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# Unique Properties of Apicomplexan Mitochondria

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## Abstract

Apicomplexan parasites constitute over 6000 species infecting a wide range of hosts. These include important pathogens such as those causing malaria and toxoplasmosis. Their evolutionary emergence coincided with the dawn of animals. Mitochondrial genomes of apicomplexan parasites have undergone dramatic reduction in their coding capacity with genes for only 3 proteins and ribosomal RNA genes present in scrambled fragments originating from both strands. Different branches of the apicomplexans have undergone rearrangements of these genes, with *Toxoplasma* having massive variations in gene arrangements spread over multiple copies. The vast evolutionary distance between the parasite and host mitochondria has been exploited for developing antiparasitic drugs, especially for treatment of malaria, wherein inhibition of the parasite mitochondrial respiratory chain is selectively targeted with little toxicity to the host mitochondria. We describe additional unique characteristics of the parasite mitochondria that are being investigated and provide greater insights into these deep branching eukaryotic pathogens.

### Keywords

Myzozoa; Plasmodium; Toxoplasma; ATP synthase; Electron transport complexes; Antiparasitic drugs

## Introduction

The emergence of eukaryotes from an exclusively prokaryotic biosphere was a momentous event that charted a divergent course for life on Earth (82). This event coincided with syntrophic adoption of mitochondria. The complexity of eukaryotes could not have existed and evolved but for the energy economy supported by mitochondrial physiology (64). Given the strong evidence for the monophyletic origin of mitochondria (37–39), it is probable that all extant mitochondria can trace their origin to that singular primordial event at the dawn of eukaryotes. Yet, evolution has resulted in a tremendous variety of mitochondria, their genomes, their regulation, and their functions, which are as diverse as the organisms they reside in, from *Paramecium* to the Pope. Each clade of eukaryotes appears to adjust

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its mitochondrial function to fit the niche in which it exists (39). Apicomplexan parasites demonstrate these evolutionary adjustments in an illuminating manner. These obligatory intracellular parasites consist of thousands of species, all making their living off a vast array of host animals (2; 3). Even corals, the deepest branching animals, have intracellular apicomplexans, suggesting that the parasitic nature of apicomplexans is as ancient as the origin of Metazoa (61; 62). The discovery of mitochondrial DNA (mtDNA) in malaria parasites as tandemly arrayed molecules with a unit length of 6 kb (51; 120; 125–127) led to the realization that a separate circular DNA of 35 kb in the parasite, believed then to be a mtDNA (34; 135), was in fact a remnant of a chloroplast genome residing in a separate organelle, now termed an apicoplast (for apicomplexan plastid). The apicoplast consists of a 4-membrane structure and thus is proposed to have originated from a secondary endosymbiotic event common to all apicomplexans, in which an algal organism was engulfed by the progenitor of both the dinoflagellates and apicomplexans (56; 57). Most extant apicomplexans have three distinct genomes: the nuclear, the mitochondrial and the apicoplast. However, the proposed secondary endosymbiotic event would have initially involved coexistence of five genomes—the progenitor's nuclear and mitochondrial DNAs plus the algal nuclear, plastid and mitochondrial DNAs—before being reduced to three (Fig. 1). The massive quantity of gene transfers and reductions that followed has resulted in apicomplexan genomes carrying a mixture of genes inherited from 5 distinct genomes (Fig. 1). This complex provenance of apicomplexans, as well as natural selection to fit a tremendous variety of parasitic niches, has resulted in many biological characteristics that are distinct from most model eukaryotes. In this review, we aim to describe such distinct aspects of the mitochondrion in apicomplexan parasites. Our goal is not to provide a comprehensive view of mitochondria in all apicomplexans but to describe their unique properties. We refer the reader to several excellent reviews that cover additional details of these organelles (11; 41–43; 79; 85; 124; 128).

## Downsizing and Diversification

Apicomplexans belong to the superphylum Alveolata, which diverged about 850 million years ago into two main branches: Ciliates and Myzozoa. Whereas the progenitor of alveolates likely contained a mtDNA of about 50 kb coding for 40 or so genes, and most ciliates continue to have a similar sized mtDNA, the Myzozoa underwent a drastic reduction in their capacity and now code for just 3 proteins (cytochrome oxidase subunits 1 and 3 (Cox1 and Cox 3) and cytochrome *b* (Cytb)) and fragmentary ribosomal RNA (rRNA) pieces (86; 128). The divergence of Myzozoa coincided with the secondary endosymbiotic acquisition of plastids (11; 49). Among the three main branches of Myzozoa, two (dinoflagellates and chromerids) consist of mostly free-living species and have plastids with photosynthesis capacity, whereas members of the third (apicomplexans) contain non-photosynthetic plastids and parasitize animals. The large reduction in the number of genes encoded by mtDNA in Myzozoa, therefore, is unlikely to be due to their dependence on an external source for their energy needs (23). One possibility could be that the progenitor of the Myzozoa, after acquiring a plastid, became more dependent on the photosynthetic supply of energy and less on the mitochondrial contribution. All through the radiation of the metazoan clade, the reduction in the gene content of their mtDNAs also appears to

be accompanied by large scale rearrangements of genes as well as of the configurations of the genomes (Fig. 2). Even among the Apicomplexans, the synteny of the genes is highly divergent and the configuration of the genomes varies among different genera, and sometimes even within the same genus (Fig. 2). Unlike the three common protein coding genes, rRNA gene fragments are highly variable among different apicomplexans. The evolutionary forces that must have driven this high degree of divergence are not clear but do suggest events that occurred at branch points in the apicomplexan phylogenetic tree coincided with massive gene rearrangements within the mtDNA. Of interest, the extent of divergence seen for the mtDNA is not observed in the apicoplast genomes of the extant apicomplexans (99; 129). This suggests differential evolutionary pressures on the two apicomplexan cytoplasmic genomes.

## Apicomplexan Mitochondrial Genomes

All apicomplexan mtDNAs encode 3 proteins and rRNA fragments but their sizes and gene arrangements vary greatly among different genera, and in some cases within a genus (Fig. 2). The size of the mtDNAs range from 6 kb (e.g., *Plasmodium* spp.) to 12 kb (*Theileria equi*) (43). In general, apicomplexan mtDNAs are present as linear molecules but in the case of *Plasmodium* spp., they appear to form a few circular DNA molecules, which are likely to be molecules undergoing a rolling circle mode of replication (105; 134). The tandem head-to-tail arrays with 6 kb unit length are the likely outcome of this mode of replication. Gene arrangements in *Plasmodium* spp. are highly conserved, with a sequence identity of >90% over an evolutionary distance among different species estimated to span millions of years based on the divergence of nuclear DNA sequences (43). For instance, the overall G+C content of the nuclear DNA varies from ~21% in *P. falciparum* to ~34% in *P. vivax*, two malaria parasites infecting humans, but both their mtDNAs have ~31% G+C content (88). It is also remarkable that, while the codon usage frequency for the nuclear genes between these species is quite divergent, it remains identical for their mtDNA-encoded genes (88).

