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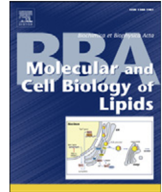

Review

The evolution of ERMIONE in mitochondrial biogenesis and lipid homeostasis: An evolutionary view from comparative cell biology☆

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ABSTRACT

The ER–mitochondria organizing network (ERMIONE) in *Saccharomyces cerevisiae* is involved in maintaining mitochondrial morphology and lipid homeostasis. ERMES and MICOS are two scaffolding complexes of ERMIONE that contribute to these processes. ERMES is ancient but has been lost in several lineages including animals, plants, and SAR (stramenopiles, alveolates and rhizaria). On the other hand, MICOS is ancient and has remained present in all organisms bearing mitochondrial cristae. The ERMIONE precursor evolved in the α-proteobacterial ancestor of mitochondria which had the central subunit of MICOS, Mic60. The subsequent evolution of ERMIONE and its interactors in eukaryotes reflects the integrative co-evolution of mitochondria and their hosts and the adaptive paths that some lineages have followed in their specialization to certain environments. By approaching the ERMIONE from a perspective of comparative evolutionary cell biology, we hope to shed light on not only its evolutionary history, but also how ERMIONE components may function in organisms other than *S. cerevisiae*. This article is part of a Special Issue entitled: The cellular lipid landscape edited by Tim P. Levine and Anant K. Menon.
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1. ERMIONE mediates membrane contact sites (MCSs) through ERMES and MICOS

Membrane contact sites (MCSs) are points of contact between two organellar membranes. MCSs exist between many different organelles and have several putative functions, including the facilitation of ion transport, non-vesicular lipid trafficking, the maintenance of membrane architecture, as well as organelle distribution, inheritance, and biogenesis (e.g., organelle division) in general [1–5]. These functions are thought to be facilitated by protein tethers that stabilize MCSs by forming a bridge between the two membrane bilayers, without allowing them to fuse. Two well-studied MCS tethers include the ER–Mitochondria Encounter Structure (ERMES), which tethers the ER-membrane to the Mitochondrial Outer Membrane (MOM), and the MItochondrial contact site and Cristae Organizing System (MICOS), which tethers the Mitochondrial Inner Membrane (MIM) to the MOM. ERMES and MICOS genetically interact in *S. cerevisiae* and form part of a larger physical-genetic interaction network with the Translocase of the Outer mitochondrial Membrane (TOM) and the Sorting and Assembly Machinery (SAM) complexes in the MOM [6,7] termed the ER–MItochondria Organizing NEtwork (ERMIONE) [8]. ERMIONE coordinates machineries for protein import and assembly, lipid metabolism, metabolite and ion transport, and mtDNA organization. Given the large size of MICOS, its multiple physical interactors, and its extended distribution in the intermembrane space (IMS) as a skeletal scaffold, van der Laan et al. (2012) [8] hypothesised that MICOS lies at the heart of ERMIONE. They suggest that MICOS: (1) limits free diffusion of proteins and/or lipids between the inner boundary membrane (IBM) and crista membrane (CM), (2) physically links the MIM to MOM, (3) interacts with components of the nucleoid, and (4) interacts genetically with ERMES, thus providing a communication path from the ER to mitochondria.

ERMIONE, therefore, is at the heart of a network of complexes and processes underlying mitochondrial biogenesis. Among the multiple processes coordinated by ERMIONE, mitochondrial lipid transfer and metabolism is a central one. Here, by focusing on MCSs, membrane organization, and lipid transport, we review what is known about the ERMIONE and its interactors and make inferences about their evolution from a comparative cell biological perspective.

2. An evolutionary comparative approach enlightens cell biology

The comparative method has a long tradition in biology: it is the foundation of historical biology, and therefore all evolutionary biology (e.g. Buffon and Darwin). It popularly finds its modern explicit formulation in the "August Krogh principle" (AKP) which states that: "For a large number of problems, there will be some [organism] of choice on which it can be most conveniently studied" [9,10]. The AKP is an implicit assumption in many cell biological investigations that make use of

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model organisms (e.g., the baker's yeast *Saccharomyces cerevisiae*) often to illuminate the biology of our own species, *Homo sapiens*. But the comparative method also encompasses comparisons that aim to discover the historical relationships between species, i.e., "phylogeny" (the descent school), and those that aim to unravel adaptations by correlating character traits with ecological factors (the guild school) [11,12].

When employed within the context of a robust phylogenetic framework, the comparison of character traits (e.g., genomic, cellular, organismal, ecological) allows the reconstruction of their evolutionary history, that is, the ancestral state of a trait and the way it has changed in different lineages throughout the course of evolution [13]. This approach can lead to deciphering what features constitute adaptive lineage-specific traits, as well as which ones are highly conserved across a broad phylogenetic spectrum and represent the ancient core aspects of the trait [14]. Thus, an understanding of the evolutionary history of a trait may illuminate aspects of its current function.

Although implicitly assuming the AKP as a core principle in its practise, the field of molecular cell biology largely lacks a phylogenetic framework to guide its investigations. Thus, one weakness of many studies that aim to establish the function of newly discovered complexes or proteins is that broad conclusions are often based upon findings from a single model organism. In this sense, although studies on models like *S. cerevisiae* have a profound role in providing functional insight to evolutionary biologists, the field of cell biology tends to overlook the vast diversity of life upon which biological inquiry is based. The majority of eukaryotes are unicellular, and this largely microbial diversity can now be grouped into five major groups, within which traditional multicellular groups (animals, land plants and fungi) are embedded (Fig. 1) [15]. The five major eukaryote groups include: opisthokonts (e.g., animals, fungi, and their close unicellular relatives), Archaeplastida (e.g., red and green algae, and land plants), amoebozoans (e.g., giant amoebae like *Amoeba proteus*, slime moulds like *Dictyostelium discoideum*, and pathogens like *Entamoeba histolytica*), excavates (e.g., the pathogens *Giardia intestinalis* and *Trypanosoma brucei*, as well as free-living *Naegleria gruberi*), and SAR, which comprises three major and diverse protist groups: stramenopiles (e.g., kelps, diatoms, and oomycetes like *Phytophthora infestans*), alveolates (e.g., ciliates like *Paramecium tetraurelia*, apicomplexans like the malaria parasite *Plasmodium falciparum*, and dinoflagellates), and rhizarians (e.g., foraminiferans radiolarians, and cercozoans). Since the majority of our model organisms are animals or fungi and these groups are contained within a single eukaryote major clade (opisthokonts), we know comparatively little about the cell biology of organisms in non-opisthokont lineages (plants and specialized parasites notwithstanding). The only way to remedy this deficiency is to begin studying cell biology from different points in the tree of life. This approach is a main goal of comparative cell biology and constitutes a central component of a newly emerging field called evolutionary cell biology.

Evolutionary cell biology aims to link cell biology to evolutionary biology and cellular diversity [16]. The model systems developed for cell biological research (namely, *S. cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and mammalian tissue culture) have been extremely successful tools for studying the intricacies of cellular mechanisms, biochemistry, and developmental biology in animals and fungi. Other models from different lineages have developed more slowly (*T. brucei*, *T. thermophila*, *D. discoideum*, and *Chlamydomonas reinhardtii*) (See Fig. 1). Thus, our knowledge of the cellular mechanisms in the fungal and animal lineages has developed much faster than our knowledge about the diversity of eukaryotic cells and their inner workings.

At the heart of comparative cell biology lies the methods of comparative genomics, which are driven and informed by questions and knowledge derived from the field of cell biology. We now have a broader sampling of sequenced genomes from across the diversity of eukaryotes that allows us to better discern which gene families are ancient and conserved in distantly related eukaryotes and therefore putatively represent genes of evolutionary and functional importance. By using the comparative approach to compare genomic sequences that determine the intricacies of the cell, both evolutionary and functional inferences can be made. This has successfully been applied in the past for diverse cellular systems, e.g., AP5 and TSET in membrane trafficking [17,18], and FtsZ and Min proteins in mitochondrial division [19], and more recently also for major protein complexes with roles in mitochondrial biogenesis (e.g., ERMES and MICOS) [20–22]. Here, in the spirit of complementing our previous efforts in reconstructing the integrative evolution of mitochondria, we hope that by providing a comparative overview of ERMIONE we will shed light not only on its evolutionary history but also how it functions in organisms other than *S. cerevisiae*. We end by reconstructing the ancestral state of the ERMIONE and its interactors in the eukaryote cenancestor (i.e., the last common ancestor of all eukaryotes) and compare the ancestral ERMIONE to that of *S. cerevisiae* and the remnants of ERMIONE present in the extremely reduced mitochondria of anaerobic protists.

