Eukaryote aggregative multicellularity – phylogenetic distribution and a case study of its proximate and ultimate cause in Dictyostelia

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Abstract

Aggregative multicellularity is a response to starvation stress that evolved many times independently across eukaryotes. This chapter summarizes their life cycles and phylogenetic distribution and examines the largest group of sorocarpic organisms, the Dictyostelia, in more detail. Regulation of the multicellular life cycle of the model *Dictyostelium discoideum* is dominated by cyclic AMP (cAMP), which acts intracellularly to induce spore and stalk cell differentiation and extracellularly to coordinate aggregation and fruiting body morphogenesis. This chapter highlights how these functions of cAMP gradually evolved from an original role as intermediate for starvation-induced encystment in a unicellular ancestor. It further reports how adaptation to a colder climate has likely driven the evolution of multicellular sporulation in Dicytostelia.

5.1 Introduction

The evolution of multicellularity provided organisms with a novel means to increase in size and to specialize cells to perform different roles. These abilities were apparently so useful that multicellularity evolved many times independently in both pro- and eukaryotes. All extant multicellular organisms spend at least a part of their life in unicellular form as gamete, zygote or spore, but otherwise a distinction can be made between organisms that feed in multicellular form and those that feed as single cells and come together in response to starvation. The first category comprises the animals, many photosynthetic pro- and eukaryotes, and fungi. A primary characteristic of these organisms is that after division of the initial progenitor cell, the daughter cells remain together and the organism feeds and spends the rest of its life in multicellular form.

The second category comprises many prokaryotes and eukaryotes. They share as a primary characteristic that after cell division the daughter cells swarm out to feed individually. They only come together when deprived of food to form a fruiting structure, inside of which all or most cells enter dormancy. This type of aggregative or facultative multicellularity has reached its highest level of complexity in the Dictyostelia, where the cells inside the multicellular agglomerate have, in addition to dormant spores, specialized into up to five somatic cell types, which lift and support the spore mass and protect it from bacterial infection. This chapter describes the known types of organisms with aggregative multicellularity and their phylogenetic distribution in eukaryotes. The evolutionary trajectory taken by the Dictyostelia to reach their current state of complexity is next discussed in more detail.

5.2 Aggregative multicellularity is evolutionary derived from unicellular encystation

The phenotype of all extant organisms is largely the end result of their existential struggle with environmental stress. A common response to stress is dormancy, which in unicellular eukaryotes is most commonly achieved by encystment. Here the stressed cell encapsulates itself in a resilient wall and shuts down metabolism. After the stress condition, usually starvation or drought, has passed, the cell leaves its capsule and starts feeding again.

Aggregative multicellularity is also triggered by starvation stress, but the aggregated cells form a fruiting structure to aerially lift the encapsulating cells, which are now usually called spores. The fruiting body is called a sorocarp and this type of multicellularity is therefore also called sorocarpic multicellularity. In prokaryotes, the myxobacteria display sorocarpic multicellularity with a wide variety of morphologically distinctive fruiting structures (Kaiser et al., 2010), while in eukaryotes sorocarpic multicellularity evolved independently in almost all major divisions (Figure 5.1).

