maintained, but the quality control systems for DNA-lacking MROs of anaerobic microorganisms are largely unknown. Mitosomes of *E. histolytica* are heterogeneous as was pointed out by previous studies (Mi-ichi *et al*., 2009, 2011) where matrix proteins, namely AS, APSK, IPP, Cpn60, and the inner membrane protein AAC, did not exhibit uniform distribution based on immunofluorescence imaging and fractionation analyses. This characteristic may hint that mitosome dynamics are in play in this organism. However, missing in *E. histolytica* are homologs of transmembrane-type dynamin-related proteins (DRPs) such as mitofusins 1 and 2, and optic atrophy 1, which are required for mitochondrial outer and inner membrane fusion. Regardless, it remains plausible that MRO fusion utilizes divergent components from Opisthokonta as demonstrated by plants, whose mitochondria fuse even in the absence of fusion-associated DRP orthologs (Arimura, 2018). The demonstration that microinjection-transplanted mitosomes containing HA-tagged APSK could fuse with the mitosomes of amoeba expressing

myc-tagged AS (Kazama *et al.*, 2017) strongly supports this scenario. More recently, ER-mitochondria tethering has been implied to promote mitochondrial fusion in tobacco, as mediated by the GTPase Miro2 (White *et al.*, 2020). Such mechanism of membrane contact site (MCS)-mediated fusion may also exist in organisms with MROs. A separate section of this review is devoted to MCS (see below).

On the other hand, mitosomes must undergo elongation and fission before being distributed to daughter cells, as they are not produced de novo. Unlike fusion, fission utilizes soluble-type DRPs. In mammals, Drp1 is recruited from the cytoplasm by DRP receptors/adaptors on the outer membrane (also known as Fis1, Mff, and Mid49/Mid51) and then forms a homo-oligomeric complex that spirals and constricts around the mitochondrion (Labbe *et al.*, 2014)) (Bui & Shaw, 2013; Osellame *et al.*, 2016). A similar process also takes place in *Entamoeba*, albeit via a heterooligomeric complex formed between two Drp homologs named DrpA and DrpB (Makiuchi *et al.*, 2017). Both DrpA and DrpB are essential to parasite proliferation as shown by growth retardation following the silencing of the DrpA- and DrpB-encoding genes, respectively (Makiuchi *et al.*, 2017). This was the first report that a heterooligomeric Drp complex is responsible for organellar (mitosome) fission. However, the corresponding Drp receptor on the outer membrane is still unidentified and the regulatory mechanism that controls mitosomal fission remains largely unknown (Makiuchi *et al.*, 2017; Santos *et al.*, 2018).

Aside from fission and fusion, mitochondrial quality control is also carried out by autodigestion specifically called mitophagy (Twig & Shirihai, 2011). However, the autophagic degradation of amoebic mitosomes has not been demonstrated, and homologs of canonical mitophagy markers including PTEN-induced putative kinase 1 (PINK1), RBR E3 ubiquitin protein ligase (Parkin), autophagy-related gene 32 (Atg32), and ubiquitin-specific peptidase 30 (USP30) are absent in the genome of this parasite.

INTERORGANELLAR CONTACT SITES INVOLVING MITOSOMES

Tethering of neighboring organellar membranes via protein–protein or protein–lipid interactions constitute membrane contact sites (MCSs) (Helle *et al.*, 2013). MCSs are regions where membranes of adjacent organelles are 30 nm as apposed to each other (Jain & Holthuis, 2017). As many lipid metabolism and lipid transport proteins (LTPs) have been reported to be involved in MCSs across a variety of eukaryotes including anaerobic parasitic protozoans, their role has been generally linked to lipid transfer between interacting membranes (Santos & Nozaki, 2021). However, MCSs also facilitate a multitude of processes in the cell including ion homeostasis (Helle

et al., 2013; Jain & Holthuis, 2017), mitochondrial (Wong *et al.*, 2018) and endosomal (Hoyer *et al.*, 2018) fission, apoptosis (Helle *et al.*, 2013; Jain & Holthuis, 2017), and immune response regulation (Helle *et al.*, 2013),