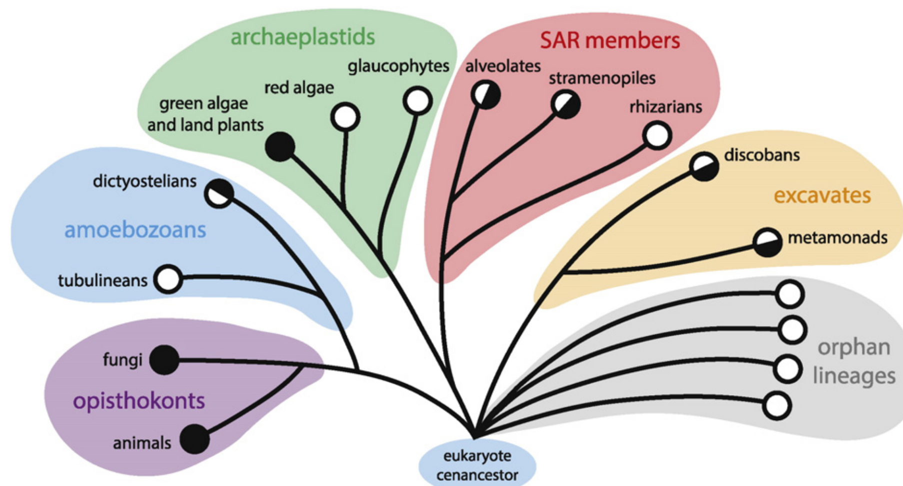

Fig. 1. Schematic representation of the phylogenetic relationships between the major eukaryote groups according to [15]. Most extant eukaryotes can now be grouped into five major supergroups: opisthokonts, amoebozoa, archaeplastids, SAR, and excavates. Some minor eukaryote lineages have yet to be unambiguously grouped into a higher order clade (orphan lineages). Black circles at the edge tips represent groups with canonical model organisms (HeLa cells, *Drosophila melanogaster*, and *Caenorhabditis elegans* among animals, *Saccharomyces cerevisiae* in the fungi, and *Arabidopsis thaliana* as a plant). Semi-filled circles represent eukaryote lineages for which alternative model organisms have been developed [*Toxoplasma gondii* (alveolates); *Phaeodactylum tricornutum* (stramenopiles); *Trypanosoma brucei* (euglenozoans); *Giardia intestinalis*, and *Trichomonas vaginalis* (metamonads)]. White circles are groups for which experimental models still need to be established.

3. ERMIONE functional modules

The ERMIONE includes many mitochondrial proteins in several complexes. These proteins have several physical and genetic interactors. Thus, ERMIONE and its interactors span from ERMES in the ER to the mitochondrial nucleoid in the mitochondrial matrix [6,7,23]. Given that ERMIONE is a network of interacting proteins at the mitochondrial envelope, it encompasses and interacts with complexes and proteins with several distinct functions in mitochondrial biogenesis. These include heterotypic membrane tethering, lipid homeostasis, membrane organization and architecture, and protein import. In order to ease our following discussion of ERMIONE and its interactors, we have loosely broken them into several different functionally-defined modules: (1) phospholipid (PL) transport between MIM and MOM (Ups1, Ups2, Ups3, and Mdm35), (2) mitochondrial MCS tethering (ERMES, vCLAMP, EMC, Lam6, Vps13), (3) MIM organization (Mdm31, Mdm32, Prohibitins, and MICOS), and (4) mitochondrial protein import and membrane permeability (TOM, TIM, and SAM complexes, MIA, and porins) (Not discussed here). In this review we will focus on the first three modules because they more directly involve the cell biology of lipids.

4. PL transport between MOM and MIM (Ups1, Ups2, Ups3, and Mdm35)

Before diving into the ERMIONE, we must first take into account the special status of mitochondrial membranes. Mitochondrial membranes do not generally partake in normal vesicular membrane trafficking (but see [24–27]) and thus their lipids must be acquired by other transport mechanisms [28,29]. Because of this, mitochondrial membranes are unique in their lipid composition. Although they contain both phosphatidylserine (PS) and phosphatidylcholine (PC), which are present in many other membranes, they contain a high proportion of non-bilayer phospholipids like phosphatidylethanolamine (PE) and the mitochondrion-specific phospholipid cardiolipin (CL). CL is unique to mitochondrial and bacterial membranes, and, along with PE, is thought to be instrumental in forming and maintaining cristae architecture and respiratory competence by stabilizing respiratory complexes [29]. Although their individual roles are unclear, it is clear that PE and CL are important as depletion of both is lethal in *S. cerevisiae* [30].

CL is synthesized in the MIM from phosphatidic acid (PA) that is imported from the ER at ER–mitochondrion MCSs. Mitochondria also synthesize PE from ER-derived PS. Thus, PS must be transported from the ER to the MIM where Psd1 (PS decarboxylase) decarboxylates PS to form PE which is then transported back to the ER for export to other cellular membranes. PE in the ER is converted to PC which is important in all membranes and constitutes the major component of mitochondrial membranes. Thus, PC follows a convoluted path during its synthesis and final mitochondrial destination. PC is derived from PS synthesized in the ER, that is then transported to the MIM, where it is converted to PE, transported back into the ER, then converted into PC, and finally transported back to mitochondrial membranes [29].

Mitochondrial membrane biogenesis depends on the correct insertion of membrane lipids in both the MIM and MOM. ER–mitochondrion MCSs ensure lipids are transferred to the MOM, but once there other transport mechanisms guarantee lipids are properly transferred from the MOM to the MIM. This is essential for the synthesis of PE and CL from the precursor PS in the MIM and to ensure that the MIM outgrows the MOM and gives rise to membrane invaginations to create cristae. Intermembrane space (IMS) proteins that have been implicated in the transfer of lipids between the MIM and the MOM in *S. cerevisiae* include Ups1, Ups2, Ups3 and Mdm35.

Ups proteins form a complex with Mdm35 and require Mdm35 for their efficient import and maintenance in the IMS [31,32]. Mdm35 is required for Ups protein function as cells lacking Mdm35 resemble *Δups1Δups2Δups3* triple knockouts [33]. Mutants lacking Ups1 are deficient in CL, while mitochondria in mutants lacking Ups2 are deficient in PE. Ups1-Mdm35 specifically facilitates the binding and transport of PA from the MOM to the MIM [34]. Due to the reciprocal phenotypes seen in mutants lacking Ups1 or Ups2, an attractive model would be that Ups1-Mdm35 catalyses transfer of PA from MOM to MIM, thereby increasing CL production, while Ups2-Mdm35 catalyses transfer of PS from MOM to MIM thereby increasing PE production. The combination of these two transfer mechanisms would therefore keep MIM composition in equilibrium. However, this does not seem to be the case as PS synthesis is not affected in cells lacking Ups2 and the specific action of Ups2 remains unclear [35].

Recent findings have shed light on the biochemical features of the Ups1-Mdm35 complex that contribute to PA extraction and transport between mitochondrial membranes [36–38]. These findings should help in understanding the mechanism of Ups2-Mdm35 and similar complexes. Interestingly, Tamura et al. [33] showed that ERMES mutants and mitochondria lacking Ups1 all had similar CL deficiencies. The deletion of an ERMES gene in Δups1 cells resulted in slightly worsened phenotypes, whereas the deletion of ERMES genes in Δmdm35 or Δups2 cells partially suppressed ERMES mutant growth and lipid defects. These interactions point to the central role of MCSs and ERMES in lipid transport. These interactions demonstrate that Ups proteins work in a parallel pathway to ERMES and affect mitochondrial lipid transport by a different mechanism.

