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Heterotrophy in dinoflagellates: components of endocytosis molecular machinery in *Prorocentrum cordatum* and *Amphidinium carterae* transcriptomes

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Summary

Heterotrophy appears to be a common and widely adopted strategy among dinoflagellates, even in predominantly photosynthetic species. To elucidate the mechanisms of switching between various trophic modes in primarily mixotrophic protists, we need the information about molecular machinery providing the uptake of nutrients of different nature. Here, we analyzed transcriptomic data available for two marine mixotrophic dinoflagellates belonging to the phylogenetically distant groups – *Prorocentrum cordatum* and *Amphidinium carterae*. This study uncovered homologs of proteins involved in endocytosis and phagosome formation, including epsin, coronin, clathrin light and heavy chains, adaptor protein complex 2 and dynamin. The enzymes, acting in the lysosomal degradation, and small GTPases regulating vesicle transport, maturation and recycling were also detected in these organisms. We provide a generalized scheme of endocytosis pathways, which includes proteins revealed in the course of this study and identified by other authors.

Key words: *Amphidinium*; dinoflagellate, endocytosis, heterotrophy, phagocytosis, *Prorocentrum*, transcrtiptome

Introduction

Heterotrophy is a widespread nutrition strategy among dinoflagellates (Stoecker, 1999). Most probably, many dinoflagellates can engulf relatively big organic molecules that cannot be delivered with the help of specific transporters. For example, vitamins that are part of the cultivation medium should be consumed by endocytosis. Moreover, these protists can feed on other microorganisms of different sizes – from bacteria to ciliates, cryptophytes, raphidophytes, prymnesiophytes, diatoms, haptophytes, and even other dinoflagellates (Jeong et al., 2005a, 2005b; Hansen, 2011; Wikfors and Fernandez, 2013; Jang et al., 2016). Particularly noteworthy is the ability of most photosynthetic dinoflagellates to use heterotrophy along with autotrophy, or mixotrophy (Stoecker, 1999). To elucidate the mechanisms of switching between various trophic modes, to determine the

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point and rate of this transition, we need information about molecular machinery providing the uptake of nutrients of different nature.

Collared pits and vesicles resembling clathrin-coated pits and vesicles were observed by transmission electron microscopy in the flagellar canals of many dinoflagellate species, for example, in *Amphidinium rhynchocephalum* (Farmer and Roberts, 1989), *Gymnodinium nolleri* (Ellegaard and Moestrup, 1999), *Gymnodinium aureolum* (Hansen, 2001), *Gyrodinium spirale* (Hansen and Daugbjerg, 2004), *Woloszynskia limnetica* (Roberts et al., 1995), *Ceratium hirundinella* (Roberts, 1989) and *Peridinium cinctum* (Calado et al., 1999). Nevertheless, the nature of these structures is not yet clear. According to transcriptomic data, clathrin-dependent endocytosis prevails in dinoflagellates (Zhang et al., 2014; Meng et al., 2019). Proteins involved in the caveola-mediated endocytosis pathway, such as caveolin and Src-kinase were not found (Zhang et al., 2014, 2019; Meng et al., 2019; Li et al., 2021). The engulfment of large food objects was studied at the level of cellular structures involved in this process, but corresponding molecular pathways remain unexplored.

Genes involved in clathrin-mediated endocytosis are significantly upregulated under nitrogen and/or phosphorus deficiency in mixotrophic dinoflagellates *Alexandrium minutum, Prorocentrum donghaiense*, and *P. shikokuense* (Meng et al., 2019; Zhang et al., 2019; Li et al., 2021). Besides nutrient limitation, endocytosis can also be regulated by illumination. It has been shown that the majority of endocytosis genes exhibited the nocturnal mode of expression (Yu et al., 2020). Moreover, actin and tubulin are abundant in the dinoflagellate metabolic profile in the mesopelagic zone where only 1% of the light reaches, which is also considered as a marker of high phagocytic activity (Cohen et al., 2021). Upregulation of cytoskeletal components could be driven by higher phagotrophic activity, since the cytoskeleton is involved in phagocytosis in dinoflagellates, particularly in *Prorocentrum cordatum* (Berdieva et al., 2020).

