

# Evolutionary crossroads in developmental biology: *Dictyostelium discoideum*

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## Summary

*Dictyostelium discoideum* belongs to a group of multicellular life forms that can also exist for long periods as single cells. This ability to shift between uni- and multicellularity makes the group ideal for studying the genetic changes that occurred at the crossroads between uni- and multicellular life. In this Primer, I discuss the mechanisms that control multicellular development in *Dictyostelium discoideum* and reconstruct how some of these mechanisms evolved from a stress response in the unicellular ancestor.

**Key words:** Evolution of multicellularity, Social amoeba, Encystation, Sporulation, *Dictyostelium*

## Introduction

The social amoebas, or *Dictyostelia*, are a group of organisms that become multicellular by aggregation and then proceed to build fruiting bodies that consist of stalk cells and spores (see Glossary, Box 1). This developmental cycle is a response to starvation, and involves both highly coordinated cell movement and a tightly controlled programme of cell differentiation. Social amoebas belong to the supergroup of Amoebozoa (see Glossary, Box 1), a sister group to the clade of Opisthokonts (see Glossary, Box 1), which contains the animals and fungi. All supergroups contain unicellular protist-like organisms (see Glossary, Box 1) and, in several groups, multicellular life forms have evolved independently (Minge et al., 2009).

The Amoebozoa supergroup consists mainly of unicellular amoeba-like organisms that have a simple life cycle. The feeding amoeba or trophozoite turns into a dormant cyst when faced with food shortage, drought or other life-threatening circumstances (Cavalier-Smith et al., 2004). Several clades of Amoebozoa have given rise to protostelid-like organisms (see Glossary, Box 1), which form a very simple fruiting body (see Glossary, Box 1) that consists of a single spore that sits on a simple stalk made by the same cell (Shadwick et al., 2009). However, only the *Dictyostelia* are able to form multicellular fruiting bodies that contain up to a million cells (Fig. 1).

There are ~120 known species of *Dictyostelia* and a molecular phylogeny has been constructed to reveal the order in which they evolved (Schaap et al., 2006). The phylogeny subdivides species into four major groups (Fig. 2), with the model social amoeba *Dictyostelium discoideum* belonging to the most recently diverged group 4. Many species in groups 1–3 can still encyst as single cells when conditions are unfavourable for aggregation. However, group 4 species have lost the ability to encyst. Strikingly, all group 4 species tested secrete cyclic adenosine monophosphate (cAMP) to

act as a chemoattractant for aggregation. This is most unusual, because almost all other organisms only use cAMP inside the cell to transduce the effect of other secreted stimuli, such as hormones, mating factors and neurotransmitters. None of the species in groups 1–3 uses cAMP for aggregation; some use glorin, a modified dipeptide of glutamate and ornithine, whereas others use folic acid, pterin or other as yet unidentified chemoattractants (Schaap et al., 2006).

In this Primer, I first explain the advantages of conducting research in this model organism and discuss the techniques that have led to the major advances in this field. I then present an

## Box 1. Glossary

**Amoebozoa.** A monophyletic supergroup of Eukaryotes that unifies lobose amoebas, pelobionts, entamoebids, dictyostelids and myxogastrids.

**Cre-loxP system.** A genetic tool to control site-specific DNA recombination. It consists of an enzyme, Cre recombinase, that catalyzes deletion of undesired DNA sequences, which are positioned between loxP sequences. The loxP sequences contain specific binding sites for Cre recombinase where recombination can occur.

**Encapsulate.** To build up a protective cell wall.

**Epistasis.** Genetic interactions in which the mutation of one gene masks the phenotypic effects of a mutation at another locus.

**Fruiting body.** A specialized spore-producing structure.

**Opisthokonts.** A broad group of eukaryotes, including the animal and fungus kingdoms and some groups of eukaryotic microorganisms that were previously assigned to the protist 'kingdom'.

**Osmolality.** The concentration of a solution expressed in osmoles per kilogram of solvent.

**Protist.** A diverse group of mostly unicellular eukaryotes of paraphyletic origins that do not have much in common besides a relatively simple organization.

**Protostelid.** A paraphyletic collection of amoebozoans that form simple fruiting bodies consisting of one or a few spores on an acellular stalk.

**Sensor histidine kinase/phosphatase.** A protein consisting of a sensor region, which, upon binding of a ligand, controls the activity of an attached histidine kinase/phosphatase enzyme. Following ligand binding, this enzyme can exhibit either autophosphorylation or autodephosphorylation activity of a histidine residue.

**Spore.** A dormant cell form, which is usually desiccated and surrounded by a resilient wall that allows a cell to survive drought, extreme temperatures and lack of nourishment for long time periods.

**Stalk.** An elongated support or structure that, in dictyostelids, supports a mass of spores.

**Supergroup.** One of the six major monophyletic groups of eukaryotes.

**Suppressor genetics.** The introduction of a mutation that restores the wild-type phenotype in an organism with a mutationally altered genotype.

overview of the model organism *D. discoideum* and the cell communication systems that control cell differentiation and morphogenetic cell movement during its development into multicellular fruiting bodies. Finally, I discuss how the important communication systems that *D. discoideum* uses evolved from a stress response in their unicellular ancestor and how the elaboration of this original response has contributed to the evolution of multicellularity in the *Dictyostelia*.

### Experimental advantages of working with social amoebas

*D. discoideum* is amenable to a broad range of experimental and genetic approaches. All known *Dictyostelia* species, including *D. discoideum*, can be cultured in the laboratory on bacterial lawns on agar, and several species can be grown as mass cultures in liquid media containing glucose and peptone, or in a defined mixture of amino acids and vitamins. This greatly facilitates the isolation and purification of cellular products for biochemical analysis and proteomics, and allows various analytical procedures to be used in *D. discoideum* research that depend on the isotopic labelling of cellular components.

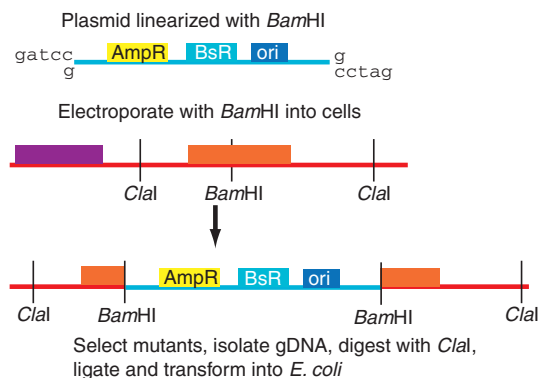
The *D. discoideum* life cycle (as discussed in more detail below) is rapid at just 24 hours, as it is for most *Dictyostelia* species. Its multicellular structures are also transparent, allowing the visualization of cell movement and of gene expression through the use of a broad range of fluorescent, enzymatic or antigenic protein tags, or by in situ hybridization (Gerisch and Muller-Taubenberger, 2003; Maeda et al., 2003).