Surprisingly, organisms outside opisthokonts (animals, fungi, and their close protist relatives) have only a single kind of Ups protein along with Mdm35 (Fig. S1) with the exception of excavates, which appear to lack these proteins altogether (although the inability to identify these proteins in this group may be due to limited sampling or short sequence length). Ups1 and Ups2 therefore appear to be the result of a lineage-specific duplication prior to the divergence of opisthokonts. Ups3 is the result of a duplication of Ups2 in the lineage leading to *S. cerevisiae* (Fig. S1). Our BLAST results suggest that most Ups proteins outside opisthokonts have a greater sequence similarity to opisthokont Ups2 than Ups1 (Fig. S1). The fact that only a single Ups protein exists in several eukaryotes outside opisthokonts suggests that, in these organisms, Ups proteins may function as promiscuous bidirectional lipid transporters. In order to better understand Ups function it is necessary to study a tractable model organism outside of opisthokonts that contains a single Ups protein (e.g., *A. thaliana*).

5. MOM-endomembrane MCS tethers (ERMES, EMC, vCLAMP, Lam6, and Vps13)

5.1. The ER–Mitochondria Encounter Structure (ERMES)

In *S. cerevisiae* ERMES tethers the ER to the MOM by the action of four core proteins [39]; Mmm2 (Mdm34) and Mdm10 localize to the MOM, Mmm1 is a single-pass ER-membrane protein, and Mdm12 is a cytosolic protein that connects the ER and MOM components. Mdm12, Mmm1, and Mmm2 are paralogues, which all contain SMP (synaptotagmin-like-mitochondrial-lipid binding protein) domains [40,41]. Mdm10 is a MOM β-barrel protein that is distantly related to two other mitochondrial β-barrels, Tom40 and porin [20]. A fifth component called Gem1 has been proposed to be part of ERMES. Gem1 is a transmembrane protein consisting of two calcium-binding EF-hand domains flanked by two Rho-like GTPases [42].Gem1 has been suggested to have a regulatory role in ERMES function [43,44]. The four core ERMES proteins generally co-occur and their phylogenetic distribution reveals that the complex is ancient, and probably ancestral to eukaryotes, despite being secondarily lost in multiple lineages [20,21] (Fig. 2). Conversely, Gem1 is nearly ubiquitous in eukaryotes and therefore is not dependent on ERMES for its function in most organisms [45]. Several excellent reviews on ERMES function have been published and the reader can refer to them for more details [46–48].

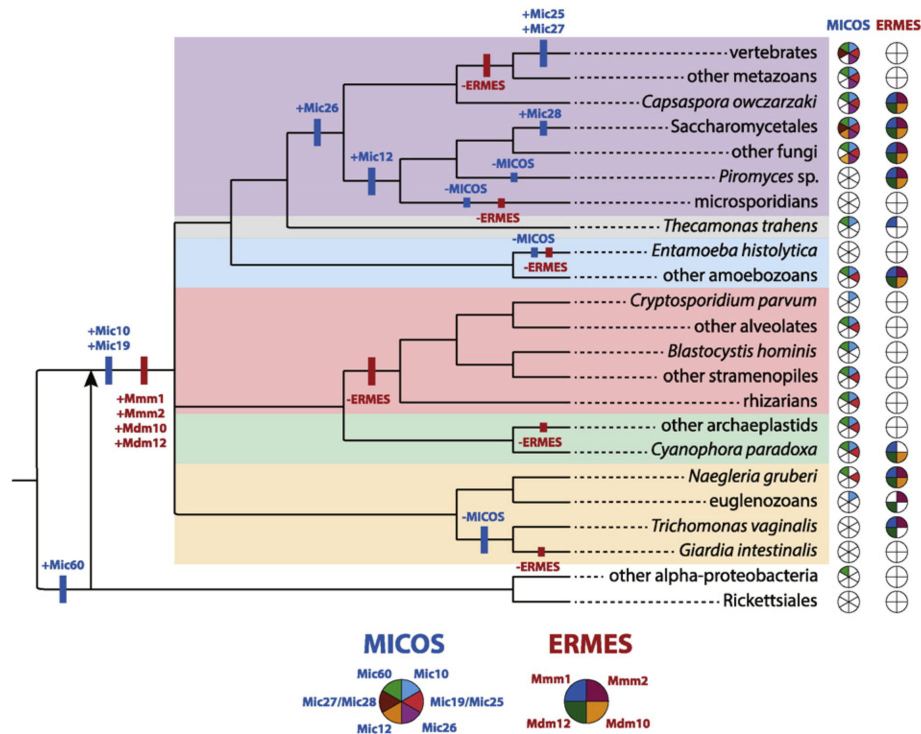

Fig. 2. The evolutionary history of MICOS and ERMES. MICOS has its origin in the probably free-living α-proteobacterial ancestor of mitochondria. ERMES has its origin at the root of eukaryotes. In the eukaryote cenancestor, but before the radiation of the major eukaryote supergroups, MICOS expanded in subunit number by acquiring Mic10 and Mic19. MICOS later expanded in opisthokonts (e.g., Mic26), fungi (e.g., Mic12) and vertebrates (e.g., Mic25 and Mic27). Both MICOS and ERMES have been secondarily lost in some eukaryote lineages. While MICOS has only been lost in those eukaryote lineages that have also lost cristae (and the respiratory chain) in their adaptation to an anaerobic lifestyle, ERMES has been lost in multiple occasions, including the extremely reduced mitosomes (e.g., *E. histolytica* and *G. intestinalis*), and some aerobic mitochondria (e.g., SAR members, most archaeplastids and metazoans). This implies ERMES has been replaced by an alternative ER-MOM tether in these aerobic lineages.

Although well-studied, the primary function of ERMES has remained elusive. Several distinct functions have been attributed to ERMES, such as ER–mitochondria lipid transfer [39,49], mitochondrial protein import [50–53], mitophagy [54,55], mitochondrial division and nucleoid segregation [56], and overall mitochondrial morphology determination [57–62]. However, studies have presented conflicting results regarding a conserved ERMES function. Some have even cast doubt on its role in lipid transfer from ER to mitochondria [63].

In order to identify the primary conserved function of a protein or protein complex, shared phenotypes of orthologue mutants in different species can be used to infer ancestral functions. In addition to *S. cerevisiae*, ERMES components have been functionally investigated in the filamentous ascomycetes *Neurospora crassa*, *Aspergillus nidulans*, and *Podospora anserina*. In *S. cerevisiae*, ERMES mutants exhibit pleiotropic effects (i.e., multiple different phenotypes), which has led to the multiple functions attributed to ERMES mentioned above. Pleiotropic effects were also observed in *N. crassa* ERMES mutants [52,53]. Only Mdm10 mutants were investigated in *P. anserina* and *A. nidulans* [57,59]. The phenotypes shared by all ERMES mutants studied in all organisms include growth defects (minor in filamentous ascomycetes) and mitochondrial morphology defects (less severe in filamentous ascomycetes). More thorough investigations into ERMES mutants were conducted in *S. cerevisiae* and *N. crassa*. Further similarities were seen as ERMES mutants in both of these fungi exhibit mitochondrial phospholipid defects (although only in MOM phospholipid to protein ratios in *N. crassa*), and defects in MOM protein import and assembly [52,53]. However, import and assembly of MOM proteins is known to be dependent on certain phospholipids [64–67], which suggests that the protein import defects seen in ERMES mutants are secondary effects caused by defects in phospholipid transport.

The similarities seen in mutant phenotypes support the notion that ERMES has a similar fundamental conserved role in different fungi. However, in *S. cerevisiae*, ERMES mutants also exhibit several other phenotypes including an inability to grow on non-fermentable carbon sources, a propensity to lose mtDNA, and defects in mitochondrial inheritance and motility [58,60–62,68–70]. These data suggest that ERMES has gained additional importance in *S. cerevisiae* compared to filamentous fungi and that its function as an ER-MOM tether might be recruited to aid in other important cellular processes (e.g., mitochondrial division/fission, nucleoid segregation and overall morphology).

