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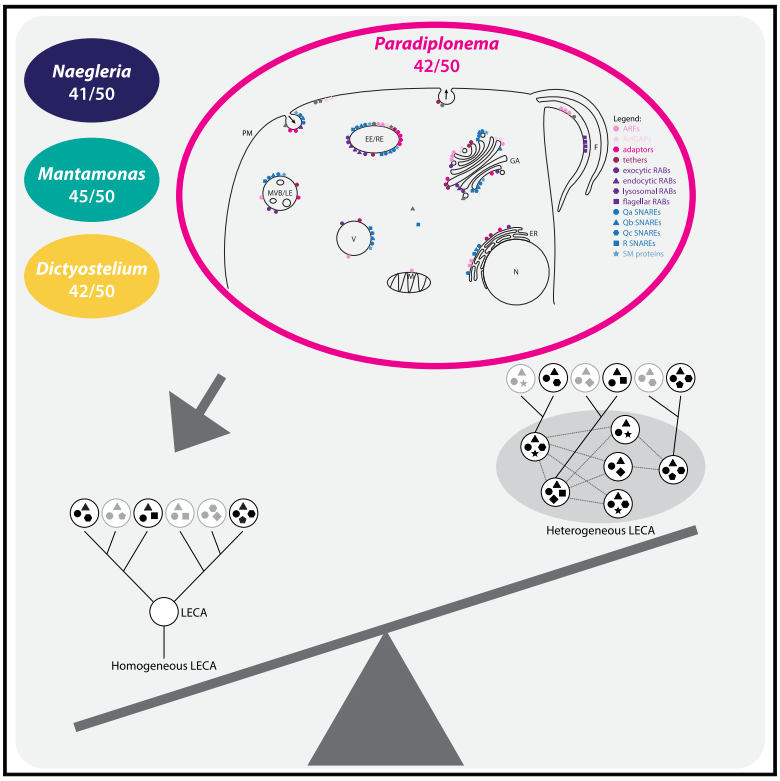
Diplonemid protists possess exotic endomembrane machinery, impacting models of membrane trafficking in modern and ancient eukaryotes

Graphical abstract



Highlights

- Diplonemids encode an extensive complement of membrane-trafficking proteins
- Heterotrophic protists possess machinery sporadically present in model organisms
- The LECA likely was a homogenous population with complex cell biology
- General cell biological frameworks should integrate data from diverse eukaryotes

Authors

Kristína Záhonová, Julius Lukeš, Joel B. Dacks

Correspondence

kika.zahonova@gmail.com (K.Z.), dacks@ualberta.ca (J.B.D.)

In brief

Diplonemids are widespread and diverse heterotrophic marine protists. Záhonová et al. report that diplonemids feature an extensive complement of membrane-trafficking machinery. The findings suggest that the last eukaryotic common ancestor had a complex cell biology and call for more generalizable models of the cell biology of extant eukaryotes.

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Diplonemid protists possess exotic endomembrane machinery, impacting models of membrane trafficking in modern and ancient eukaryotes

Q1 Kristína Záhonová,[1,2,3,4,*] Julius Lukeš,[2,5] and Joel B. Dacks[1,2,6,7,*]

[1]Division of Infectious Diseases, Department of Medicine, and Department of Biological Sciences, University of Alberta, 1-124 Clinical Sciences Building, 11350-83 Avenue, Edmonton, AB T6G 2G3, Canada

[2]Institute of Parasitology, Biology Centre, Czech Academy of Sciences, Branišovská 1160/31, České Budějovice (Budweis) 370 05, Czech Republic

[3]Life Science Research Centre, Department of Biology and Ecology, Faculty of Science, University of Ostrava, Chittussiho 10, Ostrava 710 00, Czech Republic

[4]Department of Parasitology, Faculty of Science, Charles University, BIOCEV, Průmyslová 595, Vestec 252 50, Czech Republic

[5]Faculty of Science, University of South Bohemia, Branišovská 1645/31a, České Budějovice 370 05, Czech Republic

[6]Centre for Life's Origin and Evolution, Division of Biosciences (Darwin Building), University College London, Gower Street, London WC1E 6BT, UK

[7]Lead contact

*Correspondence: kika.zahonova@gmail.com (K.Z.), dacks@ualberta.ca (J.B.D.)

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SUMMARY

Diplonemids are among the most abundant and species-rich protists in the oceans. Marine heterotrophic flagellates, including diplonemids, have been suggested to play important roles in global biogeochemical cycles. Diplonemids are also the sister taxon of kinetoplastids, home to trypanosomatid parasites of global health importance, and thus are informative about the evolution of kinetoplastid biology. However, the genomic and cellular complement that underpins diplonemids' highly successful lifestyle is underexplored. At the same time, our framework describing cellular processes may not be as broadly applicable as presumed, as it is largely derived from animal and fungal model organisms, a small subset of extant eukaryotic diversity. In addition to uniquely evolved machinery in animals and fungi, there exist components with sporadic (i.e., "patchy") distributions across other eukaryotes. A most intriguing subset are components ("jötnarlogs") stochastically present in a wide range of eukaryotes but lost in animal and/or fungal models. Such components are considered exotic curiosities but may be relevant to inferences about the complexity of the last eukaryotic common ancestor (LECA) and frameworks of modern cell biology. Here, we use comparative genomics and phylogenetics to comprehensively assess the membrane-trafficking system of diplonemids. They possess several proteins thought of as kinetoplastid specific, as well as an extensive set of patchy proteins, including jötnarlogs. Diplonemids apparently function with endomembrane machinery distinct from existing cell biological models but comparable with other free-living heterotrophic protists, highlighting the importance of including such exotic components when considering different models of ancient eukaryotic genomic complexity and the cell biology of non-opisthokont organisms.

Q2

Q5 Q4 Q3 INTRODUCTION

Marine single-celled eukaryotes, i.e., protists, form a plethora of interactions with viruses, prokaryotes, and other microorganisms, creating composite food webs.[1] They are important players in biogeochemical cycles and thus influence oceanic life and atmospheric composition.[2] Their importance in the global carbon cycle has been recognized for a long time, although mainly photosynthetic microeukaryotes were considered. The role of the most abundant heterotrophic protists started to be recognized only relatively recently,[3–5] with initiatives such as the *Tara* Oceans Expedition aiming to assess the complexity of ocean ecosystems on a global scale.[6]

One of under-appreciated groups of heterotrophic flagellates is diplonemids, a subgroup of euglenozoan protists. Diplonemids are the sister lineage to the mostly parasitic kinetoplastids,[7] together forming the subphylum Glycomonada.[8] As such, they represent an important sampling point when drawing conclusions regarding the timing of gene loss and innovation on the kinetoplastid evolutionary path to parasitism. Diplonemids were previously neglected but, based on amplicon sequencing and metagenomic studies, they are now recognized as one of the most abundant and species-rich organisms in the worlds' oceans, with much rarer occurrence in fresh waters.[9–11] Although represented by a single nuclear genome sequence (from *Paradiplonema papillatum*[12]), the introduction into culture

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of diverse diplonemids has allowed transcriptomic sampling and organelle sequencing, allowing description of massive mitochondrial genomes or uniquely edited mitochondrial transcripts and establishing them as genetically tractable organisms.[13–15] The diplonemids are divided into four main lineages: Diplonemidae, Hemistasiidae, Eupelagonemidae, and deep-sea pelagic diplonemids II (DSPD-II).[16–18] The feeding mode of diplonemids is quite variable, and they are able to switch from osmotrophy to phagotrophy to bacteriovory.[19] As heterotrophy might be one of the aspects of diplonemids' environmental success, understanding the cellular basis behind it is imperative.

