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Current Opinion in Cell Biology

# First and last ancestors: reconstructing evolution of the endomembrane system with ESCRTs, vesicle coat proteins, and nuclear pore complexes

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The eukaryotic endomembrane system is responsible for the biosynthesis and transport of proteins and lipids, and for the definition of the major subcellular compartments. Recent work indicates that the endomembrane system is ancient, with near modern complexity predating the radiation of the major eukaryotic lineages. The challenge is to look beyond the last eukaryotic common ancestor and to attempt to deduce the evolutionary steps in the rise of membrane-trafficking complexity. Relationships between the endomembrane coatomer complexes and their evolutionary connection to the nuclear pore complex are emerging. These studies, plus the realization of a role for the ESCRT complex as an alternate, but equally ancient, system for membrane deformation are providing insight into the earliest stages of endomembrane evolution.

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**Current Opinion in Cell Biology** 2009, **21**:4–13

This review comes from a themed issue on
Cell structure and dynamics
Edited by Daniel J. Lew and Michael Rout

Available online 7th February 2009

0955-0674/$ – see front matter

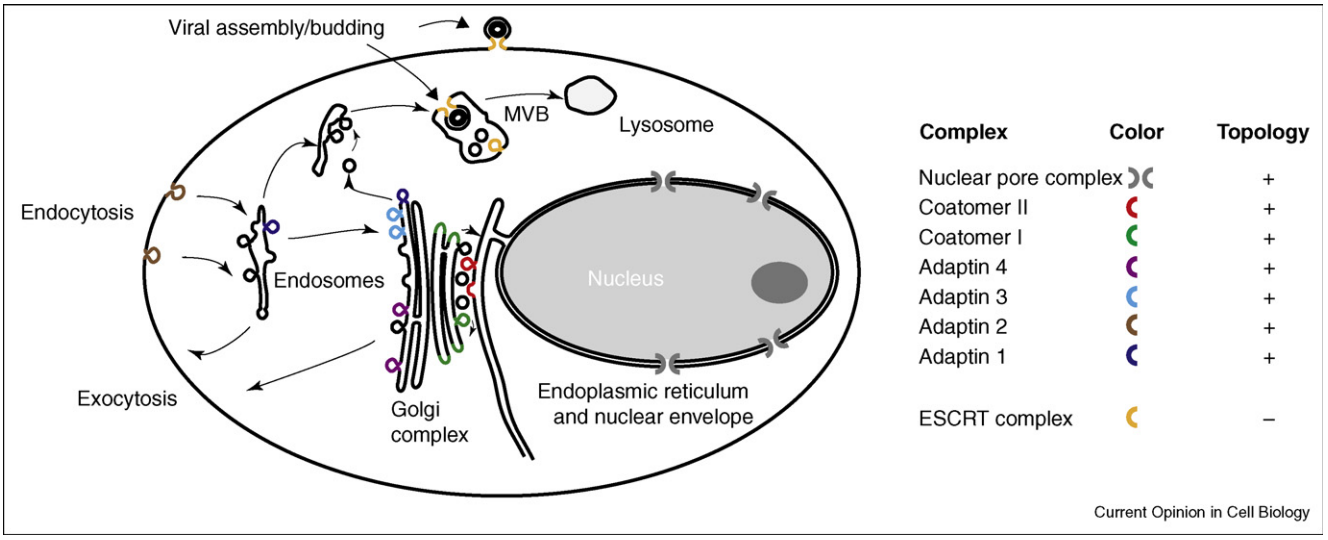

**DOI** 10.1016/j.ceb.2008.12.004

## Introduction

The defining feature of the eukaryotic cell is the presence of a nucleus, but in addition, virtually all nucleated cells also possess an endomembrane system. These organelles are dynamically connected via a series of selective vesicular transport steps that transfer proteins and lipids from one compartment to another (Figure 1). This process allows a specifically directed flow of material between compartments, but also maintains the distinct compositions of the organelles. A remarkable level of specificity is encoded within the vesicular transport system, requiring concerted action by SNARE proteins, Rab GTPases, and tethering complexes, as well as participation of the cytoskeleton and many other factors [1]. Comparative genomic and phylogenetic analyses have revealed a great deal of conservation in the machinery of membrane trafficking, not only from yeast to man, but in the broad diversity of eukaryotes. These studies have suggested that the last eukaryotic common ancestor (LECA, a hypothetical lineage that gave rise to all modern eukaryote supergroups) possessed a complex membrane-trafficking system, with a near modern array of organelles and representatives of almost all of the major protein families [2–4]. Furthermore, much of the machinery involved in vesicle-trafficking steps is composed of paralogous protein families but with organelle-specific family members [5]. This insight prompted the development of a model for organelle evolution whereby an initial endomembrane compartment in the first eukaryotic common ancestor (FECA, a hypothetical organism that was the first true eukaryotic, i.e. nucleated cell and was a direct descendent of a prokaryote and predates LECA) gave rise, via iterations of gene duplication and coevolution of organelle identity-/specificity-encoding machinery, first to basic functional division and eventually to the diversity of endomembrane organelles observed in modern eukaryotes [6•]. Specifically, several gene families are associated with multiple vesicle transport steps and constitute a specificity module governing docking of transport intermediates with target membranes or organelles. The evidence for this model, particularly with regard to the specificity machinery encoded by Rabs, SNAREs, and tethers have all been dealt with elsewhere [6•].