With the many pleiotropic effects seen in ERMES mutants, it is understandable that it has been difficult to pinpoint the primary (and likely ancestral) role of ERMES. A large number of studies have implicated ERMES in mitochondrial lipid homeostasis [33,39,71]. Some of the results seemed to be in conflict with one another [63] and the explanations for these incongruences still need to be worked out. ERMES members exhibit genetic interactions with several components of the mitochondrial lipid biosynthesis and transport pathways. These interactions are complex and the nature of the relationships is not always clear. Nonetheless, convincing genetic, cell biological, and biochemical evidence has amassed that suggests ERMES does indeed play a role in lipid transfer between the ER and mitochondrial outer membranes in *S. cerevisiae* [48]. Very recent biochemical evidence building from other studies on SMP-domains containing extended synaptotagmins/tricalbins (proteins similar to ERMES subunits) [72] shows that the SMP domains of ERMES components are capable of preferentially binding phosphatidylcholine (PC) [49]. Moreover, AhYoung et al. showed that this is likely evolutionarily conserved by demonstrating that not only Mmm1 and Mdm12 from *S. cerevisiae*, but also Mdm12 from D. discoideum can bind bacterial phospholipids. They also demonstrate that both Mmm1 and Mdm12 in *S. cerevisiae* have a strong preference for PC. Finally, they provided evidence for the stoichiometry of ERMES, suggesting that two Mmm1 molecules bind two Mdm12 molecules, thus corroborating earlier evidence from *N. crassa* that showed that Mmm1 proteins in the class Sordariomycetes form dimers in the ER by via cysteine bonding [53]. Taken together, these data definitively

point towards a primary role for ERMES in lipid transfer between the ER and mitochondria.

From these comparative data an evolutionary hypothesis can be drawn: the ancestral ERMES evolved for proper mitochondrial lipid transfer (likely PC) between ER and mitochondrial membranes. This ancestral function secondarily aids in additional processes, such as membrane protein assembly and the maintenance of mitochondrial morphology. Corroboration of this hypothesis can be attained by further investigating the function of ERMES components in alternative model organisms like *D. discoideum*, *Capsaspora owczarzaki* (a single-celled relative of animals), or *Naegleria gruberi* (a free-living amoeboflagellate excavate).

5.2. The other mitochondrial MCS tethers

ERMES is now known to have the capacity to bind and transfer lipids from the ER to the MOM [49]. However, since this process still occurs in ERMES mutants and the many organisms that lack ERMES, other transport routes must exist. Indeed, ERMES has been shown to genetically interact with two other putative MCS tethers, EMC (ER-membrane complex) and vCLAMP (vacuole and mitochondrial patch) [73–75], as well as Vps13 and Lam6, two other proteins that facilitate interaction between apposing membranes [76–78]. Some of these MCS-mediating proteins/complexes might also be involved in the transport of lipids from endomembrane compartments to mitochondria, thus making ERMES partially redundant.

5.3. The ER-Membrane Complex (EMC)

The EMC is a conserved multi-protein complex (Emc1–7 and 10 in *S. cerevisiae*, Emc1–8 and 10 in most other organisms, and Emc1–10 in vertebrates [79]) that has been implicated in ER-associated degradation [80–82], autophagosome formation [83], and the assembly of multipass transmembrane proteins [84]. EMC has also been shown to be involved in the transfer of lipids from the ER to MOM and has been proposed to be tethered to mitochondria to carry out this function via interaction with Tom5 (of the TOM complex) in the MOM of *S. cerevisiae* [73]. Indeed, absence of EMC components significantly reduced PS and its derivative PE levels in mitochondria. Therefore, the EMC, regardless of its several proposed functions, seems to functionally overlap with ERMES in *S. cerevisiae*. Interestingly, while ERMES mutants in *S. cerevisiae* are viable, Lahiri et al. showed that cells lacking all of Emc1–3,5–6 and Mmm1 are not (i.e., ERMES-EMC double mutants are inviable). These authors further demonstrated that at non-permissive temperatures cells lacking these five EMC proteins, while harbouring a temperature-sensitive allele of *mmm1*, are defective in both ER–mitochondrion tethering and lipid transfer. These experimental results might indicate that the EMC constitutes a functionally redundant complement to ERMES.

The EMC is an ancient protein complex, as its current phylogenetic distribution shows that it is ubiquitous in eukaryotes, being absent in only a few species [79]. Curiously enough, although this protein complex has existed relatively stable for ~2 billion years, five of eight complex members can be deleted in *S. cerevisiae* without very noticeable negative phenotypes. Given the absence of ERMES in several major eukaryotic lineages, it is conceivable that the more evolutionarily conserved EMC is the ER–mitochondrion tether in charge of lipid transfer between the two organelles in ERMES-lacking lineages. However, the MOM linker to the EMC is a small TOM subunit Tom5, whose importance in various organisms is quite varied [85–88]. Additionally, Tom5 is phylogenetically restricted to opisthokonts [89] (however see [90]) and has limited sequence conservation thereby casting doubt on how EMC-mediated MCSs are formed outside the animal-fungal group. One possibility hinted at by Lahiri et al. (2014) is that the EMC physically interacts with additional components of the TOM complex. Thus, it remains possible that the EMC interacts with other TOM components or other MOM proteins/complexes in other organisms. Future confirmation of the role of the EMC in lipid transfer, and existence of additional EMC interactors at the MOM, as suggested by Lahiri et al. (2014), might establish the EMC as the default complex responsible for providing mitochondria with lipids from the ER. However, the possibility remains that since the EMC may be primarily involved in other processes, its genetic interaction with ERMES could be due to secondary effects.

5.4. The VaCuoLe And Mitochondria Patch (vCLAMP)

vCLAMP connects the vacuole (the *S. cerevisiae* lysosomal compartment) to mitochondria in *S. cerevisiae* [74,75]. It comprises two trafficking proteins, the tethering protein Vps39, and the GTPase Ypt7 (Rab7). vCLAMP was discovered simultaneously by two groups, but neither group identified the mitochondrion-localized factor responsible for tethering vCLAMPs to mitochondria. As mentioned above, there is a functional connection between vCLAMP and ERMES [91–93]. Both complexes are reciprocally regulated and respond to metabolic changes. For example, ER-mitochondrial contacts made via ERMES increase in number under respiration conditions, while those made by vCLAMP decrease. This pattern is reversed under fermentable conditions subsequent to the addition of glucose [75]. Under starvation conditions, both ERMES and vCLAMPs connections are lost [94]. Furthermore, the deletion of any ERMES component causes an expansion of vCLAMPs, while the absence of vCLAMPs causes ERMES MCS expansion [74].

The two known vCLAMPs proteins, Vps39 and Rab7, are involved in many membrane trafficking processes [46–48]. Of these two, Rab7 is present in virtually every eukaryote, while Vps39 is present in most eukaryotes but absent from alveolates [95,96]. It is currently unclear whether or not vCLAMPs form in organisms other than *S. cerevisiae*. vCLAMPs formation is regulated by the phosphorylation state of Vps39 [75]. Vps39 is phosphorylated in respiring conditions when vCLAMPs are downregulated. Thus, dephosphorylated Vps39 is responsible for maintaining vCLAMPs. Interestingly, the critical phosphorylation sites that determine vCLAMPs localization in *S. cerevisiae* (S246, S247, S249, and S250) are in a poorly conserved region of the protein (Wideman, unpublished observations). This means that if vCLAMPs exist in species other than *S. cerevisiae*, they will be regulated at different phosphorylation sites or by a different mechanism altogether. These observations, taken together with the current scarcity of knowledge about vCLAMP, might suggest that vacuole-mitochondrion MCSs mediated by vCLAMP constitute a *S. cerevisiae*- or fungal-specific phenomenon. Future efforts will reveal how functionally conserved vCLAMPs are across eukaryotic diversity.

5.5. Vps13

Vps13 is a hypothesised component of vCLAMPs [43]. It has orthologues in many organisms, in which it has been shown to be involved in autophagy (in *D. discoideum* and Hela cells) [97], phosphatidylinositol metabolism (in mammals) [98], phagocytosis (in the ciliate *Tetrahymena thermophila*) [99], and TGN-endosome cycling [100] and membrane dynamics during sporulation in *S. cerevisiae* [101,102]. Any two of Vps13, ERMES or Vps39 are required for *S. cerevisiae* survival, suggesting a possible functional connection between multiple MCS-tethering complexes [76]. Vps13 dynamically localizes to nucleus-vacuole junctions or vacuole-mitochondrion junctions, depending upon metabolic conditions [76]. This, in conjunction with observations on other MCSs and tethering complexes, suggests that MCSs have a dynamic nature and that they can be bypassed by modulation of other MCSs.