Compared to *Plasmodium* spp., *Theileria* and *Babesia* mtDNAs are divergent among different species. *T. equi* mtDNA is a 12 kb linear molecule with terminal repeats, while other *Theileria* spp. have 8 to 9 kb mtDNA with genes arranged differently from those in the *T. equi* mtDNA. The size differences are also seen in *Babesia* species with *B. microti* having an 11 kb mtDNA compared to ~8 kb mtDNA in other species (43).

*Toxoplasma gondii*, and related coccidial parasites, *Hammondia* and *Neospora,* contain the most highly diverse and dramatically rearranged mtDNA among all apicomplexans (10; 96). For many years, the authentic mtDNA sequence from *Toxoplasma* could not be established. One complicating reason was the presence of multiple sequences of what appeared to be portions of mtDNA interspersed all throughout the nuclear genome of the parasite (101). Recent application of long-read DNA sequencing technology has revealed a startling complexity in the mitochondrial genome of *T. gondii* (96). Multiple molecules ranging in size from 320 to 23,600 base pairs were detected. Further analysis of all the sequences revealed that these molecules were not linear concatemers of a unit molecule but did contain 26 distinct sequence blocks that were arranged in varying configurations. Overall, coding sequences for Cox1, Cox3 and Cytb could be discerned but were arranged

differently in different molecules (96). Fragments of rRNAs could also be detected but attempts to assemble them into small and large subunit rRNAs have not been carried out (96). It is a wonder how this complex arrangement of genes can result in the generation of mitochondrial complexes necessary for the survival of the organism. It is also interesting to note that the non-cyst forming coccidial parasites of *Eimeria* species do not have this bizarre arrangement of their mtDNA but appear to consist of tandem arrays of 7 kb molecules (44; 71).

## Mitochondrial rRNA

Since the emergence of mitochondria from its alpha-proteobacterial progenitor, mitochondrial rRNAs appear to have undergone dramatic changes (24; 29; 53). This is reflected in the highly divergent structures of mitoribosomes, particularly in the increase in the ratio of protein to RNA content, suggesting the transfer of the structural role of organization and scaffolding from rRNAs to proteins in the mitoribosome (12; 106). Although, the structure of the mitoribosome of an apicomplexan parasite has not been elucidated, analysis of mitoribosomes of unicellular organisms such as *Trypanosoma brucei, Tetrahymena thermophilia*, and *Chlamydomonas reinhardtii* show a reduction in rRNA size and incorporation of new and enlarged mitoribosomal proteins. Mitoribosomal proteins form a shell that surrounds the rRNA core of the mitoribosome, stabilizing and orienting the critical regions of their fragmented rRNAs appropriately (106; 123; 132). With the much larger number of rRNA fragments encoded by apicomplexan mtDNA (see below) and the apparent absence of some of the conserved rRNA domains, the apicomplexan mitoribosomal structure, once determined, is likely to reveal many divergent and surprising features.

In contrast to most eukaryotes where mitochondrial rRNA subunits are encoded by continuous DNA sequences that are transcribed into long continuous polyribonucleotide chains, DNA sequences encoding mitochondrial rRNA in apicomplexans are fragmented and arranged in a scrambled manner originating from both strands of mtDNA (29; 125; 127). rRNA fragments have been identified in the mitochondria of all apicomplexans examined including *Plasmodium spp.* (28; 29; 125; 127), *Babesia spp.* (43; 45), *Theileria spp.* (45; 52), *Eimeria spp.* (44), and *Toxoplasma gondii* (96). However, investigations on these mitochondrial rRNA fragments have mostly been conducted on *Plasmodium spp.* parasites (29). Just as the mtDNA of each apicomplexan varies in size and organization, these mitochondrial rRNA fragments also vary in size, arrangement, and length across various apicomplexans. *Eimeria spp.* and *Plasmodium spp.* have a mtDNA of ~ 6–7 kb in length. However, most species of *Eimeria* have identified 14 large subunit (LSU) and 11 small subunit (SSU) rRNA fragments (69; 72), while *P. falciparum* has 15 LSU and 12 SSU rRNA fragments identified (29; 30). Furthermore, the DNA sequences encoding mitochondrial rRNA in *Plasmodium spp* and *Theileria spp* are arranged out of order and interspersed between protein-coding genes on either strand of the mtDNA (29; 30; 125; 127). The lengths of the mitochondrial rRNA fragments also differ vastly within each apicomplexan parasite (29; 72; 96).

Through both intra- and inter-molecular complementary base pairing, rRNA fragments in apicomplexans can be predicted to form standard RNA secondary structures, such as hairpin

loops, that are consistent with the structures formed by their continuous rRNA sequence counterparts (29; 50; 68; 125). Although these fragmented rRNAs are not linked covalently, their catalytic core is preserved so they are likely to retain their function. Systematic transcript mapping of mitochondrial rRNA transcripts in *P. falciparum* demonstrated that some are conserved across apicomplexans and can be mapped to conserved sequences in the SSU and LSU rRNAs of *E. coli* (29; 30). However, there are some portions of the rRNA in *E. coli* that could not be identified amongst the pool of rRNA transcripts in *P. falciparum*. One possibility is that these missing rRNA pieces may be imported from the cytoplasm, possibly through the machinery required for tRNA import. It is also possible that they have been lost due to the evolutionary trend of reducing mitoribosomal rRNA size and transferring its function to mitoribosomal proteins (53; 132).

The process by which rRNA fragments are processed and assembled with ribosomal proteins into a functional mitoribosome remains to be elucidated. The mitoribosome of *P. falciparum* has been shown to be associated with the inner mitochondrial membrane, and the essentiality of the mitoribosome in *P. falciparum* and *T. gondii* has been demonstrated (54; 70; 113). Based on amino acid sequence similarity to mitoribosomal proteins of other organisms, 43 proteins have been predicted to be *P. falciparum* mitoribosomal proteins, six of which have been shown to be essential for mitochondrial functions (25; 54). Recently, two proteins with RNA-binding domains that are abundant in apicomplexans, *Pf*RAP01 and *Pf*RAP21, were identified and characterized to be essential nuclear-encoded proteins that are targeted to the mitochondria where they specifically bind mitochondrial rRNAs in *P. falciparum* (46; 47). *Pf*RAP21 is involved in the control of mitochondrial rRNA expression, while both RAP proteins play a role in RNA processing, translation, and expression of mitoribosomal subunits (46; 47).

It is of interest that mitochondrial rRNAs in *P. falciparum* and *Theileria parva* have been shown to possess oligo(A) tails up to 21 nucleotides in length, which are added post-transcriptionally to their 3’ end (29; 36; 97). The length of the oligo(A) tails is transcript-specific, so it is thought their addition may serve a protective role in preventing exonucleolytic degradation of these rRNA fragments to allow for proper assembly, structure, and function of the mitoribosome. However, the roles of oligo-adenylation of specific mitochondrial rRNA transcripts in apicomplexans remain uncertain. Several investigations have revealed that the genes encoding the 16S and 12S rRNA in humans encode small open reading frames (ORFs) which are translated into “mitochondrial-derived peptides” that are involved in important cellular processes (66; 67; 90; 139). These peptides, namely Humanin, SHLPs, and MOTS-c, have been shown to have significant physiological roles and demonstrate that mitochondrial rRNA could encode peptides with important functions. Although mitochondrial rRNA-encoded peptides have not been identified in apicomplexan parasites, a possible peptide-encoding functions of small rRNA fragments could be an important topic for exploration. In addition, several small mitochondrial RNA transcripts in *P. falciparum* have been observed with no homology to known rRNA sequences and with no known functions (29). It would be of interest to assess the potential presence of small ORFs within these RNA molecules that may have the capacity to encode peptides.