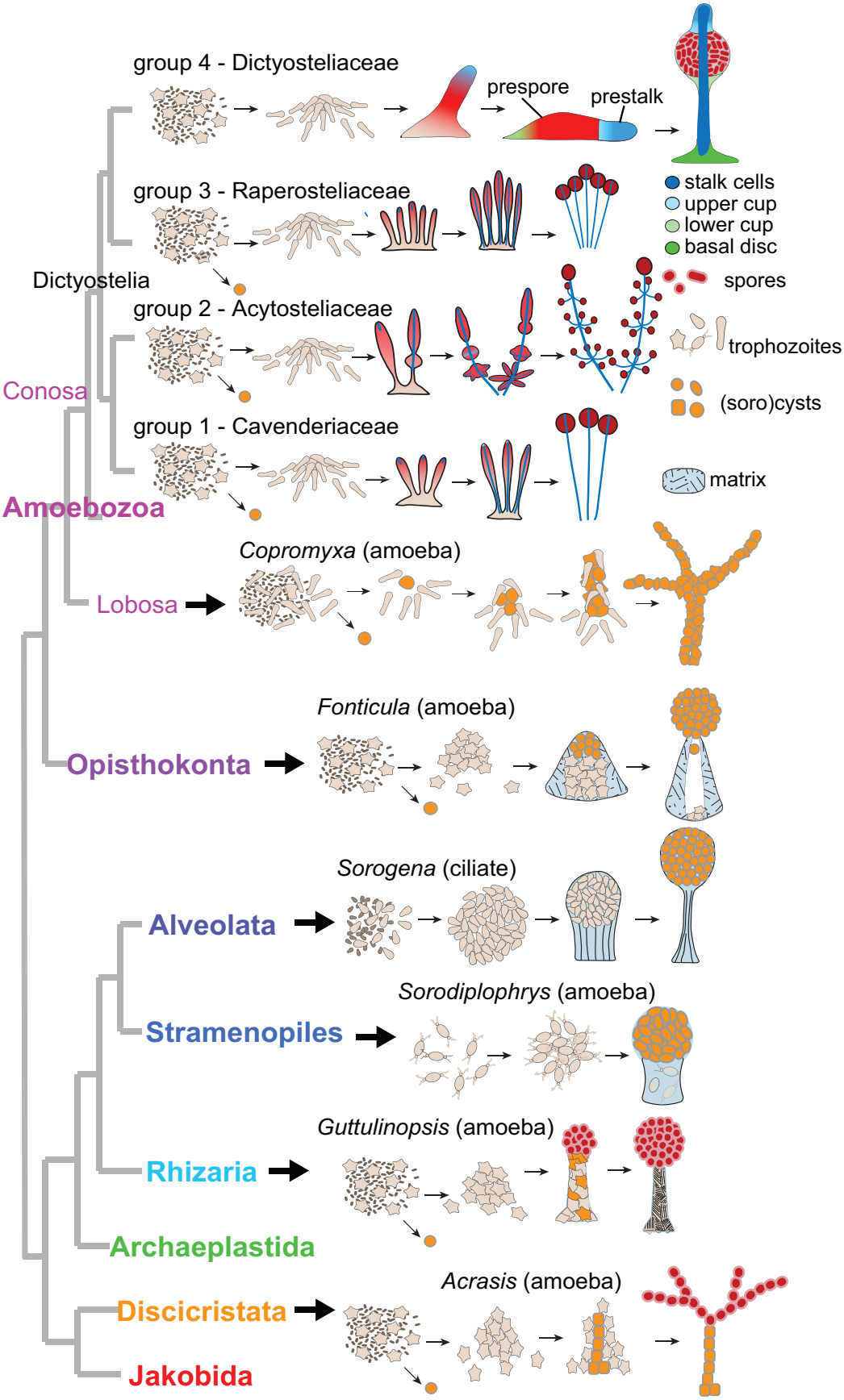
group 4 - Dictyosteliaceae
prespore
prestalk
group 3 - Raperosteliaceae
stalk cells
upper cup
lower cup
basal disc
Dictyostelia
group 2 - Acytosteliaceae
spores
trophozoites
Conosa
(soro)cysts
group 1 - Cavenderiaceae
matrix
Amoebozoa
Copromyxa (amoeba)
Lobosa
Fonticula (amoeba)
Opisthokonta
Sorogena (ciliate)
Alveolata
Sorodiplophrys (amoeba)
Stramenopiles
Guttulinopsis (amoeba)
Rhizaria
Archaeplastida
Acrasis (amoeba)
Discicristata
Jakobida

Figure 5.1. Multiple transitions to aggregative multicellularity in eukaryotes

During aggregative multicellularity cells feed and proliferate as single cells and collect into aggregates when stressed, usually by starvation. The aggregates then transform into a fruiting body (sorocarp) where the cells encapsulate and enter dormancy as either cysts or spores. This type of multicellularity evolved independently in six out of the eight major divisions of eukaryotes. Many organisms with aggregative multicellularity have retained the ability to encyst without aggregating, the survival strategy of their unicellular ancestors. Eukaryote phylogeny (grey lines) after (He et al., 2014). The Dictyostelia are the largest monophyletic group of organisms with aggregative multicellularity with the most extensive cell type specialization in taxon group 4.

In Excavates, the sorocarpic *Acrasis* and *Pocheina* amoebas form a monophyletic clade within the otherwise unicellular allovahlkampfid amoebas. Upon starvation, the amoebas either form globose cysts individually or aggregate to form a globular mound. In the mound, cells start to encapsulate as stalk cells, with others climbing on top and also encapsulating. The stalk cells assume an oblong or cuboidal shape and assemble into tiers of one or several cells wide. The ovoid spores assemble next; in *Acrasis spp.* as single or branched tiers and in *Pocheina* as a globular mass. Despite their different shapes, the cysts, spores and stalk cells all germinate to yield amoebas, when food is available again (Brown et al., 2012b).

In Rhizaria, *Guttulinopsis vulgaris* is part of a small clade of unicellular *Rosculus* amoebae (Bass et al., 2016). When starved, individual amoebas collect into loose aggregates that transform into round mounds. During sorocarp formation amoebae secrete a slimy matrix, which congeals into sheets, inside which they become progressively trapped and then disintegrate. Cells that manage to reach the top of the structure differentiate into irregularly shaped spores that combine to form a globose sorus. Amoebas of the related species *G. nivea* can also encyst individually (Brown et al., 2012a; Raper et al., 1977).