5.6. Lam6 (Ltc1)

In addition to phospholipids, sterols are also integral components of biological membranes important for their growth and proper function

(e.g., rigidity) in most eukaryotes. Like phospholipids, sterols also need to be transferred across different intracellular membranes, and this can also occur at MCSs [4]. Lam6 belongs to a paralogous set of StART-like domain-containing proteins present in *S. cerevisiae* that bind and facilitate the transport of sterols at MCSs [103]. Recent evidence implicates Lam6 in ER–vacuole and ER–mitochondrion MCSs by direct interaction with Vac8 and Tom70, respectively [77,78]. Loss of ERMES and either Tom70 or Lam6 causes severe synthetic growth defects [77,78]. Mdm10 mutants exhibit mitochondrial ergosterol defects in *S. cerevisiae* [104] and it will be interesting to see if the genetic interactions seen between ERMES and Lam6 are manifested due to synergistic defects in mitochondrial sterol trafficking. Lam6 overexpression causes expansion of ER–mitochondrion, nucleus–vacuole, and mitochondrion–vacuole MCSs [77,78]. Moreover, Lam6 directly interacts with ERMES [77,78], suggesting that Lam6 is involved in regulating the formation and expansion of MCSs. The StART domains present in Lam proteins are present across all eukaryotes [103], but the phylogenetic relationship among proteins containing the domain has not been intensely investigated. However, based on BLAST results (Wideman, unpublished observations) and preliminary phylogenetic analysis (58), Lam6 does not appear to have orthologues in *N. crassa*, *S. pombe*, early diverging fungi, or other eukaryotes.

6. Mitochondrial inner membrane organization (MICOS, Mdm31/32, Prohibitins)

6.1. MICOS

The Mitochondrial contact site and Cristae Organizing System (MICOS) is a large hetero-oligomeric complex of the MIM [6,105,106]. MICOS localizes to crista junctions (CJs) [107,108], which are neck-like membrane regions that link two functionally and structurally differentiated domains of the MIM: (a) the crista membrane (CM) harbouring the respiratory chain, and (b) the inner boundary membrane (IBM) that concentrates solute carriers and protein translocases next to the MOM [109,110]. By forming discrete foci at CJs [108,111], MICOS is believed to serve two primary functions in mitochondrial biogenesis, namely (i) the formation of CJs [112], and (ii) the tethering of MIM and MOM at sites of cristae invagination [106,113]. Synergistically, these functions contribute to the maintenance and stabilization of mitochondrial cristae (respiratory micro-compartments), and therefore the optimal respiratory output of mitochondria [114]. Although, mitochondrial cristae are largely determined by the formation of CJs and MCSs at the mitochondrial envelope through MICOS, there are additional factors responsible for the morphogenesis of cristae [115,116]. In *S. cerevisiae*, these include the MIM dynamin Mgm1 [117,118], which presumably aids in the formation of CJs (in addition to mitochodrial fusion which is its primary function), and the ATP synthase complex (as dimer and oligomers), which serves as a main morphogenetic factor by curving cristae at their tips [119, 120]. Moreover, although MICOS acts upstream in the development of cristae, the assembly and MIM distribution of respiratory complexes is affected by the absence of MICOS [111]. The proper function of the respiratory chain depends on the normal development of mitochondrial cristae, which is mostly determined by MICOS and the ATP synthase complex.

In *S. cerevisiae*, MICOS comprises six different subunits: Mic10, Mic12, Mic19, Mic26, Mic28 and Mic60 [116,121]. Functional dissection of MICOS has shown that Mic10 and Mic60 represent the core components of the complex; their disruption led to the most deleterious phenotypes, and the virtual absence of CJs and mitochondrial cristae [6,105–107,112]. The different MICOS subunits have non-redundant functions that contribute to the complex mechanism by which MICOS controls mitochondrial cristae development [111]. Among these, Mic60 is the subunit directly acting as a tether by interacting with Sam50 and Tom40 at the MOM [6,105,113,122,123]. By undergoing homotypic interactions, it is hypothesised that Mic60 also contributes to the stabilization of CJs [112]. The strong negative curvature at CJs is introduced by Mic10 oligomers, whose structural topology and function is similar to that of reticulons [124,125]. The apolipoproteins Mic26 and Mic28 bind cardiolipin and are believed to segregate this important mitochondrial structural lipid between the IBM and the CM [111,126]. Moreover, Mic19 is a soluble intermembrane subunit that aids in the assembly of the complex by bringing together MICOS subcomplexes [111]. Human mitochondria exhibit a functionally equivalent MICOS of similar structural composition. However, it differs from *S. cerevisiae*'s MICOS by lacking Mic12 (but see [127]), but containing Mic25 (a paralogue of Mic19) and Mic27 (a paralogue of Mic26) [22,121]. These observations indicate that the basic machinery that control the determination of mitochondrial ultrastructure is conserved between animals and fungi.

Our recent evolutionary analysis of MICOS revealed that this mitochondrial protein complex is not only conserved between animals and fungi (opisthokonts), but also among all cristate eukaryotes [22]. More specifically, Mic60 and Mic10 (MICOS core), and to a lesser extent Mic19, are phylogenetically widespread, whereas the other subunits are restricted to either animals or fungi (Fig. 2). Moreover, our finding of a Mic60 homologue among the bacterial progenitor group of mitochondria (α-proteobacteria) led us to infer that MICOS was acquired endosymbiotically when the ancestor of mitochondria was internalized. These observations suggested to us that the eukaryote cenancestor already possessed a relatively complete MICOS complex to organize cristae, and that its core Mic60 was directly inherited from the bacterial endosymbiont that gave rise to mitochondria [22].

More recently, we also investigated the functional evolution of MICOS by analysing the phylogenetic distribution of its physical interactors [128]. We concluded that the tethering function of Mic60 was probably already present in the bacterial ancestor of mitochondria, where MCSs between the outer and cytoplasmic membranes were created by the interaction of Mic60 and BamA (a Sam50 homologue). After the origin of mitochondria, but prior to the diversification of all modern eukaryotes, MICOS would have also acquired physical interactions with other complexes (Mia40 and TOM) to aid in the proper import of nuclear-encoded mitochondrial proteins [128]. This study strengthened the emerging view that protein complexes at the mitochondrial envelope have become more integrated throughout evolution in order to cooperate in the biogenesis of mitochondria and regulate the integration of their diverse functions with the host cell (e.g., as ERMIONE) [8,129–131].

As a MIM-MOM tether, MICOS has also been thought to create tight membrane contacts that indirectly facilitate the transport of lipids from MOM to MIM. Indeed, among the multiple genetic interactors of MICOS, ERMES subunits represent some of the strongest [6,8]. ERMES tethers the ER membrane to the MOM and one of its main functions is to aid in the import of lipids from the ER to the mitochondrial outer membrane. Next, by bringing together the two mitochondrial enveloping membranes MICOS may indirectly facilitate lipid transfer from the MOM to the MIM. This therefore suggests a functional interaction between two different MCS-tethering complexes. The functional interaction between MICOS and ERMES, and their (indirect) physical interaction through different protein/complexes at the mitochondrial envelope [e.g., TOM (Tom7) and SAM (Mdm10)] concentrates several mitochondrial biogenic processes [8]. In this way, membrane growth through lipid insertion (facilitated by ERMES, Ups-Mdm35, and MICOS) is coordinated with protein translocation and insertion into membranes (performed by TOM and TIM) at sites of respiratory complex assembly and cristae development (at or immediately next to CJs). Thus MICOS can be seen as the central scaffold that brings together the network of components responsible for the imposition of membrane structure and localization of lipids, proteins and processes at the mitochondrial envelope (the ERMIONE).

