# Phylogeny and Evolution

# 12

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**Abstract**

The concept of a phylogeny of parasites is inextricably linked to that of the phylogeny of eukaryotes. Though it can be useful to infer functional principles from similar morphologies and trophic strategies, the evolutionary histories of parasites are most accurately viewed as independent shifts to this lifestyle from a free-living state. This chapter will describe the phylogeny of eukaryotes, the evolutionary positions of various prominent parasites within this framework, and the ways in which genomics has facilitated understanding of the free-living to parasitic transition, both in terms of phylogeny and function. Two major cellular systems of parasitological relevance, mitochondrion-related organelles and endocytic systems, will be explored, highlighting where considering the genomics and molecular cell biology of parasites in the context of their emergence from free-living relatives have helped us to better understand organelle evolution.

## 12.1 Introduction

Eukaryotes have conquered many environments in their ~2-billion-year history (Eme et al. 2014), but parasites have taken on one of the most challenging of all: other eukaryotes themselves. This poses many obstacles and a diversity of environmental conditions; indeed many parasites have developed complex life cycles and a number of cellular adaptations to them, including modification of genomes and organelles.

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The success of these organisms is fascinating, and at the same time deeply disturbing, as parasites wreak havoc on plant, animal, and human populations. Diseases such as malaria and Chagas' disease cause significant annual morbidity and mortality. Other parasites, such as *Theileria* and *Phytophthora*, affect livestock and crop populations and have adverse socioeconomic impacts. The study of parasitism has traditionally focused on understanding a single parasitic organism or lineage. There is a great deal, however, to be gained from taking a comparative evolutionary approach, which, we argue, engenders understanding of how parasitism arose and the underlying biology of pathogenesis in different parasites today. The mechanism by which parasites have evolved from free-living ancestral states underlies all aspects of parasitic infection and represents an important area of study, as rapidly acquired drug resistance challenges us to identify novel infection prevention and treatment strategies.

The key to this approach is the fact that parasitic organisms do not represent a single clade, nor do they represent basal lineages or any kind of "primitive" state. Parasites are found in all major clades of eukaryotic life, each sharing a common ancestor with free-living organisms. This suggests two conclusions regarding the evolution of parasitism: it has been arrived at multiple times through convergence, and it likely involves distinct mechanisms in each lineage.

The depth of comparison has been significantly enhanced by greater availability of genomic information from organisms across the eukaryotic tree. The genome of a given organism is a tremendously powerful resource in understanding its biology. It provides an extensive, though not always complete, part list allowing predictions of what cellular systems are functional. For cellular features that possess their own DNA, such as mitochondria and plastids, the organellar genome allows for a fairly complete study of their evolution. However, autogenously derived organelles (such as the endoplasmic reticulum (ER) and Golgi body) do not contain any genetic information of their own. Rather, protein markers characteristically associated with each organelle can be used to infer the organelle's presence in the absence of morphological data. By studying the retention of these factors in the nuclear genome, it is possible to hypothetically predict organelles and therefore cellular pathways present in an organism. Additionally, identifying these markers represents a starting point for molecular cell biological analyses, such as gene product localization and disruption. These can provide the impetus for launching downstream molecular parasitological studies, and therefore it is not surprising that parasites were among the first eukaryotes sequenced and that the list of sequenced parasite genomes continues to rapidly grow.

In this chapter we will explore ways in which the recent explosion of genomic information has informed our understanding of parasite evolution. We will start with an overview of eukaryotic phylogeny, emphasizing the position of parasitic taxa. We will then examine the evolution of two cellular systems often characteristic of parasitic organisms and key to their adaptation to the host environment and/or pathogenic mechanism: mitochondrion-related organelles (MROs) and organelles of the membrane-trafficking system. Though there are multiple evolutionary paths to parasitism, comparing the parasites to their closer, free-living relatives has revealed convergent evolution of these systems.

## 12.2 Phylogeny, Parasite Evolution, and the Last Eukaryotic Common Ancestor (LECA)

Gaining a phylogenetic framework of eukaryotes, parasitic or otherwise, hinges on two separate, though related, questions: Where is the root of the eukaryotic tree, and what are the relationships of eukaryotic taxa to one another? Early studies attempted to answer these questions simultaneously by including both eukaryotic and prokaryotic sequences in single-gene phylogenies using methods, like maximum parsimony and distance matrices with simple models of sequence evolution. The resulting phylogenies showed a ladder-like radiation of eukaryotes after the prokaryote-eukaryote split, with largely parasitic protist groups like diplomonads, microsporidians, and parabasalids all branching off before the radiation of "crown groups" such as animals, plants, brown algae, and ciliates (Sogin 1991). This artifactual grouping of parasitic taxa was supported by shared characteristics, namely, the lack of classical mitochondria but also an apparent lack of introns, Golgi bodies, peroxisomes, and a sexual life cycle. Combining this evidence resulted in the formalization of the Archezoa hypothesis, whereby it was proposed that these lineages represented "early" or "ancient" eukaryotes that diverged from the crown group taxa before the acquisition of canonical eukaryotic traits such as mitochondria (Cavalier-Smith 1987, 1989).

Over several decades, the Archezoa hypothesis was shown to be false. As discussed in much further detail below, the cell biological rationale of organelle absence was refuted by the discovery, first of genetic and later of molecular cell biological evidence evolutionarily linking MROs such as hydrogenosomes and mitosomes found in nearly every "mitochondrion-lacking" organism to classical mitochondria ((Burki 2014; Müller et al. 2012), inter alia). Likewise, these data have now been reported for sexual life cycles, Golgi bodies, and introns ((Koumandou et al. 2013), inter alia).

Additionally, the Archezoa hypothesis has been refuted due to the development of more robust techniques for phylogenetic inference and an improved understanding of eukaryotic systematics. Early phylogenies did not include enough characters to robustly place the taxa within a phylogeny and in addition were flooded with incorrect relationships caused by artifacts like long-branch attraction (LBA). LBA occurs when divergent and unrelated sequences in a dataset cluster regardless of whether divergence occurred due to a rapid increase over a short time or a steady increase over a long time (Philippe 2000). By including prokaryotic taxa and long-branching taxa of some parasitic groups, early phylogenies affected by LBA suggested that these parasitic taxa were closely related to prokaryotes (Stiller and Hall 1999).

Improvement in phylogenetic methods, combined with realization and mitigation of LBA, was quickly followed by the rise of phylogenomics as a method for improving resolution of eukaryotic relationships. Trees of one or a handful of genes may still suffer from a lack of support for internal nodes due to a paucity of informative positions. Increased genomic data for diverse eukaryotic lineages allows for inclusion of more genes and taxa, which serve to mitigate these issues. Concatenation of multiple genes into large multigene sequences for comparison ("phylogenomics") is the new method of choice for large-scale inference (Delsuc et al. 2005), but

is not without flaws. It allows for reduction of stochastic errors, but does not alleviate systematic errors such as model choice and inappropriate modeling of rate heterogeneity (Philippe et al. 2011; Rodríguez-Ezpeleta et al. 2007). Though powerful, phylogenomics techniques require careful execution to obtain meaningful results. Nonetheless, based on these large-scale concatenated phylogenies and incorporating ultrastructural information, eukaryotic diversity can be divided into five or six supergroups (Fig. 12.1), with parasites in each of the major clades of eukaryotic diversity ((Adl et al. 2012), inter alia).

