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Published in:
Wellcome Open Research

DOI:
10.12688/wellcomeopenres.15142.1

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

Link to publication in Discovery Research Portal

Citation for published version (APA):
Boehm, C., & Field, M. C. (2019). Evolution of late steps in exocytosis: conservation and specialization of the exocyst complex. Wellcome Open Research, 4, 1-19. [112]. https://doi.org/10.12688/wellcomeopenres.15142.1
Evolution of late steps in exocytosis: conservation, specialization [version 1; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: The eukaryotic endomembrane system likely arose via paralogous expansion of genes encoding proteins specifying organelle identity, coat complexes and government of fusion specificity. While the majority of these gene families were established by the time of the last eukaryotic common ancestor (LECA), subsequent evolutionary events molded these systems, likely reflecting adaptations retained for increased fitness. As well as sequence evolution, these adaptations include loss of otherwise canonical subunits, emergence of lineage-specific proteins and paralog expansion. The exocyst complex is involved in late exocytosis, and possibly additional pathways, and is a member of the complexes associated with tethering containing helical rods (CATCHR) tethering complex family, which includes conserved oligomeric Golgi (COG), homotypic fusion and vacuole protein sorting (HOPS), class C core vacuole/endosome tethering (CORVET) and others. The exocyst is integrated into a complex GTPase signaling network in animals, fungi and other lineages. Prompted by discovery of Exo99, a non-canonical subunit in the excavate protist and significantly increased Trypanosoma brucei, genome sequence data, we examined evolution of the exocyst.

Methods: We examined evolution of the exocyst by comparative genomics, phylogenetics and structure prediction.

Results: The exocyst is highly conserved, but with substantial losses of subunits in the Apicomplexa and expansions in Streptophyta plants and Metazoa. Significantly, few taxa retain a partial complex, suggesting that, in the main, all subunits are required for functionality. Further, the ninth exocyst subunit Exo99 is specific to the Euglenozoa with a distinct architecture compared to the other subunits and which possibly represents a coat system.

Conclusions: These data reveal a remarkable degree of evolutionary flexibility within the exocyst complex, suggesting significant diversity in exocytosis mechanisms.

Keywords
Exocytosis, exocyst, eukaryotes, membrane transport, molecular evolution, comparative genomics
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**Author roles:** **Boehm C:** Data Curation, Formal Analysis, Software, Visualization, Writing – Original Draft Preparation; **Field MC:** Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

**Grant information:** This work was supported by the Wellcome Trust [082813], a Project Grant to MCF, and [204697], an Investigator Award to MCF.

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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**How to cite this article:** Boehm C and Field MC. *Evolution of late steps in exocytosis: conservation, specialization [version 1; peer review: 2 approved, 1 approved with reservations]* Wellcome Open Research 2019, 4:112 (https://doi.org/10.12688/wellcomeopenres.15142.1)

**First published:** 26 Jul 2019, 4:112 (https://doi.org/10.12688/wellcomeopenres.15142.1)
Introduction
A sophisticated level of cellular compartmentalisation is the major feature differentiating prokaryotic and eukaryotic cells and underpins the origins of the nucleus. Early eukaryotic ancestors possessed a complex internal membrane system, suggesting rapid evolution after the first eukaryotic common ancestor (FECA) arose and prior to origin of the major eukaryotic super-groups (Dacks & Field, 2018; Guy et al., 2014; Koumandou et al., 2013; Schlacht et al., 2014). It is becoming clear that these systems predate the origins of what would be classically recognised as eukaryotes, as some ancestral genes for constructing an endomembrane system were present in prokaryotes, and specifically Archaea (Eme et al., 2018; Spang et al., 2018) (Figure 1A).

An established theme in the evolution of membrane transport is the central role of paralogous protein families in dictating compartmental identity, specificity and supporting transport functions between compartments. These families include small GTPases, SNAREs, coat complexes and tethers. For example, SNARE and Rab paralogs associate with distinct subcellular organelles (Elias et al., 2012; Khurana et al., 2018; Zerial & McBridge, 2001), and it is likely that new organelles and/or pathways develop via emergence of novel SNARE and/or Rab paralogs through gene duplication and neofunctionalisation (Dacks & Field, 2007; Ramadas & Thattai, 2013). The evolution of some of these families has been reconstructed in some detail (Elias et al., 2012; Hirst et al., 2014; Venkatesh et al., 2017).