In Stramenopiles, the spindle-shaped *Sorodiplophrys stercorea* amoebae feed on dung by osmotrophy (uptake of dissolved organic compounds by osmosis) and move using long branched filopodia that extend from the spindle poles. When deprived of food, the cells move in streams towards aggregation centres. A relatively firm basal core develops within the aggregate that consists of gelatinous matrix and dead and degenerating cells. Amoebas that surround and surmount this core develop into sorocytes; they retract the filopods, decrease in size and form a thin but distinctive cell wall. No cysts have been observed (Dykstra and Olive, 1975; Tice et al., 2016).

In Alveolata, the ciliate *Sorogena stoianovitchae* feeds underwater on smaller colpodid ciliates. Food depletion causes the cells to collect into a loose aggregate, which next compacts by cell adhesion. The cells secrete mucous material that forms a sheath around the aggregate and the structure now rises to the water surface. Continued matrix production and contraction of the sheath causes a stalk to form that pushes up the cells, which then encyst. Aggregation occurs only in the dark, but fruiting body formation requires a light period (Olive and Blanton, 1980; Sugimoto and Endoh, 2006).

In Opisthokonta, the nucleariid amoeba *Fonticula alba* displays long filopodia while feeding on bacteria. Upon starvation, the amoebas move together individually to form a mound that

becomes surrounded by a slime sheath. The amoebas continue to secrete extracellular matrix that accumulates between the cell mass and the sheath. Starting from the top of the now volcano-shaped structure the amoebae encyst as spores, which are expelled through the top to form a globose sorus. The amoebas can also encyst individually without aggregating, with the cysts being morphologically identical to spores (Brown et al., 2009; Worley et al., 1979)

In Amoebozoa, sorocarpic multicellularity evolved in both the subphyla Lobosa and Conosa (Cavalier-Smith et al., 2015). In Lobosa, amoebae of the genus *Copromyxa* move by extending a single broad pseudopod. After depletion of their bacterial food, some amoebae encyst and become founder cells for the fruiting structure. The starving amoebas move towards an encysted founder cell, crawl on top and then also encapsulate to form sorocysts. This process continues until a tall sorocarp has formed, which is often branched. Amoebas can also encyst individually, and these microcysts are indistinguishable from the somewhat irregularly shaped sorocysts. Alternatively, they can form a round sphaerocyst after sexual fusion (Brown et al., 2011; Raper et al., 1978; Spiegel and Olive, 1978).

In Conosa, the Dictyostelia are the largest monophyletic group of organisms with sorocarpic multicellularity. The ~150 known species of Dictyostelia can be subdivided into four major groups, recently classified as the families Cavenderiaceae (group 1), Acytosteliaceae (group 2), Raperosteliaceae (group 3) and Dictyosteliaceae (group 4) (Sheikh et al., 2018). Sorocarps in groups 1, 2 and 3 consist of at most two cell types, stalk cells and spores, and are often branched or clustered. Many species in these groups have retained the ability to encyst individually, and most use the dipeptide glorin as chemoattractant for aggregation. In contrast, group 4 species lost encystation and use 3’5’-adenosine monophosphate (cAMP) as chemoattractant. They generally form large, solitary and unbranched sorocarps, which contain three novel cell types to support the stalk and spore mass. Many species throughout Dictyostelia have the additional ability to form sexual macrocysts. Here two starving cells fuse to form a zygote that subsequently attracts other starving cells and devours them to use their resources to construct a thick cell wall (Raper, 1984; Romeralo et al., 2013). The group 4 species *Dictyostelium discoideum* is a model system for research into the molecular mechanisms that control phagocytosis, motility, chemotaxis, morphogenesis, cell differentiation, and evolution of sociality and is, apart from a few other Dictyostelia, the only sorocarpic or indeed only amoeboid organism with well-developed procedures for gene modification. We therefore concentrate in the remainder of this chapter on the evolution of multicellularity in the Dictyostelid lineage.

5.3 Proximate cause of *Dictyostelium* multicellularity – how it happened

5.3.1 Regulation of multicellular development in the model D. discoideum

Over the past 40 years, the molecular mechanisms that control the developmental program of the model *D. discoideum* in group 4 have been thoroughly investigated. *D. discoideum* is one of the most morphologically complex Dictyostelia, and until recently there was only limited information on developmental control mechanisms in the other taxon groups. Research into the evolution of multicellularity in Dictyostelia therefore required a top-down approach. A well-resolved phylogeny of Dictyostelia (Schaap et al., 2006; Schilde et al., 2019), the availability of taxon group-representative genome sequences (Eichinger et al., 2005; Gloeckner et al., 2016; Heidel et al., 2011; Narita et al., 2020; Sucgang et al., 2011) and the development of genetic transformation for species outside of group 4 (Fey et al., 1995; Narita et al., 2020) were crucially important to address this problem.