Multiple mechanisms for the deformation of biological membranes by protein factors are known. For example, *in vitro* studies have demonstrated that single proteins, such as dynamin-like GTPases and proteins possessing BAR domains are capable of constricting synthetic lipid membranes or tubes effecting a membrane scission event [7,8]. However, what these minimal systems obviously lack is the ability to select cargo, and further, in the cellular context, membrane scission clearly requires participation of multiple factors. The two major systems that do appear to dominate endomembrane trafficking at the cellular level are the clathrin/coatomer vesicle coats, and the endosomal sorting complex required for transport or ESCRT. Critically, these factors exhibit distinct subcellular localizations, and all data are broadly consistent with participation in only select, *albeit* multiple pathways. Here we will discuss these two cellular systems for membrane deformation and what their evolutionary past

**Figure 1**




Vesicle coats in the eukaryotic cell. Stylized eukaryotic cell focused on the nucleus and major endomembrane compartments. Locations of the major coatomer complexes, adaptins (APs), and nuclear pore complex (NPC) are indicated by colored semicircles. MVB, multivesicular body; is the major location for the ESCRT system.

suggests about the history of membrane-trafficking organelles.

The major questions concerning the evolution of coatomer and ESCRT systems are fundamental. Firstly, how have these factors arisen and expanded to their modern complexity? Secondly, what was the original function of the vesicle coats and their protein components? In terms of membrane transport there are two competing views; that the nuclear membrane was the original membrane system, or that phagocytosis, and hence endocytosis, was the first membrane transport event [9,10]. Thirdly, at what point in eukaryotic evolution did these systems arise? The answer to this last issue is critical. Specifically, how broadly can mechanisms described in one lineage be applied across the eukaryotes, and what is the level of variation between membrane-trafficking systems at the deepest evolutionary levels? Recent improvements in genomic sampling of eukaryotic diversity and maturation of cell biology in several critical divergent organisms have contributed to a major advance in understanding the evolution of membrane trafficking.

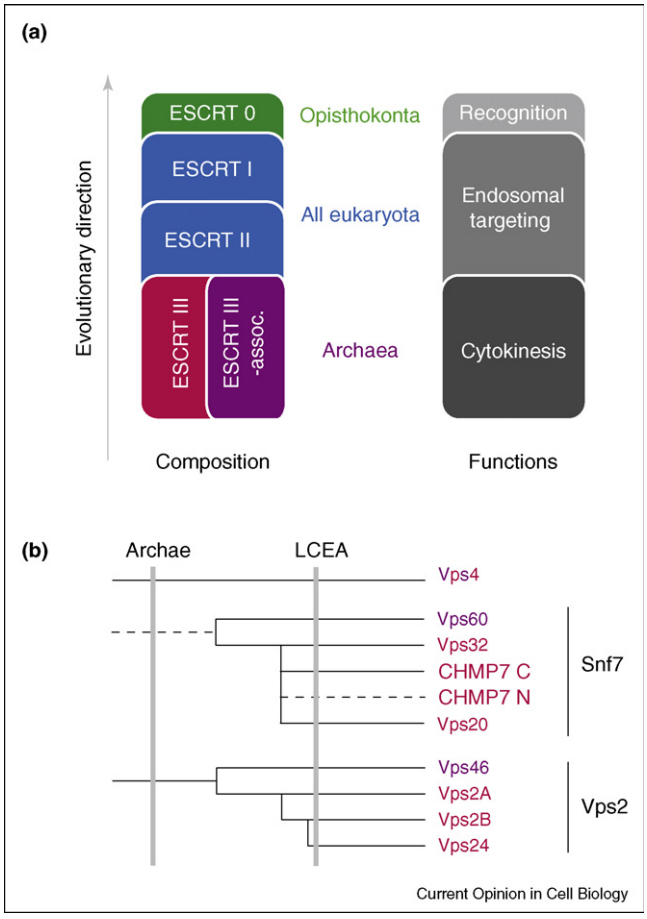
## The ESCRT complexes: ancient systems for cytokinesis and endosomal sorting