Our in silico search of membrane proteins screened from the mitosome proteome of *E. histolytica* (Santos *et al.*, 2016) aimed at finding novel protein import complexes and transporters has led to the discovery of lineage-specific mitosomal membrane proteins that mediate contact with membranes of other organelles. We reported that *Entamoeba*-specific transmembrane mitosomal protein 1 (ETMP1) interacts with a homolog of EH-domain-containing protein in *E. histolytica* (EHD1). EHDs are known to be associated in various endocytic compartments, as was also demonstrated by *E. histolytica* EHD1, suggesting a novel mitosome to endosome MCS mediated by at least these two proteins (Santos *et al.*, 2022). Although the role of this MCS has not yet been determined, we hypothesize that lipid transfer, lipid metabolism, ion transport, and quality control are possible reasons why *E. histolytica* maintains mitosome–endosome interactions (Santos *et al.*, 2022). Although a few fatty acid ligases were detected in the complex containing HA-ETMP1, direct protein–protein interaction has not yet been demonstrated, and functional characterization has not yet been completed (Santos *et al.*, 2022). Ion transport may also occur between mitosomes and endosomes through this MCS, analog to the mitochondrion–endosome contact site of epithelial cells, that facilitates iron transfer through a "kiss and run" mechanism (Das *et al.*, 2016). Finally, an alternative mode of mitosome and endosome fission may also be accomplished via this mitosome–endosome contact site, as EHD1 in HeLa cells was reported to be a novel regulator of fission in addition to rabankyrin-5 and the retromer complex (Deo *et al.*, 2018; Farmer *et al.*, 2017). The presence of an *E. histolytica* EHD1 homolog and its association with mitosomes may reflect a possible involvement of EHD1-mediated mitosome–endosome contact sites in organellar dynamics in this parasite.

Another organelle that was thought to be absent in *E. histolytica* was recently confirmed to be present in this parasite by the identification and colocalization of seven peroxin (Pex) homologs which are responsible for peroxisome biogenesis (Verner *et al.*, 2021). Amoebic peroxisomes metabolize myoinositol, similar to the anaerobic peroxisomes reported in other Archamoebae species (Verner *et al.*, 2021). Interestingly, Pex11 was demonstrated to localize dually in peroxisomes and mitosomes of *E. histolytica* (Verner *et al.*, 2021). In yeast, Pex11 interacts with Mdm34, a mitochondrial outer membrane protein involved in establishing the ER-mitochondrion encounter structure (ERMES) complex, suggesting these molecules constitute mitochondrion–peroxisome tether (Mattiazzi *et al.*, 2015). Moreover, it was also reported that the peroxisome and mitosome proteomes contain several overlapping proteins

(Verner *et al.*, 2021) which included EHI_170120, a previously characterized *Entamoeba*-specific mitosomal membrane protein (Santos *et al.*, 2016). These findings may suggest that mitosomes and the anaerobic peroxisomes of *E. histolytica* interact, similar to what has been established in the mitochondrion and peroxisomes of yeast (Shai *et al.*, 2016, 2018) and mammals (Chen *et al.*, 2020; Shai *et al.*, 2016; Xia *et al.*, 2019). Canonical mitochondrion–peroxisome contacts feature a cooperative mechanism of β-oxidation of fatty acids as well as detoxification of reactive oxidative species (Shai *et al.*, 2018), which are unlikely performed by their amoebic counterpart. Several models have been proposed for the biogenesis of peroxisomes including the growth and division model, where preexisting peroxisomes acquire proteins from the cytosol, expand, and finally undergo fission, and the de novo model, which involves the ER and its complex sorting mechanisms (Farré *et al.*, 2018). One mechanism of de novo peroxisome biogenesis demonstrated in mutant human fibroblast cells, occurs through the fusion of both mitochondrial- and ER-derived vesicles (Sugiura *et al.*, 2017). It remains to be seen whether anaerobic peroxisomes of *E. histolytica* also have mitosome origins. The role of this potential mitosome–peroxisome contact remains an open question.

Another lineage-specific mitosomal membrane protein ETMP30 was also discovered to interact with a Golgi-associated protein secretory pathway calcium ATPase (SPCA) (Rodríguez *et al.*, 2018), suggesting the existence of a mitosome-Golgi MCS (Santos *et al.*, 2019). The function of this MCS is posited to be for the exchange of Ca^{2+} and ATP between the Golgi and the mitochondria, as what has been demonstrated in pancreatic acinar cells (Dolman *et al.*, 2005). We similarly reported the dual localization of another ETMP, EHI_099350 to both mitosomes and ER, suggesting that this protein may be involved in the tethering of the two organelles (Santos *et al.*, 2016). The ER membrane network is a vital cog of MCS associations, and it is not surprising that mitosomes also form interactions with the ER, being the major source of phospholipids of organellar membranes. Mitochondria generally outsource phospholipids from the ER membrane through LTPs found along their contact sites. Although the *Entamoeba* genome is devoid of homologs for the establishment of the ERMES complex, we suppose that dual-localized membrane proteins point to the potential existence of similar tethering complexes between the ER and mitosomes. Characterization of two *E. histolytica* LTPs (LTP1 and LTP3), which belong to the steroidogenic acute regulatory protein-related lipid-transfer (START)-domain-containing LTPs, were reported (Das *et al.*, 2021), and similar to other eukaryotes, it is likely that such LTPs are involved in various amoebic organelle MCSs for the purpose of lipid exchange and membrane homeostasis (Santos *et al.*, 2022). A rigorous lipidomic and proteomic profiling of organellar membranes will further shed light on the mechanisms of lipid transfer in *E. histolytica*.