Marine mixotrophic dinoflagellates – planktonic armored species *Prorocentrum cordatum* (Ostenfeld) Dodge (syn. *Prorocentrum minimum* (Pavillard) Schiller) and benthic naked species *Amphidiniuin carterae* Hulburt – are causative agents of harmful algal blooms in the coastal and estuarine waters worldwide (Taş and Okuş, 2011; Gárate -Lizárraga, 2012; Murray et al., 2015; Telesh et al., 2016; Ajani et al., 2018). The algal blooms usually occur during and/or after the period of high stability of abiotic (physico-chemical) factors in the environment and the stably high abundance of nutrients (Telesh et al., 2021; Zhang et al., 2021). One of the possible reasons for the successful spreading and blooming of dinoflagellates in general and of *P. cordatum* and *A. carterae* in particular is their mixotrophic nutrition strategy. *P. cordatum* is able to utilize nitrate, nitrite, ammonium, and small organic molecules such as urea and glycine as nitrogen sources (Matantseva et al., 2016, 2018). The deficiency of dissolved nutrients, especially phosphate, can be compensated by phagotrophy (Wikfors and Fernandez, 2013; Johnson, 2015; Berdieva et al., 2020). *A. carterae* was shown to be able to graze on diatoms *Skeletonema costatum* in nutrient-replete conditions (Yoo et al., 2009). Vazhappilly and Chen (1998) observed heterotrophic growth of *A. carterae* in culture on acetate and glucose.

The presence and origin of genes involved in endocytosis pathways have not been described yet, neither in *P. cordatum* nor in *A. carterae* (and in *Amphidinium* species generally). In this study, we analyzed translated transcriptomes of these mixotrophic species belonging to phylogenetically distant dinoflagellate groups that are available in the Marine Microbial Eukaryote Transcriptome Sequencing Project database for the presence of homologs of the proteins that corresponded to endocytosis and phagosome formation, lysosomal degradation, and vesicle recycling in different eukaryotes. The obtained results allowed us to expand the information about dinoflagellate molecular machinery, which can be involved in heterotrophy.

Material and methods

The unannotated translated transcriptomes of dinoflagellates *Prorocentrum cordatum* (=*P. minimum*), strains CCMP1329 and CCMP2233, and *Amphidinium carterae*, strain CCMP1314, analyzed in this work were obtained from the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling et al., 2014). To gather query amino acid sequences, we used the KEGG PATHWAY database (https://www.genome.jp/kegg/pathway.html) – pathway types "Endocytosis", "Phagosome", and "Lysosome" (*Homo*

sapiens) (Ogata et al., 1999) and the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). Additionally, several amino acid sequences of *Dictyostelium discoideum* were used in the analysis. We present the full list of query amino acid sequences with identifiers in the Supporting Information (Table S1). The UniProt database was also used to search for relevant proteins and their functions (The UniProt Consortium, 2019).

The search for homologous amino acid sequences in dinoflagellates transcriptomic data was performed using BLASTP algorithm in BioEdit 7.2.5 software (Hall, 1999). The BLOSUM62 scoring matrix for amino acid substitutions was chosen for the analysis. We retained the hits with e-values ≤1e-10. Then, the obtained hits were used as queries in the reverse BLASTP search against the NCBI non-redundant protein sequence database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to re-verify their homology to corresponding proteins. Besides, we checked for the presence of specific conserved domains in the selected *P. cordatum* and *A. carterae* hits using the CD-Search NCBI tool against the CDD v3.19 – 58235 PSSMs database (Lu et al., 2020). We used the "Standard Display" mode for hits selection. The hits with e-values ≤1e-10 were considered. We included in the final result list only amino acid sequences that have passed all the verification steps.

We used a phylogenetic approach to verify the identified homologs. The candidate *P. cordatum* and *A. carterae* amino acid sequences, query sequences, and other homologs from different taxa, which were additionally sampled in the UniProt and NCBI databases, were aligned using Unipro UGENE v33.0 (MAFFT algorithm) (Okonechnikov et al., 2012). Alignments were trimmed using TrimAl v.1.3 with the "Automated 1" algorithm (http://phylemon.bioinfo.cipf.es) and then screened for non-conservative sites manually. We used the resulting alignments to select the evolutionary model for maximal likelihood analysis with MEGA X 10.2.6 (Kumar et al., 2018). Maximal likelihood phylogenetic analysis was conducted with 1000 bootstrap replications in MEGA X 10.2.6.