Both *D. discoideum* and the group 2 species *Polysphondylium pallidum* can be transformed with plasmid vectors, and genes of interest can be altered or knocked out by homologous recombination (Box 2). When required, multiple genes can be disrupted in potentially endless succession by excising the selectable marker after gene disruption with the Cre-loxP system (see Glossary, Box 1) (Faix et al., 2004). Genes can be expressed under constitutive or inducible promoters with a variety of tags for the visualization or purification of the cognate protein. Novel genes in important processes can also be identified by tagged mutagenesis (Box 2), and complex pathways and processes can be unravelled by epistasis and by suppressor genetics (see Glossary, Box 1) (Kuspa and Loomis, 1992; Shaulsky et al., 1996). The *D. discoideum* genome is completely sequenced (Eichinger et al., 2005) and a genome browser with curated and annotated gene models is available at the community resource Dictybase (<http://dictybase.org/>). This resource also holds a large collection of *Dictyostelium* species, mutants and plasmid constructs, which are provided free of charge to the community.

Owing to this experimental tractability, *D. discoideum* has become one of the foremost organisms in which to study fundamental processes in cell biology, such as chemotaxis, cytokinesis, phagocytosis, vesicle trafficking, cell motility and signal transduction. It has led the way in identifying the components that make up and control the actin microfilament system and has been extremely powerful in visualizing how these components dynamically interact with each other during chemotaxis, phagocytosis and cytokinesis (Cosson and Soldati, 2008; Shina et al., 2010; Swaney et al., 2010; Zhang et al., 2008). The *D. discoideum* genome contains many orthologues of genes that are defective in a range of human diseases and is becoming a popular model for studying how such defects impact on normal cell behaviour (Williams et al., 2006). The organism also leads the way in understanding how controlled cell movement cooperates with

### Box 2. Genetic approaches available in *Dictyostelia*

#### REMI mutagenesis

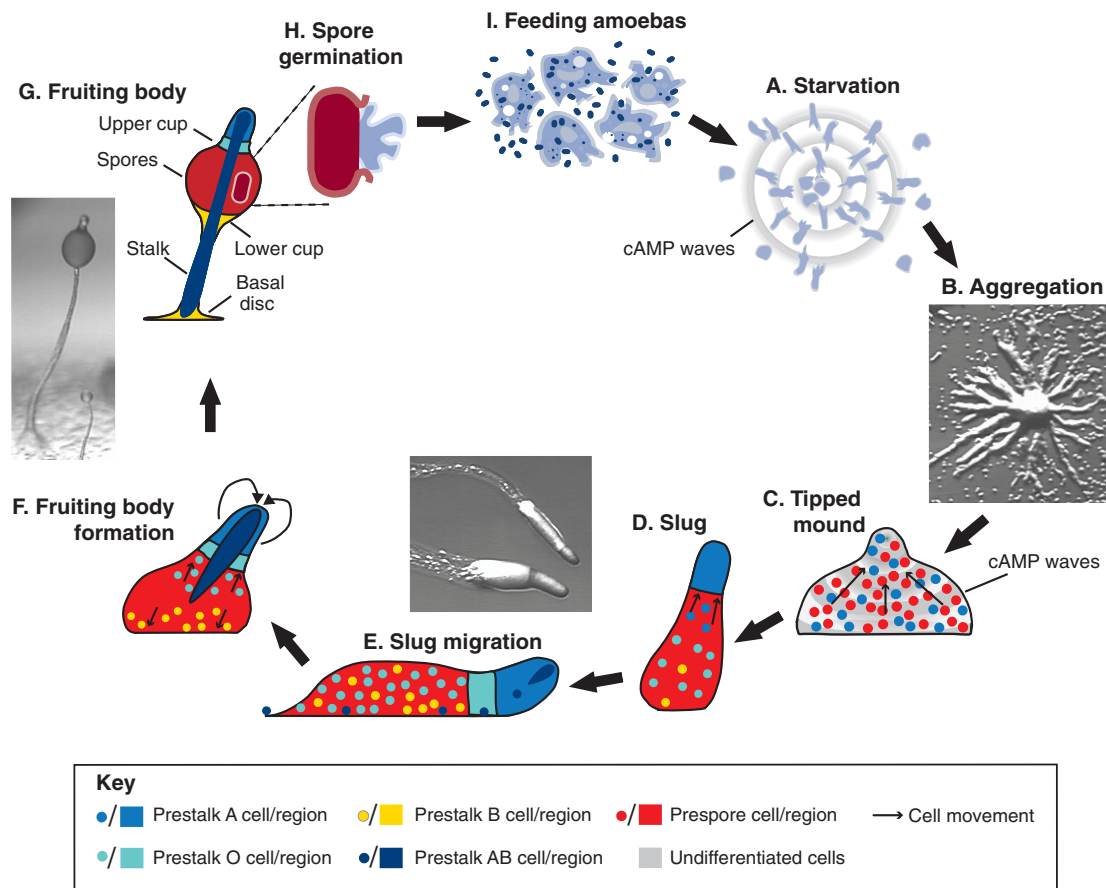


Both reverse and forward genetics approaches can be used in *D. discoideum* and in the group 2 species *P. pallidum*. In both of these species, genes can be knocked out using a standard homologous recombination approach. Populations of tagged mutants can also be generated by restriction enzyme-mediated integration of plasmid vectors (REMI) (Kuspa and Loomis, 1992). As shown in the accompanying figure, a bacterial plasmid carrying the blasticidin (*BsR*) selection cassette, in addition to its own origin of replication (*ori*) and an ampicillin resistance cassette (*AmpR*), is linearized with a restriction enzyme (such as *Bam*HI). This construct is then electroporated into *Dictyostelium* cells together with *Bam*HI or an enzyme that produces the same sticky ends. The enzyme creates cuts in the genomic DNA that are repaired by ligases within the cell, which occasionally leads to the insertion of the plasmid and the generation of a mutant. Cells that harbour plasmid insertions are selected by growth in the presence of blasticidin and can then be screened for mutant phenotypes by direct observation or using a range of selection protocols. The mutated gene is identified by cutting the genomic DNA with a suitable enzyme (*Clal* in this example), circularizing all fragments by ligation and introducing the fragments into *Escherichia coli*. Growth in the presence of ampicillin selects for those bacteria that contain the plasmid, which is then sequenced to identify the *Dictyostelium* gene fragment. The plasmid can then be reintroduced into *Dictyostelium*, where it should reproduce the mutant phenotype after homologous recombination.

regulated cell differentiation to generate shape and pattern during multicellular development (Kimmel and Firtel, 2004; Weijer, 2009; Williams, 2006).