Deep evolutionary relationships between proteins that form vesicular coats and other structures, including the COPI and II complexes, clathrin/adaptin heterotetramers and the nuclear pore complex, further supports the concept of stepwise acquisition of complexity prior to the last eukaryotic common ancestor (LECA) (Rout & Field, 2017). Perhaps most remarkable is the presence of a fully differentiated set of coat complexes and specificity-encoding machinery in the LECA, and consequentially, over a billion years separates this ancestral endomembrane system from that of extant organisms. More recently it has been speculated, based on the complexity of the architecture of nuclear

Figure 1. Evolution and structure of the exocyst. A) Cartoon representing the major supergroups, which are referred to in the text. The inferred position of the last eukaryotic common ancestor (LECA) is indicated and the supergroups are colour coordinated with all other figures. B) Structure of trypanosome Exo99, modelled using Phyre2 (intensive mode). The model for the WD40/b-propeller (blue) is likely highly accurate. The respective orientations of the a-helical regions may form a solenoid or similar, but due to a lack of confidence in the disordered linker regions this is highly speculative. C and D) Structure of the Saccharomyces cerevisiae exocyst holomeric octameric complex. In C the cryoEM map (at level 0.100) is shown and in D, the fit for all eight subunits (pdb 5yfp). Colours for subunits are shown as a key, and the orientation of the cryoEM and fit are the same for C and D. All structural images were modelled by the authors from PDB using UCSF Chimera.
pore complex subunits, that this structure, and possibly the intraflagellar transport system, arose later, during progression from FECA and LECA (Field & Rout, 2019).

A further group of proteins central to compartmentalisation are the membrane-tethering complexes (MTCs). Considerably more diverse than Rab and SNARE families in both architecture and mechanism of action, MTCs control Rab GTP cycles, as well as tether vesicles for fusion. Evidence for common evolutionary descent for some complexes and subunits has been offered (Koumandou et al., 2007; Yu & Hughson, 2010; Whyte & Munro, 2002). MTCs have splendid names that include transport protein particle (TRAPP) I, II and III, conserved oligomeric Golgi (COG), homotypic fusion and vacuole protein sorting (HOPS), class C core vacuole/endosome tethering (CORVET) (plus class C homologs in endosome-vesicle interaction, CHEVI and factors for endosome recycling and retromer interactions, FERARI), dorsalin-1 (Ds1l), Golgi-associated retrograde protein/endosome-associated recycling protein (GARP/EARP) and the exocyst. Significantly, these complexes vary considerably in the number of subunits they possess. MTCs are widely distributed among eukaryotic taxa and many subunits share the complexes associated with tethering containing helical rods (CATCHR) fold (Klinger et al., 2013; Koumandou et al., 2007; Yu & Hughson, 2010). This model is supported by the structural similarity of several exocyst subunits that share the CATCHR architecture, which is almost exclusively α-helical (Sivaram et al., 2006; Vasan et al., 2010). Further, the Saccharomyces cerevisiae CATCHR complexes, GARP, COG1–4 subcomplex of COG and HOPS share similar subunit organization (Chou et al., 2016). This is not only consistent with possible common ancestry, but also may indicate mechanistic similarities.

The exocyst, as originally described, comprised Sec3, 5, 6, 8, 10 and 15 (Novick et al., 1980). Two additional subunits, Exo70 and Exo84 were subsequently discovered and the holocomplex presented as a stable 19.5S particle (Bowser et al., 1992; Guo et al., 1999; TerBush et al., 1996) (Figure 1D). Overall, the yeast exocyst forms a loose and rather open rod, but has considerable conformational flexibility (Heider et al., 2016; Picco et al., 2017; TerBush et al., 1996) and interacts with multiple plasma membrane-located GTPases (Wu et al., 2008). CryoEM has characterized the complex and subunits to 4.4Å and demonstrated a conserved architecture for the subunits with two and four helical ‘CorEx’ bundles, together with an extended N-terminal α-helix that is critical for assembly (Mei et al., 2018). Notably, CorEx shares structural similarities with the N-terminus of COG and GARP subunits. Only Sec3 and Exo84 have an additional domain, namely a pleckstrin homology (PH) lipid interaction domain. Both structural and experimental data from yeast suggests that the exocyst is formed of two subcomplexes; Sec3, 5, 6, 8 and Sec10, 15, Exo 70 and 84. Sec3 appears critical for both assembly and disassembly (Ahmed et al., 2018) and Exo84 phosphorylation is implicated in controlling overall exocyst assembly and function. Significantly, these two subunits are components of different subcomplexes and likely interact with phospholipids through their PH domains. The exocyst has clear roles in secretion but is also implicated in disease susceptibility, host cell invasion by intracellular bacteria and development (Arasaki et al., 2018; Bommenaier et al., 2018; Lira et al., 2018) with evidence for additional roles in endocytosis/recycling also published (Boehm et al., 2017; Jose et al., 2015).

Previous comparative genomics studies identified only six of the eight canonical exocyst subunits, with Sec5 and Exo84 evading identification in all trypanosomatids (Koumandou et al., 2007), suggesting either an alternative, simplified exocyst complex in trypanosomatids or failure to uncover highly divergent orthologs. The latter possibility was demonstrated following direct identification of all eight canonical subunits as well as a ninth, Exo99, in trypanosomes (Boehm et al., 2017). Using updated methodology and genome resources, we find evidence for considerable evolutionary flexibility in exocyst retention, with essentially complete loss from some lineages and a tentative suggestion of a connection to novel coat proteins.

