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# Evolution and Natural History of Membrane Trafficking in Eukaryotes

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The membrane-trafficking system is a defining facet of eukaryotic cells. The best-known organelles and major protein families of this system are largely conserved across the vast diversity of eukaryotes, implying both ancient organization and functional unity. Nonetheless, intriguing variation exists that speaks to the evolutionary forces that have shaped the endomembrane system in eukaryotes and highlights ways in which membrane trafficking in protists differs from that in our well-understood models of mammalian and yeast cells. Both parasites and free-living protists possess specialized trafficking organelles, some lineage specific, others more widely distributed — the evolution and function of these organelles begs exploration. Novel members of protein families are present across eukaryotes but have been lost in humans. These proteins may well hold clues to understanding differences in cellular function in organisms that are of pressing importance for planetary health.

## Introduction

The membrane-trafficking system is both a prominent and essential aspect of nearly all eukaryotic cells. Composed of dynamically interconnected compartments (organelles), this system underpins the export and uptake of extracellular material, remodeling and signaling at the cellular interface, intracellular targeting, and maintenance of internal compartmentalization. In animal cells, this system allows for specialized functions, including neuronal transmission and hormonal control, as well as normal cellular activity. Disruption or dysregulation of the membrane-trafficking system in humans is associated with disease, including neurodegenerative disorders such as Alzheimer's disease [1], as well as cardiac disease and cancer [2]. The membrane-trafficking system is similarly important in other eukaryotic organisms, such as plants and fungi, which have great economic and agricultural significance.

The organelles of the membrane-trafficking system include the endoplasmic reticulum (ER), Golgi apparatus, early and recycling endosomes, multivesicular body (MVB or late endosome), lysosome/vacuole, and plasma membrane. Whether these organelles represent static fixtures or transitional stages in maturing compartments [3], transport of materials between them is largely mediated by vesicle formation from a donor organelle and vesicle fusion at a subsequent acceptor organelle. The basic process of vesicle formation is initiated when a member of the Arf family of small GTPases, primed by its guanine exchange factor (GEF), is relocated from the cytosol to the membrane of the donor organelle and recruits coat-forming proteins to this membrane. This activity is subsequently regulated through the action of a GTPase-activating protein (GAP) (see [4] for more details). Vesicle cargo proteins are bound by various cargo adaptors [5]. Following their formation, vesicles may be transported along cytoskeletal elements by the motor proteins myosin, dynein or kinesin, which interact with peptide motifs within cargo and cargo adaptors (see [6] for a description of recent advances in this area). Inbound vesicles are recognized by tethering proteins extending from the acceptor organelle. The multisubunit tethering complexes form a category of evolutionarily and functionally related tethers, which act with Rab GTPases and their respective GEFs and GAPs to coordinate membrane tethering and fusion at specific locations in the cell via interaction with effector proteins [7]. Once tethered, vesicles are docked and subsequently undergo membrane fusion with the target membrane, mediated by SNARE proteins and their regulators [8].

Much of the above described membrane-trafficking machinery is composed of protein families, with each paralog acting at different locations or pathways within the cell. The initial discovery of this machinery, and much of the functional characterization of the individual paralogs, has taken place in the experimental systems of yeast and mammalian cells. However, fungi and animals represent a small proportion of eukaryotic diversity, while the membrane-trafficking system is a feature of all eukaryotes. How well, and in what ways, does the model of vesicular transport outlined above capture the membrane-trafficking cell biology in organisms from the broader diversity of eukaryotes?

In part, this question has been answered and a great deal has been written emphasizing the conservation and commonalities in trafficking machinery across eukaryotes (for example [9,10]). This review, while acknowledging those commonalities, will first explore some of the differences in membrane-trafficking organelles in the diversity of lineages descended from the last eukaryotic common ancestor (LECA). Many of the known differences have proven functionally important and are often accompanied by the evolution of new members of paralogous membrane-trafficking protein families. Furthermore, in exploring the diversity within the membrane-trafficking system and its evolution