Results and Discussion

We revealed homologs of proteins involved in clathrin-dependent endocytosis and actin-based phagocytosis in the translated transcriptomes of two dinoflagellate species – *Prorocentrum cordatum* and *Amphidinium carterae* (44 and 41 proteins, respectively) (Fig. 1, Table S2). Identification of the hits was based on similarity to sequences of other organisms, presence of specific functional conserved domains (Table S3), and results of phylogenetic analysis (Fig. S1). *P. cordatum* and *A. carterae* have homologs of: (1) proteins employed in the initial stages – the plasma membrane invagination or protrusions formation and vesicle generation, (2) proteins acting during sorting and recycling events, (3) key players of early and late maturation of endosomes (phagosomes), (4) participants of lysosomal degradation.

The analysis revealed the amino acid sequences homologous to proteins responsible for the formation of clathrin-coated vesicles. Particularly, we obtained hits similar to epsin contributing to membrane deformation, clathrin light and heavy chains, components of adaptor protein complex 2 binding with clathrin lattice, and dynamin that provides detaching vesicles from the plasma membrane (Fig. 1, Table S2). Interestingly, *P. cordatum* and *A. carterae* possess homologs of dynamin-1, 2, and 3, which sequences were clustered with ones of other organisms from phylogenetically distant groups, and several dynamin-like proteins, which sequences formed a separate clade with dynamin-like proteins characterized in *Perkinsus* and *Symbiodinium* species (Fig. S1). The homologs of phospholipase D1 assisting in different stages of endocytosis, including membrane invagination, were also found in *P. cordatum* and *A. carterae* transcriptome assemblies. Proteins involved in the caveolar endocytosis were not revealed similarly to other studied dinoflagellate species.

There is no doubt about the presence of actin in *P. cordatum* and *A. carterae* cells, although we have not found information about the organization of the actin cytoskeleton for the latter. *P. cordatum*, in turn, possesses a well-developed cortical layer of actin filaments participating in different cellular processes (Berdieva et al., 2018, 2020; Kalinina et al., 2020). In the present work, we identified homologs of actin-associated protein coronin in both species (Fig. 1, Table S2). It induces actin polymerization and is localized in phagosomes in different organisms (Maniak et al., 1995; Rauchenberger et al., 1997; May and Machesky, 2001; Vines and King, 2019). Therefore, in the studied dinoflagellates, the engulfment of prey can be implemented through the