6.2. The ERMIONE link to the nucleoid

In addition to its essential roles in lipid and protein import into mitochondria, ERMIONE performs a more general function in mitochondrial

biogenesis [132]. As a large network that spans the ER, MOM and MIM, ERMIONE also reaches the matrix by contacting the mitochondrial nucleoid (a complex of protein-associated coiled mtDNA). Indeed, ERMES and MICOS components (e.g., Mmm1 [61], Mmm2p [62], Mic10, Mic12, Mic19, Mic28, and Mic60 [23,133]), as well as Mdm31, Mdm32 [134] and probably other Mdm proteins, are involved in the maintenance of mtDNA, as their disruption leads to mtDNA loss and instability. In *S. cerevisiae*, ERMES localizes to distinct punctae adjacent to mitochondrial nucleoids that are undergoing active replication [61,62,68,135]. Similarly, MICOS has also been shown to localize adjacent to nucleoids in *S. cerevisiae* [133]. Moreover, super-resolution microscopy has revealed the tight association between nucleoids and crista membranes in mammalian cells [136,137]. Finally, the core MICOS subunit Mic60 has been shown to physically interact with the mammalian nucleoid proteins TFAM (the main nucleoid protein, which acts both as a condenser of mtDNA and a transcription factor), TFB2M, and TFB1M, thus directly linking MICOS to mtDNA organization and replication in humans [138]. These interactions between ERMES and MICOS with mitochondrial nucleoids suggest an important function of ERMIONE in mtDNA inheritance.

Mitochondrial division (or fission) is important for mitochondrial proliferation, distribution to sites of high energy demand and the isolation of defective mitochondria for subsequent mitophagy [139]. In both *S. cerevisiae* and humans, mitochondria divide with the assistance of the ER, in a process termed ER-associated mitochondrial division (ERMD) [56]. The ER marks the sites of division, and initiates mitochondrial constriction required for the subsequent assembly of dynamin-related proteins (DRPs) that end up in complete scission into two daughter mitochondria. It is well known that in *S. cerevisiae* the tethering between ER and mitochondria during ERMD is mediated by ERMES [140]. Interestingly, as already mentioned, ERMES associates with actively replicating nucleoids, strongly interacts with MICOS, and both complexes are spatially closely associated with nucleoids as distinct foci in mitochondrial networks. It seems therefore that during ERMD, ERMIONE anchors the mtDNA nucleoid to the MIM through the action of ERMES and MICOS. The superposition of mitochondrial division sites, ERMES, and nucleoids results in the accurate segregation and inheritance of nucleoids during division, and the production of two new mitochondria, each carrying a nucleoid at its tip.

Mammalian mitochondria also divide with the assistance of the ER [140]. However, the protein complex mediating ERMD in mammals remains unknown. If the EMC acts as a tether in animals, then it emerges as a possible candidate to mediate this function, though no suggestions have been made in this regard. Although the factors involved might be different, it seems that there has been a functional continuity in mechanisms ensuring nucleoid segregation with mitochondrial division. The evolution of ERMIONE in mitochondria made this mechanistic continuity possible, and at the same time, coordinated mitochondrial behaviour with other eukaryotic organelles (e.g., the ER).

6.3. Prohibitins

Prohibitins are a family of well conserved proteins comprising Phb1 and Phb2. Although the exact mechanism and function of prohibitins are unknown, they are thought to control MIM organization and integrity by acting as protein and lipid scaffolds [141]. Phb1 and Phb2 interact with one another and form large ring-like multimeric complexes 200–250 Å in diameter [142]. A main function of prohibitins seems to be lipid organization at the MIM, as there is a relationship between prohibitin function and CL and PE levels in mitochondria, as well as interactions with lipid biosynthesis genes [71]. Moreover, prohibitins might also play a regulatory role in MIM organization, as they are required for the proper formation of cristae via regulation of OPA1/Mgm1 proteolytic processing by an m-AAA protease [143]. Like MICOS, prohibitins have been implicated in nucleoid function and have been found to interact with nucleoid components in animals [144]. In *S. cerevisiae*, ERMES and MICOS components were found to be genetically linked to the prohibitin ring complex, suggesting they collaborate in a network responsible for mitochondrial lipid metabolism [6,71,145]. Phb1 and Phb2 were also shown to be high copy suppressors of *mdm10* and *mdm12* mutations [145]. The fact that overexpression of inner membrane proteins can rescue the sick phenotype of ERMES mutants and the multiple functional associations of prohibitins with proteins at the mitochondrial envelope are indicative of the complexity of ERMIONE and its many interactors.

Similar to MICOS, prohibitins are found universally in eukaryotes, except in organisms lacking cristae (with the exception of *Piromyces* sp. which contains a single highly degenerate prohibitin) (Fig. S1). Both Phb1 and Phb2 were identified in all cristate organisms investigated with the exception of the red alga *Chondrus crispus*. The lack of Phb1 in *C. crispus* likely reflects the incompleteness of the genome rather than the actual lack of the gene. The ubiquity of prohibitins indicates that both Phb1 and Phb2 were present in the eukaryote cenanscestor and were likely dependent upon one another prior to the diversification of eukaryotes.

6.4. Mdm31 and Mdm32

The inner membrane proteins Mdm31 and Mdm32 are involved in the regulation of mitochondrial morphology and mtDNA inheritance in *S. cerevisiae* [134]. However, the precise mechanism by which these proteins exert these functions remains a mystery, and they have not been shown to be part of any major protein complex involved in mitochondrial membrane organization. Mdm31 and Mdm32 are paralogous proteins that genetically interact with ERMES in *S. cerevisiae* [146]. Any ERMES deletion combined with the deletion of either Mdm31 or Mdm32 results in synthetic lethality [134]. While both Mdm31 and Mdm32 are necessary for proper mitochondrial morphology in *S. cerevisiae* [134], other fungal species contain only one paralogue (Fig. S1). Mdm32 is the most divergent paralogue, as *S. cerevisiae* Mdm31 is much more similar to the *N. crassa* Mdm31/32 protein than to *S. cerevisiae* Mdm32 (Wideman unpublished observations). Mutants lacking Mdm31 exhibit CL deficiencies comparable to ERMES mutants [33]. Similar to what was observed for Phb1 and Phb2 [145], the overexpression of Mdm31 partially rescues the growth, lipid, and mitochondrial morphology defects in ERMES mutants even though ERMES foci are not restored [33].

S. cerevisiae cells lacking Mdm31 or Mdm32 are resistant to the drug nigericin, providing evidence that these proteins are involved in mitochondrial cation homeostasis [147]. Recent comparative data in the fission yeast *Schizosaccharomyces pombe* confirms that its single Mdm31/32 protein is a mitochondrial protein primarily involved in mitochondrial cation homeostasis as mdm31 mutants are resistant to valinomycin and nigericin [148]. Interestingly, the *S. pombe* mutant did not have altered mitochondrial morphology or lipid profiles. The *S. pombe* data suggests that, similar to ERMES mutants, many of the phenotypes seen in *S. cerevisiae* Mdm31/Mdm32 mutants are derived.

We were able to identify Mdm31 in most fungi (excluding the microsporidians), *Fonticula alba*, *Thecamonas trahens*, and all amoebozoans (excluding *Entamoeba histolytica*). This taxonomic distribution overlaps with the distribution of ERMES with the exception that ERMES is also found in several excavates and some unicellular holozoans [20,21]. This demonstrates that Mdm31 is not an ancestral eukaryotic protein but that it evolved after the major radiation of eukaryotes but before the divergence of opisthokonts from amoebozoans.

7. Evolutionary history of ERMIONE

ERMIONE encompasses a number of components, has numerous interactors, and requires complex interactions among them for the proper biogenesis of mitochondria in *S. cerevisiae*. Inevitably, its composition varies across eukaryotes, as not every ancestral component has

been retained, and some components have evolved in some lineages uniquely. The multiplicity of factors involved makes the assessment of ERMIONE evolution a challenging task. However, taking up the challenge, given our current understanding of ERMIONE in *S. cerevisiae* as a model organism (Fig. 3A), will shed light on the early evolution of the eukaryotic cell and the process by which mitochondria were transformed from a bacterial endosymbiont into a highly integrated eukaryotic organelle. By analysing the phylogenetic distribution of ERMIONE