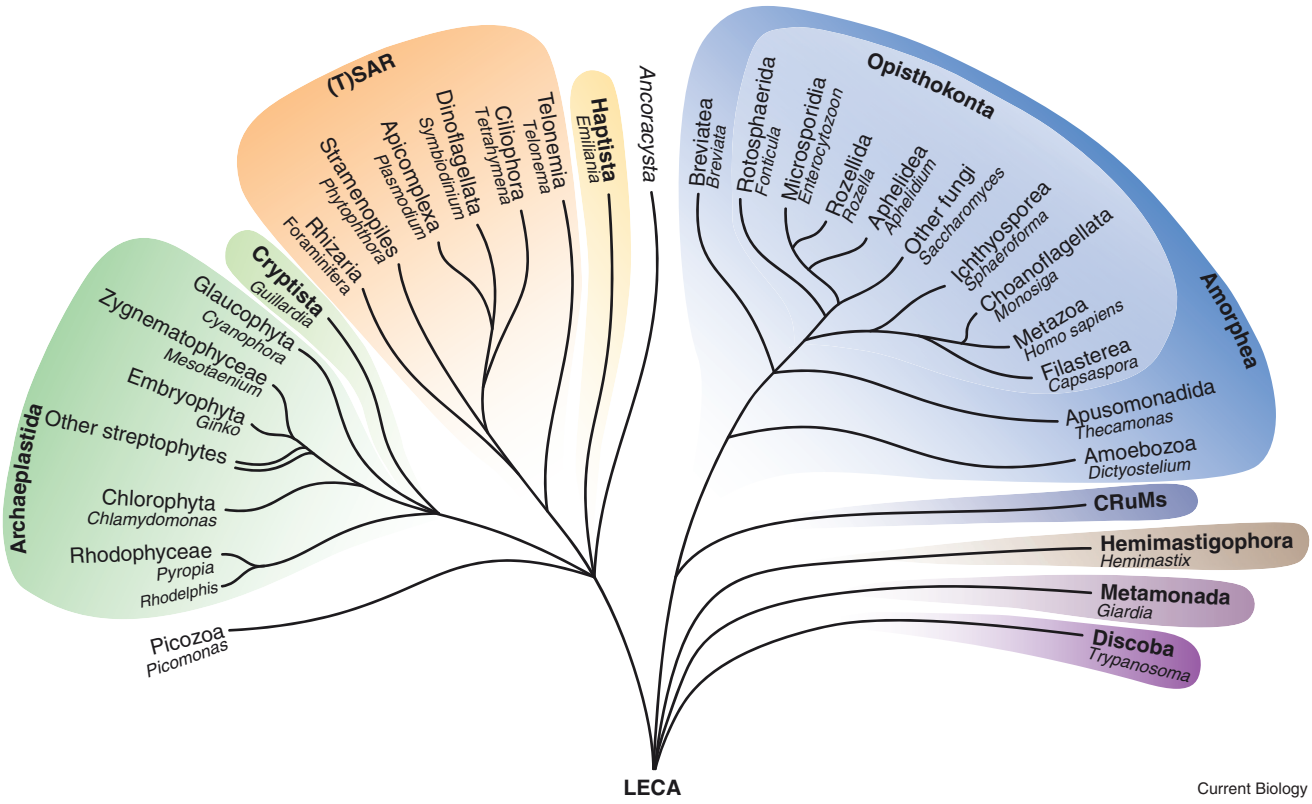


**Figure 1. Current model of the eukaryotic tree of life.**
Our current knowledge of eukaryotic diversity can be summarized in just a few 'supergroups' (in bold). Lineages within Opisthokonta and Chloroplastida are expanded to show the closest unicellular relatives of familiar multicellular organisms. See Box 1 for more information. Tree topology is largely based on [94,95], with expansions in Opisthokonta from [96,97] and Chloroplastida from [98]. Taxonomy is largely from [95]; '(T)SAR' is from [94].

(i.e. the natural history of the membrane-trafficking system) unexpected protein paralogs have been uncovered that will be the subject of the remainder of this article. These organelles and proteins provide great opportunities for acquiring new insights into the fundamental cellular process of membrane trafficking.

## Setting the Stage: Eukaryotic Diversity and Reconstructing the LECA

Exploring a trait across eukaryotes first requires an understanding of eukaryotic diversity. Eukaryotes are currently classified into high-level groupings called 'supergroups' (Figure 1). Animals, Fungi and their diverse single-celled relatives are in the Opisthokonta, which with Amoebozoa forms the supergroup Amorphea. A wide array of other familiar organisms, including plants, algae, and prominent parasites (such as *Plasmodium* and *Phytophthora*), are classified into at least five other supergroups: Archaeplastida, CRuMs (collodictyonids + Rigifilida + *Mantamonas*), (T)SAR (Telonemia, Stramenopiles, Alveolata, and Rhizaria), Cryptista, and Haptista. Two additional supergroups (Discoba and Metamonada) branch deeply and contain notable human parasites (e.g. *Leishmania* and *Giardia*, respectively). Furthermore, exciting organisms are being discovered (e.g. *Ancoracysta* [11]) or sequenced for the first time (e.g. hemimastigophorids [12]) that represent previously unknown deep-branching lineages redefining our understanding of eukaryotic diversity (see Box 1 for a larger discussion). This classification of diversity is almost entirely based on phylogenomic studies that make use of the vast amount of genomic and transcriptomic data that has recently become available from diverse eukaryotic organisms.

Contextualizing the distribution and evolution of protein families also requires knowledge of the protein complement in the LECA. The wealth of sequence data used to frame eukaryotic diversity has also been used to deduce the protein machinery present in the LECA for organelles/systems such as mitochondria [13], transcriptional regulation [14], the nuclear pore [15], the kinetochore [16], and many more. In each case, the over-riding picture has been one of a sophisticated complement of proteins, with extensive molecular machinery for each system, implying the potential for complex cell biology to have been present in this eukaryotic ancestor. However, although the LECA is often discussed as a single cell ("the LECA"), it must certainly have been a population of cells, and so a logical extension of this idea reconstructs a population of highly sophisticated heterotrophic generalists. Intriguingly, it has recently been speculated that the extensive protein complement deduced as being encoded by the LECA genome could be more compatibly explained as being a collection of genes in the genomes of an interbreeding population of organisms whose encoded complements collectively constitute a pan-genome [17].

In the case of the membrane-trafficking machinery, initial broad comparative genomic surveys and subsequent detailed

**Box 1. The vast diversity of eukaryotes.**

The diversity of eukaryotes can mostly be divided into a number of larger 'supergroups' (Figure 1), highlighted in bold in this box. Few of these supergroups have defining morphological characteristics — instead, they have been identified through concatenated phylogenies of hundreds of genes. Most supergroups are diverse assemblages of organisms with huge variation in life history, nutritional method, and body plan (for details, see [95]). The following is a summary of the current supergroups; for a thorough review of the concept, see [94].

Familiar multicellular organisms are closely related to a number of unicellular protistan lineages in the supergroups **Amorphea** and **Archaeplastida**. Within **Amorphea**, animals (Metazoa) are related to several lineages of free-living phagotrophic flagellates and amoebae (Choanoflagellata, Filasterea), as well as parasites (Ichthyosporea). Fungi, also within **Amorphea**, encompasses familiar fungi (i.e. yeast and filamentous fungi) as well as the endoparasites Microsporidia and Rozellida, and parasitoids Aphelidea. Sister to all fungi are the free-living, phagotrophic amoebae Rotosphaerida. Both these larger groups (Holozoa and Nucletmycea) form Opisthokonta, which in turn forms Obazoa with the heterotrophic flagellates Apusomonadida and Breviatea. Amoebozoa is a large group of free-living, heterotrophic amoebae (although it does include the notable human parasite *Entamoeba*), also within **Amorphea**. Land plants (Embryophyta) are found within **Archaeplastida**. Unicellular and multicellular Rhodophyceae (red algae) and Chloroplastida (land plants and green algae), as well as unicellular Glaucophyta, are all within **Archaeplastida**. Some analyses show the supergroup **Cryptista**, which contains phototrophic and phagotrophic flagellates, within **Archaeplastida** as well.

The supergroup **(T)SAR** may make up the majority of eukaryotic species diversity and is an acronym for the lineages it contains: Telonemia, Stramenopiles, Alveolata, and Rhizaria. Telonemia contains a single genus, *Telonema*, which is an abundant predator of other microbial eukaryotes in the ocean. Stramenopiles are heterotrophic or photosynthetic, including oomycete 'fungal' blights that affect many organisms (notably potatoes and frogs), algae that produce a significant amount of atmospheric oxygen, and multicellular seaweeds that form complex ecosystems in the ocean. Alveolata includes Dinoflagellata, Ciliophora, and Apicomplexa, which contain important marine algae, predators of other microbial eukaryotes, and parasites (e.g. *Plasmodium* and *Toxoplasma*). Rhizaria is mostly phagotrophic amoebae and flagellates, such as the shell-forming amoeboid foraminifera that are used as pollution indicator species in many environments. The supergroup **Metamonada** (previously grouped with **Discoba** as the supergroup 'Excavata') contains anaerobic flagellates, including human pathogens (e.g. *Giardia* and *Trichomonas*), free-living heterotrophs, and gut symbionts. **Discoba** contains many ecologically significant phototrophs (e.g. *Euglena*) and heterotrophs (e.g. diplonemids), as well as human pathogens (e.g. *Trypanosoma* and *Naegleria*).