A remarkable aspect of *D. discoideum* development is that so much of it is regulated by cAMP (Figure 5.2). As a secreted signal, cAMP controls cell movement and differentiation throughout development, while as an intracellular messenger, it mediates the effect of most other developmental signals. Intracellular cAMP is detected by cAMP-dependent protein kinase or PKA, while secreted cAMP targets G-protein coupled cAMP receptors (cARs). PKA activity is required for basal expression of aggregation genes (Schulkes and Schaap, 1995), for the expression of most prespore genes (Hopper et al., 1993), for the maturation of spores and stalk cells (Harwood et al., 1992; Hopper et al., 1993) and for keeping spores dormant in the spore head (Van Es et al., 1996). Upon starvation, PKA is upregulated by removal of the translational repressor PufA from its 3' untranslated region (Souza et al., 1999). The adenylate cyclases AcgA and AcrA activate PKA in prespore and spore cells (Alvarez-Curto et al., 2007; Soderbom et al., 1999), is activated by solute stress caused by high levels of ammonium phosphate in the spore head, while AcaA is activated by secreted c-di-GMP (Chen et al., 2017; Cotter et al., 1999; Van Es et al., 1996).

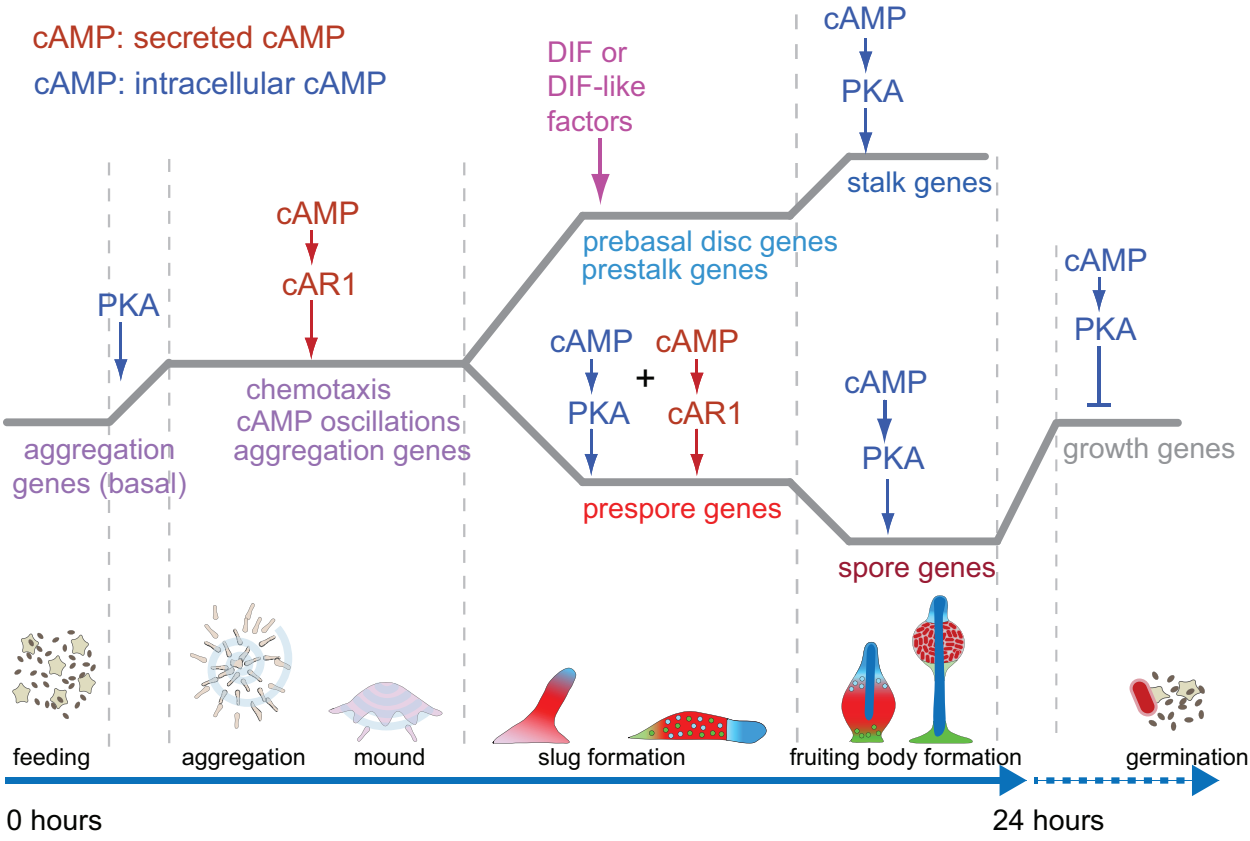

Figure 5.2. Roles of intracellular and secreted cAMP in *Dictyostelium discoideum*

Intracellular cAMP (in blue) acting on cAMP dependent protein kinase (PKA) and secreted cAMP (in red) acting on the cell surface cAMP receptor cAR1 regulate most aspects of the multicellular life cycle. See main text for a detailed description.