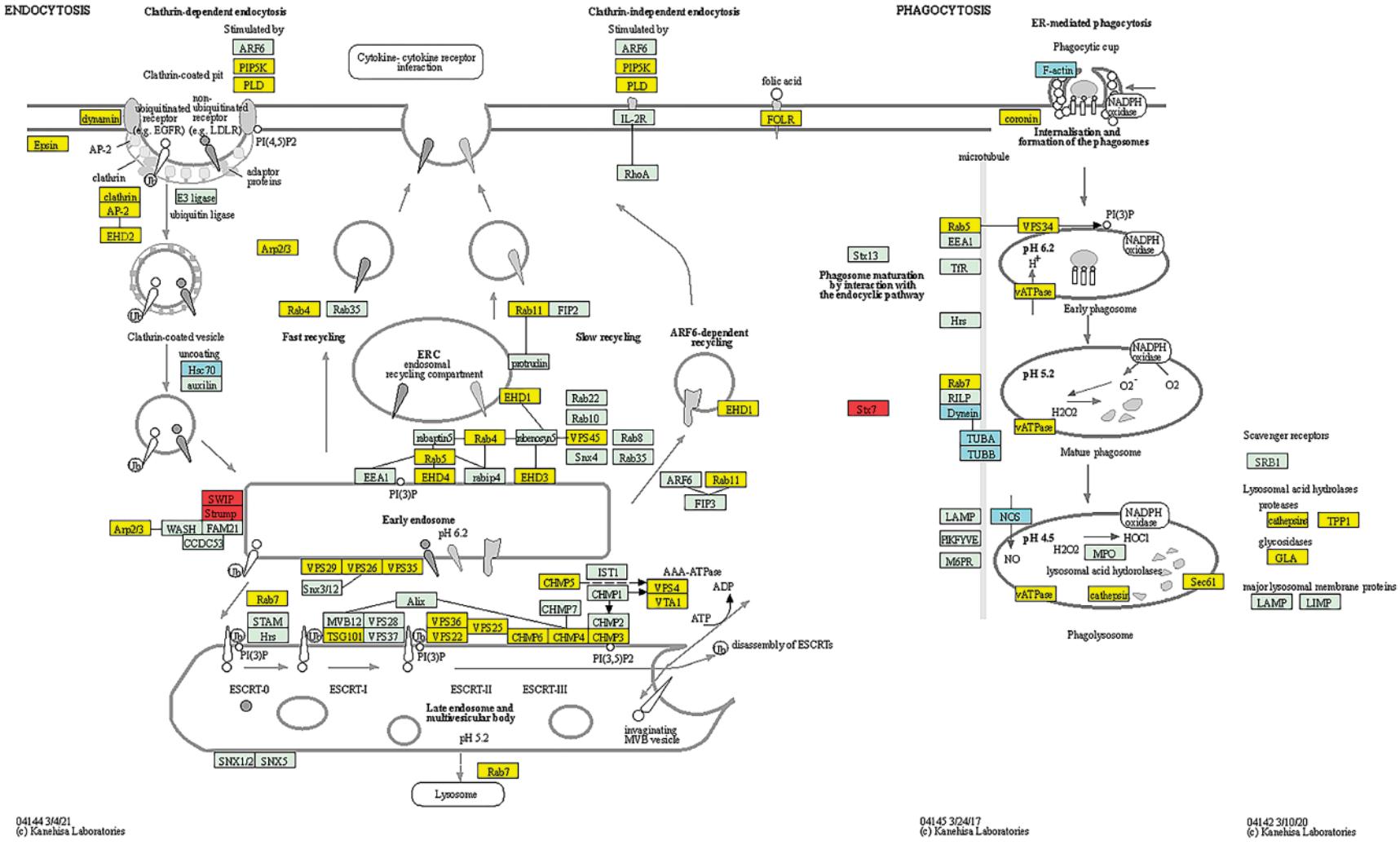

Fig. 1. Schematic map of the endocytosis pathways in mixotrophic dinoflagellates *Prorocentrum cordatum* and *Amphidinium carterae*. The proteins for which homologs have been identified both in *P. cordatum* and *A. carterae* are indicated in yellow, in *P. cordatum* only - in red. The proteins that are known for these species according to the NCBI database information and/or previous investigations are indicated in cyan. The scheme is based on the KEGG PATHWAY database (Ogata et al., 1999), with modifications.

formation of the so-called phagocytic cup, i.e., actin-based protrusions that enveloped food particles.

In both species, we detected homologs of small GTPases regulating vesicle transport and maturation (Ras-related proteins Rab5a, Rab7a) and endocytic recycling (Ras-related proteins Rab4a, Rab7a) (Fig. 1, Table S2). Besides, these dinoflagellates possess homologs of ATP- and membrane-binding EH domain-containing proteins that control membrane trafficking at the stages "plasma membrane – early endosome" (EH domain-containing protein 2), "early endosome – recycling endosome compartment", "early endosome – plasma membrane", or "early endosome – Golgi apparatus" (EH domain-containing proteins 1, 3), and transport of early endosomes (EH domain-containing protein 4) (The UniProt Consortium, 2019). In this case, some hits were identified as homologous to certain proteins, some of them were shared by any two queries, and several hits were shared by all the EH domain-containing proteins 1, 2, 3, and 4 (Table S2). On that basis, we assumed the presence of homologs of all four proteins in the studied species. The amino acid sequences similar to vacuolar ATPase (v-ATPase) were also revealed in *P. cordatum* and *A. carterae* transcriptome assemblies (Fig. 1, Table S2). The v-ATPase occurs in the endosome membrane at the early stages of endocytosis and is responsible for lumen acidification, which is crucial for the future activity of the lysosomal enzymes (The UniProt Consortium, 2019; Vines and King, 2019).

The analysis detected dinoflagellate amino acid sequences homologous to proteins of the retromer cargo-selective complex (vacuolar protein sorting-associated proteins 29 and 26) and the retriever complex (vacuolar protein sorting-associated protein 35) that are necessary for recycling of plasma membrane proteins and interact with WASP and SCAR Homolog (WASH) complex (Fig. 1, Table S2). The latter consists of several subunits and serves for recruiting and activating the Arp2/3 complex that, in turn, induces actin polymerization (Derivery et al., 2009; Gomez and Billadeau, 2009). This system provides the formation of tubules and membrane and proteins recycling. The sequences homologous to subunits of Arp2/3 complex were identified in both species studied. We found homologs of WASH complex subunits 4 and 5 in *P. cordatum* transcriptome assembly. In *A. carterae*, similar amino acid sequences were not detected. It should be noted that, according to the NCBI database, the almost full set of WASH subunits (subunits 1, 3, 4, 5) is present in *Symbiodinium* sp. Meng with coauthors (Meng et al., 2019) detected homologs of subunits 1, 4, and 5 in *Alexandrium minutum* transcriptome. No data on the presence of WASH components were provided for *Alexandrium catenella* (Zhang et al., 2014); there is only subunit 5 in *Prorocentrum shikokuense* and *P. donghaiense* transcriptomes (Zhang et al., 2019; Li et al., 2021). Such difference could be a consequence of the incompleteness of transcriptomic data, but additional search, for example, in *A. carterae* genome also did not reveal intended homologs of WASH complex elements. Meanwhile, dinoflagellates demonstrate the variety of cell organization, particularly the organization of actin and microtubular cytoskeleton (Roberts and Roberts, 1991; Roberts et al., 1992; Soyer-Gobillard et al., 1996; Villanueva et al., 2014; Berdieva et al., 2018). The question is whether this variety may be reflected in the participation of cytoskeletal elements in vesicle trafficking and determine differences in the way of endosome recycling among dinoflagellates from different groups.

