

Ca²⁺-regulated mitochondrial carriers of ATP-Mg²⁺/Pi: Evolutionary insights in protozoans[☆]

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ABSTRACT

In addition to its uptake across the Ca²⁺ uniporter, intracellular calcium signals can stimulate mitochondrial metabolism activating metabolite exchangers of the inner mitochondrial membrane belonging to the mitochondrial carrier family (SLC25). One of these Ca²⁺-regulated mitochondrial carriers (CaMCs) are the reversible ATP-Mg²⁺/Pi transporters, or SCaMCs, required for maintaining optimal adenine nucleotide (AdN) levels in the mitochondrial matrix representing an alternative transporter to the ADP/ATP translocases (AAC). This CaMC has a distinctive Calmodulin-like (CaM-like) domain fused to the carrier domain that makes its transport activity strictly dependent on cytosolic Ca²⁺ signals. Here we investigate about its origin analysing its distribution and features in unicellular eukaryotes. Unexpectedly, we find two types of ATP-Mg²⁺/Pi carriers, the canonical ones and shortened variants lacking the CaM-like domain. Phylogenetic analysis shows that both SCaMC variants have a common origin, unrelated to AACs, suggesting in turn that recurrent losses of the regulatory module have occurred in the different phyla. They are excluding variants that show a more limited distribution and less conservation than AACs. Interestingly, these truncated variants of SCaMC are found almost exclusively in parasitic protists, such as apicomplexans, kinetoplastids or animal-pathogenic oomycetes, and in green algae, suggesting that its loss could be related to certain life-styles. In addition, we find an intricate structural diversity in these variants that may be associated with their pathogenicity. The consequences on SCaMC functions of these new SCaMC-b variants are discussed.

1. Introduction

The role of Ca²⁺ in mitochondrial energy metabolism has been attributed to a single mechanism: Ca²⁺ entry in mitochondria through the Ca²⁺ uniporter complex (MCUC) and activation of mitochondrial dehydrogenases and F₁F₀-ATP synthase [1,2]. However, the identification of a kind of mitochondrial transporters belonging to the SLC25 family with Ca²⁺-binding domains facing the intermembrane space [3–6] pointed to an alternative pathway for Ca²⁺ signaling to mitochondria, not requiring Ca²⁺ entry in the matrix. Ca²⁺-dependent mitochondrial carriers (CaMCs) have a bipartite structure with a carboxyl domain common to other SLC25 members as the ADP/ATP translocases (AACs) or uncoupling proteins. These transporters are made

by three tandem repeats of 100 amino acids, each containing two transmembrane α -helices connected by a short matrix α -helix, with the N- and C-ends exposed to the cytosol (reviewed in [7]). In addition, a highly conserved signature motif, Px[DE]xx[KR], is found in the odd-numbered α -helices in these transporters [7]. In CaMCs, the transporter domain is fused to a regulatory N-terminal extension of about 180 residues containing Ca²⁺-binding domains [6,8]. The CaMCs fall on two types, the aspartate/glutamate and ATP-Mg²⁺/Pi carriers [3–6,9,10], which also show relevant differences in their regulatory N-terminal domains [8,11–13].

The ATP-Mg²⁺/Pi carriers, or SCaMCs (“Small CaMC”), are the most extended subfamily of mitochondrial transporters with five paralogs in mammals depicting one of largest MC groups together with AACs [6,8].

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This profusion reflects its relevance and increases the repertoire of AdNs transporters found in metazoans and plants organelles [14–17]. They perform the exchange of ATP—Mg²⁺ or ADP by Pi in an electro-neutral and reversible way, and, thereby, this carrier controls the net levels of adenine nucleotides (AdNs) in the matrix [18,19]. By modulating the AdNs content, it was proposed that this carrier could affect different mitochondrial functions such as respiration, the activity of ATP-dependent enzymes in the matrix [18,20–22] or even mitochondrial Ca²⁺ buffering capacity by the formation of Ca²⁺-Pi (CaP) precipitates [23,24] given that this process requires AdNs [25]. Its activity, therefore, provides a higher Ca²⁺ retention capacity and consequently a reduced sensitivity to mitochondrial permeability transition pore opening and cell death [24]. In addition, as ADP/ATP translocases, SCAmCs import glycolytic ATP that can be used for essential intra-mitochondrial pathways in situations when OXPHOS reactions are non-functional [26].

SCAmCs have a Calmodulin-like (CaM-like) N-extension containing four EF-hands in a characteristic disposition [5]. Its activity is strictly Ca²⁺-dependent at cytosolic micromolar concentration [9,19,20,24,26–28]. The structure of its regulatory Ca²⁺-binding domain has recently been resolved [11,13] providing relevant structural insights on the mechanism by which the transport of AdNs by the carrier is activated by Ca²⁺ [11,13]. Thus, under conditions of low Ca²⁺, the transport domain is blocked by its interaction with one α -helix domain that connects it with the N-regulatory domain, when Ca²⁺ increases this α -helix binds to the N-domain and opens the access to substrate [29]. Therefore, this CaM-like extension works as an autoinhibitory domain for the transport, which could possibly be regulated not by substrate availability but by Ca²⁺ binding and, therefore, be coupled with cellular Ca²⁺ signaling.

It has been proposed that SCAmCs emerged from the fusion of a CaM-like domain fused to an ancestral MC domain related to the AACs [11,29]. To date, excepting the SCAmC-3L/SLC25A41 paralogs in mammals [30], all characterized SCAmCs have identical N-extensions suggesting a common origin [5,31]. In order to gain insights about its origin now we have performed a comprehensive analysis in distantly related unicellular eukaryotes where SCAmCs remained uncharacterized. We have compiled SCAmC relatives in protozoans and established their phylogenetic relationships. We find a higher structural complexity than expected, despite of their common origin, with the existence of truncated variants lacking the regulatory CaM-like domain in parasitic protozoans and green algae. Since this CaM-like domain is essential for the autoinhibitory mechanism of the carrier, we discuss the significance of its absence in relation with the functions proposed for the ATP-Mg²⁺/Pi carriers.

2. Results and discussion

2.1. Distribution of ATP-Mg²⁺/Pi carrier orthologs in protozoans

We examined protein databases to compile SCAmC-related proteins across distantly related unicellular eukaryotes as described in the methods section. Our scrutiny has confirmed the existence of SCAmC relatives in some species belonging to the major protist groups such as Amorphea, Excavata, Archaeplastida and the TSAR (Telonemia, *Stramenopila*, Alveolata, and Rhizaria) supergroups [32], being also detected in parasitic organisms having highly reduced anaerobic mitochondria, called as mitochondria-related organelles (MROs) [37], such as *Cryptosporidium* or *Blastocystis*. In Table S1 are listed the most relevant species where we detected the presence of SCAmC orthologues. However, we found an unequal distribution of canonical SCAmCs, containing Ca²⁺-binding domains at the N-terminus, and an intricate structural diversity, highlighting the existence of shortened isoforms, thereafter named as SCAmC-b, lacking this regulatory CaM-like domain (Fig. 1A, B).

In addition to their broad distribution in Opisthokonta, previously described [31] and no longer considered here, we have identified bona-

fide SCAmC orthologs in numerous dictyostelids, the so-called cellular slime molds belonging to Amoebozoa phylum, and in a low number of protozoans included in three of the other four eukaryotic supergroups (Fig. 1B, Table S1). We detected SCAmC counterparts across distant eukaryotes as the apusozoan *Thecamonas trahens*, the discobans *Andalucia godoyi* and *Naegleria gruberi* also in the haptophyte alga *Emiliania huxleyi* or in different species belonging to SAR clade as the soil-borne obligate parasite *Plasmodiophora brassicae*, the parasitic ciliate *Ichthyophthirius multifiliis* or the non-parasitic photosynthetic alga *Vitrella brassicaformis*. However, all show a similar N-terminal extension of 160–180 residues containing the distinctive regulatory domain described in mammalian SCAmCs ([5,11,31,13]. This regulatory CaM-like module contain four EF-hands in a similar arrangement than CaM but, as distinguishable characteristic, in SCAmCs it has lost six amino acids of the flexible linker that connects the EF-1/EF-2 and EF-3/EF-4 pairs and produces a rigid structure ([5,8,11]; Yan et al., 2015) (Fig. 1C). In SCAmC, EF pairs are positioned in proximity establishing extensive hydrogen bonds and nonpolar interactions, which provides structural constraints involved in the regulation of the transport by the carrier domain (for additional information see Yan et al., 2015; [11,29]). This compact EF-2/EF-3 configuration is exclusive of SCAmCs. We failed to detect it in other known CaM-like proteins. However, this shorter link connecting EF-2 and EF-3 is found in all orthologs identified in protozoans, and, therefore, it may be considered as a featured signature for the CaM-like regulatory module of the SCAmCs (Fig. 1C). In addition, it also suggests the existence of a common mechanism of regulation by calcium among SCAmCs. As earlier described for SCAmCs in metazoans [31], the sequences of the EF-1 and EF-4 hands show a lower degree of conservation among SCAmC orthologs of protozoans (Fig. 1C), suggesting that these EF-hands may play a minor regulatory role. Indeed, spliced isoforms lacking EF-1 hand or containing a disrupted EF-4 hand have also been described in vertebrates [5,8,9]. Therefore, the presence of this highly specific “SCAmC signature” in homologues from distant evolutionary groups suggests the existence of a common origin for SCAmCs.