Many other developmental signals act on the intracellular cAMP phosphodiesterase RegA to regulate PKA activity. RegA activity requires phosphorylation of its intrinsic response regulator domain, which in turn depends on the activity of sensor histidine kinases/phosphatases (Shaulsky

et al., 1998; Thomason et al., 1998). These enzymes are the target for developmental signals, such as DhkA for the spore inducing peptide SDF-2 (Wang et al., 1999), DhkC for the stalk inhibitor ammonia (Singleton et al., 1998), DhkB and DokA for respectively discadenine and high osmolarity (Schuster et al., 1996; Zinda and Singleton, 1998), which promote spore maturation and inhibit germination.

The cAMP receptor cAR1, AcaA and the extracellular cAMP phosphodiesterase PdsA and several other proteins, amongst which PKA and RegA, form an excitable network that can spontaneously generate cAMP oscillations (Laub and Loomis, 1998). Secreted cAMP acts as a chemoattractant, and the cAMP pulses coordinate both aggregation of the starving cells (Konijn et al., 1967) and the post-aggregative cell movements that result in sorogen migration and the upward projection of the fruiting body (Singer et al., 2019). For these post-aggregative roles, *acaA* becomes specifically expressed at the tip of the emerging sorogen, which thereby becomes the organizer of morphogenesis (Verkerke-van Wijk et al., 2001). AcaA hyperactivation by c-di-GMP at the tip ensures that the *Dictyostelium* stalk is always formed at the morphogenetic organizer (Chen et al., 2017). The secreted cAMP pulses also act on cAR1 to further upregulate the expression of aggregation genes, such as *cAR1*, a*caA*, and *pdsA,* enabling rapid aggregation (Gerisch et al., 1975), while cAMP accumulates in aggregates on cAR1 to induce the expression of prespore genes (together with PKA) (Schaap and Van Driel, 1985).

Some developmental signaling events are thus far not known to require cAMP. Prespore cells secrete a chlorinated polyketide, DIF-1, that induces the differentiation of basal disc cells and contributes to robust stalk formation by increasing cytosolic Ca^{2+} (Kubohara et al., 2007; Poloz and O'Day, 2012; Schaap et al., 1996). Interaction between two highly polymorphic cell surface proteins, TgrC1 (a.k.a. LagC) and TgrB1, mediates kin recognition in *D. discoideum,* allowing only closely related strains to contribute to the same sorogen. TgrC1 acts as ligand and TgrB1 as receptor in this interaction, which results in phosphorylation of TgrB1 and eventually in expression of a set of post-aggregative genes (Benabentos et al., 2009; Dynes et al., 1994; Hirose et al., 2017).

5.3.2 The evolution of cAMP signaling in the Dictyostelia

The dominant role of cAMP in all aspects of the *D. discoideum* life cycle is suggestive of a deeper ancestral role. Such a role was likely mediated by cAMP acting on PKA rather than cARs, since PKA is present in most eukaryotes and cARs are thus far only found in Dictyostelia. Comparative genome analysis revealed that the PKA catalytic (PkaC) and regulatory (PkaR) subunits and the enzymes AcrA, AcgA, AcaA and RegA are conserved throughout the four *Dictyostelium* taxon groups (Gloeckner et al., 2016), and that PKA, AcrA and RegA are also present in the solitary Amoebozoa *Acanthamoeba castellani*, *Physarum polycephalum* and *Protostelium aurantium* (Clarke et al., 2013; Hillmann et al., 2018; Schaap et al., 2015).

Deletion of *PkaC* in the group 2 Dictyostelid *Polysphondylium pallidum,* which can both encyst individually or aggregate to form fruiting bodies, blocked not only aggregation, but also encystation. Deletion of *AcrA* and *AcgA* together also prevented encystation (Kawabe et al., 2015). Conversely, deletion of *RegA* in *P. pallidum* accelerated multicellular sporulation as it does in *D. discoideum*, and also caused precocious encystation during feeding. Pharmacological inactivation of RegA in *A.castellani* also caused amoebas to encyst precociously (Du et al., 2014). In conclusion, cAMP acting on PKA and regulated by at least RegA appears to have an ancestral role as intracellular messenger for the induction of encapsulation of starving solitary

Amoebozoa into cysts (Figure 5.3). This role persisted in Dictyostelia and became elaborated to additionally control the initiation of aggregation and the encapsulation of starving amoebas in spores and stalk cells. The genomes of the solitary *A. castellani*, *Phy. polycephalum* and *Pro. aurantium* contain many sensor histidine kinases/phosphatase, and while no biological role for any of these enzymes has yet been demonstrated, it is likely that at least some of them regulate the activity of RegA in these organisms.

