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Evolution, Genomics and Cell Biology

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Preface

Dictyostelid social amoebae are a unique group of organisms with a life cycle that includes both unicellular and multicellular stages. They belong to the eukaryotic supergroup Amoebozoa, which is the sister group to Opisthokonta (animals and fungi). At the unicellular stage the amoebae prey on other microorganisms, mostly bacteria, by phagocytosis and multiply by binary fission. When food becomes scarce, dictyostelids have developed different responses to survive, including a remarkable process whereby a multicellular structure is formed by aggregation of individual amoebae. These aggregates behave as true multicellular organisms, showing processes of cell differentiation and morphogenesis that are reminiscent of those of metazoans. This unique multicellular stage and the position of the dictyostelids in the eukaryotic tree of life led to the sequencing of several genomes across the group. The availability of these genomes, as well as those from other non-dictyostelid sorocarpic amoeba, will allow us to gain insight into one of the great mysteries of evolutionary biology, the evolution of multicellularity across the tree of life. The social behavior of the dictyostelids also offers a unique opportunity to address the roles that multicellularity plays in the evolution of cooperation in complex organisms.

After aggregation, *Dictyostelium* development is governed by a complex interplay of extracellular signals between the different cell types. Despite being initially regarded as a simple system, development in *Dictyostelium* has proved to be more complex than previously expected. The molecular network that regulates cell motility and chemotaxis during the aggregation stage is a major topic in *Dictyostelium* research, which leads the field with astonishing discoveries about the "molecular compass" that allows eukaryotic cells (regardless if they are amoeba or human cells) to move in response to external gradients. A delicate and interconnected regulatory network of gene expression is now emerging as a result of massive transcriptomic analyses, which have helped to illuminate general principles in the evolution of development. One of the species in the group, *Dictyostelium discoideum,* has been used as a model organism since the 1960s. This species is highly amenable to experimental manipulations as well as genetic approaches, which together allow for a deep analysis of the function of genes. Conservation of the molecular mechanisms underlying the most fundamental cellular processes among distant taxa reinforces the use of *D. discoideum* as a

suitable experimental system to address not only basic questions of cell biology, but also issues of importance to human health and disease. These include the study of pathogen infections, mitochondrial diseases, cell motility-related diseases, and developmental and neurodegenerative diseases, among others.

This book highlights some of the most important recent discoveries in dictyostelid cell and molecular biology as well as the latest information about dictyostelid ecology, life cycle, and genomics. We also include a chapter on the study of the multiple independent origins of aggregative multicellularity across eukaryotes, which highlight the complexity of the evolution of cooperation among microbes. The ongoing active research on dictyostelids and the application of high throughput technologies suggest a promising future for the understanding of fundamental questions about living organisms, as inferred from the workings of the humble but wise social amoeba.

Maria Romeralo
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Contents

Dictyostelium discoideum as a Model in Biomedical Research

Sandra Muñoz-Braceras, Ana Mesquita and Ricardo Escalante

Abstract The simple eukaryote *Dictyostelium discoideum* has been traditionally used to understand basic principles of cell and developmental biology and now has also become a useful system in biomedical research. What are the similarities and differences between *D. discoideum* and other simple microbial models such as *Saccharomyces cerevisiae*? Which aspects are more advantageous to address in *D. discoideum*? Are there any processes or specific proteins present in *D. discoideum* that are difficult or impossible to study in other systems? Does it make sense to use such a simple organism in biomedicine? These and other questions will be addressed in this chapter, together with some specific examples in which *D. discoideum* has proved its potential to model human disease.

Keywords *Dictyostelium discoideum* · Model organisms · Biomedical research · Human disease · Pathogens · *Pseudomonas* · *Legionella* · Chemotaxis · Development · Cell motility · Autophagy · Molecular pharmacology · Mitochondrial disease · Sphingolipids · Cisplatin · Bipolar disorder

1 Introduction

A model organism is used in experimental biology to understand particular biological processes with the expectation that the knowledge obtained is applicable in other organisms, often especially in humans. Regarding the study of human disease, a model organism is chosen on the basis of being simple and amenable to experimental manipulations, but also, and obviously, for its similarity to humans. These two aspects usually run in opposite directions as exemplified by microbial

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models, which are the best suited for genetic manipulations but are distantly related to mammals. Fortunately, many processes affected in human diseases, such as those concerning basic cellular functions, are well conserved in evolution and thus can be addressed in simple but powerful microbial models. Historically, fungal models have been the most widely used non-metazoan eukaryotic organisms for their simplicity, genetic manipulability, and the profound knowledge of their biology. However, the fungal lifestyle has specific characteristics that make them different from animal cells. The social amoeba *Dictyostelium discoideum* is not a fungus and lacks the complexity of simple metazoan models such as the nematode *Caenorhabditis elegans* and the insect *Drosophila melanogaster* but it shares with them many cellular properties and a simple developmental cycle that can be used to model some basic aspects of human diseases.

Dictyostelid social amoebae are a group of eukaryotic soil microbes that prey on bacteria and yeast. Taxonomically, they belong to the Amoebozoa, a sister group of animals and fungi. Individual amoebas are highly motile cells and multiply by binary fission while nutrients are available. When food is scarce the cells become chemotactic and aggregate to form a simple multicellular organism. Cell motility is regulated by complex signaling pathways that render the cells responsive to an extracellular chemotactic molecule, which is produced and secreted by the cells themselves. Eventually, the cells become polarized and form streams, which converge into aggregative centers forming a mound. At this point, a developmental program involving cell movement and cell differentiation leads to the formation of a fruiting body formed by a stalk of vacuolated cells supporting the spores. There are around 150 known dictyostelid species and the latest studies group them into 8 divisions based on molecular phylogenic analyses (Romeralo et al. 2011). The best-studied species is *D. discoideum*, which has become a model organism in many different laboratories (Fig. 1).

2 What Makes Social Amoebas Different?

As mention above, a number of the model systems belong to the fungus kingdom and as such have specific characteristics, which are different from animal cells or *D. discoideum* cells. One of these fungal traits is the presence of rigid cell walls made of chitin which, like the cellulose walls present in plants, provide structural support and protection. In contrast, vegetative *D. discoideum* cells do not have cell walls and this allows the flexible plasma membrane to perform processes typically present in animal cells. These processes include phagocytosis (uptake of solid particles), macropinocytosis (uptake of liquid), cell motility and chemotaxis, which are accomplished by the formation of pseudopods (protrusions of the cell membrane generated by the mechanical force exerted by the cytoskeleton). The formation of the multicellular structure involves well-defined morphogenetic movements and cell differentiation processes that also share more similarities with animal development than with fungal mycelia (Escalante and Vicente 2000).

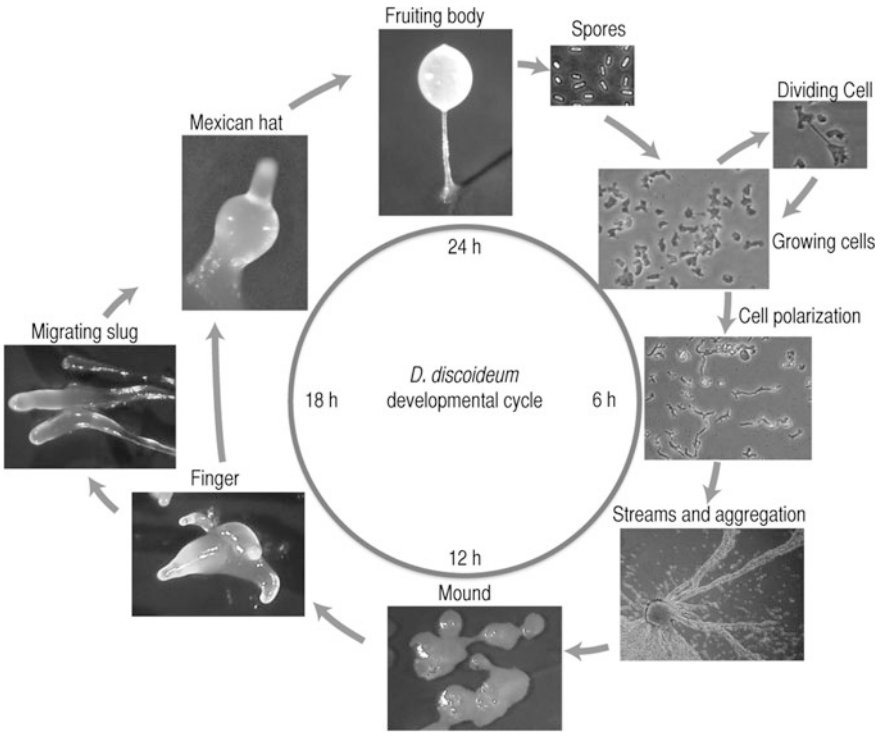

Fig. 1 *Dictyostelium discoideum* life cycle. Representative pictures of vegetative and developmental stages

All these parallelisms with animal cells have their reflection in the encoded proteins. Although social amoebas diverged before fungi, *D. discoideum* has many more proteins in common with animals than does the yeast model *S. cerevisiae* (Fig. 2). It is believed that many proteins present in the common ancestor of fungi, animals, and amoebas have been lost during fungal evolution. The similarities at the cellular level between amoebas and animal cells must have been the driving force for maintaining most of these proteins. Obviously, this has a profound impact on the utility of *D. discoideum* as a model system, since the function of many proteins and certain molecular pathways cannot be studied in fungal models.

One good example of this principle is the group of SH2 domain-containing proteins. These domains mediate protein–protein interactions and were thought to be present exclusively in animal cells. Surprisingly, *D. discoideum* possesses 13 SH2 proteins and 4 of them (STATa-d proteins, Signal Transducer and Activator of Transcription) have been characterized and found to be involved in development and cell type differentiation (Williams 2003; Langenick et al. 2008; Kawata 2011). The mammalian STATs are phosphorylated at specific tyrosine residues by JAK receptors and this phosphorylation creates sites for interaction with SH2 domains of other STATs, mediating their dimerization. The active dimers are then

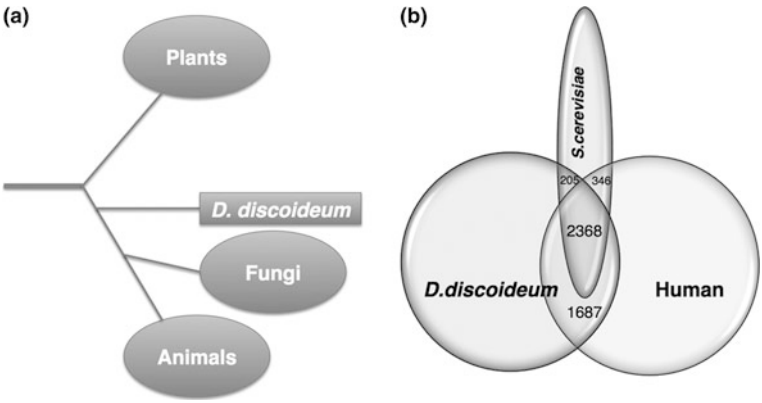

Fig. 2 Comparative genomics in *D. discoideum*. **a** A simplified representation of the evolutionary relationships of *D. discoideum* with plants, fungi and animals. **b** Proteome comparison between *D. discoideum*, human and *S. cerevisiae* using the on-line program PROCOM (http://procom.wustl.edu/). The intersections show the number of similar proteins with an E-value $<10^{-20}$. *D. discoideum* shows more conserved proteins in common with human than the yeast model does. Specifically, 1,687 proteins are present in human and *D. discoideum* but absent in *S. cerevisiae*, which makes the social amoeba a suitable system to study the function of new human proteins

translocated to the nucleus where they regulate the expression of developmental genes. As occurs in the worm *C. elegans*, which also possesses a STAT homolog (Wang and Levy 2006), no canonical JAK kinases have been found in *D. discoideum*, suggesting the presence of other tyrosine kinases involved in STAT activation. A recent study identified the kinase that activates STAT in *D. discoideum*. This kinase belongs to a group of kinases known as tyrosine-like kinases (TKLs) suggesting an atypical and possible ancestral regulation of STAT proteins (Araki et al. 2012). The study of this class of proteins in the *Dictyostelium* model is shedding light on their function and evolution (Soler-Lopez et al. 2004).

Another example of animal-like proteins in *D. discoideum* is illustrated by the recent discovery of the first lamin-like protein in a non-metazoan organism. Lamins are involved in severe inheritable diseases, including muscular dystrophy, cardiomyopathy, lipodystrophy, and progeria (Batsios et al. 2012), and are required for chromatin organization, gene expression, and cell cycle progression.

Along these lines, a comparative and functional genomic study aimed at characterizing proteins of unknown function conserved between *D. discoideum* and humans, but absent in the fungi *S. cerevisiae* and *S. pombe*. Of the total number of proteins, 41 were identified fulfilling these criteria and 28 were successfully disrupted by homologous recombination (Torija et al. 2006a, b). The sequences of the encoded proteins did not show recognizable functional motifs that would allow a prediction of their function. Two of these proteins turned out to be fundamental for cell function. One of them is Vacuole membrane protein 1 (Vmp1), whose disruption in *D. discoideum* leads to defects in autophagy and

other membrane traffic processes (Calvo-Garrido et al. 2008; Calvo-Garrido and Escalante 2010). The other protein is Mitochondrial dysfunction protein A (MidA), which is involved in *D. discoideum* and human cells in mitochondrial bioenergetics acting as an assembly factor of Complex I of the oxidative phosphorylation chain (Carilla-Latorre et al. 2010).

3 From the Soil to the Laboratory: The Early Times of *D. Discoideum* Research

The species *D. discoideum* can be considered as a newcomer in science history, since it was discovered not so long ago by Raper in 1933 in the Craggy Mountains of Western North Carolina (Raper 1935, 1936). At that time, several fundamental characteristics of dictyostelids, such as the first description of phagocytosis phenomena, had already been studied in other species of this group. It was revolutionary when Vuillemin realized that *Dictyostelium mucoroides* engulfed and digested bacteria intracellularly (Vuillemin 1903), in a way totally different from fungi, which obtain their nutrients by extracellular digestion. Later on, Raper went beyond mere observation and applied experimental biology to these organisms. Once *D. discoideum* could be grown to large quantities using a lawn of bacteria (Raper 1936, 1939), Raper addressed fundamental questions of cell biology and development. He was the first to study systematically the capacity of *D. discoideum* cells to ingest and kill pathogenic bacteria, anticipating the great importance of this model for the study of infectious diseases (Raper and Smith 1939). He discovered that the proportion of stalk and spore cells is kept nearly constant irrespective of the size of the developing structure (Raper 1940b). Another important aspect of development concerns cell fate specification, and he addressed this with elegant grafting experiments showing that the front of the slug, upon culmination, gives rise to the stalk and that the rear gives rise to the spores (Raper 1940a, b). All these early studies showed the enormous potential of this organism for developmental biology (Escalante and Vicente 2000) and set the stage for its success as an experimental system.

One of the first observations of directional cell movement in eukaryotic cells was made by Leber in 1888, when he observed streams of leukocytes moving toward an injured retina in rabbit (Leber 1888). Although the underlying cause of the movement was suggested to be mediated by extracellular molecules, experimental proof of chemotaxis and the identification of these molecules had to wait for several decades (McCutcheon 1946). The groundbreaking studies in *D. discoideum* by Bonner in 1947 (Bonner 1947) definitively settled the chemotaxis hypothesis and convinced others that the aggregative movement of *D. discoideum* cells toward the aggregation centers was a wonderful opportunity to characterize this phenomenon. As described below, the opportunity was not wasted and now this organism has become a leading model in cell motility and chemotaxis research.

The mysterious chemotactic molecule in dictyostelid movement was named acrasin and its chemical nature was identified in 1968 (Konijn et al. 1968). Surprisingly, the molecule turned out to be cyclic adenosine monophosphate (cAMP), discovered only a few years earlier by Sutherland as an intracellular second messenger in hormonal action (Rall and Sutherland 1958). cAMP is not the only acrasin identified; other dictyostelid species use different molecules such as glorin or folate for the same purpose. The identification of the first leukocyte chemotactic molecule took another several years (Yoshimura et al. 1987; Walz et al. 1987). These mammalian chemotactic molecules (called chemokines) are small peptides, different from dictyostelid acrasin molecules but they act through conserved receptors and signaling pathways.

The ease with which an organism can be grown and handled in the laboratory is an important factor in the advancement of new experimental tools. Significant efforts were invested to grow *D. discoideum* in liquid media; first in association with live or dead bacteria (Gezelius 1962; Sussman 1961) and later, by the isolation of strains capable of growing in axenic liquid broth medium (Sussman and Sussman 1967; Loomis 1971). Two independent strains, AX1 (which later gave rise to AX2) and AX3 (which later gave rise to AX4), were isolated from NC4 (a natural isolate of the species *D. discoideum*) in two different laboratories by clonal selection or mutagenesis. While the original NC4 isolate can only grow by phagocytizing bacteria, these strains were able to grow axenically by macropinocytosis, engulfing large amounts of liquid media. These new possibilities allowed the manipulation of large amounts of cells for diverse purposes and made it possible to synchronize development by washing off the nutrients and depositing the cells in a moist filter.

The isolation and study of mutants has been of central importance in the history of experimental biology. By studying the abnormal functioning of a given process, one might infer the mechanisms controlling the normal function. Sussman's group was the first to isolate *D. discoideum* mutants affected in growth and in different stages of development by exposing cells to UV-light. These mutants allowed the first synergistic studies and led to the realization of the complex networks underlying developmental processes, even in simple organisms (Sussman 1954; Sussman and Sussman 1953; Sonneborn et al. 1963). In many cases, a mixed population of two mutant strains could achieve normal development suggesting the presence of non-cell autonomous defects that can be mutually complemented in the mixed population. The particular genes affected in these mutants could not be identified at that moment, but these studies anticipated the future advances of developmental biology using this simple model.

Biochemical studies of enzyme activities in normal and mutant strains obtained by chemical mutagenesis opened new frontiers for the characterization of developmental processes, not only at the morphological but also at the biochemical level. An important example is the level and activity of enzymes involved in polysaccharide synthesis (Loomis et al. 1976; Loomis 1969a, b, 1970; Loomis and

Sussman 1966; Sussman 1965; Ashworth and Sussman 1967). The idea of a complex regulation of the level of expression of certain genes at transcriptional and translational levels during development was then clearly established (Roth et al. 1968; Newell et al. 1969).

4 The Advent of Molecular Genetics Propelled *D. discoideum* into the Post-genomic Era

In the 1970s, the development of recombinant DNA technology and DNA sequencing (Maxam and Gilbert 1977) allowed molecular genetics to flourish in several experimental systems. The first analyses of the *D. discoideum* genome were accomplished by Firtel and Sussman, who analyzed its size and realized its extreme AT-richness (Sussman and Rayner 1971; Firtel and Bonner 1972), and about a decade later, some *D. discoideum* genes were sequenced (Peffley and Sogin 1981; Hori et al. 1980; Poole et al. 1981). But the real breakthrough concerning the potential of *D. discoideum* as a genetic system came with the finding that *D. discoideum* cells could be transformed with exogenous DNA, which allows a direct manipulation of specific genes (Hirth et al. 1982). DNA is integrated randomly in the genome as tandem repeats of varying numbers of copies, but currently non-integrative extrachromosomal plasmids are also available (Gaudet et al. 2007; Veltman et al. 2009a, b; Firtel et al. 1985). This prompted the development of a wide array of techniques that have, nowadays, become routine in *D. discoideum* laboratories, including the expression of tagged proteins and reporter genes, gene inactivation by homologous recombination, insertional mutagenesis, and many other applications aimed at studying specific genes and their functions (Eichinger and Rivero 2006; Meima et al. 2007; Dubin and Nellen 2010; Levi et al. 2000).

Among these techniques, special attention must be paid to the generation of knock-out (KO) strains of specific genes by homologous recombination, which in *D. discoideum* is very efficient and since this organism is haploid, the consequences of the mutation can be determined directly in the clonal isolate without further manipulation. Myosin heavy chain and alpha-actinin were the first genes disrupted using homologous recombination in 1987 (De Lozanne and Spudich 1987; Witke et al. 1987), revealing the surprising finding that myosin is not essential for cell viability and that it is required for cytokinesis and development. Recent advances have facilitated the generation of large collections of strains carrying mutations in selected genes, opening the future possibility to achieve the systematic disruption of any single gene in this system (Torija et al. 2006a; Wiegand et al. 2011). Multiple KO mutants can now be obtained using the Cre-loxP system, and a remarkable example is the generation of a sextuple mutant lacking five type-1 phosphoinositide 3-kinases and the PTEN phosphatase; this mutant was used to investigate the role of phosphoinositide signaling in cell motility (Hoeller and Kay 2007).

A new step forward was the application in 1992 of another powerful technique: insertional mutagenesis by restriction enzyme-mediated integration (REMI) (Kuspa 2006; Kuspa and Loomis 1992). Briefly, a linearized plasmid containing a selection marker is electroporated together with a restriction enzyme into the cells. The restriction enzyme will create compatible integration sites in the genome, facilitating the integration of the plasmid. For example, the restriction enzyme *Dpn*II (which recognizes the 4-base sequence GATC and generates GATC overhangs) will allow the integration of a plasmid cut with the compatible restriction enzyme *Bam*HI (that recognizes the 6-base sequence GGATCC and generates GATC overhangs). The frequency of *Dpn*II-sites in the genome is much higher than that of *Bam*HI-sites, thus covering a wider spectrum of potential target genes. If the insertion interrupts a gene necessary for development, the resulting phenotype can be easily identified, and the strain isolated for further manipulation. This technique proved to be very efficient for generating mutants affecting diverse aspects of development. The difference with mutants generated by chemical mutagenesis or UV-light is that this technique enables the rapid identification of the disrupted gene. Identification of the mutated gene is accomplished by plasmid rescue and sequencing of the regions flanking the insertion (Fig. 3).

The potential of this technique was clearly illustrated by the work of Loomis, who isolated a collection of 80 REMI mutants affected in development (http://www-biology.ucsd.edu/labs/loomis/REMI/index.html). The study of some of these mutants has revealed essential signal transduction pathways controlling development and cell differentiation. The REMI technique also allows the identification of genes specifically involved in a given process using the appropriate phenotypic selection (Adachi et al. 1994; Nagasaki and Uyeda 2008).

The insertional mutagenesis has also been used in other screening strategies such as the powerful second-site suppressor analysis, which allows the identification of components of complex signal transduction pathways. It is based on the identification of a second mutation that overcomes the defect of a primary mutation. Second-site mutagenesis is performed on the mutant background using REMI, which later will allow the recovery of the gene acting as a suppressor. After antibiotic selection, transformants are screened for clones that display total or partial recovery of the phenotype. The disrupted gene is expected to act as a negative component downstream of the primary defect in a given signal transduction pathway (Shaulsky et al. 1996). For example, the mutation of *tagB*, which encodes a multidrug resistance transporter (MDR), blocked sporulation, and a second-site suppressor screening performed on the *tagB* mutant revealed several genes whose disruption partially bypassed the sporulation defect. One of these suppressors of *tagB* mutation was found to be a phosphodiesterase, which plays an essential role in the control of terminal differentiation (Shaulsky et al. 1996).

With all these new technical possibilities at hand, the next challenge was to obtain the whole sequence of the genome. This would allow comparative and functional genomic studies and fully exploit all the mentioned advantages.