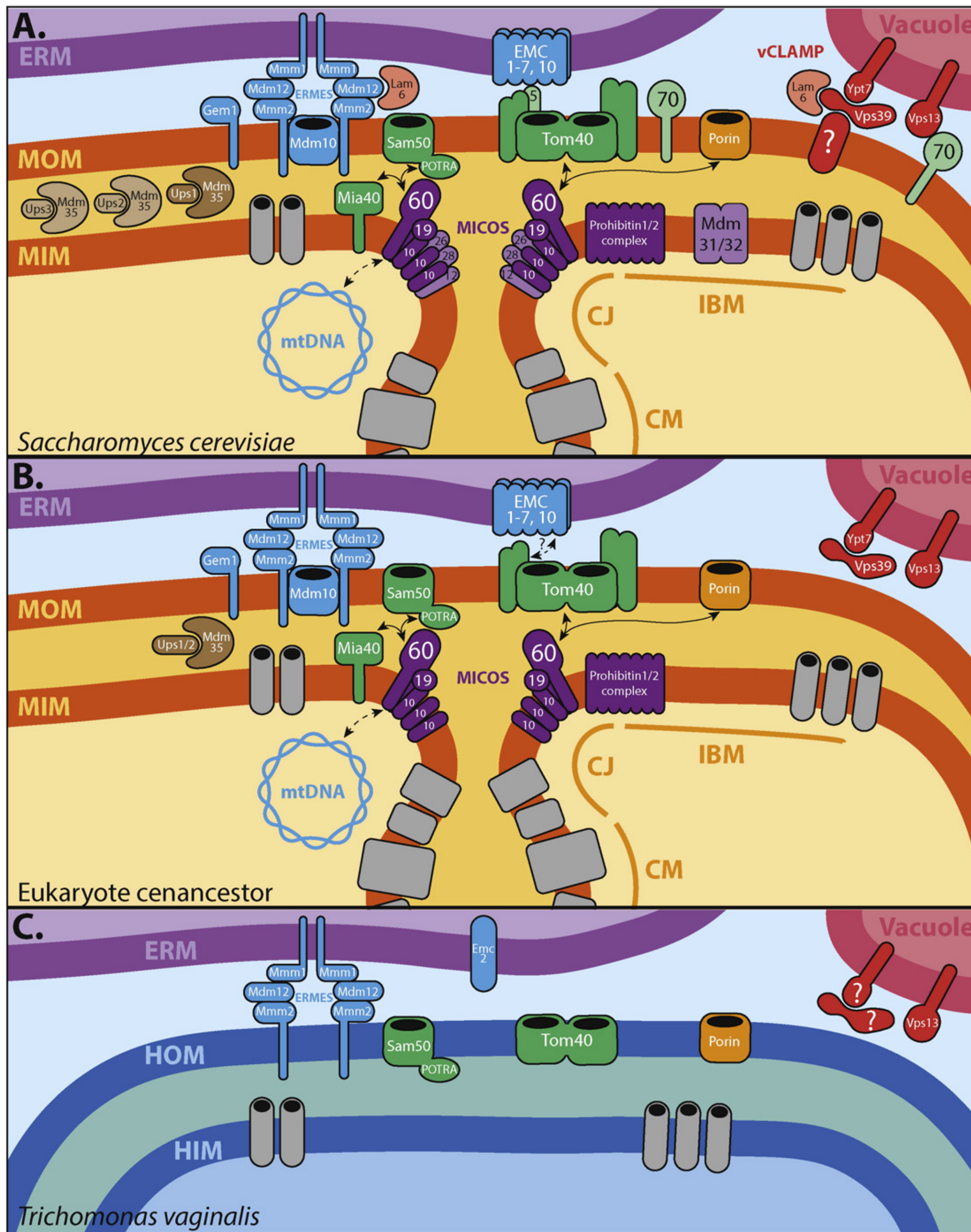

Fig. 3. The ancestral ERMIONE shown in contrast to extant organisms. ERMIONE and interactors in A. *S. cerevisiae*, B. the eukaryote cenancestor, and C. a secondarily reduced anaerobic mitochondrion, the hydrogenosome of *T. vaginalis*. Major mitochondrial envelope complexes are coloured according to their function. Protein complexes involved in MIM architecture and organization (MICOS and the prohibitin ring complex) are in purple; those that participate in protein import (TOM, SAM and MIA) are in green. ER-MOM tethering complexes (ERMES and EMC) are in blue, whereas vCLAMP components, which also function as a vacuole-mitochondrion tether in *S. cerevisiae*, are in red. Porin is in orange, and the lipid transfer/metabolism proteins Ups and Mdm35 are in brown. The MIM respiratory chain and solute carriers are shown in grey. The *S. cerevisiae* mitochondrion expresses the most complex ERMIONE, as it has been characterized and defined in this organism. *S. cerevisiae* has a functionally characterized vacuole-mitochondrion tether (the vCLAMP), Tom5, Tom70, Lam6, Mdm31, Mdm32, Ups2, Ups3, Mic12, Mic26, and Mic28 as additional ERMIONE components. In contrast to mammalian mitochondria (see section The ERMIONE link to the nucleoid), nucleoid proteins that directly interact with ERMIONE have not been investigated in *S. cerevisiae*. Light colours indicate differences between the *S. cerevisiae* and cenancestor models. The *T. vaginalis* hydrogenosome has a drastically simplified ERMIONE. It has lost cristae, and with them the respiratory chain, MICOS and prohibitins. It has furthermore lost Mia40, Ups proteins, Mdm35, and most EMC proteins. Interestingly, it still retains an almost complete ERMES complex (only lacking Mdm10), suggesting an active role of the ER in hydrogenosomal membrane lipid biosynthesis. Further reduction has occurred in organisms such as *G. intestinalis* which contains mitosomes instead of hydrogenosomes. The *G. intestinalis* further lacks ERMES and identifiable porin and Sam50. Arrows indicate direct physical interactions between proteins. Dashed arrows imply hypothetical interactions, for which direct evidence is lacking. ERM, endosplasmic reticulum membrane; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; IBM, inner boundary membrane; CJ, crista junction; CM, crista membrane; HOM, hydrogenosomal outer membrane; HIM, hydrogenosomal inner membrane.

components in modern groups we are able to infer the composition of ERMIONE in the eukaryote cenancestor and how it has changed since the origin of eukaryotes. Finally, by extrapolating the current functional knowledge on ERMIONE, and assuming functional conservation based on the phylogenetic co-occurrence of interaction partners, our comparative approach allows us to construct a historical narrative to explain the integrative evolution of mitochondria.

Mitochondria evolved approximately 1.5–2 billion years ago from an α-proteobacterium, whose exact phylogenetic identity remains controversial. The modern mitochondrial proteome is a mixture of both bacterial and eukaryotic proteins; however, among ERMIONE constituents, only Sam50 and Mic60 (MICOS′ core) can so far be traced back to the α-proteobacterial ancestor of mitochondria. Congruent with the idea that MICOS constitutes the scaffold structure that keeps ERMIONE together, it is its most ancient component. ERMIONE therefore finds its precursor as Mic60 in α-proteobacterial envelopes, where, in partnership with BamA (Sam50 homologue in eubacteria), most probably created MCSs between the cytoplasmic membrane and the outer membrane, and anchored intracytoplasmic membranes to the cytoplasmic membrane.

After internalization, the endosymbiont gradually became more integrated into the economy of its host cell. Components of the ancestral protein export machinery of the α-proteobacterium were exapted and, with the addition of new eukaryotic inventions, transformed into the protein import machinery that defined the new organelle. Two important ERMIONE components appeared during this transformation: (i) TOM, the MOM translocator of cytosol-translated proteins which evolved from β-barrel precursors, and (ii) MIA, the oxidative import (and disulphide bond-introducing) machinery at the IMS, that replaced the ancestral analogous bacterial system at the periplasm. Soon after, MICOS established interactions (via Mic60) with both complexes (Tom40 and Mia40) to increase the efficiency of protein import (see [128]), thereby increasing control over mitochondrial biogenesis.

The increased specialization and reduction of the endosymbiont as a respiratory ATP-producing organelle [after the acquisition of AAC (ADP/ATP carrier) to tap ATP supply] was facilitated by the expansion of MICOS through the eukaryotic invention of subunits Mic10 and Mic19. Mic10 bent the MIM at sites of cristae invagination, whereas Mic19 probably evolved to mediate the interaction between Mic10 and Mic60, and therefore create CJs and further differentiate the IBM from the CM. MICOS created cristae micro-compartments that optimized the efficiency of aerobic respiration.