One set of organelles and molecular machinery underlying heterotrophy is the membrane-trafficking system (MTS), which facilitates transport of macromolecules between organelles within the cell and between the cell and its surrounding environment. The process itself is most simply described as a relatively linear sequence of vesicle initiation, budding, and scission at the donor organelle, through to uncoating, tethering, docking, and fusion of the vesicle at the acceptor organelle. These processes are enabled by a range of protein machineries, each acting at various stages. Wrapping of cargo into the vesicle is facilitated by GTPases of the Arf/Sar protein family, whereas vesicle budding and its transport involves adaptor and coat proteins. Tethering of vesicles with the acceptor organelle is mediated by multisubunit tethering complexes and Rab GTPases, and final vesicle fusion is facilitated by SNARE proteins.[20] Many of these proteins belong to broader protein families with paralogs operating in different locations of the cell. Though plant cell biology is increasingly becoming integrated and mainstream,[21] this general modeling of membrane trafficking was first derived from, and remains heavily based on, data from animal and fungal (i.e., opisthokont) model organisms. Given that Opisthokonta is a small proportion of extant eukaryotic diversity, yet its cell biological frameworks are often held as generally applicable to the eukaryotic cell, a question arises as to how well our existing descriptions of eukaryotic cell biology truly apply.

Comparative genomics and molecular evolutionary analysis of protist genomes have provided an answer to some extent. The major protein families and many of their subfamilies described above are indeed present in the diversity of eukaryotes. Overall, the opisthokont-derived model holds in broad strokes but with clear, exciting exceptions of opisthokont novelty and losses across different eukaryotic lineages.[22] In nearly every system examined from a comparative molecular evolutionary perspective, the extent of component retention within paralogs of each family has ranged from highly conserved through to less frequently present, albeit still broadly distributed, components, i.e., those with a patchy distribution.[23] This includes cases when the loss has taken place in the lineage of animals and fungi, from where most of the information about eukaryotic cells is derived. There is a growing list of the jötnarlogs, i.e., cellular components, usually paralogs of characterized machinery that show a pattern of being widely present across diverse eukaryotes but are missing in the most common model organisms such as yeast and mammalian cells.[24] Sixteen such examples have been reported in the MTS alone.[24,25] The key question is whether such components are rare and exotic curiosities that need not be integrated into general understanding of eukaryotic cell biology and evolution or whether they represent under-appreciated core cellular machinery in microbial eukaryotes.

Though the relatively pedestrian—but nonetheless important—specter of false negatives due to incomplete data or methods at the initial time of reporting, and the extent to which horizontal gene transfer (HGT) impacts the distribution of cellular machinery, deserves consideration, the presence of patchy proteins has been interpreted as evidence for a highly sophisticated complement of cellular machinery in the last eukaryotic common ancestor (LECA).[26] In this interpretation, the LECA is envisioned originally as a single ancestor[27] and, more recently,[28] as a population of highly complex heterotrophic generalists, and losses from the descendant, often more-specialist lineages explain this patchy distribution. Other interpretations, however, also exist. Tantalizingly, the existence of proteins with a patchy distribution (both those found in animals or fungi and jötnarlogs) is central to the hypothesis of an ancient pan-genome with a small core and large variable complement in the LECA population.[27] As the MTS protein families are well documented to include a range of taxonomic retentions across eukaryotes, from extensively distributed to sporadic components and jötnarlogs,[23,24] this cellular system is particularly well suited to test these two hypotheses regarding the complexity of the LECA population.

In order to address the following three orthogonal but intersecting knowledge gaps, namely (1) the unexplored status of the endomembrane complement of abundant and functionally impactful marine heterotrophic protists and how this compares with kinetoplastids, (2) the nature of LECA's genomic vs. pangenomic nature, and (3) the extent to which opisthokont-based cellular models need to be extended to take patchily distributed proteins into account (including the relative contribution of jötnarlogs), we have undertaken a comprehensive molecular evolutionary analysis of the protein families underlying the MTS in diplonemid flagellates.

RESULTS

Dataset and rationale

To assess the complement of membrane-trafficking machinery encoded by diplonemid flagellates (Figure S1), we assembled a dataset encompassing 13 euglenozoan genomes/transcriptomes. The diplonemid dataset was composed of *Paradiplonema papillatum* (the only species with a high-quality genome assembly available),[12] *Diplonema japonicum*, *Rhynchopus humris*, *Lacrimia lanifica*, and *Sulcionema specki* (Diplonemidae), as well as *Artemidia motanka* and *Namystynia karyoxenos* (Hemistasiidae), for which only incomplete genomes and transcriptomes were published.[15] Because the MTS has been studied in detail in sister kinetoplastids,[29] we chose *Trypanosoma brucei*, *Leishmania major*, and *Bodo saltans* to cover their phylogenetic diversity.[30] Euglenids were represented by *Euglena gracilis*,[31] *Euglena longa*,[32] and *Eutreptiella gymnastica*,[33] with the MTS complement of the former having been analyzed previously but the latter two being analyzed for the first time here. This composite dataset was queried by BLAST or HMMER (Data S1A–S1E), and orthology of specific components was validated by phylogenetics when relevant (Figures S2–S6). These methods allowed us to query the presence of 173 proteins, covering the secretory and endocytic machineries. Overall, the diplonemid

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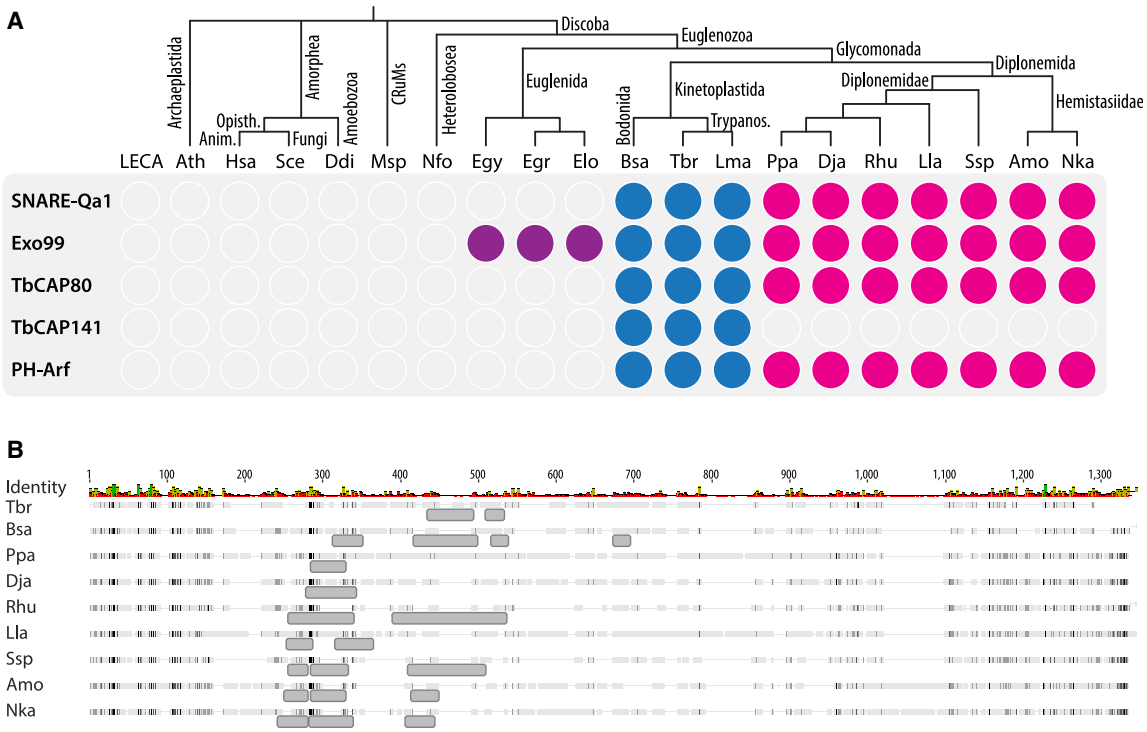