We also revealed components of the endosomal sorting complexes required for transport (ESCRT) I–III that participate in the multivesicular body formation and sorting of cargo for further degradation – tumor susceptibility gene 101 protein, vacuolar protein sorting-associated proteins 25, 36, SNF8, charged multivesicular body proteins 3, 5, and 6 (Fig. 1, Table S2). The homologs of vacuolar protein sorting-associated protein 4, which interacts with ESCRT-III and catalyzes its disassembly, and its cofactor VTA1 were also detected in *P. cordatum* and *A. carterae* transcriptomes. ESCRT complexes are considered to be participants of such cellular processes as endocytosis and biogenesis of multivesicular bodies, autophagy, and viral budding (Vietri et al., 2020). Nevertheless, ESCRT-III components have been shown to be localized in the phagosomes in *Entamoeba histolytica* cells (Avalos-Padilla et al., 2018). Therefore, ESCRT machinery can be involved in the degradation of the engulfed food in the dinoflagellate cells.

The homologs of proteins providing final lysosomal degradation of material were also detected in the studied organisms. In particular, the member of the SNARE family – syntaxin-7 – that mediates the endocytic trafficking from early endosomes to late endosomes (phagosomes) and lysosomes was revealed in *P. cordatum*. Both species possessed homologs of phosphatidylinositol 3-kinase catalytic subunit type 3 involved in the transport of lysosomal enzyme precursors to lysosomes and homologs of