Several new major lineages of eukaryotes have been described in recent years. Some of these are within, or sister to, previously established supergroups (e.g. Telonemia sister to **SAR**, a few heterotrophic lineages within **Cryptista** and **CRuMs**, the phagotrophic *Rhodelphis* sister to Rhodophyceae). Others have been formed from known groups that were previously thought to be unrelated, including the supergroups **CRuMs**, containing heterotrophic flagellates and amoebae, and **Haptista**, containing haptophyte algae and centrohelid amoebae. Others robustly branch external to previously established supergroups, such as **Hemimastigophora**, Picozoa, and *Ancoracysta.*

molecular evolutionary analyses ([13] and references therein) confirmed the universality of the fundamental model of vesicle formation and fusion as described in animal and fungal model systems. In many cases this universality extends to the individual paralogs acting at specific organelles. Functional characterization in model organisms across eukaryotic diversity largely confirms the functional homology of the orthologs, which act at similar locations and perform similar functions in distantly related organisms ([18] and references therein). This finding has two implications. Firstly, the basic model of membrane trafficking holds across eukaryotes, but with interesting exceptions that we explore below. Secondly, because much of the molecular machinery comprises protein families that diversify through gene duplication, and because the different members of the families (i.e. paralogs) act at specific locations or trafficking pathways within the cell, cooperatively encoding specificity, this lends itself to a proposed mechanism of evolution for the membrane-trafficking machinery and the endomembrane system itself. It is postulated that iterative rounds of gene duplication and co-evolution of interacting, specificity-encoding proteins for the endomembrane organelles (e.g. SNAREs, Rabs, vesicle coats, and their regulators, such as GEFs, GAPs, and Sec1/Munc18 (SM) proteins) could have yielded the sophisticated complement that has been deduced as being present in the LECA [19]. Furthermore, theoretically this process of endomembrane organelle and machinery evolution should continue to shape cellular diversity after divergence from the LECA, with new paralogs of trafficking machinery accompanying the emergence of endomembrane organelles in the descendant lineages. Indeed, ample lineage-specific innovations in both organelles and trafficking machinery exist, representing exciting new frontiers for cell biology.

## Lineage-Specific Organelles

### *Parasites and Their Arsenal*

Some of the most illustrative examples of lineage-specific membrane-trafficking organelles and protein expansions come from parasites. Perhaps the best understood are found in the Apicomplexa ((T)SAR; Figure 1), the phylum of unicellular parasites that includes *Plasmodium*, the causative agent of malaria. This phylum derives its name from the apical complex, a cytoskeletal structure underlying the plasma membrane at the apical end of the cell during certain life-cycle stages of many species [20]. The apical complex is thought to act as a gateway for the regulated secretion of apicomplexan-specific organelles that contribute to motility and host cell invasion, micronemes and