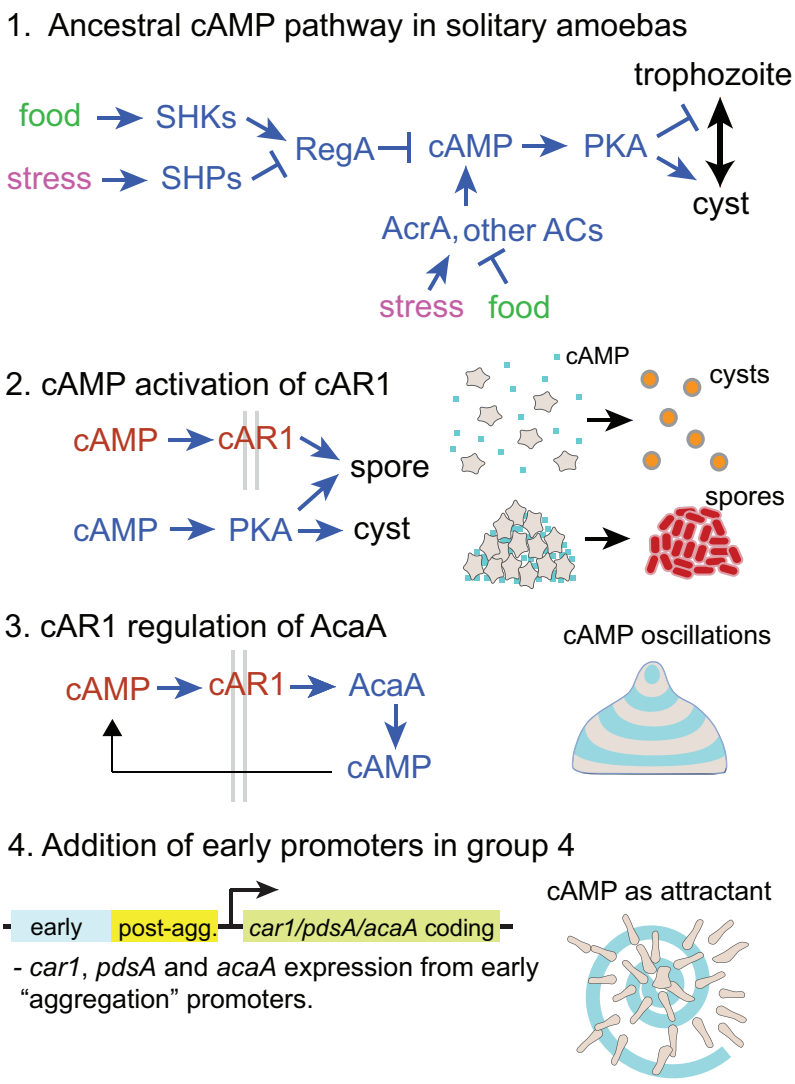

Figure 5.3. Evolution of cAMP signalling in Dictyostelia

Putative scenario for the evolution of intra- and extracellular cAMP signalling in Dictyostelia. See main text for description. SHK: sensor histidine kinase; SHP sensor histidine phosphatase. Drawing revised from (Kawabe et al., 2019)

While dictyostelids in groups 1, 2 and 3 do not use cAMP as an attractant to aggregate, cARs and PdsA are conserved in these groups, suggesting at least some roles for extracellular cAMP. Loss of its two *cAR* genes or its *pdsA* gene had no effect on aggregation in *P. pallidum,* but

massively disrupted fruiting body morphogenesis, with stalk cells differentiating in random clumps (Kawabe et al., 2009; Kawabe et al., 2012). Sp-cAMPS, a non-hydrolysable cAMP analog that disrupts oscillatory cAMP signaling, equally disrupted fruiting body morphogenesis in group 1, 2 and 3 species (Romeralo et al., 2013), indicating that cAMP oscillations ancestrally emerged to coordinate post-aggregative morphogenesis. Oscillatory cAMP signalling then moved forward in development in group 4 to organize aggregation as well. Addition of distal "early" promoters to proximate "late" promoters of *cAR1*, *pdsA* and *acaA* genes likely enabled the use of cAMP as attractant for aggregation (Alvarez-Curto et al., 2005; Faure et al., 1990; Galardi-Castilla et al., 2010; Louis et al., 1993).

Organisation of morphogenesis may however not have been the earliest role for secreted cAMP. The *P. pallidum* cAR-less cells made cysts instead of spores in their aberrant fruiting bodies and lost cAMP-induced prespore gene expression. Encystment only requires intracellular cAMP acting on PKA, while sporulation additionally requires extracellular cAMP acting on cARs. Without cARs, the *P. pallidum* presumptive spore cells reverted to the ancestral process of encystation (Kawabe et al., 2009) and points to what might be the most ancestral role for secreted cAMP. All starving Amoebazoa synthesize cAMP to activate PKA and encyst. Dictyostelia secrete most of their cAMP and may originally have used accumulation of secreted cAMP in aggregates to instruct the starving cells to form spores and not cysts.