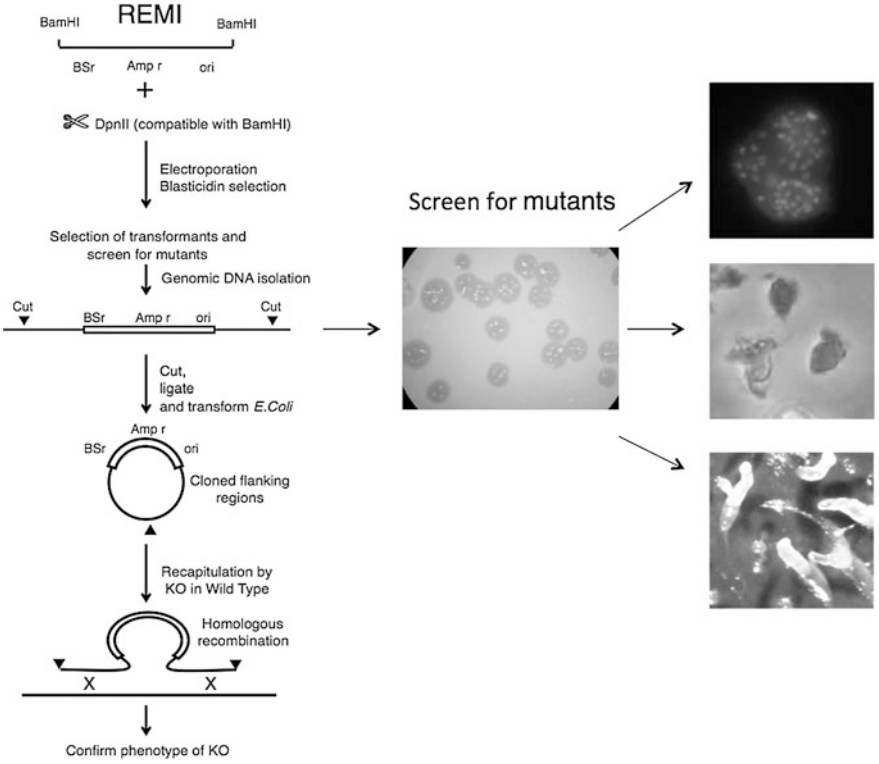

Fig. 3 Insertional mutagenesis in *D. discoideum* using REMI. A restriction enzyme (for example *Dpn*II) is used to create insertion sites in the genome for a plasmid containing compatible ends (in this case, cut with *Bam*HI). Transformants can be screened for abnormal cell morphology or developmental defects. Three mutants are shown as examples: multinucleated cells stained with DAPI, cells with aberrant polarization during aggregation and a mutant arrested at the slug stage. The disrupted gene can be identified by plasmid rescue of the genomic regions flanking the locus of insertion. Recreation of the mutant phenotype can be obtained by homologous recombination using the same construct

The sequence of the *D. discoideum* genome was published in 2005 (Eichinger et al. 2005). The genome is 34 megabases (Mb) in size, with six chromosomes encoding an estimated 12,500 proteins. This makes it quite small and compact, similar to the genome of the yeast model *S. cerevisiae*. The genome sequences of other dictyostelid species (*D. purpureum*, *D. fasciculatum,* and *Polysphondylium pallidum*) are now also available (Sucgang et al. 2011; Heidel et al. 2011). A central resource for dictyostelid genomics, dictyBase (http://www.dictybase.org/), provides invaluable tools, including full annotation, expression analysis, and associated bibliography.

5 A New Frontier in *D. discoideum* Research: Modeling Human Disease

Genome analyses have shown the presence of disease-related genes that are conserved between *D. discoideum* and human (Eichinger et al. 2005). Many of these genes are involved in processes that have been well conserved in evolution allowing their study in the social amoeba in a meaningful way, as has indeed been proved in many recent studies (Escalante 2011). In the past years, the number of studies in *D. discoideum* aimed at understanding genes or processes directly related to human disease has increased steadily as illustrated in the next sections of this chapter.

5.1 Pathogen–Host Interactions in D. discoideum

The bacterivore nature of dictyostelids and other protists imposes a strong selection pressure on the soil microbes. As a result, it is believed that some bacteria have developed during evolution strategies to survive the attack of these predatory amoebae. Pathogenic bacteria might have transformed these defense mechanisms into virulence traits to survive the attack of professional phagocytes in the human body: the macrophages and neutrophils. Since phagocytosis and endocytic traffic are highly conserved in evolution, it is not surprising that the essential mechanisms used by bacterial pathogens to evade and infect humans have been conserved in the interactions with *D. discoideum* and can be conveniently studied in this model (Lima et al. 2011; Steinert 2011).

D. discoideum cells can be easily co-cultured with pathogenic bacteria in agar plates. If the amoeba is able to ingest and kill the bacteria, clear plaques appear. Conversely, the absence of clear plaques reveals resistance to *D. discoideum* predation and might indicate the presence of virulence mechanisms (Fig. 4). This simple assay allows large-scale analysis of virulence levels among different isolates, or genetic screening to identify host or pathogen determinants. In 1978, an extensive study by Depraitere and Darmon (1978) tested up to 78 different gram-positive and gram-negative bacterial species for their capacity to support *D. discoideum* growth. They found that most of them were normally ingested and killed, but also found some species that showed different degrees of toxicity for the social amoeba. Since then, *D. discoideum* has been used as an infection model for a still growing list of human pathogens. Some of them are able to grow intracellularly and parasite the cell host, such as *Legionella* and *Mycobacterium* (Fig. 4); others are extracellular pathogens able to kill the host cells using a variety of toxins, such as *Pseudomonas aeruginosa*. The list of pathogens tested in the *Dictyostelium* model includes species such as *Salmonella typhimurium, Yersinia pseudotuberculosis, Burkholderia cenocepacia, Vibrio cholerae, Stenotrophomonas maltophilia*, *Acinetobacter baumannii,* and *Pseudomonas fluorescens* (Steinert 2011; Sperandio et al. 2012; Iwashkiw et al. 2012).

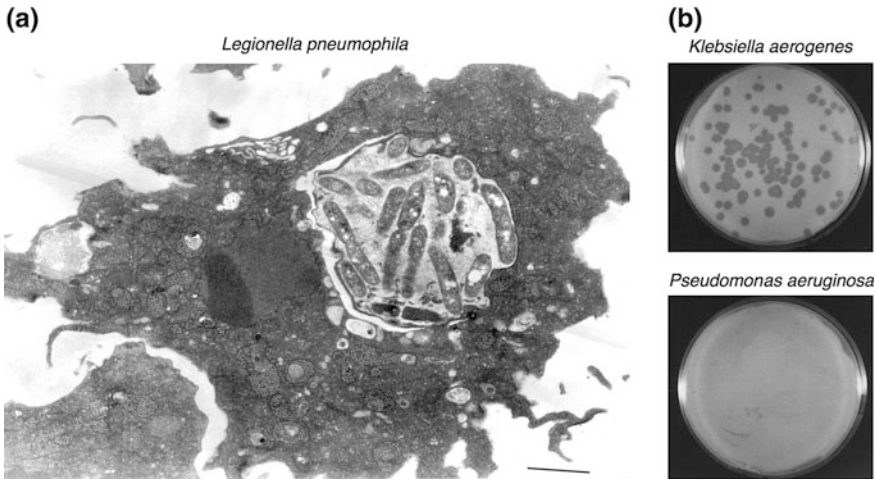

Fig. 4 *Dictyostelium discoideum* and bacterial pathogens. **a** Transmission electron micrograph of an *L. pneumophila*-infected *D. discoideum* cell. Bacteria are concentrated within a single vacuole. This photograph was reproduced with permission (Steinert 2011). **b** *Pseudomonas aeruginosa* virulence tested in a simple plating assay. *D. discoideum* cells are mixed with the non-pathogenic bacteria *K. aerogenes* or the pathogenic *P. aeruginosa* and plated on agar plates. *D. discoideum* is only able to feed on the non-pathogenic bacteria and, after a few days, clear plaques are formed

Pseudomonas aeruginosa is an opportunistic environmental bacterium that is particularly dangerous in patients with severe burns and compromised immunity, and it is one of the most common pathogens of nosocomial infections. It is also responsible for frequent chronic infections in patients suffering from cystic fibrosis. *Pseudomonas* is intrinsically resistant to antibiotics and it has developed a large array of virulence factors. Flagella and pili facilitate the contact of bacteria with their target cells and enhance their adhesion, which is a critical step in infection. After contact, the type III secretion system is capable of injecting into the cell cytoplasm an arsenal of cytotoxic molecules, which act at different levels and interfere with as yet unidentified host cofactors (Kipnis et al. 2006). Other products, such as elastase, alkaline phosphatase, and exotoxin A, are secreted into the extracellular space by secretion systems I and II. The expression of many of these systems is regulated in the bacterial population by a mechanism mediated by extracellular signals, the "quorum-sensing" (QS) (Van Delden and Iglewski 1998). Mutations affecting these virulence systems in *Pseudomonas* makes the bacteria more sensitive to *D. discoideum* predation and the correlation with data obtained in other virulence systems (worms, rats, and mice) is near-perfect, suggesting that the mechanisms of infection have been conserved (Lima et al. 2011; Cosson et al. 2002; Pukatzki et al. 2002).

Measuring the virulence of opportunistic bacteria in mammalian models is difficult since it requires immune-compromised mice or rats. This is very

expensive and even impractical when the number of tests is high such as those required for studies of clinical isolates or collections of mutated bacteria. The reproducibility of such assays is also questionable since the response of an individual to an infection depends on many different factors, which are often difficult to control. Consequently, the use of simple experimental systems such as the worm *C. elegans* and *D. discoideum* has become a reliable alternative in the research of *Pseudomonas* and other opportunistic pathogens.

A nice example of such use is the study of the evolution of *Pseudomonas* virulence within infected patients, as this requires massive testing of many isolates. Clinical populations of *P. aeruginosa* from acute infections show a wide range of virulence that can be conveniently tested in *D. discoideum* assays (Janjua et al. 2012). Another recent study has revealed that during the course of chronic infections in cystic fibrosis patients *Pseudomonas* accumulates mutations in virulence genes such as the *lasR* gene involved in QS response. *D. discoideum* was used to measure the virulence of hundreds of clinical isolates (Lelong et al. 2011; Bradbury et al. 2011). A decrease in virulence was observed over the time of infection, suggesting that the persistence of high virulence is not required to maintain a chronic infection. Interestingly, there was no correlation between the loss of lasR and the loss of virulence, indicating that other virulence traits must be affected.

A recent report analyzed the impact of the Crc protein, a global regulator of metabolism. A *P. aeruginosa* strain lacking Crc showed defects in type III secretion and motility and showed a less virulent phenotype in *D. discoideum*. These results suggest that Crc might be a good target in the search for new antibiotics (Linares et al. 2010).

Despite the functional and genomic similarities among different *P. aeruginosa* strains (Alonso et al. 1999; Morales et al. 2004), some differences in their pathogenicity have been observed (Lee et al. 2006). This is clearly exemplified by two clinical isolates commonly used as model strains, PAO1 and PA14. As occur in other models, *D. discoideum* is more sensitive to PA14 than to PAO1 (Carilla-Latorre et al. 2008). To investigate the origin of these differences, *D. discoideum* cells were exposed to either *P. aeruginosa* PAO1 or *P. aeruginosa* PA14 and after 4 h *D. discoideum* RNA was extracted. Transcriptome analyses by microarrays showed the existence of common and specific responses of *D. discoideum* cells to infection. The expression of 364 genes changed in a similar way upon infection with one or the other strain, whereas 169 genes were differentially regulated depending on whether the infecting strain was *P. aeruginosa* PAO1 or PA14. Effects on metabolism, signaling, stress response, and cell cycle can be inferred from the genes affected. These results indicate that the infective process of bacterial pathogens can be strain-specific and more complex than previously thought (Carilla-Latorre et al. 2008).

Intracellular pathogens interfere with the sequence of events leading to internalization by phagocytosis and intracellular digestion of the bacteria. In *D. discoideum* and mammalian cells, phagocytosis is initiated by bacterial adhesion to the cell surface. This triggers an actin cytoskeleton rearrangement at the site of

ingestion that plays an essential role in the internalization and transport of the phagosome vesicle into the cells. The signaling events involve heterotrimeric and monomeric G proteins, phospholipases, PI kinases, phosphatases, and calcium ions (Bozzaro et al. 2008). The following steps of maturation include acidification of the phagosome by fusion with acidic vesicles containing a V-H^+ATPase, and fusion with non-acidic vesicles containing Nramp1, a divalent metal transporter involved in the depletion of divalent metals that prevent bacterial growth (Peracino et al. 2006, 2010, 2012). Successive recruitment of lysosomal hydrolases eventually leads to bacterial digestion. Undigested material is then exocytosed to the exterior of the cell. The challenge is to understand how different pathogens interfere with this sequence of events.

Legionella pneumophila is a pathogenic gram-negative bacterium responsible for Legionnaires'disease, a severe life-threatening pneumonia. *Legionella* is common in the natural environment where it lives within different amoeba species. After ingestion, the bacteria are able to establish an intracellular niche evading lysosomal degradation. This *Legionella* replicative environment allows the bacteria to grow and replicate inside amoebae and human cells. The establishment of this intracellular niche is very complex and still poorly understood, but it is known to involve the recruitment of mitochondria, endoplasmic reticulum (ER), and smooth vesicles, and to block the fusion with lysosomes. The use of *D. discoideum* mutants has revealed that *Legionella* uptake takes place by conventional phagocytosis requiring heterotrimeric G proteins (Steinert 2011). *Legionella* expresses a number of effector proteins that are translocated to the cytoplasm by the secretion system Icm-Dot, interfering with diverse signaling pathways involved in membrane traffic. Some of these effectors include regulators of small GTPases (GEFs, guanine exchange factors, and GAPs, GTPase activating proteins), interactors of phosphatidylinositol 4-phosphate, a regulator of Arf1, and a protein involved in vesicle transport from the Golgi to the ER.

The expression in *D. discoideum* of another *Legionella* effector, LegC3, leads to the disruption of organelle trafficking by the accumulation of endosome-like structures containing undigested material (de Felipe et al. 2008). Ankyrin B (AnkB), another *Legionella* effector required for its replication, is essential for the recruitment of polyubiquitinated proteins to the *Legionella* vacuole. The AnkB effector achieves this by mimicking the action of host F-box proteins, thus hijacking the conserved SCF ubiquitin ligase complex in macrophages and *D. discoideum* (Price et al. 2009).

5.2 Pathobiology of Cell Motility and Chemotaxis

Cell motility and chemotaxis are essential for many aspects of the life cycle of eukaryotes. From the earliest stages of development, cell migration is necessary for the formation of the embryo. A paradigmatic example is that of the neural crest cells, a multipotent, migratory cell population. After gastrulation, these cells are

specified at the border of the neural plate. They move from there to colonize different regions of the embryo, and then give rise to different cell types such as connective tissue cells, cartilage, skeletal tissue, melanocytes, etc. (Trainor 2005). Their movement is regulated by extracellular signals that act as attractants and repellents of their migration (Jones and Trainor 2005). Cell migration is also essential in wound healing, where fibroblasts must move to repair the wound (Kole et al. 2005). The immune system is another example where white blood cells are attracted to sites of infection and inflammation by chemotaxis to exert their protective function (Parent 2004; Jin et al. 2008). Chemokines are extracellular molecules essential for guiding these immune cells to the site of infection. Abnormal secretion of chemokines may lead to excessive recruitment of leukocytes contributing to several inflammatory diseases such as chronic obstructive pulmonary disease (COPD), multiple sclerosis (MS), atherosclerosis, inflammatory bowel disease, and endocrine autoimmune disease. Another, no less important, motility-dependent pathological process is cancer, where the migratory capacity of transformed cells promotes their exit from the tissue of origin and the invasion of new areas (Roussos et al. 2011; Condeelis et al. 1992). It is therefore not surprising that many diseases are related with cell migration defects.

The motility behavior of leukocytes and *D. discoideum* cells is very similar in response to their chemoattractants (chemokines and cAMP, respectively). Cells elongate and polarize the cytoskeleton in the direction of the gradient. In both cell types, the signal is transmitted into the cells by G-protein coupled receptors (GPCRs) and the molecular parallelisms of signal transduction and the regulation of the chemotactic machinery are wonderfully conserved as described in other chapters of this book (Swaney et al. 2010; Wang et al. 2011a; King and Insall 2009; Jin et al. 2008).

The actin cytoskeleton is finely regulated during cell motility and chemotaxis since it is responsible for generating the forces necessary for cell movement. A number of diseases are associated with dysfunction of actin or its regulation, such as defects in immunity, neuronal development, degenerative diseases, cancer, and pathogen infection. *D. discoideum* has contributed to understanding the basic principles of some of these diseases. A relevant example is the Wiskott-Aldrich syndrome, an X-linked recessive immunodeficiency characterized by thrombocytopenia, eczema, and recurrent infections. The disease is caused by mutations in the *WAS* gene encoding WASP, a protein expressed preferentially in the hematopoietic system. This protein regulates the actin nucleation complex Arp2/3 and thus the formation of new actin filaments. Another member of the WASP family, the actin nucleation promoting protein SCAR/WAVE, which is essential for the generation of pseudopods, was first identified and characterized in *D. discoideum* (Bear et al. 1998; Veltman et al. 2012; Ura et al. 2012).

Lissencephaly, which literally means smooth brain, is a severe developmental brain defect caused by abnormal migration of neurons. This defect is caused by mutations in either one of the two proteins Lissencephaly 1 (LIS1) and Doublecortin (DCX), which interact with the microtubule and actin cytoskeleton. LIS1 and DCX have been characterized in *D. discoideum* and these studies have lead to

surprising results about the function of these important proteins (Meyer et al. 2011). Expression of a hypomorphic lissencephaly causing allele of LIS1 in *D. discoideum* led to severe abnormalities at the cellular level such as disruption of the microtubule cytoskeleton, disorganization of the Golgi apparatus, detachment of centrosomes from the nucleus, and reduced F-actin content. Moreover, a potential relationship with the actin cytoskeleton was suggested by the interaction with a potential actin regulator, Rac1A (Rehberg et al. 2005). DCX and LIS1 physically interact both in mammalian and in *D. discoideum* cells. Remarkably, ablation of DCX in the mutant strain expressing the hypomorphic LIS1 leads to an additional phenotype not present in the single mutants, a defect in the formation of streams during aggregation. These double mutants were able to respond normally when a few wild-type cells were added in the experiment, suggesting that the defect in aggregation is not due to abnormal cytoskeleton rearrangement but to defects in extracellular cAMP signaling. These and other additional experiments led to the conclusion that DCX and LIS1 cooperate in a cytoskeleton-independent manner to regulate cAMP signaling and open the question whether a similar cooperation might also be present during brain development (Meyer et al. 2011).

Shwachman-Bodian-Diamond syndrome (SDS) is a severe hereditary disease characterized by skeletal abnormalities, pancreatic insufficiency, bone marrow failure, and increased sensitivity to infections. The observation that leukocytes taken from SDS patients show abnormal orientation during chemotaxis led to the hypothesis that cell motility might play a role in the etiology of the disease. The function of the protein mutated in this syndrome (SBDS) is unknown and it has no primary sequence similarity to any other protein or structural domain that would indicate a possible function. Interestingly, the homologous *D. discoideum* protein fused to GFP showed an enrichment of the protein in the pseudopods during cell migration, suggesting a direct role in the chemotactic machinery (Wessels et al. 2006). However, recent work in *D. discoideum* led to unexpected results, since the SBDS protein was found to be directly involved in ribosome assembly. These results support the hypothesis that SDS is a ribosomopathy caused by abnormal ribosome maturation (Wong et al. 2011).

5.3 Autophagy and Protein Aggregation Disorders

Autophagy is a lysosomal degradation pathway of the cell's own material and is highly conserved in all eukaryotes. Three forms have been described: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. They differ in their physiological functions and the mechanisms to carry out the degradative process. During CMA, specific proteins are recognized by chaperones and translocated through the lysosome membrane for degradation (Koga and Cuervo 2011; Arias and Cuervo 2011). Microautophagy is a poorly defined process involving invagination of the lysosome membrane; the resulting small vesicles containing cytoplasmic material are degraded inside the lysosome (Mijaljica et al. 2011).

The best known and the most conserved is the third type, macroautophagy (called 'autophagy' hereafter for simplicity) and it is the main cellular mechanism involved in protein and organelle degradation (Yang and Klionsky 2010; Calvo-Garrido et al. 2010).

Autophagy is triggered by starvation conditions such as nutrient or growth factor depletion, but it is also induced in circumstances requiring for example the elimination of protein aggregates and defective organelles, or in response to bacterial pathogens. A double-membrane vesicle called autophagosome is formed in the cytoplasm engulfing the cargo. This is followed by fusion of the autophagosome with lysosomes and the subsequent degradation of the vesicle and its content. The simple molecular constituents are released during this degradation process to be recycled or used for ATP production.

During aging and neurodegeneration, cells accumulate abnormal protein aggregates, misfolded proteins, and defective organelles that need to be removed by autophagy, since they would otherwise interfere with normal cell function and contribute to the increasing risk of suffering several disorders (Xilouri and Stefanis 2011). The enormous impact of autophagy on pathology and aging has just begun to be recognized and it has started to attract the interest of medical research. Its therapeutical manipulation might be of great importance to fight cancer and degenerative diseases, to mention just two of the most devastating illnesses affecting millions (Yang et al. 2011; Xie et al. 2011). Understanding the molecular mechanisms of the autophagy machinery and its regulation is essential for its potential use as a therapeutic target and we are still far from understanding it in sufficient detail.

The first autophagy proteins were identified in yeast (coined Atg, for "autophagy-related") and grouped into functional complexes that are required for the initiation, elongation, and completion of the autophagosomes, although the precise mechanism of action of many of these proteins and the way they are regulated are not completely understood. Three signaling complexes regulate the initial inductive stage: Tor 1 kinase, Atg1 kinase, and the class III PI3Kinase complex. Vesicle expansion and completion require two ubiquitin-like conjugation systems. In the first conjugation reaction Atg12 is covalently bound to Atg5, a reaction catalyzed by the E1-type enzyme Atg7 and the E2 enzyme Atg10. After this, Atg16 interacts with the Atg12-Atg5 complex localized in the elongating membrane. This step facilitates the second conjugation reaction involving Atg8 (known as LC3 in mammals), which is attached to the expanding autophagosome membrane by conjugation to phosphatidylethanolamine (Fig. 5). Other proteins have been recognized to be involved in autophagy but their functions are still poorly defined. Examples are the membrane proteins Vmp1 and Atg9, which are believed to play a role in autophagosome membrane initiation. For further details see the following reviews (Inoue and Klionsky 2010; Yang and Klionsky 2010; Calvo-Garrido and Escalante 2010).

The origin and the mechanisms of formation of the autophagic double membrane are still open questions. It seems that different organelles including the ER and mitochondria can mediate the formation of the autophagosome membrane, but

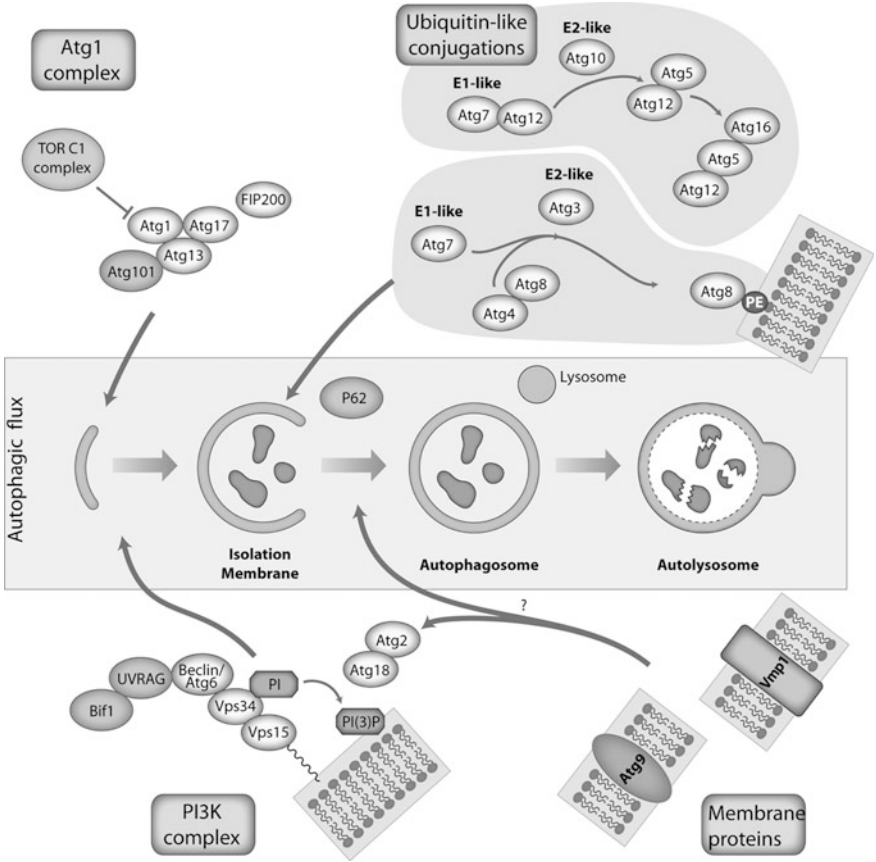

Fig. 5 Autophagic machinery in *D. discoideum*. The isolation membrane is a double membrane that enlarges and finally engulfs parts of the cytoplasm, aggregated proteins or organelles. After fusion with lysosomes, the content is degraded and recycled. The predicted *D. discoideum* autophagic proteins are organized into functional complexes using the information available from the yeast *S. cerevisiae* and mammalian cells. Some proteins such as Atg101, UVRAG, Bif 1 and Vmp1 are present in *Dictyostelium* and higher eukaryotes but seem to be absent in *S. cerevisiae*

how these membranes are recruited and specified to become an autophagosome is unknown (Reggiori 2006; Hailey et al. 2010). The PI3Kinase complex generates phosphatidylinositol 3-phosphate (PtdIns3P) at the site of autophagosome formation and this signaling is essential for the recruitment of autophagic proteins. Many other issues remain to be characterized, such as the mechanisms of membrane elongation and completion, the regulation of the fusion with lysosomes, and the link between the inductive signaling pathways, and the autophagic machinery. The autophagy process is so complex that other unknown autophagy-related proteins are expected to exist.