Modification of surface receptors and other molecules by ubiquitin conjugation is an important endocytic mechanism [11]. Ubiquitylated molecules are delivered to the multivesicular body (MVB), a late endocytic compartment functioning before lysosomal degradation. This pathway is responsible for receptor downregulation, antigen presentation, and is exploited by several viruses for assembly [12]. Critically, inward budding of membrane creates vesicles contained within the endosomal membrane [13] and requires participation of the ESCRT complex (8). The ESCRT system consists of 5 subcomplexes, confusingly numbered 0, I, II, III, and III-associated, and together these comprise over 20 distinct polypeptides. With one major exception, these factors are well conserved across the eukaryotic lineage [14••], indicating an origin predating the LECA (Figure 2). Most striking, ESCRT 0, which functions in recognition of ubiquitylated cargo, is opisthokont-specific, suggesting both a distinct recognition mechanism is present in the majority of eukaryotes and a later origin for this subcomplex. Clues to the manner in which the ESCRT system arose during eukaryotic evolution have been gained from several sources.

Firstly, most data suggest a modular ESCRT system architecture. Initial membrane targeting is achieved through recognition of endosomal phosphatidylinositol 3-phosphate (PI3P) and ubiquitin on the cargo polypeptides by Vps27p/Hrs, an ESCRT 0 subunit [15,16]. ESCRT 0 then recruits ESCRT I to endosomal compartments [15]. Subsequent binding of ESCRT complexes II, III, and III-associated is required for the formation of inward-budding membranes. Finally the ESCRT complexes are disassembled by the AAA-ATPase Vps4 [16]. Vps4 is also critical in the generation of inward-budding membrane structures.

Secondly, minimal ESCRT function, specifically membrane deformation, can be achieved by the III and III-associated complexes alone. Although the precise mech-

**Figure 2**




From cell division to garbage disposal; evolution of the ESCRT system. Panel **(a)** evolutionary and functional context of ESCRT subcomplexes. Left schema: ESCRT III and III-associated appear very ancient and are common with the Archaea, while ESCRT I and II (blue) are shared by all eukaryotic lineages and therefore were present in the LECA. ESCRT 0 is specific to opisthokont taxa. Right schema: functions of ESCRT complexes. The earliest and most primitive function appears to be in cytokinesis, and evidence that this is retained between the eukaryotes and Archaea is now strong. ESCRT I and II are likely mainly involved in endocytic activities, suggesting that evolution of this complex co-opted the earlier ESCRT III system to a new role. Finally, the emergence of the ESCRT 0 complex may be important for increased levels of cargo selection or recognition; this final contention is not yet supported by experimental data. Arrow indicates evolutionary direction. Panel **(b)** evolutionary relationships between subunits of the ESCRT III and III-associated subcomplexes, noting the probable archaeal origin for the AAA-ATPase Vps4 and the Vps2 subfamily. Dotted line for the Snf7 family indicates uncertainty and no archeal homolog has been identified to date, and dotted line for CHMP7 N-terminal domain indicates uncertainty. Subunits are color-coded for ESCRT III in magenta and ESCRT III-associated in purple.

anism of membrane deformation is unknown, multiple copies of ESCRT III and III-associated factors can form lattices on the endosomal membrane [16]. Overexpression of ESCRT III factors Snf7/CHMP4 and Vps4 in mammalian tissue culture cells lead to protrusions of the plasma membrane [17•]. This functionality is further supported by recent studies describing self-assembly *in vitro* into helical structures of ESCRT III proteins Vps2/CHMP2A, and Vps24/CHMP3, which may be disassembled by Vps4 [18]. Furthermore, ESCRT subcomplexes I and II are absent from multiple apicomplexan lineages [14••], but the expression of an ATPase-inactive Vps4 mutant in the apicomplexan *Toxoplasma gondii* results in MVB-like structures in the cytoplasm located close to early endosomes, consistent with the minimal ESCRT III and III-associated complexes in this organism being capable of biosynthesis of MVB-like structures and critically, membrane invagination [19]. This model of a principle, membrane-deforming role for Vps2, Vps24, and Vps4 is considerably strengthened by recent evidence for both Vps4-like and Vps2-like family homologues in Archaea, consistent with an ancestral ESCRT function arising from these subunits, and remarkably, predating eukaryogenesis [20••]. To date, no additional archaeal ESCRT factors have been identified.

Thirdly, the very recent discovery that ESCRT factors are associated with the mammalian midbody during cytokinesis has profound implications. Specifically, the ESCRT I (Vps23/Tsg101) and ESCRT III factors (Vps2/CHMP2, / Snf7/CHMP4, Vps60/CHMP5), Alix and Vps4 have all been localized to the midbody, in addition to their classical endosomal locations. Significantly, interactions with known midbody/cytokinesis factors have also been detected, strengthening the model that midbody localizations of ESCRT factors is functionally relevant [21•,22••]. Further, dominant-negative Vps4 mutants inhibit the abscission step associated with the final steps of cytokinesis, suggesting a critical role for the ATPase in cell division. Most provocatively, recent evidence suggests that, in Archaea, participation of the Vps2/Vps4-related factors in cytokinesis is a conserved function. In contrast there is unlikely to be a role in MVB trafficking as a classical endocytosis system is absent from these organisms [23]. These observations raise the exciting possibility that the original function of the ESCRT system has been glimpsed, allowing us to reconstruct evolution of both MVB biogenesis and cytokinesis beyond FECA and LECA.