FUTURE PERSPECTIVES

Since the discovery of mitosomes in *E. histolytica*, we have slowly expanded our grasp of this enigmatic MRO in this parasite. It is not surprising that the roles and makeup of this organelle are almost entirely unique to its genus. Only mitosomes of *Entamoeba* and the MROs of *M. balamuthi* and *P. schiedti* carry out compartmentalized sulfate activation. It is expected that universal processes including protein and metabolite transport, dynamics, and mitophagy demonstrated in mitochondria and other MROs are carried out by noncanonical or novel proteins unique to this parasite. Advanced in silico prediction tools are valuable for mining candidate proteins that may be utilized for such processes. As a result of our screening of mitosomal membrane proteins initiated from in silico prediction of the transmembrane domain (Santos *et al.*, 2016), we confirmed several proteins to be integrated onto the membranes of mitosomes that led to the discovery of novel interactions with other organelles (Santos *et al.*, 2019, 2022; Santos & Nozaki, 2021). Previous to this, nothing was known about the molecular compositions, mechanisms, and roles of interorganellar membrane tethers in *Entamoeba*, making it imperative to conduct further investigation into this matter. A more detailed morphological analysis of the organelles of *E. histolytica*, including its mitosomes could be provided by focused ion beam scanning electron microscopy analysis, as was performed in mammals (Wu *et al.*, 2017), in yeast (Quon *et al.*, 2018), and in *G. intestinalis* (Zumthor *et al.*, 2016). Such sophisticated imaging methods will provide 3D reconstitution snapshots that show the dynamic interplay of organelles and help deepen our understanding of MCSs in *E. histolytica*.

Advances must also be made to fill the void, particularly on the current knowledge about lipid metabolism and transport mechanisms in *E. histolytica*. The precise lipid transfer mechanisms involving LTPs, including their potential involvement in the observed MCSs, should be elucidated. The schemes of amoebic lipid transfer can be further clarified by extensive lipidomic profiling of *E. histolytica* whole cells, subcellular fractions, and organelles. Furthermore, lipid metabolism within the mitosome remains unexplored. At least one gene encoding a PRELI-like domain-containing protein (EHI_143630) was detected in the *E. histolytica* genome (Das & Nozaki, 2018) and may potentially participate in mitosomal lipid homeostasis, as demonstrated in the aerobic mitochondria of higher eukaryotes (Miliara *et al.*, 2015; Tatsuta & Langer, 2017).

A more sophisticated proteomic analysis such as data-independent acquisition (DIA) may help in identifying undiscovered components of the transport

machineries in the mitosomes of *E. histolytica*. To help us identify protein translocase system components that operate within the mitosome, our group had expressed the matrix protein AS fused with the promiscuous biotin ligase BirA, (Roux *et al.*, 2012) in *E. histolytica* trophozoites. A protein interactome analysis based on DIA proteomics was conducted by our group, which is a more suitable approach to identify less abundant proteins that may have been missed by our previous attempts that relied on data-dependent acquisition (DDA) proteomics. Although DDA remains a very reliable approach for proteomics analysis, it is limited by its selection of only the most prominent peptide ions for fragmentation; thus, less abundant peptides are not identified in the tandem mass spectrometry results (Rinschen *et al.*, 2018; Sukumaran *et al.*, 2021). In contrast, DIA aims to detect every peptide by fragmenting them together regardless of their abundance, which leads to more complicated spectra, with a higher number of detected peptides that require a dedicated software (Rinschen *et al.*, 2018; Sukumaran *et al.*, 2021). Indeed, DIA can improve the identification of peptides and data reproducibility, but the interpretation of the resulting complex spectra could be difficult (Christopher *et al.*, 2021). We are currently conducting further investigation and characterization of several candidate proteins identified from DIA-based proteomic analysis of the AS-BirA interactome.