Next, we examined the presence of the classical AACs in these species. It has been shown in yeast that Sal1p, the ATP-Mg²⁺/Pi carrier orthologue, becomes essential in mutant cells lacking the classical ADP/ATP translocases [26,38,39]. Therefore, we were interested in determining if SCAmCs co-exist or not with AACs in protozoans. As expected by their pivotal role in mitochondrial bioenergetics, we found a broader distribution for AAC orthologs in protozoans (Fig. 1B and Table S1). Moreover, only in one of the species devoid of AACs, the anaerobic parasite *Blastocystis hominis*, we have detected the presence of a SCAmC-b homologue (see below, Table S1). In other parasitic protozoans that lack typical AACs, such as the amoebozoan *Entamoeba histolytica* or the excavate *Trichomonas vaginalis*, we did not find SCAmC orthologs. In fact, in these species other putative AdNs transporters, different from AAC or SCAmC, have been identified [40–42]. Nevertheless, some of them, as those identified in the MRO of *T. vaginalis* (Hmp-31, TVAG 051820, TVAG 196220 and TVAG 262210) [41,43,44], show a significant homology with SCAmC proteins, specially TVAG 196220, and with the plastidic AdN uniporter BT1 (Brittle1 transporter, [16,45]).

Phylogenetic analysis indicates, as expected, that SCAmC and AAC orthologues form unrelated entities confirming their independent evolutionary origin (Fig. 2). Furthermore, AAC orthologs display shorter branch lengths compared to SCAmCs, which form a more divergent group with long branches (Fig. 2), demonstrating a higher degree of conservation than for SCAmCs, as was observed between the corresponding human orthologues [31].

2.2. A new variant devoid of the regulatory Ca²⁺-binding domain is found in protozoan parasites

Next, we analysed the distribution of the shortened SCAmC-b variants to determine whether they may represent an ancestral precursor or

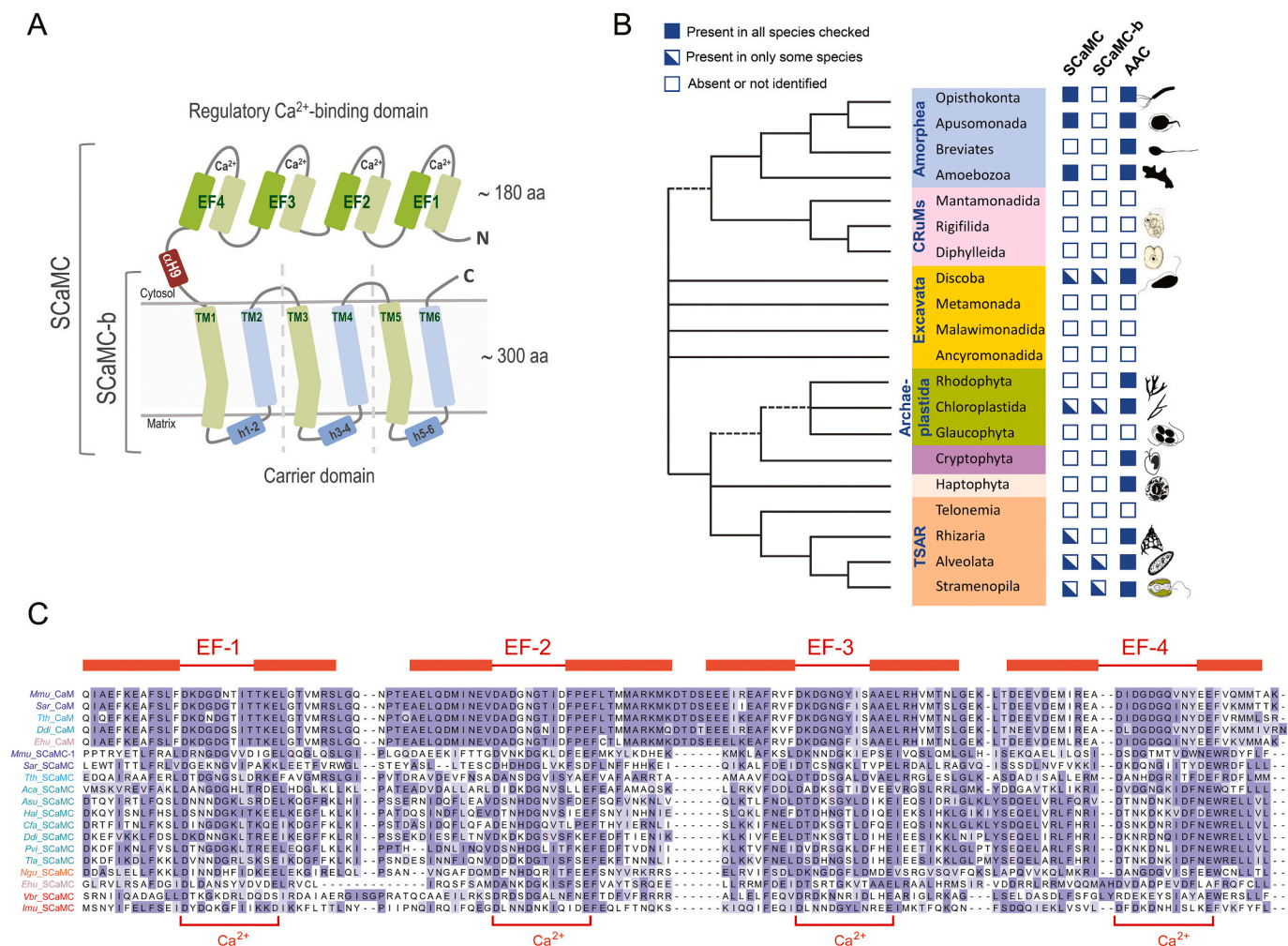


Fig. 1. Characteristics and distribution of ATP-Mg²⁺/Pi carriers, SCaMCs, in eukaryotes. **A**) Scheme of the three structural domains composing SCaMCs; an N-terminal Calmodulin-like calcium-binding domain, a carrier domain homologous to MCs both connected by a short regulatory α -helix (α H9) [11]. Truncated SCaMC-b variants found in protozoans lack of regulatory N-domain. **B**) Distribution of SCaMC, SCaMC-b and AAC carriers in eukaryotes. The schematic tree was drawn as a consensus of recent phylogenomic analyses ([32,33], and references therein), the positions that remain unresolved are indicated by dashed lines. Silhouettes were taken from Wikipedia and www.phylogenic.org. **C**) Alignment of the N-terminal regulatory domains of selected SCaMC and CaM orthologues. Only the region covering the EF-hands has been aligned. Sequences are aligned using MUSCLE and formatted for display in Jalview. The positions of each calcium binding EF-hand indicating their flanking α -helices (rectangles) and the calcium binding loop (line) identified by PSIPRED [34] are shown. Species abbreviations used are: Mmu, *Mus musculus*; Sar, *Sphaeroforma arctica* JP610; Th, *Thecamonas trahens*; Aca, *Acanthamoeba castellanii*; Asu, *Acytostelium subglobosum*; Hal, *Heterostelium album* PN500; Cfa, *Cavendishia fasciculata*; Ddi, *Dictyostelium discoideum*; Ppa, *Polysphondylium violaceum*; Tla, *Tieghemostelium lacteum*; Ngr, *Naegleria gruberi*; Ehu, *Emiliania huxleyi*; Pbr, *Plasmodiophora brassicae*; Vbr, *Vitrella brassicaformis*; Imu, *Ichthyophthirius multifiliis*. Species were coloured according to phylogeny showed in Fig. 2. Sequence IDs of SCaMC homologues are provided in Additional Table S1.

whether the loss of this Ca^{2+} -binding domain represents a subsequent event. We detected SCaMC-b variants in green algae and in various parasitic protists belonging to different supergroups of eukaryotes in which archetypal SCaMCs were also identified in some of their species (Figs. 1B, 2, Table S1). Nevertheless, we never observed the presence of both variants together. Phylogenetic analysis indicates that both SCaMC-b and SCaMCs variants are clustered together in each phyla excluding that SCaMC-b may represent an ancestral form of the ATP-Mg²⁺/Pi carriers (Fig. 2). SCaMC-b sequences show high similarity to each other, around 60–65% at the carrier domain confirming that they are certainly N-truncated SCaMC variants (Fig. 3), but they show short extensions at the N-termini ranging from 30 to 150 amino acids that do not present any homology each other or with those showed by typical SCaMCs (see Fig. 3).

Putative SCaMC-b homologues are detected in some species of the Alveolata and Stramenopila clades englobed in the TSAR supergroup (Fig. 1B and Table S1). Among alveolates, there are SCaMC-b variants in some species of parasitic apicomplexans belonging, among others, to the

genera *Cryptosporidium*, *Plasmodium* or *Toxoplasma*. They are found in the gastric *Cryptosporidium*, *C. muris* and its closely related *C. andersoni*, but are not in the intestinal *C. parvum*, *C. hominis* and *C. ubiquitum*. It should be noted that comparative analyses between *Cryptosporidium* spp. genomes have revealed an asymmetric reduction in the mitosome genome and consequently in their metabolic pathways among *Cryptosporidium* spp. [46,47]. As a result, *C. muris* and *C. andersoni* can use both the TCA cycle and glycolysis for energy metabolism and have a near conventional oxidative phosphorylation system whereas *C. parvum* and *C. hominis*, with a major reduction in gene content, lack the TCA cycle and possess a reduced electron transport system [47]. Interestingly, AACs homologues detected in gastric and intestinal cryptosporidians also show significant differences. Thus, despite its high homology, *C. parvum* AAC has shown broader substrate specificity in transport assays than eukaryotic AACs, a strictly ADP/ATP exchanger, which has been associated to a specific cysteine residue in the putative translocation pathway [48]. However, in AACs of gastric *Cryptosporidium* spp. a serine residue, as in standard AACs, is found at the same position (not shown).

