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Opinion

Encystment of Free-Living Amoebae, So Many Blind Spots to Cover

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Abstract: Due to frequent variations in environmental conditions, free-living amoebae adapt through differentiation into different states. Hence, favorable conditions enable the formation of a feeding and proliferative form named "Trophozoïte" whereas unfavorable situations drive differentiation into resting and resistant single forms such as cysts, spores, or multicellular structures. Transformation into cyst, named "encystment" or "encystation", is a common feature found in testate, naked, or flagellated free-living amoebae. Although much effort has been made to understand encystment, several blind spots are still present. This short opinion paper highlights some difficulties impeding a better understanding of encystment.

Keywords: encystment; encystation; free-living amoebae

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1. Introduction

The physiology of free-living amoebae is a topic of interest at different levels. Results obtained on aggregative and fruiting amoebae reveal a number of mechanisms in terms of evolution [1], cell biology [2], and social behavior [3]. To face stressful conditions, free-living amoebae differentiate into cysts which ensure they survive, due to their high resilience. Amoebae contain several human pathogens, such as *Entamoeba histolytica*, responsible for amoebiasis; *Naegleria fowleri* causing meningoencephalitis; and *Acanthamoeba castellanii* inducing keratitis. Understanding the cell differentiation process of encystment is of substantial importance to eliminate amoebae when needed. Indeed, the presence of components such as a proteinaceous, calcareous, or siliceous shell, or a cyst wall composed of cellulose or chitin, allows the persistence of amoebae in adverse conditions. Encysted amoebae resist biocides, radiation, and immune cell attacks [4–6]. Due to the high resistance of cysts, amoebae are also vectors and protection sites for numerous intra-amoebal bacteria [7].

Although morphological modifications that occur during encystment are well described in several organisms, we face many barriers to investigating the molecular pathways involved in this process. For a long time, the lack of genomes and genetic tools for an efficient down-regulation of gene expression rendered the study of gene regulation incomplete for most of the free-living amoebae. These gaps are slowly being filled with more annotated genomes and transcriptomes being published and the use of both efficient transfection solutions and of the CRISPR/Cas technology [8–10] (https://doi.org/10.1101/2022.08.18.504477; https://doi.org/10.1101/2022.12.01.518696). Today, it seems clear that the multitude of amoeba types (naked, protected by a shell, flagellated, single, with the ability to become multicellular) and the absence of integrated omics data represent the future major challenges to establishing signaling pathways for encystment of amoebae.

1.1. Free-Living Amoebae: A Multitude of Forms and Models to Study Encystment

Free-living amoebae exhibit diverse morphological forms resulting in naked, shelled, and/or flagellated amoebae.

Shelled, or testate, amoebae can build an "external skeleton". They belong to different phylogenetic groups. Arcellinida, which extend lobose pseudopodia through the aperture, are classified among Tubulinea class (Amoebozoa supergroup) whereas Euglyphida that protrude filose pseudopodia are found in cercozoans (SAR supergroup) [11,12]. Testate amoebae are abundant in moist soils, freshwater, and wetlands. They present a protective shell with an aperture that protects amoebae against desiccation and predation. The building of the siliceous shell occurs just before cell division and it involves microtubules that support silica deposition vesicles (SDV). Siliceous scales formed within SDV are secreted at the aperture to form a new shell where the daughter cell will move [13]. Testate amoebae display a morphological variability that seems to be associated with environmental factors such as temperature, turbidity, mineral composition of their living medium, or the depth at which they are found [14]. In the soil, there is increasing evidence showing the active role of testate amoebae in silica and nitrogen cycles [15]. In certain conditions, they could consume close to 55 % of the silica absorbed by microorganisms [16]. Preservation of shells in sediments of testate amoebae seems to be a valuable element for paleoecological reconstitution [17]. If the shell is sufficient to ensure the survival of testate amoebae under unfavorable conditions is unclear. Some testate amoebae in the genus *Lecythium* can encyst by building a cyst wall around the protoplast (cell whose wall has been removed) but inside their shell [18]. Interestingly, Cienkowski's drawings suggest that the testate *Chlamydophrys stercorea* exits its shell, and becomes a naked amoeba to build a cyst wall around the protoplast [19]. These two different examples re-enforce the necessity to investigate the encystment process in testate amoebae.

The flagellated phenotype is another state that can be observed in free-living amoebae. Amoebae from the Heterolobosoa can differentiate transiently into flagellate form. Such an event can occur when amoebae are washed off the agar surface to a liquid medium [20]. In nature, that would probably be the flooding of the habitat with water or snow melt. Amoeboflagellates, such as *Naegleria gruberi*, seem to lack a cytoplasmic microtubule cytoskeleton in the growing phase. Under stress, the amoeba transforms into a flagellate state with a typical cytoplasmic cytoskeleton [20]. Interestingly, the presence of flagella is used as a marker for taxonomy. For example, the molecular analysis suggested the origin of Penardeugenia (Cercozoa) in the flagellate class of *Thaumatomonadida*. However, because no flagellated stage was found, *Penardeugenia* could represent a closely related genus to *Thaumatomastix*, which has apparently lost its ability to exhibit flagella [21]. The flagellate state can revert into trophozoïte which can encyst. However, can flagellate amoebae directly form cysts? If not, why? The possible putative connection between the two processes (encystment and degradation of flagella) is worth exploring.