A number of studies in *D. discoideum* have revealed the similarities (both morphological and molecular) of its autophagic machinery with that of animal cells and, importantly, the presence of homologous autophagic proteins in *D. discoideum* and mammals that are absent in *S. cerevisiae* such as Atg101, FIP200, and Vmp1 (Calvo-Garrido et al. 2010; King et al. 2011; King 2012). In *D. discoideum*, as in mammalian cells, nascent autophagosomes originate in the cytoplasm from multiple origins and fuse with lysosomes, which are also animal-like. In contrast, in the yeast model *S. cerevisiae*, as in other fungi, these structures are concentrated and assembled in a single location of the cytoplasm near the single huge lysosome, the so-called vacuole.

Several markers have been optimized to study different stages of autophagosome formation as well as techniques to monitor autophagic flux (Calvo-Garrido et al. 2011; King et al. 2011), allowing a good level of molecular definition of the autophagic process in the *Dictyostelium* model. A number of mutants in *D. discoideum* have been generated affecting genes coding for at least one component of each of the referred functional complexes and the membrane proteins Atg9 and Vmp1 (Otto et al. 2003, 2004; Calvo-Garrido and Escalante 2010; Tung et al. 2010). A common phenotype in *D. discoideum* autophagy mutants is aberrant development and, interestingly, the severity of the phenotype depends on the mutated gene. The strongest phenotypes correspond to mutations in the kinase Atg1 and the transmembrane proteins Atg9 and Vmp1. Vmp1 is an ER protein that also co-localizes with autophagosomes, suggesting a role in the initial stages of autophagosome formation from the ER, modulating the spatial and temporal dynamics of PtdIns3P signaling (unpublished results). Apart from affecting autophagy, ablation of the protein causes defects in other membrane-dependent processes such as protein secretion and the functioning of the contractile vacuole, an organelle responsible for osmoregulation in social amoebas. Interestingly, Vmp1 has been proposed to have a role in pancreatitis and cancer (Ropolo et al. 2007; Grasso et al. 2011).

Another remarkable trait of autophagy mutants in *D. discoideum* is the presence of ubiquitinated protein aggregates, which are also common features in pathologies like Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease (HD), etc. The above-mentioned differences in the severity of the developmental phenotypes among autophagic mutants correlate quite well with the size and number of ubiquitin-positive protein aggregates (Calvo-Garrido and Escalante 2010). An attractive hypothesis is that the observed phenotypes are aggravated by the presence of these aggregates. The protein composition of the aggregates was partially analyzed in a mutant strain lacking Vmp1 and showed the presence of P62 (also known as Sequestosome 1), an adaptor protein involved in autophagic clearance of several substrates and also implicated in diverse protein aggregation disorders and infectious diseases (Zheng et al. 2011; Mostowy et al. 2011). The study of the formation and degradation of these aggregates is clinically important as it might provide opportunities for effective therapies.

It is still debated if protein aggregates in neurodegenerative diseases are the cause of the disease or a cell defense mechanism to accumulate and separate

abnormal proteins that otherwise would be toxic. Several studies in *D. discoideum* suggest that the protein aggregation phenotype might be deleterious for cell function, perhaps acting as a sink for other normal proteins that are attracted to the aggregate and thus lose their normal localization. This phenomenon was recently described in *D. discoideum* when VASP, an actin-binding protein, was expressed with an endosomal targeting signal. The mis-targeting of this protein leads to the formation of huge actin aggregates reminiscent of Hirano bodies, often present in neurodegenerative diseases. These actin aggregates were found to sequester a number of actin-binding and endosomal proteins promoting their disappearance from the cytoplasm, their normal location (Schmauch et al. 2009). Interestingly, the strong defect in the cytoskeleton observed in *D. discoideum* cells with Hirano bodies can be mimicked by disruption of these sequestered proteins, opening the possibility that protein sequestration might also contribute to neuronal malfunction in these pathologies. Hirano body-like aggregates can also be induced in *D. discoideum* by the overexpression of a truncated form of a 34 kDa actin-binding protein (Maselli et al. 2002). A recent report showed that both autophagy and the proteasome pathway contribute to the degradation of Hirano bodies in *D. discoideum*. Moreover, the autophagosome marker protein GFP-Atg8 co-localized with Hirano bodies in wild-type *D. discoideum* cells, but not in cells deficient in the autophagic proteins Atg5 or Atg1 (Kim et al. 2009).

D. discoideum is also a promising model to understand the normal physiological role of the presenilins, which are key proteins in Alzheimer's disease. Presenilins are the catalytic moiety of γ-secretase, a protease that cleaves a wide variety of integral membrane proteins. Inappropriate processing of amyloid precursor protein (APP) by this protease is associated with familial Alzheimer's disease. The analysis of null mutants indicates that presenilin complexes regulate cell differentiation and phagocytosis in *D. discoideum*. Remarkably, *D. discoideum* cells are able to process ectopically expressed human APP, and thus it might serve as a valuable model to understand the pathology and to apply high-throughput screening of new therapeutic drugs (McMains et al. 2010).

HD is a neurodegenerative genetic disorder caused by the presence of polyglutamine tracts in the protein huntingtin. *D. discoideum* has a huntingtin homolog whose disruption leads to pleiotropic defects in different cellular processes including cell motility, adhesion, and cytokinesis suggesting that huntingtin, as also suspected in human, is a complex multifunctional protein (Myre et al. 2011; Wang et al. 2011b).

Unexpected connections of human diseases with autophagy might arise from studies in *D. discoideum*. This is the case of the human *VCP* (p97) gene, whose mutations cause IBMPFD (inclusion body myopathy with early onset Paget's disease of bone and frontotemporal dementia), ALS14 (amyotrophic lateral sclerosis), and HSP (hereditary spastic paraplegia). This protein is conserved in *D. discoideum* and the expression of the most prevalent mutation leads to defects in growth, development, proteasomal activity, and autophagy. Interestingly, a novel relationship of VCP with the core autophagic protein Atg9 has been discovered, which is based on mutual inhibition (Arhzaouy et al. 2012).

5.4 The D. dictyostelium Model of Mitochondrial Disease

The mitochondrion is the organelle responsible for the generation of most cellular ATP through oxidative phosphorylation, and thus plays a key role in cellular bioenergetics. Similar to impaired autophagy, mitochondrial dysfunction affects the energy status of the cell and affects multicellular development. It should be noted that mitochondria are also involved in other important functions such as apoptosis, calcium homeostasis, lipid synthesis, generation of reactive oxygen species, aging, etc. (Sanz et al. 2006; Barja 2004; Satrustegui et al. 2007; Lopez-Lluch et al. 2008; Cadenas 2004; Fernandez-Moreno et al. 2007; Kompare and Rizzo 2008; Suen et al. 2008). Mitochondrial diseases are caused by genetic mutations in proteins encoded in the mitochondrial genome or in the nuclear genome. The pathological phenotypes of mitochondrial diseases are very complex and include blindness, deafness, epilepsy, heart disease, muscle, and neurological disorders, etc. Although many different associated mutations have been identified, the relationship between genotype and phenotype is still poorly understood. For example, the same genetic defect might result in different symptoms and the opposite is also true, similar outcomes might be originated by different genetic lesions (Debray et al. 2008; DiMauro and Schon 2008). Mitochondrial dysfunction is also believed to be involved in neurological disorders such as Parkinson's disease and Alzheimer's disease (Santos et al. 2011). The inefficient clearance of damaged mitochondria by mitophagy, a specific form of autophagy, is believed to be responsible for the accumulation of defects in mitochondria leading to cell malfunction and pathology during aging and neurodegenerative diseases (Xilouri and Stefanis 2011; Winslow and Rubinsztein 2011).

D. discoideum is a suitable model to study mitochondrial diseases for several reasons. It combines a powerful genetic tractability with a unique life cycle that provides a great variety of reproducible phenotypes associated with mitochondrial dysfunction (Francione et al. 2011). As occur in humans, mitochondrial disease in *D. discoideum* is characterized by what is known as pathological thresholds, so that some phenotypes appear more sensitive than others to the level of mitochondrial dysfunction. A number of different strategies are available in *D. discoideum* to generate sub-lethal mitochondrial defects such as RNAi (RNA interference) (Morita et al. 2005), antisense inhibition (Kotsifas et al. 2002), heteroplasmic disruption of mitochondrial genes (Francione and Fisher 2011), and disruption of nuclear genes encoding mitochondrial proteins (Torija et al. 2006b).

Traditionally, it has been assumed that low levels of ATP are the principal factor in mitochondrial diseases. However, recent studies in the *Dictyostelium* model suggest that some of the symptoms might be the consequence of abnormal regulation of signaling pathways. The relationship between the AMP-activated protein kinase (AMPK), a critical regulator of the energy status of the cell, and mitochondrial diseases has been clearly established in *D. discoideum* (Bokko et al. 2007). AMPK is highly sensitive to the AMP/ATP ratio and it is activated when ATP levels decrease as a consequence of mitochondrial dysfunction. Once

activated, AMPK tries to re-establish the cellular energy levels by activating pathways that generate ATP while inhibiting others that consume it (Hardie 2011). Antisense inhibition of the mitochondrial chaperonin 60 produces defective photo/thermotaxis at the slug stage and affects growth and morphogenesis in *D. discoideum*. Interestingly, these defective phenotypes are suppressed when AMPK expression is knocked-down in this mutant. Conversely, the expression of a constitutively active form of AMPK in wild-type cells mimics the observed phenotypes. These data strongly suggests that AMPK is chronically activated in *D. discoideum* cells with mitochondrial dysfunction (Bokko et al. 2007; Francione et al. 2009). Interestingly, recent reports indicate that abnormally activated AMPK is accumulated in cerebral neurons in Alzheimer's disease and other neurodegenerative disorders (Vingtdeux et al. 2011; Choi et al. 2010).

An added value of *D. discoideum* is the presence of complex I (CI), the largest complex of the respiratory chain that couples the oxidation of NADH to the reduction of ubiquinone and the transport of protons across the mitochondrial inner membrane. This complex is not present in some yeast models such as *S. cerevisiae* or *S. pombe*, although it is present in others such as *Yarrowia lipolytica*. CI has an enormous impact on human disease, since about 40 % of inherited mitochondrial disorders involve isolated or combined deficiencies in CI activity. CI contains 45 protein subunits in human and *D. discoideum* codes for most of these proteins (Francione et al. 2011). Despite its complexity, relatively few assembly factors have been described to assist in the correct assembly and stability of this huge complex. Studies in *D. discoideum* have led to the discovery of MidA (also known as PRO1853) and its role in CI function. Mutant *D. discoideum* cells where MidA has been ablated show a specific defect in CI activity that has also been confirmed in human HEK293T cells where MidA was down-regulated by RNAi. Moreover, both *D. discoideum* and human MidA interact with the essential CI subunit NDUFS2 (Carilla-Latorre et al. 2010). MidA deficiency not only causes the typical AMPK-dependent defects seen in other mitochondrial mutants, but also produces additional phenotypes related to phagocytosis and macropinocytosis that highlight the complexity of mitochondrial diseases even in this simple model. All these data suggest that MidA is a good candidate to be involved in human mitochondrial disease.

5.5 Molecular Pharmacology in D. dictyostelium

Understanding the mechanism of action of drugs and toxic compounds at the cellular and physiological levels is fundamental in clinical research. This difficult task is in part hampered by the use of complex models, for example mammalian cell lines, which are very different in origin and properties and often harbor mutations and chromosome duplications. Along the same line, and apart from the inherent ethical problems, the use of mammalian models (mice and rats) is

expensive and the necessity to use large numbers of animals (for example in drug screening) increases the costs even more.

But above all, in such complex models it is impossible to use unbiased genetic screening to clonally isolate mutant strains and the analysis of single and multiple KOs is far from simple. These problems can be partially circumvented by using simple and genetically tractable organisms such as *D. discoideum*. In spite of the evolutionary distance, recent research has shown an amazing conservation of the mechanisms of action of some drugs between the social amoeba and humans.

One example in which *D. discoideum* has proved useful is in deciphering the mechanisms underlying the resistance and sensitivity to chemotherapeutic drugs such as cisplatin. Only in Spain, approximately, 200,000 people are diagnosed with cancer every year and many of them are treated with chemotherapeutic drugs, which are cytotoxic molecules that are more efficient in killing rapidly dividing cancer cells than normal cells. However, one of the most important problems is that tumors often become resistant to the drugs. The mechanisms of such resistance are not completely understood and this knowledge is essential to design new anticancer drugs and new strategies. A blind genetic screening in *D. discoideum* for selection of mutants resistant to cisplatin opened new avenues for the research into the mechanisms of resistance (Alexander and Alexander 2011). Mutagenesis was performed by REMI to identify genes involved in cisplatin resistance. None of the identified genes were previously implicated in cisplatin action (Li et al. 2000). One of them was the gene encoding the enzyme sphingosine-1-phosphate (S-1-P) lyase, a highly conserved enzyme of the sphingolipid metabolic pathway (Li et al. 2001) (Fig. 6a). This discovery led to further characterization of this pathway using KOs and overexpression strains, which demonstrated that multiple enzymes of this biochemical pathway are involved in the mechanism of action of cisplatin and other chemotherapeutic drugs in the *Dictyostelium* model and, most importantly, also in human cells (Alexander and Alexander 2011). These studies suggest that the balance between the signaling sphingolipids ceramide and S-1-P determines whether a cell dies or lives in the presence of the drug. Thus, modulation of the activity of sphingolipid metabolizing enzymes is a potential new target for improving cancer therapy.

Bipolar disorders are complex pathological conditions leading to extreme changes in mood. Current treatments for bipolar disorder include, among others, valproic acid (VPA) and lithium (Li). The effects of these compounds are complex and seem to affect diverse signaling pathways, but their precise sites of action and the signaling pathways involved are largely unknown (Ketter 2010). In both *D. discoideum* and mammalian cells, VPA and Li acting at different levels are able to increase the level of activation of the MAP kinase ERK2 by increasing its phosphorylation (Einat et al. 2003). MAPK pathways are involved in neuronal differentiation and plasticity and thus are good candidates to be involved in the disease.

Extracellular signal-regulated kinases (ERKs) are a class of MAP kinases functioning in many different signaling pathways. In *D. discoideum* there are two ERK kinases (ERK1 and ERK2). ERK2 is involved in the chemotactic response to

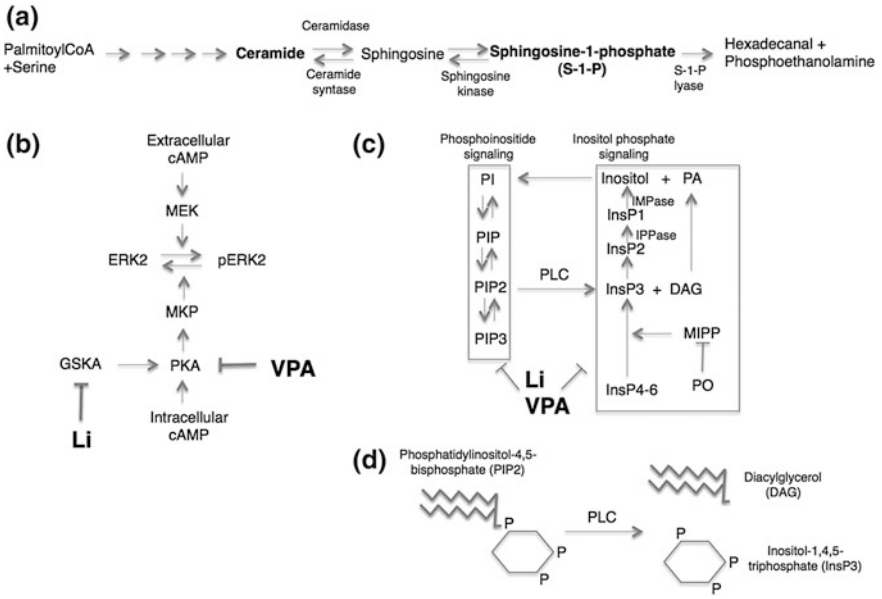

Fig. 6 Molecular pharmacology in *D. discoideum*. **a** Schematic representation of the sphingolipid metabolic pathway involved in cisplatin resistance. **b** MAPK regulation by VPA and Li, used to treat bipolar disorders. ERK2: extracellular regulated kinase 2; pERK2: phosphorylated ERK2; MKP: MAPK phosphatase; MEK: MAP kinase kinase; PKA: protein kinase A; GSK3: glycogen synthase kinase 3. **c** Inositol phosphate and phosphoinositide signaling regulation by bipolar disorder treatments. PO: prolyl oligopeptidase; MIPP: multiple inositol polyphosphate phosphatase; IPPase: inositol polyphosphate phosphatase; IMPase: inositol monophosphatase. **d** Schematic representation of the structure of PIP2 (phosphatidylinositol 4,5-biphosphate) and its cleavage by PLC (phospholipase C) to generate DAG (diacylglycerol) and InsP3 (Inositol 1,4,5-triphosphate). See the text for a complete description of the pathways

cAMP and cell differentiation during development (Nguyen et al. 2010). The use of KO strains and overexpressors suggests the model depicted in Fig. 6b (Ludtmann et al. 2011). Treatment of *D. discoideum* cells with cAMP gives rise to a transient phosphorylation of ERK2 and the use of Li or VPA induces a significant increase in this effect. However, they do so by affecting different pathways. VPA-induced ERK2 phosphorylation can be mimicked by decreasing intracellular cAMP or PKA activity and by pharmacological inhibition of phosphotyrosine phosphatase activity, suggesting that VPA acts by inhibiting the de-phosphorylation of ERK2 (Boeckeler et al. 2006). The precise mechanism and the crosstalk between the cAMP pathways and ERK2 are not fully defined and require further studies in *D. discoideum* and mammalian cells. The effect of Li is believed to involve inhibition of GSKA since GSKA ablation reduces the lithium effect on ERK2 phosphorylation, while VPA has an increased effect in this mutant suggesting that VPA acts through a GSKA-independent mechanism.

VPA and Li have also been shown to modulate another fundamental and well-conserved signaling pathway, the inositol phosphate pathway (Fig. 6c). Li attenuates inositol recycling by inhibiting the enzymes inositol monophosphatase (IMPase) and inositol polyphosphate 1-phosphatase (IPPase) (Williams et al. 2002; Allison et al. 1980; Hallcher and Sherman 1980; Berridge et al. 1989). Additional studies by REMI mutagenesis identified prolyl oligopeptidase (PO) as the gene disrupted in a mutant resistant to the block in development exerted by Li (Williams et al. 1999). PO mutants had elevated InsP3 levels, suggesting an effect on this signaling pathway. In agreement with this, the effect of Li, VPA and carbamazepine (another mood-stabilizing drug) on development was reversed by addition of exogenous inositol. A similar situation was also observed in mammalian neurons (Williams et al. 2002; Eickholt et al. 2005). Although PO is supposed to be involved in the cleavage of small peptides containing prolyl residues, a recent study established a clear link between PO and the enzyme multiple inositol polyphosphate phosphatase (MIPP) involved in the formation of InsP3 by the breakdown of InsP4, InsP5, and InsP6 (King et al. 2010). As expected, the ablation of MIPP results in reduced InsP3 levels and its overexpression leads to the opposite effect. Moreover, the PO-dependent effect on inositol signaling requires active MIPP. Taken together, these results suggest that PO is an inhibitor of MIPP activity. Importantly, a significant decrease in PO activity has been reported in bipolar disorder patients undergoing Li treatment (Breen et al. 2004).

Another layer of complexity is the presence in *D. discoideum* of a gene expression regulatory mechanism where the activation of MIPP induces the expression of inositol-regulatory genes including IMPase and IPPase, which are targets of Li, and inositol synthase 1 (INO1), involved in the synthesis of inositol. Interestingly, these observations have been reproduced in human cell culture (King et al. 2010).

A related signaling pathway, the phosphoinositide pathway, has also been implied in the mechanism of action of bipolar disorder drugs thanks to the work in *D. discoideum* (Xu et al. 2007; Chang et al. 2012). VPA treatment reduces phosphoinositide levels and this observation led for the first time to the suggestion that elevated phosphoinositol signaling might be an important factor in the development of the disease. Remarkably, this idea is supported by the observation of increased levels of phosphatidylinositol-4,5-bisphosphate (PIP2) in untreated patients and its down-regulation by the drugs (Soares et al. 1997, 2000). Further observations in *D. discoideum* implicate phosphoinositides as a target for Li treatment. Translocation of protein kinase B (PkbA) to the membrane is mediated by the interaction of its PH domain with PIP3, thus serving as a marker of PIP3 levels. Li was found to reduce PkbA translocation, suggesting a defect in PIP3 signaling. Once more, these observations were reproduced in human cells (King et al. 2009). In summary, the results in the *Dictyostelium* model support the hypothesis that Li and VPA exert their action through inositol depletion but also through attenuation of phosphoinositide signaling.

6 Concluding Remarks

The studies reviewed in this chapter justify the interest in *D. discoideum* as a model system to study several aspects of biomedical sciences and its potential to open new avenues in the study of mechanisms of human disease. A recent bibliometric analysis has shown that more than 75 % of research still focuses on the 10 % of proteins that were known before the human genome was sequenced (Edwards et al. 2011). This conservative tendency is still stronger for proteins of unknown function. Some of these proteins are highly conserved among evolutionarily distant species suggesting that they play relevant roles, perhaps related to human diseases. The inherent difficulty in the study of a disease-related protein of unknown function or the complexity of signaling networks in pathology can be partially overcome by the powerful genetics of simple models, as illustrated in this review. These models may serve to give us new and unexpected insights.

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Genome Analysis of Social Amoebae

Gernot Glöckner

Abstract Genomics is now an indispensible part of the biological sciences. Today a species description without genome information is incomplete. This chapter describes the current knowledge on the genome of the model species *Dictyostelium discoideum*. A comparison with other social amoebae genomes covering the whole breadth of this branch of evolution carves out driving forces of speciation and the common toolkit of all social amoebae. The vast evolutionary distance within this branch makes ortholog detection difficult. While the coding capacity of all social amoebae is largely conserved, species specific gene family expansions of proteins for environmental sensing, signaling, and secondary metabolites provide for diversification. The sequences of the functional chromosomal elements (telomeres and centromeres) are not conserved, rather they seem to have underwent severe modifications. Nucleosome patterns link the social amoebae to other, more sophisticated multicellular systems. Comparative curated databases make this wealth of genome information accessible and play an important role for the dissemination of the knowledge on this evolutionary branch.

1 Genomics

Genomics has revolutionized the biologist's view on taxonomy, species, and traits in the last few years. Molecular data provide an unambiguous data source for the analysis of these features and allow an unprecedented detailed view on the evolutionary history of organisms. Initially, only a handful of model organisms were selected for a full genome analysis since in the early years of genomics sequencing was expensive and standard methods for computational analysis were still in

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development. Among this selection was *Dictyostelium discoideum*, a primary basic genetic model system for the analysis of development, signalling, and the cytoskeleton. It soon became evident however, that only one species as representative for whole eukaryote evolutionary branches is not enough to get insight into evolutionary events and trajectories in the eukaryote tree. To be able to discern species specific from branch specific inventions, genomes of several species must be deciphered and compared to each other. Thus, immediately after the completion of the *D. discoideum* genome in 2005 (Eichinger et al. 2005; Glöckner et al. 2002), several comparative genomics projects were launched. The previously calculated detailed phylogeny of the main groups of Dictyostelids (Schaap et al. 2006) enabled the selection of species from each of the four main groups of social amoebae for comparative purposes.