The Amoebozoa and Opisthokonta comprise a larger clade, alternately named Unikonta, Amorphea, or most recently Opimoda. The Opisthokonta (Fig. 12.1, purple) includes animals and their unicellular relatives (Holozoa), along with fungi (Nucletmycea). Amoebozoa (Fig. 12.1, blue) contain a large number of amoeboid organisms along with slime molds. Parasites are found in numerous amorphean lineages; for example, microsporidians are basal fungi that parasitize animals, and *Entamoeba histolytica* is an important human parasite.

SAR and Archaeplastida, together with Cryptophyceae, Centrohelida, Telonemia, and Haptophyta, comprise the Diaphoretickes. The Archaeplastida (Fig. 12.1, teal) contain

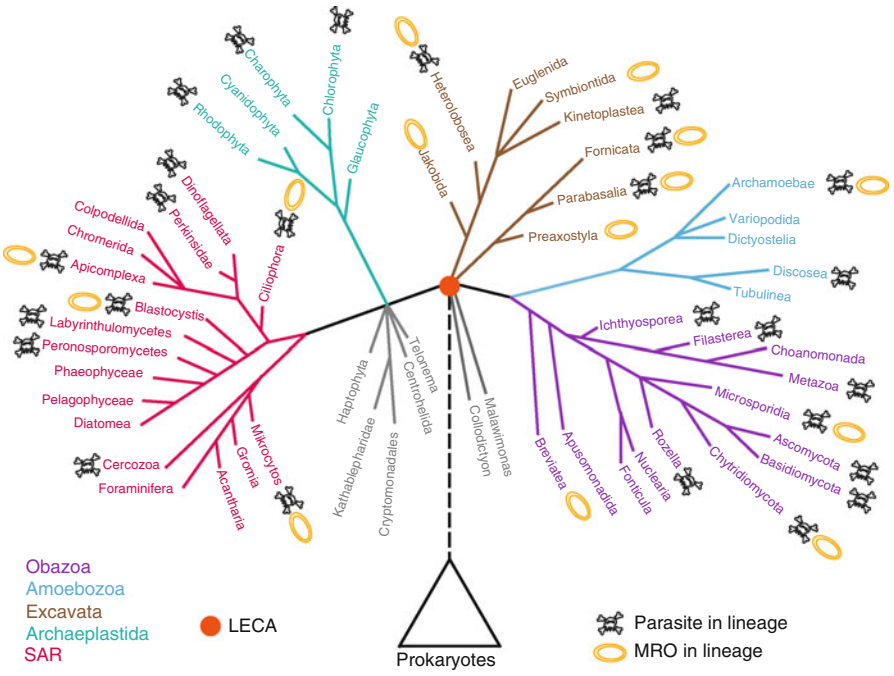



**Fig. 12.1** Eukaryotic phylogeny. The current view of eukaryotic phylogeny, rooted as recently proposed on an excavate-like lineage. Large clades are color-coded and the distribution of mitochondrion-related organelles (MROs) and known parasitic organisms are denoted by *symbols* as per figure legend. Though these traits are often present in the same lineage, they do not demonstrate perfect correlation

land plants, mosses, as well as green, red, and glaucophyte algae, all united by the acquisition of a plastid through a single primary endosymbiotic event with a cyanobacterium.

SAR (Fig. 12.1, fuchsia) contains three main lineages, the stramenopiles, alveolates, and rhizarians. The rhizarians diverged first and are arguably the least understood major eukaryotic group. Stramenopiles can range from unicellular heterotrophs and photoautotrophs to multicellular kelps. Notable parasitic members include *Blastocystis*, an opportunistic pathogen of humans, along with *Phytophthora*, species of which cause potato blight and sudden oak death. Alveolates include the familiar ciliates *Tetrahymena* and *Paramecium* but also important fish pathogens like *Ichthyophthirius multifiliis*. Apicomplexa are almost exclusively parasitic, including the causative agents of malaria, *Plasmodium* spp., and cryptosporidiosis (diarrheal disease), *Cryptosporidium* spp.

The Excavata (Fig. 12.1, brown) is a diverse group of predominantly heterotrophic flagellates, many of which live in oxygen poor environments and/or are important parasites. Two main divisions exist: metamonads are generally endosymbionts/parasites and lack classical mitochondria, while discobids are generally free-living and have classical mitochondria. Within the metamonads reside some of the original Archezoa "amitochondriates," such as *Trichomonas vaginalis* and *Giardia intestinalis*. Within the discobids are important human parasites such as *Leishmania* and *Trypanosoma*. This group contains the only photosynthetic excavate lineage, euglenophytes, as well as the recently described deep-sea dwelling lineage of Symbiontida (e.g., *Calkinsia aureus*) and diplonemids, one of the most abundant groups in ocean plankton (Lukeš et al. 2015). The monophyly of Excavata was originally proposed based on shared morphological characteristics, essentially the presence of a ventral feeding groove "excavated" from the cell, along with the underlying cytoskeletal structures (Simpson 2003). Although the two main divisions of Excavata likely form a monophyletic clade, the relation of these to other lineages possessing the "excavate apparatus" is uncertain. This includes the originally proposed excavate *Malawimonas* and the newly characterized *Collodictyon* (Brugerolle et al. 2002; Zhao et al. 2012). Indeed, lineages such as apusomonads and breviates both possess two basal bodies, and their flagellar apparatuses are similar to the excavate-like state (Heiss et al. 2013a, b). These two lineages, however, are convincingly established as paraphyletically basal to the opisthokonts (Brown et al. 2013), forming the clade Obazoa (Fig. 12.1, purple).

Though our understanding of eukaryotic phylogeny is vastly improved from the days of the Archezoa hypothesis, the rooting remains uncertain. Various potential options are proposed, but none are overwhelmingly supported. The most recent evidence suggests a possible root either on an excavate lineage, between the Excavata and all others (He et al. 2014), or dividing Opimoda and Diphoda, consistent with the previous Bikont-Unikont hypothesis (Derelle et al. 2015). The lack of a concrete rooting hypothesis, however, does not change the notion that parasitism has multiple independent origins, nor does it hamper our ability to reconstruct the cellular state of the ancestral eukaryote. Consistent with current rooting hypotheses, a recent overview of ultrastructural data from microtubule organizing centers reconstructed a remarkably "excavate-like" state for the ancestral eukaryote (Yubuki and Leander 2013).

In the last decade, considerable effort has been put into sequencing and analyzing the genomes of diverse eukaryotes. These studies have resulted in a reconstruction of the last eukaryotic common ancestor (LECA), the last organism through which all extant eukaryotes draw their evolutionary path. This reconstruction has revealed a surprisingly sophisticated set of cellular components in the LECA, including nuclear architecture, the endomembrane system, mitochondria, cytoskeleton, and metabolism (reviewed in (Koumandou et al. 2013)).

Though the utility of such a reconstruction may not be immediately obvious to those outside the field of evolutionary cell biology, it is inherently applicable to the study of any cellular system of interest. The LECA can be thought to serve as a control group in the study of extant eukaryotes, allowing us to generate a null hypothesis about the evolution of a system: assuming no change to a system, it follows that the system should resemble that of the LECA, while changes represent potentially important innovations for that lineage. This evolutionary history provides essential context to the study of parasite/free-living pairs, including whether a given morphological or genomic character is an adaptive feature concurrent with parasitism or is preadaptive and hence present in both the parasite and its free-living relative(s).