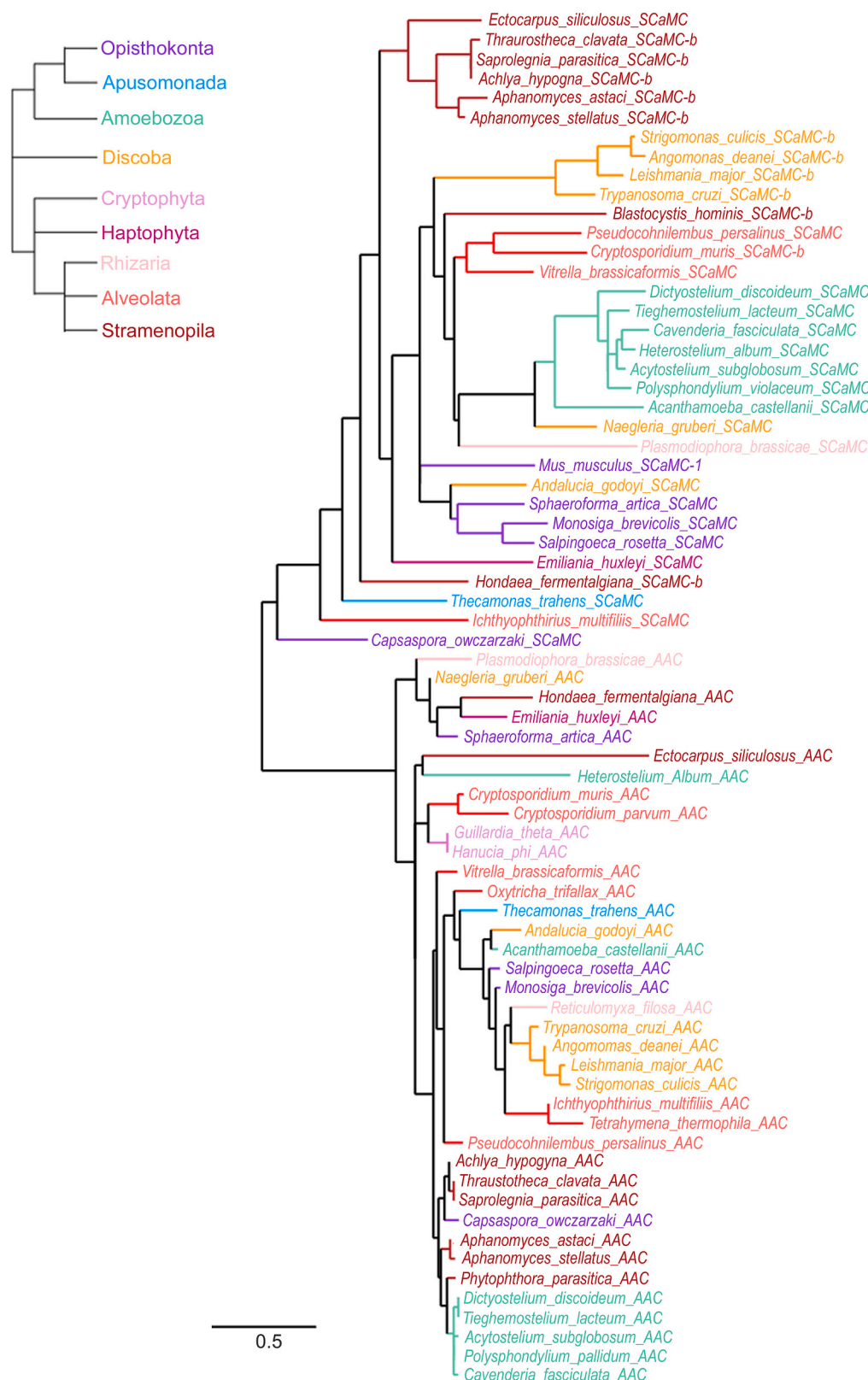


Fig. 2. Phylogenetic analysis of AACs and SCaMCs in protozoans. The tree was generated using PhyML and drawing using TreeDyn in the Phylogeny.fr platform with default parameters [35] using the maximum likelihood method [36] implemented in the PhyML program (v3.1/3.0 aLRT). The WAG substitution model was selected assuming an estimated proportion of invariant sites (of 0.049) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 0.798). The scale bar indicates the inferred number of substitutions per site. Taxa names for the AAC/SCaMC/SCaMC-b sequences are color-coded according to the Eukaryote Tree of Life (eToL, [32]) supergroups as indicated. Sequence IDs are provided in Table S1.

If these differences between cryptosporidian AACs could be related to the absence of additional AdNs transporters is currently unknown. SCAmC-b relatives are also observed in numerous *Plasmodium* spp., as well as in *Toxoplasma gondii* and other related apicomplexans. However, these SCAmC-b homologues show remarkable differences from those of *Cryptosporidium* spp. (see below).

In Stramenopiles, SCAmC-b orthologs are found in oomycetes and, as indicated, in the atypical stramenopile *Blastocystis hominis*. This human parasite has MROs but still shows cristae, membrane potential and organellar genome [49]. In addition, although *Blastocystis* shows a reduced number of MCs [49,50], a SCAmC-b counterpart, which could be involved in the exchange of AdNs with the cytosol, is retained. Also, a putative ADP/ATP carrier was previously identified in the MRO of *Blastocystis* (GSBLH_T00004392001; [50]), however, this protein shows a higher homology with the orthologs of SLC25A43, an orphan member of the MCF related with the AdNs transporters. *Blastocystis* SCAmC-b is the shorter variant, it has only 315 amino acids as it shows a very short N-extension (see Fig. 3).

In the fungi-like oomycetes, SCAmC-b homologues of around 350–370 amino acids are detected in species from genera *Achlya*,

Aphanomyces and *Saprolegnia*, all with an animal-pathogenic lifestyle, whereas are totally undetectable in other oomycetes as *Pythium*, *Phytophthora* or *Albugo* spp. that are plant pathogens. Interestingly, in oomycetes the expansion of genes encoding proteins involved in transport has been associated with pathogenicity [78]. Indeed, a gene encoding SLC25A43-related carrier was found amplified in *Aphanomyces euteiches* (Ae_97AL5378; [51]), and we also detected an increased number of gene copies for this carrier in *Achlya hypogyna*, *Saprolegnia parasitica* and *Aphanomyces* spp., [51]. Therefore, these observations indicate that these AdNs transporters may participate in the pathogenicity of oomycetes and *Blastocystis*.

A third group of protozoan parasites showing this shortened transporter is made by flagellated trypanosomatids that belong to the Kinetoplastea class of Excavates (Figs. 1B, 2). SCAmC-b orthologues are detected in *Strigomonas culicis*, *Angomonas deanei* and in numerous *Leishmania* and *Trypanosoma* spp. Interestingly, SCAmC-b counterparts in trypanosomatids show insertions of amino acid stretches, of variable lengths, at similar positions. These insertions mostly elongate the loops connecting the transmembrane segments (TM) facing to cytosol, TM2/TM3 and TM4/TM5, and occasionally the matrix-exposed loop