2 Nuclear Genomes of Social Amoebae

2.1 The Model Organism D. discoideum

Assembly of sequences ranging from only 100 bases to a little bit more than 1 kb normally yields only draft genomes, i.e. genomes represented by a large number of unordered contigs separated by gaps of unknown length. If higher order organizational features of a genome should be analyzed these contigs must be brought into their natural order and as many gaps as possible should be filled. For *D. discoideum* a genetic map of the genome already existed (Kuspa et al. 1992; Loomis et al. 1995) although routine crosses with sufficient progeny for linkage analysis are rarely successful (Francis 1998). This genetic map was based on mutants with a visible phenotype and therefore of low resolution. To get a marker dense map of the genome a physical mapping method [happy mapping (Dear and Cook 1993)], which does not rely on sexual reproduction, was employed with this species (Konfortov et al. 2000; Williams and Firtel 2000). Both the genetic and the "Happy" map generally agreed thus providing the basis for the reconstruction of whole chromosomes. The maps also showed that the 34 Mb of the genome are distributed over six chromosomes.

Dictyostelium discoideum has a genome highly biased towards A and T nucleotides. With a mean A/T content of 78 % (Table 1) it exhibits the second highest bias so far observed, only surpassed by *Plasmodium falciparum* (Gardner et al. 2002). Later comparisons between these highly biased genomes revealed, that they employ different strategies to achieve such high A/T contents. The A/T bias influences the codon usage more pronounced in *D. discoideum* than in *P. falciparum*, while *P. falciparum* extends its intergenic regions with long A/T stretches (Szafranski et al. 2005). Long stretches of A/T rich codons encoding the same amino acid (mainly asparagine) contribute significantly to the overall A/T content in *D. discoideum* and considerably alter also the amino acid sequence of

Table 1 Genome properties of social amoebae. Only completed genomes are shown

	DD	DP	DL	PP	DF
Contigs	226	1,213	54	52	33
Supercontigs	6	838	54	41	25
Total nucleotides (Mbp)	35	33	23	33	31
Average contig length (kbp)	155	27	433	634	1,064
Overall nucleotide frequency (A/T %)	77.6	75.4	70.2	68	66.2
Palindrome arm size (kb)	45	26	14	15	28
Mitochondrial genome size (kb)	55	52	47	48	56
Chromosome numbers	6	nd	8 (or more)	7	6
Repeat content(%)	~10	3.4	<1	<1	<1
Telomere repeat structure	Palindrome arm	Palindrome arm?	TAGGG + palindrome arm	TAAGGG	TTAGGG
Predicted coding sequences (CDS)	13,433	12,410	10,958	12,373	12,173
Average gene length	1,579	1,689	1,596	1,552	1,672
Gene density (CDS per Mb)	396	376	470	375	392
Nucleotide frequency in CDS (A/T %)	72.6	69.9	67.8	63.8	63.2
Predicted tRNAs	401	375	61	273	198

DD: *Dictyostelium discoideum*; DP: *D. purpureum* (both group 4); DL: *D. lacteum* (group 3); PP: *Polysphondilium pallidum* (group 2); DF: *D. fasciculatum* (group 1)

the encoded proteins. No functions so far could be assigned to those homopolymer runs and later genome wide comparisons revealed that the position of these runs within a protein is not conserved (Sucgang et al. 2011). Thus, the only purpose of the homopolymer runs is seemingly to drive the A/T content of the genome to higher extremes. The A/T bias also prevented the usage of large insert size vectors like fosmids or bacterial artificial clones (BAC) since recombination in the bacterial host frequently affects the inserts resulting in deletions in the inserts of these clones (Glöckner et al. 2002).

The analysis of the coding capacity of the *D. discoideum* genome brought a big surprise: It encodes more than twice the protein coding genes than the unicellular budding yeast *Saccharomyces cerevisiae* (Goffeau et al. 1996). Presumably, the higher complexity of the cytoskeleton, the amoeboid mobility, sophisticated signalling systems, complex environments, and the developmental cycle require this higher amount of genetic information in *D. discoideum*.

The *D. discoideum* genome harbours comparably many transposable elements (TEs). With a total of 10 % of the whole genome this is by far more than in other unicellular species observed (Glöckner et al. 2001). The TEs comprise retrotransposons and DNA transposons. Additionally, some smaller repeated sequences were found. The TEs exhibit a non-random distribution in the genome. Some TEs (the TREs) use tRNAs as target sequences for integration and thus are mainly found (with a few exceptions) in a defined vicinity of tRNAs. Dictyostelium

intermediate repeat sequence (DIRS) elements on the other hand are restricted to the tips of the chromosomes where they form extensive clusters interspersed with some DNA elements. Three of these clusters could be reconstructed by making use of read pair and positional information on single nucleotide polymorphisms (SNPs) (Glöckner and Heidel 2009). These clusters are up to 300 kb long and, according to the analysis of the distribution of SNPs in DIRS elements, exchange these elements or parts of clusters among each other either by homologous recombination or transposition. It remains enigmatic why a genome densely packed with protein coding genes enables the spreading of TEs. Possibly, the restriction of TEs to certain regions of the genome ameliorates the adverse effects of transposition events. On the other hand increased mortality of strains harbouring TEs at uncommon positions could be responsible for clearance of these strains from the population (Winckler et al. 2002, 2005).

Previous genetic analyses had shown that many protein components indispensible for multicellular life forms in the Opisthokontha branch of eukaryote life (comprising Metazoa, Amoebozoa, Fungi, and some unicellular flagellated species) are also present in *D. discoideum*. A genetic analysis using mutants however is hampered by the fact that functions or parts of functions are often encoded redundantly in genomes. The complete genome then gave additional insight into the composition and extent of gene families. Several gene families, myosins, actins, rho related proteins, rasGEFs, kinesins, etc. (Joseph et al. 2008; Kollmar 2006; Kollmar and Glöckner 2003; Rivero et al. 2001; Wilkins et al. 2005), were analysed separately yielding information on basic eukaryote family sets and species specific expansions.

As a surprise came the finding that transcription factors are rare in this genome and that basic helix-turn-helix motifs are missing. This indicates that transcription either plays a minor role in regulation of the life and development of *D. discoideum* or that specifically adapted not yet identified transcriptional regulators are encoded in this genome.

2.2 Comparative Sequencing of Other Social Amoebae

The development of next generation sequencing (NGS) technologies over the last few years made genome analysis much cheaper. This enabled the sequencing of additional species within the social amoebae. To date at least a genome of one species in each of the four main branches of social amoebae is available in draft or complete state (Heidel et al. 2011; Sucgang et al. 2011; Felder et al. 2013). Here, genomes are considered complete, if all or nearly all contigs could be ordered and oriented or gap closure procedures were employed to close as many remaining gaps as possible. This is only possibly if additionally to the NGS data large insert size clones are used to order and orient contigs in their natural order along the chromosomes. Since the A/T bias is not so extreme in the other social amoebae groups it was possible to construct fosmid libraries enabling long range mapping

of contigs (Heidel et al. 2011). Table 1 lists relevant features of the available 5 genomes. Intriguingly, the total genome size ranges from 23 Mb for *D. lacteum* from group 3 to 34 Mb for *D. discoideum* in group 4. Part of the larger size of *D. discoideum* compared to all other social amoebae is attributable to the exceptional amount of TEs in *D. discoideum*, but another part is due to more extensive gene family expansions and larger intergenic regions. Thus, all genomes have nearly the same coding capacity, emphasizing the establishment of a life style early in evolution with only a few modifications required for the differentiation into lineages.

Orthologs between the species can be identified, if the similarity is not decayed below a certain threshold. Over the whole range of species in the social amoebae only roughly half of the genes have clear orthologs (Heidel et al. 2011). If no similar protein can be detected in another species, but an encoded protein contains an identifiable domain, this domain might be a hint that functional relationships exist between otherwise unrelated proteins. For example only a minority of transcription factors has identifiable direct orthologs, but the transcription factor domains are equally present in all genomes. Possibly, the lack of similarity is here due to the adaptation of these factors to a specific genomic environment.

Alignments of coding DNA between species are possible, if the coding region in question represents a highly conserved protein and the protein sequence is used as a guide for the alignment (Fig. 1). The third position of each codon can be altered often without changing the amino acid sequence in the protein. The alignment shows that this is the case at almost all third positions, which indicates a saturation with mutations. This fact emphasizes the large evolutionary distance between the species analysed. Synteny is scarce between groups, only a few genes have the same neighbours in species of different groups. Moreover, the location on a certain chromosome as it is the case in Drosophila species (Heger and Ponting 2007) is also not conserved. This indicates a frequent reshuffling of the genetic material irrespective of the chromosomal position.

The analysis of gene families revealed that only some specific families are affected by differential family expansions, others have nearly the same number of members in each genome with clear orthology relationships among the members. One of the most prominent examples of species specific differences is the polyketide synthase family (PKS). This family has more than 15 members in each species. A phylogenetic analysis revealed that only a few PKS family members had orthologs in other species, the majority of the members cluster together in species specific clades. Thus, while this family has a common origin, species specific amplifications contribute to species diversification.

Taken together, families encoding proteins required for environmental sensing, defence, or production of secondary metabolites seem to be driving forces for speciation, while the basic repertoire for the vegetative and developmental cycle remained remarkably stable.

Interestingly, chromosomal structures are variable within the social amoebae. The DIRS element is present as a cluster at each tip of the chromosomes of *D. disoideum* and here constitutes the centromeres (Dubin et al. 2010). This might

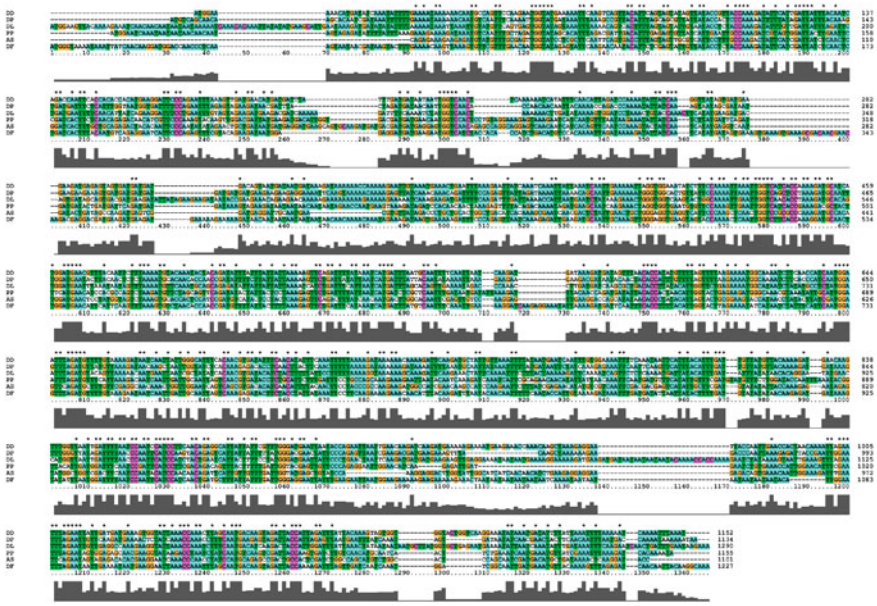

Fig. 1 DNA alignment of gene cdc123 between six social amoebae of which the genomic sequence is known. Coding sequences were retrieved from the respective databases and aligned in MEGA5 (Tamura et al. 2011) based on the amino acid sequence. Thus the alignment was built from three-base blocks (codons). Identical residues are marked with an *asterisk* above the alignment. The overall conservation is depicted as *blocks* below the alignment. DD *Dictyostelium discoideum*; DP *D. purpureum* (both group 4); DL *D. lacteum* (group 3); PP *Polysphondilium pallidum*, AS *Acytostelium subglobosum* (both group 2); DF *D. fasciculatum* (group 1)

also be true for *D. fasciculatum*, where also, albeit smaller, DIRS clusters were found at chromosomal tips (Fig. 2). All other genomes as the nearly complete assemblies of *P. pallidum*, *D. lacteum*, and *D. fasciculatum* show, do not contain enough DIRS sequences to endow each chromosome with at least one DIRS element. Instead, in case of *D. lacteum* a short species specific sequence is located at chromosome tips, whereas *P. pallidum* has a complex pattern of sequences at each end of the chromosome (Fig. 2). It is not clear, which sequences fulfil the role of centromeres in these species since no functional analysis was done so far. We only can state that obviously the functional elements of centromeres are different from those in *D. discoideum*. Likely, DIRS elements were already the centromeres in the last common ancestor of social amoebae, since the most divergent groups 1 and 4, represented by *D. fasciculatum* and *D. discoideum*, have such centromeres. The other species analysed have engaged other sequences to fulfil these functions. The telomere sequences also differ between species (Fig. 2). While group 1–3 species have normal eukaryote telomere structures with differing telomere repeat sequences at chromosomes (Table 1 and Fig. 2), group 4 species seem to have replaced these with long sequences stemming from the rDNA palindrome (see below). The rDNA palindromes normally have the same structures at the ends as

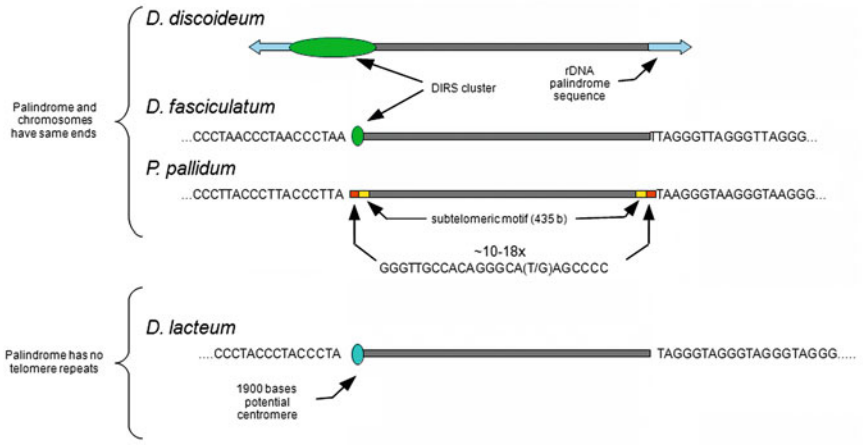

Fig. 2 Schematic representation of chromosome structures and palindromes. Only species for which the structures were investigated in detail are shown

the chromosomes of the species. Only in case of *D. lacteum* these structures differ between chromosomes and rDNA palindrome. While the chromosomes are endowed with normal telomere repeat sequences, the palindrome has structures resembling those in *D. discoideum*. This situation could be interpreted as an intermediate stage between that with the basic eukaryote telomere sequences seen in groups 1 and 2 and the complete replacement of those sequences by a specific sequence stretch as in *D. discoideum* and presumably also in *D. purpureum*, since no eukaryote telomere sequences could be found in this assembly (own analysis).

Taken together, the original centromeres and telomeres seem to have been exchanged several times in the social amoebae clade so that each species has its unique outfit with such features.

3 Plasmids, rDNA Palindrome, and Mitochondrial Genomes

Besides the nuclear genome in social amoebae there exist extrachromosomal elements and plasmids in the nucleus and organellar genomes in mitochondria.

3.1 Plasmids

It is unknown which role plasmids play in the life of social amoebae. No function besides for plasmid maintenance itself could so far be assigned to one of the

protein coding genes of the deciphered plasmid sequences. Plasmids seem also to be dispensable since some strains harbour them and some not without any obvious differences in fitness or other features (Rieben et al. 1998).

3.2 rDNA Palindromes

Extrachromosomal elements encode the rRNA genes on an inverted repeat with a small centre region of only a few bases. Such an organisation is also called a palindrome. The purpose of the palindrome is to provide the organism with an amplified number of rRNA genes just like the repeated arrays of rRNA genes in chromosomes of other organisms (Pendas et al. 1993). Besides the genes the palindrome contains repeated sequence stretches, which are species specific. Thus, the palindromes cannot be aligned to each other outside the genic regions. The extent of the repeats of the non-coding part of the palindrome arms is responsible for the varying length of the palindrome in the different species from a mere 14 kb in *D. lacteum* to more than 80 kb for one arm in *D. discoideum* (Heidel et al. 2011; Sucgang et al. 2003). Since the palindromes are presumably being replicated like chromosomes they also harbour species specific chromosomal end structures (Fig. 2).

3.3 Mitochondria

The mitochondrial genomes are around 45 kb in length (Table 1). They all encode the same genes mainly in the same order. Only one segmental shift occurring in group 4 could be observed (Heidel and Glöckner 2008). Comparison to the *Acanthamoeba castellanii* mitochondrial genome revealed extensive syntenic regions also to this distantly related genome within the Amoebozoa (Glöckner and Noegel 2013). The mitochondrial genomes are thus stable in terms of coding capacity and gene order, yet the nucleotide sequence is so divergent that alignments outside the coding regions are impossible. The coding parts of the mitochondrial genomes were also used to calculate a molecular phylogeny. The tree obtained differed from the previously calculated rDNA and some protein coding genes based phylogeny of social amoebae, but agreed with the second best possible tree (Schaap et al. 2006). Later calculations based on large concatenated data sets from complete genomes confirmed the correctness of the mitochondrial tree. Thus, the revised social amoebae phylogeny shows a bifurcation of groups 1/2 and 3/4 rather than a sequential emergence of the major branches.

4 Regulation of Transcription on a Global Scale

Efforts are underway to characterize the whole transcriptomes during the developmental cycle of all social amoebae of which the genomes are currently known. So far, only data on the transcriptomes of *D. discoideum* and *D. purpureum* are available. Initially, for *D. discoideum* microarrays were used to produce expression profiles of all at that time available genes (Booth et al. 2005; Sasik et al. 2002). Later, RNASeq (Oshlack et al. 2010) was also employed, which is a more sensitive method for transcriptome analysis.

A comparison of transcription of *D. discoideum* and *D. purpureum* during the developmental cycle revealed that this program is largely conserved between the two species (Parikh et al. 2010). Given the large evolutionary distance of the two species, which is estimated to be between 300 and 400 million years, this is remarkable. It remains to be shown that this grade of conservation is also present in the more distantly related groups. Yet, at least the core components remained presumably unchanged since the establishment of the whole clade some 600 million years ago.

Only in the last few years it became apparent that besides proteins also small RNAs play a pivotal role in regulation of expression. Some species of these RNAs play roles in silencing transposons. Indeed, a large number of such RNAs were found to regulate DIRS and skipper elements in *D. discoideum* (Hinas et al. 2007). Furthermore, also some developmental genes seem to be regulated by such RNAs. For other social amoebae species currently are no data on small RNAs available.

Methylation of DNA or histones is another way to regulate transcription. The genomes of social amoebae only harbour DNA-methyltransferase, which methylates Cs in a context independent manner. These methylases are involved in silencing of some, but not all transposon species (Kuhlmann et al. 2005). Thus, regulation of transposon activity is either achieved by siRNAs (DIRS and skipper) or DNA methylation (TRE elements). Histone methylation was also proven to play a role in developmental timing in *D. discoideum* (Chubb et al. 2006).

Gene transcription is also influenced by the packaging state of the DNA around a certain locus (Farkas et al. 2000; Ito 2003). Thus, the analysis of nucleosome positions around genes is an indispensible step towards the complete understanding of gene regulation in an organism. Global analysis of nucleosomes can be done using next generation sequencing technology. In short, DNA is cross-linked with the proteins attached to it and is subsequently digested using endonucleases. This way, only DNA covered by proteins remains intact and can be, after selective enrichment and reversal of the cross-link, sequenced. Such analyses in different eukaryote model organisms revealed that non- coding portions of a gene are normally depleted of nucleosomes. Not surprisingly, this same pattern seemingly common to all eukaryotes was found in *D. discoideum*. In *D. discoideum* the transcripts have poly-T tracts at their 5' end and poly A-tracts at their 3' end. These tracts are associated with nucleosome free regions. The nucleosome border regions are precisely marked with homo- and heteropolymeric tracts of A and T

nucleotides (Chang et al. 2012). The nucleosomes do not change their position upon entry into the developmental cycle as revealed by the comparative analysis of nucleosome positions between the vegetative state and 4 h into the developmental cycle. One interesting finding was that the transcriptional start site is marked by nucleosomes at the +1 position indicative of a paused RNA polymerase in the same way as in most multicellular systems in contrast to unicellular species. Thus, a multicellular stage seems to depend not only on coding capabilities of an organism but also on DNA occupancy properties. Since currently nucleosome data are available only for a handful of species this finding must remain preliminary until more species have been examined.

5 Curation of Genomes and Databases

Genome analyses should not end with the completion of a first draft annotation of a genome. Rather, annotation must proceed and ameliorated by integrating literature, the latest genome sequence, new data like expression analysis, and information on mutation phenotypes. Fortunately, in the early days of the *D. disoideum* genome project a database system was set up to fulfil all these purposes (Fey et al. 2009). Now, dictybase (http://dictybase.org) has curated all *D. discoideum* predicted genes and made them publicly available. Other genomes are being integrated so that this database will give a comprehensive overview on all available genomes of social amoebae. Another database has been used to initially annotate the genomes of three social amoebae (*D. lacteum*, *D. fasciculatum*, and *P. pallidum*; http://sacgb.fli-leibniz.de) (Felder et al. 2013). This database also provides alignments of all genes to their best bidirectional hits and tools for the analysis of synteny between genomes. A further database provides information on the draft and yet incomplete genome of *Acytostelium subglobosum* (http://acytodb.biol.tsukuba.ac.jp/).

6 Future Directions of Genomics in Social Amoebae

Social amoebae likely diverged at least 600 million years ago (Heidel et al. 2011). This is a larger time span than the evolution of vascular plants needed, and only a little bit less than that for the evolution of Metazoa. Given the big differences in genome size and coding capacity observed in these evolutionary branches, it is surprising that these features remained stable in the social amoebae. Since we now have genomes from all major social amoebae branches in hand we can extrapolate that this is true for most, if not all social amoebae. The small genome size thus makes it easy to sequence additional genomes at very low costs. However, this approach only makes sense if species specific inventions are under investigation, since the common genetic outfit of social amoebae has been described already in

detail (Heidel et al. 2011). The genome sequence of new species must be also endowed with transcriptome data. Of great interest will be to investigate the regulation and timing of transcription in diverse social amoebae taxa. This will foster our understanding of the evolution of regulatory circuits. Systems biology approaches then can elucidate why the developmental cycle remained stable and whether this stability is an intrinsic feature of the sophistication of the cycle.

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Signalling During Dictyostelium Development

Cornelis J. Weijer

Abstract Dictyostelium has become an important model system to study the molecular details of the signalling pathways controlling gradient sensing and cell polarisation that control localised activation of the actin–myosin cytoskeleton responsible for evolutionary highly conserved mechanisms of chemotactic cell movement up chemoattractant gradients. 3′-5′ cyclic AMP is the chemoattractant that controls the chemotactic cell movements that result in aggregation of up to several hundred thousand cells, slug formation, migration and fruiting body formation. The coordination of these complex cell movements require long-range cAMP mediated cell–cell signalling based on periodic initiation of cAMP signals in the aggregation centre and slug tip and relay by surrounding cells, resulting in highly dynamic patterns of cAMP wave propagation. Model calculations have shown that the dynamic feedbacks between autocatalytic cell–cell cAMP signalling and cAMP-mediated collective chemotactic cell movement result in emergent properties that readily explain multicellular morphogenesis. cAMP signalling not only controls cell movement but also acts as a key morphogen to control cell differentiation, which in turn affects cell type specific cell–cell signalling and cell movement, adding an additional layer of feedback. To fully understand the multicellular morphogenesis of this organism at the level of cell behaviours, it will be needed to integrate the detailed cell type proportioning mechanisms in models describing cell–cell signalling and movement. Dictyostelium is likely to be the first eukaryotic organism where it will be possible to quantitatively understand how multicellular development and morphogenesis arise as emergent properties from a few relatively simple collective cell behaviours.