### The habitat and life cycle of *Dictyostelia*

The *Dictyostelia* social amoebas are found in a wide range of soil habitats, ranging from arctic to tropical regions and from desert to rainforest. They are most prevalent in the leaf litter of tropical to temperate forests, which is the richest source of their bacterial food (Swanson et al., 1999). Individual species can utilize one of three survival strategies when food runs out. (1) They can encapsulate (see Glossary, Box 1) individually to form a dormant cyst called a microcyst. This process is favoured in dark and wet conditions, or if ammonia levels or osmolality (see Glossary, Box 1) are high (Raper, 1984). (2) They can fuse to form a zygote. This usually requires amoebas of opposite mating types. The zygote then attracts and cannibalizes the surrounding amoebas and uses their resources to build a thick-walled sphere, the macrocyst. This sexual



**Fig. 1. The life cycle of *Dictyostelium discoideum*.** (A) Starving *D. discoideum* amoebas secrete pulses of cyclic adenosine monophosphate (cAMP, indicated by grey rings) that (B) cause the chemotactic aggregation of cells into mounds. Cells in the mound move upwards in response to continued cAMP emission from its top, (C) causing the formation of a tipped mound. The differentiation of cells into two cell types, prespore and prestalk A, begins in these mounds. (D) A mound then forms a slug, in which further differentiation of cells into prestalk O, B and AB cells occurs. (E) The slug then falls over and starts to migrate. (F) Finally, it undergoes fruiting body formation, during which the different cell types migrate to specified locations in the fruiting body and (G) ultimately differentiate into spores, stalk cells and the structures that support the stalk and spore head. (H) After their dispersal to nutrient-rich habitats, spores germinate and (I) resume proliferation as individual amoebae. Modified with permission from Schaap, 2007 (Schaap, 2007).

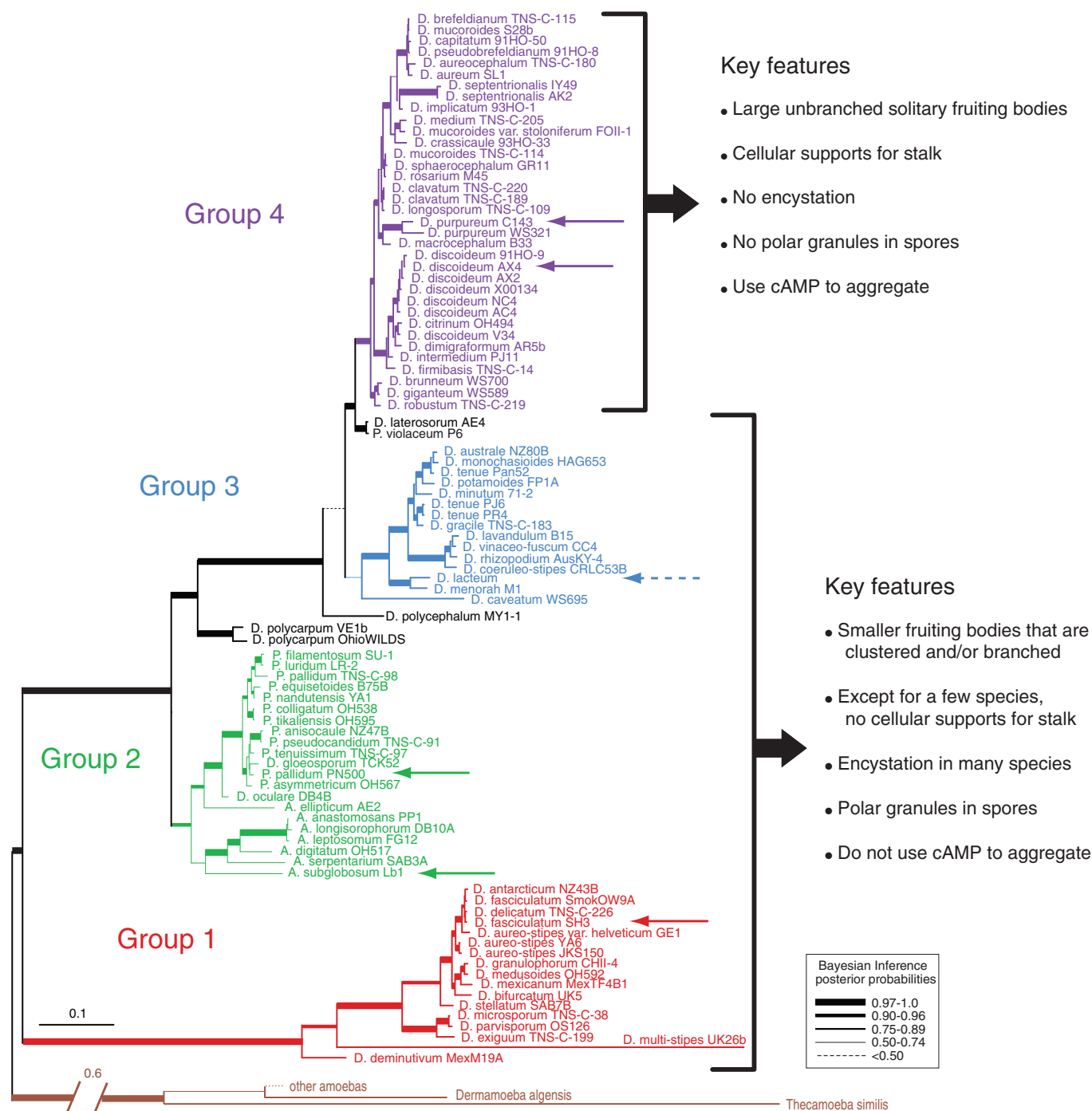
life cycle also occurs under dark and wet conditions, and is triggered by ethylene produced by the cells (Amagai et al., 2007). (3) They can come together to form a fruiting structure, in which a proportion of cells are sacrificed to build the stalk and the remainder differentiate into resilient dormant spores. This multicellular life cycle (Fig. 1) is common to all *Dictyostelids*. It consists of an interconnected programme of cell movement and cell differentiation, and has been intensively studied in the model organism *D. discoideum*.

#### From unicellular amoeba to fruiting body

During growth, unicellular *D. discoideum* amoebas monitor their cell density relative to that of their bacterial food source by secreting a glycoprotein called prestarvation factor (PSF) at a constant rate. When the ratio of PSF relative to that of bacteria exceeds a certain threshold, cells stop proliferating and initiate the expression of genes that are required for their aggregation (Clarke and Gomer, 1995). Early on during aggregation, gene expression requires the activity of cAMP-dependent protein kinase (PKA) (Schulkes and Schaap, 1995), and a high PSF to food ratio is thought to upregulate the translation of the PKA catalytic subunit PKA-C (Souza et al., 1999; Souza et al., 1998).