Figure 1. Previously identified kinetoplastid-specialized MTS components

(A) Their presence in selected eukaryotes. Relationships among the eukaryotes are shown by a schematic tree above the figure. LECA, last eukaryotic common ancestor; Ath, *Arabidopsis thaliana*; Hsa, *Homo sapiens*; Sce, *Saccharomyces cerevisiae*; Ddi, *Dictyostelium discoideum*; Msp, *Mantamonas sphyraenae*; Nfo, *Naegleria fowleri*; Egy, *Eutreptiella gymnastica*; Egr, *Euglena gracilis*; Elo, *Euglena longa*; Bsa, *Bodo saltans*; Tbr, *Trypanosoma brucei*; Lma, *Leishmania major*; Ppa, *Paradiplonema papillatum*; Dja, *Diplonema japonicum*; Rhu, *Rhynchopus humris*; Lla, *Lacrimia lanifica*; Ssp, *Sulcionema specki*; Amo, *Artemidia motanka*; Nka, *Namystynia karyoxenos*; Anim., animals; Opisth., Opisthokonta, Trypanos., Trypanosomatida.

(B) The alignment of TbCAP80 shows that diplonemid candidate orthologs possess relatively conserved N termini followed by disordered domains with coiled-coils (gray boxes), similar to kinetoplastids.[36]

See also Figures S1–S3 and S7, Table S1, and Data S1.

complement is remarkably complete (e.g., 158 present out of 173 MTS proteins examined in *P. papillatum*). These data provided insights into evolutionary trends in the lineage, with relevance across eukaryotes.

Diplonemid genomes inform understanding of the kinetoplastid MTS complement

We began framing the diplonemid MTS complement by juxtaposing it with that of kinetoplastids. Previous analyses of the diverse kinetoplastid MTS have revealed a progressive loss of components from various systems,[31,34] as well as five components proposed as specialized innovations in this lineage. Starting with the SNARE proteins, syntaxin Qa1 was previously identified as a kinetoplastid-specific sub-family of the membrane-fusing SNARE proteins,[34,35] yet we find it also in diplonemids (Figures 1A and S2; Data S1E). Second, and expectedly, a subunit of the exocyst complex, Exo99, which mediates fusion of secretory vesicles at the plasma membrane in *Trypanosoma* spp.[37] and is encoded in the *E. gracilis* genome,[38] we find to be well conserved across euglenids and diplonemids (Figure 1A; Data S1C). Third, TbCAPs are proposed as kinetoplastid-specific clathrin-binding proteins.[36] Although we were unable to detect orthologs of TbCAP141, we identified a putative homolog of TbCAP80 (Figures 1A and 1B; Data S1B).

Finally, PH-Arf is a lineage-specific Arf-related GTPase,[25] previously described in kinetoplastids but here also detected in diplonemids (Figures 1A, 2, and S3). Our phylogenetic analyses resolved it with moderate support as a sister clade to Arl16 (Figures 2 and S3), which is strongly correlated with flagella across eukaryotes.[39] Although it has flagellar localization and is implicated in a Golgi-to-cilia trafficking pathway in human cells,[39] Arl16 is a cytosolic protein in *T. brucei* (http://tryptag.org/?query=Tb927.7.4630).[40] However, PH-Arf has a strong localization to the flagella-associated hook complex in *T. brucei* (http://tryptag.org/?query=Tb927.10.9670). Curiously, diplonemids have orthologs of PH-Arf, but not Arl16 (Data S1A). Although the placement of PH-Arf as basal to the entire pan-eukaryotic Arl16 clade could be interpreted as a pre-LECA duplication and loss in all non-glycomonad lineages, we prefer the scenario of a glycomonad-specific duplication of Arl16, with a phylogenetic artifact drawing PH-Arf outside the Arl16 clade. In this scenario, PH-Arf retained flagellar function, allowing degeneration and subsequent loss of Arf16 as a redundant duplicate in diplonemids.

Diplonemids thus possess the MTS components previously thought to be specialized for kinetoplastids, showing that this machinery was acquired already before the transition of trypanosomatids to parasitism. This evolutionary shift can also be

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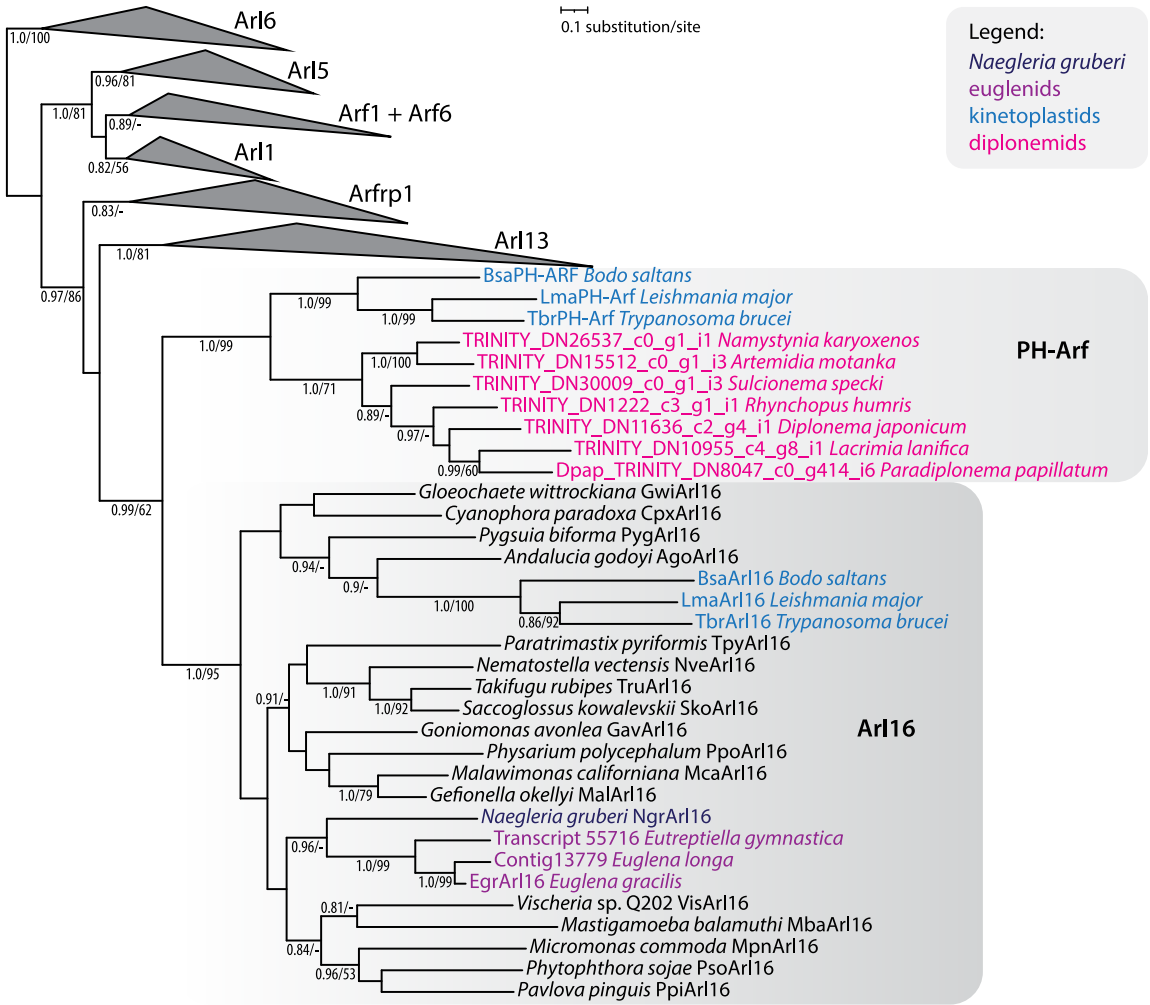

Figure 2. Phylogenetic analysis of Arfs

MrBayes topology of phylogenetic tree is shown, onto which posterior probabilities (PP)/bootstrap support (BS) values from RAxML are overlaid. Support values for PP < 0.8 and BS < 50% are denoted by a dash (–) or not shown in case of both supports below these thresholds. Several clades were collapsed for visualization purposes (for full tree, see Figure S2B).