It has been established that mitosomes majorly contribute to the biology of *Entamoeba histolytica* by way of the connection between sulfate activation and trophozoite-cyst stage conversion. As encystation of *E. histolytica* in vitro has only been demonstrated recently (Wesel *et al.*, 2021), mitosomes have only been characterized in trophozoites and not in cysts. The reptilian parasite species *Entamoeba invadens* is often used as a model of amoebic encystation, since in vitro stage conversion is readily inducible (Siegesmund *et al.*, 2011). Mitosomes were detected as abundant, punctate compartments by IFA using anti-Hsp60 (synonymous with Cpn60) and antimitochondrial-type Hsp70 antibodies in *E. invadens* cysts. Comparative analysis revealed no significant difference in mitosome abundance and distribution between *E. invadens* trophozoites and cysts. The results imply that mitosomes may play a role in *E. invadens* cysts (Siegesmund *et al.*, 2011); however, the finding remains to be validated. It is important to investigate the role of mitosomes in cysts, as well as during en/excystation, once en/excystation of *E. histolytica* becomes robustly demonstrable (Wesel *et al.*, 2021). One alternative possibility is to study amoebic cell differentiation using organoids derived from the human colon. A similar approach has been developed to study the life cycle of *Cryptosporidium parvum* using mouse and human small intestinal and lung organoids (Heo *et al.*, 2018). Combining these approaches with single-cell genomics, transcriptomics, and proteomics will give us profound insights not limited to mitosomes, but into the biology, biochemistry, parasitism, and evolution of the parasite *E. histolytica*. Overall, the collection of novel and unanticipated findings summarized in this review has opened new perspectives and highlighted the fact that the adaptation of *Entamoeba* to a parasitic lifestyle caused the secondary loss of canonical mitochondrial processes and components and drove it to tailor-specific and minimalistic machineries to maintain a reinvented role fit and unique to itself.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ORCID
Herbert J. Santos https://orcid.org/0000-0003-4322-0145
Tomoyoshi Nozaki https://orcid.org/0000-0003-1354-5133

REFERENCES

Arimura, S.I. (2018) Fission and fusion of plant mitochondria, and genome maintenance. *Plant Physiology*, 176, 152–161.

Arisue, N., Sánchez, L.B., Weiss, L.M., Müller, M. & Hashimoto, T. (2002) Mitochondrial-type hsp70 genes of the amitochondriate protists, *Giardia intestinalis*, *Entamoeba histolytica* and two microsporidians. *Parasitology International*, 51, 9–16.

Bajaj, R., Jaremko, Ł., Jaremko, M., Becker, S. & Zweckstetter, M. (2014) Molecular basis of the dynamic structure of the TIM23 complex in the mitochondrial intermembrane space. *Structure*, 22, 1501–1511.

Baker, K.P., Schaniel, A., Vestweber, D. & Schatz, G. (1990) A yeast mitochondrial outer membrane protein essential for protein import and cell viability. *Nature*, 348, 605–609.

Budzinska, M., Galganska, H., Karachitos, A., Wojtkowska, M. & Kmita, H. (2009) The TOM complex is involved in the release of superoxide anion from mitochondria. *Journal of Bioenergetics and Biomembranes*, 41, 361–367.

Bui, H.T. & Shaw, J.M. (2013) Dynamin assembly strategies and adaptor proteins in mitochondrial fission. *Current Biology*, 23, R891–R899.

Bykov, Y.S., Rapaport, D., Herrmann, J.M. & Schuldiner, M. (2020) Cytosolic events in the biogenesis of mitochondrial proteins. *Trends in Biochemical Sciences*, 45, 650–667.

Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T. & Pfanner, N. (2009) Importing mitochondrial proteins: machineries and mechanisms. *Cell*, 138, 628–644.

Chan, K.W., Slotboom, D.J., Cox, S., Embley, T.M., Fabre, O., van der Giezen, M. et al. (2005) A novel ADP/ATP transporter in the mitosome of the microaerophilic human parasite *Entamoeba histolytica*. *Current Biology*, 15, 737–742.

Chen, C., Li, J., Qin, X. & Wang, W. (2020) Peroxisomal membrane contact sites in mammalian cells. *Frontiers in Cell and Development Biology*, 8, 1–9.

Chen, H., Detmer, S.A., Ewald, A.J., Griffin, E.E., Fraser, S.E. & Chan, D.C. (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *The Journal of Cell Biology*, 160, 189–200.

Christopher, J.A., Stadler, C., Martin, C.E., Morgenstern, M., Pan, Y., Betsinger, C.N. et al. (2021) Subcellular proteomics. *Nature Reviews Methods Primers*, 1, 1–24.

Clark, C.G. & Roger, A.J. (1995) Direct evidence for secondary loss of mitochondria in *Entamoeba histolytica*. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 6518–6521.

Colombini, M. (2004) VDAC: the channel at the interface between mitochondria and the cytosol. *Molecular and Cellular Biochemistry*, 256–257, 107–115.

Das, A., Nag, S., Mason, A.B. & Barroso, M.M. (2016) Endosome-mitochondria interactions are modulated by iron release from transferrin. *The Journal of Cell Biology*, 214, 831–845.

Das, K. & Nozaki, T. (2018) Non-vesicular lipid transport machinery in *Entamoeba histolytica*. *Frontiers in Cellular and Infection Microbiology*, 8, 1–14.