A second glycoprotein, CMF (conditioned medium factor), is secreted during starvation. CMF stimulates gene expression in parallel with PSF, and both signals potentiate cAMP signalling by inducing genes involved in cAMP synthesis and detection (Clarke and Gomer, 1995; Deery and Gomer, 1999). For example, among the newly activated genes are those encoding adenylate cyclase A (ACA, which synthesizes cAMP), cAMP receptors (cARs, G-protein coupled receptors that detect cAMP) and the extracellular cAMP phosphodiesterase PdsA (which hydrolyzes cAMP). These proteins, together with PKA and RegA, an intracellular cAMP phosphodiesterase with response regulator, form a biochemical network that generates pulses of cAMP (Laub and Loomis, 1998).

Once these proteins are functioning, a few starving cells start to emit cAMP pulses (Fig. 1A). Surrounding cells respond by moving towards the cAMP source and by relaying the pulse to more distant cells. Chemotaxis and signal relay together then cause the rapid aggregation of cells into multicellular mounds (Fig. 1B,C). The top of the mound continues to emit cAMP pulses and is pushed upwards by the movement of cells underneath (Siebert and Weijer, 1995). The emerging column of cells topples over and is now called 'the slug' (Fig. 1D). Guided by signals, such as light and warmth, the slug migrates to the top layer of the soil, where it proceeds to build the



**Fig. 2. Phylogeny of *Dictyostelia*.** All known species of social amoebas can be subdivided into four major groups based on phylogenetic analysis of their small subunit ribosomal RNA sequences (Schaap et al., 2006). Group 1 (red) is most closely related to the solitary amoebozoan ancestors. Groups 1-3 have retained the ancestral survival strategy of encystation of individual amoebas. This strategy is lost in Group 4 (purple), the most recently diverged group, which contains the model organism *D. discoideum*. Group 4 species also show other features, such as the use of cyclic adenosine monophosphate (cAMP) as a chemoattractant and cellular supports for the fruiting body, that are not (or seldomly) displayed by species in Groups 1-3. Coloured arrows point to species with completely sequenced genomes. Sequencing of the *D. lacteum* genome (group 3, indicated by a dashed arrow) is still in progress. Scale bar: 0.1 substitutions per site. Line thicknesses of branches correlate to the probability that nodes that connect immediate descendants are correct, as determined by Bayesian inference (Ronquist and Huelsenbeck, 2003). Modified with permission from Schaap et al. (Schaap et al., 2006).

fruiting structure (Fig. 1E,F) (Bonner and Lamont, 2005). This structure (Fig. 1G) consists of a tapered column of stalk cells that carries a spherical mass of spores aloft. The stalk is supported by a

basal disc and the spore mass is prevented from sliding down the stalk by flanking support structures, which are called the upper and lower cup (Fig. 1G).

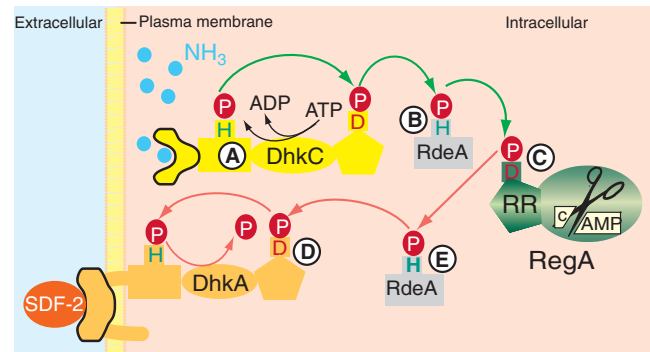


The presumptive stalk and spore cells can be recognized as early as in the aggregate, where they first appear to be interspersed with undifferentiated cells (Ozaki et al., 1993; Thompson et al., 2004). The prespore cells become less chemotactically sensitive to cAMP (Matsukuma and Durston, 1979; Traynor et al., 1992) by reducing their expression of certain proteins, such as cAR1 and PdsA, that are essential for generating and sensing steep chemotactic gradients (Schaap and Spek, 1984; Weening et al., 2003). This causes the prestalk cells to move selectively to the oscillating tip (Fig. 1C). During fruiting body formation, the prestalk cells first lay down a central cellulose tube. They then crawl into the tube and differentiate into stalk cells. This differentiation process involves massive vacuolization of the cells and the construction of a cellulose cell wall (Raper and Fennell, 1952). The prespore cells have, meanwhile, synthesized the first layer of the spore wall and further spore wall precursors in Golgi-derived prespore vesicles. The prespore cells climb up the newly formed stalk and initiate spore formation by rapid fusion of the prespore vesicles with the plasma membrane. The remainder of the spore wall is then synthesized in situ from the secreted precursors (West, 2003).

### cAMP regulation of fruiting body formation

Research in the 1980s showed that extracellular cAMP acts on cARs to induce prespore differentiation in aggregates (Schaap and Van Driel, 1985; Wang et al., 1988). In the 1990s, it became clear that, in addition to extracellular cAMP, intracellular cAMP acting on PKA was also required for prespore differentiation and was, furthermore, essential for spore and stalk maturation and for the control of spore germination (Harwood et al., 1992; Hopper et al., 1993; Van Es et al., 1996). For stalk and spore maturation, cAMP is produced by adenylate cyclase R (ACR), whereas cAMP for induction of prespore differentiation and control of spore germination is produced by adenylate cyclase G (ACG) (Soderbom et al., 1999; Van Es et al., 1996; Alvarez-Curto et al., 2007). ACR harbours seven transmembrane domains and is embedded in the nuclear membrane of prestalk cells (Soderbom et al., 1999; Alvarez-Curto et al., 2007; Chen et al., 2011). ACG harbours two transmembrane domains that separate a sensor domain from the adenylate cyclase domain, which faces the cytosol. ACG is translationally upregulated in prespore cells, where it localizes to the membrane of the prespore vesicles. During spore formation, ACG localizes to the spore surface when the prespore vesicles fuse with the plasma membrane (Alvarez-Curto et al., 2007). The cAMP phosphodiesterase RegA also plays a crucial role in regulating intracellular cAMP levels (Shaulsky et al., 1996; Shaulsky et al., 1998; Thomason et al., 1998).