## Mitochondrial Proteomes in Apicomplexans

Except for the three mitochondrial electron transport chain (mtETC) subunits encoded by the mtDNA, all other mitochondrial proteins are encoded by the nucleus and imported into the mitochondrion. A variety of approaches have been used to generate a predicted compilation of mitochondrial proteomes in *P. falciparum* and *T. gondii*. Bioinformatic (18) and manual (17) annotations initially generated a list of about 400 proteins likely to be imported into the *Plasmodium* mitochondrion. Since the mitochondrion contains multiple sub-compartments with multiple import mechanisms, comprehensive lists are inevitably difficult to complete. Current manual curation suggests a proteome size of about 500 proteins associated with the *P. falciparum* mitochondrion; the *T. gondii* proteome may be slightly larger given its more complex metabolism. Experimental validations of the predicted mitochondrial proteome have been limited but are gradually accumulating. A mitochondrially-targeted proximity biotinylation followed by enrichment and mass spectrometry approach was used to identify uncharacterized *P. falciparum* proteins putatively targeted to the mitochondrion (63). A list of 122 putative mitochondrial proteins was generated with many proteins that were unannotated and likely to be essential for parasite survival (63). However, biotinylated proteins identified in this study also included apparently cytoplasmic proteins, thereby pointing to the limitations of this approach.

Proteomic evidence suggesting a unique architecture of apicomplexan respiratory chain complexes was reported in *T. gondii* using mitochondrially-targeted proximity biotinylation approaches (111). In addition to five canonical subunits of the cytochrome *c* oxidase (Complex IV) (Cox1, Cox2, Cox3, Cox5b, and Cox6b), this analysis identified 11 apicomplexan-specific Complex IV subunits (111). Complexome profiling, in which label-free quantitative mass spectrometry is paired with microscale fractionation of large complexes in blue native gels, has the power to identify novel protein subunits that co-migrate with such complexes (107; 136). This approach has recently been applied to *P. falciparum* mitochondria-enriched samples and led to identification of novel components of mitochondrial respiratory chain complexes (27). Orthologues of all 11 previously reported Complex IV subunits were identified in the *P. falciparum* complexome profile, confirming the unique Complex IV architecture in these organisms (27). Five uncharacterized, mostly myzozoan-specific, putative subunits also co-migrated with Complex IV, which were termed respiratory chain Complex 4 associated proteins 1–5 (C4AP1–5). In most eukaryotes, the Complex IV subunit Cox2 is encoded by the mtDNA. Uniquely, in apicomplexans and other myzozoans, the gene is split into two nuclear-encoded genes, Cox2a and Cox2b (131). Both of these proteins were detected in the *P. falciparum* complexome profile, confirming their mitochondrial targeting (27).

Canonically, at least four subunit proteins compose succinate dehydrogenase (Complex II): succinate dehydrogenase subunit A (SDHA), SDHB, SDHC, and SDHD. Only SDHA and SDHB are experimentally validated in *Plasmodium* (121; 122), but candidates for SDHC (PF3D7_0611100) and SDHD (PF3D7_1010300) have been proposed (91). In the *Plasmodium* respiratory chain complexome profile, SDHA and SDHB were observed to be co-migrating together in a ~530 kDa complex, but the previously proposed SDHC and SDHD proteins were not found to co-migrate with this complex (27). However, the authors

identified five putative subunits that all shared a common dominant band and assigned one as a candidate for *Pf*SDHC (PF3D7_1448900). This assignment was based upon the conserved “DY” motif found in SDHC in many species (93). The other putative Complex II subunits were termed respiratory chain complex 2 associated proteins 1–4 (C2AP1–4). One of these proteins, PF3D7_0808450, is restricted only to myzozoans and was previously reported as a mitochondrion-localized protein essential for malaria transmission (59).

Complexome profiling has also been carried out on *T. gondii* mitochondria (78). In this study, 60 proteins were identified and assigned to Complexes II, IV, and the $F_1F_o$-ATP synthase (Complex V). The details of recent work investigating $F_1F_o$-ATP synthase complexes in apicomplexans will be discussed in detail below. Sixteen of these 60 respiratory chain complex proteins had not been previously identified. The study also determined the composition of *T. gondii* Complex III, a known drug target. In doing so, two new homologous subunits and two parasite-specific subunits were identified. All four of these proteins were found to be essential for Complex III stability and parasite viability. In addition, depletion of these four subunits led to collapse of the mitochondrial membrane potential, consistent with their assignment as Complex III subunits (78). Similar to *P. falciparum,* the *T. gondii* Complex II subunit SDHB was also observed to migrate as a ~500 kDa complex (78), a much larger size than observed in yeast and mammalian Complex II (~130 kDa) (109; 110). This size was confirmed using endogenous tagging of *Tg*SDHB (TGGT1_215280) and subsequent blue native PAGE analysis, making it a larger Complex II than reported in any other well studied organisms outside of apicomplexans. Seven other proteins co-migrated with *Tg*SDHB, all of which are annotated as hypothetical proteins that bear no obvious protein features indicating function, highlighting the unique nature of Complex II in apicomplexans.

Nucleus-encoded mitochondrial matrix proteins often have N-terminal targeting peptides that are cleaved during import by mitochondrial processing peptidases (MPP) (33; 60). In yeast and mammals, MPPs are heterodimers of subunits α and β, which are located in the mitochondrial matrix (33). In addition to these matrix MPPs, the cytochrome $bc_1$ complex (Complex III) in eukaryotes contains Core1 and Core2 subunits with high amino acid sequence homology to MPP subunits α and β (137). In contrast, plant mitochondria do not have matrix resident MPPs. It has therefore been suggested that, in plants, Complex III has two functions: ubiquinol oxidation and processing of mitochondrially targeted proteins (15; 16). The *P. falciparum* genome encodes annotated α and β MPP subunits that were clearly shown to be part of Complex III (27). This suggests a unique aspect of the malaria parasite mitochondrion in which MPPs are part of Complex III as in plants, playing a role in its structural assembly. In addition to co-migration of all canonical components of Complex III, four additional proteins co-migrated with the complex in the *P. falciparum* mitochondrial complexome profile (27). Three of these putative subunits are represented almost exclusively in apicomplexa, lacking no obvious sequence homology with characterized subunits in other phyla.