Das, K., Watanabe, N. & Nozaki, T. (2021) Two StAR-related lipid transfer proteins play specific roles in endocytosis, exocytosis, and motility in the parasitic protist *Entamoeba histolytica*. *PLoS Pathogens*, 17, 1–27.

Deo, R., Kushwah, M.S., Kamerkar, S.C., Kadam, N.Y., Dar, S., Babu, K. et al. (2018) ATP-dependent membrane remodeling links EHD1 functions to endocytic recycling. *Nature Communications*, 9, 5187.

Dolezal, P., Dagley, M.J., Kono, M., Wolynec, P., Likic, V.A., Foo, J.H. et al. (2010) The essentials of protein import in the degenerate mitochondrion of *Entamoeba histolytica*. *PLoS Pathogens*, 6, e1000812.

Dolman, N.J., Gerasimenko, J.V., Gerasimenko, O.V., Voronina, S.G., Petersen, O.H. & Tepikin, A.V. (2005) Stable Golgi-mitochondria complexes and formation of Golgi Ca²⁺ gradients in pancreatic acinar cells. *The Journal of Biological Chemistry*, 280, 15794–15799.

Elgass, K., Pakay, J., Ryan, M.T. & Palmer, C.S. (2013) Recent advances into the understanding of mitochondrial fission. *Biochimica et Biophysica Acta*, 1833, 150–161.

Fahey, R.C., Newton, G.L., Arrick, B., Overdank-Bogart, T. & Aley, S.B. (1984) *Entamoeba histolytica*: a eukaryote without glutathione metabolism. *Science*, 224, 70–72.

Farmer, T., Reinecke, J.B., Xie, S., Bahl, K., Naslavsky, N. & Caplan, S. (2017) Control of mitochondrial homeostasis by endocytic regulatory proteins. *Journal of Cell Science*, 130, 2359–2370.

Farré, J.C., Mahalingam, S.S., Proietto, M. & Subramani, S. (2018) Peroxisome biogenesis, membrane contact sites, and quality control. *EMBO Reports*, 20, e46864.

Garg, S.G. & Gould, S.B. (2016) The role of charge in protein targeting evolution. *Trends in Cell Biology*, 26, 894–905.

Ghosh, S., Field, J., Rogers, R., Hickman, M. & Samuelson, J. (2000) The *Entamoeba histolytica* mitochondrion-derived organelle (crypton) contains double-stranded DNA and appears to be bound by a double membrane. *Infection and Immunity*, 68, 4319–4322.

van der Giezen, M., León-Avila, G. & Tovar, J. (2005) Characterization of chaperonin 10 (Cpn10) from the intestinal human pathogen *Entamoeba histolytica*. *Microbiology*, 151, 3107–3115.

Helle, S.C.J., Kanfer, G., Kolar, K., Lang, A., Michel, A.H. & Kornmann, B. (2013) Organization and function of membrane contact sites. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1833, 2526–2541.

Heo, I., Dutta, D., Schaefer, D.A., Iakobachvili, N., Artegiani, B., Sachs, N. et al. (2018) Modelling *cryptosporidium infection* in human small intestinal and lung organoids. *Nature Microbiology*, 3, 814–823.

Hill, K., Model, K., Ryan, M.T., Dietmeier, K., Martin, F., Wagner, R. et al. (1998) Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature*, 395, 516–521.

Hoppins, S. (2014) The regulation of mitochondrial dynamics. *Current Opinion in Cell Biology*, 29, 46–52.

Hoyer, M.J., Chitwood, P.J., Ebmeier, C.C., Striepen, J.F., Qi, R.Z., Old, W.M. et al. (2018) A novel class of ER membrane proteins regulates ER-associated endosome fission. *Cell*, 175, 254–265.

Jain, A. & Holthuis, J.C.M. (2017) Membrane contact sites, ancient and central hubs of cellular lipid logistics. *Biochimica et Biophysica Acta – Molecular Cell Research*, 1864, 1450–1458.

Jedelsky, P.L., Dolezal, P., Rada, P., Pyrih, J., Smid, O., Hrdy, I. et al. (2011) The minimal proteome in the reduced mitochondrion of the parasitic protist Giardia intestinalis. *PLoS One*, 6, e17285.

Karnkowska, A., Vacek, V., Zubáčová, Z., Treitli, S.C., Petrželková, R., Eme, L. et al. (2016) A eukaryote without a mitochondrial organelle. *Current Biology*, 26, 1274–1284.

Kawano-Sugaya, T., Izumiyama, S., Yanagawa, Y., Saito-Nakano, Y., Watanabe, K., Kobayashi, S. et al. (2020) Near-chromosome level genome assembly reveals ploidy diversity and plasticity in the intestinal protozoan parasite *Entamoeba histolytica*. *BMC Genomics*, 21, 1–8.