The activity of RegA itself is closely regulated by extracellular signals that control the phosphorylation state of its attached response regulator domain (Fig. 3). This process is mediated by so-called sensor histidine kinases/phosphatases (see Glossary, Box 1), which either phosphorylate or dephosphorylate themselves on a histidine residue when a signal molecule binds to their sensor domain. The phosphoryl group is then transferred in two steps towards or away from the response regulator domain of RegA, thereby activating or inactivating its cAMP-phosphodiesterase activity, respectively (Thomason et al., 1999). For instance, ammonia, which inhibits the onset of fruiting body formation and stalk maturation, activates the histidine kinase activity of sensor histidine kinase C (Dhkc), resulting in RegA activation, hydrolysis of cAMP and inhibition of PKA activity (Singleton et al., 1998). Spore differentiation factor 2 (SDF-2) activates the histidine phosphatase activity of DhkA (histidine phosphatase A), which



**Fig. 3. Regulation of the cAMP phosphodiesterase RegA.** RegA controls intracellular cAMP levels in *D. discoideum* cells and, thus, the level of PKA activity. The schematic shows the extracellular and intracellular environment of a *D. discoideum* cell and how RegA (green) is activated by phosphorylation (P) of an aspartate (D) in its response regulator (RR) domain. This phosphorylation is regulated by a sensor histidine kinase, DhkC. (A) The binding of ammonia (NH<sub>3</sub>) to the sensor domain of DhkC (outlined in black) leads to the activation of its histidine kinase activity and to the autophosphorylation of a DhkC histidine (H) residue. The phosphoryl group is then transferred to an aspartate in the same protein, and from there to a histidine in the phosphotransfer intermediate RdeA (B), and finally to RegA (C). By contrast, the binding of SDF-2, which is present extracellularly, to the sensor domain of DhkA (outlined in black), causes histidine-mediated dephosphorylation of an attached aspartate residue (D) and reverse phosphotransfer via RdeA (E). This leaves RegA without a phosphoryl group, thereby inactivating cAMP hydrolysis. ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DhkA, histidine phosphatase A; DhkC, histidine kinase C; PKA, cAMP-dependent protein kinase A; RdeA, phosphotransfer intermediate Rapid Development A; RegA, intracellular cAMP phosphodiesterase with response regulator; SDF-2, spore differentiation factor 2.

results in the inactivation of RegA, the accumulation of cAMP and in PKA activation, which subsequently causes spore maturation (Wang et al., 1999).

The adenylate cyclase ACG is also regulated by external stimuli. As mentioned above, ACG is localized on the spore surface, where it is directly activated by its extracellular osmo-sensing domain (Van Es et al., 1996). High levels of ammonium phosphate are released during spore maturation. These high ammonium phosphate levels raise osmolality in the spore mass and keep spores dormant while still in the fruiting body (Cotter et al., 1999). The effect of high osmolality is mediated by ACG acting on PKA (Van Es et al., 1996).

The multitude of essential roles of both extracellular and intracellular cAMP is the most striking aspect of *D. discoideum* development. Identification of the origin of cAMP function in the Amoebozoa and how this function was elaborated during *Dictyostelia* evolution therefore holds the key to understanding how this form of multicellularity evolved.

### Recent insights into developmental signalling in *D. discoideum*

In this section, I discuss recent insights into the regulation of the stalk and spore cell differentiation pathways, and how these pathways might have evolved in *Dictyostelia*.

### The stalk pathway

The non-prespore cell population in slugs is traditionally subdivided into four cell types based on the expression of the prestalk genes *ecmA* and *ecmB* (Jermyn et al., 1989; Jermyn and Williams, 1991; Ceccarelli et al., 1991; Early et al., 1993). Prestalk AB cells, which express both *ecmA* and *ecmB*, occupy the core of the tip. During fruiting body formation, these cells differentiate into stalk cells. During slug migration, they are sloughed off and replaced by the forward movement of more posteriorly located cells. These are the prestalk A and prestalk O cells, which express *ecmA* from the proximal 'A' and distal 'O' region of its promoter, respectively, and occupy the front and back half of the prestalk region (Fig. 1C,D). Once the prestalk A cells have reached the tip, they also start to express the *ecmB* gene. Prestalk O cells can also be found in the prespore region and these cells move forward to replenish the prestalk O region. Finally, prestalk B cells, which weakly express the *ecmB* gene, move downward to form the basal disc and lower cup (Jermyn et al., 1996) (Fig. 1F,G).

In the 1980s, a secreted polyketide called DIF-1 (differentiation inducing factor 1) was identified and it was shown that this polyketide could induce prestalk differentiation and stalk maturation in cell monolayers in the presence of a PKA activator (Kay and Jermyn, 1983; Morris et al., 1987). Research over the past decade has improved our understanding of the synthesis and roles of DIF-1.

The first step in the synthesis of the chlorinated polyketide DIF-1 is catalysed by the polyketide synthase *StlB* (Austin et al., 2006). DIF-1 then undergoes intermediate chlorination by the flavin-dependent halogenase *ChlA* (Neumann et al., 2010) and becomes methylated by a methyltransferase, *DmtA* (des-methyl-DIF-1 methyltransferase) (Thompson and Kay, 2000). DIF-1 can induce all prestalk subtypes and stalk cell differentiation in cell monolayers (Morris et al., 1987; Williams, 2006). However, studies of *stlB*- and *dmtA*-null mutants have shown that the role of DIF-1 in the intact organism is much more restricted. In *stlB*-null mutants, only the prestalk B cells are missing from slugs and, consequently, the basal disc and lower cup of the fruiting body fail to form (Saito et al., 2008). In *dmtA*-null slugs, prestalk O cells are reduced in number (Thompson and Kay, 2000) but this is due to the accumulation of des-methyl-DIF-1, the substrate of *DmtA*, which inhibits prestalk O cell differentiation (Saito et al., 2008).

These findings leave us short of the factors that are required for prestalk O, prestalk A and stalk cell differentiation. Secreted DIF-like factors that can also induce prestalk and stalk cell differentiation in the monolayer system have been fully or partially identified (Saito et al., 2006; Serafimidis and Kay, 2005), although their role in normal *D. discoideum* development is yet to be established.

### The spore pathway

The first step in spore formation, the differentiation of prespore cells, requires both extracellular cAMP acting on cARs and intracellular cAMP acting on PKA (Hopper et al., 1993; Schaap and Van Driel, 1985; Wang et al., 1988). cAMP for both roles is provided by ACG. ACG is expressed after aggregation in the posterior prespore region of the slug (Alvarez-Curto et al., 2007). The next step, spore maturation, requires a second phase of PKA activation (Mann et al., 1994), as does the maturation of stalk cells (Harwood et al., 1992). The formation of *Dictyostelium* fruiting bodies is entirely dependent on cell movement, which is most likely to be mediated by cAMP waves emerging from the tip (Dormann and Weijer, 2001; Schaap et al., 1984). Because both stalk and spore maturation involve a complete loss of cell motility, these final differentiation stages require

very precise spatiotemporal regulation. Such regulation is provided by parallel cascades of signals that are exchanged between the maturing prestalk and prespore cells. These signals ultimately control the activation state of PKA (Fig. 4).