Results

Identifying exocyst subunits across the eukaryotes

The earlier failure to identify Sec5 and Exo84 in excavates by comparative genomics (Koumandou et al., 2007), and subsequent identification in trypanosomes by immunosolation and mass spectrometry, indicated that the earlier study lacked sensitivity, and suggested other false negatives within the dataset (Boehm et al., 2017). Furthermore, the distribution of the newly identified Exo99 subunit has not been investigated systematically. Considerable genome sequencing has taken place in the period since that earlier analysis, as well as availability of superior search algorithms.

We screened for genes encoding the eight canonical exocyst subunits and the newly identified subunit Exo99 in 87 eukaryotic genomes by BLAST, inspection of alignments and phylogenetic reconstruction. This increased the size of our genome panel ~five-fold and took advantage of the increased quality of these resources. Furthermore, we were able to harness high quality phylogenetic reconstruction to validate our data. Only subunit predictions that passed reciprocal BLAST, phylogenetic validation and were predicted to be within a similar length as the query, together with homology that extended over more than 50% of the sequence (to avoid calls based exclusively on conservation of small architectural features) were annotated as ‘found’. Example phylogenetic trees for three subunits (Sec15, Exo99 and Exo70) are shown in Figure 2 and the overall distribution in Figure 3. Phylogenetic trees for the remaining subunits, as well as accession numbers of found orthologs are included as Underlying data (Table S1) and Extended data (Figures S1-6).

Distribution of the canonical octamer subunits

The eight canonical exocyst subunits are well conserved, reflecting their ancient origins, and specifically detected in representatives of all five eukaryotic supergroups. Phylogenetic analysis indicates that these sequences are likely bona fide orthologs and, as the topology of the gene and taxon trees are highly similar, there is no evidence for lateral gene transfer (Figure 2). Given the noted similarities in structures of these subunits and the
Figure 2. Phylogenetic reconstructions for Sec15, Exo99 and Exo70. Trees show the best Bayesian topology for reconstructions of A) Sec 15, B) Exo99 and C) Exo70. Numerical values at the nodes indicate statistical support from analysis with MrBayes and PhyML. Values for highly supported nodes have been replaced by symbols as indicated in the legend. Species names are coloured for recognised supergroups: Opisthokonta (blue), Amoebozoa (pink), Archeoplastida (green), Stramenopile-Alveolate-Rhizaria (SAR, orange), Excavata (purple). The same colour convention is used throughout this figure and phylogenetic trees.

We found expansion of Sec6 and 15 in vertebrates and in a close relative Brachiostoma (commonly lancelets), where multiple subunits have expanded. This pattern may reflect tissue complexity, but the absence of an obvious interaction between Sec6 and 15 suggests this is unlikely to be associated with specific subfunction. However, the locations of these two subunits, contributing to opposite ends of the octameric complex, may suggest that this facilitates variation in interactions between exocytic vesicles and plasma membrane docking sites in different tissues (Heider & Munson 2012; Heider et al., 2016). Sec10 is also expanded in the fungi, and there is complete absence of the complex in Rhizopus, the only such example in

clear sharing of extensive α-helical bundles revealed by cryoEM, this suggests that the exocyst octamer indeed arose prior to the LECA and most likely by paralog expansion from an ancestral subunit complement.

We found little partial subunit loss, which may indicate that some of our examples of loss of single subunits are artefactual. This pattern of retention also argues against functionality for the two subcomplexes, as does the absence of paralog expansion of specific exocyst subunit cohorts, with the exception of some streptophytes and widely dispersed taxa. Overall, this is consistent with recent functional studies (Ahmed et al., 2018).
Figure 3. Coulson plot of distribution of exocyst components across eukaryotic lineages. Calls are based on a combination of BLAST, alignments and phylogenetic reconstruction. Filled circles indicate the presence of the protein, open circles that an ortholog was not found and grey indicates that the sequence could not be fully verified by phylogenetics. The numbers in the circles indicate multiple paralogs and the number of individual genes identified. Accession numbers for all reported exocyst subunits are given in Table S1 (Underlying data). Species names are coloured by supergroup as in Figure 2. The eight canonical exocyst subunits are conserved throughout the eukaryotic lineage, with the exception of the Alveolata. Some of the plants have greatly increased numbers of Exo84 and especially Exo70 genes in their genomes. The presence of Exo99 is restricted to the Excavata.
the Opistokhonta sampled. As this taxon of fungi lack the ability for septation, this may explain the loss of the entire exocyst.