The most parsimonious model for ESCRT evolution is therefore that a primordial complex comprised a single Snf7-like and Vps2-related ancestral factor, with the latter at least being derived from an archaeal protein that is involved in cell division. Because several ESCRT III and III-associated subunits are related, and comprise two families of proteins defined by the Snf7 and Vps2 domains (Figure 2) [11,15], duplications of the ancestral Snf7 and Vps2 genes allowed functional diversification into ESCRT III and III-associated complexes. This occurred before LECA [14••]. The similarity in size and coiled-coil architecture of the Snf7 and Vps2 families may also

suggest a so far undetected common ancestry for these factors. Additional duplications of Snf7 genes gave rise to Vps32 and Vps20 and two duplications producing first Vps2A and then Vps2B and Vps24 [14••]. Subsequent recruitment of ESCRT I and II subunits, predating the LECA, produced the general ESCRT system, with ESCRT 0 arising later in a lineage-specific manner. Critically, despite participation in endocytic trafficking, it appears that an ancestral role in cytokinesis has been retained, at least by some eukaryotic lineages. Nonetheless, the topology of ESCRT complex membrane deformation is positive, and production of cytosolic vesicles requires negative membrane curvature. A different system, the coatomer, which is able to deform membranes with just such topology, dominates the endomembrane system.

## Coatomers and adaptins

Multiple distinct vesicle coats are required for the movement of material between the compartments of the endomembrane system (Figure 1). Coatomer complex two (COPII)-coated vesicles mediate anteriograde movement from the ER to the *cis*-Golgi, while COPI-coated-vesicles mediate transport of material in the opposite direction as well as movement of material through the Golgi body [5]. The clathrin/adaptin (AP) coats mediate transport in the endocytic and late secretory system, with AP 1 involved in *trans*-Golgi network (TGN) to endosomal trafficking, AP2 in endocytosis at the cell surface, AP3 involved primarily in endosomal transport, and AP4 involved in the movement of material from the TGN to the plasma membrane [24]. AP1 and AP3 are also thought to be involved in endosomal to TGN transport [24]. Retromer is a multisubunit complex participating in retrograde transport and containing sorting nexins that bind PI3P-rich membranes and three Vps proteins that recognize cargo. AP1, AP3, and retromer all play similar roles, *albeit* each trafficking rather discrete cargo [25]. In all cases, these vesicle coats are heteromeric complexes composed of at least five different proteins. Comparative genomics, as well as functional studies, has demonstrated the presence of the key components for each vesicle coat in a wide variety of eukaryotes [2,3,26,27]. This finding implies, with reasonable confidence, that these vesicle coats were each already present by the time of the LECA (Figure 3).

Despite their involvement in highly distinct trafficking pathways, these complexes of the coatomer class share a common mechanism of vesicle formation. While the details of retromer mechanism are less clear, for the COPI, COPII, and clathrin/AP coats, a small GTPase of the Arf/Sar family binds to the membrane of the organelle from which transport will take place, initiating the vesicle budding process. Both guanine-exchange factors (GEFs) and GTPase activating proteins (GAPs) facilitate the GTP cycle of Arf/Sar and serve to target the GTPase to the correct compartments. Cargo adaptors select proteins for transport via recognition of specific amino acid motifs and concentrate them in the vicinity of the burgeoning vesicle. Membrane deformation and scission then take place with the aid of additional coat proteins [5,28]. The vesicle then buds away and migrates to its target organelle. This commonality of mechanism could simply be the product of biophysical constraints on vesicle formation, that is there is only one way to make a vesicle. However, the alternate mechanism utilized by ESCRTs in MVB formation argues against that model. Instead, mechanistic similarity is the first piece of evidence implying homology between these seemingly disparate vesicle coats.

The second form of evidence comes directly from shared components and detectable sequence homology. Both the clathrin/AP and COPI coats employ an Arf protein as the nucleating GTPase [5]. COPII uses Sar1, a GTPase most closely related to Arf. Both are the products of a gene duplication predating the LECA [9]. Clathrin and components of both the COPI and COPII complexes all possess WD40 beta-propeller domains detectable by homology-searching algorithms [3]. More compellingly, however, the cargo adaptor subcomplex of COPI (F-COP) and the APs are all clearly related at the primary sequence level [29]. The APs and F-COP are heterotetrameric complexes of two large, one medium, and one small subunit. Phylogenetic analysis of large and medium subunits has demonstrated that almost all of the gene duplications giving rise to the various complexes predate the LECA [30•,31].