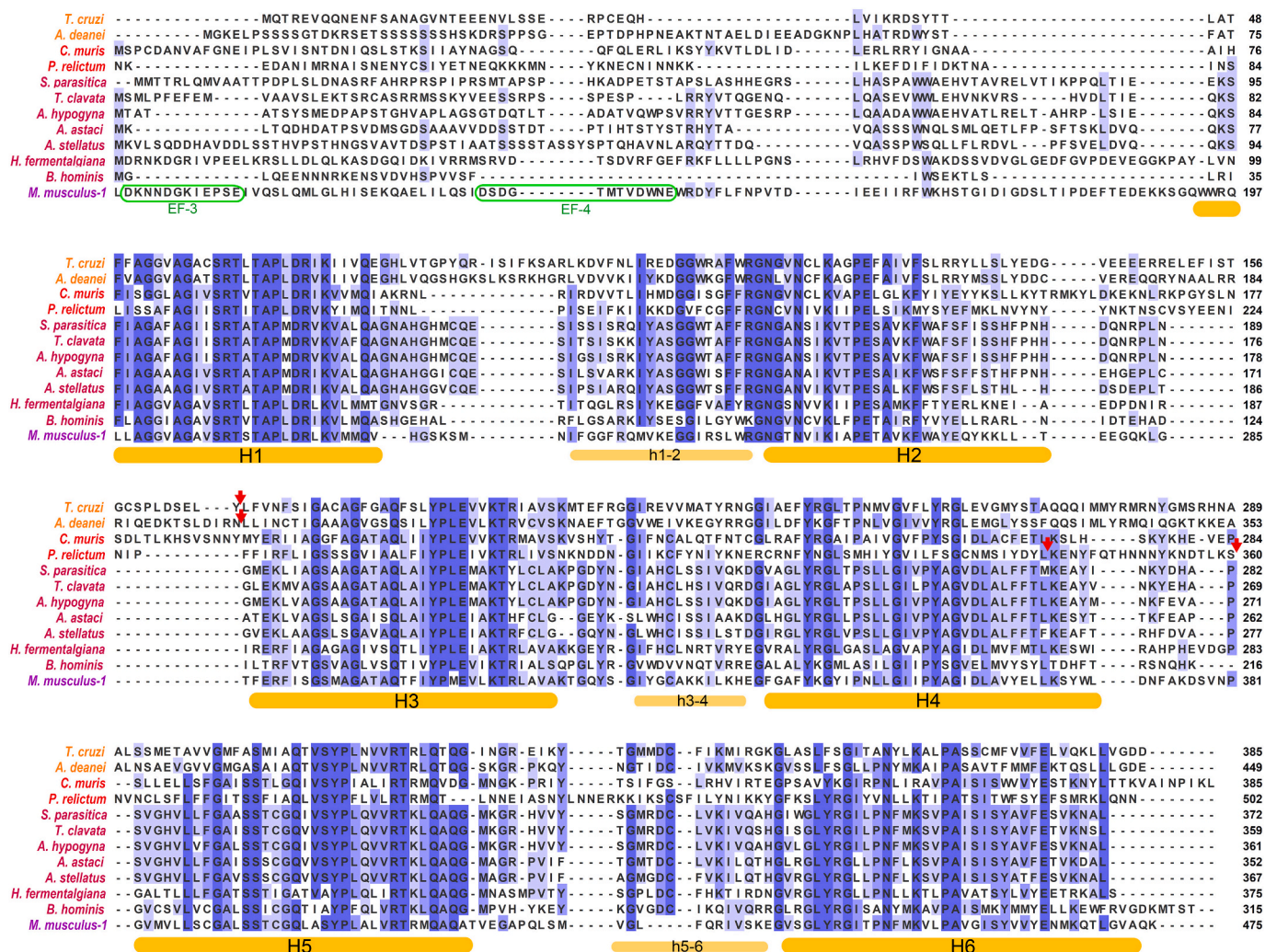


Fig. 3. Multiple sequence alignment of protozoan SCAmC-b proteins. Multiple alignment of selected SCAmC-b variants and the *Mus musculus* SCAmC-1 orthologue are shown. Sequences are aligned using MUSCLE and formatted for display in Jalview. Conservation of residues is indicated colouring in accordance with percentage identity (Jalview; darker blue is more conserved). The positions of the transmembrane α -helices (H1–H6) and matrix α -helices (h1–2, h3–4, and h5–6), identified by PSIPRED [34] in the *M. musculus* SCAmC-1 orthologue, are showed. By arrows are indicated the positions where contiguous amino acids stretches are removed from cytosolic loops in *Trypanosoma cruzi*, *Angomonas deanei* and *Plasmodium falciparum* SCAmC-b orthologues. EF-3 and EF-4 domains in the N-terminal extension of *Mus musculus* SCAmC-1 are indicated. Species were coloured according to phylogeny showed in Fig. 2. Sequence IDs of SCAmC-b homologues are provided in Additional Table S1.

connecting TM1/TM2. Equivalent insertions are also found in the SCaMC-b orthologs of some apicomplexan parasites as *C. muris*, *C. andersoni*, *Toxoplasma gondii*, *Neospora caninum*, *Cyclospora cayetanensis* and in different *Plasmodium* and *Eimeria* species, and in the SCaMC isoform of the Alveolate *V. brassicaformis*. However, some differences are observed between both groups. Indeed, the longer insertions are found in the TM2/TM3 connector in trypanosomatids and in *Cryptosporidium* and *V. brassicaformis*, whereas in the rest of apicomplexans they are detected in the cytosolic loop TM4/TM5 with the exception of *T. gondii* where long insertions are found in both cytosolic loops. Furthermore, mainly in apicomplexan SCaMC-b proteins, these insertions contain homorepeats, repeats of a single amino acid residue, mainly made up of glutamine (*C. cayetanensis*), alanine (*Eimeria mitis*), serine (*N. caninum*) or asparagine (*Plasmodium falciparum*), amino acids that are often found in homorepeats in eukaryotes [52]. These homorepeats are also found in other regions of SCaMC-b, mainly those near to

the N-termini. In *P. falciparum*, although numerous proteins that contain homorepeats have been identified [53], their presence was not previously observed in other MCs [54]. Interestingly, the homorepeats are associated with intrinsically disordered regions (IDRs) [55], which are frequently found in the proteins involved in host/parasite interactions in *P. falciparum* [56]. Indeed, it has been proposed that the structural plasticity provided by these IDRs may contribute, by different ways, to the invasion and survival of the parasite within the host [56].

2.3. A new MC containing a CaM-like regulatory domain is detected in green algae

Finally, a more complex situation is found in unicellular green algae, in the Chlorophyta clade, in agreement with the expansion of transporters of adenine nucleotides and derived compounds found in cytosolic organelles in plants [14–17,45]. In this group we found two types of

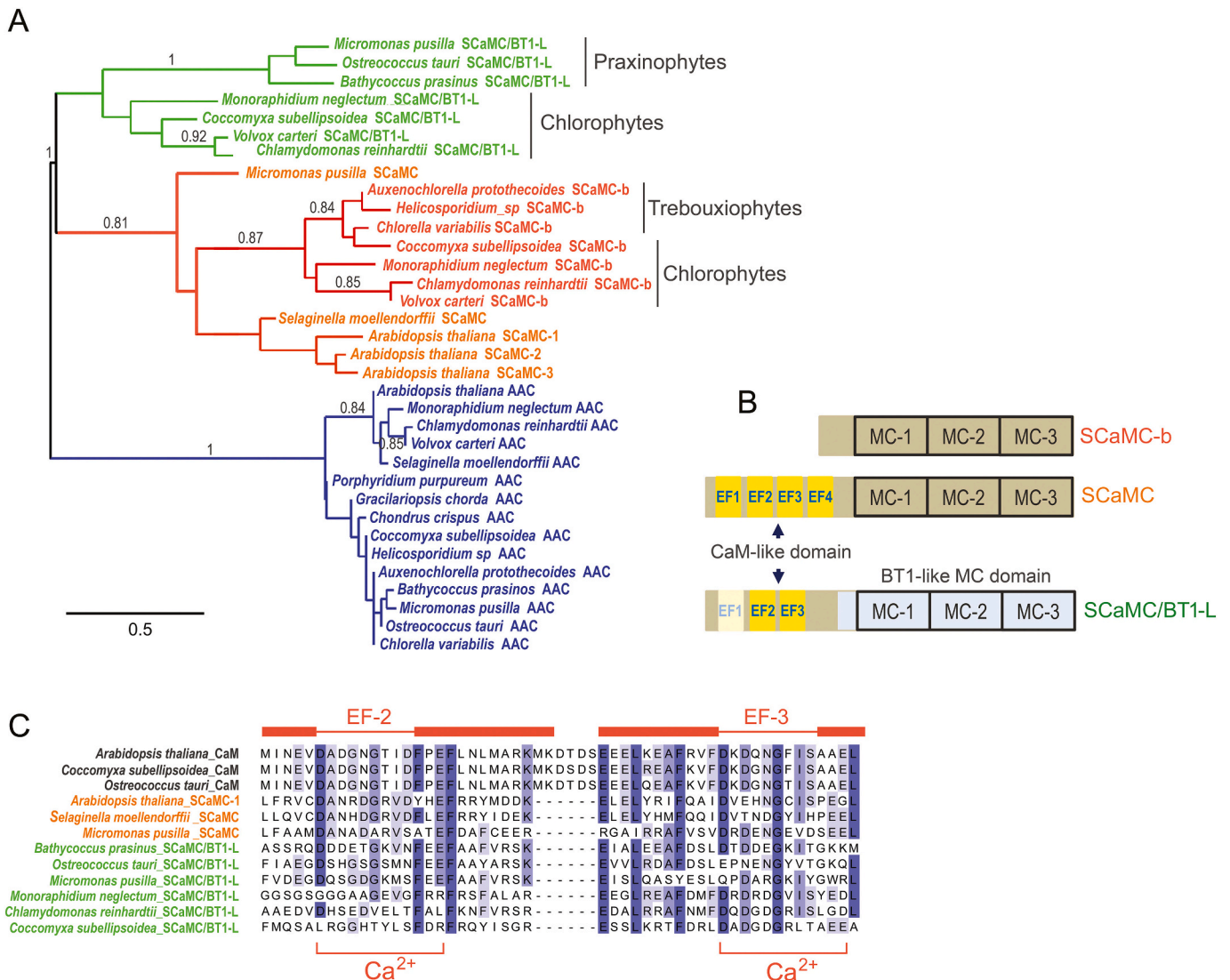


Fig. 4. SCaMC-related variants in unicellular green algae. A) Phylogenetic relationships between SCaMC-related proteins and AACs in green algae. The tree unrooted was generated as indicated in Fig. 2. The WAG substitution model was selected assuming an estimated proportion of invariant sites (of 0.000) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 0.903). Taxa names are coloured according to their belonging to each AAC/SCaMC/SCaMC-b/SCaMC/BT1-L branch. The numbers indicate bootstrap values for each branch above 80% from PhyML. 1 = 100% of 100 bootstrap replicates. The scale bar indicates the inferred number of substitutions per site. B) Scheme of the distinctive domains present in SCaMC-b, SCaMC and SCaMC/BT1-L proteins. The canonical EF-hand domains (EF) at the regulatory N-terminal region are indicated, the low conserved EF-domain is shadowed C) Alignment of the calcium-binding sites corresponding to the EF-2 and EF-3 hands and its connecting linker of CaM, SCaMC and SCaMC/BT1-L proteins from the indicated species. The sequences are aligned using MUSCLE and formatted for display in Jalview. The positions and structural features of EF-hand are indicated as in Fig. 1C. Sequence IDs of SCaMC and SCaMC/BT1-L proteins are provided in Table S1.

SCaMC-related proteins, ones containing a CaM-like N-terminal domain but fused to a transport domain homologous to the plastidic AdN uniporter BT1 (named here as SCaMC/BT1-L “Small Calcium-binding Mitochondrial Carriers BT-1 Like”) [16,45] and a second type lacking the regulatory N-extensions and whose transport domain displays a remarkable homology with that of the *Arabidopsis thaliana* ATP-Mg²⁺/Pi carriers (Table S1) [28,57,58]. SCaMC/BT1-L would represent a novel plastidial Ca²⁺-dependent AdNs transporter, or of structurally related compounds, different to those previously described in green algae and land plants [58]. On the other side, the shorter carriers are SCaMC-counterparts that show a significant similarity, around 60%, with these isoforms. Phylogenetic analysis confirms their clustering in two differentiated sorts of transporters entirely independent from each other and, as showed for SCaMCs, from that formed by AACs (Fig. 4A). These novel SCaMC-related proteins are probably exclusive of green algae, as we were unable to detect them in other members of the Chlorophyta clade such as Embryophytes or Charophytes. Orthologues for both “SCaMC” classes are detected in several species of chlorophytes as *Volvox carteri*, *Coccomyxa subellipsoidea* or *Chlamydomonas reinhardtii*, whereas SCaMC/BT1-L transporters are only found in the early diverging prasinophytes as *Bathycoccus prasinos* or *Ostreococcus tauri* (Fig. 4A, Table S1). However, SCaMC-b was only found in trebouxiphytes, included the parasitic/pathogenic genus *Helicosporidium*, and in chlorophytes (Fig. 4A).