## 12.3 Mitochondrion-Related Organelles (MRO) and Parasitism

One such system where a comparative evolutionary and genomic approach has been particularly insightful is the diversity of mitochondrion-related organelles. Dramatic differences in morphology of mitochondria in microbial eukaryotes under light and electron microscopy (shape, size, cristae morphology, number, etc.) have been known for decades. Textbook aerobic mitochondria are well characterized and act as the main sites of aerobic ATP generation, but also play pivotal roles in other aspects of cellular metabolism like apoptosis, amino acid metabolism, and synthesis of folate and iron-sulfur clusters, which are functional groups of some essential enzymes.

Some eukaryotes, including many prominent parasites, recognized as amitochondriates were classified as Archezoa (Cavalier-Smith 1987). However, the lack of mitochondria observed in Archezoa taxa was contradicted by the discovery of smaller double-membrane-bound enclosures lacking cristae, which were characterized as either hydrogenosomes or mitosomes. The notable exception is the lineage of oxymonads that appears to have lost the mitochondrion completely (Karnkowska et al. 2016). Accumulating genomic data have helped to decipher the evolutionary history of mitochondria, and it is now broadly accepted that mitosomes, hydrogenosomes, and other compartments, collectively known as MROs, originated from aerobic or facultatively aerobic mitochondria present in the LECA by adaptation to environments with low oxygen concentration (Embley and Martin 2006).

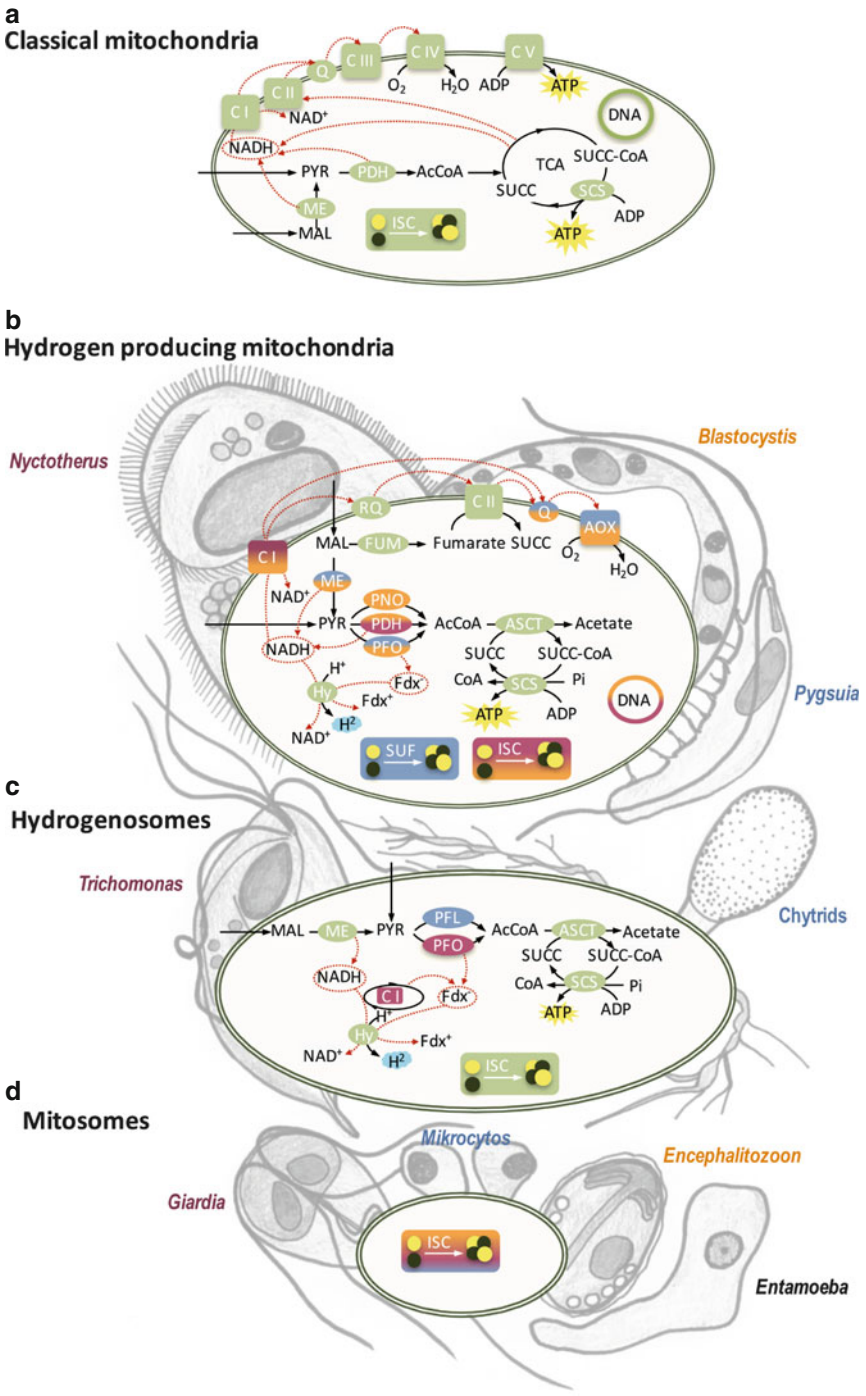
The patchy distribution of MROs on the tree of eukaryotes (Fig. 12.1) reflects the fact that several lineages have undergone such adaptation independently. Some clades contain only a few representatives with MROs (e.g., ciliophora, heterolobosea, Euglenozoa, apicomplexa), while other clades are composed entirely of

MRO-containing organisms (e.g., microsporidia, fornicata, parabasalia, archamoebae). Many organisms containing MROs are intestinal, mucosal, or intracellular parasites or commensals, but there is growing evidence of free-living lineages lacking classical mitochondria.

MROs represent various outcomes and transitional stages of a gradual evolutionary process, which greatly complicates our efforts to classify them. Müller et al. (2012) identify five classes of mitochondria and MROs: (1) classical mitochondria produce Adenosine Tri-Phosphate (ATP) by oxidative phosphorylation on an electron transport chain (ETC) using $O_2$ as the terminal electron acceptor (Fig. 12.2a); (2) anaerobic mitochondria produce ATP on an ETC in hypoxic environments and use other electron acceptors like fumarate or nitrate (not shown in Fig. 12.2); (3) hydrogen-producing mitochondria (HPMs) produce ATP by substrate-level phosphorylation (SLP) yet still possess a truncated ETC and produce $H_2$ (Fig. 12.2b); (4) hydrogenosomes produce ATP by SLP, lack an ETC, and produce $H_2$ (Fig. 12.2c); and (5) mitosomes do not produce ATP at all; organisms with mitosomes produce all ATP via cytosolic SLP (Fig. 12.2d). This classification reflects biochemical aspects of MRO diversity but does not reflect their evolutionary history; related taxa often possess different MROs and each class of MROs is distributed across unrelated clades. MROs in unrelated eukaryotes show striking convergent features, suggesting that there are a limited number of ways by which mitochondria may evolve to function under low oxygen concentration. These involve replacement of the pyruvate dehydrogenase complex (PDH) by anaerobic analogs, truncation or loss of the Electron Transport Chain (ETC), and involvement of [FeFe]-hydrogenase (H2ase) ((Hampl et al. 2011), inter alia).