See also Figures S1, S3, and S4 and Data S1.

understood from the 11 instances where diplonemids possess proteins lost in kinetoplastids after their divergence from diplonemids, as detailed below.

Diplonemids encode substantial complements of patchy proteins

As well as understanding glycomonad biology, examining the diplonemid MTS complement allows us to test current hypotheses about the nature of the LECA. These hypotheses differ in their interpretation of the complement of patchy proteins in modern eukaryotes, i.e., whether this is a signature of a pan-genome in the LECA population[27] (Figure 3A). However, if one or more modern eukaryotes possessed a near-complete set of the candidate patchy proteins, this would support the idea of a sophisticated cellular form in the LECA population and speak against the idea of a pan-genome with a small core set (Figure 3B).

The complement of MTS machinery encoded in diplonemids speaks directly to this point. We chose to examine 50 individual proteins designated as having patchy distributions, with the criteria set out in More et al. of "at least three losses in two supergroups, where a loss is defined as at least two absences in the genomes of closely related species."[24] Starting with 34 patchy proteins that are present in human cells (i.e., not jötnarlogs), 29 were found in multiple diplonemid datasets (Figure 4; Data S1A–S1E), suggesting their presence at a deep node within the eukaryotic tree. Several informative examples come from the endosomal system.

The vesicle formation process is largely mediated by coat complexes, allowing for cargo selection and membrane deformation. The most pervasively involved of these across the cell are the adaptin-related coats.[41] Although the AP-1 and AP-2 coats are highly retained across eukaryotes, others, such as the adaptor protein complexes AP-3, AP-4, and AP-5, have a substantially more sporadic distribution.[24] AP-3 is involved in trafficking from the *trans*-Golgi network (TGN) and early endosomes to late compartments within the endosomal system

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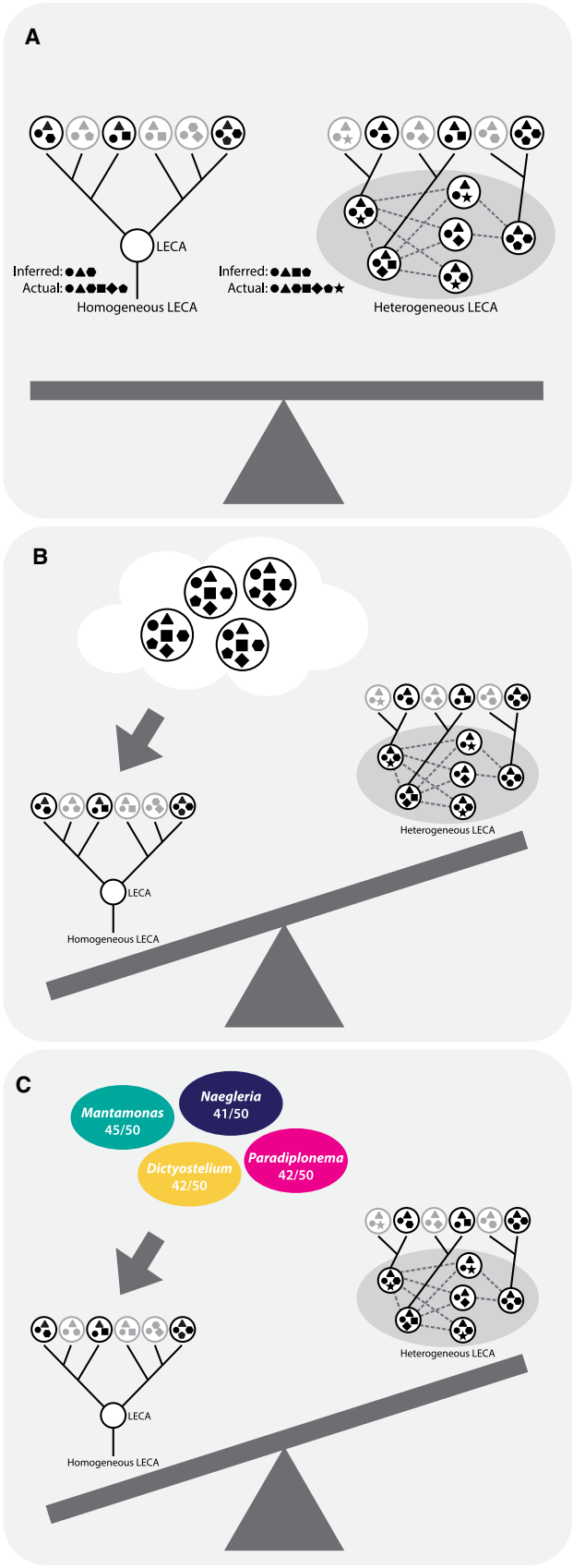

Figure 3. Patchy protein complements and the nature of genomic complexity in the LECA

(A) Two interpretations of the presence of proteins with patchy distribution across eukaryotes. In both scenarios, the proteins (denoted as different shapes) are found sporadically in different eukaryotes, with the circle and triangle inferred confidently. The homogenous LECA hypothesis postulates a highly complex ancestor with extensive subsequent losses. In the heterogenous LECA population hypothesis, a pan-genome existed that gave rise to the distribution. These hypotheses are equally likely when many organisms exist with non-overlapping sets of patchy proteins.

(B) If there are multiple examples of organisms whose genomes encode many or all of the patchy proteins, then the hypothesis of a homogenous LECA population[28] becomes much more likely.

(C) The complement of patchy proteins of the four heterotrophic protists are shown as a value out of 50 components assessed. Taking into account the additional proteins considered as broadly conserved, the data are most consistent with a much more confidently inferred, extensive set of membrane-trafficking proteins than was previously inferred when considering organisms with more derived endomembrane systems, e.g., kinetoplastids, yeasts, plants, and humans. These new data favor the hypothesis of a homogenous LECA population with a confidently inferred, extensive set of the MTS machinery—or at least that the core portion of the pan-genome becomes so large as to make the variable portion trivial and rendering the two hypotheses nearly synonymous. Redrawn with modifications.[27]

See also Figures S1 and S4.

(late endosomes, phagosomes, and lysosome-related organelles),[42,43] whereas AP-4 is responsible for transport from the TGN to endosomes, being also involved in autophagy.[44,45] Finally, in mammalian cells, AP-5 is responsible for retrograde movement from the late endosomes to the early endosomes and is associated with lysosomal transport and signaling.[45–47] We find that all AP-3 and AP-4 components are present in all sampled diplonemids, whereas the AP-5 components are identified in all five representatives of Diplonemidae but are missing in Hemistasiidae (Figures 4, 5, and S4; Data S1B), implying presence in the diplonemid ancestor but loss from this sub-lineage.