The regulatory domain of SCaMC/BT1-L shows less homology to CaM proteins than SCaMCs. The EF-4 hands are hardly detectable and the EF-1 hands are also lost in the orthologs of chlorophytes. However, in spite of its lower homology, this CaM-like domain shows remarkable similarities with those present in SCaMCs. Thus, the length of the N-extension and the organization of its EF-hands 1 to 3 are very similar. Notably, the organization and the composition of the central EF-2 and EF-3 hands are well conserved between SCaMC/BT1-L and SCaMCs, included the featured shortening of the loop connecting the EF-2/EF-3 hands (Fig. 4B; C). Therefore, a mechanism similar to that proposed for SCaMCs can also be involved in the regulation by Ca²⁺ of SCaMC/BT1-L. Furthermore, the identification of this novel SCaMC-related transporter indicates that the regulation by cytosolic Ca²⁺ of MCs may be a mechanism more widespread than it is believed to date.

2.4. Evolutionary insights from SCaMC-b variants in protozoans

From this scenario in protozoans some questions arise regarding these new SCaMC-b variants such as their origin and the functional consequences of the regulatory module loss. Given that both paralogs, classical and shorter SCaMC variants, coexist in most of the eukaryotic supergroups it is tempting to propose that truncated SCaMC-b types could have evolved independently in each lineage and, consequently, the loss of the regulatory domain would represent an adaptive acquisition occurred recurrently associated to their specific lifestyles. Indeed, SCaMC-b homologues from chlorophytes, apicomplexans, oomycetes or trypanosomatids appear clustered indicating a close evolutionary relationship inside each branch (Fig. 2). Moreover, out of Opisthokonta phyla, and with the exception of dictyostelids, kinetoplastids and some species of oomycetes, we detected SCaMC relatives in a reduced number of species scattered throughout the different groups of protists, that contrasts with the widespread presence of AACs (Fig. 1B, Table S1). Their distribution indicates that they were probably lost several times during the evolution of protozoans. Consequently, it is reasonable to propose that the redundancy with other AdNs transport systems may have endorsed its loss. This fact may also explain the less degree of conservation observed between SCaMC orthologues (Fig. 2). Nevertheless, we cannot rule out that their presence in some groups may be due to horizontal gene transfer (HGT) events, widely demonstrated as an important mechanism by which unicellular eukaryotic organisms adapt to new environments [33,50,59].

Interestingly, while the repertoire of MCs in parasitic protists is

markedly reduced [37,60,61], reflecting the decrease of the metabolic capabilities of their mitochondria, ATP-Mg²⁺/Pi carriers are maintained in some of these groups as *Blastocystis* or some *Cryptosporium*. In addition, they maintain mostly the SCaMC-b variants, and, consequently, probably it will not require the binding of Ca²⁺ to carry out the transport of nucleotides. This loss will be substantial for its functionality because it could now allow the “free” import of ATP from cytosol by the mitochondria or MROs. This activity has been proposed for AACs operating in reverse mode in the mitochondria of these parasites [40,60] and for members of the family of bacterial nucleotide transports (NTT) resident in the mitosomes of the microsporidia *Encephalitozoon cuniculi* [62,63]. Although transport assays for SCaMC-b proteins have not been yet carried out, their high homology with metazoan homologues suggests that they could indeed transport AdNs. Moreover, the loss of the regulatory domain probably does not preclude its transport activity as reported for mouse SCaMC-3L/Slc25a41, a shorter isoform lacking this domain [30], neither for shortened recombinant versions of human SCaMCs reconstituted into proteoliposome [9]. Similarly, a constitutive activation is observed in some isoforms of the calcium-dependent protein kinases (CDPKs) after the loss of a regulatory module containing EF-hands [64]. CDPKs are cytosolic serine/threonine protein kinases found in plants and apicomplexans that contain a regulatory CaM-like domain which provides an activation mechanism upon Ca²⁺-binding similar to that described for SCaMCs [29,65,66]. Nevertheless, its regulation may also be subjected to additional mechanisms. Indeed, as previously observed for CDPKs in *T. gondii* [67], the reexpression of a truncated version devoid of the Ca²⁺-binding domain does not reverse the phenotype caused by the absence of functional SCaMC alleles in *Drosophila* [27].

On the other hand, in contrast to its reduction in protozoans, archetypal SCaMCs, are nearly the only form in opisthokonts [31], where they are part of the invariant repertoire of MCs presents in mitochondria [5,30,31,68]. Its prevalence, especially in multicellular metazoans, could reflect its functional relevance in the transduction of Ca²⁺ signals to mitochondria as previously proposed [6,20–22,24,26,27,38]. It is well recognized that its activation by cytosolic Ca²⁺ signals stimulates the mitochondrial respiration and the activity of matrix enzymes [18,20,22,26]. Therefore, it appeared reasonable to think that SCaMC-b could not fulfill this function. However, the substrate transported by SCaMCs is AdNs which can also facilitate the formation of CaPi precipitates [23,25] and, as a result, modulate the levels of free calcium in the matrix and the mitochondrial Ca²⁺ buffering capacity as has been reported for SCaMCs in mammals [22,24]. Indeed, SCaMC-3/SLC25A23 deficiency decreases mitochondrial Ca²⁺ uptake and reduces cytosolic Ca²⁺ clearance after histamine stimulation [69].

In protozoans, intracellular Ca²⁺ signaling is essential for cell mobility, invasion and egress of the host cell and cell differentiation [70]. However, except for the Ca²⁺-sensitive pyruvate dehydrogenase phosphatase described in some *Trypanosome* spp [71], the knowledge of the roles of Ca²⁺ in mitochondria are more limited than in mammals because Ca²⁺ regulated dehydrogenases are either not present or remain uncharacterized [70,72,73]. Nevertheless, *Trypanosome* and *Plasmodium* mitochondria are also capable of acting as effective buffers of cytosolic Ca²⁺ [70,73,74]. For this reason, we hypothesized that SCaMC-b could also be involved in mitochondrial calcium homeostasis and we were interest in knowing if its distribution coincides with that of MCUC, the highly selective channel involved in mitochondrial Ca²⁺-uptake [75,76]. To address this, we examined the presence of MCUC components in those protozoans showing SCaMCs. Homologues of MCU, the channel-forming protein, and MICU, the Ca²⁺-sensing component of the complex [1], were compiled from a recent survey [76] and from databases. Although, MCU and MICU orthologs are not found in certain protozoans and fungal lineages [73,75,76], we detected them in all those protist species that show typical SCaMCs (Table S1). In addition, we also found the presence of both MCUC components in unicellular green algae, kinetoplastids and oomycetes, all of them groups containing

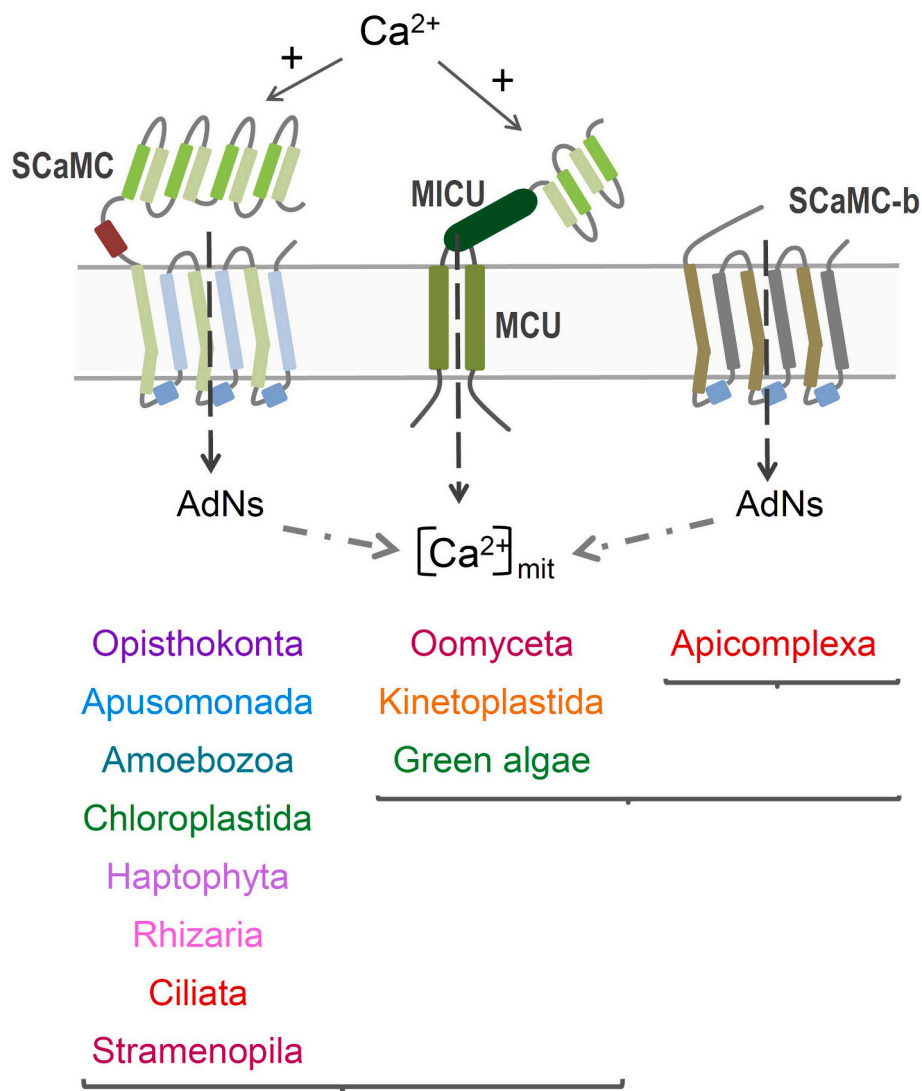


Fig. 5. Distribution of the MCUC components and the SCaMC variants in protozoan groups. A scheme of both SCaMCs and MCU and MICU proteins, two components the MCUC [1] is shown. Their transport activity regulates the capacity of Ca^{2+} buffering in mitochondria. Under the scheme are indicated the protozoan groups that display SCaMC, SCaMC-b and the mentioned MCUC components. Taxa names are color-coded as in Fig. 2.