Another approach of spatial proteomics called, “hyperplexed localization of organelle proteins by isotope tagging” (hyperLOPIT) was used to determine the steady-state subcellular localization of thousands of *T. gondii* proteins (9). The hyperLOPIT technique

capitalizes on specific abundance-distribution contours that organelles and other subcellular structures form after being subjected to density-gradient centrifugation (21; 95). This study identified 193 mitochondrial membrane-associated proteins and 274 soluble mitochondrial proteins, further enriching the proteomic landscape of *Toxoplasma* mitochondria (9).

## Mitochondrial Contributions to Metabolism

Since apicomplexans are phylogenetically distant from mammals and other model organisms, it is not surprising that their mitochondria exhibit significant differences from those of model organisms. They are also a large phylum containing diverse parasitic species existing in numerous host species and environments. Hence, there is a wide range of bioenergetic capabilities and metabolism associated with apicomplexan mitochondria and related organelles (86). Expression of mitochondrial activities also frequently varies significantly between parasite life cycle stages. Several metabolic pathways common in more familiar model organisms are not found in apicomplexan mitochondria, such as beta oxidation of fatty acids and portions of amino acid and steroid biosynthesis. Fig. 3 provides a schematic description of mitochondrial functions in *P. falciparum*, a parasite for which the most extensive information is available. Apicomplexan parasites of vertebrates, such as *Plasmodium* and *Toxoplasma* have a nearly complete mtETC (lacking only Complex I) and an ATP synthase complex, providing robust oxidative phosphorylation, at least in some life stages. The metabolic partner of the oxidative phosphorylation (OxPhos) ATP generation pathway is the oxidative TCA cycle that provides reducing equivalents to the mtETC. In *Plasmodium* and *Toxoplasma*, a repurposed branched-chain alpha-keto acid dehydrogenase (BCKDH) complex in the mitochondrion, rather than a direct ortholog of the pyruvate dehydrogenase found in model organisms, provides acetyl-CoA to the cycle (100), and a set of five ubiquinone-dependent dehydrogenases provide reducing equivalents to the mtETC. Among the dehydrogenases, only dihydroorotate dehydrogenase is essential in asexual blood stage *Plasmodium,* as a key enzyme in the pyrimidine biosynthesis pathway, and critical for virulence in *Toxoplasma*, even though the later parasite has a salvage pathway for pyrimidines (31; 40; 102). In these parasites 2-oxoglutarate (from glutamine), in addition to glycolytic pyruvate, is a major contributor to carbon flux through the TCA cycle (55; 80; 81). In *Toxoplasma*, the presence of a γ-aminobutyric acid shunt pathway allows for additional input to the TCA cycle of succinate derived from glutamine. Two of the TCA cycle enzymes are novel relative to those in more familiar organisms, malate-quinone oxidoreductase in place of NADH-dependent malate dehydrogenase, rendering this step essentially irreversible, and in place of the more familiar metal free class II fumarase, a class I fumarate hydratase containing an iron-sulfur cluster [Fe-S], rendering the parasite enzyme sensitive to reactive oxygen species. The very low oxidation-reduction potential maintained in the parasite mitochondrion should, however, help protect Fe-S clusters in fumarate hydratase, aconitase and other Fe-S containing proteins (92). Combined genetic and metabolomic experiments have found that acetyl-CoA from the *Plasmodium* mitochondrion is required for acetylation of histones and other cytoplasmic and nuclear proteins, which is provided by BCKDH, or by alpha-ketoglutarate dehydrogenase if BCKDH is ablated (22). Since *Plasmodium* spp., unlike animals and *Toxoplasma*, lack ATP-citrate lyase, which cleaves citrate exported from the mitochondrion to acetyl-CoA and oxaloacetate, it is

presently unclear how acetyl-CoA made in the *Plasmodium* mitochondrion reaches other compartments.

In the most virulent malaria parasite species *P. falciparum*, expression of the enzymes of most mitochondrial pathways including oxidative phosphorylation, is greatly reduced in the asexual blood stage, and the enzymes of the TCA cycle are dispensable (55). Nevertheless, the mtETC and ATP synthase complexes remain essential in this stage, as well as in the insect and liver stages. Expression of these complexes is greatly increased in the sexual blood stage gametocytes (27), although this stage is not fast growing and was thought to be less metabolically active. Stable isotope metabolomic experiments, however, suggest that gametocytes actually exhibit increased utilization of glucose with increased glycolytic flux directed through the TCA cycle (80). This increased expression of mitochondrial enzymes may be in preparation for the increased oxidative phosphorylation activity needed in the following insect stages. In *Toxoplasma*, on the other hand, the fast-growing, invasive tachyzoite stage has relatively robust oxidative phosphorylation activity, but upon stress from the immune system or treatment with certain drugs, including inhibitors of the mtETC, *Toxoplasma* undergoes stage conversion to the slow-growing bradyzoite cyst form with decreased reliance on mitochondrial oxidative phosphorylation and TCA cycle activities (20; 103).

Many *Cryptosporidium* parasites of the mammalian lower intestine and gregarine parasites of invertebrates have extremely reduced mitochondria related organelles called mitosomes that lack internal structure, as well as a TCA cycle, mtETC complexes and ATP synthase, but do contain a small number of ubiquinone-dependent dehydrogenases, such as NADH dehydrogenase and malate-quinone oxidoreductase, and an ubiquinol-utilizing alternative oxidase (1; 73; 86; 138). It is not clear how such a reduced mitosome would energize its membrane to provide for protein import, which, at a minimum, is required to carry out the common essential function of all mitochondria and mitosomes, i.e. iron-sulfur cluster ([Fe-S]) biogenesis. One possibility is a combination of external proton release concomitant to oxidation of ubiquinol and electrogenic exchange of $ADP^{3-}$ for $ATP^{4-}$ via the mitochondrial adenine nucleotide transporter.

## Mitochondrial Targets for Antiparasitic Drugs

Early investigations using preparations enriched in mitochondria from malaria parasites showed that an antimalarial hydroxynaphthoquinone under development, subsequently named atovaquone, inhibited Complex III of the parasites (32). An examination of the sequence of *P. falciparum* cytochrome *b,* the central subunit of Complex III, revealed subtle but significant differences in the ubiquinol oxidation ($Q_o$) and ubiquinone reduction ($Q_i$) sites of the parasite cytochrome *b* compared to its host counterpart, which were proposed as the basis for selective toxicity of antimalarial drugs such as atovaquone (127). Atovaquone treatment also caused a collapse of the membrane potential across the inner membrane of parasite mitochondria (116). Subsequent studies revealed that mutations surrounding the $Q_o$ site in the parasite cytochrome *b* were responsible for resistance to atovaquone (58; 83; 87; 115). Structural studies using heterologous Complex III have supported the $Q_o$ site as the area of interaction with atovaquone (13). Because cytochrome *b* is largely conserved among