Fruiting body formation initiates when the migrating slug projects its tip into the air. In nature, this occurs in response to incident light when the slug reaches the top level of the soil. This allows ammonia to be lost from the slug tip by gaseous diffusion. Ammonia is produced in large quantities from protein degradation in the starving cells and acts to block both the slug-to-fruiting body transition and the maturation of stalk cells (Schindler and Sussman, 1977; Wang and Schaap, 1989). Both processes require PKA activity, and ammonia indirectly inactivates PKA by activating DhkC (Singleton et al., 1998), which in turn activates cAMP hydrolysis by RegA (as shown in Fig. 3). Loss of ammonia from the slug tip thus leads to PKA activation and to the initiation of stalk encapsulation (Fig. 4A).

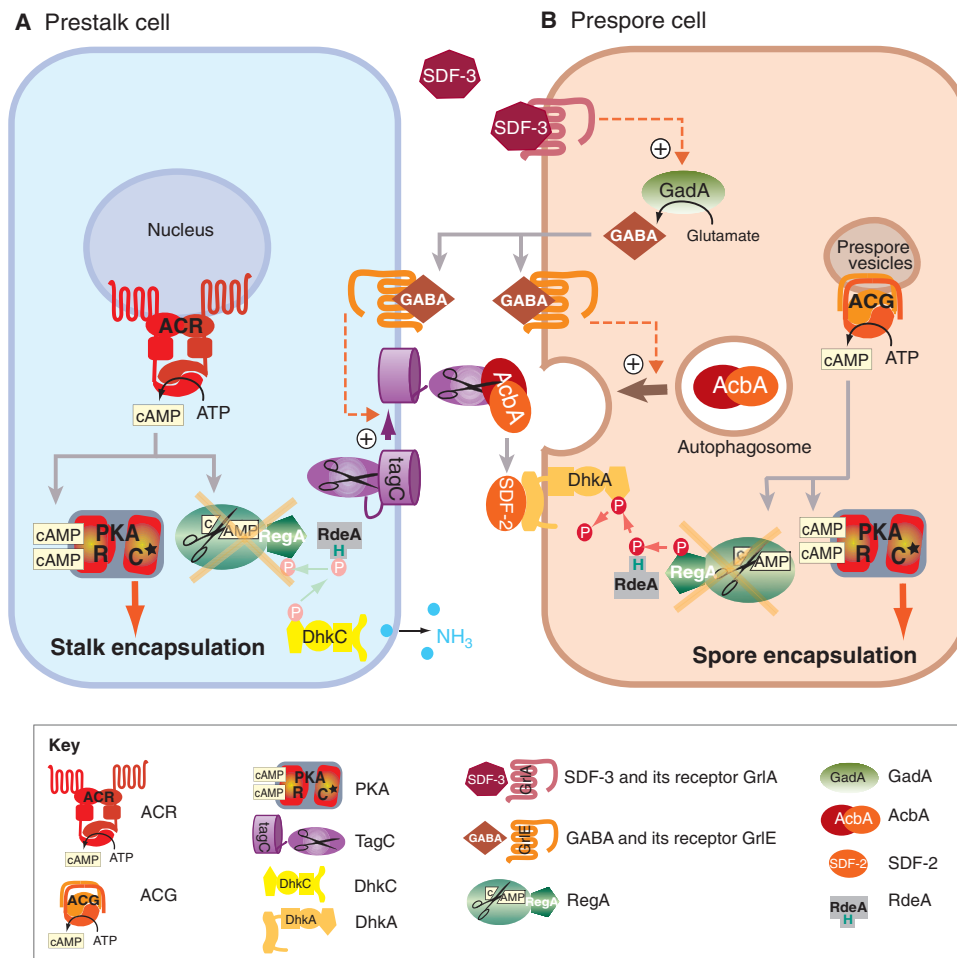
Next, the steroid SDF-3 (spore differentiation factor 3) is released, which triggers the production of GABA ( $\gamma$ -aminobutyric acid) by prespore cells (Fig. 4B) (Anjard et al., 2009). GABA has two effects: first, it triggers the secretion of AcbA (acyl-coenzyme A binding protein) from prespore cells; and second, it causes the TagC (tight aggregate C) serine protease domain to be exposed at the cell surface. TagC then cleaves secreted AcbA to form SDF-2 (Anjard and Loomis, 2006). SDF-2 activates the histidine phosphatase DhkA of prespore cells, which dephosphorylates, and thereby inactivates, RegA (Fig. 3). cAMP levels then increase, causing PKA activation and spore maturation (Wang et al., 1999).

In addition to this cascade, two other signals are required for spores to mature. SDF-1, another secreted peptide, acts on ACG to activate PKA (Anjard and Loomis, 2008), and discadenine acts on the histidine kinase DhkB and ACR to upregulate PKA. Discadenine also acts in parallel to high osmolality to keep spores dormant in the spore head (Anjard and Loomis, 2008; Zinda and Singleton, 1998). This redundancy of regulatory pathways highlights the crucial importance of making and germinating spores only at the correct time and place.

### Evolutionary origins of cAMP signalling

The most fascinating aspect of the transition of *D. discoideum* amoebas into multicellular fruiting bodies is that so much of this process is regulated by cAMP and that, apart from DIF, most other developmental signals exert their effects by modifying the detection, production or degradation of cAMP. Until recently, it was not clear to what extent the extensive use of cAMP was common to all *Dictyostelia*, but recent studies have investigated the conservation and change in cAMP signalling genes and the associated functions of cAMP throughout the *Dictyostelia* phylogeny.

ACG, the osmolyte-activated adenylate cyclase that crucially regulates prespore differentiation and spore germination in *D. discoideum*, is functionally conserved throughout the entire *Dictyostelia* phylogeny (Ritchie et al., 2008). Many early diverging species have retained the ancestral mechanism of encystation (Fig. 2). Similar to spore germination, cyst germination is also inhibited by high osmolality but, unlike spore formation, encystation can be directly induced by high osmolality. For soil amoebas, high osmolality is probably a signal of approaching drought, which increases the concentration of soil minerals. Osmolyte-induced encystation is mediated by cAMP production and by PKA activation (Ritchie et al., 2008), indicating that the roles of intracellular cAMP and PKA in spore differentiation and germination are evolutionarily derived from a similar role in the encystation of solitary amoebas (Fig. 5).