the SCaMC-b variants. However, as previously reported [75,76], we failed to detect MCUC components in apicomplexans despite that in some apicomplexan species such as *P. falciparum* an increase in mitochondrial Ca^{2+} concentration was observed in response to signals that cause increases in cytosolic Ca^{2+} levels [70]. In sum, these data indicate that, excepting apicomplexans, the distribution of MCUC components matches well that of SCaMC paralogs, of both together, suggesting that their activities could cooperate in the control of Ca^{2+} homeostasis in mitochondria (Fig. 5).

3. Conclusions

SCaMCs are an alternative system to AACs for AdNs transport across the inner mitochondrial membrane that allow to regulate the AdNs levels in the matrix favoring oxidative phosphorylation reactions, the activity of ATP-dependent enzymes or the capacity of mitochondrial Ca^{2+} buffering. Furthermore, unlike other MCs, they have an extra domain that permits to regulate transport activity not by the availability of substrates but by Ca^{2+} signals, and therefore, coupling its activity to cell physiology.

To investigate its evolutionary origin, we have examined their

orthologs in unicellular eukaryotes. We have identified archetypal SCaMCs homologues in most eukaryotic groups, indicating that their primitive form was already like a Ca^{2+} -dependent transporter. However, our analyses also reveal an unanticipated structural diversity in some groups of parasitic protozoans, as kinetoplastids or apicomplexans, and in green algae. These distant groups show shortened variants lacking the regulatory domain. This reveals an adaptative capacity in SCaMCs probably due to their distinctive modular structure. Interestingly, although these variants could be functional alternatives to the typical SCaMCs, this loss could have functional effects favoring a constitutive activity of the transporter and providing benefits to the parasitic life-style. Therefore, its appearance could be related with the pathogenicity of these groups, suggested by the recurrent losses of the regulatory CaM-like domain in different groups of parasitic protozoans. In accordance with its involvement in pathogenicity, some of them show features common to proteins involved in pathogenicity as the presence of homorepeats. This study in protozoans completes the characterization of the SCaMCs, the ATP- Mg^{2+} /Pi transporters, one of the Ca^{2+} -dependent mitochondrial carriers, proving its relevance and discovering new variants. The specificity of these SCaMC-b variants makes them potential targets to for the development of new strategies against these pathogenic

protozoans.

4. Methods

4.1. Sequence retrieval and analysis

To identify *SCaMC* related proteins in unicellular eukaryotes we perform a homology- and domain-based sequence analysis using characterized *SCaMC* and AAC proteins as query [5,20,26,38]. Searches in protein databases were performed using the BLASTP and BLAT algorithms. We used NCBI (<http://blast.ncbi.nlm.nih.gov>), Ensembl (www.ensembl.org) and EnsemblProtists (<https://protists.ensembl.org>). For all BLAST searches low-complexity regions in the query sequence (default parameter) were filtered out to minimize the number of false positives. Sequences derived from the most closely related phylogenetic species were used as query in each search. Protein homology was determined using the BLASTP algorithm.

Secondary structure prediction was performed using PSI-blast based secondary structure Prediction (PSIPRED) (<http://bioinf.cs.ucl.ac.uk/psipred/>) [34]. The presence of homorepeats was analysed by using the dAPE program [77].

4.2. Phylogenetic analysis

Multiple sequence alignment was performed using CLustalW or MUSCLE server of EBI (<https://www.ebi.ac.uk/Tools/msa/>) [78] and visualized using Jalview (www.jalview.org). The phylogeny analysis was done by using the webtool Phylogeny.fr (<https://www.phylogeny.fr/>) in advanced mode [35]. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The WAG substitution model was selected with an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

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CRedit authorship contribution statement

Silvia García-Catalán: Performing the data/evidence collection.

Luis González-Moreno: Data curation, Writing-Original Draft Preparation.

Araceli del Arco: Conceptualization, Writing- Reviewing and Editing.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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References

- [1] D. De Stefani, R. Rizzuto, T. Pozzan, Enjoy the trip: calcium in mitochondria Back and forth, *Annu. Rev. Biochem.* 85 (2016) 161–192.
- [2] M.R. Duchen, Mitochondria, calcium-dependent neuronal death and neurodegenerative disease, *Pflugers Arch.* 464 (2012) 111–121.
- [3] A. del Arco, J. Satrustegui, Molecular cloning of Aralar, a new member of the mitochondrial carrier superfamily that binds calcium and is present in human muscle and brain, *J. Biol. Chem.* 273 (1998) 23327–23334.
- [4] A. Del Arco, M. Agudo, J. Satrustegui, Characterization of a second member of the subfamily of calcium-binding mitochondrial carriers expressed in human non-excitable tissues, *Biochem. J.* 345 (2000) 725–732.
- [5] A. del Arco, J. Satrustegui, Identification of a novel human subfamily of mitochondrial carriers with calcium-binding domains, *J. Biol. Chem.* 279 (2004) 24701–24713.
- [6] J. Satrustegui, B. Pardo, A. Del Arco, Mitochondrial transporters as novel targets for intracellular calcium signaling, *Physiol. Rev.* 87 (2007) 29–67.
- [7] J.J. Ruprecht, E.R.S. Kunji, The SLC25 mitochondrial carrier family: structure and mechanism, *Trends Biochem. Sci.* 45 (2020) 244–258.
- [8] A. del Arco, L. Contreras, B. Pardo, J. Satrustegui, Calcium regulation of mitochondrial carriers, *Biochim Biophys Acta* 1863 (2016) 2413–2421.
- [9] G. Fiermonte, F. De Leonardi, S. Todisco, L. Palmieri, F.M. Lasorsa, F. Palmieri, Identification of the mitochondrial ATP-Mg/Pi transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution, *J. Biol. Chem.* 279 (2004) 30722–30730.
- [10] L. Palmieri, B. Pardo, F.M. Lasorsa, A. del Arco, K. Kobayashi, M. Iijima, M. J. Runswick, J.E. Walker, T. Saheki, J. Satrustegui, F. Palmieri, Citrin and Aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria, *EMBO J.* 20 (2001) 5060–5069.
- [11] S.P. Harborne, J.J. Ruprecht, E.R. Kunji, Calcium-induced conformational changes in the regulatory domain of the human mitochondrial ATP-Mg/Pi carrier, *Biochim. Biophys. Acta* 1847 (2015) 1245–1253.
- [12] C. Thangaratnarajah, J.J. Ruprecht, E.R. Kunji, Calcium-induced conformational changes of the regulatory domain of human mitochondrial aspartate/glutamate carriers, *Nat. Commun.* 5 (2014) 5491.
- [13] Q. Yang, S. Brüsche, J.J. Chou, A self-sequestered calmodulin-like Ca²⁺ sensor of mitochondrial SCA_{MC} carrier and its implication to Ca²⁺-dependent ATP-Mg/Pi transport, *Structure* 22 (2014) 209–217.
- [14] I. Haferkamp, S. Schmitz-Esser, The plant mitochondrial carrier family: functional and evolutionary aspects, *Front. Plant Sci.* 3 (2012) 2.
- [15] A. Nunes-Nesi, J.H.F. Cavalcanti, A.R. Fernie, Characterization of in vivo function (s) of members of the plant mitochondrial carrier family, *Biomolecules* 10 (2020) 1226.
- [16] M.R. Toleco, T. Naake, Y. Zhang, J.L. Heazlewood, A.R. Fernie, Plant mitochondrial carriers: molecular gatekeepers that help to regulate plant central carbon metabolism, *Plants (Basel)* 9 (2020) 117.
- [17] J. Traba, J. Satrustegui, A. del Arco, Adenine nucleotide transporters in organelles: novel genes and functions, *Cell. Mol. Life Sci.* 68 (2011) 1183–1206.
- [18] J.R. Aprile, Regulation of the mitochondrial adenine nucleotide pool size in liver: mechanism and metabolic role, *FASEB J.* 2 (1988) 2547–2556.
- [19] J.L. Joyal, J.R. Aprile, The ATP-Mg/Pi carrier of rat liver mitochondria catalyzes a divalent electroneutral exchange, *J. Biol. Chem.* 267 (1992) 19198–19203.
- [20] I. Amigo, J. Traba, M.M. González-Barroso, C.B. Rueda, M. Fernández, E. Rial, A. Sánchez, J. Satrustegui, A. Del Arco, Glucagon regulation of oxidative phosphorylation requires an increase in matrix adenine nucleotide content through Ca²⁺ activation of the mitochondrial ATP-Mg/Pi carrier SCA_{MC}-3, *J. Biol. Chem.* 288 (2013) 7791–7802.
- [21] R.P. Anunciado-Koza, J. Zhang, J. Ukropce, S. Bajpeyi, R.A. Koza, R.C. Rogers, W. T. Cefalu, R.L. Mynatt, L.P. Kozak, Inactivation of the mitochondrial carrier SLC25A25 (ATP-Mg²⁺/Pi transporter) reduces physical endurance and metabolic efficiency in mice, *J. Biol. Chem.* 286 (2011) 11659–11671.
- [22] C.B. Rueda, J. Traba, A. Amigo, I. Llorente-Folch, P. González-Sánchez, B. Pardo, J. A. Esteban, A. del Arco, J. Satrustegui, Mitochondrial ATP-mg/pi carrier SCA_{MC}-3/Slc25a23 counteracts PARP-1-dependent fall in mitochondrial ATP caused by excitotoxic insults in neurons, *J. Neurosci.* 35 (2015) 3566–3581.
- [23] P. Hernansanz-Agustín, C. Choya-Foces, S. Carregal-Romero, E. Ramos, T. Oliva, T. Villa-Piña, L. Moreno, A. Izquierdo-Álvarez, J.D. Cabrera-García, A. Cortés, A. V. Lechuga-Vieco, P. Jardiya, E. Navarro, E. Parada, A. Palomino-Antolín, D. Tello, R. Acín-Pérez, J.C. Rodríguez-Aguilera, P. Navas, Á. Cogolludo, I. López-Montero, Á. Martínez-Del-Pozo, J. Egea, M.G. López, J.W. Elrod, J. Ruiz-Cabello, A. Bogdanova, J.A. Enríquez, A. Martínez-Ruiz, Na⁺ controls hypoxic signalling by the mitochondrial respiratory chain, *Nature* 586 (2020) 287–291.
- [24] J. Traba, A. Del Arco, M.R. Duchen, G. Szabadkai, J. Satrustegui, SCA_{MC}-1 promotes cancer cell survival by desensitizing mitochondrial permeability transition via ATP/ADP-mediated matrix Ca(2+) buffering, *Cell Death Differ.* 19 (2012) 650–660.
- [25] E. Carafoli, C.S. Rossi, A.L. Lehninger, Uptake of adenine nucleotides by respiring mitochondria during active accumulation of Ca⁺⁺ and phosphate, *J. Biol. Chem.* 240 (1965) 2254–2261.
- [26] J. Traba, E.M. Froeschauer, G. Wiesenberger, J. Satrustegui, A. Del Arco, Yeast mitochondria import ATP through the calcium-dependent ATP-Mg/Pi carrier Sal1p, and are ATP consumers during aerobic growth in glucose, *Mol. Microbiol.* 69 (2008) 570–585.
- [27] A. Hofherr, C. Seger, F. Fitzpatrick, T. Busch, E. Michel, J. Luan, L. Osterried, F. Linden, A. Kramer-Zucker, B. Wakimoto, C. Schütze, N. Wiedemann, A. Artati, J. Adamski, G. Walz, E.R.S. Kunji, C. Montell, T. Watnick, M. Köttgen, The