Kazama, M., Ogiwara, S., Makiuchi, T., Yoshida, K., Nakada-Tsukui, K., Nozaki, T. et al. (2017) Behavior of DNA-lacking mitochondria in *Entamoeba histolytica* revealed by organelle transplant. *Scientific Reports*, 7, 1–10.

Kmita, H. & Budzinska, M. (2000) Involvement of the TOM complex in external NADH transport into yeast mitochondria depleted of mitochondrial porin1. *Biochimica et Biophysica Acta*, 1509, 86–94.

Labbe, K., Murley, A. & Nunnari, J. (2014) Determinants and functions of mitochondrial behavior. *Annual Review of Cell and Developmental Biology*, 30, 357–391.

Léon-Avila, G. & Tovar, J. (2004) Mitosomes of *Entamoeba histolytica* are abundant mitochondrion-related remnant organelles that lack a detectable organellar genome. *Microbiology*, 150, 1245–1250.

Lill, R. & Kispal, G. (2000) Maturation of cellular Fe–S proteins: an essential function of mitochondria. *Trends in Biochemical Sciences*, 25, 352–356.

Mai, Z., Ghosh, S., Frisardi, M., Rosenthal, B., Rogers, R. & Samuelson, J. (1999) Hsp60 is targeted to a cryptic mitochondrion-derived organelle ("Crypton") in the microaerophilic protozoan parasite *Entamoeba histolytica*. *Molecular and Cellular Biology*, 19, 2198–2205.

Makiuchi, T., Mi-ichi, F., Nakada-Tsukui, K. & Nozaki, T. (2013) Novel TPR-containing subunit of TOM complex functions as cytosolic receptor for *Entamoeba* mitosomal transport. *Scientific Reports*, 3, 1129.

Makiuchi, T. & Nozaki, T. (2014) Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa. *Biochimie*, 100, 3–17.

Makiuchi, T., Santos, H.J., Tachibana, H. & Nozaki, T. (2017) Hetero-oligomer of dynamin-related proteins participates in the fission of highly divergent mitochondria from *Entamoeba histolytica*. *Scientific Reports*, 7, 13439.

Maralikova, B., Ali, V., Nakada-Tsukui, K., Nozaki, T., van der Giezen, M., Henze, K. et al. (2010) Bacterial-type oxygen detoxification and iron-sulfur cluster assembly in amoebal relict mitochondria. *Cellular Microbiology*, 12, 331–342.

Martinez-Palomo, A. (1986) Biology of *Entamoeba histolytica*. In: *Amebiasis (human parasitic diseases vol. 2.)*. Amsterdam: Elsevier Science Publishers B.V. (biomedical division), pp. 11–43.

Mattiazzi, U.M., Brložnik, M., Kaferle, P., Žitnik, M., Wolinski, H., Leitner, F. et al. (2015) Genome-wide localization study of yeast Pex11 identifies peroxisome-mitochondria interactions through the ERMES complex. *Journal of Molecular Biology*, 427, 2072–2087.

Mi-ichi, F., Makiuchi, T., Furukawa, A., Sato, D. & Nozaki, T. (2011) Sulfate activation in mitosomes plays an important role in the proliferation of *Entamoeba histolytica*. *PLoS Neglected Tropical Diseases*, 5, e1263.

Mi-ichi, F., Miyamoto, T., Takao, S., Jeelani, G., Hashimoto, T., Hara, H. et al. (2015a) *Entamoeba* mitosomes play an important role in encystation by association with cholesteryl sulfate synthesis. *Proceedings of the National Academy of Sciences*, 112, E2884–E2890.

Mi-ichi, F., Miyamoto, T. & Yoshida, H. (2017) Uniqueness of *Entamoeba* sulfur metabolism: sulfolipid metabolism that plays pleiotropic roles in the parasitic life cycle. *Molecular Microbiology*, 106, 479–491.

Mi-Ichi, F., Nozawa, A., Yoshida, H., Tozawa, Y. & Nozaki, T. (2015b) Evidence that *Entamoeba histolytica* mitochondrial carrier family links mitosomal and cytosolic pathways through exchange of PAPS and ATP. *Eukaryotic Cell*, 14, 1144–1150.

Mi-ichi, F. & Yoshida, H. (2019) Unique features of *Entamoeba* sulfur metabolism; compartmentalization, physiological roles of terminal products, evolution and pharmaceutical exploitation. *International Journal of Molecular Sciences*, 20, 1–13.

Mi-ichi, F., Yousuf, M.A., Nakada-Tsukui, K. & Nozaki, T. (2009) Mitosomes in Entamoeba histolytica contain a sulfate activation pathway. *Proceedings of the National Academy of Sciences*, 106, 21731–21736.