Both major Amoebozoa taxa retain a full complement of exocyst subunits in the majority of lineages. The absence of several subunits from Entamoeba invadens and one from E. mutabilii is noteworthy, as is the expansion of Sec10, a feature shared with the fungi, and raises the possibility that a duplication of Sec10 occurred at the root of the unikonts. However, this is not supported by phylogenetic reconstruction, which suggests independent, but likely basal, origins for the fungal and Entamoeba paralogs (Figure S5, Extended data).

The major subunit expansion within the entire sampled genomes is present amongst the Streptophyta plant lineage. Interestingly, in the closely allied algal Chlorophytes, loss is the dominant evolutionary trend, with producing meroiota and Ostreococcus tauri lacking sufficient subunits to build a function exocyst, which may suggest alternate functions or mechanisms, at least for O. tauri and Micromonas where only four subunits could be identified. Interestingly, for the Chlorophytes lacking many subunits, Sec6 and Sec10 are retained, which significantly are components of distinct subcomplexes and unlikely to physically interact (Munson review, Ahmed et al., 2018). A small number of plants also have multiple Sec10 paralogs and, in common with other taxa (see above), the origin of the duplication was likely taxon-specific (Figure S5, Extended data).

The most extreme expansions within Streptophyta subunits are Exo70 and Exo84, with at least 26 copies detected in Populus trichocarpa and 18 in rice. We are aware that the total number of genes we have predicted for all eight exocyst subunits in plants varies to some extent from those previously published, mostly because we did not distinguish between genes with ≥99% sequence homology. However, this does not affect the overall consensus between our and previous studies; namely, that all exocyst subunits in plants are expanded to some extent. The precise functions of some of these multiple exocyst subunits indicate that exocyst complexes with different Exo70 paralogy have specialized functions in differentiated plant tissues (Takemoto et al., 2018). Phylogeny indicates a complex evolutionary pathway for Exo84, and while our reconstruction suggests that most paralogs arose via lineage-specific expansions, the absence of good statistical support makes this conclusion equivocal (Figure S6, Extended data). By contrast, it is clear that Sec15 and Exo70 expansions began at the root of the Streptophyta and, in the case of Exo70, this has continued in a lineage-specific manner to create a family of paralogs of considerable diversity (Figure 2). Live imaging in mammalian cells suggests that Exo70 is the first subunit to contact the plasma membrane (Ahmed et al., 2018), and hence, the presence of so many Exo70 variants is likely a result of tissue-specific and/or plasma membrane-domain target specificity.

All of the Apicomplexa, including Plasmodium falciparum and Toxoplasma gondii, lack the entire exocyst complex, while other alveolates within the Ciliata lineage have retained a subset. Since the retained subunits vary between ciliate species, albeit for both taxa sampled, including Exo70 and Exo84, this suggests individual losses rather than a stepwise loss of exocyst function during the evolution of the alveolates and raises the question for the existence of an exocyst-independent exocytic pathway in these organisms. It is, however, clear that the loss from the Apicomplexa is an ancestral event. Apicomplexa are known for a patchy distribution of the endocytic machinery and other tethering complexes like COG, GARP, Dsl1 and TRAPP-II (Koumandou et al., 2007), and may reflect specific simplifications of trafficking systems in these obligate intracellular parasites. Many Apicomplexa possess unique secretory organelles, including micronemes and rhoptries, that are essential for host cell invasion, but these organelles appear not to require the canonical tether machinery for biosynthesis (Tomavo, 2014).

Amongst Stramenopiles, there is a complex pattern of retention and loss. There is near full retention amongst the Oomycota, which contrasts with the many losses in the sister taxon Ochrophyta. It may be significant that in these organisms, Exo84 and, in a more limited manner, Exo70 are most commonly absent, similar to the ciliates. A limited number of expansions are also detected, principally in Sec3, Sec5 and Sec6, which may suggest more diversity within vesicular cargo transport than at the plasma membrane, as all three of these subunits are components of a single subcomplex that likely interfaces with the incoming vesicle (Ahmed et al., 2018).

Only single orthologues of all exocyst subunits were found in the kinetoplastids, with possible duplications in Bodo saltans and Trypanoplasma borelli. The few apparent losses, for example in T. cruzi and Phytomonas HART are most likely the result of incomplete sequence data/assembly, with the suggestion that for these taxa, the composition of the canonical octomeric exocyst component is essentially invariant.

Exo99, a taxon-restricted subunit with distinct structure
The exocyst was originally identified via yeast secretory mutant screens, which uncovered six subunits (Sec3, 5, 6, 8, 10 and 15), and interaction between Sec15 and Sec4, a small GTPase at the plasma membrane required for secretion and orthogonal to Rab11 (Novick et al., 1980). Two additional subunits, Exo70 and Exo84, were subsequently described, and the entire system demonstrated by biochemical and multiple interactome analyses to be a stable 19.5S complex, albeit with evidence for the presence of additional forms (Bowser et al., 1992; Guo et al., 1999; Morgera et al., 2012).