- mitochondrial transporter SLC25A25 links ciliary TRPP2 signaling and cellular metabolism, *PLoS Biol.* 16 (2018), e2005651.
- [28] M. Monné, D.V. Miniero, T. Obata, L. Daddabbo, L. Palmieri, A. Voza, M. C. Nicolardi, A.R. Fernie, P. Palmieri, Functional characterization and organ distribution of three mitochondrial ATP-Mg/Pi carriers in *Arabidopsis thaliana*, *Biochim. Biophys. Acta* 1847 (2015) 1220–1230.
- [29] S.P. Harborne, M.S. King, P.G. Crichton, E.R. Kunji, Calcium regulation of the human mitochondrial ATP-Mg/Pi carrier SLC25A24 uses a locking pin mechanism, *Sci. Rep.* 7 (2017) 45383.
- [30] J. Traba, J. Satrustegui, A. del Arco, Characterization of SCA-MC-3-like/slc25a41, a novel calcium-independent mitochondrial ATP-Mg/Pi carrier, *Biochem. J.* 418 (2009) 125–133.
- [31] S.P.D. Harborne, E.R.S. Kunji, Calcium-regulated mitochondrial ATP-Mg/Pi carriers evolved from a fusion of an EF-hand regulatory domain with a mitochondrial ADP/ATP carrier-like domain, *IUBMB Life* 70 (2018) 1222–1232.
- [32] F. Burki, A.J. Roger, M.W. Brown, A.G.B. Simpson, The new tree of eukaryotes, *Trends Ecol. Evol.* 35 (2020) 43–55.
- [33] M.W. Gray, G. Burger, R. Derelle, V. Klimeš, M.M. Leger, M. Sarasin, Č. Vlček, A. J. Roger, M. Eliáš, B.F. Lang, The draft nuclear genome sequence and predicted mitochondrial proteome of *Andalucia godoyi*, a protist with the most gene-rich and bacteria-like mitochondrial genome, *BMC Biol.* 18 (2020) 22. D.
- [34] D.W.A. Buchan, D.T. Jones, The PSIPRED protein analysis workbench: 20 years on, *Nucleic Acids Res.* 47 (W1) (2019) W402–W407.
- [35] A. Dereeper, V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J.F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J.M. Claverie, O. Gascuel, Phylogeny.fr: robust phylogenetic analysis for the non-specialist, *Nucleic Acids Res.* 36 (Web Server issue) (2008) W465–W469.
- [36] S. Guindon, O. Gascuel, A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood, *Syst. Biol.* 52 (2003) 696–704.
- [37] H.J. Santos, T. Makiuchi, T. Nozaki, Reinventing an organelle: the reduced mitochondrion in parasitic Protists, *Trends Parasitol.* 34 (2018) 1038–1055.
- [38] S. Cavero, J. Traba, A. Del Arco, J. Satrustegui, The calcium-dependent ATP-Mg/Pi mitochondrial carrier is a target of glucose-induced calcium signalling in *Saccharomyces cerevisiae*, *Biochem. J.* 392 (2005) 537–544.
- [39] Chen X.J. (2004) Sal1p, a calcium-dependent carrier protein that suppresses an essential cellular function associated with the Aac2 isoform of ADP/ATP translocase in *Saccharomyces cerevisiae*. *Genetics* 167:607–17.
- [40] K.W. Chan, D.J. Slotboom, S. Cox, T.M. Embley, O. Fabre, M. van der Giezen, M. Harding, D.S. Horner, E.R. Kunji, G. León-Avila, J. Tovar, A novel ADP/ATP transporter in the mitosome of the microaerophilic human parasite *Entamoeba histolytica*, *Curr. Biol.* 15 (2005) 737–742.
- [41] S.D. Dyall, C.M. Koehler, M.G. Delgadillo-Correa, P.J. Bradley, E. Plümper, D. Leuenberger, C.W. Turck, P.J. Johnson, Presence of a member of the mitochondrial carrier family in hydrogenosomes: conservation of membrane-targeting pathways between hydrogenosomes and mitochondria, *Mol. Cell. Biol.* 20 (2000) 2488–2497.
- [42] F. Mi-ichi, A. Nozawa, H. Yoshida, Y. Tozawa, T. Nozaki, Evidence that the *Entamoeba histolytica* mitochondrial carrier family links Mitosomal and cytosolic pathways through exchange of 3'-phosphoadenosine 5'-phosphosulfate and ATP, *Eukaryot. Cell* 14 (2015) 1144–1150.
- [43] N.C. Beltrán, L. Horváthová, P.L. Jedelský, M. Sedinová, P. Rada, M. Marcinčíková, I. Hrdý, J. Tachezy, Iron-induced changes in the proteome of *Trichomonas vaginalis* hydrogenosomes, *PLoS One* 8 (2013), e65148.
- [44] P. Rada, P. Doležal, P.L. Jedelský, D. Bursac, A.J. Perry, M. Sedinová, K. Smíšková, M. Novotný, N.C. Beltrán, I. Hrdý, T. Lithgow, J. Tachezy, The core components of organelle biogenesis and membrane transport in the hydrogenosomes of *Trichomonas vaginalis*, *PLoS One* 6 (2011), e24428.
- [45] D. Hu, Y. Li, W. Jin, H. Gong, Q. He, Y. Li, Identification and characterization of a plastidic adenine nucleotide Uniporter (OsBT1-3) required for chloroplast development in the early leaf stage of Rice, *Sci. Rep.* 7 (2017) 41355.
- [46] S. Liu, D.M. Roellig, Y. Guo, N. Li, M.A. Frace, K. Tang, L. Zhang, Y. Feng, L. Xiao, Evolution of mitosome metabolism and invasion-related proteins in *Cryptosporidium*, *BMC Genomics* 17 (2016) 1006.
- [47] Z. Xu, Y. Guo, D.M. Roellig, Y. Feng, L. Xiao, Comparative analysis reveals conservation in genome organization among intestinal *Cryptosporidium* species and sequence divergence in potential secreted pathogenesis determinants among major human-infecting species, *BMC Genomics* 20 (2019) 406.
- [48] M.S. King, S. Tavoulari, V. Mavridou, A.C. King, J. Mifsud, E.R.S. Kunji, A single cysteine residue in the translocation pathway of the Mitosomal ADP/ATP carrier from *Cryptosporidium parvum* confers a broad nucleotide specificity, *Int. J. Mol. Sci.* 21 (2020) 8971.
- [49] A. Stehman, K. Hamblin, V. Pérez-Brocal, D. Gaston, G.S. Richmond, M. van der Giezen, C.G. Clark, A.J. Roger, Organelles in *Blastocystis* that blur the distinction between mitochondria and hydrogenosomes, *Curr. Biol.* 18 (2008) 580–585.
- [50] F. Denoed, M. Roussel, B. Noel, I. Wawrzyniak, C. Da Silva, M. Diogon, E. Viscogliosi, C. Brochier-Armanet, A. Couloux, J. Poulain, B. Segurens, V. Anthouard, C. Texier, N. Blot, P. Poirier, G.C. Ng, K.S. Tan, F. Artiguenave, O. Jaillon, J.M. Aury, F. Delbac, P. Wincker, C.P. Vivarès, H. El Alaoui, Genome sequence of the stramenopile *Blastocystis*, a human anaerobic parasite, *Genome Biol.* 12 (2011) R29.
- [51] E. Gaulin, M.A. Madoui, A. Bottin, C. Jacquet, C. Mathé, A. Couloux, P. Wincker, B. Dumas, Transcriptome of *Aphanomyces euteiches*: new oomycete putative pathogenicity factors and metabolic pathways, *PLoS One* 3 (2008), e1723.
- [52] N.G. Faux, S.P. Bottomley, J.A. Lesk, J.R. Morrison, M.G. Banda, J.C. Whiststock, Functional insights from the distribution and role of homopeptide repeat containing proteins, *Genome Res.* 15 (2005) 537–551.
- [53] G.P. Singh, B.R. Chandra, A. Bhattacharya, R.R. Akhouri, S.K. Singh, A. Sharma, Hyper-expansion of asparagines correlates with an abundance of proteins with prion-like domains in *Plasmodium falciparum*, *Mol. Biochem. Parasitol.* 137 (2004) 307–319.