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1 Introduction

One of the central aims of the study of development is to understand how distinct cellular behaviours e.g. division, differentiation, apoptosis and shape changes and movement are coordinated in space and in time to result in emergent properties at the tissue and organism level. The coordination of these cellular behaviours requires extensive signalling between cells of different types and cells and their environment. Signalling is mostly mediated by chemical and mechanical signals, although other forms of communication through electrical fields and light are also known. In slime moulds, signalling between cells and the environment plays an essential role in processes such as the location of food through sensing of metabolites and quorum sensing, during which process the cells determine their own density relative to the available level of food supplies (Kessin 2001; Gole et al. 2011). The latter process controls the decision to either keep on looking for nutrients or to start the starvation induced multicellular developmental cycle to make gametes or spores. Food is located through chemotaxis towards metabolites such as folic acid secreted by bacteria. During the vegetative phase of the life cycle, cells secrete chemorepellent signals that results in dispersal of cells, increasing their probability of finding new food reserves (Kakebeeke et al. 1979). These repellents have been poorly characterised and little is known about the signal transduction pathways. Once the cells start to develop, they signal each other and this results in the aggregation of hundred to several hundred of thousands of cells which depending on the prevailing environmental conditions can either result in the entry in the sexual cycle which includes the formation of macrocysts through cell fusion followed by meiosis and hatching as described elsewhere in this volume (O'Day 1979; Lewis and O'Day 1985) or in the entry of the asexual developmental cycle which results in the formation of fruiting bodies. In this chapter, we will describe cell–cell signalling mechanisms that the cells use to coordinate their movement behaviour during development, discuss briefly how they contribute and interact with signals controlling differentiation and we will highlight how the interactions between cell–cell signalling movement and differentiation control the emergent morphogenesis at the organismal level (Weijer 2004). We will also discuss key intra-cellular signalling mechanisms and pathways that coordinate the molecular mechanism underlying cell–cell signalling and chemotactic cell movement.

2 Cell Movement and Signalling to the Cytoskeleton

Cell movement is characterised by a series of complex behaviours. It is generally thought that cell movement involves cycles of pseudopod or lamellipod extension at the front end of a migrating cell, coupled with retraction in the rear end of the cell (Fig. 1a). During these processes new cell-substrate contacts are made in the

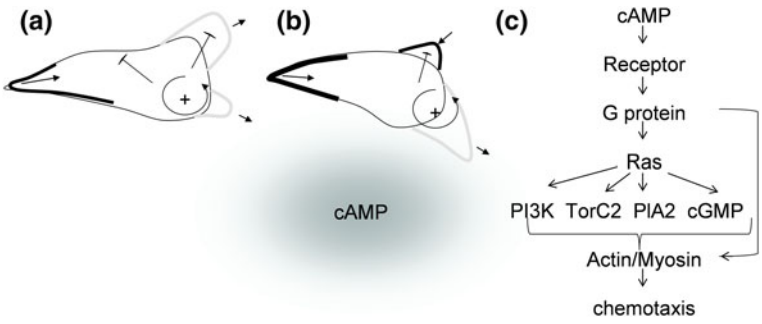

Fig. 1 **a**, **b** Cells moving in response to cAMP gradient. Pseudopods extend in the direction of the gradient extend. Pseudopods extending away chemoattractant source retract. Extension is driven by actin polymerisation (*green*). Retraction is driven by assembly of Myosin II filaments (*blue*). Direction of extension and retraction are indicated by *small black arrows*. The *circular arrow* in the extending pseudopod indicates a strong local autocatalytic feedback mechanism operating between signal detection by cAMP receptors and actin polymerisation at levels of highest external signal. The *blocking arrows* indicate long-range inhibition resulting in retraction of pseudopods pointing away from the chemoattractant source possibly resulting from a competition for limited resources. **c** Simplified signal transduction scheme from receptor to chemotaxis. cAMP binds to the cAMP receptor. This activates a heterotrimeric G protein, which in turn activates Ras. Downstream of Ras there are at least four parallel signal transduction pathways involving PI3 kinase signalling, TorC2 signalling, signalling through PlA2 and through cAMP to the actin–myosin cytoskeleton. There may also be Ras-independent signalling pathways, possibly through the Dock180/Elmo family of Rac Gef's

front and broken in the back. These behaviours are primarily brought about by dynamic changes in the actin–myosin cytoskeleton and interacting components such as transmembrane adhesion molecules (Ridley et al. 2003). Extension is believed to be driven by localised polymerisation of actin filaments resulting in a pushing force to the membrane. Once the membrane is extended new contacts with the substrate need to be made and this involves the formation of transient focal adhesions. In the back of the cells, actin filaments depolymerise and contacts with the substrate need to be broken. This requires force and this involves the action of myosin II mini filaments which are involved in contracting the actin filament network, which could also play a role in stimulating actin filament depolymerisation. Since the membrane is not able to stretch much, it appears likely that continuous membrane synthesis needs to be coordinated with actin polymerisation and pseudopod or filopod extension, while depolymerisation may be coupled to localised membrane resorption (Bretscher and AguadoVelasco 1998). Indeed fast migrating cells such as Dictyostelium have been shown to turn over their membranes every 10 min and mutants defective in membrane secretion are defective in locomotion (Bretscher and AguadoVelasco 1998; Zanchi et al. 2010). There is likely an important role for different classes of unconventional myosins that link the cytoskeleton to the membrane, in both tension sensing and movement of components of the cytoskeletal machinery along the membrane (Chen et al. 2012). Deletion of several of these myosins especially of class I myosins have been

shown to result in severe movement defects (Fukui et al. 1989; Wessels et al. 1991; Dai et al. 1999; Falk et al. 2003). At the same time that the cell is extending, the cellular contents of the cell needs to be displaced and this involves likely transport of material along both the actin and microtubule network involving different classes of motor proteins. The cytoskeletal machinery appears always to be running as shown by the fact that cells extend and retract pseudopods essentially continuously. Non-stimulated cells extend pseudopods in random locations around the cell, resulting in a very low persistence of directional migration resulting in what is known as a random walk (Soll et al. 2003; Sasaki et al. 2007).

3 Cellular Behaviours During Chemotaxis

There is evidence that cells can modulate the rate of cytoskeletal dynamics in response to extracellular factors and can move faster or slower depending on the factor and its local concentration (Rifkin and Goldberg 2006; Song et al. 2006). This process is known as chemokinesis. Chemokinesis will result in cells avoiding areas of these factors if they stimulate movement and in accumulation of cells if they inhibit movement. In case where movement stimulating factors are secreted in an autocrine manner, this will result in cell dispersal. Through these mechanisms, cells of different types can also influence each others behaviours and they may well play a role during the development of Dictyostelium. However, another more efficient mode of control of cell motility is well established during the development of Dictyostelium and this is chemotaxis. During chemotaxis in Dictyostelium, cells measure a gradient of a chemoattractant and then use this information to bias the extension or persistence of protrusions up or down the direction of the gradient (Wang et al. 2011; Cai et al. 2012). Although chemotaxis can result from a temporal measurement of the concentration at different locations, the evidence suggests that in Dictyostelium the cells measure a spatial gradient over the length of the cell, although due to the adaptation in the chemosensory systems the temporal component also plays a role in chemotaxis in vivo during aggregation as will be discussed in more detail later (Fisher et al. 1989; Iijima et al. 2002).

Starving Dictyostelium cells have been shown to be able to respond accurately to steep but also to very shallow gradients of the chemoattractant 3′-5′cyclic-AMP (cAMP) (Andrew and Insall 2007; Fuller et al. 2010). One view of the underlying mechanism of chemotaxis is that in the presence of an external gradient of a chemoattractant such as cAMP, the cells persistently extend successive pseudopods in the direction of rising cAMP concentration, while at the same time by some global inhibition mechanism suppressing the extension of lateral pseudopods (Van Haastert and Devreotes 2004). In this view, there is a direct coupling between the concentration of the chemoattractant and the local rate of actin polymerisation driving the formation of a new pseudopod. A competing view is that cells extend pseudopods a constant cell specific internal rate of around three pseudopods per minute and that this process is not dependent on the chemoattractant concentration

(Andrew and Insall 2007). Furthermore, the direction of pseudopod extension is essentially random, although it has been suggested that pseudopods and new pseudopods form preferentially close to the site where the preceding pseudopod was initiated. This process results in a cell having several pseudopods at any given time and chemotaxis results from the fact that the pseudopod in a higher local cAMP concentration persists, while a pseudopod in a region of lower cAMP concentration is retracted. This process will result in a gradual movement of the cell up a chemoattractant gradient (Andrew and Insall 2007; King and Insall 2009). In this model, cells move in the right direction essentially by an error correction process, continuously updating their directionality in response to the signal. The latter has been shown to work remarkably well in detailed model computations (Neilson et al. 2011) making the assumption that there is an internal competition for available resources (Fig. 1a, b). These models include local activation and local and global inhibition working on different time scales.

4 Signalling During Chemotaxis

Much current research is directed towards understanding the molecular mechanism by which cells detect cAMP gradients, polarise their cytoskeleton and move in response to cAMP gradients (Fig. 1c) (Chen et al. 2007; Franca-Koh et al. 2007; Insall and Andrew 2007). Cells can respond very reliably to very shallow gradient of cAMP, where it is estimated that the difference in occupied cAMP receptors between the front and the back is just few percent, while the total number of cAMP receptors occupied is very low (Ueda et al. 2001). Therefore, it is assumed that an internal amplification mechanism exists that amplifies the weak external signal gradient into a large internal signal gradient that results in a reliable polarisation of the actin–myosin cytoskeleton and directional movement up the cAMP gradient (Fig. 1a, b). It is currently though that there exists a gradient sensing mechanism, the chemical compass, which is followed by an internal amplification mechanism that controls the polarisation of the cytoskeleton, which may well involve the cytoskeleton itself (Bourne and Weiner 2002; Franca-Koh and Devreotes 2004). This view is based on the observation that the initial signalling events, receptor activation and G protein activation occur in the presence of inhibitors of actin polymerisation, but that for full-blown activation of downstream pathways such as Ras and phosphatidyl-inositide 3 kinase (PI3 kinase) activation among others, actin polymerisation is required (Devreotes and Janetopoulos 2003). To explain the differences in cytoskeletal organisation especially actin polymerisation between the front and the back of the cell it has been suggested that the compass is based on a local excitation global inhibition (LEGI) model (Ma et al. 2004). In this model, a local stimulus results in the local production of an excitation variable (receptor occupancy, G protein activation) of limited range as well as a long-range inhibitory signal. The difference between excitation and inhibition is assumed to be proportional to an effector output such as actin polymerisation, thus resulting in

a front-to-back gradient of activation (Iglesias and Devreotes 2008). Another property of this system is that the excitation normally increases faster in time than the inhibitor; the LEGI mechanism can provide perfect adaptation, but it does not necessarily provide any amplification (Iglesias 2012; Wang et al. 2012). Since the response of individual cells to a standard stimulus has been shown to be essentially bimodal, i.e. some cells respond where others do not. This has been taken to imply that the LEGI module is followed by an amplification module with cell specific variable thresholds. This results in individual cells in a population responding at different concentrations.

Much work has gone into identifying the components of the compass, the mechanism of short-range activation and long-range inhibition as well as the source of signal amplification. It has become clear that there is no amplification, for instance through ligand-induced clustering and redistribution at the level of the receptors. The receptors remain homogenously distributed in the membrane in the presence of a chemotactic gradient (Ueda et al. 2001). The receptors have been shown to diffuse in the membrane, where they activate G heterotrimeric proteins, but there is also no evidence for differential distribution of G proteins in the membrane in response to a chemoattractant gradient (Jin et al. 2000). Furthermore, there is no evidence for amplification nor for adaptation at the level of G protein activation, as measured by dissociation of G_{α} and $G_{\beta\gamma}$ subunits using a fluorescence resonance energy transfer (FRET)-based approach (Janetopoulos et al. 2001). The next step in the cascade is activation of the Ras oncogenes RasG and RasC (Kae et al. 2004; Sasaki et al. 2007; Sasaki and Firtel 2009). There may be some amplification of the signal at this stage. The activation of Ras is thought to result from the balance by activation of a RasGef (Guanine Nucleotide Exchange Factor activating Ras) and inactivation by a RasGap (GTPase Activating Protein) (Takeda et al. 2012). This has been suggested to act as an incoherent feed-forward mechanism. Activated Ras is then involved in the activation of several downstream pathways such as the activation of the TORC2 complex and PI3 kinase which in turn result in the activation of PkB (Charest et al. 2010). There is a strong amplification at the level of the activation of PI3 kinase, which involves translocation of PI3 kinase to the membrane in a Ras-dependent manner and a dissociation of PTEN from the membrane (Iijima and Devreotes 2002). The difference between these competing reactions, synthesis and degradation results in an amplification of the response, which may be further amplified by a feedback of actin polymerisation of PI3 kinase localisation. Activated PkB in combination with PkBR1 result in the phosphorylation of several targets and may play a role in the activation of actin polymerisation and inhibition of myosin thick filament assembly in the front (Chung and Firtel 1999; Chung et al. 2001; Kamimura et al. 2008). Surprisingly, it has been shown that knockout of all PI3 kinase signalling (five PI3 kinases and Pten) results in cells that can still do chemotaxis rather well (Hoeller and Kay 2007; Kay et al. 2008). This has led to investigation of further parallel pathways, involving other phospholipases such as Phospholipase A2 as well provide further evidence of a role for cGMP signalling in cell polarisation and chemotaxis (Chen et al. 2007; Veltman et al. 2008; Kamimura and Devreotes 2010) (Fig. 3). Furthermore, there is

recently increasing evidence that parallel signalling pathways to the cytoskeleton may involve members of unconventional Gefs of the Dock180/Elmo families (Para et al. 2009; Pakes et al. 2012; Yan et al. 2012). It remains to be discovered how the activation of these components are coupled to the cAMP receptors, and whether the responses are G protein dependent.

Activation of Rac has been shown to destabilise the Wave/Scar complex which in turn results in the formation of the Arp2/3 complex and nucleation of new actin filaments from existing filaments (Bear et al. 1998; Blagg and Insall 2004; Ibarra et al. 2005). These new filaments are capped by capping proteins and cross-linked to each other and membranes by a host of proteins (Eddy et al. 1997). Localised actin polymerisation in the leading edge then results in the generation of protrusive forces as described above.

Actin polymerisation in a broad front results in the formation of lamellipodia, however, cells are known to form large numbers of filopodia, fine protrusive structures. Filopodia may be involved in gradient sensing or in force generation and assist in movement, since deletion of components that results in ablation of filopodia often also result in defects of cell migration. It appears that actin polymerisation in filopodia is dependent on that local activation of formins, rather than being dependent on the activation of the Arp2/3 complex and these are shown to have interaction with wave (Schirenbeck et al. 2005a, b). Unconventional myosins, especially myosin VII, play a key role in bundling and possibly transport of components along the actin bundles in the filopodia as well as in the control of cell substrate adhesion via interactions with talin (Maniak 2001; Faix and Rottner 2006; Galdeen et al. 2007).

Cells move by extending pseudopods at their leading edge, a process driven by localised actin polymerisation which requires the action of members of the myosin I family and inhibition of the formation of myosin II thick filaments through phosphorylation of the tail of the myosin heavy chain on several threonine residues (Yumura et al. 2005; Bosgraaf and van Haastert 2006; Goldberg et al. 2006). To move, cells need to pull up their back ends and suppress the extension of lateral pseudopods. This involves the formation and contraction of actin–myosin II thick filament networks, actin depolymerisation and is dependent on internal cAMP levels (Falk et al. 2003; Zhang et al. 2003). To move forward, the cells must gain traction from the substrate on which they are moving. This requires the formation of multiple transient (10–20 s) cell-substrate contact sites that are actin rich and have been shown to transduce traction forces to the substrate (Bretschneider et al. 2004; Uchida and Yumura 2004). It appears that cells may undergo alternating phases of actin driven extension at the front and myosin II driven contraction at the back (Iwadate and Yumura 2008). Much work is directed towards the investigation of the molecular mechanisms resulting in signal detection and cell polarisation, this has been extensively reviewed elsewhere recently and will not be covered here in detail (Willard and Devreotes 2006; Janetopoulos and Firtel 2008). In addition to this, more recent methods have been developed to investigate the traction and motive forces produced by migrating cells and to use these methods to characterise the molecular mechanisms that translate cell polarisation into directed movement

(Del Alamo et al. 2007; Meili et al. 2010; Bastounis et al. 2011). This is a complex area of research that will undoubtedly gain in importance in the future, since it allows a functional quantitative characterisation of how signalling is translated into motive forces underlying directed cell movement.

5 Cell–Cell Signalling Controlling Cell Movement During Multicellular Development

Since Dictyostelium development takes place in the absence of food, under starvation conditions only limited cell divisions occur during multicellular development. Morphogenesis therefore primarily results from the movement of individual differentiating cells into a relatively complex structure, the proportions of which are essentially independent of how many cells exactly aggregate. Key questions are, which signals guide the movement behaviour of thousands of cells during development, which signals control differentiation and how do cell–cell signalling, movement and differentiation interact to form a fruiting body?

6 Aggregation

Starvation induces changes in the gene expression programme that results in the cells acquiring the ability to respond to cAMP gradients by chemotaxis through mechanisms described above, but importantly they also acquire the ability to produce and secrete and degrade cAMP (Saran et al. 2002; Iranfar et al. 2003). Aggregation is caused by periodic cAMP synthesis and secretion by cells in the aggregation centre. The cells initially become chemically excitable and will produce cAMP when stimulated with cAMP. Binding of cAMP to the serpentine transmembrane cAMP receptor results in stimulation of signal transduction cascade that leads to the activation of an adenylylcyclase (ACA), that within tens of seconds produces cAMP part of which is secreted to the outside (Fig. 2). The secreted cAMP binds to the receptor and thus is part of an autocatalytic feedback loop resulting in a rapid increase of cAMP production. However, stimulation of the receptor also activates an adaptation process that with a small time delay results in the inhibition of ACA activation and a cessation of cAMP production. Since cAMP diffuses away into the extracellular medium and is also degraded by secreted cAMP phosphodiesterases, this results in a drop in cAMP levels, which in turn results in de-adaptation of the cells (Fig. 2). Both excitation and adaptation depend on the receptor-dependent activation of a heterotrimeric G protein, resulting in activation of Ras, which in turn activates PI3 kinase, which phosphorylates phosphatidylinositide(4,5)phosphate (PIP2) to phosphatidyl-(3,4,5)-phosphate (PIP3). PIP3 then activates many downstream pathways leading to

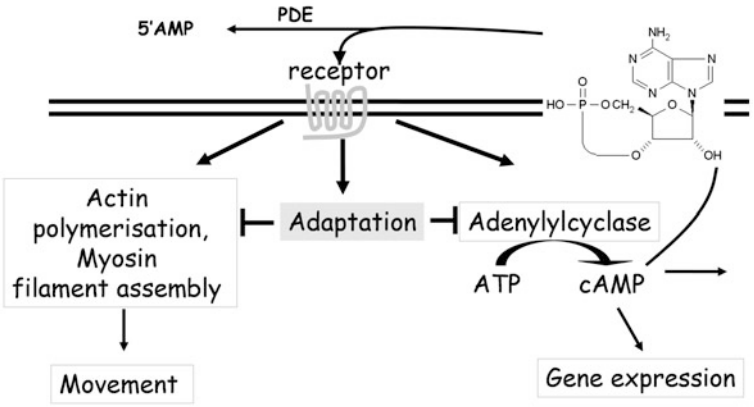

Fig. 2 cAMP signalling activates cAMP relay and chemotaxis. Extracellular cAMP binds to a transmembrane serpentine cAMP receptor and activates two distinct signal transduction pathways. One pathway leading to organisation of the actin–myosin cytoskeleton and chemotaxis. The second pathway results in activation of the aggregation stage adenylylcyclase (ACA). Activation of ACA results in cAMP production part of which is secreted and binds to the receptor to form a positive feedback loop where a little extracellular cAMP results in the production of more. Binding of cAMP to the receptor also activates an adaptation process that inhibits both ACA activation and the chemotactic signal transduction pathway. Once adaptation reaches a full response, the production of cAMP ceases and cAMP secretion stops. Since cAMP is continuously degraded by an extracellular cAMP phosphodiesterase, extracellular cAMP levels fall which allow the cells to de-adapt and start a new cAMP response

chemotaxis and ACA activation (Mahadeo and Parent 2006). The activation of ACA is critically dependent on the PIP3-dependent binding of cytosolic regulator of adenylyl cyclase (CRAC) to the membrane where it is activated which is required for adenylyl cyclase activation and chemotaxis (Comer et al. 2005).

There are no special cells that form aggregation centres and they form in a stochastic manner. Aggregation centres from cells will produce and secrete cAMP continuously at a low but increasing level. Most of this secreted cAMP will be degraded by the secreted cAMP phosphodiesterase. However, since gene expression is heterogeneous, some cells will produce and secrete a little more cAMP than others. Due to this heterogeneity in gene expression and stochastic distribution of cells, by chance there will be an area where some cells can just produce enough cAMP to start the amplification of the signal through positive feedback (Gregor et al. 2010; Kamino et al. 2011). This locally produced cAMP diffuses to neighbouring cells, which now detect an above threshold signal to which they can respond and therefore amplify the signal and pass it on to their neighbours, resulting in the formation of travelling waves of cAMP. Since the cAMP waves direct the chemotactic movement of the cells to their source, this will result in an increased local cell density in the region that initiated the signal, making it more likely that the cells in this region will fire again once they are de-adapted. Through

these feedbacks, the group of cells that started signalling will establish itself as an aggregation centre. cAMP receptor mutants with lower affinity for cAMP show altered patterns of wave propagation but also in extreme cases result in mutants that can still propagate waves, but these wave fragments do not set up aggregation centres. This is caused by the fact that the time it takes between two oscillations is too long and the cells will disperse again by random movement resulting in the generation of waves in other random locations (Dormann et al. 2001b). Several centres will arise in random locations and compete to attract cells. Faster oscillating centres will encroach on slower oscillating centres and finally can wipe them out. Successful centres will typically send out a series of around 20 cAMP waves that will attract up to several hundred thousands of other cells to the initial aggregation centre to form a mound.

Detection and amplification of this signal by surrounding cells coupled with desensitisation of the cAMP producing cells results in the propagation of waves of cAMP away from the aggregation centre (Fig. 3). Cells detect the rising phase of the wave and move in the direction of increasing cAMP concentration, once the waves passes; the cells are adapted and are insensitive to the falling phase of the wave and therefore do not turn around and chase the wave once it has passed. These cAMP waves therefore guide the cells towards the aggregation centre, where they accumulate into a three-dimensional aggregate, the mound (Dormann and Weijer 2001, 2003). During the synchronised chemotactic movement phase cells elongate, while during the falling phase of the waves, the cells are amoeboid in shape. The large-scale spatiotemporal patterns of cells behaviour can be visualised as changes in light scattering since moving elongated cells scatter more light (Fig. 3a). These wave can thus successfully be used to visualise and measure the spatiotemporal dynamics of wave propagation at all stages of development (Fig. 3b–d). Recently, this method has been used to perform a high throughput analysis of cAMP signalling mutants (Sawai et al. 2007). Initially, the cells move towards the aggregation centre as individuals, but after 10–20 waves have passed they form bifurcating aggregation streams, in which the cells make head to tail contacts via a calcium-independent adhesion molecule, contact site A and side to side contacts via a calcium-dependent cadherins (Wong et al. 2002; Harris et al. 2003). Stream formation is dependent on the localisation of ACA in the rear of the aggregating cells, resulting in polarised cAMP secretion from the back of the cells (Kriebel et al. 2003). cAMP wave propagation can be observed at the individual cell level by following the localised translocation of PIP3 at the leading edge of the cell (Dormann et al. 2002, 2004). The number of cells in aggregation streams appears to be controlled by the local concentration of a secreted extracellular high molecular weight protein complex, counting factor, which through modulation of movement and adhesion may control the numbers of cells that stably migrate in an aggregation stream (Jang and Gomer 2008).