The most recently identified vesicle coat is the retriever complex, which mediates transport from the early endosome to the cell surface. This complex is found in animals, plants, and some protist lineages,[48] although not in fungi and only sporadically in kinetoplastids. It has been recently found in the parabasalid lineage, with the first experimental demonstration of functionally homologous retriever localization in an organism other than animals.[49] Here, we identified all subunits of the retriever complex across all diplonemids sampled (Figures 4 and S4; Data S1B).

Though increasingly implicated in bridging vesicle formation and fusion, the Rab GTPases are best known for interacting with effectors early in the process of vesicle tethering, in advance of cargo delivery.[50] The LECA complement of Rabs is inferred as being between 19 and 24 components, depending on analyses and cutoff values.[51–53] Based on a previous analysis,[51] Rabs 4, 14, 20, 21, 22, 23, 24, 32A, and 34 were observed to show a patchy distribution. Of these, Rabs 4, 14, 21, 22, and 23 are present in both Glycomonada lineages, whereas only diplonemids also possess Rabs 32A and 34 (Figures 4 and S5; Data S1D). These latter two Rab proteins are each implicated in processes at endosomal compartments.[54,55]

Diplonemids clearly possess an extensive set of membrane-trafficking machinery. However, to fully understand this complement, one final set of proteins from this system needed to be examined.

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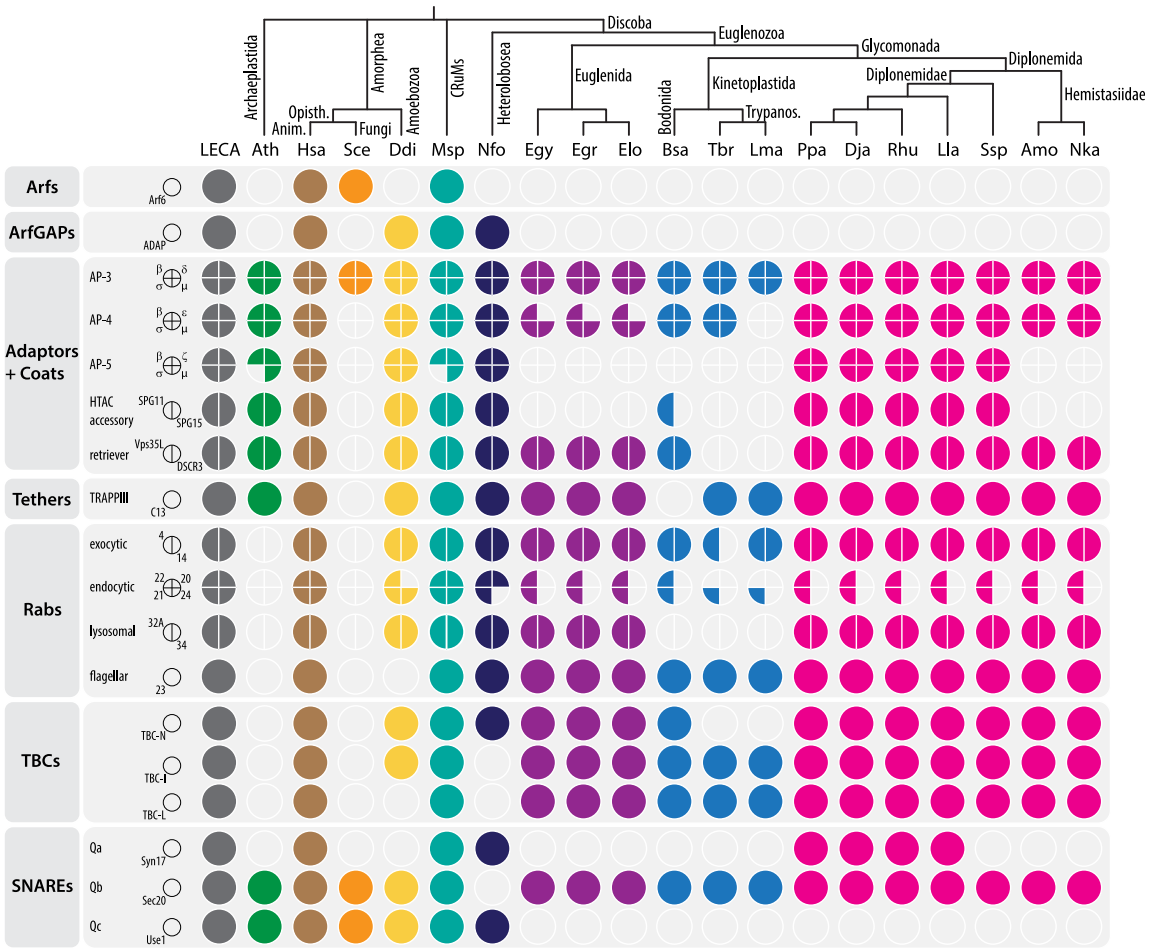

Figure 4. Presence of pan-eukaryotic patchy proteins of the MTS in selected eukaryotes

Relationships among the eukaryotes are shown by a schematic tree above the figure. Abbreviations as in Figure 1.
See also Figures S1–S7, Table S1, and Data S1.

Jötnarlogs are encoded in diplonemids

To address the question of the relevance of exotic membrane-trafficking proteins in modern (e.g., diplonemid) and LECA cell biology, we next turned our attention to proteins with a jötnarlog distribution. For example, the TSET complex is a primary mediator of endocytosis in plants[56] and is also implicated in this process in the slime mold *Dictyostelium discoideum*.[57] Its distribution spans the eukaryotic diversity but has been reduced from at least six subunits to a single divergent protein (FCHO) in animals and yeast.[57] Although the TSET complex is absent in kinetoplastids, it is fully retained in the examined diplonemid genomes and transcriptomes (Figures 5, 6, and S4; Data S1B). Other key jötnarlogs are the Qb and Qc-SNARE proteins NPSN and Syp7, respectively.[58] These proteins are well conserved in most eukaryotic groups and implicated in secretion to the cell surface in plants, forming the cell wall during division,[59] whereas NPSN at least functions in the contractile vacuole of *D. discoideum*.[60] Syp7 also shows a partial endoplasmic reticulum (ER) localization in *Arabidopsis*.[61,62] NPSN and Syp7 are missing in animal and most fungal lineages, but all diplonemid genomes encode both orthologs (Figures 6 and S2; Data S1E).

Overall, our analyses identified a high number (13 of 16) of jötnarlogs investigated to be widely present in diplonemids (Figure 6). Notably, these include several proteins where their function is merely speculated, based on paralogy with a characterized relative (e.g., SarB vs. Sar in COPII formation[63] and Rab32B vs. Rab32A in endosomal function[50,51]). It also includes some examples where the protein appears to be fairly widespread in eukaryotes (e.g., RabTitan[51] and Arl18[25]), but its function remains entirely untested. These examples highlight the importance of jötnarlogs and the unexplored biology of membrane trafficking in this highly abundant and diverse group of marine eukaryotes.