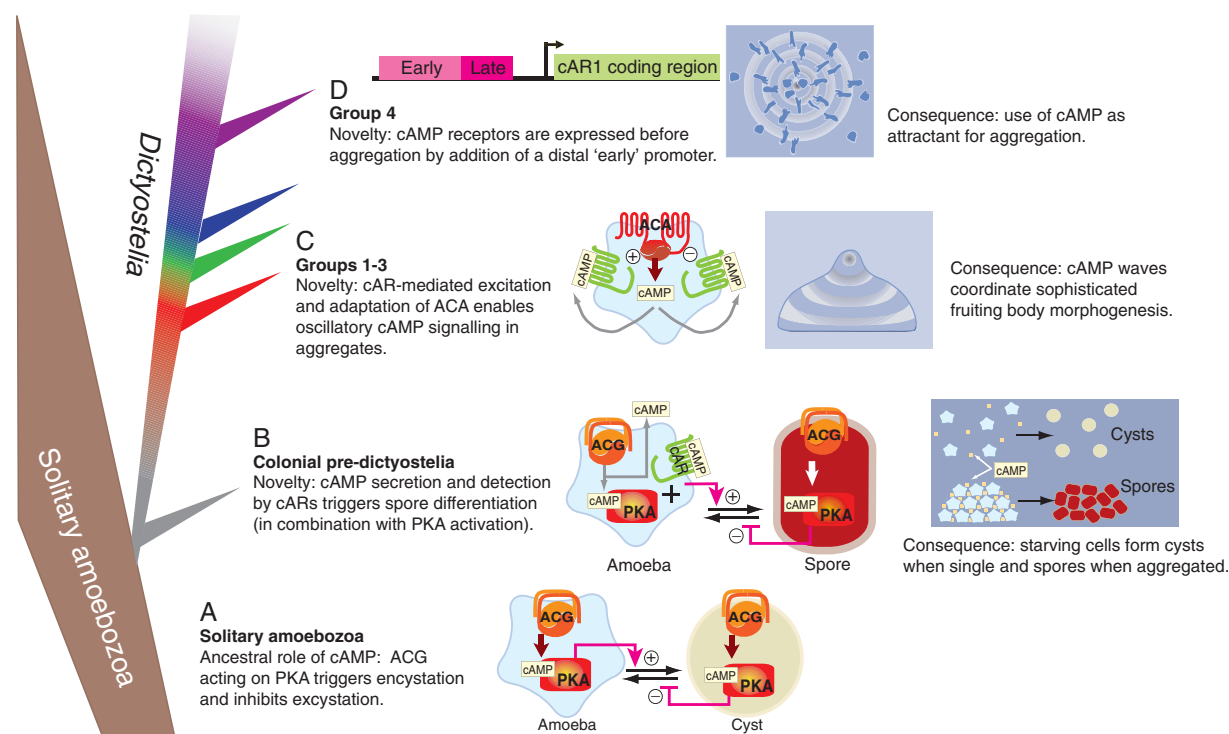
**Fig. 4. Coordination of *D. discoideum* stalk and spore maturation by cAMP.** (A) In prestalk cells at the slug tip, cAMP is synthesized by ACR and can either be hydrolysed by RegA or bind to PKA to trigger stalk encapsulation, which normally occurs when the tip projects upward at the onset of fruiting body formation and loses ammonia ( $\text{NH}_3$ ) by gaseous diffusion.  $\text{NH}_3$  inhibits PKA by indirectly activating cAMP hydrolysis via DhkC-RdeA-mediated phosphorylation of RegA. (B) In prespore cells, PKA activation occurs as a consequence of a signalling cascade that is activated by SDF-3, which triggers the synthesis of GABA by prespore cells. Secreted GABA then acts on prestalk cells to induce the cell-surface exposure of the serine protease domain of the ABC transporter TagC. GABA also acts on prespore cells to induce secretion of AcbA, which in turn is cleaved by the TagC protease to produce the peptide SDF-2. SDF-2 next activates the histidine phosphatase activity of DhkA, causing inactivation of cAMP hydrolysis by RegA. This allows cAMP to accumulate and activate PKA, which in turn induces spore encapsulation. ABC, ATP-binding cassette; AcbA, acyl-coenzyme A binding protein; ACR, adenylate cyclase R; ATP, adenosine triphosphate; DhkA, histidine phosphatase A; cAMP, cyclic adenosine monophosphate; DhkC, histidine kinase C; GABA,  $\gamma$ -aminobutyric acid; GadA, glutamate decarboxylase A; GrIA, G-protein coupled receptor-like protein A; GrIE, G-protein coupled receptor-like protein E; PKA, cAMP-dependent protein kinase A, with R and C denoting the regulatory and catalytic subunits, respectively, and a star to denote that the catalytic subunit is active in the presence of cAMP; RdeA, phosphotransfer intermediate Rapid Development A; RegA, intracellular cAMP phosphodiesterase with response regulator; SDF-2, spore differentiation factor 2; SDF-3, spore differentiation factor 3; TagC: tight aggregate C, an ABC transporter with intrinsic serine protease domain.

The cAMP receptor cAR1 and therefore extracellular cAMP signalling, are also conserved throughout the *Dictyostelia* phylogeny. In group 4 species, such as *D. discoideum*, cAR1 is expressed from separate promoters during aggregation and post-aggregative development (Louis et al., 1993). Remarkably, in groups 1-3, cAR1 is only expressed after aggregation (Alvarez-Curto et al., 2005). The inhibition of oscillatory cAMP signalling blocks aggregation and fruiting body morphogenesis in group 4 species but only disrupts fruiting body morphogenesis in group 1-3 species (Alvarez-Curto et al., 2005). This suggests that all *Dictyostelia* use oscillatory cAMP signalling to coordinate cell movement during slug and fruiting body formation. This role of oscillatory cAMP signalling therefore evolved first and its additional role in coordinating aggregation appeared more recently in *Dictyostelia* evolution.

cAR gene disruption in the group 2 species *P. pallidum* not only disorganizes fruiting body morphogenesis but also results in stunted structures that form cysts instead of spores in the spore head (Kawabe et al., 2009). This is because the cAR-null mutant no longer expresses prespore genes in response to cAMP stimulation (Kawabe et al., 2009). As discussed above, sporulation and encystation both require intracellular cAMP acting on PKA, but sporulation additionally requires extracellular cAMP acting on cARs. With the latter pathway ablated, the cAR-null cells revert to the ancestral survival strategy of encystation.