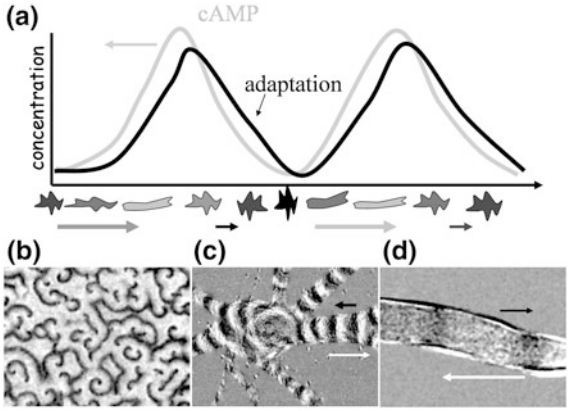

Fig. 3 Optical density waves reflect cAMP waves at different stages of development. **a** Aggregation centres initiate cAMP waves. These propagate from the centre outward (*black arrow*). Cells detect the rising phase of the cAMP wave and move in the direction of higher cAMP concentrations (*bottom figures and arrows*, *colour of cells and arrows* indicates degree of light scattering). During their chemotactic movement, the cells elongate and this changes their light scattering properties. When during the rising phase of the wave, many cells move in synchrony, this results in increased light scattering waves reflecting the rising phase of the cAMP signal. **b** Spiral optical density waves during the early aggregation phase, when the cells are still in a monolayer on agar. **c** Optical density waves in a streaming aggregate. In the body of the aggregate, multi-armed spiral waves rotate counter clockwise throwing off individual wave-fronts that propagate down the streams to the periphery of the aggregate *white arrow* indicates the direction of wave propagation, the *black arrow* indicates the direction of cell movement. **d** A slug migrating to the right, showing two dark optical density waves that travel from right to left (*white arrow*), cells move to the right following the tip (*black arrow*)

7 Mound and Slug Formation

After the cells have aggregated they form a hemi-spherical structure, the mound. Mounds are characterised by rotating waves of cAMP that direct the counter rotational periodic movement of the cells. Cells start to differentiate into prespore and prestalk cells during aggregation, based on physiological biases like nutritional state and cell cycle position at the time of starvation already present in the population before aggregation (Weeks and Weijer 1994; Araki et al. 1997). As a result, there is little correlation between the time of arrival in the mound and differentiation fate. Therefore, initially the prestalk and prespore cell types display a salt and pepper distribution in the mound (Fig. 5a). A subpopulation of prestalk cells sort out to form the tip and the slug tip guides the movement of all other cells thus acting as an organiser (Weijer 2004). External cAMP has been shown to be able to affect cell sorting of neutral red labelled prestalk cells at the mound stage (Matsukuma and Durston 1979; Sternfeld and David 1981). More recently, use of a temperature-sensitive ACA mutant has shown that ACA activity is required in vivo for cells to be able to sort to the tip (Patel et al. 2000). The tips action as an

organiser can be mimicked by the periodic injection of cAMP pulses of the right frequency and duration (Matsukuma and Durston 1979; Dormann and Weijer 2001), suggesting that the tip is a source of periodic cAMP waves, in agreement with the fact that prestalk cells express ACA and the extracellular cAMP phosphodiesterase pdeA (Verkerke-van Wijk et al. 2001; Weening et al. 2003). More recently, it has become clear that prestalk cells likely generate more force in response to a chemotactic signal than prespore cells. This can be the result of the increased myosin expression and assembly by cells in the prestalk zone (Eliott et al. 1991, 1993). More recently, it has been shown that it likely also involves differential regulation of the actin cytoskeleton especially through filamin (Blagg et al. 2011). Furthermore, it is possible that differential expression of adhesion molecules such as DdCad the Dictyostelium cadherin analogue may contribute to cell sorting (Wong et al. 2002; Sriskanthadevan et al. 2011).

It is not yet known which signals control tip cell fate (see below), but it is becoming clear that to proceed from the aggregate to the mound stage cell–cell adhesion and or contact start to play an important role. Mutants defective in the putative single pass transmembrane contact molecules lagC, lagD cannot proceed beyond the aggregation stage and are defective in tip formation (Kibler et al. 2003). There is evidence that Dictyostelium may possess several integrin-like adhesion molecules (Cornillon et al. 2008) and it has been known that mutants in talin, paxillin and a lim domain protein which are thought to couple adhesion molecules to the actin cytoskeleton are all defective in cell sorting (Chien et al. 2000; Tsujioka et al. 2004; Bukharova et al. 2005). Sorting of prestalk cells towards the tip requires the invasive movement of prestalk cells through a tightly packed mass of other (Tasaka and Takeuchi 1979; Weijer 2004; Kay and Thompson 2009). Myosin II is absolutely required for progression beyond the mound stage and it is known that prestalk cells express higher levels of myosin II (Eliott et al. 1991; Springer et al. 1994). For prestalk cells to sort, they need to assemble more myosin thick filaments than prespore cells (Singer and Dormann Weijer 2013). There is also evidence that cell type specific changes in the organisation of the actin cytoskeleton may affect cell sorting, since mutants affecting the cell type specific regulation of filamin, a major actin cross-linking protein and RapGapA which controls Rap1 activity involved in control of adhesion both affect cell sorting out of prestalk cells (Parkinson et al. 2009; Blagg et al. 2011). Together all these observations suggest that tip formation is the result of cellular properties specific to tip forming cells. Tip cells express high levels of ACA resulting in increased signalling ability, therefore allowing them to set up a new independent signalling centre that can outcompete the aggregation centre organising the mound. They also assemble more myosin II thick filaments, critical to their ability to produce more force in response to a cAMP signal (Clow et al. 2000). This latter property, allows them to push other cells aside and re-aggregate within the mound to form the tip.

In slugs, optical density waves can be seen to propagate from the middle of the prestalk zone to the back, reflecting the periodic movement of the cells forward (Fig. 3). These optical waves are strictly dependent on the tip. Cells in the tip often

rotate perpendicular to the direction of slug migration, especially when it is lifted from the substrate. In the back of the slug, the cells move periodically forward and all cells move on average with slug speed. It has been shown that the assumptions cAMP wave propagation and chemotaxis in response to these waves is in principle sufficient to explain morphogenesis from single cell via aggregation, stream and mound formation to cell sorting and slug formation. The interactions between cell signalling and cell movement can be described by relatively simple mathematical models in a robust way and it would appear that these process are sufficient to explain Dictyostelium morphogenesis (Fig. 4) (Vasiev and Weijer 2003; Umeda and Inouye 2004). However, the situation is almost certainly more complex since strains lacking the aggregation stage ACA can still form slugs, when they over-express the catalytic subunit of protein kinase A, suggesting either that there either exists an ACA-independent mechanism to produce periodic cAMP signals, for instance involving cAMP generation by other adenylylcyclases ACB and or ACG and the recently discovered cAMP stimulated cAMP phosphodiesterase (Meima et al. 2003) or that there exists altogether different mechanisms that can control cell movement such as contact following (Umeda and Inouye 2002). The latter mechanism does, however, not explain which signals direct the movement of the cells in the tip. The hypothesis that prestalk cells generate much of the force for migration in the slug has been supported by traction force measurements produced by measuring the local deformation of elastic gels by migrating slugs (Rieu et al. 2005). These measurements show that the posterior region of the slug generates much of the motive force during migration. Measurements of forces in slugger mutants have further suggested that the anterior-like cells provide the motive force for the prespore zone of the slug (Rieu et al. 2009). These results all suggest that cell-type specific differences in cell mechanics differences are critical for cell sorting and slug migration.

8 Differentiation

It is well established that Dictyostelium slugs can form from as few as a few hundred cells and can contain up to several million cells, while the proportions between spore and stalk cells remain relatively constant (Rafols et al. 2001; Maruo et al. 2004). A major goal is to understand the relationship between cell movement and the signals that control differentiation. These signals must be able to maintain the correct proportioning of the prespore and prestalk celltypes in an environment of extensive cell movement and changes in shape of the slug. In the slug, the different cell types are arranged in a simple axial pattern, pstA cells in the tip, a band of pstO cells that form the upper and part of the lower cup of the fruiting body, prespore cells, precursors for spores, which are intermingled anterior-like cells and rearguard cells precursor to the lower cup and basal disk in the back of the slug (Fig. 5a) (Williams 2006; Yamada et al. 2010). It seems evident that this requires adaptive signalling dynamics, but the signals and the details of their

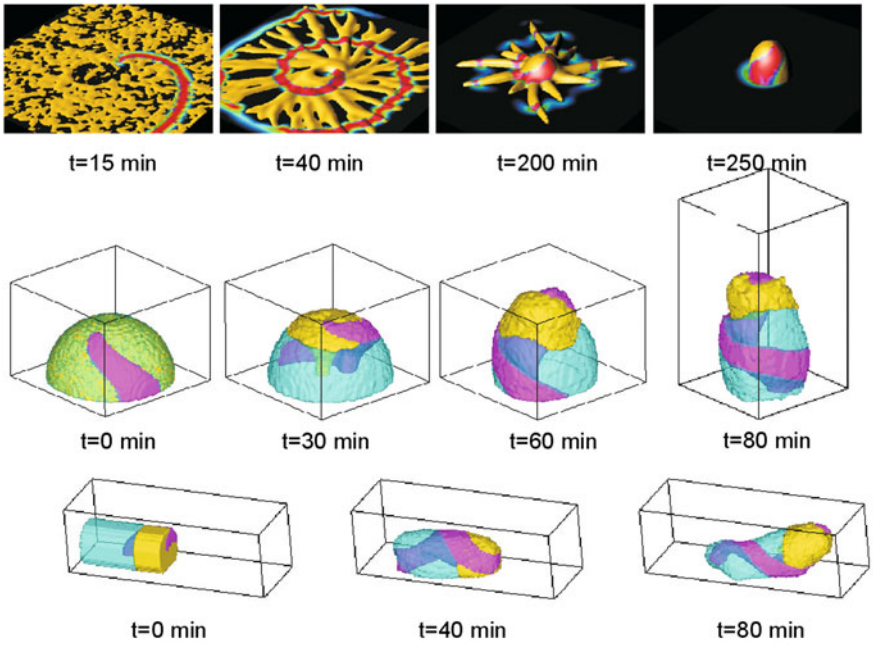

Fig. 4 Model calculation of wave propagation and cell movement from aggregation to slug migration using a hydrodynamic model. The top row depicts the aggregation up to the mound stage. The first image starts with the randomly distributed cells (*yellow*) which are organised by a spiral wave of cAMP (*red*). They form aggregation streams and finally a hemispherical mound (Vasiev et al. 1997). The *middle row* shows cell sorting and the formation of a slug. The mound consists of two cells types: 20 % *yellow* prestalk cells and 80 % *blue* prespore cells. They are initially randomly mixed. The cAMP waves (*purple*) organise the movement of the cells. In the model, the assumption is that the prestalk cells are more excitable and develop more movement force in response to a cAMP wave. As a result of this, they move towards the centre of the mound and up to form the tip. The separation of the cells feeds back on the signal propagation resulting in the formation of a twisted scroll wave. This leads to an intercalation of the cells and an upward extension of the slug (Vasiev and Weijer 1999). The *bottom row* shows that a slug organised by a scroll wave can move (Vasiev and Weijer 2003)

regulation are not yet understood in detail (Fig. 5b). cAMP pulses control the expression of aggregation stage genes necessary for cAMP relay and cell–cell contact and cAMP is necessary for prespore gene expression in later development (Saran et al. 2002; Iranfar et al. 2003). Prespore cells in turn produce DIF (differentiation inducing factor, a small chlorinated hexaphenone), which controls the differentiation of pstO cells (Kay and Thompson 2001; Maeda et al. 2003; Thompson et al. 2004; Saito et al. 2006). DIF spreads by simple diffusion from the prespore zone in adjacent regions where it controls the differentiation of prestalk O cells and possibly rearguard cells (Fukuzawa et al. 2003). Cells in the pstA zone express ACA and studies investigating the cyclic AMP-dependent nuclear translocation of the transcription factor statA have shown that cAMP levels are high in

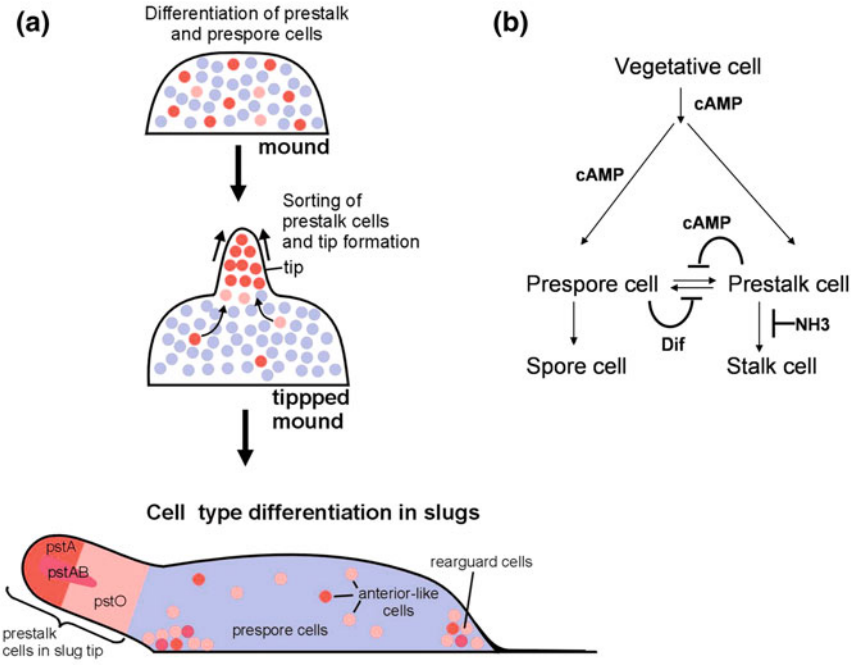

Fig. 5 Cell types and cell type proportioning in Dictyostelium. **a** During aggregation cells start to differentiate into prestalk (*dark grey*) and prespore cells (*light grey*). Since cells arrive in the mound in random order they from a salt and pepper distribution of cell types. After a variable time of movement, the prestalk cells re-aggregate to form the tip and the initial prestalk zone. The tip guides the movement of all the other cells and the structure elongates to from a slug that topples over and migrates away. In the slugs, there are at least four cell types. The tip is made up of PstA (*dark grey*) cells, followed by a cohort of PstO (*light grey*) cells, which together form the prestalk zone. The prestalk zone is followed by the prespore zone in which mainly prespore cells are intermingled with anterior like cells, cells of prestalk character that do not sort but express ACA and relay the cAMP signal. In the back of the slug, the rearguard cells are found. **b** Prespore and prestalk cells differentiate from vegetative stage cells. The early differentiation in aggregation stage cells requires cAMP pulses. The cells then differentiate into prestalk and prespore cells. Prestalk cells initiate cAMP waves and extracellular cAMP is needed for prespore gene expression. Prespore cells make DIF which is necessary for PstO cell differentiation. Prestalk cells secrete DIF-ase that inactivates DIF. Prestalk cells differentiate into stalk cells and prespore cells differentiate into spores. Stalk differentiation is inhibited by NH_3. An open question is how cell-type proportioning works quantitatively which is the subject of further modelling studies

the tip, while cAMP is lower elsewhere in the slug (Dormann et al. 2001a; Verkerke-van Wijk et al. 2001), compatible with the idea that all cells in the tip relay the cAMP signal and only the anterior-like cells in the rest of the slug. The signals and signalling pathways that control the expression of functionally important tip enriched molecules such as ACA and myosin II presumably involve signalling through the Stat pathway (Wang and Williams 2010; Araki and Williams 2012), but the exact details remain to be established. It also remains to be seen how

accurate cell type proportioning is achieved in slugs despite their vastly different sizes and dynamic changes in shape.

The switch from migrating slugs to culmination appears to be controlled by a fall in ammonia concentration. The identification of a number of ammonia transporters some of which are expressed in the very tip and when deleted show a slugger phenotype supports the importance of ammonia as a morphogen (Kirsten et al. 2005, 2008; Singleton et al. 2006). Ammonia signals most likely through the histidine kinase DhkC to the response regulator domain of the internal cAMP phosphodiesterase RegA, which is a major determinant in the control of intracellular cAMP levels (Singleton et al. 1998; Saran et al. 2002). High ammonia is expected to result in activation of regA and low internal cAMP levels. A drop in ammonia is expected to result in a rise of intracellular cAMP and stalk cell differentiation.

In conclusion, Dictyostelium is besides being a system of choice to investigate the molecular mechanisms underlying cell polarity and chemotaxis also as an excellent model system to investigate the basic cell–cell signalling mechanisms that underlie multicellular tissue formation and morphogenesis.

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The Chemotactic Compass

Dawit Jowhar and Chris Janetopoulos

Abstract Cells have an amazing ability to sense very shallow gradients of chemoattractants and move directionally. This fundamental process is critical for development and numerous disease states. *Dictyostelium* has emerged as one of the best understood model systems for elucidating the complex signaling pathways that drive chemotaxis. This review focuses on the signaling mechanisms regulating directed migration and discusses the role of polarity and development on our current understanding of this process. We highlight new findings using a second chemoattractant, folic acid and suggest that this chemical cue should be used when a developmental defect is suspected. We also speculate on recent studies which suggest that researchers should use our new understanding of the temporal and spatial relationships of signaling and cytoskeletal proteins to guide future experiments.

1 Chemotaxis

Chemotaxis is the directed migration of cells up or down a chemical gradient. This process is critical during many eukaryotic processes including development and wound healing (Wood et al. 2006; Frost et al. 2009; Schneider et al. 2010), numerous disease states including cancer (Di Gennaro and Haeggström 2012; Varani 1982; Roussos et al. 2011; Müller et al. 2001; Condeelis and Segall 2003) and the immune response (Liu et al. 2012; Franciszkiewicz et al. 2012). While bacterial cells use a well-defined temporal mechanism to migrate directionally

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(Baker et al. 2006), eukaryotic cells are capable of interpreting spatial cues across the length of a cell. The mechanism regulating this eukaryotic "chemotactic compass" has remained more elusive. When a cell is exposed to a chemical signal, numerous processes occur before it migrates in response to the gradient. First, there is signal interpretation and integration. The external cues are detected by transmembrane receptors which trigger a set of signaling events that initiate cell migration (Ridley 2001). This binding of chemoattractants on the external surface of the cell must be "transduced" to the interior of the cell. While conformational changes in the receptor trigger the activation of downstream responses, many of the initial events that occur after chemoattractant binding are still not well understood. What is clear is that migratory cells have an ability to amplify many of the "upstream" signals.

Numerous biosensors have been developed to visualize these events in living, migrating cells (Augsten et al. 2006; Chiu et al. 2002; Kae et al. 2004; Parent et al. 1998; Funamoto et al. 2002; Bretschneider et al. 2002; Rehberg et al. 2005). Signaling cascades and effector molecules are preferentially activated and localized to where the chemoattractant concentration is higher, while others segregate and become active at the low side of the cell. An important part of this rearrangement involves the cytoskeleton, which plays a critical role as the cell begins to establish polarity, and has a defined leading and trailing edge. These localized responses at the front and rear of the cell make up the engines that drive migration, so that the cell can move directionally. Once a cell begins to migrate in the proper direction, the cell often enhances its polarity and feedback mechanisms further contribute to a defined leading edge and rear of a cell (Devreotes and Janetopoulos 2003). This reinforces pseudopodia extension in the correct direction. Asymmetric distributions of cytoskeletal components such as F-actin will occur at the leading edge, with myosin filament assembly at the rear of the cell (Van Haastert and Devreotes 2004).

For many mammalian cells, the basic steps of migration occurs with the initial extension of lamellipodia, the forming of new adhesions, the contraction of the cell body, and the detaching of the rear of the cell from the substrate (Ridley 2001). The development of many new biophysical methodologies has been used to further our understanding of the physics that regulate these movements and cell shape changes (Hur et al. 2009; Curtis et al. 2007; Schoen et al. 2010; Kraning-Rush et al. 2012). Similar techniques, combined with modeling approaches are being developed to understand the biophysics underlying the movement of the simple eukaryote *Dictyostelium discoideum* (Alonso-Latorre et al. 2011; Xiong et al. 2010; Takeda et al. 2012; Nishimura et al. 2012; Semplice et al. 2012; Driscoll et al. 2012). As discussed below, much progress has been made in understanding eukaryotic chemotaxis, but we still have a long way to go, with the social amoeba leading the way in our understanding of this complex process.

2 *Dictyostelium* as Model System for Chemotaxis

Dictyostelium has proven to be an excellent model system for the study of chemotaxis. While there are over 100 discovered species of Dicytostelia (Bonner 2009), most of the work on *Dictyostelium* chemotaxis has been focused on *D. discoideum* (Maria et al. 2011). It has been more than 75 years since the discovery of *D. discoideum* by K. B. Raper (Raper 1935), and there has been a tremendous amount of work performed on this genetically and biochemically tractable system to understand the different behaviors these social amoeba display in response to chemical signals. Nonessential genes in these cells are highly amendable to genetic manipulation, with targeted gene disruptions obtainable in a few weeks. Its relatively small genome is sequenced and RNAi and insertional mutagenesis can be used to identify the function of novel genes (Landree and Devreotes 2004; Garcia et al. 2009; Eichinger et al. 2005). Pathways regulating chemotaxis are conserved throughout evolution, as was discovered early on when investigations using *D. discoideum* first showed that eukaryotic cells use serpentine receptors coupled to the heterotrimeric G proteins to undergo chemotaxis (Devreotes 1994; Parent et al. 1998). It was only after this discovery that a similar signaling cascade was found in mammalian neutrophils during directed migration (Kim et al. 1996; Franca-Koh et al. 2009).

3 cAMP and Folic Acid

D. discoideum respond to a number of chemoattractants depending on the stage of their lifecycle. During vegetative growth, amoeba will respond to folic acid (FA) when grown in axenic media (Palmieri et al. 2000), but respond more robustly when grown in the presence of the bacterium *Escherichia coli* (Pan et al. 1972; Srinivasan et al. 2013). Cyclic adenosine monophosphate (cAMP), on the other hand, is the chemoattractant during aggregation and development (Konijn and Meene 1967). Upon starvation, cells enter a developmental program, and after several hours, they begin to secrete cAMP at 5–6 min intervals. This periodic regulation of cAMP is a consequence of the ability of the cells to bind cAMP and synthesize more, and thus propagates the signal. cAMP receptors (cARs) sense cAMP, and activate adenylyl cyclase, which converts cAMP from ATP (Dinauer et al. 1980). An adaptation response occurs which is independent of cAR1 phosphorylation and cAMP release is halted. cAMP phosphodiesterases, both intracellular and extracellular are activated and lower the cAMP levels and the cells ready themselves for the next wave (Snaar-Jagalska et al. 1990; Theibert and Devreotess 1986; Reymond et al. 1995; Insall et al. 1994; Pupillo et al. 1992; Bader et al. 2007). Individual cells aggregate by cAMP-mediated chemotaxis to form first mounds, and then multicellular structures called slugs, which are phototactic and will ultimately give rise to fruiting bodies that contain spores (Kessin

2001; Kay 1982). Compromises in the cAMP signaling pathway can lead to developmental delay or arrest, and cells will remain as smooth monolayers when grown on a non-nutrient surface (Pitt et al. 1992; Garcia et al. 2009). For phenotypic analysis, clonal populations can be grown on a bacterial lawn where they form plaques and starve. Developmental and migratory mutants can be readily identified by the size of the plaque and the ability to form fruiting bodies. Work derived from understanding this developmental process has provided much insight into the role of many molecules critical for cell signaling and motility. However, mutants that appear to be defective in aggregation should also be assayed for their ability to chemotax to FA, which does not require a developmental program. This will rule out a developmental defect that results in the cells not being competent to respond to cAMP (see below). This lack of responsiveness can be a problem since many signaling molecules that are used during chemotaxis are also critical for the signal relay necessary for early development and cell polarization.

4 The *Dictyostelium* cAMP Receptors

Dictyostelium have transmembrane cAMP receptors that belong to the G Protein coupled receptor superfamily (Xiao et al. 1997; Strader et al. 1995). There are four cAMP receptors in *Dictyostelium* (designated cARs 1–4) that are expressed at different stages of the development life cycle (Klein et al. 1988; Saxe et al. 1991). cAR1 is expressed during early development while cAR3 is expressed later during aggregation and reaches maximal expression during the mound stage of slug formation (Johnson et al. 1993). cARs 2 and 4 are expressed in the prestalk cells during slug formation, and later fruiting body stages, respectively (Johnson et al. 1993; Saxe et al. 1991; Insall et al. 1994). The affinities of these receptors for cAMP are also different with cAR1 having the highest affinity and cAR 3 having the least affinity (Dormann et al. 2001). cAR3 shares 56 and 69 % amino acid sequence identity with cAR1 and cAR2, respectively (Johnson et al. 1993).

5 cAR Structure and Localization

The structure of the cAMP receptors reveals that they have seven transmembrane domains with an extracellular amino terminus and an intracellular carboxy tail (Klein et al. 1988). Ligand induced as well as basal phosphorylation occurs on the C-terminal tail of the cytoplasmic region of cAR1, which contains several clusters of serine residues (Hereld et al. 1994). Work from the Devreotes lab identified the first cluster of cytoplasmic serine residues as being responsible for a reduced affinity for cAMP (Caterina et al. 1995). In addition, dominant negative cAR1 mutants, which are constitutively phosphorylated, block development, possibly through persistent activation of mechanisms that normally regulate cAR1 signaling

(Zhang et al. 2005). Further characterization of cAR1 phosphorylation demonstrated that it was not essential for terminating processes such as adenylyl cyclase activation and actin polymerization (Kim et al. 1997). By genetically encoding cAR1 with the green fluorescent protein (GFP), it was found that these receptors appear to be distributed uniformly on the plasma membrane (PM) during gradient sensing (Xiao et al. 1997). The same phenomenon has also been shown in mammalian neutrophils during chemotaxis (Servant et al. 1999). This work suggested that the signal amplification, as seen by a number of in vivo biosensors, must occur "downstream" of receptor activation.