The final evidence for homology comes through tertiary structural studies. The large subunit components of the clathrin/AP, COPI, and COPII coats are composed of either alpha-solenoid, or beta-propellor domains, or a combination of both [32•]. Vps35, a major component of retromer, also possesses an alpha-solenoid structure [33•]. As all of these components are involved in either membrane deformation or cargo selection, the common structure suggests homology beyond that detectable at primary structure level. This homology also appears to extend beyond the membrane-trafficking system in an intriguing way.

Specifically the alpha-solenoid, beta-propellor domain architecture is seen in at least two additional transport systems. Several components of intraflagellar transport (IFT), the IFT proteins, contain a beta-propellor domain at the N-terminus, followed by a Tpr helix–turn–helix configuration, related to the alpha-solenoid [34]; there is also some rather weak sequence homology between several IFT subunits and coatomer components, while many IFT proteins themselves appear to have been present in the LECA [35]. Mechanistically IFT is related to vesicle transport and requires participation of IFT27, a Rab-like small GTPase [36]. However, IFT itself is not a membrane-deforming event and IFT particles appear to move

along the pre-existing cilium or flagellar membrane. The evidence for structural similarity to coatomer is indirect and *in silico*, but highly intriguing. The second example, the nuclear pore complex, is supported by stronger data at this time, and is described in more detail below.

## The nuclear pore complex

Regardless of the scope of membrane trafficking in the LECA, eukaryotes are nucleated by definition. In modern eukaryotes a double-membrane nuclear envelope (NE) functions as a barrier between the nucleoplasm and the cytoplasm. The NE is punctured by nuclear pores traversing both membranes which are occupied by the nuclear pore complex (NPC). This ~50 MDa structure mediates exchange between nucleoplasmic and cytoplasmic compartments. Morphologically, NPCs consist of a core containing eight spokes joined by rings and surrounding a central transport orifice. The NPC composition of ~30 nucleoporins (NUPs) has been well defined for two opisthokonts, *Saccharomyces cerevisiae* [37] and *Rattus norvegicus* [38,39].

Attempts to determine the NPC composition of additional species by *in silico* methods have been only partially successful. An early attempt concluded that the conserved core of the NPC was likely very limited, although a later study suggested a more extensive, but still restricted, level of conservation [40,41]. This would imply that the majority of NUPs likely arose after eukaryotic radiation and are lineage specific. However, an extreme level of divergence among even established NUP orthologs suggests that many factors have not been identified. One class of scaffold NUPs, constituting the bulk of NPC mass, form the NPC central tube. Most relevant here is the recognition that these scaffold NUPs contain only two definable tertiary folds, alpha-solenoid and beta-propeller domains [32•], an architecture remarkably similar to a subset of coatomer factors with precisely this arrangement; common ancestry is now suspected [42•,43•]. Further, the beta-propeller protein, Sec13, is a constituent of both the NPC and COPII.

Nonetheless, the addition of an NPC composition from a divergent organism is vital for establishing the state of the NPC in the LECA, and has recently been achieved by deGrasse *et al*. by directly identifying candidate NUPs in the excavate *Trypanosoma brucei*. Combined proteomics, *in silico* structure prediction and localization identified the majority of trypanosome NUPs (JA DeGrasse, KN DuBois, D Devos, N Siegel, A Sali, MC Field, MP Rout, BT Chait, The establishment of nuclear pore complex architecture occurred early in evolution, unpublished data). Significantly, the trypanosome NPC shares a remarkable level of architectural and compositional similarity with the opisthokonts. While primary structures are indeed divergent, eukaryotes have retained a restricted NUP fold architecture, particularly within the scaffold group. Concordance between number, molecular weight, and domain architecture of trypanosome and opisthokont scaffold NUPs is apparent. Given the evolutionary distance between these lineages, this evidence supports structural conservation of an NPC core scaffold, arguing for the establishment of the majority of the NPC before the LECA.