The role of DIF-1 in basal disc and robust stalk cell differentiation is not conserved outside group 4, despite the fact that the genes encoding the enzymes, StlB, DmtA and ChlA, which synthesize DIF-1, are present throughout Dictyostelia. However, a group 2 *dmtA* fails to restore a *D. discoideum dmtA* null mutant, indicating that it has another molecular function in group 2 (Mohri et al., 2014), while deletion of *stlB* in *Polyspondylium violaceum*, a sister species to group 4, causes more instead of fewer stalk cells to differentiate (Narita et al., 2020). Since basal disc cells are unique to group 4, it is likely that Dictyostelia co-evolved this trait with the signal (DIF-1) that induces basal disc differentiation.

GSK3 (glycogen synthase kinase 3) which, as component of the wingless/wnt pathway, determines cell fate decisions in animal development (Forde and Dale, 2007), was also reported to control cell fate choice in *D. discoideum*, by preventing the DIF-1 induced transition of prespore cells into basal disc cells (Harwood et al., 1995; Schilde et al., 2004). This role is not conserved in *P. pallidum*, where instead active GSK3 favours entry of starving cells into aggregation rather than encystation (Kawabe et al., 2018). While group 4 species such as *D. discoideum* lost encystation, remnants of this role in early development are still evident in a GSK3 requirement for expression of some early genes (Strmecki et al., 2007) and for efficient chemotaxis (Teo et al., 2010). Like the role of DIF-1, the role of GSK3 in basal disc differentiation appears to have co-evolved with the emergence of this novel cell type in group 4.

In conclusion, the dominant roles of secreted and intracellular cAMP in controlling *D. discoideum* multicellular development emerged from an ancestral role of intracellular cAMP as an intermediate for stress-induced encystation (Figure 5.3). A requirement for secreted cAMP acting on cARs to induce sporulation in aggregates (as opposed to encystation) may have been the first role of secreted cAMP. Next, incorporation of cARs, PdsA, AcaA, RegA and PKA into an excitable network yielded the cAMP waves that enabled morphogenesis of well-structured fruiting bodies, while finally pre-aggregative expression of *cARs*, *pdsA* and *acaA* enabled the use of cAMP as a chemoattractant in group 4.

5.4 Ultimate cause of *Dictyostelium* multicellularity – why it happened

Similar to many sorocarpic organisms, Dictyostelia evolved from a unicellular ancestor that survived starvation by differentiating into a cyst. The fact that sorocarpic multicellularity evolved so frequently across eukaryotes suggests an advantage to sporulating in fruiting bodies as opposed to unicellular encystment. An obvious benefit is improved spore dispersal from the aerially lifted spore head. This is particularly true for hydrophobic spores, such as those of fungi, which are carried by the wind. However, hydrophilic spores, such as those of Dictyostelia, depend for dispersal on sticking to soil invertebrates (Smith et al., 2014), or on being washed away by rain or snowmelt, the latter also causing dispersal of the soil-bound cysts. The aerial uplift of the spore agglomerate may further protect spores from decomposing influences in soil and predation by larger protists. Particularly for Dictyostelia with their small cells, predation may initially have encouraged starving amoebas to stick together, while going through encystation. Aggregation of solitary starving amoebas prior to encystation is reported for the holozoan amoeba *Capsaspora owczarzaki* (Sebe-Pedros et al., 2013), while the unicellular alga *Chlamydomonas reinhardtii* was shown to evolve multicellular structures when exposed to predation by ciliates for many generations (Herron et al., 2019).

For most sorocarpic protists, the spores only differ from cysts in being aloft, but in Dictyostelia the two forms are also morphologically distinct. Spores are predominantly elliptical with dense cytoplasm and a three-layered cell wall, while cysts are round and less dense with a two-layered wall (Hohl and Hamamoto, 1969; Hohl et al., 1970). This suggests that physiological differences between spores and cysts may have contributed to consolidation of the multicellular lifestyle of Dictyostelia. Long term survival experiments of spores and cysts of representative species of each taxon group showed that spores and cysts, resuspended in water, survived temperatures above 20^{o}C equally well, but that spores outperformed cysts at 4^{o}C and -20^{o}C. Cysts showed particularly poor survival when subjected to dry frost. Among taxon groups, group 4 spores were the most cold resistant. At the ultrastructural level, this was correlated with group 4 spores displaying a combination of dense cytoplasm and a thick spore wall. Global distribution data showed that group 4 species were frequently isolated from alpine and arctic zones, which was rarely the case for species in groups 1, 2 and 3. A fossil-calibrated phylogeny of Amoebozoa sets the split between the two major branches of Dictyostelia at 0.52 billion years ago, following the global glaciations known as “snowball earth”. Combined, these observations suggest that *Dictyostelium* sporulation in multicellular fruiting bodies was an adaptation to cold climate (Lawal et al., 2020).