The key process of mitochondrial biochemistry is the conversion of pyruvate to acetyl-CoA. In classical mitochondria this process is performed by the Pyruvate dehydrogenase PDH complex, but in MROs, three alternative enzymes are known to fulfill this role: pyruvate-ferredoxin oxidoreductase (PFO), pyruvate-NADP oxidoreductase (PNO), and pyruvate-formate lyase (PFL). The other key aspect is the reoxidation of reduced cofactors (NADH and ferredoxins). In classical and anaerobic mitochondria, this is done by the ETC, although the chain is truncated in the latter. In hydrogenosomes this function is performed by H2ase that reduces protons to $H_2$. HPMs represent a combination of the two ways. H2ases, PFOs, and PFLs of eukaryotes are apparently not directly related to homologues from the putative α-proteobacterial mitochondrial ancestor and have a patchy distribution across eukaryotes (Hackstein 2005; Hug et al. 2010; Meyer 2007; Stairs et al. 2011). The most probable explanation for these observations is one or a small number of gene transfer events from prokaryotes to eukaryotes followed by several eukaryote-to-eukaryote transfers.

In the course of evolution, many eukaryotic lineages adopted a parasitic lifestyle and entered new niches. Parasites, and gut endoparasites particularly, often have to survive in an environment that is low in oxygen, yet rich in organic nutrients. This is usually reflected by changes in their mitochondrial metabolism, as can be demonstrated by the anaerobic mitochondria of parasitic worms (Müller et al. 2012). These modifications are observed not only between species but also within lifecycles of a single species such as *Trypanosoma brucei*. The tsetse fly stage of *T. brucei* has a biochemically potent mitochondrion with effective ATP production. Though


a
Classical mitochondria
C I
C II
C III
C IV
C V
Q
NAD+
NADH
PYR
PDH
AcCoA
TCA
SUCC-CoA
SUCC
SCS
ADP
ATP
ME
MAL
ISC
DNA
b
Hydrogen producing mitochondria
Nyctotherus
Blastocystis
Pygsuia
RQ
MAL
FUM
Fumarate
SUCC
AOX
H2O
PNO
PFO
ASCT
Acetate
CoA
Pi
Hy
Fdx
Fdx+
H2
SUF
c
Hydrogenosomes
Trichomonas
Chytrids
PFL
d
Mitosomes
Mikrocytos
Encephalitozoon
Giardia
Entamoeba

unrelated to the presence/absence of oxygen, transformation to the bloodstream stage involves a downregulation of mitochondrial metabolism to such an extent that the mitochondrion no longer generates, and in fact consumes, ATP concurrent with a role in cytosolic NADH oxidation. The parasite's energetic demands are fully satisfied by glycolytic ATP production (Vanderheyden et al. 2000).

Further and permanent modifications can be seen in HPMs of the gut parasite/commensal stramenopile *Blastocystis* (Denoeud et al. 2011; Stechmann et al. 2008), the cockroach hindgut symbiont ciliate *Nyctotherus ovalis* (Boxma et al. 2005, 2007), and the free-living breviate *Pygsuia biforma* (Stairs et al. 2014) (Figure 12.2B). Unlike *Nyctotherus* that converts pyruvate to acetyl-CoA by the PDH complex, *Pygsuia* uses PFO and *Blastocystis* appears able to produce acetyl-CoA from pyruvate in three ways: by the PDH complex, PFO, and PNO. All three HPMs contain a partial Krebs cycle and similarly truncated respiratory chains with fumarate as the terminal acceptor. *Blastocystis* and *Pygsuia* also contain a gene encoding a noncanonical respiratory chain complex called alternative oxidase (AOX), known also from mitochondria of plants, trypanosomes, *Euglena*, and *Cryptosporidium parvum* (Putignani et al. 2004). AOX can receive electrons from complexes I and II and pass them to the terminal acceptor $O_2$, allowing adaptation to oxygen stress and maintenance of the NADH/NAD balance. These three examples represent modifications of classical mitochondria to an anaerobic lifestyle, but without concurrent loss of some features typical for mitochondria. Most notably some of these MROs still possess their own genomes. It is hypothesized that the genome has to be retained since it codes for particular pieces of the ETC that cannot be encoded entirely in the nucleus. Conditions under which an MRO may lose its genome are currently unclear (Allen 1993; Björkholm et al. 2015; Popot and de Vitry 1999), but hydrogenosomes and mitosomes, which we will discuss below, no longer possess any genetic material.

Hydrogenosomes (Fig. 12.2c) were discovered in parasitic trichomonads (Lindmark and Müller 1973), but are also found in other metamonads (e.g., *Paratrimastix*, formerly *Trimastix,* and *Spironucleus*), as well as in chytridiomycete fungi, ciliates, archamoebae, and heteroloboseans. In the *T. vaginalis* hydrogenosome, pyruvate is oxidized to Acetyl-CoA by a PFO; an H2ase reoxidizes the ferredoxin reduced in this reaction. Acetyl-CoA is then converted to acetate by an acetate-succinate CoA transferase (ASCT), and the resulting succinyl-CoA is used by succinyl-CoA synthetase (SCS) to generate ATP by SLP (Hrdy et al. 2004).

**Fig. 12.2** Reductive evolution of mitochondria. In each panel a cartoon of the organelle and its accompanying biochemistry is shown, along with drawings of example organisms possessing the organelle. Mitochondrial evolution: (**a**) classical mitochondria, (**b**) hydrogen-producing mitochondria (HPM), (**c**) hydrogenosomes, and (**d**) mitosomes. Note that anaerobic mitochondria are not shown here as they are not yet described from microbial eukaryotic parasites. *AcCoA* acetyl-CoA; AOX, alternative oxidase, *ASCT* acetate/succinate CoA transferase, *C I* complex I, *C II* complex II, *C III* complex III, *C IV* complex IV, *C V* complex V (ATP synthase), *Fdx* ferredoxin, *FUM* fumarase, *Hy* [Fe]-hydrogenase, *ISC* iron-sulfur cluster pathway, *MAL* malate, *ME* malic enzyme, *PDH* pyruvate dehydrogenase complex, *PFO* pyruvate/ferredoxin oxidoreductase, *PNO* pyruvate/NADP oxidoreductase, *PYR* pyruvate, *Q* quinone, *RQ* rhodoquinone, *SCS* succinyl-CoA synthetase, *SUF* sulfur mobilization system, *SUCC* succinyl, *SucCoA* succinyl-CoA, *TCA* tricarboxylic acid cycle; *red dotted line* indicates electron transport

Other hydrogenosomes also produce $H_2$ and ATP via SLP, but the biochemistry varies. For example, hydrogenosomes of the symbiotic chytridiomycetes, *Neocallimastix* and *Piromyces*, lack PFO and instead contain PFL and their H2ase reoxidizes NADH produced by malic enzyme (Hackstein et al. 2008). The presence of hydrogenosomes not only in unrelated lineages of parasites but also in mutualists (e.g., *Neocallimastix*, the ciliate *Dasytricha*) and free-living organisms (e.g., the ciliate *Trimyema*, probably the excavate *Paratrimastix* and the holozoan *Loricifera*), indicates that, as with HPMs, hydrogenosomes have evolved multiple times by convergent evolution and do not represent a specific feature of parasites.