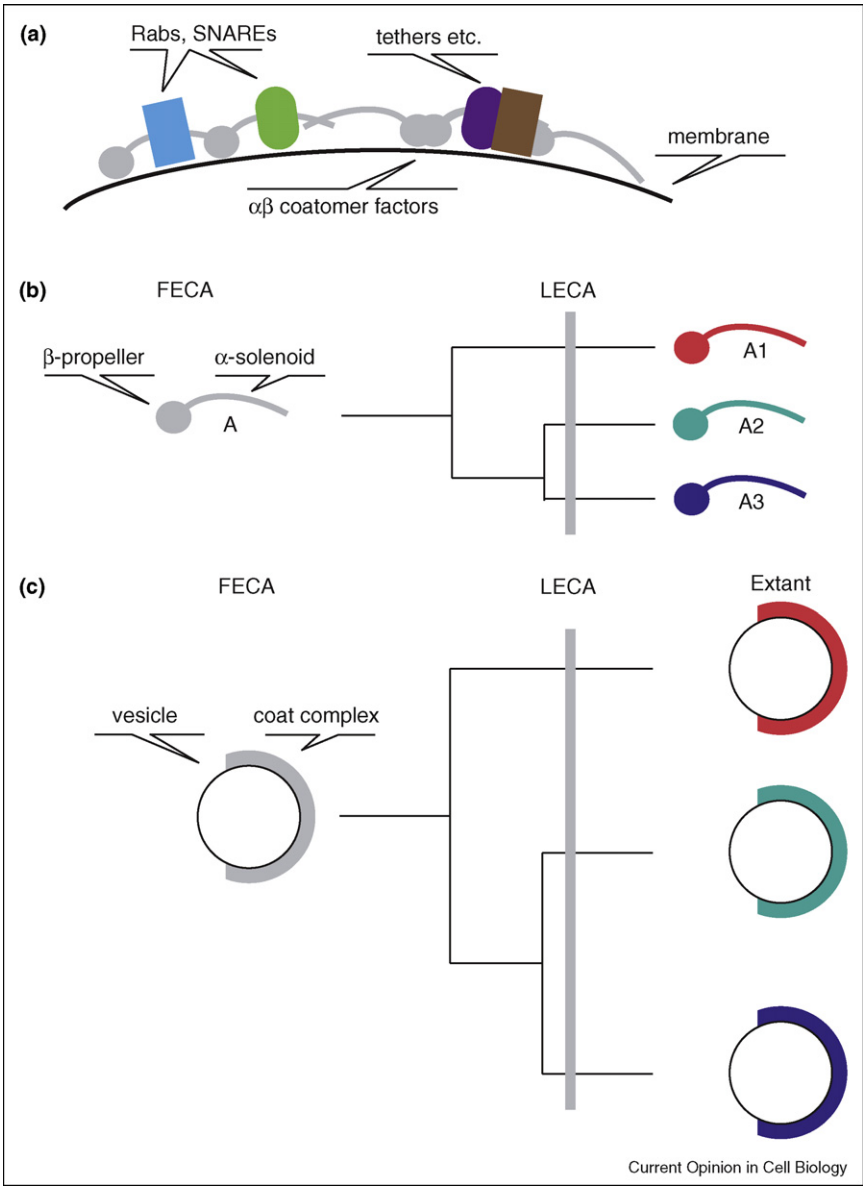
Without a complete survey from a divergent organism, the formal possibility that morphological similarities in NPC structure arose by convergent evolution, while unlikely, could not be discounted. Further, given structural relationships between opisthokont scaffold NUPs and coatomer subunits, the timing of divergence of NUPs and vesicle coats was unclear. Convergence can now be robustly rejected as a credible evolutionary pathway for these complexes, and data support a model for a common origin from a complex NPC, followed by extensive divergent evolution. It follows that the LECA likely possessed an NPC structurally analogous to the contemporary NPCs found in extant taxa (Figures 3 and 4).

## From first to last eukaryotic common ancestors

The idea of a protocoatomer that evolved to give rise to the NPC, COPs, clathrin/AP and perhaps even retromer raises an interesting opportunity to look at evolution before the LECA. If the relationships between these coats can be deduced, then the corresponding relationships of their associated organelles may also be uncovered. Shared factors between coats are one way of establishing relationships. The model for organelle evolution by gene duplication and coevolution of specificity machinery provides another tractable method of investigation, by phylogenetic analysis. While the order of divergence of the protocoatomer families has not been established with confidence, some intermediate progress has been achieved. Striking sequence similarity between the F-COP and AP subunits suggests their closer homology relative to the other coats, yet phylogenetic analysis demonstrates clear separation between coatomer and AP clades. It is clear that AP1 and AP2 are most recent relatives with the beta-subunit being common to both complexes in LECA, and in many extant taxa; subsequent duplications producing AP1B and AP2B were the result of parallel evolution [30•]. Analyses of SNAREs, Rabs, and endocytic cargo adaptors are also consistent with this pattern [30•,44]. Although all steps in the transition from a single to multiple endomembrane compartments have not been resolved, several interesting features have emerged (Figure 4a):

First, as the NPC and COPII complexes share Sec13, and are located on a contiguous endomembrane compartment, these two complexes likely share close common ancestry. On the basis of current functions and location, their evolution would have been associated with differentiation in machinery and specialization of function into biosynthetic