A ninth subunit, Exo99, was identified by affinity isolation in African trypanosomes. Exo99 phenocopies Sec15 under knockdown, indicating that it is a bona fide member of the complex (Boehm et al., 2017). Here, we find that Exo99 is present in all kinetoplastids and also the related bodonids (Bodo saltans), suggesting a unique aspect in export pathways in these organisms.
The predicted structure of Exo99 is highly distinct from the canonical exocyst subunits and possesses a confidently predicted seven blade β-propeller at the N-terminus, together with an α-helical C-terminus (Figure 1B). The topology for several short stretches of the C-terminal region are predicted as disordered, preventing prediction of the overall architecture of the α-helical region. Hence, it is unclear if this region adapts a fold similar to the CATCHR family and other exocysts subunits or is distinct. Very weak homologs were also found in *Naegleria gruberi*, *Trichomonas vaginalis*, *Giardia lamblia* and social amoeba. Structure prediction suggests that the *N. gruberi* sequence may well share architecture with the kinetoplastida (Data archive 2, Extended data), but that the other possible orthologs do not, indicating likely restriction to Euglenozoa, as well as at least one heterolobosid.

It is, however, tempting to speculate that Exo99 is a divergent member of the protocoatomer family, which populate the endomembrane system. These proteins are associated with vesicular transport and related functions and bear the β-propeller N-terminus as well as an α-helical C-terminal domain, perhaps best recognized in the heavy chain of the endocytosis coat protein clathrin (Rout & Field, 2017). However, in that instance the helices form a coiled-coil solenoid, a specific type of higher order architecture, whilst for Exo99 it is unclear if this is the case. Clearly, more precise structural data are required to evaluate this possibility, as well as the location of Exo99 within the trypanosome exocyst. It is also unclear if additional coat-like components are associated with the trypanosomatid exocyst, but not captured in the affinity isolation. Most significant however, is the presence of this divergent subunit, which evaded detection by *in silico* methods due to its novelty, opens the possibility of additional lineage-specific exocyst components in other species. Direct isolation of such complexes is a pre-requisite to the identification of such potential factors.

**Discussion**

Cellular complexity was revolutionised by development of an endomembrane system during eukaryogenesis. In the period since, losses and gains have moulded the ancestral transport system into the huge variety present in living cells. Secondary loss of ancestral components is common, while expansion of individual paralogs is also a high frequency event. It is less common to uncover the birth of completely new components or complexes.

The exocyst forms an open, monomeric rod, with each component present as a single copy. Several complexes appear to be required for vesicle fusion (Ahmed et al., 2018). All canonical subunits share the CorEx secondary structure that is predominantly α-helical and suggests a stepwise pathway for exocyst origins. This model is supported by the similar predicted and atomic structures of several exocyst and additional MTC subunits (Sivaram et al., 2006; Vasan et al., 2010), all of which share the CATCHR architecture. Based on prolific interactions with multiple Ras-superfamily GTPases, the exocyst has been proposed to act as a tether to bring secretory vesicles to the plasma membrane, and recent *in vivo* imaging supports this model (Ahmed et al., 2018; Picco et al., 2017).

There is considerable variability between exocyst gene complement in different organisms, with many examples of complete loss or spectacular expansion (Figure 4). In terms of loss, representation is quite broad, with examples in fungi, plants and multiple protozoan lineages. Some, such as *C. merolae*, reflect known unusual biology, and the complete absence from the Apicomplexa is also a feature consistent with highly unusual and reduced secretory systems in a predominantly parasitic taxon. For example, there is repeated loss of adaptins and degeneration of the Golgi complex in Apicomplexa (Nevin & Dacks, 2009), which correlates well with the loss of much of the COG, Dsl and TRAPPII complexes (Klinger et al., 2013),...
while loss of the exocyst dates back to the origins of the chromists (Woo et al., 2015).

Expansions tend to focus on about half of the subunits, with Sec6, 10 and 15 duplicated across more than one supergroup, a pattern indicating independent events. Larger scale expansions are Exo74 and 80 in the higher plants. Significantly, Exo70 in Arabidopsis thaliana carries multiple motifs for interactions with Atg8 (Cvřeková & Zárský, 2013) and Exo70 has been subjected to extreme paralogous expansion in Streptophyte plants. Tissue-specific expression, together with distinct interactomes, may explain these differential roles (Takemoto et al., 2018), although precise functions are unclear as yet. Significantly, plants also possess an expanded Rab11 (RabD) family (Rutherford & Moore, 2002), but it is not known if there is a specific relationship between Rab11 and Exo70 paralogs. Retention of the exocyst is, in the main, all-or-nothing, indicating that the complex functions essentially as a single unit, and loss of even one subunit compromises function, consistent with the structure of the complex (Ahmed et al., 2018; Heider et al., 2016; Mei et al., 2018). Overall, despite considerable conservation, there is remarkable sculpting of the complex, which suggests a range of functional roles remaining to be uncovered.