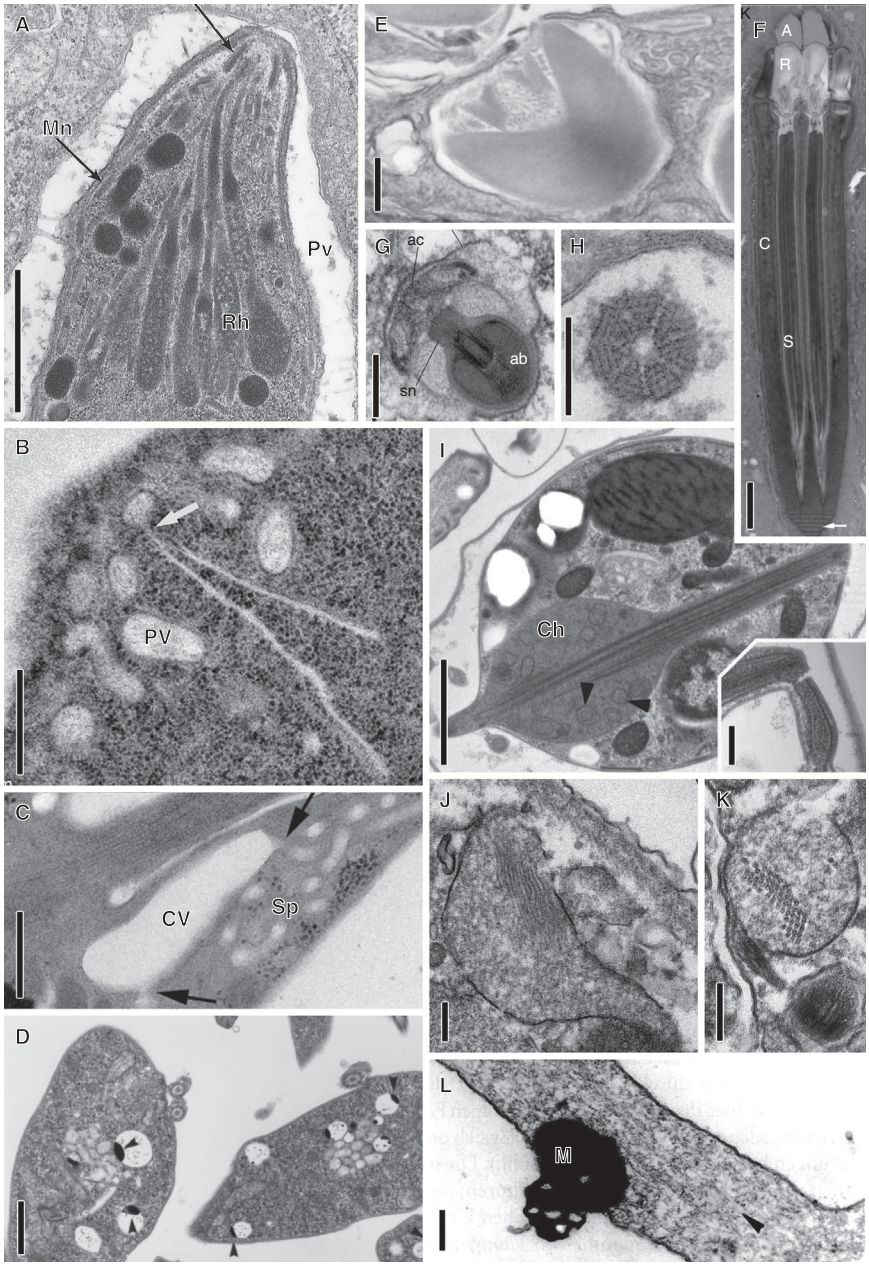


**Figure 2. Transmission electron microscopy of intriguing and/or understudied organelles in protists.**
(A) Rhoptries (Rh) and micronemes (Mn) of *Toxoplasma gondii* (Apicomplexa, (T)SAR) within a parasitophorous vacuole (Pv); reproduced from [23] with permission from American Society for Microbiology. (B) Peripheral vacuoles (PV) of *Giardia lamblia* (Metamonada) showing contiguity with the ER (arrow); reproduced with permission from [99] (CC BY 4.0). (C) Contractile vacuole (CV) and tubular water-gathering canals (spongiome; Sp) of *Trypanosoma cruzi* (Discoba); reprinted by permission from Springer Nature [100] © 2012. (D) Acidocalcisomes (large vesicles containing electron-dense inclusions; arrowheads) of *Trypanosoma brucei* (Discoba); reproduced from [101] with permission from John Wiley and Sons. (E–H) Various extrusomes: (E) ejectosome of *Cryptomonas* sp. (Cryptista), reprinted by permission from Springer Nature [102] © 2017; (F) nematocyst of *Nematodinium* sp. (Dinoflagellata, (T)SAR), reprinted with permission of AAAS from [51]. © The Authors, some rights reserved (CC BY-NC 4.0); (G,H) ancoracyst of *Ancoracysta twista* (orphan lineage), images from [11]. (I) Chromerosome (Ch) of *Chromera velia* (Apicomplexa, (T)SAR) with serial section through the proboscis (inset). Note the multimembranous vesicles (arrowheads) and fibres [54]. (J,K) Telonemosome of *Telonema subtilis* (Telonemia, (T)SAR): (J) in longitudinal section and (K) transverse section. Note the tubular structures within the telonemosome. Images in (I–K) from [55]. (L) Burst membranosome (M) within the filopodium of *Vampyrella lateritia* (Rhizaria, (T)SAR) showing putative membrane loops. Image from the IWF Knowledge and Media gGmbH report associated with [103], provided by Technische Informationsbibliothek (TIB). Scale bars are 100 nm (H), 200 nm (C,E,G,J–L), 500 nm (B,I-inset), 1 μm (A,F,I-main), and 5 μm (D).

rhoptries (Figure 2A) [21,22]. The apical complex is one of the few break points in the cell pellicle, which is formed from the plasma membrane and the underlying network of fused double-membrane-bound compartments termed the inner membrane complex (IMC) [23]. The IMC is thought to be homologous to the flattened membranous sacs (or alveoli) of other alveolates, while micronemes and rhoptries are likely homologous to, and potentially additional derived specializations of, endolysosomes found throughout eukaryotes [24,25].

Importantly, it is hypothesized that the presence of such organelles would likely necessitate expansions of trafficking machinery [18], and at least some examples in the literature support this notion. Some apicomplexans, including *Toxoplasma gondii*, possess three Rab5 paralogs [26], two of which (TgRab5A and TgRab5C) function in trafficking to micronemes and rhoptries [27]. Orthologs of TgRab5C appear to be specific to apicomplexans and dinoflagellates (C.M. Klinger, M. Meissner and J.B. Dacks, unpublished observations), which both possess apical complex trafficking organelles [28]. Furthermore, two Rab11 paralogs (TgRab11A and TgRab11B) traffic to the IMC and/or plasma membrane in *T. gondii* [29,30]. Rab11B orthologs form a clade that is an alveolate-specific expansion, suggesting that it may have arisen concurrently with alveoli [30].

Although their function remains unclear, some paralogs of trafficking machinery are restricted to specific groups including the Apicomplexa. This includes a Rab1 paralog (Rab1A) found only in the SAR clade (plus an ortholog in the cryptistan *Guillardia theta*) [31]. Although one study localized this paralog in *T. gondii* throughout the early and late secretory pathways (through colocalization with diverse markers), no function was determined [27]. Additionally, the Tre-2/Bub2/Cdc16-Sec7 (TBS) protein in alveolates [32] is a fusion between a cytohesin-derived Sec7 Arf GEF domain and TBC-N Rab GAP domain, both of which act at endolysosomes in model systems [33].

Unfortunately, no data have been published regarding either the localization or function of TBS proteins. Apicomplexans possess further unusual endomembrane organelles, including an atypical vacuolar compartment [23,34–36], as well as subpopulations of micronemes [27] and dense granules [37].

Apicomplexans are not alone amongst parasites in possessing unusual membrane-trafficking organelles. The diarrheal pathogen *Giardia* (Metamonada) lacks conventional endosomes or lysosomes, but instead possesses a poorly understood organelle with characteristics of both — the peripheral vacuole (Figure 2B) ([38] and references therein). Even the ER is unconventional in *Giardia*, with the colocalization of both standard ER and non-conventional endocytic markers in the same organelle [39]. Kinetoplastids (Discoba) possess modified endocytic machinery that is integral to their evasion of host immune systems [40]. *Entamoeba* (Amoebozoa, Amorphea) relies on both secretion and phagocytosis for the pathogenesis of amoebic dysentery. It lacks distinctive stacked Golgi bodies, but has a highly expanded paralogous complement of trafficking proteins that is being fruitfully explored (e.g. [41]).

### *Lesser-Known Protistan Endomembrane Organelles*

Though the unusual organelles of parasites are among the best-studied examples, there is a tantalizing array of underexplored membrane-trafficking organelles in protists. The contractile vacuole (CV) is an osmoregulatory organelle (Figure 2C), reported in most freshwater lineages across eukaryotes [42]. CVs are rarely described in closely related marine or parasitic taxa, though the CVs of marine ciliates and some parasitic trypanosomatids (Discoba) are notable exceptions [43]. CVs are best studied in four distantly related model protists: *Dictyostelium discoideum* (Amoebozoa, Amorphea), *Paramecium tetraurelia* (Ciliophora, (T)SAR), *Chlamydomonas reinhardtii* (Chlorophyta, Archaeplastida), and *Trypanosoma cruzi* (Discoba). Molecular and cell biological analyses have identified roles in CV function for various membrane-trafficking components. Often, these are paralogs associated with endolysosomal compartments in other organisms, such as Rab11, and most recently Vps8 in the ciliate *Tetrahymena* [44,45]. Vacuolar ATPases, water-transporting aquaporins, and calcium-binding proteins such as calmodulin have also been implicated [45]. The acidocalcisome (Figure 2D) is a calcium and pyrophosphate storage organelle present in diverse free-living and parasitic eukaryotes [46]. Though acidocalcisomes are comparatively poorly characterized, there are intriguing similarities in the organellar reaction to pyrophosphate between acidocalcisomes and well-explored compartments such as the yeast vacuole [47]. Notably, the acidocalcisome is one of the targets of miltefosine [48], a drug commonly used to treat leishmaniasis, trypanosomiasis, and primary amoebic meningoencephalitis.