mitochondriate apicomplexan parasites, atovaquone has been shown to be also effective against parasites such as *Toxoplasma* and *Babesia* (4; 19). While atovaquone is a potent drug, resistance to it arises rapidly when administered as a monotherapy (76). Therefore, a synergistic combination of atovaquone with a biguanide, proguanil, has been used for malaria prophylaxis and treatment (75; 77). The synergy between atovaquone and proguanil results from the ability of proguanil to inhibit a secondary pathway for generating membrane potential across the inner mitochondrial membrane in malaria parasites (117). Another series of compounds called endochin-like quinolones (ELQs) have been extensively investigated with one of them, ELQ-331, being designated a clinical candidate (89; 98; 118). ELQ-331 is a prodrug that is converted to ELQ-300, which has been shown to work at the $Q_i$ site of the parasite cytochrome *b* (89; 98). Other ELQ compounds have been shown to inhibit the growth of *Toxoplasma* and *Babesia* (26; 65). Medicinal chemistry exploration of the ELQ compounds has revealed that subtle differences in their structure can result in compounds that target either the $Q_i$ or $Q_o$ site (118). A combination of ELQ-300 and atovaquone to treat rodent malaria was found to be highly effective without engendering the emergence of resistant parasites (119). Such a combination holds promise for a malaria treatment with a greatly reduced chance of succumbing to the development of drug resistance. Long-lasting implants of atovaquone or ELQ-331 in mice have been shown to provide protection lasting months against experimental malaria (5; 114). Such a strategy has the potential for mass drug administration to control and eliminate malaria in endemic regions.

*Plasmodium* spp. are unable to salvage pyrimidines for their nucleic acid synthesis and must generate pyrimidines de novo (112). As mentioned earlier, an essential role of the parasite mtETC is to regenerate ubiquinone which is used as the electron acceptor for mitochondrially localized dihydroorotate dehydrogenase (DHODH), a critical enzyme in pyrimidine biosynthesis (102). Thus, DHODH has been shown to be an attractive target for antimalarial drug discovery (8). Several compounds have been shown to be potent inhibitors of parasite growth and a few have undergone clinical trials (74; 104). As with atovaquone, resistance to DHODH inhibitors also arise rapidly (133) and thus will require partner drugs to mitigate this risk.

## Unique Features of Apicomplexan ATP synthase

F-type ATP synthases are large rotary molecular machines that use electrochemical potential across a membrane to synthesize ATP, playing a central role in the energy economy of a wide range of organisms (14; 130). They are constituted from two subcomplexes: the soluble $F_1$ portion and the membrane -associated $F_o$ portion. When the complete genome sequences of malaria parasites were assembled, genes encoding most of the $F_1$ subcomplex could be detected but surprisingly highly conserved and essential genes encoding the $F_o$ subcomplex subunits, with the exception of the subunit-c and OSCP, could not be detected through similarity searches (35); this was also observed in other apicomplexan genomes (84). In *P. falciparum* ATP synthase was found to form dimers and genes encoding its subunit were found to be refractory to disruption (7). More recent work, however, revealed the apicomplexan $F_1F_o$ ATP synthase to be highly divergent from its counterpart in other classes of organisms. Proteomic analyses of isolated ATP synthase from *T. gondii* carried out by two independent groups revealed the presence of multiple proteins in addition to the canonical $F_1$

subunits and the subunit-c (48; 108). Nearly all these new proteins were conserved in other apicomplexan parasites as well as other Myzozoans, except *Cryptosporidium* and gregarine spp. that lack mtDNA (48; 108). Remarkably, these newly discovered ATP synthase subunits did not have apparent orthologous proteins in ciliates (6; 123). These studies indicated that the Myzozoa branch of the Alveolata clade possess a central bioenergetic complex that had a divergent evolutionary provenance. These myzozoa-specific subunits of the ATP synthase are divergent even from their closest sister clade of ciliates. One possible origin of these subunits could be from the ATP synthase that was part of the mitochondrion or the plastid belonging to the alga that was engulfed by the progenitor of myzozoans at the transitory stage (Fig. 1A). We acknowledge that there is no phylogenetic evidence supporting this proposition, but that could be due to an absence of sequence information from the algal progenitor. Chimeric origins of critical pathways are known to exist among unicellular parasites.

The unusual nature of the ATP synthase of these organisms became more apparent with the determination of a high-resolution cryogenic electron microscopic (cryo-EM) structure of the *T. gondii* complex (Fig. 4) (94). The complex was observed both as dimers and hexamers (trimers of dimers). There were 32 different proteins in each monomer, 15 of which could be discerned as structural equivalents of canonical subunits seen in ATP synthases of model organisms. Remarkably, 17 subunits were Apicomplexa-specific (identified as ATPTG1–17, for ATP synthase subunit from *T. gondii*). While 14 such ATPTG were observed in previous proteomic studies, 3 were identified directly from the cryo-EM structure (ATPTG1, ATPTG7 and ATPTG16). A gentler extraction revealed the dimers come together to form trimers, thus constituting hexamers, which were more akin to their native configuration in the parasite mitochondria. In the *T. gondii* ATP synthase dimer, the angle between the monomers is a low 19°--very different from the 100° angle seen in yeast and mammalian complexes. Because dimerization of ATP synthase is responsible for inducing curvature in the inner mitochondrial membrane, this narrow angle in the *T. gondii* dimer would induce reduced curvature compared to the yeast and mammalian membranes. Cryo-electron tomography of mitochondrial membranes showed bulbous vesicles that were decorated with icosahedral arrangements of ATP synthase hexamers to form pentagonal pyramids localized to the regions of curvatures in the membranes, indicating that hexamer packing is likely the primary determinant of cristae morphology in *T. gondii*. Overall, this work (94) constitutes the first structural elucidation of any mitochondrial complex from an apicomplexan parasite and reveals in remarkable detail the divergence and intricacies of a critical molecular machine. Further studies using cryo-EM and cryo-electron tomography to investigate mitochondrial complexes and their arrangements in situ are likely to reveal further amazing details.

## Unresolved Aspects of Apicomplexan Mitochondria

The unusual features of apicomplexan mitochondria summarized here raise many additional questions. These involve the machinery used by the parasites to manage the separate genetic systems relegated to the mitochondria. The remarkable conservation of the mtDNA in *Plasmodium* spp. would suggest a robust system of DNA replication, repair and recombination. While some of the components of this system have been identified, much

remains to be elucidated. For instance, drug resistance mutations that arise in the mtDNAs of both *Plasmodium* and *Toxoplasma* quicky achieve fixation in the multiple copies of the genome such that wild-type sequences are no longer detected; such mutations are also very stable with no reversion to the wild type. This suggests a "copy-correction" machinery that remains unexplored. *Plasmodium* mtDNA molecules in linear arrays with intermediates suggesting strand invasions were observed in an early study. This would suggest extensive gene conversion events among the multi-copy mtDNA molecules, requiring a variety of enzymes that would mediate this process. At this point none of these mediators have been identified or investigated. Transcription regulation and processing of RNA encoded by mtDNA have also been underexplored. About 40 distinct RNA molecules are generated from the 6 kb mtDNA of *Plasmodium* spp. Enzymes involved in precise processing of these molecules are not known. The finding that a large number of proteins with RNA binding motifs amplified in Apicomplexa (RAP proteins) appear to be localized to the parasite mitochondrion suggest their potential role in post-transcriptional processing of mitochondrial RNA molecules. The most intriguing aspect of mitochondrial functions in apicomplexans is the manner in which the 3 mtETC proteins encoded by the mtDNA are translated. The structure of the mitochondrial ribosomes remains unresolved as is the process by which such ribosomes are assembled from multiple rRNA fragments and a large number of mitoribosomal proteins imported from the cytoplasm. With the availability of powerful new technologies, we anticipate many of these questions will be addressed in coming years.