- [54] A. Nozawa, D. Ito, M. Ibrahim, H.J. Santos, T. Tsuboi, Y. Tozawa, Characterization of mitochondrial carrier proteins of malaria parasite *Plasmodium falciparum* based on in vitro translation and reconstitution, *Parasitol. Int.* 79 (2020) 102160.
- [55] S. Chavali, A.K. Singh, B. Santhanam, M.M. Babu, Amino acid homorepeats in proteins, *Nat. Rev. Chem.* 4 (2020) 420–434.
- [56] Z. Feng, X. Zhang, P. Han, N. Arora, R. Anders, R. Norton, Abundance of intrinsically unstructured proteins in *P. falciparum* and other apicomplexan parasite proteomes, *Mol. Biochem. Parasitol.* 150 (2006) 256–267.
- [57] A. Lorenz, M. Lorenz, U.C. Vothknecht, S. Niopek-Witz, H.E. Neuhaus, I. Haferkamp, In vitro analyses of mitochondrial ATP/phosphate carriers from *Arabidopsis thaliana* revealed unexpected Ca(2+)-effects, *BMC Plant Biol.* 15 (2015) 238.
- [58] S. Stael, A.G. Rocha, A.J. Robinson, P. Kmiecik, U.C. Vothknecht, M. Teige, *Arabidopsis* calcium-binding mitochondrial carrier proteins as potential facilitators of mitochondrial ATP-import and plastid SAM-import, *FEBS Lett.* 585 (2011) 3935–3940.
- [59] D. Moreira, P. López-García, Protist evolution: stealing genes to gut it out, *Curr. Biol.* 27 (2017) R223–R225.
- [60] P. Dean, P. Major, S. Nakjang, R.P. Hirt, T.M. Embley, Transport proteins of parasitic protists and their role in nutrient salvage, *Front. Plant Sci.* 5 (2014) 153.
- [61] P.L. Jedelský, P. Doležal, P. Rada, J. Pyrih, O. Smíd, I. Hrdý, M. Sedinová, M. Marcinčíková, L. Voleman, A.J. Perry, N.C. Beltrán, T. Lithgow, J. Tachezy, The minimal proteome in the reduced mitochondrion of the parasitic protist *Giardia intestinalis*, *PLoS One* 6 (2011), e17285.
- [62] E. Heinz, C. Hacker, P. Dean, J. Mifsud, A.V. Goldberg, T.A. Williams, S. Nakjang, A. Gregory, R.P. Hirt, J.M. Lucocq, E.R. Kunji, T.M. Embley, Plasma membrane-located purine nucleotide transport proteins are key components for host exploitation by microsporidian intracellular parasites, *PLoS Pathog.* 10 (2014), e1004547.
- [63] A.D. Tsoulos, E.R. Kunji, A.V. Goldberg, J.M. Lucocq, R.P. Hirt, T.M. Embley, A novel route for ATP acquisition by the remnant mitochondria of *Encephalitozoon cuniculi*, *Nature* 453 (2008) 553–556.
- [64] M. Boudsocq, M.J. Droillard, L. Regad, C. Laurière, Characterization of *Arabidopsis* calcium-dependent protein kinases: activated or not by calcium? *Biochem. J.* 447 (2012) 291–299.
- [65] G. Freymark, T. Diehl, M. Miklis, T. Romeis, R. Panstruga, Antagonistic control of powdery mildew host cell entry by barley calcium-dependent protein kinases (CDPKs), *Mol. Plant-Microbe Interact.* 20 (2007) 1213–1221.
- [66] A. Villalobo, M. González-Muñoz, M.W. Berchtold, Proteins with calmodulin-like domains: structures and functional roles, *Cell. Mol. Life Sci.* 76 (2019) 2299–2328.
- [67] J.R. Ingram, K.E. Knockenhauer, B.M. Markus, J. Mandelbaum, A. Ramek, Y. Shan, D.E. Shaw, T.U. Schwartz, H.L. Ploegh, S. Lourido, Allosteric activation of apicomplexan calcium-dependent protein kinases, *Proc Natl Acad Sci U S A* 112 (2015) E4975–E4984.
- [68] I. Amigo, J. Traba, J. Satrustegui, A. del Arco, SCA-MC-1Like a member of the mitochondrial carrier (MC) family preferentially expressed in testis and localized in mitochondria and chromatin body, *PLoS One* 7 (2012), e40470.
- [69] N.E. Hoffman, H.C. Chandramoorthy, S. Shanmughapriya, X.Q. Zhang, S. Vallem, P.J. Doonan, K. Mallankaraman, S. Guo, S. Rajan, J.W. Elrod, W.J. Koch, J. Y. Cheung, M. Madesh, SLC25A23 augments mitochondrial Ca²⁺ uptake, interacts with MCU, and induces oxidative stress-mediated cell death, *Mol. Biol. Cell* 25 (2014) 936–947.
- [70] P.H. Scarpelli, M.F. Pecenin, C.R.S. Garcia, Intracellular Ca²⁺ signaling in protozoan parasites: an overview with a focus on mitochondria, *Int. J. Mol. Sci.* 22 (2021) 469.
- [71] N. Lander, M.A. Chiurillo, M.S. Bertolini, M. Storey, A.E. Vercesi, R. Docampo, Calcium-sensitive pyruvate dehydrogenase phosphatase is required for energy metabolism, growth, differentiation, and infectivity of *Trypanosoma cruzi*, *J. Biol. Chem.* 293 (2018) 17402–17417.
- [72] M.V. Dubinin, K.N. Belosludtsev, Taxonomic features of specific Ca²⁺ transport mechanisms in mitochondria, *Biochemistry (Moscow), Suppl A: Membr Cell Biol.* 13 (2019) 194–204.
- [73] G. Huang, R. Docampo, The mitochondrial Ca(2+) uniporter complex (MCUC) of *Trypanosoma brucei* is a hetero-oligomer that contains novel subunits essential for Ca(2+) uptake, *mBio.* 9 (2018) e01700–e01718.
- [74] M.S. Bertolini, M.A. Chiurillo, N. Lander, A.E. Vercesi, R. Docampo, MICU1 and MICU2 play an essential role in mitochondrial Ca²⁺ uptake, growth, and infectivity of the human pathogen *Trypanosoma cruzi*, *mBio.* 10 (2019) e00348–19.
- [75] A.G. Bick, S.E. Calvo, V.K. Mootha, Evolutionary diversity of the mitochondrial calcium uniporter, *Science* 336 (2012) 886.
- [76] A.A. Pittis, V. Goh, A. Cebrian-Serrano, J. Wettmarshausen, F. Perocchi, T. Gabaldón, Discovery of EMRE in fungi resolves the true evolutionary history of the mitochondrial calcium uniporter, *Nat. Commun.* 11 (2020) 4031.
- [77] P. Mier, M. Andrade-Navarro, dAPE: a web server to detect homorepeats and follow their evolution, *Bioinformatics* 33 (2017) 1221–1223.
- [78] F. Madeira, Y.M. Park, J. Lee, N. Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A. R.N. Tivey, S.C. Potter, R.D. Finn, R. Lopez, The EMBL-EBI search and sequence analysis tools APIs in 2019, *Nucleic Acids Res.* 47 (2019) W636–W641.
- [79] R.H. Jiang, I. de Bruijn, B.J. Haas, R. Belmonte, L. Löbach, J. Christie, G. van den Ackerveken, A. Bottin, V. Bulone, S.M. Díaz-Moreno, B. Dumas, L. Fan, E. Gaulin, F. Govers, L.J. Grenville-Briggs, N.R. Horner, J.Z. Levin, M. Mammella, H.J. Meijer,

P. Morris, C. Nusbaum, S. Oome, A.J. Phillips, D. van Rooyen, E. Rzeszutek, M. Saraiva, C.J. Secombes, M.F. Seidl, B. Snel, J.H. Stassen, S. Sykes, S. Tripathy, H. van den Berg, J.C. Vega-Arreguin, S. Wawra, S.K. Young, Q. Zeng, J. Dieguez-Urbeondo, C. Russ, B.M. Tyler, P. van West, Distinctive expansion of potential

virulence genes in the genome of the oomycete fish pathogen *Saprolegnia parasitica*. PLoS Genet. 9 (6) (2013) e1003272, <https://doi.org/10.1371/journal.pgen.1003272>.