Although encystment results in the encapsulation of a single cell, several amoebae such as Dictyosteliids and Protosteloid behave differently, forming highly complex forms of aggregative multicellular structures stalked with one or several spores [22]. Both encapsulation of a single cell and the construction of a multicellular structure can also be observed in a single organism. For example, under starvation, the amoeba *Dictyostelium discoideum* builds stalked fruiting bodies that allow the dispersal of spore cells in fruiting bodies but also forms sexual amoebae that will mate and create a zygote. The latter cannibalizes aggregated neighbor amoebae to form a cellulose-coated macrocyst [23]. The existence of fruiting spores could concern a larger group of amoebae as a sporocarpic fruiting amoeba has been reported in Acanthamoebidae that was previously thought to encyst only by encapsulation of a single cell [24]. Sexual reproduction during macrocyst formation seems absent in single asexual amoebae even if an expression of meiosis-specific genes was detected. These meiotic genes could have acquired new functions calling for an investigation of their role in encystment [25,26].

1.2. Multi-Omics Data Integration Approaches Have to Be Considered to Reconcile Seemingly Contradictory Data

The advent of high-throughput technology is increasing the availability of a large dataset in genomics, transcriptomics, and proteomics. These high throughput 'omics' technologies are welcome as they can help to decipher underlying encystment molecular mechanisms in so many organisms. Compared to previous experimental results, such massive data could reveal an apparent contradiction that needs another 'omic' level to be solved. For example, we recently investigated the pathways modulated during *Acanthamoeba* encystment [27]. As expected, using the transcriptomics approach, we found that expressions of transcripts related to mitosis were down-regulated during encystment. The transcript level of the cell division control protein 2b (CDC2b), which was suggested to control the cell cycle in *A. castellanii* [28], decreased in encysting cells. Our data were consistent with the Jantzen et al. conclusion on an incompatibility between the progression of the cell cycle and encystment [29]. Indeed, encystment occurs in cells under a cell cycle arrest [30]. Interestingly, although the CDC2b transcript was down-regulated at the beginning of encystment, using proteomics data, we observed that the protein level remained unchanged [27]. The phosphoproteomics approach helped to understand the fine regulation of CDC2b during encystment. In brief, the threonine residue 21 (Thr21) of the CDC2b protein (L8HG09) was less phosphorylated compared to non-encysting cells. This residue aligns with the Thr14 of the cyclin-dependent kinase cdc2, for which dephosphorylation activates the kinase activity [28,31]. Although the CDC2b level remained unchanged at the beginning of encystment, dephosphorylation of the Thr14 could induce the activity of CDC2b before the drop of the protein level a few hours after. Hence, our data could explain how encystment competence slightly precedes the peak of p34cdc2 kinase activity [32] and why encysting cells do not cycle [30]. The emergence of single-cell omics technologies will solve the problems associated with small sample sizes and allow investigation of more and more amoeba species.

1.3. Bacteria Can Teach Us How Free-Living Amoebae Encyst

The use of bacteria to respond to questions in cell biology has tremendously increased our understanding of eukaryotic functions [33,34]. For example, the toxin C3 of *Clostridium botulinum* was a valuable tool to decipher the pathways regulated by the Rho proteins. Free-living amoebae are infected by numerous bacteria that are able either to inhibit [35–37] or to activate encystment [38–41]. Thus, the bacterium *Legionella pneumophila* was shown to inject in *Acanthamoeba polyphaga* an amylase which catalyses glycogenolysis and glycogen depletion impairing the ability of the amoeba to form a mature cyst [30]. In the case of the bacterium *Parachlamydia acanthamoebae*, while it has been shown to inhibit *Acanthamoeba* encystment at a very early stage, its mode of action remains unknown. Authors have suggested that the proliferating parachlamydiae could deprive their host of the resources that are needed for encysting [37]. For bacteria inducing encystment, we still do not know the mechanisms that occur in the amoeba. Deciphering the cross-talk between bacteria and free-living amoebae would also help to understand how amoeba symbionts such as *Protochlamydia* or *Simkania* survive in amoeba cysts [42,43]. With only a few exceptions [36,37], there is no information on the host pathways disturbed by those bacteria which may affect amoebae encystment. Giant viruses which can infect trophozoïte but not cyst represent another actor able to disturb amoeba encystment. Indeed, through an undetermined mechanism, mimivirus could avoid the *Acanthamoeba* encystment by reducing the expression of the encystment-mediating subtilisin-like serine proteinase [44]. Interestingly, it was shown that infected *Vermamoeba vermiformis* are able to trap viruses and to induce encystment of neighbor cells to prevent virus dissemination [45]. Investigating these interactions will undoubtedly provide a considerable number of features that will enrich our picture of encystment pathways existing in free-living amoebae.

2. Conclusions

Encystment is a common stress response of free-living amoebae. Regarding the multitude of organisms capable of encystment, the number of models and the experimental approaches need to be diversified to build, if possible, a complete map of encystment routes.

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