Finally, extrusomes (Figure 2E,F) are a common and understudied component of the organellar arsenal of free-living protists. Though extrusomes have a diversity of names depending on the lineage in which they are found and their function (which includes hunting, defense, encystation, host cell invasion and more [49,50]), they share a common function of extruding substances into the environment. The harpoon-like nematocysts of some dinoflagellates ((T)SAR) are a particularly impressive example [51], with transmission electron microscopy revealing a complex organization belying their "ballistic" nature (Figure 2F). However, due to the tractability of molecular cell biological analyses, our molecular understanding of extrusomes is much better developed in another alveolate lineage, Ciliophora ((T)SAR). Endolysosomal trafficking machinery, such as SNAREs and adaptor protein (AP) complexes [52], as well as ciliate-specific expansions of subunits from the multisubunit tethering complex class C core vacuole/endosome transport (CORVET) [53] are involved in extrusome (mucocyst) biogenesis in the ciliate *Tetrahymena* and more recently have been associated with several other of its endolysosomal compartments [44]. This overlapping protein complement has led to the hypothesis that extrusomes are homologous to lysosome-related organelles of other eukaryotes, including animals [52].

In each of the cases above, some degree of molecular or detailed microscopic investigation has been undertaken. However, there are many organelles that have scarcely been studied beyond their initial ultrastructural description. The ancoracyst of the orphan taxon *Ancoracysta twista* (a heptaradially symmetrical structure; Figure 2G,H) may well be an extrusome, on the basis of ultrastructural similarities to those organelles as well as an image of a structure interpreted as an expulsed ancoracyst [11]. Less clear are functions of organelles including: the chromerosome of *Chromera velia* (Apicomplexa, (T)SAR), which is a compartment that grows to span the entire cell length and contains a striated fibrous structure and multimembranous vesicles (Figure 2I); the telonemosome of *Telonema subtilis* (Telonemia, (T)SAR), a membrane-bound organelle containing thin, bundled tubules (Figure 2J,K); and the membranosomes of vampyrellid amoebae (Rhizaria, (T)SAR) [54–56]. These last organelles (Figure 2L) are spherical granules (0.5–1.5 μm) that are rapidly shuttled along the filose pseudopodia of *Vampyrella lateritia* [57]. Due to the loops that form when these organelles are burst (Figure 2L), it has been suggested that they act as membrane reserves [56], but they may be extrusive organelles involved in encystation or predation, or have another function altogether.

Some of the underexplored membrane-trafficking organelles of protists appear to be lineage-specific derivations of canonical (possibly endolysosomal) organelles. These organelles, best described in apicomplexans and ciliates, also appear to have been accompanied by novel paralogs of membrane-trafficking machinery. Other organelles, like the CV and acidocalcisome, appear to be widespread in eukaryotes, but are somewhat obscured from our attention, being frequently not observed in our best-studied experimental models. There are echoes of this phenomenon at the molecular level as well.

## The Evolution of Membrane-Trafficking Protein Paralogs

### *Wide Taxonomic Distribution but Sparsely Present: A 'Patchy' Distribution*

In addition to the conserved core set of trafficking machinery and lineage-specific expansions, a third evolutionary pattern emerged from molecular evolutionary analyses: proteins that were both broadly distributed and frequently lost or unidentified, i.e. a patchy distribution. One interpretation of the many observed proteins with such a distribution is that the LECA population had a highly complex — and potentially redundant — complement of membrane-trafficking machinery. This complexity could then have been reduced upon subsequent lineage-specific specialization [9]. The recent pan-genome

interpretation of the LECA also addresses the patchy distribution of cellular machinery [17], particularly for paralogs that are variably present at deep nodes in the eukaryotic tree. Given this, the relative roles of loss of machinery versus distribution by paralog sorting from a pan-genome warrant exploration on a case-by-case basis as taxonomic sampling gaps are filled in by genome sequencing.

Examples of membrane-trafficking proteins with a patchy taxonomic distribution can be found among nearly all of the main vesicle-trafficking protein families described. These patchily distributed proteins are frequently paralogs from protein families where highly conserved LECA paralogs and lineage-specific expansions are also reported (Table 1). An example of particular medical relevance is the AP5 complex, where components were first identified in a high-throughput patient screen for the neurodegenerative disorder hereditary spastic paraplegia [58]. Mutations in the subsequently described AP5 pathway have recently been proposed as defining a novel category of lysosomal storage disorders [59]. Characterization in HeLa cells showed that AP5-coated vesicles act at a previously unknown retrieval pathway from the late endosome to the *trans*-Golgi network (TGN) [60]. The AP5 complex is found in humans, plants and a variety of prominent parasites, but is also missing in a wide variety of eukaryotic lineages [61]. Other recent examples of complexes or components with a patchy distribution include the WDR11 complex. Composed of WDR11, FAM91A1 and C17orf75, the WDR11 complex captures endosome-derived AP1-coated vesicles and facilitates their delivery to the TGN [62]. The complex is widely distributed across eukaryotes but is missing from fungi, red algae, stramenopiles and alveolates [62]. Another example is TSSC1, a protein that interacts with the GARP (Golgi-associated retrograde protein) and EARP (endosome-associated recycling protein) complexes and is required for the fusion of endosome-derived vesicles with the TGN and recycling endosomes, respectively. TSSC1 is found in humans and in representatives of the major supergroups, although has not been identified in *Saccharomyces cerevisiae* and the parasites *Giardia lamblia* (Metamonada) and *Entamoeba histolytica* (Amoebozoa) [63]. Finally, C10orf76 is a recently described protein that binds to the GEF GBF1 and plays a role in Golgi maintenance. It is found in humans, as well as all supergroups sampled, but was not identified in several key model organisms, including *S. cerevisiae*, *Arabidopsis thaliana*, and *Caenorhabditis elegans* [64].