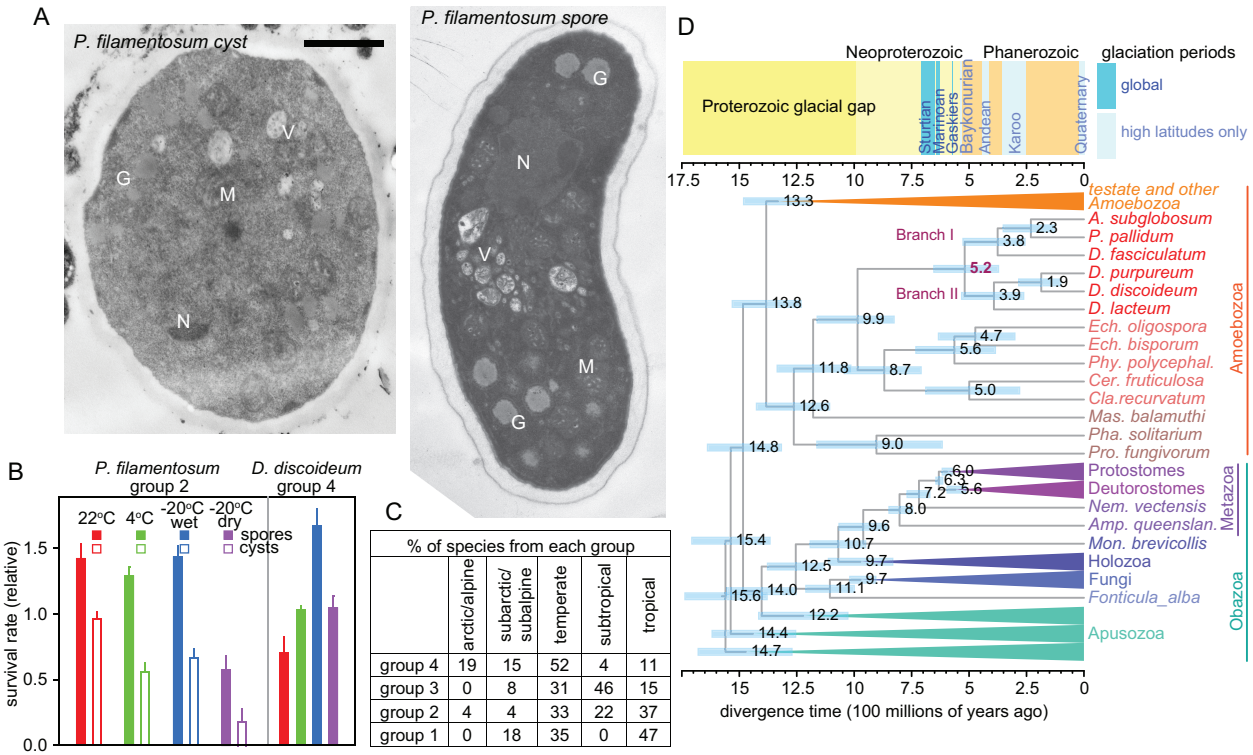

Figure 5.4. A cooling climate likely triggered multicellular sporulation in Dictyostelia

Spores are more compact than cysts and have a 3-layered instead of maximally 2-layered cell wall (A). Spores are more frost-resistant than their cysts with group 4 spores being most resistant (B), which is correlated with group 4 species being more frequently isolated from arctic and alpine habitats (C). The two major branches of Dictyostelia diverged 0.52 billion years ago, following several global glaciation periods (D). Data taken from (Lawal et al., 2020). Bar in (A): 1 µm; N: nucleus; M: mitochondria; V: vesicles; G: granules.

Whether climate change also played a role in the evolution of other sorocarpic protists is unknown. The fact that spores and cysts of *Fonticula alba*, *Copromyxa spp.* and *Acrasis spp.* are morphologically identical makes this unlikely. For these organisms, ultimate causes for their transitions to multicellularity may yet be found in protection from predation and/or improved spore dispersal and preservation. An important factor is the geological time at which the multicellular forms first appeared. Dictyostelia likely evolved on land since their aggregation and fruiting body formation require an air-water interface. They emerged when animal life was still bound to the oceans, invalidating the argument for improved spore dispersal by soil invertebrates. However, this may still have been a factor in the evolution of aggregative multicellularity in other organisms and in the later evolution of Dictyostelia (Smith et al., 2014).

The question remains why dictyostelid cysts did not simply evolve higher frost resistance on their own. The answer may lie in the stalk cells, which undergo extreme autolysis, not merely functioning to lift the spores, but also to provide them with metabolites for spore wall synthesis. This would be analogous to the sexual macrocysts of Dictyostelia cannabalizing their brethren to construct their very substantial cell walls. A flux of metabolites from stalk to spore cells has however yet to be demonstrated.

Inferring events that happened in the distant past is fraught with uncertainty. However, since the events that triggered multicellularity will also have contributed to shaping the mechanisms that control multicellular development, it is important to investigate ultimate cause, if only to have it questioned and analysed further by other workers.

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