Mitosomes (Fig. 12.2d) represent the most highly and permanently reduced forms of MROs, typically not involved in energy generation (Katinka et al. 2001; Loftus et al. 2005; Morrison et al. 2007). Mitosomes not only lack genomes but also most known mitochondrial proteins. They probably lost their energy production capacity concurrently with adaptation to an endobiotic lifestyle and opportunity to scavenge energy-rich biomolecules from the host. This notion is supported by the fact that mitosomes, unlike other MROs, are not identified in free-living microbial eukaryotes. Some of them, like diplomonad *Giardia* and amoebozoan *Entamoeba*, are cavity or tissue parasites invading the intestinal tract, while others, like *Cryptosporidium*, Microsporidia, and *Mikrocytos mackini*, invade the cytoplasm of host cells. Due to their extreme simplification, examination of mitosomes may aid in answering the question of whether there are any universal functions of MROs and mitochondria.

*Cryptosporidium* spp. MROs produce ATP by SLP and, in the case of *Cryptosporidium muris*, also by oxidative phosphorylation using the Krebs cycle with an unusual respiratory chain and ATP synthase (reviewed in (Mogi and Kita 2010)). They lack a genome and $H_2$ production and so may be classified as mitosomes (Mogi and Kita 2010; Müller et al. 2012) or more generally as MROs (Keithly 2008), a view which we favor. The functions of mitosomes in other lineages are unclear. The mitosome of the microsporidian *Encephalitozoon cuniculi* probably contains two subunits of the E1 component of a PDH complex encoded by the nuclear genome, but this complex cannot be functional without the other two unidentified components (Katinka et al. 2001). They also likely contain glycerol 3-phosphate dehydrogenase, an enzyme that is involved in the transport of electrons from the cytosol to mitochondria, and manganese-containing superoxide dismutase, which forms part of the system that protects the cell from oxidative stress (Burri et al. 2006). A proteomic study of *E. histolytica* mitosomal fractions (Mi-ichi et al. 2009) revealed the presence of several enzymes involved in sulfate activation: ATP sulfurylase (AS), adenosine-5-phosphosulfate (APS) kinase (APSK), and inorganic pyrophosphatase (IPP), as well as a sodium/sulfate symporter involved in sulfate uptake. This suggests that sulfate activation is the major function of these mitosomes. Proteomic analysis of the mitosomal fraction of *Giardia* (Jedelský et al. 2011) revealed 139 proteins, but only 20 were confirmed to localize in the organelle. A study using biochemical tagging revealed presence of many other, mostly *Giardia*-specific, proteins in the mitosome (Martincová et al. 2015). Functions of these proteins are largely unknown and the only biochemical activity detected so far

is limited to the Fe-S cluster synthesis (Tovar et al. 2003). The recently discovered mitosome of the rhizarian *Mikrocytos mackini* is also most likely involved mainly in the Fe-S cluster assembly (Burki et al. 2013). Hence, formation of Fe-S clusters appears to be a common function of virtually all MROs with the exception of *E. histolytica* mitosomes.

Transcriptomic and genomic investigations of free-living relatives of some parasitic anaerobes or microaerophiles suggest that they also contain anaerobic derivates of mitochondria. Hence, free-living ancestors of these parasite/free-living pairs were already adapted to an anaerobic niche, allowing some extant lineages to colonize anaerobic environments such as intestinal tracts. Two major clades of microbial eukaryotes have been particularly useful in understanding the connection between the free-living and parasitic members.

Metamonads are a group of excavates known to inhabit anaerobic or microaerophilic niches like animal guts. Well-known metamonad parasites like *T. vaginalis* and the salmonid fish pathogen *Spironucleus salmonicida* (Jerlström-Hultqvist et al. 2013) contain hydrogenosomes, while *G. intestinalis* contains a mitosome. *Trichomonas* and *Spironucleus* hydrogenosomes have relatively similar biochemistry, despite the fact that *Spironucleus* is more closely related to *Giardia*. Comparison and phylogenetic analyses of *Trichomonas* and *Spironucleus* hydrogenosomal genes suggest derivation from a common ancestor, with *Giardia* mitosomes representing further degeneration (Jerlström-Hultqvist et al. 2013). A more complete understanding of the evolution of these parasite MROs has arisen due to recent sequencing projects focused on their free-living relative *Paratrimastix*. A transcriptome survey has revealed that it possesses a hydrogenosome-like MRO with a mitochondrial pathway involved in amino acid metabolism, as well as H2ase and a lack of all ETC complexes (Hampl et al. 2008; Zubáčová et al. 2013). Analysis of these four organisms clearly shows that hydrogenosomes appeared in the free-living ancestor of Metamonada.

The recently described hydrogenosome of the free-living archamoebae *Mastigamoeba balamuthi* (Nývltová et al. 2013, 2015) provides an opportunity for similar comparative studies with the mitosome-bearing *Entamoeba*. The *M. balamuthi* hydrogenosome contains complex II (but no other ETC complexes) as well as a H2ase and a PFO (Gill et al. 2007). Genomic data have also demonstrated the presence of genes involved in sulfate activation (AS, APSK, and IPP), a property shared with *E. histolytica.* The second unusual feature shared by those two taxa is the replacement of the mitochondrial type Fe-S cluster assembly machinery (ISC) by ε-proteobacterial enzymes NifS and NifU. Genes for these enzymes have been duplicated in *M. balamuthi*, with the product of one paralog localized in the cytosol and the other in the MRO. *E. histolytica* has only one copy, products of which are localized in the cytosol and putatively also in the mitosomes, but the evidence for mitosomal localization is ambiguous (Maralikova et al. 2010; Mi-ichi et al. 2009). The presence of common features between *E. histolytica* and *M. balamuthi* MROs suggests that the loss of aerobic mitochondrial metabolism and acquisition of anaerobic enzymes, sulfate activation pathways, and ε-proteobacterial Fe-S cluster assembly proteins occurred in a free-living ancestor of these amoebae.

Genomic and transcriptomic data, provided in increasing amounts by high-throughput sequencing techniques, can be used for in silico prediction of MROs based on their known properties (Burki et al. 2013), even for organisms without established cultures. Features related with adaptation for an anaerobic lifestyle, namely, modification or loss of ETC and presence of Fe-S cluster-containing proteins like PFO, are exploited in specific treatments by metronidazole. This nitroimidazole antibiotic is activated to its cytotoxic form by reduction, which takes place in the presence of low redox potential electron transporters (ferredoxins, PFO) and in the absence of the high redox potential acceptor $O_2$ and so has little effect on human cells (Upcroft and Upcroft 2001).

Comparative genomics of parasitic protists and their free-living relatives are profoundly impacting our understanding of reductive evolution of mitochondria. It endorses the idea that the evolution of parasitism was in some cases preceded by adaptation to anaerobic environments, including major mitochondrial modifications. A likely model is that free-living organisms first evolved the capacity to survive in anaerobic environments like animal guts, and some subsequently evolved further to become endobionts and, in some cases, parasites. However, extreme reduction exemplified by mitosomes is probably only possible in parasites or endobionts, as they are able to use their host to fulfill energetic requirements.

Reduction of classical mitochondria to MROs exemplifies a situation in which similar environmental pressures constrain evolution toward convergent features. The evolution of membrane trafficking in diverse parasites follows similar trends, including modification in gene complement and localization/function, but often results in very different systems depending on parasite, environmental, and host factors.