6 Signaling After Receptor Activation

The binding of chemoattractants to the receptors catalyzes the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) and allow both the GTP-bound α-subunit and free $\beta\gamma$ complexes to signal to downstream effectors (Gilman 1987; Bourne 1997). The first in vivo look at G protein dynamics in response to a stimulus was performed using *D. discoideum* (Janetopoulos et al. 2001). The $\alpha 2$ and $\beta\gamma$-subunits rapidly dissociated and reassociated upon addition and removal of cAMP, respectively. Interestingly, the G proteins appeared to remain in an active conformation in the presence of cAMP, even though responses had declined. This suggested that adaptation must be downstream or independent of heterotrimeric G protein activation. Upon dissociation of the G$\beta\gamma$ subunit from Gα, indirect evidence has shown that G$\beta\gamma$ leads to the activation of the small GTPase Ras (Jin 2011). Ras proteins serve as molecular switches whose activity is regulated by Ras GEFs (Guanine nucleotide Exchange Factor) and GAPS (GTPase activating Proteins) (Bourne et al. 1991; Boguski and McCormick 1993; Bolourani et al.2006). In addition, Ras activity does not increase in response to chemoattractants in cells lacking functional heterotrimeric subunits (Kae et al. 2004; Sasaki and Firtel 2006).

Ras is the first marker to show an amplified asymmetric response during migration and is followed closely at the PM by changes in the level of the enzymes that synthesize and degrade the phosphoinositides (PIPs) that include PI(3,4)P2, PI(3,4,5)P3, and PI(4,5)P2 (Whitman et al. 1988; Sasaki et al. 2004, 2007). In *D. discoideum*, PI 3-kinases and the tumor suppressor PTEN relocate to the front and back of the cell, respectively (Iijima and Devreotes 2002). Similar mechanisms have been shown in numerous mammalian systems (Ménager et al. 2004; Weiss-Haljiti et al. 2004). While enhanced motility may contribute to the metastatic properties of mammalian cells, where PIPs are improperly regulated (Vazquez and Sellers 2000; Samuels et al. 2004; McCubrey et al. 2006; Hollestelle et al. 2007; Fang et al. 2007; Berns et al. 2007; Pandolfi 2008), numerous studies have also shown that these components are not critical for chemotaxis, and in some instances, the phenotypes are actually rather marginal (Wang 2009; Stephens et al. 2008; Kay et al. 2008). Interestingly, it has also been shown that Ras and PI3K

activity, along with PTEN, are reciprocally regulated and along with many other components, play an important role in the asymmetric shape changes occurring during cell division (Janetopoulos et al. 2005; Janetopoulos and Devreotes 2006). The critical role of these signaling molecules in cell division makes it likely that there are redundant pathways and/or compensation of other pathways that insures that a cell propagates if one signaling pathway is perturbed. For instance, in *D. discoideum* when the small G protein Ras G is disrupted, expression levels of Ras D and B increase substantially (Bolourani et al. 2010). Ras D expression has also been found to go up in cells lacking Ras C and G (Srinivasan et al. 2013). When observed from an evolutionary context (Fig. 1), these findings suggest that the regulatory elements controlling cell shape changes evolved first, and only after the

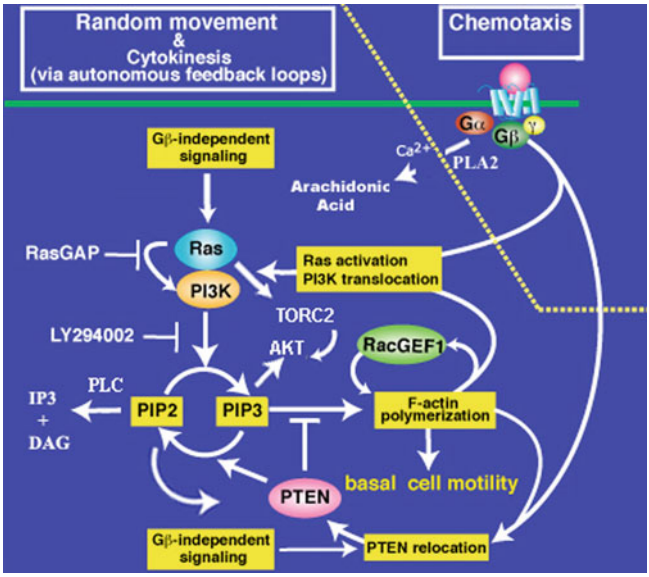


Fig. 1 Evolutionary model for random movement, cytokinesis, and chemotaxis. Since cells divide and move randomly in the absence of heterotrimeric G protein function, a feedback loop has been proposed that is activated autonomously and without extracellular stimuli and evolved prior to the introduction of serpentine receptors and heterotrimeric G proteins. This loop requires cytoskeletal and plasma membrane regulators that simultaneously recruit PI3K and delocalize PTEN from the protrusion site and the rear of the cell. It is assumed that the regulators for Ras/PI3K/PTEN and F-actin polymerization/disassembly can influence the initiation and decay of the circuit. As the process is stochastic, it is speculated that an increase in the level of any of the responses over a threshold level may be sufficient to trigger the feedback loops and pseudopod formation, while components such as GAPs and phosphatases regulate the threshold and level/time of activation. Chemoattractants activate receptors and heterotrimeric G proteins and these molecules, through mostly unknown mechanisms, are able to bias the circuit. Interestingly, polarity does partially silence the circuit as random pseudopods and macropinocytosis are down regulated. This may help provide amplification that induces cell polarity and supports directed migration. Adapted from Janetopoulos and Firtel (2008), FEBS

development of serpentine receptors and heterotrimeric G proteins did a cell "compass" develop (Janetopoulos and Firtel 2008; Sasaki et al. 2007).

There are many mutants identified by screens over the years that have identified components that clearly are involved in cell motility in *D. discoideum*, yet there are only a few dozen at most that appear to be pure chemotaxis mutants. Many of these mutants were identified by their inability to aggregate and form fruiting bodies after starvation, a process that requires the proper timing of cAMP oscillations. If these waves are not proper, cells do not upregulate the cAMP machinery and fail to fruit. Unfortunately, in many cases these mutants are developmental and not chemotaxis mutants. If a cell has not upregulated the cAMP receptors, then of course it will not migrate well to a cAMP gradient. Similarly, if a cell line is not polarizing properly, then it is likely not going to migrate in response to cAMP.

It may be difficult to generate new mutants of key components since many of these molecules also regulate cell division. We have tested many available mutants to migrate directionally to the chemoattractant FA. FA chemotaxis becomes extremely robust when vegetative cells are grown in the presence of bacteria. The upregulation of FA receptors does not require a developmental program (Srinivasan et al. 2013). To date, we have found only two mutants that are unable to chemotax to FA; those lacking the Gα4 subunit that presumably couples to the unknown FA receptor, and cells lacking the Gβ subunit. Cells lacking PI3Ks, PLC, Ras C and G, PKBA and PKBR1 (Srinivasan et al. 2013), and numerous TorC components including Rip3, Lst8, and Pianissmo (Ricktor) all chemotax to FA (Janetopoulos lab, unpublished results). Several mutants tested have been shown to have severe chemotaxis defects, including Ras C/G double nulls. Ras C/G double nulls were reported to be completely blind in a cAMP gradient (Bolourani et al. 2010). We tested multiple Ras C/G null strains and they are all defective in development, but chemotax well to FA. The latter results and the fact that the TorC components all chemotax to FA are consistent given that cells lacking both PKBA and PKBR1 chemotax to FA, although their overall motility appeared compromised and they are aberrant as compared to wild type cells. The above findings, coupled with the critical nature of many of these molecules in cell division, likely mean that genetic screens targeting chemotaxis and polarity mutants performed up to this point are not saturated. There may be components critical for both cell division and migration that have not been identified because they grow slowly or not at all and suggest that conditional mutants may be necessary to find other core regulatory elements critical for chemotaxis.

7 Temporal Responses to Chemoattractants Provide Valuable Spatial Information

The biosensor PH-GFP, a marker for PI(3,4)P2 and PI(3,4,5)P3 rapidly redistributes to the PM in response to uniform cAMP or FA stimulation (within a few sec) (Parent et al. 1998). PI3K is also rapidly recruited to the PM, while PTEN

follows a reciprocal time course and moves from the PM to the cytosol (Funamoto et al. 2002). Other leading edge proteins such as actin binding proteins and coronin display similar localizations (Bretschneider et al. 2002). It has also been found that the localization of cortexillin, which contains a PI(4,5)P2 binding motif (Faix et al. 2001), mirrored that of Myo2-GFP and PTEN-GFP and moved from the PM to the cytosol (Srinivasan et al. 2013). These findings suggest that the uniform activation of the heterotrimeric G proteins leads to a dramatic reorganization of the PM, and that the quiescent state is more similar to the rear of a cell, while the PM at the peak of the response is most representative of the leading edge. The cytoskeletal and signaling molecules that show polarized distributions in migrating cells also redistribute between the PM and cytosol when cells were given a uniform stimulus of chemoattractant (Srinivasan et al. 2013). These findings suggest that the isolation of membrane-associated proteins in response to a uniform stimulus could be a powerful method for isolating leading edge and rear proteins of polarized cells. Similarly, global changes in lipids and other metabolites in response to a temporal stimulus of chemoattractant may reveal other molecules that are spatially localized during directed cell migration.

8 The Role of Polarity in Migration

The morphology and behavior of unpolarized and polarized cells were investigated during gradient sensing and gradient switching. Most polarized cells make big U-turns when a gradient is reoriented (Srinivasan et al. 2013). In contrast, vegetative cells extend multiple pseudopods that tend to be oriented toward the initial FA source. When the gradient is switched, unpolarized cells stopped immediately and extended pseudopods toward the new direction of the gradient. These findings demonstrated that the apparent biased random walk of unpolarized cells is more efficient at rapidly changing directions than fully polarized cells. This may be a useful characteristic so that feeding *D. discoideum* can rapidly reorient to the correct direction of food sources. In addition, unpolarized cells have the capacity to generate phagosomes along the entire periphery of the cell (Srinivasan et al. 2013).

Unpolarized cells taken from bacterial lawns and treated with Latrunculin-A were unable to form a stable PH-GFP crescent toward a FA source. PI(3,4,5)P3 oscillations were observed all over the PM regardless of direction of the gradient. However, in polarized cells, the PI(3,4,5)P3 crescent was stably localized toward the high side of the cAMP gradient. Interestingly, highly unpolarized cells, whether responding to FA or cAMP, were unable to make stabilized PI(3,4,5)P3 responses as was determined by examining cells that have been starved for 4 h (Srinivasan et al. 2013). It may be that these underlying “random” crescents in vegetative and in 4 h starved unpolarized cells are generated by an oscillatory mechanism that is independent of the heterotrimeric G proteins and can only be biased to some extent by the chemotactic signal transduction system. Polarized

cells appear to be able to suppress these basal oscillations and focus the PI(3,4,5)P3 synthesis in the direction of the gradient and redistribute PTEN to the rear of the cell. This ability to dampen the random activity provides a focusing mechanism that likely helps cells to migrate up a concentration gradient. It is likely that unpolarized cells responding to FA are still performing spatial sensing and integrate the signal across the entire periphery of the cell. This may increase the probability of random pseudopods on the high side of the gradient. However, it is also possible that the pseudopods are reinforced in the correct direction and that FA-mediated signaling is not capable, by itself, of generating a pseudopod (Andrew and Insall 2007).

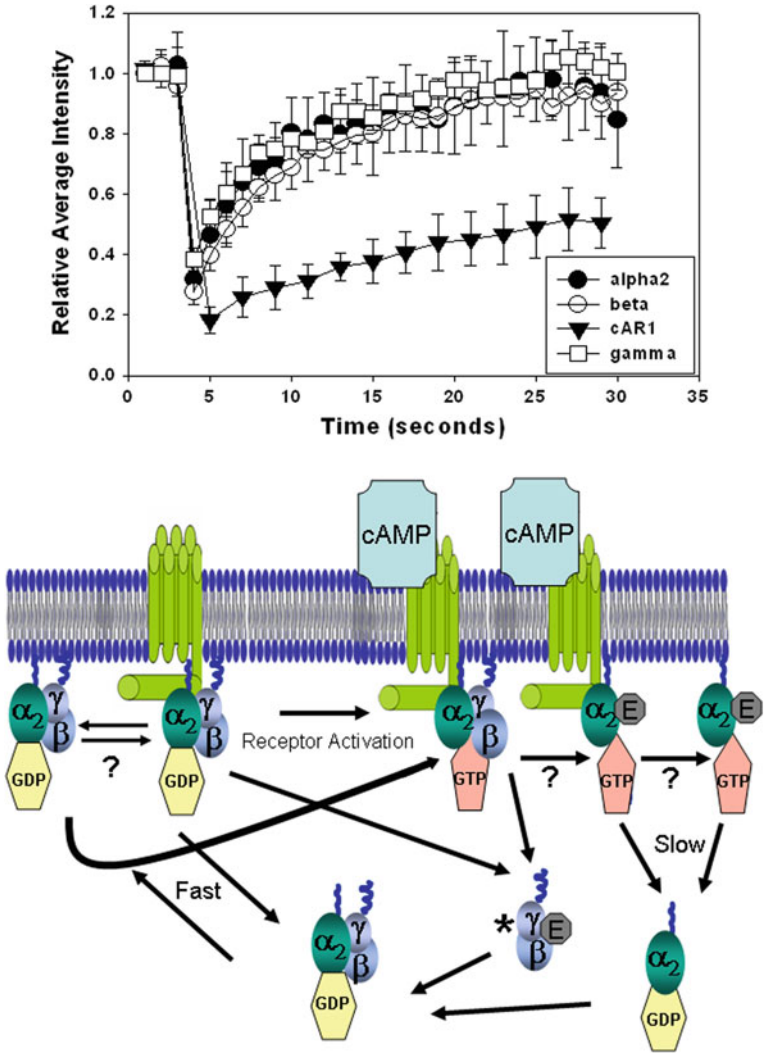
9 *Dictyostelium* Heterotrimeric G Proteins and Functions

The putative FA receptor and the *Dictyostelium* cAMP receptors are coupled to heterotrimeric G proteins (Gα, β and γ) which mediate the majority of the signaling responses (Bagorda and Parent 2008). To date, 14 Gα subunits, 1 Gβ (although there is another putative Gβ subunit of unknown function) and a single Gγ subunit have been identified in the *D. discoideum* genome (Eichinger et al. 2005). The Gα2 subunit couples to the cAR1 receptor and is essential for development and chemotaxis toward cAMP, while the Gα4 subunit is critical for FA-mediated chemotaxis (Kumagai et al. 1991; Hadwiger et al. 1994; Janetopoulos et al. 2001). The Gα4 subunit is homologous to the Gα2 subunit and has 41 % amino acid sequence identity (Hadwiger et al. 1991).

Cells lacking Gβ fail to aggregate or display typical chemoattractant induced responses. In addition, Gβ is required for receptor G protein coupling (Wu et al. 1995). Workers in the Devreotes lab also investigated the structure and function of the *D. discoideum* Gγ and demonstrated that this subunit is necessary for anchoring Gβ to the membrane. Deletion of a CSVL motif in the carboxy terminal of Gγ lead to the relocation of Gβ to the cytosol. In addition, cells with this truncation were unable to sense a cAMP gradient (Zhang et al. 2001).

Biophysical approaches including Förster resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) showed that the G protein subunits continuously shuttle from a cytosolic pool to the plasma membrane whether they were active or inactive (Fig. 2, top) (Elzie et al. 2009). Interestingly, the Gα2 subunit demonstrated a longer half time on the plasma membrane when cells were in the presence of cAMP. The resident time on the plasma membrane of the G$\beta\gamma$ complex did not change, showing very strong evidence for the complete dissociation of the heterotrimer when in the active state. It is still unclear whether the Gα2, presumably in an active GTP-bound state, is coupled to the receptor or another binding partner at the inner leaflet of the plasma membrane when cAMP is present (Elzie et al. 2009).

This data suggests that that the Gα-subunit might locally activate downstream effectors, whereas diffusible G$\beta\gamma$ subunits could potentially act as inhibitory



molecules. This fits a model proposed by Levine et al. (2006), which suggested that the G proteins themselves make good candidates for the activator and inhibitor molecules that they propose underlie the large gain seen in the responses at the leading edge during gradient sensing. As depicted in the cartoon, the data presented in the Elzie et al. manuscript suggest that the Gα subunit remains local and the G$\beta\gamma$ subunits diffuse away (see Fig. 2, bottom for model). Further support for this idea comes from single-molecule analysis of cAR1-YFP, which was shown to have two different receptor populations in the absence of signaling. Some were immobile and some were mobile, suggesting that some cAR1 is not precoupled to

◀ **Fig. 2** G protein heterotrimers cycle between the cytosol and plasma membrane. **a** FRAP experiments were conducted on Gα2-null cells expressing Gα2-CFP, Gβ-null cells expressing β-CFP, AX2 cells expressing γ-YFP and cAR1/3-null cells expressing cAR1-YFP. Gα2-CFP, β-CFP, and γ-YFP had similar recovery rates, whereas cAR1-YFP recovery was significantly slower. **b** Model for G protein cycling in *Dictyostelium discoideum*. Inactive heterotrimers continuously cycle between the cytosol and cAR1 or the *inner* leaflet of the plasma membrane. It is likely that the lipid modifications of both Gα2 (palmitoylation and myristoylation) and Gγ (isoprenylation) play a significant role in the heterotrimer interacting with the plasma membrane. Upon binding of cAMP to the receptor, there is a change in receptor conformation that might increase the affinity of the receptor for the Gα2 subunit. The conformational change in cAR1 simultaneously triggers the exchange of GDP for GTP on the Gα2 subunit and the heterotrimer dissociates. Because the Gβγ subunit does not apparently change its residency time on the plasma membrane, time spent in the empty-pocket conformation is probably extremely fast and the Gα2 subunit might remain coupled to the receptor in the GTP-bound state. It is also possible that both GDP- and GTPbound Gα2 subunits interact with both receptor and the plasma membrane, or effector molecules (E) in the latter case, when activated. This is supported by data showing that the Gα2 subunits still interact with the plasma membrane in cells lacking cAR1 and cAR3. In either situation, this dissociation is complete, and the active Gβγ subunit diffuses away from the membrane. The intrinsic GTPase activity of the Gα2 subunit hydrolyzes the bound GTP and the receptor, membrane or effector molecule and Gα2 subunits dissociate. Because we have been unable to measure changes in FRET in the cytosol, liberated Gβγ subunits probably find free Gα2 subunits and reform the heterotrimer. This model can explain both the loss of G protein FRET and the lack of a change in membrane Gβ-subunit intensity upon receptor activation. From Elzie et. al. (2009)

the G protein (De Keijzer et al. 2008). The authors further found that the immobile fraction was almost identical in chemotaxing cells to that seen at the leading edge of cells lacking the Gα2 subunit. They interpreted this to mean that there was uncoupling of the receptor from the G proteins in response to cAMP. Taken together with the FRAP results, the single molecule results suggest that the Gα2 subunits might be interacting with the receptor directly, but binding the membrane or proteins on the membrane after receptor activation. This local increase of Gα2 subunits at the front of a cell may in turn provide the molecular component for the initial linear amplification that occurs during gradient sensing.

We have recent data that suggests that cAR1 partially redistributes to the rear of the cell during chemotaxis, overturning the long held belief that receptors are uniformly localized during gradient sensing (unpublished results, Janetopoulos lab). Our results suggest that activated receptors migrate to the rear of the cell while naïve receptors localize to the front of the cell. There is a large fractional increase in the number of receptors in the rear of the cell, yet the cell continues to move up the gradient. This suggests that these receptors are either desensitized or uncoupled from the heterotrimeric G proteins. Uncoupling would fit the data described above for the quantitation of the cAR1 immobilization fraction at the leading edge of a chemotaxing cell. This might also explain the single molecule data obtained by Ueda and colleagues showing that cAMP binding at the leading edge differed dramatically from the kinetics at the rear of a cell (Ueda et al. 2001). It is exciting that the molecular characterization of the chemotactic compass has returned back to one of the earliest discovered components. It will be interesting to

see what roles cAR1 plays in amplifying the internal cellular responses and whether this work will also translate to our understanding of directed migration in mammalian systems.

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Transcriptional Regulators: Dynamic Drivers of Multicellular Formation, Cell Differentiation and Development

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Abstract In this chapter, we examine what is known about the roles of individual transcription regulators in mediating development in *Dictyostelium discoideum.* We present a broad review of the field, covering genetic, biochemical, molecular, and bioinformatic experiments that illuminate transcriptional regulation in the context of developmental events. We highlight evidence for evolutionary conservation where it exists, and have sought to underscore the power of RNA sequencing as a tool for comparative studies and global analysis. We believe that as next generation, omics approaches are more widely applied, we may paint a more complete picture of the gene regulatory networks governing dictyostelid development, and gain insight into general evolutionary processes that shape developmental biology.

1 Introduction

Dictyostelid amoebae inhabit the forest soil, consume bacteria through phagocytosis, and grow as solitary unicellular organisms when resources are abundant. Once food is depleted, they initiate a tightly regulated developmental program that culminates in the formation of a multicellular fruiting body (summarized in Kessin

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2001). Among dictyostelid species whose genomes are available, *Dictyostelium discoideum* (Raper 1935) and *Dictyostelium purpureum* (Olive 1901) belong to the same clade (group 4) in the *Dictyostelium* phylogeny (Schaap et al. 2006). They have similar sized, AT-rich genomes encoding for about 12,000 predicted proteins, of which an estimated 7,619 proteins share significant reciprocal amino acid sequence similarity (Eichinger et al. 2005; Sucgang et al. 2011). The primary nucleotide sequences of their genomes, meanwhile, are highly divergent, reflecting an evolutionary distance of roughly 400 million years, comparable to that between humans and jawed fish (Sucgang et al. 2011).

Nevertheless, these congeneric amoebae display a striking similarity in the morphological progression of their developmental programs. They both initiate their developmental programs upon starvation, secreting cAMP, and aggregating toward the signal by chemotaxis (Konijn et al. 1969). Through a series of morphogenic changes, the multicellular organism eventually culminates to form a fruiting body: a droplet of spore cells (the sorus) held atop a cellular stalk (Raper 1940). Various differences in *D. purpureum* development relative to *D. discoideum* include: *D. purpureum* developmental structures take shape 4 h later; *D. purpureum* begins stalk formation during migration, rather than as part of culmination; *D. purpureum* constructs triangular cellular supports for its sporophore, rather than basal discs; and the color of the *D. purpureum* sorus is, eponymously, purple rather than translucent yellow (Schaap et al. 2006). Despite these differences, development of these species results in fruiting bodies of similar shape at the end of the 24 h developmental cycle.

The observation that ontogeny is largely conserved over deep evolutionary time suggests that the genetic mechanisms regulating development are conserved as well. One powerful approach to dissecting the molecular physiology of development is to measure global changes in gene expression over time. In eukaryotes, transcription is governed by the interplay of several classes of proteins (Fig. 1) (Krebs et al. 2013). General transcriptional machinery including RNA polymerase assembles near the start of transcription, in many cases binding TATA-box sequences through a component TBP (TATA-binding protein). Certain transcription factors bind cis-DNA elements in the promoters of genes in a sequence-specific manner, activating or repressing the transcription of target genes. These regulatory acting transcription factors ("Transcription Factors/Regulators" in Fig. 1) are the main subject of this chapter, herein interchangeably referred to as TFs. TFs communicate with the general transcriptional machinery via mediators. Additionally, chromatin remodelers and histone modifiers play essential roles in determining the accessibility of DNA for these other molecules to bind. The particular interactions regulating transcription will be somewhat different in organisms throughout the eukaryotic evolutionary tree, affected by differences in genomic organization, promoter and gene structures, and the complement of regulatory proteins, yet the overall scheme is highly conserved.