Exo99 is currently the sole example of a lineage-specific exocyst subunit, and may be part of a larger coat complex, based on similarity to protocoatomer. The presence of Exo99 further highlights evolutionary modifications to membrane trafficking pathways and underscores the flexibility of these pathways across evolution, as well as suggesting that there may be additional exocyst components in other lineages (Manna et al., 2017; Rout & Field, 2017).

The exocyst’s many functions include: formation of lamellipodia; interaction with WASH to control actin at endosomes; targeting Rab11 to the mammalian cleavage furrow; an endoplasmic reticulum to vacuole pathway analogous to autophagy; disease susceptibility; development; host cell invasion by intracellular bacteria; and endocytic recycling (Arasaki et al., 2018; Boehm et al., 2017; Bonnemaier et al., 2018; Jose et al., 2015; Lira et al., 2018; Monteiro et al., 2013; Synek et al., 2014). Further, Rab11 has extensive localisation within mammalian, plant and protozoan cells (Zulkifli et al., 2019), suggesting activity in transport beyond simple tethering of secretory vesicles to the plasma membrane. The direct assessment of an exocyst role in secretion is surprisingly not that well documented (Luo et al., 2014), despite evidence for localisation of the exocyst to regions of the plasma membrane active in exocytosis for a broad range of species. With both the detailed structure of the complex, together with an understanding of the evolutionary variation of exocyst subunits, it will be of great interest to uncover these functions in molecular detail.

Methods

Comparative genomics of exocyst components
Candidate exocyst components were identified by scanning a panel of eukaryotic predicted proteomes (Table S1, Underlying data; Field, 2019) with known exocyst component sequence queries using BLAST (Altschul et al., 1990). For each subunit, one query sequence was selected from each of the following predicted proteomes: Homo sapiens, Saccharomyces cerevisiae, Trypanosoma brucei, Dictyostelium discoideum, Phytophthora sojae, Phytophthora ramorum, Arabidopsis thaliana and Selaginella moellendorfii (accession numbers for initial queries are provided in Table S1, Underlying data; Field, 2019). For each subunit, the top BLAST hits from each of these scans were pooled in a neighbour-joining tree after alignment with ClustalW with default parameters to remove erroneous sequences (Thompson et al., 1994). The gene IDs on the tree were then annotated with predicted protein length (based on) alignments with known exocyst components, pfam domain predictions (pfam server default parameters and notes of which (if any) of the six initial query sequences detected the ID as a reciprocal best BLAST hit. This annotation allowed the identification of a cluster of robust candidates by neighbour joining (NJ). Off-target matches were identified by manual inspection of both the annotated NJ tree and the underlying alignment and these were excluded. Furthermore, the overall length of the predicted protein and the region of homology were considered, to exclude proteins that were likely only related through possession of a common domain.

In cases where a candidate was not found, additional datasets were queried by web-based BLAST searches at TriTrypDB, JGI and NCBI as appropriate. Alignments were created using MUSCLE (Edgar, 2004). Only unambiguous homologous regions were retained for phylogenetic analysis, performed by two separate methods. To obtain the Bayesian tree topology and posterior probability values, the program MrBayes version 3.2.2 was used (Ronquist & Huelsenbeck, 2003) with 100 bootstrap replicates. Nodes with better than 0.95 posterior probability and 80% bootstrap support were considered robust, and nodes with better than 0.80 posterior probability and 50% bootstrap support are shown.

Structure prediction

The structures of both T. brucei and N. gruberi Exo99 proteins were predicted using the Phyre2 server running under intensive mode (Kelley et al., 2015, full output available as Extended data). The data for this, as well as for the exocyst octameric complex of S. cerevisiae, were visualized using UCSF Chimera (Pettersen et al., 2004). Data for experimentally determined structures were retrieved from PDB.

Graphic production

The Coulson plot in Figure 3 was produced using Coulson Plot Generator (Field et al., 2013). All images were prepared for final production in Adobe Illustrator 23.0.3.