**Figure 3**



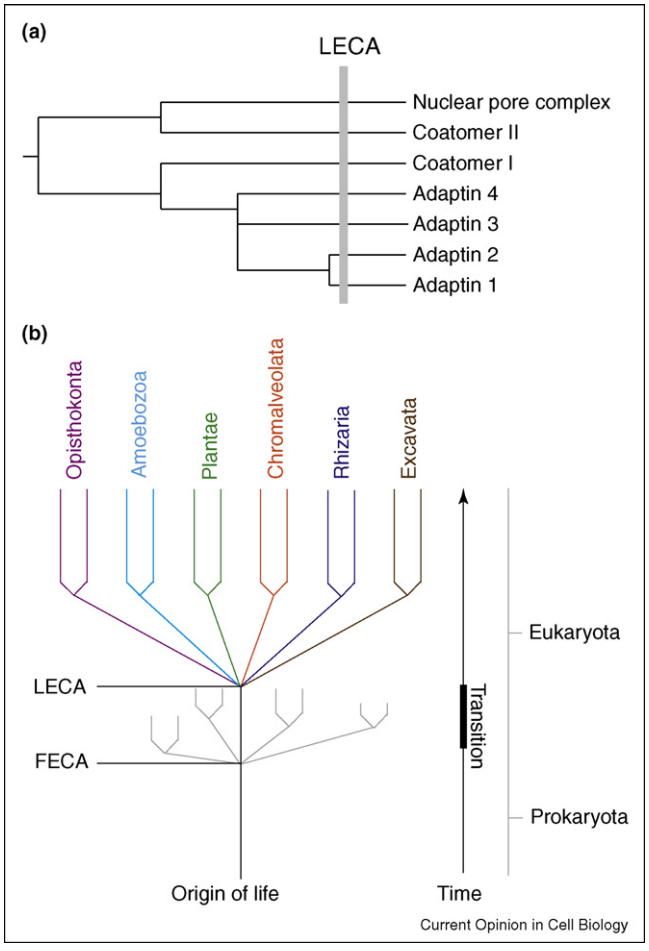

Paralogous expansion and evolution of coat systems of many colors. Panel **(a)** coatomers, nuclear pore complexes (NPCs) and clathrin coat systems all contain proteins with a beta-propeller/alpha-solenoid architecture (in gray), and which likely represents basic building blocks for at least one class of membrane deformation complexes. Specificity, cargo selection, and interaction with other cellular systems in modern cells is in part provided by the presence of additional proteins within the coatomer complex (colored blocks; Rabs, SNAREs, tether factors, and additional proteins). However, this potentially modular configuration allows a route to the evolution of additional coats through paralogous duplication of the alpha/beta factors [Panel **(b)**]. Accumulation of mutations by the resulting paralogs facilitates interaction and recruitment with a distinct set of additional factors, leading to functional differentiation and ultimately the generation of distinct vesicle and/or organellar structures [Panel **(c)**]. Importantly, as coatomer, the NPC, and clathrin/AP systems are widely distributed across the eukaryotes, the expansion of the vesicle coats, and hence the alpha/beta factors that define them, occurred before the LECA (gray bars). We assume that as endomembrane systems, and hence vesicle coats, appear restricted to the eukaryotes, that the alpha/beta coat configuration must have arisen in the FECA, and was elaborated during the transition period (see Figure 4).

export and gated transport across the NE from an undifferentiated NE/endoplasmic reticulum organelle.

Second, COPI and clathrin/AP, which both utilize Arf as their GTPase and comprise components most recently derived from an ancestral subcomplex, that is F-COP and AP, must also share close common ancestry. On the basis of associations of these complexes with *cis*/intra-Golgi transport and TGN function, respectively, we speculate that this set of gene duplications was associated with differentiation of function of a primordial Golgi complex into *cis*-faces and *trans*-faces.

Third, AP1 and AP2 are closest relatives and were only partially differentiated in the LECA. Evolution of AP1 and AP2 may have been associated with recruitment of AP2 to endocytosis, because AP1, AP3, and AP4 are involved, at least to some degree, with trafficking at the TGN.

**Figure 4**




FECA to LECA and modern eukaryotic diversity: a highly stylized tree of life. Panel **(a)** likely evolutionary relationships between APs, vesicle coats and the NPC. Note an alternate topology, whereby the NPC diverged from the other complexes first, rather than being monophyletic with coatomer II, is also possible. It is essential to recognize that only a minority of subunits within these complexes likely share a common evolutionary origin. Panel **(b)** extant lineages are represented as the major supergroups, and in color. These all originated from the last common eukaryotic ancestor (LECA), which is now widely acknowledged to have been a sophisticated organism, and which surprisingly possessed many of the modern cellular-trafficking systems, and more importantly, the basic core in its entirety. The presence of a simpler cellular state, representing the first nucleated cell, but lacking many of the LECA's additional systems, is presumed here to have been the first common eukaryotic ancestor (FECA) and was a direct product of a prokaryotic lineage. Critically a transition period between FECA and LECA would presumably have given rise to many lineages (gray) that are not represented among modern eukaryotes. The chronological time between LECA and FECA may have been very brief or more protracted. Importantly, this view implies a single lineage breaking through to the next level of sophistication, reminiscent of the 'hopeful monster' paradigm, or an extreme case of punctuated equilibrium [51–53].