### Enter the Jotnar

In the above cases, human proteins provided insights into membrane trafficking in the rest of the eukaryotes. But what about instances where this cannot possibly be true? One of the more surprising findings to come from exploring the natural history of the membrane-trafficking system across eukaryotes has been the identification of paralogs that are broadly distributed, but either lost or obscured by change in our own lineage. At least ten membrane-trafficking proteins have this basic evolutionary distribution and there is every reason to think that the molecular machinery of other cellular systems will show this phenomenon as well, as illustrated by the discovery of bacterial-like division machinery composed of FtsZ and Min proteins that mediate mitochondrial division in some eukaryotes [65]. Proteins with this distribution will provide insight into how various eukaryotic lineages have changed from the LECA and possibly inform our general models of eukaryotic cell biology. Therefore, in order to facilitate future discussion, we are hereby coining a term for proteins with this evolutionary distribution. A 'jotnarlog' is a homolog with the evolutionary pattern of being ancient but hidden from view, as with the dark or walled-off ancient world of Jotunheim in Norse mythology. Specifically, genes are jotnarlogs if found in sufficiently diverse eukaryotic taxonomic supergroups to infer a common origin concurrent with or pre-dating the LECA, but were hidden from previous cell biological investigation due to loss or divergence in yeast and animal model systems. This may, but does not necessarily, reflect absence in Opisthokonta as a lineage.

The existence of jotnarlogs is a reminder that the evolutionary processes that drive change, and possibly loss, in other lineages shape our cells as well. Moreover, it suggests that, despite the overall commonalities of protein components and function, differences do exist between current models of membrane trafficking based on animals and fungi and those that are generalizable to the entirety of eukaryotes.

### Plants and Trypanosomes as Models for Understanding Membrane Trafficking

Plant cell biology models, particularly *Arabidopsis*, have brought to light several broadly distributed proteins or complexes not found in animals or fungi. The TPLATE protein, known since 2006 for its role during cytokinesis in membrane trafficking at the cell plate [66], was initially thought to be a plant-specific paralog of an AP or coat protein I (COPI) beta subunit. However, elegant and convincing work in 2014 elucidated the entire TPLATE complex (TPC) in *Arabidopsis* [67], acting in endocytosis. Very shortly afterwards, the TPC was shown to be the embryophyte version of what was, in fact, a pan-eukaryotic trafficking complex [68] and was given the overarching name TSET (Figure 3A). This complex was also shown to act at the cell surface, likely in endocytosis, in *Dictyostelium* (Amoebozoa). The only component of TSET that remains in opisthokonts is the medium subunit (in vertebrates termed the muniscin), which has been elaborated upon with an amino-terminal F-BAR membrane curvature domain and acts as a monomeric clathrin adaptor. While the TPC in plants is emerging as a regulator of membrane-localized cellulose synthases through clathrin-mediated endocytosis [69], TSET remains to be integrated into a general model of endocytosis.

Echoing this narrative of jotnarlogs identified in plants are the SNARE proteins NPSN and Syp7. Proteins of the SNARE superfamily are essential for mediating membrane fusion [8]. Each SNARE contributes one domain to a heterotetrameric coiled-coil bundle, which comprises an R, Qa, Qb, and Qc SNARE domain. Early characterization demonstrated that SNARE proteins are a common feature of the eukaryotic membrane-trafficking machinery [70–72], with detailed work from 2006 to 2009 establishing hypotheses for which organelle-specific R, Qa, Qb, and Qc-SNARE family members, known from standard model organisms, were likely to be present in the LECA (Table 1) [73–76]. Given their absence from human and yeast genomes, the initial suggestion was that NPSN and Syp7 were plant specific, as their names imply (novel plant syntaxin and syntaxin of plants 7, respectively). However, as early as 2006, comparative genomics based on similarity-searching methods identified

**Table 1. Evolutionary distribution of membrane-trafficking components in the last common eukaryotic ancestor (LECA) and the lineages leading to animals.**

| Protein family | Role | LECA | Amorphea | Opisthokonta or Obazoa[a] | Holozoa[b] | Metazoa[c] | References |
|---|---|---|---|---|---|---|---|
| ArfGAPs | Recruitment of Arf GTPases to initiate GTP hydrolysis and terminate Arf activity | SMAP, ArfGAP1, ArfGAP2/3, ACAP, AGFG, ***ArfGAPC2*** | AGAP | ASAP | ADAP, GIT, ARAP | | [87] |
| Adaptor proteins | Vesicle adaptor coats at various intracellular locations | AP1, AP2, *AP3*, *AP4*, *AP5*, **TSET** complexes | | GGA | | Stonin | [61,68,106–110] |
| SNAREs | Recognition and fusion between membrane-bound compartments | Bet1/Stf1, Bos1, Gos1, **NPSN**, *Rd*, *Sec20*, Sec22, SNAP25, **Syp7**, Syx5, Syx6, Syx8, Syx16, *Syx17*, Syx18, SynE, SynPM, *Use1*, *VAMP7*, Vti1, Ykt6 | VAMP4 | SNAP23 | | VAMP1, 2, 3, 8, VAMP5/6, Vti1a | [73,75–77,111] |
| TBCs | Rab-binding domains, often (but not exclusively) in RabGAPs | TBC-B, TBC-D, TBC-E, TBC-F, TBC-G[d], *TBC-I*, TBC-K, *TBC-L*, TBC-M, *TBC-N*, TBC-Q, ***TBC-RootA*** | TBC-A, TBC-O, TBC-P, TBC-T | TBC-R | TBC-S, TBC-X | TBC-J, TBC-V | [104] |
| Rab GTPases | Regulation and binding of effectors | Rab1, Rab2, Rab4, Rab5, Rab6, Rab7, Rab8, Rab11, *Rab14*, Rab18, *Rab20*, *Rab21*, *Rab22*, *Rab23*, Rab28, *Rab32A*, ***Rab32B/7L***, *Rab34*, ***Rab50/X1***, *RabL4/IFT27*, ***RabTitan*** | Rab24, Rab29[e] | | Rab9 | Rab3, Rab10, Rab15, Rab19, Rab26, Rab27, Rab30, Rab33, Rab35, Rab37, Rab39, Rab40, Rab43, Rab44, Rab45, RabX4, RabX6, RabZ3 | [89–91] |