Other free-living protists also possess elaborate MTS machinery

The extensive endomembrane complement in diplonemids reflects a trend that has also been anecdotally observed in some other free-living heterotrophic protists. For example, individual analyses of genomes of *D. discoideum* (Amoebozoa) and the flagellate *Mantamonas sphyraenae* (CRuMs),[64] as well as the amoeboflagellate *Naegleria fowleri* (Discoba),[65] all show a similar

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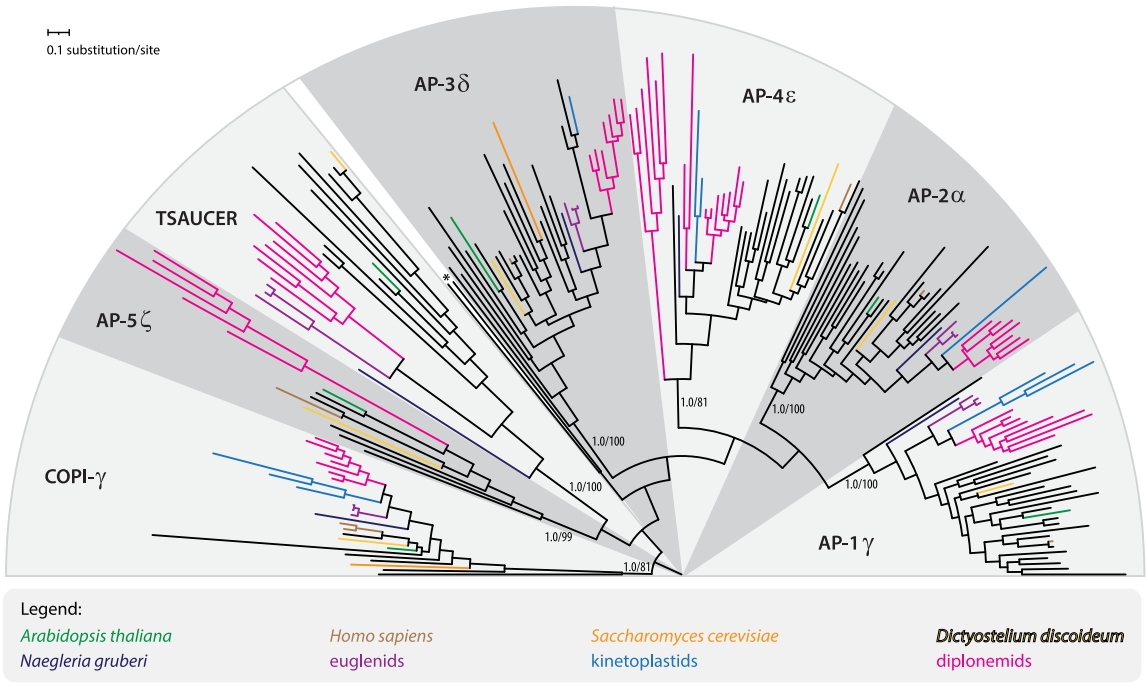

Figure 5. Phylogenetic analysis of adaptin-related large EGADZ (ε/γ/α/δ/ζ) subunits

MrBayes topology of phylogenetic tree is shown, onto which PPs/ BS values from RAxML are overlaid. Supports are shown only for major clades (for full tree, see Figure S4B). Asterisks mark the AP-3δ and AP-4ε sequences of *Plasmodium falciparum* and *Entamoeba histolytica*, respectively, that fell outside of the corresponding clades.

See also Figures S1 and S4 and Data S1.

pattern of high proportions of patchy proteins (including jötnarlogs) (Figures 4, 6, and S1). In order to more systematically compare these with our diplonemid datasets, we used the literature, where possible,[25,51,57,64–69] and performed additional homology searches (Table S1) and phylogenetic analyses (Figure S7) to bring the datasets into concordance. Specifically, we analyzed the complement of Arf GTPase family members and Rab GTPases as a whole, as well as dissected the endocytic Rabs from *M. sphyraenae*.

Consistent with past reports, we observed rich endomembrane system complements in the three abovementioned genomes, particularly in patchy machinery and jötnarlog. Although *D. discoideum* encodes 42 of 50 patchy proteins (13 of 16 jötnarlogs), *N. fowleri* encodes 41 of 50 patchy proteins (12 of 16 jötnarlogs), and *M. sphyraenae* retained the most of any group examined so far, namely 45 of 50 patchy proteins (12 of 16 jötnarlogs). These are in line with the totals that we observe from diplonemids, with *P. papillatum* retaining 42 of 50 patchy proteins (13 of 16 jötnarlogs).

DISCUSSION

In this work, we report that diplonemids encode an extensive set of MTS machinery. This includes the expected complement of conserved trafficking machinery, components that had been previously proposed as kinetoplastid specific, and patchily distributed MTS proteins, both those found in the opisthokont models and jötnarlogs, which are not. Finally, we provide new analyses that confirm and extend reports of similarly extensive MTS complements in other, unrelated free-living heterotrophic flagellates.

There are limitations to our work to be discussed up front. Although we found a large majority of the patchy components in diplonemids, some were nonetheless not identified. Although the possibility for detection failure exists in any given instance, the overall consistency of the results across the diplonemid datasets likely speaks against this as a substantial source of error (we note that the missing TTRAY2 of the TSET complex in *A. motanka*, Rab32B in *L. lanifica*, or Qa SNARE protein Syn17 in *S. specki* likely stem from the incompleteness of the datasets). Indeed, we were able to infer that in all cases of patchy proteins where the component was absent in all diplonemids, the respective losses of the components took place upstream in the larger lineage, whether in the Euglenozoa (ADAP, ArfGAP_C2, Rab8, Rab20, Rab50, and Use1) or even further back in the Discoba (Arf6, Arl17, ACAP, and Rab24) (Figure 7A). Moreover, we examined only a single cellular system—that of membrane trafficking. Although we draw conclusions about ancient eukaryotic evolution and modern cellular models, we acknowledge that our conclusions are based on a small snapshot and should be tested by examinations of other cellular systems. Nonetheless, our data have implications for the three questions raised at the beginning of our work: what is the diplonemid endomembrane complement and how does it compare with that in kinetoplastids, how to interpret the observed patchy proteins across eukaryotes for inferences about the nature of the LECA, and are patchy and jötnarlog proteins exotic but trivial curiosities or core machinery for models of eukaryotic membrane trafficking?

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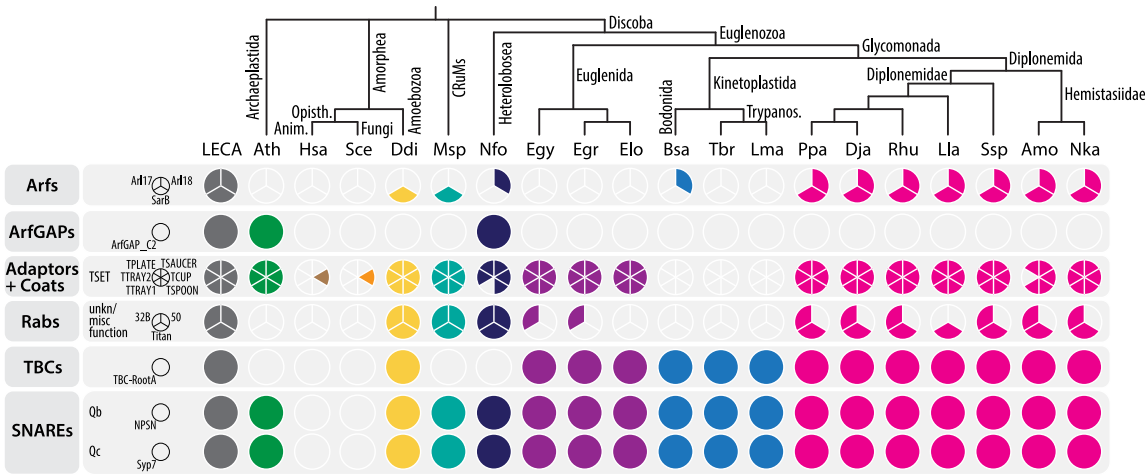

Figure 6. Presence of jötnarlogs of the MTS in selected eukaryotes

Relationships among the eukaryotes are shown by a schematic tree above the figure. Abbreviations as in Figure 1.
See also Figures S1–S5 and S7 and Data S1.