Selective forces for increased regulatory control over the biogenesis of the new respiratory organelle probably led to interactions between mitochondria and other endomembrane compartments. Due to its extensive network distribution within the cell, and important biosynthetic roles, ER–mitochondrion interactions were perhaps the first to evolve. This is consistent with the multiplicity of MCSs between ER and mitochondria observed in modern cells. ERMES and the EMC then evolved to mediate these ER–mitochondrion MCSs and ancestrally facilitate lipid transport from the ER to mitochondria. Concomitantly, the origin of Ups1/2 and Mdm35, as well as the prohibitin ring complex to organize MIM lipids occurred. Only then, could the endogenous pathways for lipid synthesis be lost from the ancestral mitochondrion/endosymbiont. Once established for lipid transfer, MCSs between ER and mitochondria were co-opted for different functions such as calcium homeostasis and ERMD. This drastically expanded the pre-existing mitochondrial biogenic network, and established the core ERMIONE (i.e., ERMES and MICOS) by linking ER, MOM and MIM.

The mitochondrion of the last eukaryote common ancestor would then already have in place a complex regulatory network in control of its biogenesis (Fig. 3B). Its ERMIONE was already in possession of ERMES and MICOS, as well as TOM, MIA and SAM. Furthermore, it contained the prohibitins Phb1 and Phb2, Ups1/2 and Mdm35, and porin (which also evolved from β-barrel precursors). In addition to these core ERMIONE parts, the eukaryote cenancestor also had Lam domain-containing proteins and Vps39, Rab7, and Vps13, and a complete EMC (Emc1–8 and 10). However, as these proteins seem to be primarily involved in other cellular processes not directly related to mitochondrial biogenesis (i.e., endomembrane vesicular trafficking, or membrane protein assembly), it is plausible that vCLAMPs and other endomembrane–mitochondrion MCSs did not evolve until much later in *S. cerevisiae* and other derived lineages. Most eukaryotic innovation at the level of mitochondrial biogenesis, however, occurred before the diversification of all eukaryotes. Therefore, it is conceivable that a complex network of interactions was already in place in the eukaryote cenancestor.

After the diversification of the major eukaryote groups from the eukaryote cenancestor, some lineages expanded ERMIONE protein complexes, and acquired new interactions among them (e.g., Fig. 3A). On the other hand, ERMIONE also lost components during evolution in a lineage-specific manner (e.g., Fig. 3C). For instance, Mic26 was added to MICOS at the origin of opisthokonts. After opisthokonts diverged into Holozoa (animals and unicellular relatives) and Holomycota (fungi and unicellular relatives), Mic12 was added to MICOS in the holomycotan line of descent (but see [127]), whereas ERMES was lost at the origin of animals. Other MICOS subunits were recently acquired in more derived groups, such as Mic25 and Mic27 in vertebrates and Mic28 in the Saccharomycetales. Similarly, the ancestral Ups1/2 duplicated to give rise to Ups1 and Ups2 in opisthokonts; Ups2 would duplicate once more to make Ups3 in *S. cerevisiae*. Groups outside animals and fungi very likely also acquired new specific components, although this awaits further experimental confirmation. The repeated evolution of some mitochondrial paralogues might be suggestive of specific selective regimes in certain lineages, or simply a combination of neutral ratchet-like processes followed by purifying selection.

Curiously, although MICOS has been relatively well preserved during eukaryote evolution, only being lost when its function in cristae compartmentalization can be dispensed with, ERMES has been lost in multiple lineages (Fig. 2). These include animals, the SAR group, red and green algae (including land plants), and the mitosome-bearing species *Entamoeba histolytica*, *Encephalitozoon cuniculi* (a microsporidion), and *Giardia intestinalis*. This is indicative of alternative ER–mitochondrion tethers in these lineages that might compensate for ERMES absence.

8. MCSs have a high degree of evolutionary plasticity

In principle, the existence and functions of MCSs and their associated tethering complexes are essential for the cell. MCSs ensure non-vesicular communication and trafficking of diverse lipids (and solutes like signalling molecules, e.g., calcium) between organelles. However, the multiplicity of different MCSs, sometimes even involving the same organelles, suggests a certain degree of functional redundancy within the cell. This might help explain how ERMES, a protein complex critical in *S. cerevisiae*, could be lost in several different lineages. Similarly, some MCS-tethering complexes might be lineage-specific innovations (e.g., vCLAMPs) evolved for particular adaptive needs, and not being ancestrally required for the basic functioning of the eukaryotic cell.

The dynamic interaction between different MCS-tethering complexes, as in the case of ERMES and vCLAMPs, and the maintenance of more than one MCS-tethering complex for the same pair of organelles (e.g., ERMES and EMC) lead us to suggest that although endomembrane–mitochondrion interactions are necessary, several redundant pathways have evolved. This might reflect slightly different functional (either structural or regulatory) requirements under various environmental conditions (e.g., ERMES expansion during respiratory growth and the converse expansion of vCLAMPs during fermentative growth). An alternative explanation is that the eukaryote cenancestor was already in possession of multiple inter-organellar connections (e.g., ERMES and EMC, both of which appear to be ancestral to eukaryotes) and several of its descendant lineages followed different evolutionary trajectories, some losing one mechanism (e.g., ERMES) while maintaining another (e.g., EMC). Cell biology has

shown that *S. cerevisiae* has several mitochondrion-endomembrane MCSs and tethers. It will be interesting to see what analogous features will be found in diverse eukaryotes, and what MCSs and tethering complexes represent the ancestral repertoire of the eukaryotic cell.

9. The reduction of ERMIONE in anaerobic eukaryotes

The incredibly diversifying evolution of eukaryotes has led to the adaptation of several lineages to hypoxic or anoxic habitats. This niche specialization has been accompanied by the reduction of mitochondria and their modification into anaerobic energy-producing organelles (e.g., anaerobic mitochondria and hydrogenosomes). This is seen in the simplification of ERMIONE in some anaerobic eukaryotes that have lost MICOS as they have dispensed with a complete respiratory electron chain and cristae (Fig. 3C). All acristate species have lost MICOS, but not all have lost ERMES. ERMES has been retained by the hydrogenomes of *Piromyces* sp., and *Trichomonas vaginalis*. The retention of ERMES in these mitochondrion-derived organelles suggests that their hydrogen-based anaerobic energy metabolism might still be carefully regulated by ER-hydrogenosome interactions responsible for lipid transfer and membrane growth. Other anaerobic species (e.g., *E. histolytica*, *E. cuniculi*, and *G. intestinalis*) that have further reduced their mitochondria into energy-consuming organelles like mitosomes (retained for iron–sulphur cluster synthesis) have dispensed with ERMES altogether (as well as factors involved in lipid transport and organization like Ups, Mdm35 and prohibitins). This implies that the loss of oxidative phosphorylation has released the anaerobic host cell of some of the tight regulatory control necessary for aerobic mitochondria, although it also raises questions as to how mitochondrion-derived mitosomes obtain their membrane lipids. ER-mitosome connections mediated by alternative mechanisms might have evolved in these highly reduced anaerobic parasites. Indeed, numerous questions remain unresolved regarding mitosome biogenesis, given that ERMIONE has almost completely disappeared in these organelles.

10. Concluding thoughts

Although we are beginning to understand the genetic networks that control mitochondrial morphology and biogenesis in *S. cerevisiae*, we are only beginning to piece together the molecular mechanisms that explain these genetic interactions. It is apparent that the picture is complex, and different mechanisms may be at play in different organisms; however, there are general lessons that we can learn from ERMIONE. Mitochondrial morphology is a complex trait, and in order for it to be properly maintained several processes must successfully occur including: (1) lipid transfer between ER and MOM, (2) lipid transfer between MOM and MIM, (3) ER to MOM tethering, (4) MIM to MOM tethering, (5) MIM lipid organization, and (6) cristae formation and MIM structural maintenance. The underlying mechanisms that control these basic processes can be specifically sought in various more phylogenetically informative organisms in order to identify the components involved. In this way, searching for known processes may lead to the identification of important proteins in diverse lineages.

By presenting our current knowledge on ERMIONE in a comparative manner we hope to have clearly outlined the similarities and differences in the ERMIONE network in various lineages in order to reconstruct its evolutionary history. More specifically, we have drawn attention to (1) the major transitions that have occurred in diverse lineages, (2) the sources of stability, that is, the evolutionarily ancient components of the network, and (3) the precariousness of conclusions drawn from studies on a single model organism. We do this in order to derive novel hypotheses about the conserved and ancient functions of this network, as well as to develop novel explanations about how and why components of the network have been lost in several lineages while retained in others.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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