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* (Nývtová 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 (Nývtová et al., 2013). In *B. balamuthi*, the NiFe and NiFe subunits were demonstrated to localize in both cytosol and its MRO, the hydrogenosome, (Nývtová et al., 2013). In the case of *P. schiedti*, NiFe (two out of three homologs), but not NiFe, was demonstrated to be targeted to yeast mitochondria by heterologous localization experiments (Záhonová et al., 2022). The localization of *Entamoeba* NiFe and NiFe 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 (APS), and inorganic pyrophosphatase (Patron 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* (Nývtová 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). EhIPP and one of three IPP paralogs in *M. balamuthi*, *MbIPP*-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 *MbIPP*-2 and *MbIPP*-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 Eukarya 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′-phosphoade nosine-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; Zársky *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; Zársky *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 sulfotases (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).
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, 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 (mHsp70) 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) synthetized 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.

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 to 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 (mHsp70) 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) synthetized 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.

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(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. schiedtii (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 EhAS is a feature that is different from the AS homolog of *M. bala- muthi*, 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, EhMPPβ 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 (mitosomal) 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 30nm 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 protozoa, 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 (ETMPL) interacts with a homolog of EH-domain-containing protein in E. histolytica (EHDI). EHDS are known to be associated in various endocytic compartments, as was also demonstrated by E. histolytica EHDI, 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-ETMPL, 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 EHDI 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 EHDI homolog and its association with mitosomes may reflect a possible involvement of EHDL-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 Archaeomae 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 (Mattiazi 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 Ca²⁺ 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 steriodogenic 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 (Siegemund 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 (Siegemund 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.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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