The machinery that we identify as encoded in diplonemids suggests that these organisms possess an elaborate endomembrane system, particularly with endosomal complexity (Figure 7B). This is consistent with the extensive set of endomembrane compartments recently visualized in the free-living *P. papillatum*[63] and *Lacrimia vacuolata*[71] and, at the same time, contrasts with the somewhat simplified and divergent endosomal system in the parasitic *T. brucei*.[72] The path toward parasitism can involve acquisition of traits upstream that lay the groundwork for the shift to a new niche, changes that are concurrent with the transition, and further adaptations that are specialization to specific hosts. The three proteins—previously proposed as kinetoplastid specific—that we identified in diplonemids (i.e., Qa1, TbCAP80, and PH-Arf) represent upstream events to the parasitic shift and could be pre-adaptive (Figure 7A). The loss of components also often accompanies the shift to parasitism, and we identified 10 instances—counting the loss of the TSET and AP-5 complexes as one instance each—that we confidently ascribe as losses in the kinetoplastids. Several of these are inferred as acting in the endosomal system (AP-5, TSET, Vps35L, DSCR3, and C10), others in the further modification of the peroxisome to its specialized function as a glycosome (Dsl1 and Tip20), and others still with untested cell biological function (SarB, Rab32B, and RabTitan). All of these inferences beg testing in the now experimentally tractable model species *P. papillatum*.[14] Examinations of the endomembrane system components in this organism have thus far been restricted to proteins shared by diplonemids and kinetoplastids, revealing unexpected instances of shared complexity as compared with other model organisms.[63] However, the roles of the machinery confined to diplonemids remain to be explored and should help us to better understand these important microbial players in the world's oceans.

The elaborate endomembrane complement found in diplonemids is echoed in that found in the other heterotrophic organisms spanning eukaryotic diversity that we examined as well. This generally speaks against the patchy distribution of MTS proteins being explained by HGT. Although HGT should not be ruled out in cases of sparse taxonomic distribution, the more prevalent the component's distribution, and the more components that are all found together in the same genomes, the more cumbersome an HGT-based explanation becomes. With respect to the inferences of LECA, even if its population did have a pan-genome structure, our data are consistent with the core genome being relatively large and complex, encompassing the machinery for a sophisticated endomembrane capacity. However, the high proportion of patchy proteins (including jötnarlogs) in distantly related organisms, each fairly close to the latest candidate placement for the root of eukaryotes,[73,74] does not support the pan-genomic model (Figure 3C). Rather, it is consistent with a complex MTS in the LECA (and more speculatively to other systems for a complex LECA), sculpted by loss in its descendant specialist lineages. This is bolstered by the many MTS components that are found consistently (i.e., weakly or unsporadically) across eukaryotes. Overall, the LECA likely had a sophisticated set of endomembrane organelles and pathways, including redundant machinery (e.g., AP-2 and TSET).

Finally, with respect to producing generalizable cell biological models of membrane trafficking, the extensive diplonemid endomembrane complement is likely not exceptional but reflective of a norm in free-living microbial eukaryotes. This contrasts with the specialized phototrophic, parasitic, or multicellular organisms that served as major models for eukaryotic cell biology. We argue that diplonemids (and most likely other heterotrophic flagellates) may be more representative model organisms for studies of the MTS than many of our current systems and are eminently tractable.[14] That the MTS complement in protists as abundant and diverse as diplonemids differs so widely from that of the model opisthokonts underlines the importance of including seemingly exotic components such as patchy and jötnarlog proteins when considering the cell biology of non-opisthokont organisms. It also speaks to the potential impact and promise of searching for more such exotic examples in cellular systems outside of membrane trafficking.

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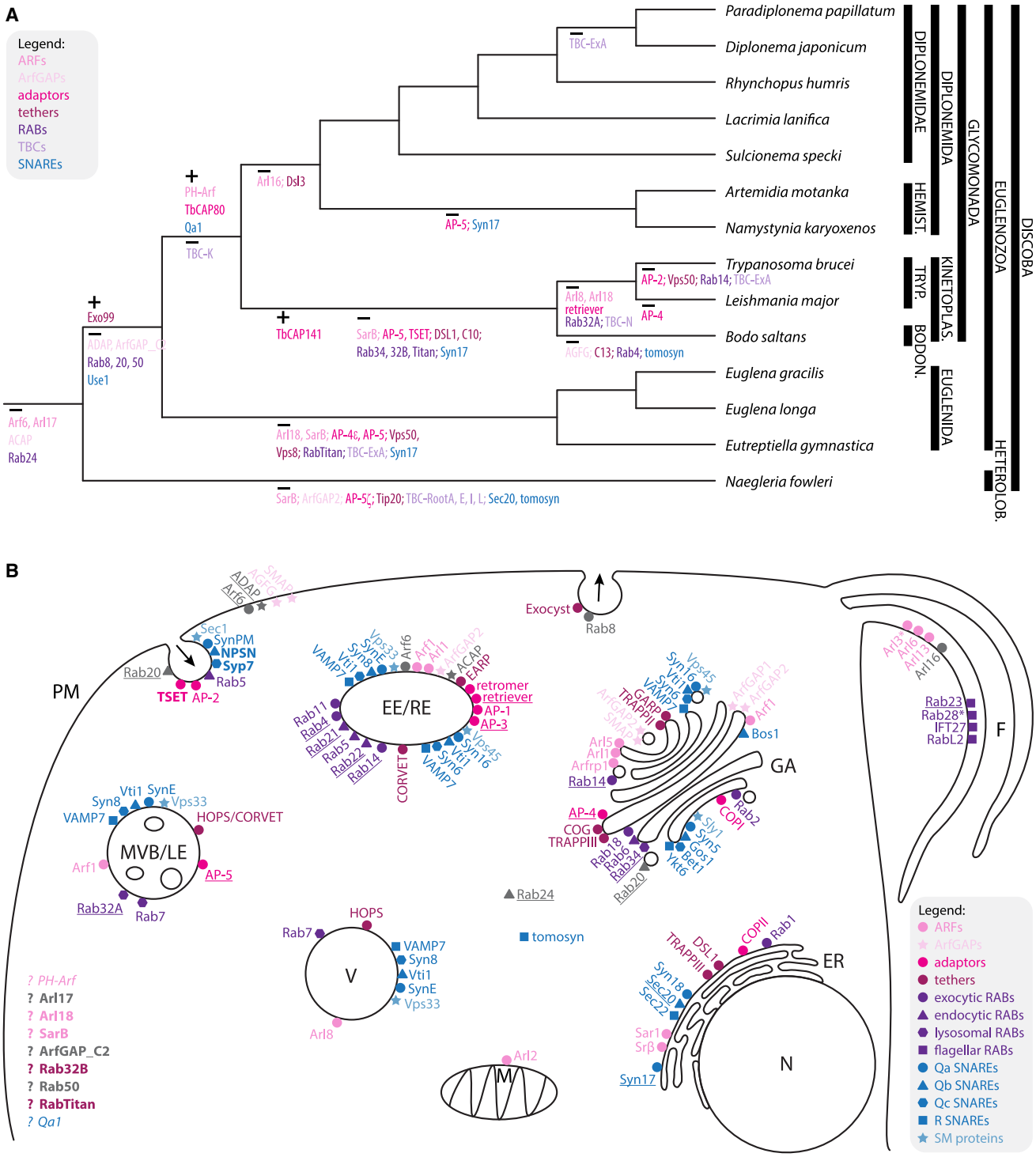

Figure 7. Membrane-trafficking components in Discoba and their evolution

(A) Schematic phylogenetic tree of euglenozoans with mapped gains (+) and losses (−) of the MTS components. Hemist., Hemistasiidae; Tryp., Trypanosomatida, Bodon., Bodonida; Kinetoplas.; Kinetoplastida; Heterolob., Heterolobosea.