Patchily distributed proteins (here defined as at least three losses in two supergroups, where a loss is defined as at least two absences in the genomes of closely related species) are in italics. Jotnarlogs are in bold.
[a]Opisthokonta + Apusomonadida + Breviatea.
[b]Metazoa + Choanoflagellata + Filasterea + Ichthyosporea. Due to availability of genomic information, for many of the earlier papers this was limited to Metazoa + Choanoflagellata or Metazoa + Choanoflagellata + Filasterea.
[c]See relevant citations for vertebrate-specific expansions.
[d]Depending on the root of the tree.
[e]Additionally found in *Naegleria gruberi* (Discoba) in Klöpper *et al*. [90].

putative orthologs of both the Qb-SNARE NPSN and the Qc-SNARE Syp7 in protist genomes, including those of *D. discoideum* (Amoebozoa) and *Batrachochytrium dendrobatidis* (basal Fungi) [73,75,76]. Comprehensive phylogenetic analysis of an updated genome set confirmed and extended the conclusion that orthologs of both NPSN and Syp7 are, in fact, present in the majority of extant eukaryotes (Figure 3B), despite having been lost independently in animals and fungi [77]. In plants, these proteins play roles in exocytosis, promoting vesicle fusion at the cell surface [78]. However, there has been little analysis outside the plant system, with a single characterization of a Syp7 ortholog in the pathogenic protist *Trypanosoma brucei* (Discoba) that suggested a juxtanuclear localization [79].

Another instance where the trypanosome model system has been insightful is in the characterization of a Tre-Bub-Cdc16 (TBC) domain-containing protein. The TBC family proteins are the major GAPs of the Rab GTPases. A pan-eukaryotic analysis of TBC proteins revealed at least 10 paralogs present in the LECA (Table 1), as well as expansions at various levels of taxonomic diversity. Animals, and the supergroup Amorphea in general, showed the most novel paralogs (Table 1), but plant-specific paralogs were also found. Notably, there was at least one previously unknown paralog, TBC-RootA, identified in members of Discoba, (T)SAR, and Amoebozoa, but not in members of Opisthokonta and Archaeplastida sampled in this analysis (Figure 3C). In *T. brucei*, this protein was identified in an interactome with the mRNA export factor Mex67 and has been proposed to be the GAP for the nuclear transport GTPase Ran [80]. Although here TBC-RootA is involved in nuclear transport, and not membrane trafficking *per se*, there is a well-established evolutionary connection between proteins associated with the nuclear pore complex and membrane trafficking [81]. Nonetheless, as the authors point out, trypanosomes have highly unusual mechanisms for gene expression and mRNA export that differ from animals, yeast and plants [80]. It is therefore unclear whether a role for TBC-RootA at the nuclear pore complex is

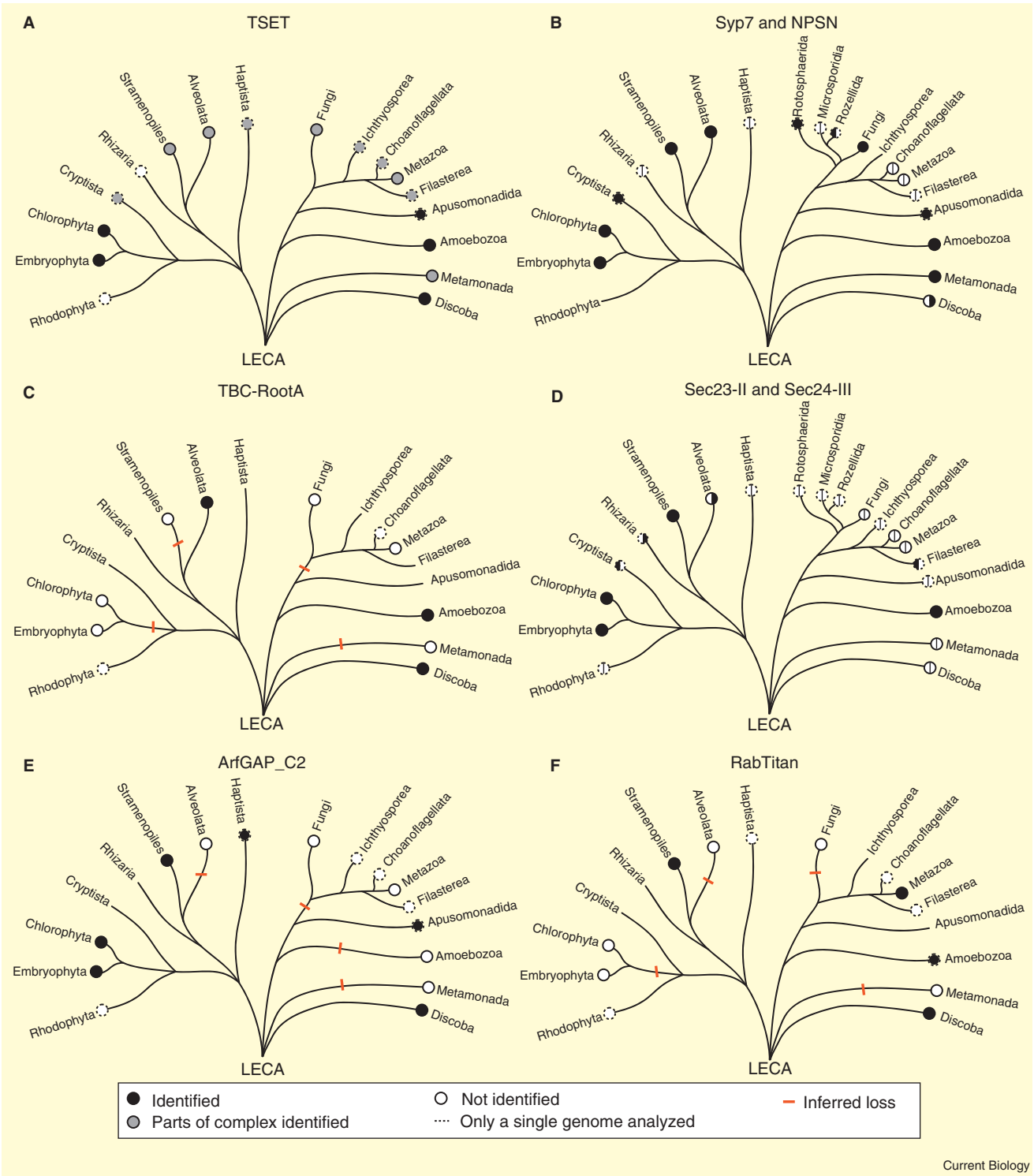


**Figure 3. Membrane-trafficking jotnarlogs.**
Many membrane-trafficking protein families contain uncharacterized paralogs that are absent from traditional model organisms yet present in diverse eukaryotes, including: (A) TSET, an adaptor protein complex; (B) the SNAREs Syp7 and NSPN (in the left and right halves of the circles, respectively); (C) TBC-RootA; (D) the COPII components Sec23-II and Sec24-III (in the left and right halves of the circles, respectively); (E) ArfGAP_C2; and (F) RabTitan. Inferred losses (red bars in C–E) are based on the absence of a protein in two or more genomes within a lineage. N.B. Presence in a lineage does not mean that every organism in the lineage has the protein. For example, RabTitan is found in some metazoans but has not been identified in the genomes of *C. elegans*, *Drosophila melanogaster*, or *Homo sapiens*. Distributions are based on the following: RabTitan [91], TSET [68], TBC-RootA [104], NPSN and Syp7 [77], ArfGAP C2 [87], Sec23-II and Sec24-III [86], and updated with [105] for Metamonada and [88] for Discoba.