Significantly this differentiation preceded elaboration of additional endocytic pathways as only the clathrin-dependent route appears present in the LECA [44].

There remains much more to learn from the evolution of protocoatomer families. Relative relationships between the coats described above with each other and to retromer are unclear. The point at which the ESCRTs were incorporated is not known, but the restricted role of ESCRTs compared to the dominant roles of the protocoatomer system potentially suggests that ESCRTs came later and hence may have been recruited to the late endosomal system following establishment of the basic pathway.

Most critically, these new data do not yet allow resolution of the contentious issue of the order of acquisition of phagocytosis and the NE in the protoeukaryote; attempts at using molecular phylogenetics to resolve this point have provided no resolution (Dacks and Field unpublished). Nonetheless, it does establish a model for further investigation, and places the debate in a molecular context. Investigations aimed at resolving the order of the other molecular complexes encoding organelle identity/vesicle trafficking specificity, that is SNAREs, GAPs, and Rabs, will certainly help in the larger search for endomembrane origin and evolution.

Major systems for membrane deformation in a cellular context now appear restricted to either the protocoatomer or ESCRT systems. These systems were both fully present in the LECA and, because they do not appear to share common ancestry, the ability to produce membrane vesicles must have arisen on two separate occasions, from independent sources.

## Challenges

Our understanding of eukaryotic diversity has been revolutionized by genome sequencing, structural biology, increased activity in empirical analysis of divergent model organisms, and improved computation. These have facilitated reconstruction of the configurations of membrane-trafficking systems across eukaryotes and the realization that major systems, including NPCs, a diversified endocytic pathway, the Golgi apparatus, MVBs, and the vesicle coat complexes were already present in the LECA. This conceptual organism was remarkably sophisticated, leading to suggestions of a revolutionary jump accompanying eukaryogenesis. This model requires coevolution of multiple systems and poses several critical questions.

Firstly, how much of the system is truly eukaryote-specific? Previously essentially all membrane-trafficking factors were viewed as unique to eukaryotes, but the clear recognition of dynamin [45], some ESCRT, and retromer components [20••,46] suggests prokaryotic ancestors. It remains to be seen as to how extensive prokaryotic origins may be, but it can be anticipated that even more detailed

pre-LECA models for the origins of membrane trafficking will be possible in the future.

Secondly, the origins of specificity remain unaddressed. Although the manner in which Rab and SNARE proteins arose by paralagous expansion is clear, the precise mechanisms and levels of conservation between different transport steps remain unclear. The possibility of a module able to swap out components to generate novel specificity is interesting, but requires experimental validation [47].

Thirdly, what evolutionary drivers underpin evolution of the endomembrane system? Clearly the basic configuration is remarkably robust, being retained by essentially all taxa, but examples of extreme expansion (e.g. *Trichomonas* [48,49]), multiple lineage-specific expansions (e.g. Rab5 [30•] or exocytotic SNAREs [50]), lineage-specific innovations (caveolin, ESCRT 0 [14••,35]), and secondary losses (Rab4) are common [44]. In some cases multicellularity is a likely contributory factor, but in others the primary cause is less clear; detailed dissection and comparisons of such systems where, for example, opisthokonts possess additional factors compared to other taxa will be essential.

Fourthly, what were the original functions of these systems before the LECA? For example the ESCRT system is involved in cytokinesis, but for coatomer an ancestral function is unclear. This is especially relevant to the debate concerning the order of acquisition of the nucleus, the advent of phagocytosis/membrane trafficking, and other systems within the protoeukaryote.

Finally, how big was the evolutionary jump associated with eukaryogenesis and is it necessary to invoke a massive cellular revolution occurring in a short period of time [51–54] Figure 4? It is unclear when this occurred; life on Earth began ~3.5 Bya, and recognizable eukaryotes, that may be assigned to recognizable extant lineages date back at least 1.2 billion years [54]. Unclassifiable eukaryotic fossils are up to 500 million years older still, providing a protracted chronological period during which such a transition could have occurred. While the concept of the LECA is well supported by multiple lines of evidence, understanding how the first common eukaryotic ancestor arose from, and its relationship with, prokaryotes is less clear. As the duration of the transition is unknown, potentially FECA could have given rise to many lineages that were then outcompeted by LECA and its descendants (Figure 4b). In such a scenario FECA could have been a very much simpler system, allowing a more gradual acquisition or elaboration of the endomembrane system.

## Acknowledgements

Much of the work from the authors' laboratory has been funded by the Wellcome Trust, which is gratefully acknowledged. We are indebted to Michael P Rout, Brian T Chait (The Rockefeller University), and Nicholas Butterfield (The University of Cambridge) for discussions.

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- • of special interest
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