Data availability

Underlying data
Figshare: Extended data. https://doi.org/10.6084/m9.figshare.8167724.v3 (Field, 2019)
This project contains the following underlying data:
- Table S1.xlsx (accession numbers and URL links, or sequences where those were unavailable, for exocyst subunit orthologs, as shown in Figure 3)

Extended data
Figshare: Extended data. https://doi.org/10.6084/m9.figshare.8167724.v3 (Field, 2019)

This project contains the following extended data:
- Figure S1 – Figure S6 (Phylogenetic reconstructions for Sec3, Sec5, Sec6, Sec8, Sec10 and Exo84, respectively)
- TbExo99.zip (Structure prediction of T. brucei Exo99 from Phyre2. Data are available as a zipped webarchive)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Grant information
This work was supported by the Wellcome Trust [082813], a Project Grant to MCF, and [204697], an Investigator Award to MCF.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Version 1

Reviewer Report 28 October 2019

https://doi.org/10.21956/wellcomeopenres.16521.r36430

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Joshua H. Lipschutz
Department of Medicine, Medical University of South Carolina (MUSC), Charleston, SC, USA

The manuscript, “Evolution of late steps in exocytosis: conservation, specialization”, addresses an interesting question, namely the evolution of the exocyst complex. I think the authors do a good job of describing the comparative genomics, phylogenetics and structure prediction of the exocyst.

Where the authors could do better is describing the many cellular functions of the exocyst and offering a possible mechanism for this. For example, in the abstract the authors write, “The exocyst complex is involved in late exocytosis, and possibly additional pathways, and is a member...”. In the introduction, the authors describe some of the other additional pathways, “The exocyst has clear roles in secretion but is also implicated in disease susceptibility, host cell invasion by intracellular bacteria and development (Arasaki et al., 2018; Bonnemaijer et al., 2018; Lira et al., 2019) with evidence for additional roles in endocytosis/recycling also published (Boehm et al., 2017; Jose et al., 2015). This is not a complete list as the exocyst has also been shown to be centrally involved in basolateral protein transport (Grindstaff et al, Cell, 1998), ciliogenesis (Zuo et al, Mol Biol Cell, 2009), and protein translocation in the ER (Toikkanen et al, J Biol Chem, 2003; Lipschutz et al, J Biol Chem, 2003). How does the exocyst perform these myriad functions? The authors allude to a possible mechanism when they write, “Based on prolific interactions with multiple Ras-superfamily GTPases, the exocyst has been proposed to act as a tether to bring secretory vesicles to the plasma membrane, and recent in vivo imaging supports this model (Ahmed et al., 2018; Picco et al., 2017). The exocyst has actually been shown to be regulated by multiple GTPases of the Arf (e.g. Seixas et al, Mol Biol Cell, 2016; ), Ral (e.g. Moskalenko et al, Nat Cell Biol, 2002, Sugihara et al, Nat Cell Biol, 2002; Brymora et al, J Biol Chem 2001), Rab (Knodler et al, PNAS, 2010), and Rho (Phiel et al, J Biol Chem, 2001; Choi et al, J Am Soc Nephrol, 2013) families. A possible model is that the exocyst is like a Swiss army knife and has the ability to accomplish multiple tasks depending on which small GTPase is regulating it. I think this kind of discussion would strengthen the manuscript.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** I have been studying the exocyst complex for over 20 years and have generated novel reagents (cell lines, zebrafish and mice) and described multiple novel functions of the exocyst.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 08 October 2019

https://doi.org/10.21956/wellcomeopenres.16521.r36431

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Viktor Žárský

1 Department of Experimental Plant Biology, Faculty of Science, Charles University, Prague, Czech Republic
2 Laboratory of Cell Biology, Institute of Experimental Botany CAS, Prague, Czech Republic
Mark Field and colleagues opened the field of phylogenetic analyses of tethering complexes evolution more than ten years ago and this report, using five fold increase of well selected genomes, enhances the resolution of the exocyst status in extant eukaryotes.

p.4. - In Novick et al. (1980) bunch of first sec mutants were described (including sec subunits of the exocyst), but exocyst was stepwise discovered later (culminating in TerBush et al. 1996 EMBO J). Similarly on p.7 – interaction of Sec4p with Sec15p containing particle is described in Bowser et al. (1992, JCB) not in Novick et al. (1980).

In the description of the complex in the Intro it would be important to mention, that it is effector of both RAB and RHO GTPases, and that also EXO70 contributes a crucial direct interaction with the membrane lipids.

The extent of the expansion of EXO70 family in land plants is not comparable to any other subunit in any other organism (e.g. 23 in Arabidopsis and 47 in rice) and possibly would be correct to highlight it also in the Abstract (nicely demonstrated by the Fig. 2C).

Evolution of plant EXO70s was first addressed by Marek Eliáš in Synek et al. (2006) clearly indicating deep early land plant EXO70 divergence into three subfamilies. Existence of these three subfamilies was fully corroborated by Cvrčková et al. (2012) – liverwort Marchantia has three EXO70 paralogs, each in one subfamily (Cvrčková in Rawat et al. 2017). The existence of different plant cell cortical secretory domains and exocyst complexes based on differential EXO70 participation is described in Žárský et al. (2009 and 2013). Importantly data in plant cells specific transcriptomes and proteomes as well as experimental data clearly indicated that in single plant cell several EXO70 paralogs (and therefore exocyst complexes) might operate – tissue specific expression does not explain full multiplicity of EXO70s in land plants. The major driving force of evolution esp. in EXO70.2 land plants subfamily seems to be competition with parasites.