## 12.4 Membrane Trafficking in Parasites

Membrane trafficking is crucial to the ability of diverse parasites to grow and proliferate. It contributes to processes including motility, nutrient acquisition, host invasion, immune evasion, and secretion. Generally, membrane trafficking is the process by which protein and lipid components are shuttled between intracellular compartments and by which cells take up and release materials into their environment. These diverse roles follow a similar scheme, roughly divided into phases of cargo selection and coat recruitment, vesicle formation and scission, transport from donor to acceptor compartment, and tethering and fusion with the target membrane (Bonifacino and Glick 2004). Despite the importance of trafficking to parasite biology, knowledge of membrane trafficking function in many taxa lags behind that of model systems such as yeast and mammalian cells.

Studies in recent years have revealed that the LECA possessed a complex membrane trafficking system (MTS), including an ER, Golgi body, and at least one form of endocytic organelle, such as an endosome or lysosome ((Koumandou et al. 2013) inter alia). These studies have also revealed unexpected losses of ancestral MTS components in our model systems. Examples include three ancestral Rab proteins not found in the human genome (Elias et al. 2012), a novel ArfGAP family completely

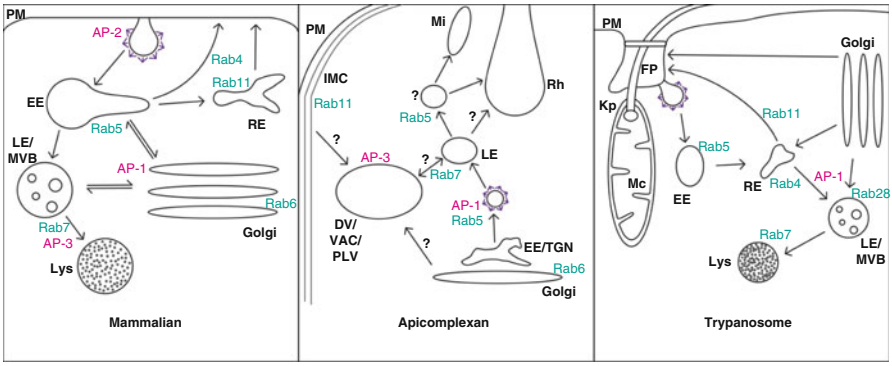



**Fig. 12.3** Membrane trafficking in parasites. General trafficking pathways present in mammalian, apicomplexan, and trypanosomes are shown. *Bold* type labels organelles, while adaptor protein (*AP*) and Rab proteins are listed separately; *question marks* denote uncertainty in pathways and/or machinery. Clathrin coats are denoted by *purple triskelions*. *Abbreviations*: *PM* plasma membrane, *TGN* trans-Golgi network, *EE* early endosome, *RE* recycling endosome, *LE* late endosome, *MVB* multivesicular body, *Lys* lysosome, *IMC* inner membrane complex, *Mi* microneme, *Rh* rhoptry, *DV* digestive vacuole, *VAC* vacuolar compartment, *PLV* plant-like vacuole, *FP* flagellar pocket, *Kp* kinetoplast, *Mc* mitochondrion. Note that though apicomplexans like *T. gondii* possess stacked Golgi, morphology differs in other members of the phylum and a single cisterna is shown for simplicity. Also note that diagrams are not to scale

absent from animals and yeast (Schlacht et al. 2013), and a unique adaptor protein-related complex known as "TSET", one remaining component of which gave rise to the muniscin cargo adaptor in metazoa (Hirst et al. 2014). Thus, only by studying trafficking in diverse eukaryotes will we obtain a generalizable model of MTS function and be able to characterize divergent systems, including those of parasites.

One process of particular interest is post-Golgi trafficking, including endocytosis, exocytosis, and movement of cargo through endosomal organelles (Fig. 12.3). Uptake of extracellular material frequently occurs by clathrin-mediated endocytosis (CME), which involves numerous adaptor proteins that link cargo and clathrin at the plasma membrane (PM). Accessory proteins, together with clathrin, bend and stabilize the nascent vesicle, which eventually buds away from the PM and is separated by dynamin (Rao et al. 2012; Reider and Wendland 2011; Traub and Bonifacino 2013). Cargos are internalized and subsequently enter the endosomal system. All endocytic, as well as some exocytic, cargoes converge at the early endosome (EE) for sorting. Recycled cargo accumulates in tubular extensions for subsequent transport to the PM (either through recycling endosomes, or more rapid direct recycling), or back to the trans-Golgi network (TGN). Otherwise, it accumulates in larger volume domains for transit to the late endosomal system. Formation of intraluminal vesicles to form multivesicular bodies (MVBs) begins at the EE and continues throughout the endosomal maturation process. During this process, the organelles acidify and undergo changes in marker proteins. Late endosomes (LEs) form and subsequently fuse with lysosomes to form hybrid endolysosomes, in which cargo is finally degraded by acidic hydrolases (Huotari and Helenius 2011; Scott et al. 2014).

The study of parasites has led to a better understanding of the plasticity of this system. *T. vaginalis* adheres to host cells as a critical step in pathogenesis. This is mediated by adhesins such as lipoglycans that bind host galectin-1 and numerous other secreted components of the lipophosphoglycan surface matrix (Bastida-Corcuera et al. 2005; Okumura et al. 2008). The presence of prominent Golgi bodies (parabasal apparatus) is well documented (Honigberg et al. 1971), and multivesicular bodies (MVB)-derived exosomes were recently described (Twu et al. 2013). Consistent with secretion of many virulence factors, numerous trafficking families, including adaptor and coat proteins involved in vesicle formation, and tethering and fusion proteins involved in vesicle fusion are expanded several fold compared to humans (Carlton et al. 2007). *E. histolytica* lacks many identifiable endomembrane structures, such as stacked Golgi, but does possess numerous vacuolar structures of likely endolysosomal origin (Perdomo et al. 2015). Pathogenicity relies on efficient phagocytosis, and many proteins are recruited to the phagosome that function in endocytic trafficking in model systems ((Juárez-Hernández et al. 2013), inter alia). This amoeba encodes all common coat complexes, as expected, but similar to *T. vaginalis*, it possesses large expansions of GTPases known to regulate trafficking (Loftus et al. 2005). By contrast, the diplomonad *G. intestinalis* represents a case of extreme MTS divergence. Materials move from the ER to peripheral vacuoles, compartments that appear to perform all the functions of diverse endosomes/lysosomes, directly with no intermediate sorting compartment such as a Golgi (reviewed in (Faso and Hehl 2011)); enclosures termed encystation-specific vesicles present during part of the lifecycle have been suggested to represent a "Golgi-like" compartment. Unlike *T. vaginalis* and *E. histolytica*, *G. intestinalis* encodes a reduced set of trafficking factors compared to close relatives and the LECA.

The two parasitic lineages best studied, Apicomplexa and trypanosomes, have modified their MTS to facilitate very different modes of parasitism. Apicomplexa include obligate intracellular parasites of humans and domestic animals. They represent a system in which the endocytic functions of post-Golgi trafficking have been reduced in favor of increased secretive capabilities. Apicomplexans possess a cytoskeletal apparatus at the apical end of the cell known as the apical complex, along with secretory organelles involved in host cell invasion known as micronemes and rhoptries (Baum et al. 2008). These organelles are derived from endosomes/lysosomes (Klinger et al. 2013b; Ngô et al. 2004), yet the effect of these additional endosomal compartments on the parasite's MTS is incompletely understood. Despite extensively reduced metabolic functions, and a proposed reliance on uptake of host cell metabolites for growth and proliferation (Abrahamsen et al. 2004; Cassera et al. 2008; Plattner and Soldati-Favre 2008; Woodrow et al. 2000), classical endocytosis has yet to be demonstrated in these organisms (Pieperhoff et al. 2013). Indeed, it appears that apicomplexan parasites have co-opted machinery traditionally involved in endocytosis and recycling to function in building their invasion organelles (reviewed in (Tomavo 2014)).