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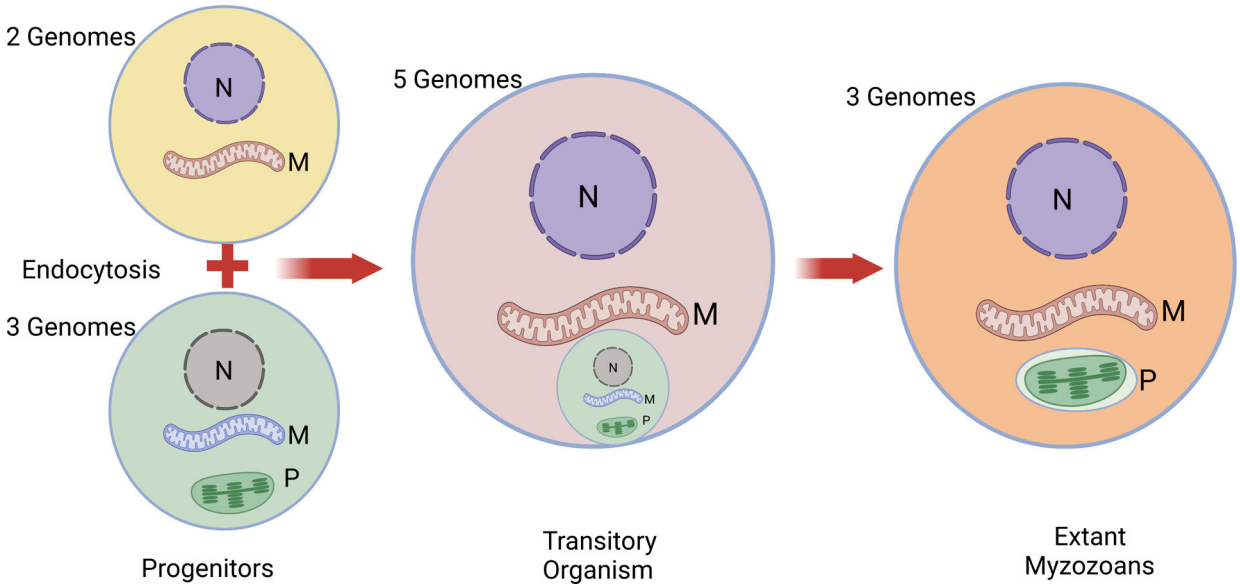



**Figure 1. Schematic representation of the secondary endosymbiotic event involving the progenitor of myzozoans with two genomes and an alga with three genomes.**

Engulfment of the alga by the progenitor of Myzozoa resulted in a transitory organism with five genomes. Massive gene transfers and losses led to the elimination of the algal nucleus and mitochondrion, thus resulting in the three genomes seen in extant Myzozoa, which remain a conglomerate of five genomes.