Miliara, X., Garnett, J.A., Tatsuta, T., Abid, A.F., Baldie, H., Pérez-Dorado, I. et al. (2015) Structural insight into the TRIAP 1/PRELI -like domain family of mitochondrial phospholipid transfer complexes. *EMBO Reports*, 16, 824–835.

Nývltová, E., Šuták, R., Harant, K., Šedinová, M., Hrdy, I., Paces, J. et al. (2013) NIF-type iron-sulfur cluster assembly system is duplicated and distributed in the mitochondria and cytosol of *Mastigamoeba balamuthi*. *Proceedings of the National Academy of Sciences*, 110, 7371–7376.

Nývltová, E., Stairs, C.W., Hrdý, I., Rídl, J., Mach, J., Paçes, J. et al. (2015) Lateral gene transfer and gene duplication played a key role in the evolution of *Mastigamoeba balamuthi* hydrogenosomes. *Molecular Biology and Evolution*, 32, 1039–1055.

Osellame, L.D., Singh, A.P., Stroud, D.A., Palmer, C.S., Stojanovski, D., Ramachandran, R. et al. (2016) Cooperative and independent roles of the Drp1 adaptors Mff, MiD49 and MiD51 in mitochondrial fission. *Journal of Cell Science*, 129, 2170–2181.

Patron, N.J., Durnford, D.G. & Kopriva, S. (2008) Sulfate assimilation in eukaryotes: fusions, relocations and lateral transfers. *BMC Evolutionary Biology*, 8, 39.

Quon, E., Sere, Y.Y., Chauhan, N., Johansen, J., Sullivan, D.P., Dittman, J.S. et al. (2018) Endoplasmic reticulum-plasma membrane contact sites integrate sterol and phospholipid regulation. *PLoS Biology*, 16, e2003864.

Rinschen, M.M., Limbutara, K., Knepper, M.A., Payne, D.M. & Pisitkun, T. (2018) From molecules to mechanisms: functional proteomics and its application to renal tubule physiology. *Physiological Reviews*, 98, 2571–2606.

Rodríguez, M.A., Martínez-Higuera, A., Valle-Solis, M.I., Hernandes-Alejandro, M., Chávez-Munguía, B., Figueroa-Gutiérrez, A.H. et al. (2018) A putative calcium-ATPase of the secretory pathway family may regulate calcium/manganese levels in the Golgi apparatus of *Entamoeba histolytica*. *Parasitology Research*, 117, 3381–3389.

Roux, K.J., Kim, D.I., Raida, M. & Burke, B. (2012) A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *The Journal of Cell Biology*, 196, 801–810.

Saidha, T., Na, S.Q., Li, J.Y. & Schiff, J.A. (1988) A sulphate metabolizing centre in Euglena mitochondria. *The Biochemical Journal*, 253, 533–539.

Saidha, T., Stern, A.I., Lee, D.H. & Schiff, J.A. (1985) Localization of a sulphate-activating system within euglena mitochondria. *The Biochemical Journal*, 232, 357–365.

Santos, H.J., Chiba, Y., Makiuchi, T., Arakawa, S., Murakami, Y., Tomii, K. et al. (2020) Import of Entamoeba histolytica mitosomal ATP sulfurylase relies on internal targeting sequences. *Microorganisms*, 8, 1–17.

Santos, H.J., Hanadate, Y., Imai, K. & Nozaki, T. (2019) An Entamoeba-specific Mitosomal membrane protein with potential association to the Golgi apparatus. *Genes (Basel)*, 10, 367.

Santos, H.J., Hanadate, Y., Imai, K., Watanabe, H. & Nozaki, T. (2022) *Entamoeba histolytica* EHD1 is involved in mitosome-endosome contact. *mBio*, 13(2), e0384921.

Santos, H.J., Imai, K., Hanadate, Y., Fukasawa, Y., Oda, T., Mi-ichi, F. et al. (2016) Screening and discovery of lineage-specific mitosomal membrane proteins in *Entamoeba histolytica*. *Molecular and Biochemical Parasitology*, 209, 10–17.

Santos, H.J., Imai, K., Makiuchi, T., Tomii, K., Horton, P., Nozawa, A. et al. (2015) A novel mitosomal β-barrel outer membrane protein in *Entamoeba*. *Scientific Reports*, 5, 1–10.

Santos, H.J., Makiuchi, T. & Nozaki, T. (2018) Reinventing an organelle: the reduced mitochondrion in parasitic protists. *Trends in Parasitology*, 34, 1038–1055.

Santos, H.J. & Nozaki, T. (2021) Interorganellar communication and membrane contact sites in protozoan parasites. *Parasitology International*, 83, 102372.

Shai, N., Schuldiner, M. & Zalckvar, E. (2016) No peroxisome is an Island – peroxisome contact sites. *Biochimica et Biophysica Acta – Molecular Cell Research*, 1863, 1061–1069.