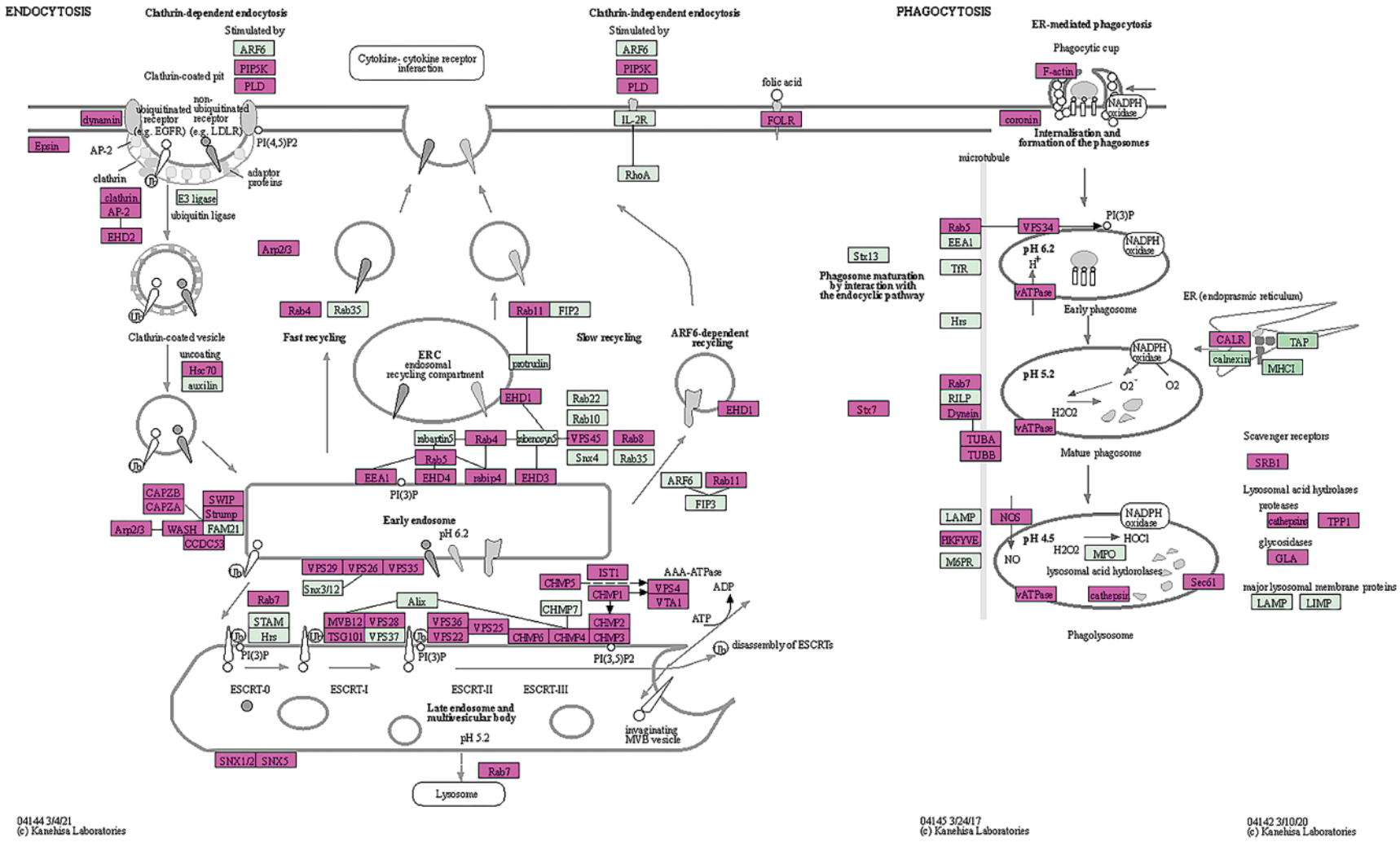

Fig. 2. Generalized schematic map of the endocytosis pathways in the studied dinoflagellates based on this work, results obtained by Zhang et al. (Zhang et al., 2014, 2019), Meng et al. (Meng et al., 2019), Li et al. (Li et al., 2021), and the NCBI database. The identified proteins are indicated in purple. The scheme is based on the KEGG PATHWAY database (Ogata et al., 1999), with modifications.

Sec61. The latter is an endoplasmic reticulum-resident protein complex whose presence in the phagosomes has been discussed (Rock, 2006). We also succeeded in identifying homologs of several enzymes – cathepsins L, D, tripeptidyl-peptidase 1 (lysosomal pepstatin insensitive protease), and alpha-galactosidase (Fig. 1, Table S2).

Therefore, two pathways of internalization – clathrin-dependent endocytosis and actin-based phagocytosis – can take place in the life strategy of mixotrophic dinoflagellates *P. cordatum* and *A. carterae*. Apparently, they are used for dissolved nutrients uptake or internalization of plasma membrane components and prey capture, respectively. The core set of found proteins was similar to that in the other studied dinoflagellate species (Zhang et al., 2014, 2019; Meng et al., 2019; Li et al., 2021); however, we succeeded to complement the data. To consolidate our findings and information available in the literature, we provide a generalized scheme that includes proteins revealed in the course of this study and detected by other authors (Fig. 2).

Obviously, dinoflagellates can possess their own molecular machinery providing endocytosis, including engulfment and degradation of prey. Nevertheless, a set of genes and proteins, which was characterized initially in mammals and some model objects among invertebrates and yeasts, has been identified in these protists; therefore, this apparatus appears to be broadly conserved across eukaryotic groups. The recent studies aimed at the assessment of the transcriptomic response of dinoflagellate cells to changes in the accessibility of nutrient elements in culture medium support involvement of these genes in nutrition processes (Zhang et al., 2019; Li et al., 2021). The investigation of endocytosis molecular apparatus in dinoflagellates from different groups having different trophic strategies is an agenda for the future research.

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Supplementary materials

Figure S1. Maximum likelihood inferences of amino acid sequences inventoried in *Prorocentrum cordatum* CCMP1329 and CCMP2233, and in *Amphidinium carterae* CCMP1314.

Table S1. The full list of proteins involved in endocytosis pathways that have been used as queries in the analysis.

Table S2. The amino acid sequences homologous to proteins involved in endocytosis pathways that have been inventoried in *Prorocentrum cordatum* CCMP1329 and 2233, and *Amphidinium carterae* CCMP1314 transcriptome assemblies.

Table S3. The conserved domains identified in selected hits from *Prorocentrum cordatum* CCMP1329 and 2233, and *Amphidinium carterae* CCMP1314 transcriptome assemblies.

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