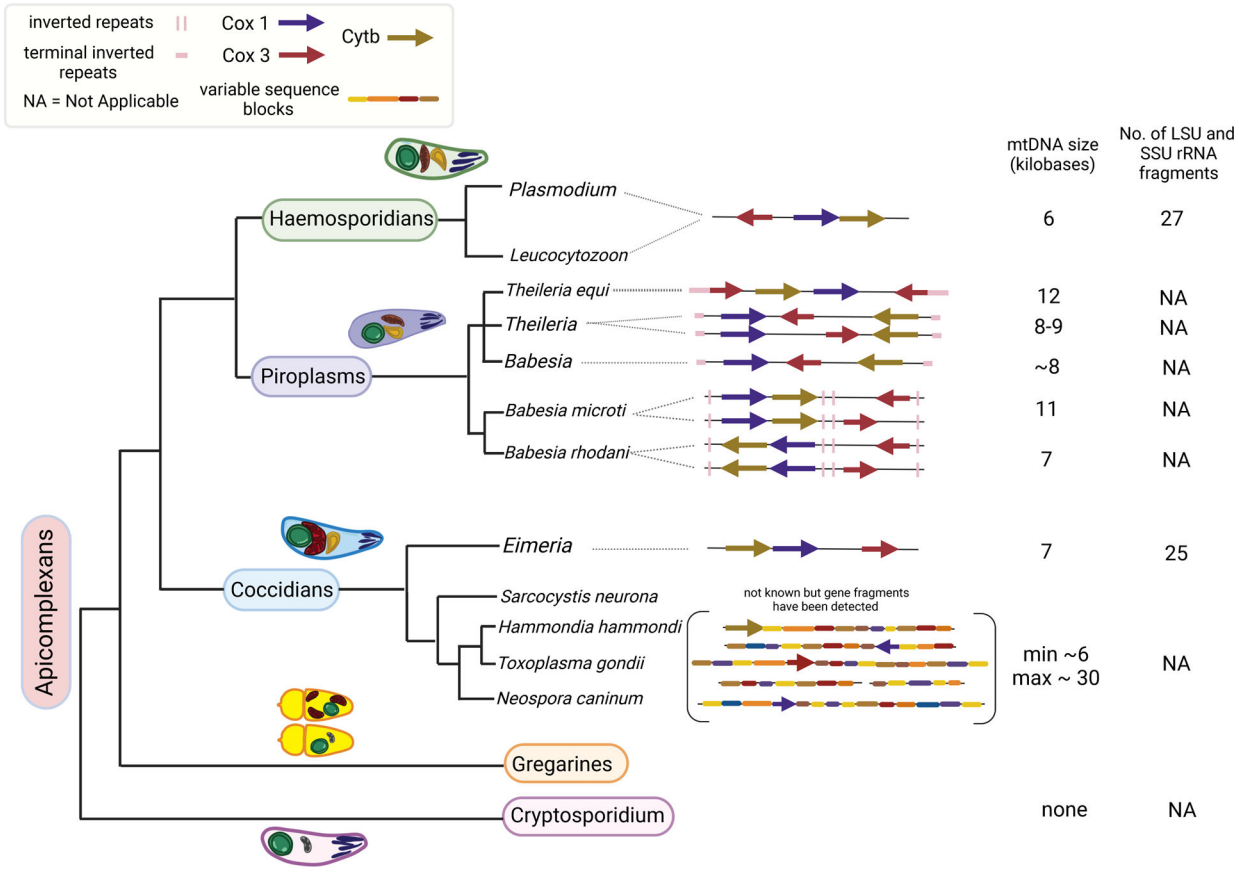
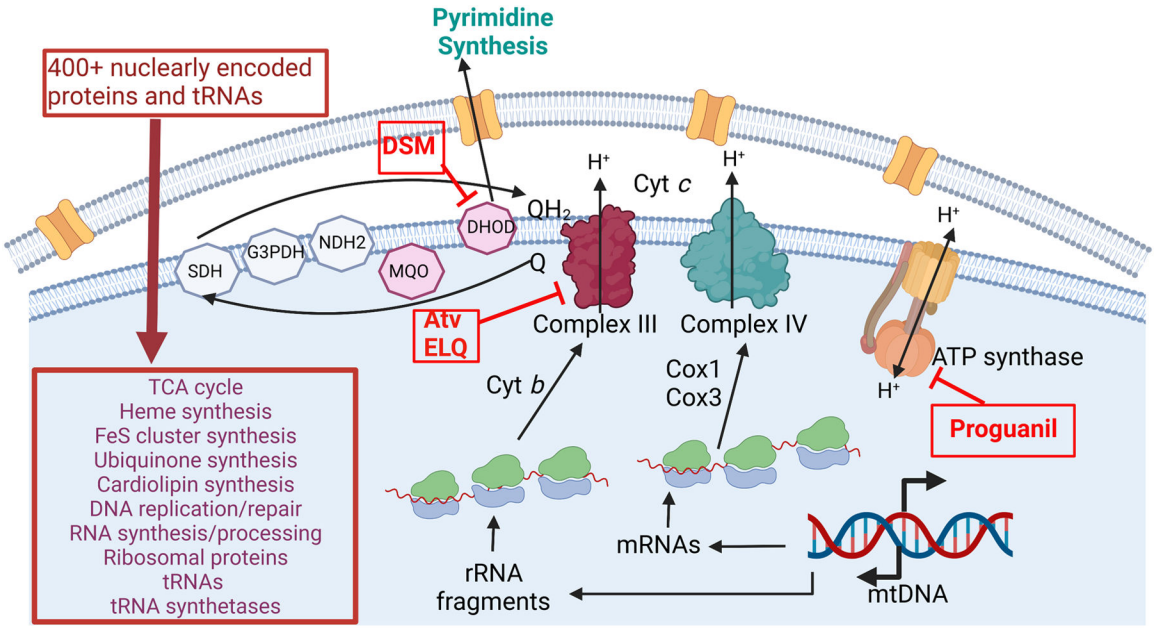
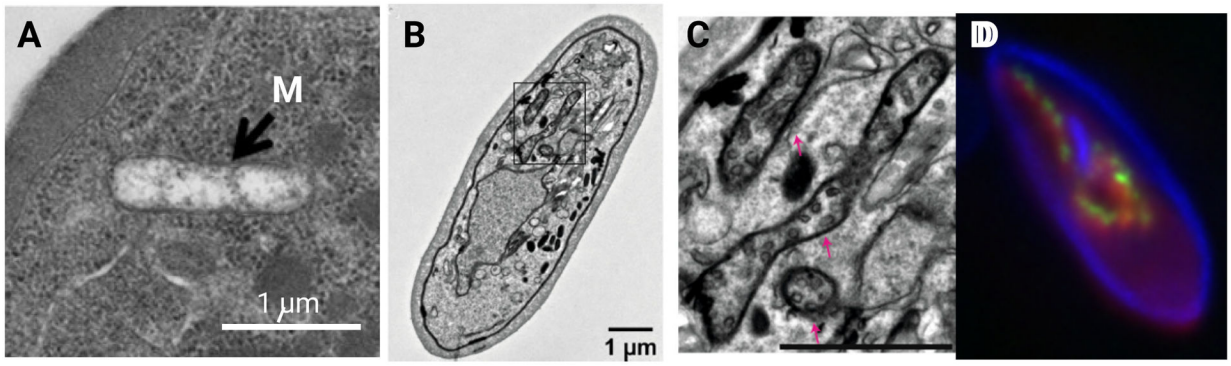
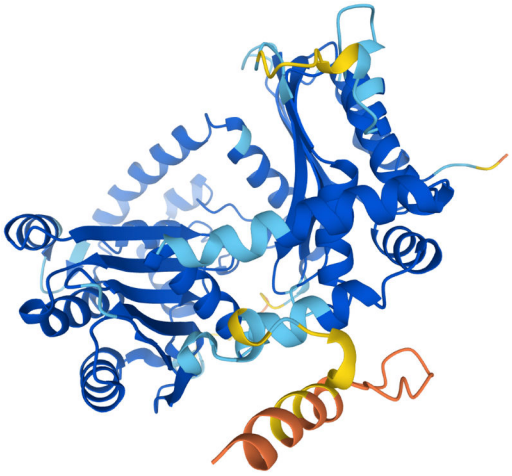


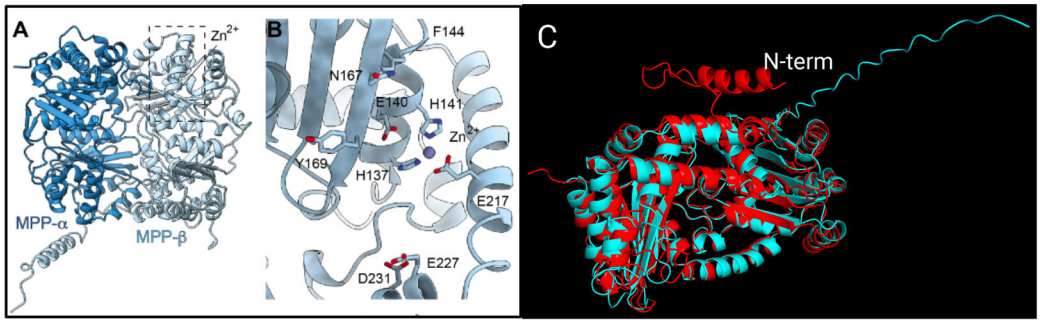

**Figure 2. Phylogenetic tree illustrating the evolutionary relationship between the subclasses of apicomplexans (branch lengths are not drawn to scale).**

The synteny of protein-coding genes and their directionality in the apicomplexan mitochondrial genome of organisms in each subclass is shown as arrows. The size of the mitochondrial DNA as well as the known number of fragmentary mitochondrial large subunit and small subunit rRNA transcripts in each subclass are indicated to the right of the tree. This figure is modified from Figure 1 from Berná et al. (ref. 10).