Shai, N., Yifrach, E., Van Roermund, C.W.T., Cohen, N., Bibi, C., Ijlst, L. et al. (2018) Systematic mapping of contact sites reveals tethers and a function for the peroxisome-mitochondria contact. *Nature Communications*, 9, 1761.

Siegesmund, M.A., Hehl, A.B. & van der Giezen, M. (2011) Mitosomes in trophozoites and cysts of the reptilian parasite *Entamoeba invadens*. *Eukaryotic Cell*, 10, 1582–1585.

Sogin, M.L., Edman, U. & Jornvall, H. (1989) A single kingdom of eukaryotes. In: Fernholme, B., Bremer, K. & Jornvall, H. (Eds.) *In the Hierarchy of Life*. Amsterdam: Elsevier Science, pp. 133–143.

Stairs, C.W., Leger, M.M. & Roger, A.J. (2015) Diversity and origins of anaerobic metabolism in mitochondria and related organelles. *Philosophical Transactions of the Royal Society B*, 370, 1–13.

Sugiura, A., Mattie, S., Prudent, J. & Mcbride, H.M. (2017) Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature*, 542, 251–254.

Sukumaran, A., Woroszchuk, E., Ross, T. & Geddes-McAlister, J. (2021) Proteomics of host–bacterial interactions: new insights from dual perspectives. *Canadian Journal of Microbiology*, 67, 213–225.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M. et al. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, 397, 441–446.

Tatsuta, T. & Langer, T. (2017) Intramitochondrial phospholipid trafficking. *Biochimica et Biophysica Acta – Molecular and Cell Biology of Lipids*, 1862, 81–89.

Tovar, J., Fischer, A. & Clark, C.G. (1999) The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. *Molecular Microbiology*, 32, 1013–1021.

Twig, G. & Shirihai, O.S. (2011) The interplay between mitochondrial dynamics and mitophagy. *Antioxidants & Redox Signaling*, 14, 1939–1951.

Verner, Z., Žárský, V., Le, T., Narayanasamy, R.K., Rada, P., Rozbeský, D. et al. (2021) Anaerobic peroxisomes in *Entamoeba histolytica* metabolize myo-inositol. *PLoS Pathogens*, 17, e1010041.

Wesel, J., Shuman, J., Bastuzel, I., Dickerson, J. & Ingram-Smith, C. (2021) Encystation of E*ntamoeba histolytica* in axenic culture. *Microorganisms*, 9, 873.

White, R.R., Lin, C., Leaves, I., Castro, I.G., Metz, J., Bateman, B.C. et al. (2020) Miro2 tethers the ER to mitochondria to promote mitochondrial fusion in tobacco leaf epidermal cells. *Communications Biology*, 3, 1–8.

Wong, Y.C., Ysselstein, D. & Krainc, D. (2018) Mitochondria-lysosome contacts regulate mitochondrial fission via RAB7 GTP hydrolysis. *Nature*, 554, 382–386.

Wu, Y., Whiteus, C., Xu, C.S., Hayworth, K.J., Weinberg, R.J., Hess, H.F. et al. (2017) Contacts between the endoplasmic reticulum and other membranes in neurons. *Proceedings of the National Academy of Sciences of the United States of America*, 114, E4859–E4867.

Xia, M.F., Zhang, Y.Z., Jin, K., Lu, Z.T., Zeng, Z. & Xiong, W. (2019) Communication between mitochondria and other organelles: a brand-new perspective on mitochondria in cancer. *Cell & Bioscience*, 9, 1–19.

Yousuf, M.A., Mi-Ichi, F., Nakada-Tsukui, K. & Nozaki, T. (2010) Localization and targeting of an unusual pyridine nucleotide transhydrogenase in *Entamoeba histolytica*. *Eukaryotic Cell*, 9, 926–933.

Záhonová, K., Treitli, S.C., Le, T., Škodová-Sveráková, I., Hanousková, P., Čepička, I. et al. (2022) Anaerobic derivates of mitochondria and peroxisomes in the free-living amoeba *Pelomyxa schiedti* revealed by single-cell genomics. *BMC Biology*, 20, 1–16.

Žárský, V., Klimeš, V., Pačes, J., Vlček, Č., Hradilová, M., Beneš, V. et al. (2021) The *Mastigamoeba balamuthi* genome and the nature of the free-living ancestor of *Entamoeba. Molecular Biology and Evolution*, 38, 2240–2259.

Zumthor, J.P., Cernikova, L., Rout, S., Kaech, A., Faso, C. & Hehl, A.B. (2016) Static clathrin assemblies at the peripheral vacuole—plasma membrane interface of the parasitic protozoan *Giardia lamblia. PLoS Pathogens*, 12, 1–33.