unique to kinetoplastids or indicative of a more widely conserved role. Enticingly, however, in a recent high-throughput CRISPR screen of *Toxoplasma gondii* [82], the TBC-RootA gene (TGGT1_226550) had a phenotypic score of –4.82, suggesting that the gene is very much on the essential side. Whatever TBC-RootA is doing in this globally prevalent parasite, this function clearly cannot be compensated for by any of *Toxoplasma*'s other 17 TBC proteins.

### *Paralogs from the Unknown*

The cases described above involve proteins that have been well characterized in at least one model system — just not in opisthokonts. However, comparative approaches have also yielded examples for which almost no functional information exists.

The COPII coat is involved in vesicle formation for transport from the ER to the *cis*-Golgi [83]. It is composed of seven subunits, with Sar1 serving as the nucleating GTPase, and Sec23 and Sec12 serving as the GAP and GEF proteins, respectively. Sec16 serves as a regulator and a scaffold recruiting other coat members [84], while Sec24 acts as a cargo adaptor protein, and Sec13 and Sec31 form the vesicle cage. Sec13, Sec23, Sec24, Sec31 and Sar1 are all highly conserved across eukaryotes, while Sec12 and Sec16 have a much patchier distribution [85]. Notably, however, there are paralogs of both Sec23 and Sec24 that have a jotnarlog distribution. While the initial paper reporting Sec23-II [86] was circumspect about declaring a new paralog in the LECA, a well-supported clade does contain representatives from the Opisthokonta, Amoebozoa, (T)SAR, and Archaeplastida, though we recognize the possibility that this is due to long branch attraction. The Sec 24-III paralog is slightly less broadly distributed but is supported by more comprehensive phylogenetic analysis (Figure 3D).

ArfGAP-domain-containing proteins allow for GTP hydrolysis in the Arf GTPases, which have no inherent GTPase activity of their own. Of the ten known human ArfGAP families, at least five were identified as being present in the LECA (Table 1) in a pan-eukaryotic assessment of ArfGAP evolution [87]. In this same analysis, a jotnarlog (ArfGAP_C2) was identified as an eleventh member of the ArfGAP family and a sixth LECA paralog. Found quite sparsely in the initial study, it was nonetheless identified in *A. thaliana* and the model moss *Physcomitrella patens* (Archaeplastida), the plant pathogen oomycete *Phytophthora sojae* ((T)SAR), *Emiliania huxleyi* (Haptista), and *Thecamonas trahens* (Amorphea). Additionally, an ArfGAP_C2 homolog has been identified in *Naegleria* (Discoba) [88], bringing its distribution to at least five supergroups (Figure 3E). With a C2 lipid-binding domain carboxy-terminal to the main ArfGAP activity domain, and conservation of 17 out of the 18 residues involved in ArfGAP activity, there is good reason to think that the protein is functional. The variable expression of ArfGAP_C2 genes in *Arabidopsis* tissues under different nitrogen sources, at the very least, confirms its biological relevance (AT4G21160.4, AT4G05330.1 and AT3G07940.1; https://phytozome.jgi.doe.gov/pz/portal.html).

Finally, there are several examples of Rab GTPase jotnarlogs. Analyses in 2011 and 2012 examined the pan-eukaryotic evolution of the Rab GTPases by phylogenetic and similarity-searching methods [89–91]. Estimates from these analyses converged on between 15 and 22 Rab paralogs in the LECA, depending on the method and whether some families such as RabL2/RTW and RabL4/IFT27 are included within the Rab family. Nonetheless, all three analyses agreed that the LECA Rab complement was strikingly complex and larger than that of several of the notable extant model organisms; some paralogs were found to be widely distributed and frequently present, while others have a much more patchy taxonomic distribution (Table 1). All three analyses also noted lineage-specific expansions in the line leading to metazoans (Table 1), more so than other major taxonomic groups (discussed thoroughly in [89,90]). While extensive overall elaboration was not observed in other lineages, the reduction of the Rab complement in the archaeplastids and then subsequent expansion of remaining Rabs in embryophyte plants is well described and remarkable [92]. The Rabs reconstructed as present in the LECA include three jotnarlogs that are absent from humans and yeast as well as *Arabidopsis*: Rab32B/7L, Rab50/X1, and RabTitan. Rab32B, though also identified as Rab7L, is robustly resolved as a paralog from an ancient gene duplication in the Rab32 family, while Rab50 is a member of a deep clade of Rabs with endocytic function [91]. Their relationships to functionally characterized Rabs provide starting hypotheses to test for potential cellular functions. RabTitan is present in at least four supergroups, spanning the diversity of eukaryotes (Figure 3F). In addition to the Rab domain, RabTitan also possesses a carboxy-terminal extension, often with an SH2 domain [91], and has been confirmed as being differentially expressed under varying conditions in *D. discoideum* (http://dictybase.org).

## Onward

While molecular cell biological and molecular evolutionary studies have drawn an increasingly detailed picture of how the membrane-trafficking system functions and evolves, there are still surprises to be had and proteins and organelles that beg exploration. This exploration is best achieved by molecular evolutionary biologists and molecular cell biologists working together to explore the natural history of the membrane-trafficking system. Such an approach is now more feasible than ever, thanks to the rapid development of tractable genetic tools in a wide diversity of organisms [93].

Understanding the factors described already, particularly those that are absent from our currently well-characterized model organisms, is important for comprehending the evolutionary patterns and processes that have resulted in extant cellular diversity. Moreover, generating accurate and complete models of the cell biology underlying trafficking is also of pressing importance. Such studies will result in a deeper understanding of organisms that drive ecosystem-level processes in our oceans and of parasites that are of increasing global health relevance as humanity tackles the challenges of climate change and disease.

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