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                          137  141                 217
                           |    |                   |
ScMPP-beta   AHFLEHMIFKGT...VILREMEEVE
             **********...**********
PfMPP-beta   AHFLEHMIFKGT...VILREMEEVE
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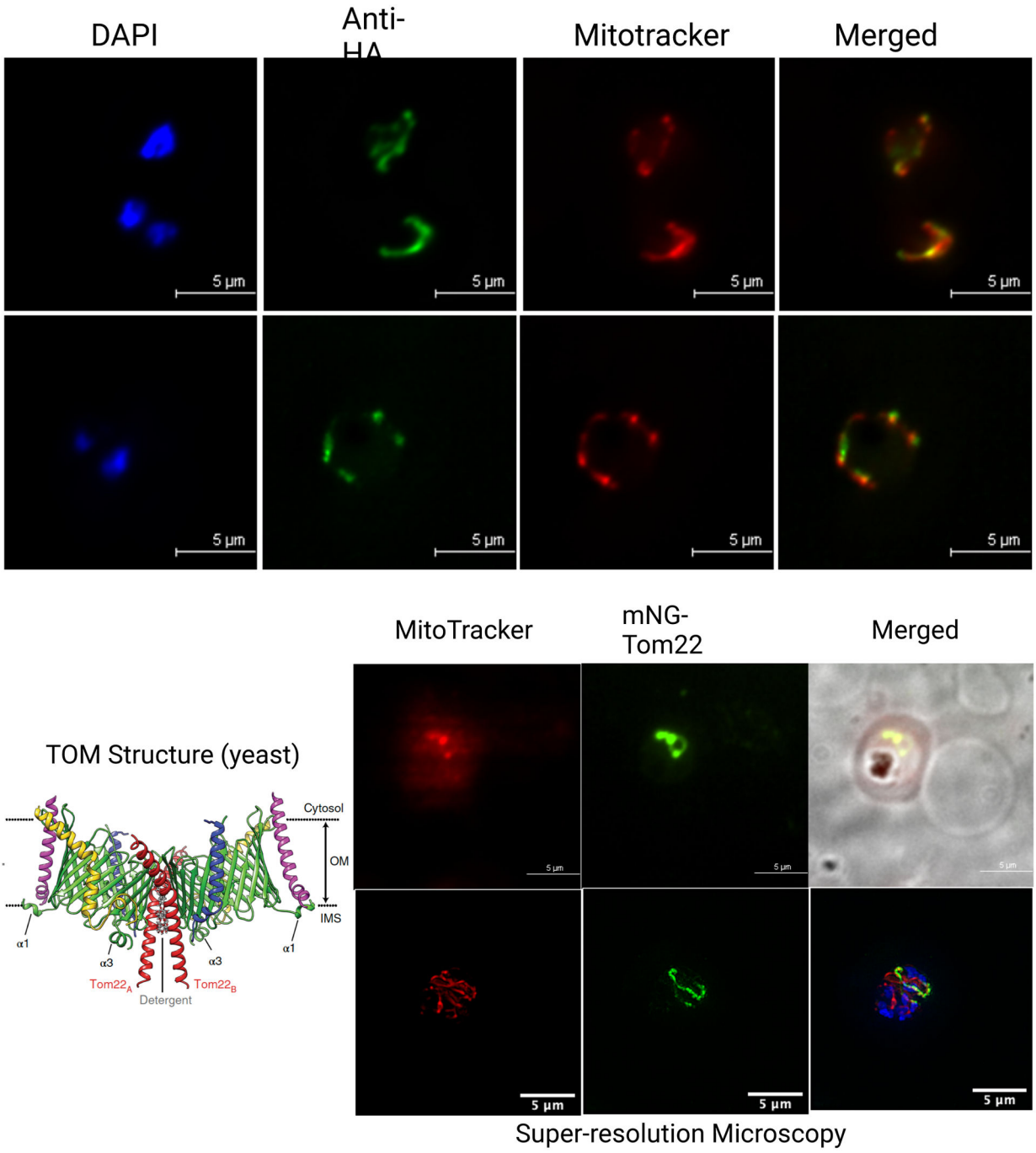


**Figure 3. A schematic of mitochondrial functions in *P. falciparum* with associated drug targets.**

Maroon boxes depict the estimated 400+ nuclearly encoded proteins targeted to the mitochondrion and their associated biological processes. Drugs that target respiratory chain proteins are shown in red. Reduced ubiquinone ($QH_2$) is generated by 5 dehydrogenases, of which SDH, MQO, G3PDH and NDH2 were found to be not essential in blood stages but DHOD is essential for its role in pyrimidine biosynthesis and is the target of DSM antimalarial compounds. $QH_2$- is oxidized by Complex III, a step that is inhibited by atovaquone, ELQs and other inhibitors. Complex III transfers electrons to cytochrome *c,*

which is then oxidized by Complex IV. Both Complexes III and IV pump $H^+$ across the inner membrane, generating protonmotive force. Only 3 components of these complexes are encoded by the mtDNA: Cyt *b*, Cox1, and Cox3. These are translated by unusual ribosomes assembled from fragmented rRNAs encoded by mtDNA and ribosomal proteins encoded on the nuclear genome and imported into the mitochondrion. ATP synthase does not seem to be a significant source of ATP in blood stages but can work in reverse by hydrolyzing ATP to pump $H^+$. This step is proposed to be targeted by proguanil, a component of antimalarial drug Malarone. *rRNA= ribosomal RNA. Cyt b=cytochrome b. Cox1/3= cytochrome c oxidase 1/3. SDH = Succinate Dehydrogenase. G3PDH= glycerol-3-phosphate dehydrogenase. NDH2= type II NADH dehydrogenase. MQO= malate quinone oxidoreductase. DHOD= dihydroorotate dehydrogenase. ELQs = Endochin-Like Quinolones*.

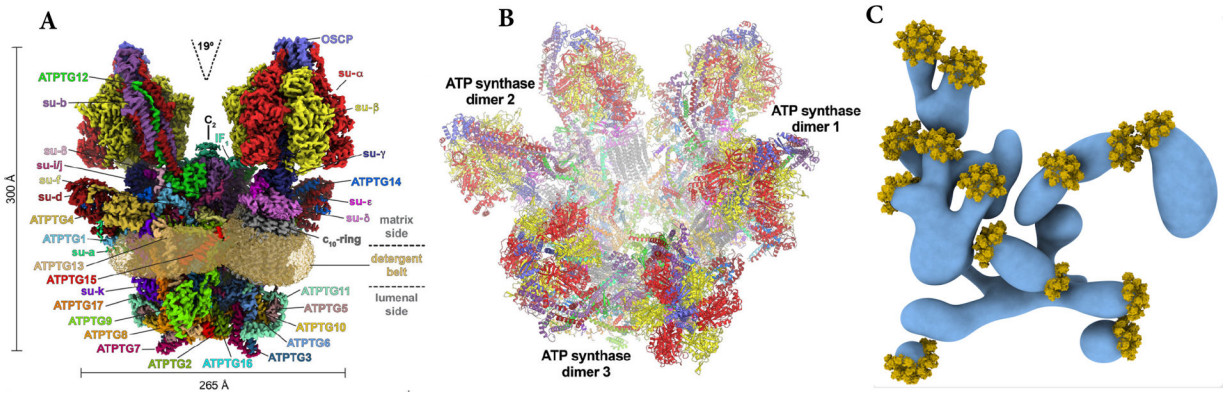


**Figure 4. Cryo-EM structure of the ATP synthase complex from *T. gondii*.**

(A) A dimer of *T. gondii* ATP synthase. There are 32 subunit proteins in each monomer. Fifteen of these have structural equivalents in other ATP synthases and are identified with prefix “su-“; 17 other subunits are unique to *T. gondii* and are identified as ATPTG1 to ATPTG17. The monomers form a 19° angle relative to each other in the dimer, compared to 100° angle seen in the yeast and mammalian ATP synthase. (B) In native state, *T. gondii* ATP synthases form hexamers (trimers of dimers). (C) The hexamers associate at the bulbous mitochondrial inner membranes in pentameric pyramids that provide the curvature to the cristae. The figure is adapted from images in Muhleip et al. (ref. 94).