The presence of a plant-like vacuolar lysosome (VAC) in *Toxoplasma gondii* suggested the potential for degradation of endocytosed materials (Miranda et al. 2010). Recent work has elucidated such a role for the VAC, yet the mechanism

through which uptake occurs remains elusive (Dou et al. 2014). There have been numerous reports of plasma membrane invaginations and endocytic activity (Botero-Kleiven et al. 2001; Coppens et al. 2000; Gross et al. 1993; Nichols et al. 1994), but no mechanistic data has yet emerged. Endocytic uptake has been visualized extensively in *P. falciparum*-infected red blood cells, where the process of internalization and digestion of hemoglobin in an acidified digestive vacuole (DV) represents a drug target (Sigala and Goldberg 2014).

In comparison, endocytosis is comparatively well studied in African trypanosomes and many mechanistic details have been elucidated. *T. brucei* relies extensively on endocytosis for its continued survival as an extracellular parasite, including extensive endocytosis and recycling of variable surface glycoproteins (VSGs) (Cross 1975; Field et al. 2009; Manna et al. 2014; Vickerman 1969). While Apicomplexa are polarized toward the apical end of the cell, trypanosomes are polarized posteriorly. All endocytic traffic occurs in the small region between the nucleus and the kinetoplast (the mitochondrial genome)/flagellar pocket, and all endo- and exocytic events occur in the pocket itself (reviewed in (Field and Carrington 2009)). All the hallmarks of a canonical endocytic system are present, including early, recycling, and late endosomes/MVBs, as well as a terminal lysosome for degradation. Endocytosis is essential to *T. brucei* survival, and perturbation of endocytic functions leads to pleiotropic effects on morphology and cytokinesis that are usually lethal (Allen et al. 2003; Manna et al. 2014).

Endocytic uptake between these two groups does share some features. Uptake of material involved in DV formation in *P. falciparum* and endocytosis in *T. brucei* both rely on actin, yet clathrin is dispensable for the former and not the latter (Elliott et al. 2008; García-Salcedo et al. 2004; Lazarus et al. 2008). Vesicle fission also differs from current models. Endocytosis in *T. brucei* occurs without dynamin or a dynamin-related protein (DRP) (Morgan et al. 2004), and though multiple DRPs have been found in Apicomplexa, these are involved in biogenesis of invasion organelles and fission of endosymbiotic organelles during cell division (Breinich et al. 2009; Charneau et al. 2007; Li et al. 2004; van Dooren et al. 2009). Trypanosomes and apicomplexans, like the vast majority of eukaryotes, lack Epsin, which is a specific innovation of the opisthokonts (Field et al. 2007). Instead, *T. brucei* uses a single "ENTH" domain-containing protein, EpsinR, to mediate cargo selection and clathrin recruitment (Gabernet-Castello et al. 2009). EpsinR proteins, although present in Apicomplexa, have not been functionally characterized.

Though this direct comparison clearly demonstrates a classical form versus function argument, comparison of genomic, morphological, and functional data between these groups and their close relatives can help to understand the evolutionary origins behind these fascinating MTS alterations. Two trafficking protein families, the adaptor protein (AP) complexes and Ras from brain (Rab) GTPases, have been particularly useful in this manner.

AP complexes are heterotetrameric complexes, composed of adaptin subunits, involved in cargo selection and coat recruitment during vesicle biogenesis (Robinson 2004). All five known AP complexes were present in the LECA, though AP-5 has been secondarily lost in numerous extant lineages (Hirst et al. 2011). In model

systems, AP-1 and AP-2 interact with clathrin, whereas AP-4 and AP-5 do not; interaction of AP-3 with clathrin is uncertain (Dell'Angelica 2009; Hirst et al. 2013; Robinson 2004). AP-1 is involved in trafficking between the Golgi and endosomes, AP-2 primarily functions in endocytosis at the plasma membrane, and AP-3 is involved in biogenesis and maintenance of lysosomes and related organelles. AP-4 and AP-5 functions are less well established, but appear to involve endosomal trafficking.

Salivarian trypanosomes encode AP-1, AP-3, and AP-4, though they lack AP-2 and AP-5 (Manna et al. 2013). The loss of AP-2 is associated with the gain of the Variant Surface Glycoprotein (VSG) coat, while the loss of AP-5 is more deeply derived within the kinetoplastids. *Leishmania*, another important disease-causing kinetoplastid, has independently lost the AP-4 complex. AP-1 was shown to be essential, yet it is dispensable for correct localization of the lysosomal marker protein p67 (Allen et al. 2007). AP evolution in Apicomplexa is somewhat more convoluted. AP-3 has been secondarily lost in the piroplasmids and *Cryptosporidium*, together with multiple losses of the complete AP-5 complex or AP-5 subunits in the above groups and *Plasmodium* (Nevin and Dacks 2009; Woo et al. 2015). The pattern of AP-3 loss suggests lineage-specific functions related to the *Plasmodium* DV and *T. gondii* VAC; this has been suggested by a recent study of chemotherapeutics in *T. gondii* (Fomovska et al. 2012). The only well-studied complex in Apicomplexa is AP-1. Consistent with model systems, where AP-1 is associated with TGN-endolysosome trafficking, early studies provided convincing localization for AP-1 at rhoptries and endosomal compartments in *T. gondii* (Ngô et al. 2003). More recent evidence points to a role for AP-1 in trafficking to micronemes and rhoptries, together with a sortilin-like receptor for cargo recognition (Kibria et al. 2015; Krai et al. 2014; Sloves et al. 2012; Tomavo et al. 2013).

Rabs are members of the Ras family of small GTPases involved in trafficking processes. They perform their roles by acting as organizational hubs for a multitude of upstream and downstream effectors, including tethers, SNAREs, SM proteins, PI kinases, and motor and cytoskeletal elements (Angers and Merz 2011; Brighouse et al. 2010; Stenmark 2009). Recent analyses demonstrate that the Rab complement of the LECA was extensive (23 Rabs), but multiple expansions and reductions have occurred post-LECA (Elias et al. 2012).

Apicomplexa and trypanosomes represent a reduction from the LECA Rab complement; trypanosomes have 16 Rabs (Ackers et al. 2005), whereas Apicomplexa possess between 15 (*T. gondii*) and eight (*C. parvum*) (Langsley et al. 2008). Three Rabs have been well characterized in both systems: Rab5, Rab7, and Rab11, which mediate EE, LE/lysosome, and recycling traffic in mammalian cells, respectively. *T. brucei* (Tb) Rab5 plays a role very similar to that in mammalian cells, being involved in facilitating EE traffic (Hall et al. 2004). *T. gondii* (Tg) Rab5A and 5C (two of three Rab5 paralogs encoded, an expansion from the primordial Rab5 in the LECA) play a different role and are essential for proper biogenesis of invasion organelles (Kremer et al. 2013). TbRab7 is involved in trafficking to lysosomes (Silverman et al. 2011), while Rab7 in *T. gondii* and *P. falciparum* localizes to putative LEs (Krai et al. 2014; Parussini et al. 2010). TbRab11, as well as TbRab4 in procyclic

parasites, is involved in recycling of material from intermediate endosomes to the PM (Hall et al. 2005; Pal et al. 2003). Apicomplexan homologues have been co-opted for a different function, as the Rab11 paralogs, Rab11A and 11B, are involved in formation and maintenance of the inner membrane complex (IMC) (Agop-Nersesian et al. 2009, 2010). Roles for additional *T. brucei* Rabs in membrane trafficking have been elucidated and appear to function similarly to their characterized homologues in model systems; TbRab28 is involved in retromer-dependent trafficking processes (Lumb et al. 2011) and TbRab21 is involved in intermediate endocytic trafficking (Ali et al. 2014). A detailed understanding of the function of other Rab proteins in Apicomplexa remains elusive. A recent screen in *T. gondii* failed to define precise roles for a large number of the remaining Rabs, but localized the majority in the early or late secretory system (Kremer et al. 2013).