Two exocyst subcomplexes (4+4) mostly work together, however work on exocyst in autophagy (Bodemann et al 2011; Kulich et al. 2013) indicates possibility of functional exocyst subcomplexes specialization.

“Octamer” instead of octomer – as in Fig. 4 – see root of the tree.

Typo p. 9 not EXO74 and 80, but EXO70 and 84.

This bioinformatics analysis highlight post-LECA evolutionary destiny of exocyst vesicle tethering complex marked by both local expansions as well as gene/whole complex losses well correlating with lineage specific biological contexts.

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**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Plant cell and development biology with the focus on the secretory pathways regulation.

*I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.*
This study investigates the evolution of the exocyst using comparative genomics, phylogenetics and structure prediction. The focus is the evolutionary flexibility of the exocyst complex, which was prompted by the initial difficulty in identifying all of the eight canonical exocyst subunits in Trypanosomatids along with the recent discovery of an additional 9th component, Exo99, in these organisms. The challenge is to identify those orthologues that have diverged significantly and as well as the implications of this additional 9th subunit in African trypanosomes.

The results, nicely presented and summarised in Figs. 3 & 4, reveal the degree of flexibility in exocyst evolution across eukaryotic lineages. There are examples of expansion, notably Exo70 but also Sec 10, absence/loss of individual components, Sec 8 in Entamoeba, as well as complete loss in Apicomplexa. The other notable finding is that Exo99 appears to be a taxon-restricted subunit most likely with a distinct structure.

The message of the study is that the conventional view of the exocyst, as a conserved octameric protein complex, needs to be more nuanced. The authors speculate that subtleties in exocyst complex composition (loss as well as presence of new components) suggest a diversity in exocytosis mechanisms. This seems a reasonable proposition, as pointed out here and elsewhere Apicomplexa possess unique secretory organelles that do not appear to require the canonical tether machinery for biosynthesis, while the presence of Exo99 all kinetoplastids (and also the related bodonids), suggests possibly unique elements in export pathways in these organisms.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes
**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular and biochemical parasitology; specifically African trypanosomes

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Comments on this article**

**Version 1**

Reader Comment 08 Aug 2019

**Viktor Zarsky**, Charles University and Inst. Exp. Bot CAS, Prague, Prague, Czech Republic

I am truly grateful to my fellow colleague Ivan Kulich for his suggestions to this submission, which might be summarized - in single plant cell there are around 5 different EXO70 isoforms expressed. I only like to further highlight non-canonical function of exocyst in autophagy - both in animals and plants (Kulich et al. 2013 - doi: 10.1111/tra.12101 and Bodeman et al. 2011 - doi: 10.1016/j.cell.2010.12.018.) - certainly an important feature to understand exocyst evolution. In respect to plant exocyst I like to recommend for comparative discussion (incl. gene predictions) reports from our lab: Cvrckova et al. 2012 - (doi: 10.3389/fpls.2012.00159.) and Rawat et al. 2017 (doi: 10.1111/nph.14548.).

**Competing Interests:** No competing interests were disclosed.

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Reader Comment 08 Aug 2019

**Ivan Kulich**, Charles University, Prague, Czech Republic

Takemoto et al. 2018 does not say anything about functionality of EXO70 as authors claim here. This is a SNARE paper. Moreover, from this review it seems that the expression pattern is the main difference among plant EXO70 paralogs. But this is not the case.

We have shown that Arabidopsis EXO70 paralogs differ a lot. For example, exo70H4 mutant phenotype can not be complemented by any other paralog and EXO70H4 still interacts and colocalizes with the exocyst core subunits (DOI: https://doi.org/10.1104/pp.17.01693). Moreover EXO70H4 and EXO70A1 show different localization within the same cell due to different lipid binding capacities (https://doi.org/10.3390/ijms20153803), showing that multiple secretory domains labelled by different EXO70 can coexist within the same cell. Similar thing was shown for ntEXO70A1 and ntEXO70B1 in the growing pollen tube DOI: https://doi.org/10.1104/pp.16.01709. Pollen tube also contains strange EXO70C2 paralogs, which lost their interaction with the exocyst complex and possibly adopted novel functions in the control of the pollen tube tip growth (doi:10.1104/pp.16.01282).

There is also interesting literature to be found on EXO70s in the immune responses - for example that TN2 guards EXO70B1 https://doi.org/10.1371/journal.pgen.1004945 and that RIN4 can recruit EXO70B1, but not EXO70B2 to the plasma membrane https://doi.org/10.1093/jxb/erx007.

**Competing Interests:** No competing interests were disclosed.