(B) Location of membrane-trafficking components in diplonemids. Identified MTS components were mapped onto a schematic diplonemid cell based on data from model organisms. Components with unknown function and localization are marked with a question mark and placed in the periphery of the cell, with the exception of the TBCs, which are not shown here, as the corresponding substrates are unknown in many cases.[54,70] Note that adaptors and tethers are represented by whole complexes, whereas other components are represented by sole proteins. The MTS components are color-coded and types of particular

(legend continued on next page)

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joel B. Dacks (dacks@ualberta.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Profile HMMs, full and trimmed alignments, and phylogenetic trees in Newick format have been deposited at Figshare: https://figshare.com/projects/Membrane-trafficking_system_of_diplonemids/234203.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.L. and J.B.D.; data curation, K.Z.; formal analysis, investigation, and results interpretation, K.Z. and J.B.D.; funding acquisition, J.L. and J.B.D.; project administration, J.B.D.; supervision, J.B.D.; visualization, K.Z.; writing – original draft, K.Z. and J.B.D.; writing – review & editing, all authors. All the authors have seen and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- METHOD DETAILS
  - Homology searches
  - Phylogenetic analyses

SUPPLEMENTAL INFORMATION

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components are in different shapes, as inset. Proteins present in the LECA but not identified in diplonemids are in gray. Patchy proteins, jötnarlogs, and Euglenozoa/Glycomonada-specific proteins are underlined, in bold, and in italics, respectively. EE/RE, early/recycling endosome; ER, endoplasmic reticulum; GA, Golgi apparatus; F, flagella; M, mitochondrion; MVB/LE, multivesicular body/late endosome; N, nucleus; PM, plasma membrane.
See also Figures S1–S6 and Data S1.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
BLAST v2.9.0+	Altschul et al.[75]	RRID: SCR_004870; https://blast.ncbi.nlm.nih.gov/Blast.cgi
HMMER v3.3	Eddy[76]	RRID: SCR_005305; http://hmmer.org
MAFFT v7.458	Katoh and Standley[77]	RRID: SCR_011811; https://mafft.cbrc.jp/alignment/software/
AMOEBAE	Barlow et al.[78]	https://github.com/laelbarlow/amoebae
InterProScan	Jones et al.[79]	RRID: SCR_005829; https://www.ebi.ac.uk/interpro/search/
trimAl v1.4.rev15	Capella-Gutiérrez et al.[80]	RRID: SCR_017334; http://trimal.cgenomics.org/
MrBayes v3.2.7a	Ronquist et al.[81]	RRID: SCR_012067; https://nbisweden.github.io/MrBayes/
RAxML v8.2.8	Stamatakis[82]	RRID: SCR_006086; https://github.com/stamatak/standard-RAxML

METHOD DETAILS

Homology searches

Components of studied machineries were retrieved by BLAST v2.9.0+[75] searches using proteins previously identified in discobans, human, and yeast as query sequences (listed in Tables S1 and S2).

Proteins with clear roles in membrane-trafficking were included as queries. Additionally, a few proteins that are very clearly members of the homologous protein family and have peripheral or poorly documented roles in trafficking to other organelles, most frequently flagella, were also assessed. We did this to rely more heavily on the clear homology of the proteins and to encompass the undercharacterized and interdependent nature of organelle interactions and crosstalk. These were searched against previously published transcriptomes of diplonemids *Paradiplonema papillatum*,[83] *Diplonema japonicum*, *Rhynchopus humris*, *Lacrimia lanifica*, *Sulcionema specki*, *Artemidia motanka*, and *Namystynia karyoxenos*,[15] and euglenids *Euglena gracilis*,[31] *Euglena longa*,[32] and *Eutreptiella gymnastica*[33] (reassembly available at https://doi.org/10.5281/zenodo.257410), and the genomes of the kinetoplastids *Trypanosoma brucei*,[84] *Leishmania major*,[85] and *Bodo saltans*,[86] the discoban *Naegleria fowleri*,[65] the CRuMs protist *Mantamonas sphyraenae*,[64] the amoebozoan *Dictyostelium discoideum*,[87] and the archaeplastid *Arabidopsis thaliana*.[88] Absent genes from the *P. papillatum* and the rest of diplonemids' transcriptomes were confirmed by searches in the recently published high-quality genome[12] and the incomplete genomes produced previously,[15] respectively. Sequences that were found with an E-value threshold 0.005 were used to search the genome of a reference organism to confirm the orthology with an E-value threshold 0.05. More divergent sequences were identified by HMMER v3.3[76] employing profile hidden Markov models (HMMs) for more sensitive searches (E-value threshold 0.001). The profile HMMs were built from datasets of protein sequences identified in previous publications[34,36,57,67,89–91] and by BLAST, namely glycomonads' AP-4ε, diplonemids' SPG11 and SPG15, euglenids' and diplonemids' TSET subunits and Dsl1, and euglenids' Sec39. The sequences were aligned by MAFFT v7.458[77] under L-INS-I strategy and profile HMMs were built. In case of identifying proteins in only a subgroup of euglenozoans, these were added to the alignment to build an additional, subsequent HMM, specifically diplonemids' AP-5 subunits, diplonemids' and euglenids' C10 and Tip20. This updated HMM was then used to search in the remaining subgroups. For HMMs of Rab32B, C12, and C13, euglenozoan-specific profiles were built from protein sequences identified by BLAST searches. The AMOEBAE workflow[78] was also used to identify homologous sequences using default E-value thresholds (forward search: 0.0005, reverse search: 0.05). Protein domains were identified by InterProScan.[79] Classification of SNARE sequences into higher groups (i.e., Qa, Qb, Qc, or R) was done by the SNARE database (http://bioinformatics.mpibpc.mpg.de/snare/snareSubmitSequencePage.jsp).

Phylogenetic analyses

Identified sequences were added to the previously published datasets of Arfs,[25] ArfGAPs,[68,92] adaptor proteins,[57] Dsl1 and Tip20 proteins of the DSL1 complex,[90] Vps3 and Vps39 proteins of the HOPS/CORVET complexes,[93] Rabs,[34,51] TBCs,[34] SNAREs,[34,91] and SM proteins[66] to perform phylogenetic analyses (Figures S2–S7). Either all sequences were aligned by MAFFT v7.458 under L-INS-I strategy or identified sequences were added to the aligned dataset using MAFFT—add option. Poorly aligned positions were removed by

trimAl v1.4.rev15[80] using -gt 0.8. Bayesian inference was done by MrBayes v3.2.7a[81] under a mixed amino acid model, with at least 10 million Markov Chain Monte Carlo generations and 4 gamma rate categories. Sampling frequency was set to every 1,000 generations. The first 25% of the runs were discarded as burn-in. Tree convergence was ensured when average standard deviation of split frequency values fell below 0.01. Maximum likelihood (ML) phylogenetic analyses were done by RAxML v8.2.8[82] using the LG4X model and the number of rapid bootstrap replicates (-f a) determined by the program as necessary for obtaining stable support values (-N autoMRE_IGN). Bootstrap support values were overlaid onto the MrBayes tree topology with posterior probabilities. Final trees were visualized by FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and arbitrarily rooted for visualization purposes.