Many of the unique features noted in Apicomplexa are shared with out-group taxa, such as ciliates and dinoflagellates, suggesting an origin in a common free-living ancestor. The alveolate-specific Rab11B mediates IMC formation in Apicomplexa and likely serves a similar role in maintenance of the homologous alveoli in other members of this group (Agop-Nersesian et al. 2010; Klinger et al. 2013b). Many alveolates have specialized secretory granules of uncertain origin referred to generally as extrusomes (reviewed in (Rosati and Modeo 2003)). Though it is unclear if these are homologous to secretory organelles of Apicomplexa, the use of sortilins as sorting receptors for secretory organelle proteins is a feature shared with the ciliate *T. thermophila* and likely with other alveolates as well (Briguglio et al. 2013). Additionally, *T. thermophila* uses CME for uptake of extracellular materials, but this process does not depend on actin and uses a DRP that does not group with classical dynamin in phylogenetic analyses (Elde et al. 2005). Due to a lack of knowledge for many related taxa, it is unclear if the free-living ancestor of Apicomplexa completed endocytic uptake with a DRP or not.

Closer still within the alveolates, Apicomplexa are sisters to the dinoflagellates, perkinsids, colpodellids, and chromerids, forming a single clade Myzozoa. Every group of myzozoans contains organisms that possess a structure morphologically similar, and potentially homologous, to the apical complex of Apicomplexa, including the conoid (peduncle in dinoflagellates), and organelles similar to micronemes and rhoptries ((Okamoto and Keeling 2014), inter alia). It is believed that the apical complex is a plesiomorphic feature of the Myzozoa and that it originally served for myzocytosis (phagocytosis of prey cytoplasm), as in extant lineages such as *Colpodella*. Apicomplexa later modified the function of this apparatus to facilitate host cell invasion (Cavalier-Smith and Chao 2004). Determining which changes in the apicomplexan MTS are concurrent with modification of this complex and which are specific adaptations to parasitism is important to understanding the evolution of the apicomplexan lifestyle. From our own results, it is clear that at least some changes, such as the loss of the exocyst complex (Klinger et al. 2013a), a tethering complex involved in regulated secretion, are common to all myzozoan taxa studied so far and may be associated with the acquisition of the apical complex. Other changes, such as widespread loss of AP-3, are restricted to Apicomplexa and hence may be concurrent with their parasitic lifestyle.

Analysis of the genome of *Naegleria gruberi,* a free-living heterolobosean related to kinetoplastids, revealed that the common ancestor of these taxa possessed a full complement of AP and Rab components, comparable to the LECA (Elias et al. 2012; Manna et al. 2013). Furthermore, the loss of AP-2 in salivarian trypanosomes is restricted to this subset of *Trypanosoma spp.*, suggesting this was a recent event. It is also concurrent with the acquisition of the Variant Surface Glycoprotein (VSG) family and has been suggested as a modification to enable extremely rapid, though less selective, endocytic processes (Manna et al. 2013). In addition to the aforementioned departures from classical CME, a recent proteomics-based study found novel clathrin-interacting proteins (CLASPS) specific to kinetoplastids of the genera *Trypanosoma* and *Leishmania* (Adung'a et al. 2013). Though further characterization is ongoing, it is clear that African trypanosomes have evolved to specialize in rapid, though not necessarily selective, endocytic events in order to survive the ravages of host immune responses. Only some of these traits are shared with their close relatives and may relate to mode of parasitic infection, as, for instance, *Leishmania* spp. are intracellular parasites.

One major challenge to understanding MTS evolution and function is the considerable plasticity in the system. Reductions and expansions of machinery do not seem to correlate well with functional complexity. *P. falciparum* encodes a set of membrane trafficking machinery that is reduced from, or at best comparable to, the LECA, and yet in infected red blood cells, the parasite must traffic components to at least ten distinct locations. *T. vaginalis* encodes large numbers of paralogs for most gene families, yet is not noticeably more complex than the human host it infects. Even within commonly used model systems, endocytic trafficking follows distinct pathways. Plants appear to lack an EE and use the TGN instead. *Saccharomyces* does not possess the same complexity of endosomal structures as mammalian cells and use distinct retromer-dependent trafficking pathways.

This extensive plasticity makes reconstructing an accurate ancestral MTS challenging and limits the use of any given organism as a model system. However, both hurdles may be overcome by continuing to grow our repertoire of genomic sequence and functional knowledge of cellular pathways in parasites and related free-living taxa. The study of parasite membrane trafficking has and will continue to lead to new avenues of discovery and understanding, not just of universal eukaryotic features but also of key differences between host and parasite biology. Through a thorough understanding of general eukaryotic cell biology, we gain a better appreciation for what is novel and hence therapeutically exploitable.

## Conclusion

Comparison of parasites to multiple free-living out-groups improves the resolution of character evolution, but was infeasible prior to an increase in the number of available genomes. Throughout the mid-2000s chain-termination (Sanger) sequencing was gradually replaced by 454's pyrosequencing and Illumina's method of sequencing by synthesis, which were far less expensive and more practical for large sequencing projects. These technologies allowed for many genomes to be sequenced by individual labs or small consortia, increasing the number of taxonomic sampling points available. These technical advances have

not only increased the number of parasite/free-living pairs for direct evolutionary comparison but have also provided a wealth of sequence information for large-scale dissection of phylogenetic relationships, placing these pairs in context.

The recognition that parasites represent independent convergences on similar modes of life from a free-living ancestor has been an important factor in shaping the evolving landscape of parasitological research. It is crucial that parasitologists interpret parasite biology in the context of closely related, free-living organisms in order to understand how parasitism arose in any given lineage. Though some characteristics are shared, such as decreased aerobic function of mitochondria and modification of the membrane trafficking system, these represent cases of convergence through occupying similar environmental niches.

Recently published genomes of additional free-living relatives of parasitic groups, such as Chromera velia and Vitrella brassicaformis (Woo et al. 2015), as well as Bodo saltans (Jackson et al. 2016), in addition to others currently in progress including Mastigamoeba, Paratrimastix, and Carpediemonas, will allow for even more comparative studies of the transition to parasitism. Increased efforts to develop model parasitic systems show promise for molecular characterization, driven by the hypotheses produced from comparative studies. In this way, it will be possible to combine putative targets from bioinformatic studies with detailed biochemical studies to understand the modification of cellular systems that give parasites the ability to exploit their hosts. In the course of these studies, we expect to identify a number of ways in which parasites may gain traction in their new niches; these may well represent new opportunities to be exploited in combating parasitic threats to human health and well-being.

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