Together, these results suggest a possible scenario for the evolution of cAMP signalling in *Dictyostelia* that starts with cAMP functioning as an intracellular signal that transduces the perception of environmental stress into an encystation response in the solitary





**Fig. 5. Evolution of cAMP signalling in *Dictyostelia*.** A tentative hypothesis for the evolution of developmental cAMP signalling in *Dictyostelia* from a cAMP-mediated stress response in the solitary ancestor. The four groups of *Dictyostelia* (see Fig. 2) are roughly estimated from accumulated changes in their small subunit ribosomal RNA and  $\alpha$ -tubulin sequences to have diverged from their last common amoebozoan ancestor about 1 billion years ago (Schaap et al., 2006). (A) The amoebozoan ancestor is likely to have used cAMP as an intracellular second messenger for stress-induced encystation. During the long era of *Dictyostelia* evolution, the mechanisms associated with synthesis, production and degradation of cAMP increased in complexity and acquired novel roles in cell differentiation and control of cell movement. (B) Accumulation of secreted cAMP was probably first used as a signal in aggregates to prompt cells to form spores. (C) Next, pulsatile cAMP secretion evolved to coordinate cell movement during fruiting body formation. (D) Finally this process was brought forward in development to coordinate the aggregation process. ACA, adenylate cyclase A; ACG, adenylate cyclase G; ACR, adenylate cyclase R; cAMP, cyclic adenosine monophosphate; cAR, cAMP receptor; PKA, cAMP dependent protein kinase A.

ancestor (Fig. 5A). Basal dictyostelids do not use cAMP to aggregate, and at least one species (*D. minutum*) uses the same attractant (folic acid) for food-seeking as it does for aggregation. The first colonial amoebas might, therefore, have adapted their food-seeking strategy for aggregation, while still using cAMP intracellularly to trigger encystation. *Dictyostelia* secrete most of the cAMP that they produce but can only accumulate the micromolar concentrations that are required for prespore differentiation (Schaap and Van Driel, 1985) once they have aggregated. Micromolar cAMP therefore signals the aggregated state and prompts cells to form spores and not cysts (Fig. 5B).

Oscillatory cAMP secretion, which requires cAR-mediated positive and negative feedback on cAMP synthesis by ACA (Devreotes, 1994), evolved initially to form architecturally sophisticated fruiting bodies and then evolved further to coordinate aggregation in the most recently diverged group 4 (Fig. 5C,D). Analysis of the promoters of *cAR1* and *PdsA*, which are essential for oscillatory cAMP signalling, suggests that this occurred through the insertion of a distal 'early' promoter next to the existing 'late' promoter that directs the post-aggregative expression of these genes (Faure et al., 1990; Louis et al., 1993; Alvarez-Curto et al., 2005).

### Limitations and future directions

Although the evolutionary reconstruction of cAMP signalling has been facilitated by the well conserved nature of the cAMP signalling genes, which makes them easy to amplify by polymerase chain reaction from different species, the complete evolutionary

reconstruction of the associated control mechanisms and of the other signalling pathways that mediate *Dictyostelium* development requires a phylogeny-wide genomics approach. In addition to the genome of the model *D. discoideum* (Eichinger et al., 2005), the genomes of *D. fasciculatum* (a group 1 species) and *P. pallidum* (group 2) have now also been completely sequenced (A. J. Heidel, H. M. Lawal, M. Felder, C. Schilde, N. R. Helps, B. Tunggal, R. Rivero, U. John, M. Schleicher, L. Eichinger, M. Platzer, A. A. Noegel, P.S. and G. Glöckner, unpublished). Draft sequence data for *D. purpureum* (group 4) are also available (see below) and the genome of a group 3 species *Dictyostelium lacteum* is currently being assembled. The *D. fasciculatum*, *P. pallidum* and *D. purpureum* genomes are now in the public domain (<http://sacgb.fli-leibniz.de/cgi/index.pl>; <http://genomes.dictybase.org/purpureum>) and their availability opens up tremendous opportunities to identify conservation and change in all developmentally relevant genes. Comparative genomics can also be used to correlate trends in gene and genome evolution with trends in phenotypic evolution. The modification of candidate genes to either an earlier or later form in suitable test species followed by observation of the phenotypic consequences can reveal how changes in genotype have caused phenotypic evolution. The genetically tractable *Dictyostelia* are eminently suitable for investigation of this fundamental biological question.

In addition to these comparative approaches, much is still to be learnt about the signals and associated pathways that control *D. discoideum* development. For example, the molecules that induce



prestalk and stalk cell differentiation remain to be identified. And, although some of the developmental signals of *D. discoideum* are known, the signal transduction pathways involved have only been partially characterized. While collecting in aggregates, cells become strongly cohesive and there is good evidence that direct cell-cell interactions are essential for post-aggregative gene expression (Kibler et al., 2003). However, it is not known how the perceived cell-cell interaction signal is transduced to affect gene regulation.

Although in theory the experimental and genetic approaches that are available in *D. discoideum* are well suited to investigate its signalling pathways, in practice these approaches are time-consuming and technically challenging, making progress slow. The development of high-throughput approaches that allow the genome-wide disruption or knockdown of genes could greatly alleviate this problem, but mutant analysis and elimination of false positives will remain time-consuming bottlenecks (Mohr et al., 2010). Computational approaches, such as Genepath, which constructs genetic networks from mutant data (Juvan et al., 2005), and epistatic analysis of global transcriptional phenotypes (Van Driessche et al., 2005) have been used successfully in *D. discoideum* to gain more rapid and novel insights into signalling pathway architecture. The development of improved computational tools and instrumentation for large-scale genome, transcriptome and proteome analysis will probably have the strongest impact on this field of biological research within the next ten years.

## Conclusions

The excellent accessibility of *D. discoideum* to both genetic and biochemical approaches and to imaging techniques has made it an outstanding model for resolving fundamental questions in cell and developmental biology. Research in this and other organisms highlights the complexity of the signalling processes that control cellular functions and developmental programmes, with interacting and parallel pathways providing both fine-tuning and redundancy in their regulation. The interlinked pathways that control spore maturation in *D. discoideum* are a good example of this complexity.

Both crosstalk and redundancies between signalling pathways can often obscure how a signal actually flows through a given pathway. Because any process in biology is the end product of opportunistic events that occurred during evolution, the only intrinsic logic in the pathway is the order in which its component parts evolved. A comparative analysis of cAMP signalling in social amoebas has made it possible to reconstruct how the role of cAMP in morphogenesis and differentiation in the model *D. discoideum* evolved from a stress response in its unicellular ancestor. This approach has increased our understanding of the evolution of multicellularity but has also provided insight into the hierarchy of the signalling pathways that mediate the effects of cAMP, with cAMP acting on PKA at the top of this hierarchy. In addition to experimental and genetic approaches, evolutionary reconstruction is, therefore, a powerful tool to unravel and understand signalling processes in all multicellular organisms.

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## Competing interests statement

The author declares no competing financial interests.

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