Comparative transcriptomics of *D. discoideum* and *D. purpureum* using RNA-seq continues to shed light on the genetic interactions that regulate transcription, and how these affect development (Parikh et al. 2010b). In *D. discoideum*, 243

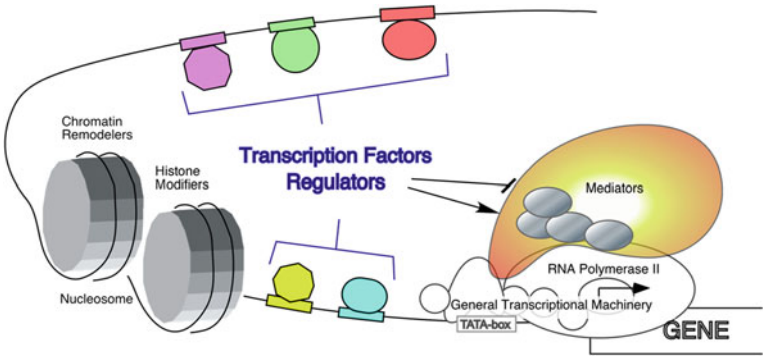

Fig. 1 Cartoon schema of transcriptional regulation in *Dictyostelium*

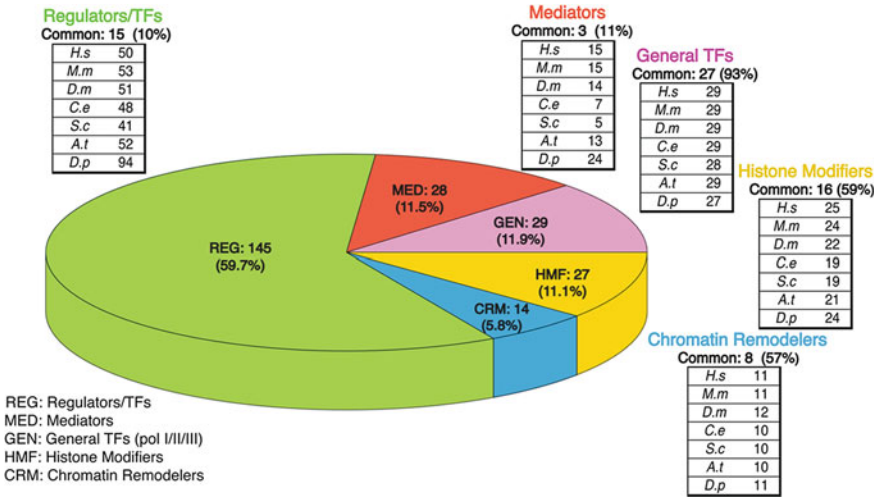

Fig. 2 Functional categories of *D. discoideum* proteins involved in transcriptional regulation, as annotated in dictyBase. For each segment, the numbers of orthologs found in each model organism, or common to all listed, are tabulated. *H.s*: *Homo sapiens, M.m*: *Mus musculus*, *D.m*: *Drosophila melanogaster*, *C.e*: *Caenorhabditis elegans*, *S.c*: *Saccharomyces cerevisiae*, *A.t*: *Arabidopsis thaliana*, *D.p*: *Dictyostelium purpureum*

genes encode proteins predicted to regulate transcription, including components of general transcriptional machinery (29), transcriptional regulators (145), mediators (28), chromatin remodelers (14), and histone modifiers (27) (Fig. 2). A significant number (180) of these have orthologs in *D. purpureum* (Fisher's test, p value $= 2.49 \times 10^{-6}$), and 69 retain significant amino acid similarity between *D. discoideum* and other eukaryotic model organisms over 1 billion years removed. In addition to amino acid sequence similarity, coordinated expression of transcriptional regulators during specific developmental events suggests orthologs

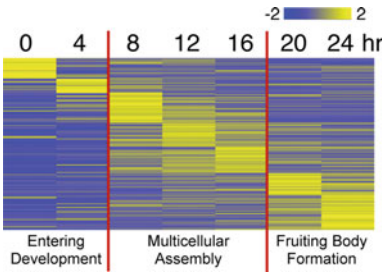

Fig. 3 Expression profile of transcriptional regulator genes during development. Heat map represents standardized mRNA levels (determined by RNA-seq) of 145 genes of all predicted transcriptional regulators in *D. discoideum*. There are two major transitions (*red lines*) with three broad developmental stages: (*1*) entering development, (*2*) multicellular assembly, and (*3*) culmination and fruiting body formation. Within the first stage, two distinct blocks of upregulated genes might reflect two developmental events, starvation response (0–4 h), and aggregation from unicellularity to multicellularity (4–8 h). Changes in regulatory gene expression during subsequent morphogenic events are less dramatic and partly-overlapped but still display a molecular signature associated with developmental progression

retain conserved functions. Comparing the transcriptional profiles between these species over the 24 h developmental period, more than 50% of the transcriptional regulation-related orthologs show almost identical expression trajectories (Pearson's correlation > 0.5). The conservation of transcriptional programs in *D. discoideum* and *D. purpureum* helps explain the similarity in their developmental progression and morphologies. We describe transcriptional regulators and their roles in *D. discoideum* development in the following three sections divided based on major developmental events and transitions in transcriptional profiles (Fig. 3).

2 Entering Development

Perhaps the most curious aspect of *Dictyostelium* biology, commonly invoked as a subject for investigation, is its faculty to grow as single celled amoebae, only to later aggregate and undergo multicellular development. Starvation provides the environmental cue to exit vegetative growth in favor of the developmental program leading to spores that may disperse to more hospitable environments. In the earliest stages of *D. discoideum* development, the first transcriptional changes occur before cells generate pulsatile cAMP. Cell density sensing factors such as prestarvation factor (PSF) and conditioned medium factor (CMF) initiate the expression of numerous early developmental genes via the protein kinase A (PKA) pathway (Gomer et al. 1991; Mann and Firtel 1989; Rathi and Clarke 1992; Schulkes and Schaap 1995). The subsequent establishment of cAMP pulsing depends on an initial low-level accumulation of the high affinity cAMP receptor cAR1, and the production of cAMP by adenylate cyclases. These early expressed

genes, *carA* (encoding cAR1) and *acaA* (encoding adenylate cyclase A), enable cAMP pulse-dependent gene expression. Nanomolar levels of cAMP oscillate due to the complex interplay of cAMP receptors, adenylate cyclases, phosphodiesterases, and other factors (Aubry and Firtel 1999; Laub and Loomis 1998). This oscillatory signal coordinates the chemotactic aggregation of hundreds of thousands of amoebae from a wide area, as cells migrate toward the aggregation center and relay the cAMP signal to other cells behind in the field. Thus, the progression from starvation into development depends on *carA* and *acaA* expression, which is regulated directly or indirectly by multiple transcription factors, including MybB, CbfA, and SrfB.

MybB is a myeloblastosis (MYB) domain-containing protein that belongs to the 27-member *D. discoideum myb* gene family. Expression is observed almost immediately after starvation and peaks around 12 h (Parikh et al. 2010b). The *mybB* gene was identified by a restriction enzyme-mediated integration (REMI) screen for "synag" mutants, those with synergizable aggregation defects (Otsuka and Van Haastert 1998). These mutants have defective cAMP signaling, but development proceeds normally when mutants are co-developed with wild-type cells or when cAMP pulses are provided exogenously. The *mybB* null exhibits an aggregation minus (agg-) phenotype and shows greatly reduced *carA* and *acaA* expression, preventing cAMP-mediated chemotaxis and cAMP relay. These *mybB*$^{-}$ defects are rescued by ectopic expression of *acaA*, but not of *carA*. These data suggest MybB is involved in regulating cAMP synthesis.

Like MybB, C-module binding factor A (CbfA) is essential for the initiation of *acaA* expression, and therefore the initiation of development (Siol et al. 2006; Winckler et al. 2004). CbfA contains an amino-terminal jumonji-C domain characteristic of chromatin modifiers, a non-canonical zinc-finger containing region, and a carboxy (C)-terminal domain with an AT-hook DNA-binding motif (Horn et al. 1999). An amber-mutation knockdown strain (*cbfA*^{am}), producing $\sim$5% of wild-type CbfA protein, failed to enter development (Winckler et al. 2004). The *cbfA*^{am} mutant does not express *acaA* and thus its aggregation defects can be explained by the inability to synthesize cAMP. The *cbfA* mutant shares many features with the *mybB* null. Mixing *cbfA*^{am} cells with wildtype, or pulsing exogenous cAMP in suspension, partially restores the developmental phenotype and the expression of early developmental genes like *acaA*, but not of postaggregative genes such as *tgrC1* (formerly *lagC*, for "loose aggregate"). This implies that the *cbfA*^{am} mutant retains the capacity to respond to cAMP. Similar to the *acaA*$^{-}$ mutant, development is restored by the constitutive expression of the catalytic subunit of PKA (Wang and Kuspa 1997). RNA-seq data reveal that *cbfA* expression increases post-starvation, peaking around 12 h in *D. discoideum*, suggesting a possible role in later development as well (Parikh et al. 2010b). The C-terminal domain of CbfA is highly conserved in all dictyostelids, and was sufficient to regulate most CbfA-dependent gene expression in vegetative cells (Schmith et al. 2013). The domain's function is conserved such that expression of C-terminal of distantly related *Polysphondylium pallidum* CbfA restored aberrant gene expression in a *D. discoideum* mutant. This work implies that the C-terminal

domain and jumonji-C/zinc-finger domain have distinct evolutionary roles and could function independently for vegetative and developmental gene regulation.

Unlike the requirement of MybB and CbfA for initiating development and cAMP pulsing, *srfB*⁻ shows precocious development (Galardi-Castilla et al. 2008). The *srfB* gene encodes the second *D. discoideum* protein found to have similarity with MADS-box transcriptional factors of the serum response factor (SRF) family. In a *srfB* null, *acaA* and *carA* are expressed 2 h prior to wildtype, suggesting SrfB might play a regulatory role by repressing these genes. In spite of early expression of these genes, the *srfB* mutant fails to relay cAMP, though they are capable of responding to ectopic cAMP. Further, the *srfB* mutant exhibits a complex set of expression patterns of vegetative and early developmental genes. For example, expression of vegetative *ponA* (ponticulin) persisted into development, while *ctsD* (cathepsin) and *alyB* (lysozyme) were atypically downregulated in the mutant. EDTA-stable adhesion, which is established at 6 h of normal development, is highly impaired in the *srfB* null. Since this defect was restored by ectopic cAMP pulsing, it is likely that *srfB*⁻ cells under-express early developmental genes required for aggregation, such as *csaA* (adhesion protein). Taken together, the evidence suggests SrfB contributes to regulating the starvation response and early developmental events.

Once cAMP pulses begin, *carA* and *acaA* expression rapidly increases as part of a positive feedback mechanism to maintain the oscillatory signal (Firtel 1996). cAR1 transcription factor (Crtf), which is present throughout development, is likely one of the transcriptional regulators that establish this loop (Mu et al. 2001; Parikh et al. 2010b). Mobility shift assays and DNA affinity chromatography showed Crtf is a zinc-dependent binding protein of a *carA* promoter element responsible for early expression of cAR1. The *crtf* null suffered severely impaired aggregation and displayed only basal levels of *carA* expression, as well as abrogated expression of *csaA* and *gpaB* (G-protein α2). Ectopic cAMP pulsing rescued the aggregation defect and restored expression of early developmental genes. However, delayed and asynchronous development could not be rescued by constitutive *carA* expression, indicating *crtf*⁻ misregulates additional cAR1-independent or global pathway(s) (Mu et al. 2001, 1998).

During normal development, cells chemotax along a pulsatile cAMP gradient, entering streams that lead to an aggregation center. After aggregation, a multicellular mound forms, cell differentiation begins in the mound, and extracellular cAMP accumulates to constant micromolar levels (Abe and Yanagisawa 1983). This continuous high concentration of cAMP (in contrast to nanomolar pulses) suppresses early aggregative genes, like *carA*, and activates expression of post-aggregative genes, including *tgrC1* (adhesion molecule), *cprB* (cysteine protease), and *rasD* (Ras GTPase). Crtf may also be important for the sharp transition to cell-type-specific gene expression when extracellular cAMP rises (Mu et al. 2001). In *crtf*⁻ cells, differentiation markers such as *ecmA* and *cotB* were expressed even in the absence of high levels of cAMP, suggesting Crtf may repress some cell differentiation genes to regulate proper developmental progression. Later still, low spore viability persisted despite 8-Br-cAMP (a membrane permeable cAMP

analog) activation of PKA, suggesting Crtf plays another role in sporulation either apart from, or downstream of, PKA. In sum, Crtf directly activates early *carA* expression, is essential for the expression of other early developmental genes, and appears to have additional targets in later stages of development.

G-box binding factor (GBF) is a well-characterized TF that regulates postaggregative and cell-type-specific gene expression (Brown and Firtel 2001; Schnitzler et al. 1994). GbfA was identified by affinity purification of the nuclear activity bound to the G-box in the promoter of the prespore(psp)-specific gene *cotC* (Powell-Coffman et al. 1994; Schnitzler et al. 1994). G-box motifs that contain two GT/CA-rich sequences were found enriched in postaggregative and cell-type-specific gene promoters (Haberstroh et al. 1991; Hjorth et al. 1990; Pears and Williams 1987; Powell-Coffman et al. 1994). Prior to cloning the *gbfA* gene, G-box motifs were shown to be essential for the expression of the prestalk (pst) genes *cprB* and *ecmB*, and required in concert with another poly-AT cis-element for proper psp gene induction (Ceccarelli et al. 1992; Hjorth et al. 1990; Hjorth et al. 1989). Expression of *gbfA* increases in the early hours after starvation, with continual, high levels that suggest GbfA may be active throughout development, possibly playing numerous roles (Parikh et al. 2010b; Schnitzler et al. 1994). The *gbfA*$^{-}$ mutant fails to express both postaggregative and cell-type-specific genes during development on solid media and in suspension with exogenous cAMP pulses. The null phenotype is characterized by arrested development at the loose aggregation stage, wherein mounds repeatedly disperse and reaggregate. This defect is cell-autonomous and thus not rescued by co-development with wild-type cells (Schnitzler et al. 1994).

Physical cell–cell contact is also required for proper TF modulation of developmental genes. For example, TgrC1, though not a TF, is essential for normal expression of postaggregative genes and subsequent cell-type-specific genes. TgrC1 is a cell surface immunoglobulin-like domain-containing protein that mediates cell–cell interactions and allorecognition with its binding partner TgrB1 (Benabentos et al. 2009; Dynes et al. 1994; Hirose et al. 2011). Null mutants of *tgrC1* and *gbfA* have similar developmental phenotypes. Phenotypic rescue and global expression analyses define the relationship between *tgrC1* and *gbfA* as a feed-forward loop (Iranfar et al. 2006; Sukumaran et al. 1998). Both transcriptional regulation by GbfA and some unidentified intracellular signaling derived from TgrC1-mediated cell–cell interactions are necessary for the developmental switch from aggregation to cell differentiation stages.

3 Multicellular Assembly

Following aggregation, *Dictyostelium* cells undergo a series of morphogenetic changes in response to inter- and intracellular signaling molecules, including cAMP and the chlorinated hexaphenone DIF-1. Complex gradients of signaling molecules potentiate and antagonize one another and induce the progression of

development. These signals are generated by, and relayed to, gene products under the precise control of transcription factors. Genes representing bZIP, Myb, STAT, and GATA family TFs are active in various cell types in *Dictyostelium*, integrating dynamic information regarding cellular identities, molecular signals, and spatial position. As the mound tightens and begins to change shape, becoming tipped and then forming a slug (or finger), cells migrate and sort, giving rise to a structure with distinct cellular populations. Classic studies of slugs stained with the vital dye neutral red indicated the presence of two distinct cell populations, one capable of generating spores, and the other stalk (Bonner 1959; Raper 1940). These populations are readily mapped in the developing structure. Both progenitor cells are found scattered throughout the mound initially, but once the slug forms, psp and pst populations show precise spatial and numerical proportioning. Pst cells constitute the front 20% of the slug while psp cells occupy the rear 80%. Psp cells are generally viewed as a fairly homogeneous population, while pst cells are a heterogeneous class containing multiple sub-types including pstA, pstO, pstAB, pstB, and pstU (reviewed in Fukuzawa 2011).

The pst subtypes can be distinguished by reporter gene expression driven by cell-type-specific promoter regions, especially of genes *ecmA* and *ecmB,* encoding extracellular matrix proteins that contribute to the stalk tube. For example, promoter analysis of the pst-specific gene *ecmA* delineated two regulatory regions that differentially direct cell-type-specific expression. The region proximal to the *ecmA* transcription start site controls its expression in pstA cells, located in the anterior part of the pst region. The distal *ecmA* promoter region directs expression in two cell types, pstO cells located in the posterior part of the pst region adjacent to the psp population and anterior-like cells (ALC) scattered throughout the psp region (Early et al. 1993). Expression from the *ecmB* promoter marks the pstB cells along the ventral side of the slug, and also pstAB cells arranged in a cone-like group in the anterior region (Ceccarelli et al. 1991). The very tip of the anterior pstA region contains cells that seem important for slug migration and culmination, and display expression from the proximal fragment of the *cudA* promoter (Fukuzawa and Williams 2000). The most recently described type, pstU, derives from a subset of ALCs and is marked by expression from the promoter of *rtaA* (lipid exporter gene) (Yamada et al. 2010). During culmination, the various pst cells differentiate and migrate to form the stalk proper, the basal disc, as well as lower and upper cup structures. The fact that the various cell populations are discernible based on differential gene expression suggests that each type possesses a distinct molecular physiology.

DIF-1 deficient mutants fail to differentiate some pst cell types and do not develop proper stalks (Saito et al. 2008; Thompson and Kay 2000). Screening mutants in submerged monolayers exposed to DIF-1 can reveal genes essential for stalk cell differentiation. One such assay isolated *dimA* (DIF-1 insensitive mutant), encoding a basic leucine zipper (bZIP) transcription factor (Thompson et al. 2004). In the *dimA* mutant, DIF-1 neither induces stalk formation nor suppresses spore production. Since bZIP TFs are known to dimerize, Huang et al. (2006) searched the *D. discoideum* genome for candidates that might interact with DimA. They

found DimB based on the similarity of predicted dimerization domains and spatiotemporal co-expression with *dimA*, and confirmed that the proteins can interact *in vitro*. Another class of TF was also identified by a similar screening as DimA. GtaC (alternatively, DimC) belongs to the zinc-finger domain-containing GATA family, which canonically bind to a core GATA motif (Keller and Thompson 2008).

Both *dimA*$^-$ and *dimB*$^-$ mutants developed long slugs lacking the pstO region and eventually formed fruiting bodies with a compromised basal disc and lower cup. In contrast, *gtaC*$^-$ mutant formed small slugs and tiny fruiting bodies in which the basal disc was incomplete. DIF-1 induces the nuclear accumulation of all three Dim proteins, although the nuclear accumulation of DimA is dependent on DimB. In a monolayer assay in the presence of both 8-Br-cAMP and DIF-1, wild-type amoebae form stalk cells, however, *dimA*$^-$ formed spores. Under the same condition, *gtaC*$^-$ cells remained as undifferentiated amoebae, while *dimB*$^-$ and *dimA*$^-$/*dimB*$^-$ double mutants entirely failed to differentiate, ultimately succumbing to non-vacuolar cell death. DIF-1 functions concurrently in two pathways: stalk induction and spore suppression. DimA may mediate DIF-1 suppression of spore production, while GtaC might mediate DIF-1 stalk induction. The severe response of the *dimB* mutant to these stimuli might be related to DimB's proposed multifunctionality (described below). Taken together, DimA, DimB, and GtaC play distinct roles in the DIF-1 signaling cascade.

DimB was also independently purified by another group with affinity chromatography of two fragments in the *ecmA* promoter (Zhukovskaya et al. 2006). Beta-galactosidase reporter assays and ChIP analysis revealed that DIF-1 induces DimB binding to the promoter core sequence of *ecmA* responsible for normal pstO/ALC-specific expression, consistent with the observation that DimB induces *ecmA* expression in pstO, but not in pstA (Fukuzawa 2011; Huang et al. 2006; Zhukovskaya et al. 2006). DimB was found to accumulate in the nucleus specifically in pstB cells and bind to the *ecmB* promoter to activate its expression. In the *dimB*$^-$ background, the expression pattern of *ecmB* remained unchanged in the anterior region, but was measurably lower in the ventral region of the slug, and very few pstB and lower cup cells could be detected (Yamada et al. 2011). DimB also acts as a repressor, binding CCCCAC sequences in the promoter of the psp-specific gene *pspA* to silence its expression in pstO cells (Nuñez-Corcuera et al. 2012). DimB seems to be a multifaceted TF for cell-fate determination, regulating *ecmA* expression in pstO/ALC *ecmB* in pstB cells, as well as repressing *pspA* in pstO cells.

Signal transducers and activators of transcription A (STATa) was identified by mass spectrometry as a protein that bound the repressor regions of the *ecmB* promoter (Kawata et al. 1997). In mammalian systems, post-translational modifications and interactions with protein inhibitor of activated STAT (PIAS) family members are key regulatory mechanisms of STAT activity. Kawata and colleagues have described the first example of non-metazoan STAT pathway involving *Dictyostelium* STATa and PIAS (Kawata et al. 2011; reviewed in Kawata 2011). STATa, the first of four *Dictyostelium* STAT proteins, is encoded by *dstA* and developmentally regulated, with its nuclear localization dependent on extracellular

cAMP levels mediated at least in part by cAR1 (Araki et al. 1998; Dormann et al. 2001). The *dstA*$^{-}$ mutant exhibits complex morphological defects during development including: delayed aggregation, partially explained by decreased cAMP chemotaxis; a prolonged migratory slug stage; abnormal culmination; an inability to clearly delineate its pst region; and irregularly shaped fruiting bodies with columnar structures containing undifferentiated amoebae and very few vacuolated stalk cells (Mohanty et al. 1999). The complexity of the null phenotypes suggests STATa involvement in regulating a variety of pathways including the spatial and temporal expression of key pst genes such as *ecmB* and tip-organizer *cudA*.

Another member of the STAT family, STATc (*dstC*), was cloned via low stringency hybridization to the SH2 domain of the *dstA* gene. In response to DIF-1, STATc appears to translocate to the nucleus in pstO cells and ALCs, requiring the tyrosine kinase-like protein SplB/Pyk2 for activation (Araki et al. 2012; Fukuzawa et al. 2001). The *dstC*$^{-}$ mutant displays accelerated early development and prolonged slug migration. In *dstC*$^{-}$ slugs, *ecmA* expression is dramatically higher throughout the pst region, and the expression, normally balanced between pstA and pstO, shifts higher in pstO suggesting that STATc acts as a repressor of *ecmA* in the pstO cells (Fukuzawa et al. 2001).

MybE, another Myb domain-containing TF, was purified biochemically using a distal fragment of the *ecmA* promoter (Fukuzawa et al. 2006). Expression of *mybE* expression displays a monotonic increase until 12 h of development and a late peak around 20–24 h (Parikh et al. 2010b). The *mybE* mutant phenocopies the morphological defects of DIF-1 insensitive mutants and shows reduced expression of *ecmA* in both pstO cells and ALCs. Intriguingly, unlike the *dim* mutants, the expression of *ecmB* is distributed randomly throughout the *mybE*$^{-}$ slug, suggesting de-repression of *ecmB* in various cell types or the mislocalization of pstAB cells. Either way, MybE appears to play a key role in the expression of cell-type-specific genes, and in the proper partitioning of these subpopulations (Fukuzawa et al. 2006).

More recently, a myelin regulatory factor (MrfA) was found to directly associate with a proximal fragment of the *ecmA* promoter (Senoo et al. 2012). *MrfA* expression peaks around the aggregation stage (Parikh et al. 2010b). The *mrfA*$^{-}$ cells show delayed development and reduced pstA-specific gene expression in the slugs (Senoo et al. 2012). While this family of TFs is not well understood outside of the metazoans, the identification of another transcriptional regulator in *Dictyostelium* that affects the *ecmA* cell-type marker underscores the complexity of overlapping gene networks.

Transcription factors play critical roles in shaping cellular populations within the multicellular *Dictyostelium* organism. They integrate numerous chemical signals, like DIF-1 and cAMP, and convert this information into specific cellular behaviors. The DIF-1 signaling pathway appears to be highly conserved evolutionarily—orthologs of all proteins involved in the production of DIF-1 in *D. discoideum* were identified in the *D. purpureum* genome, and key proteins (i.e., DmtA, des-methyl-DIF-1 methyl transferase) have been shown to retain function between these species (Motohashi et al. 2012). A major role was just discovered

for c-di-GMP in *D. discoideum* stalk differentiation at culmination (Chen and Schaap 2012). This molecule is the product of diguanylate cyclase, encoded by *dgcA*. While *dgcA* does not have a predicted ortholog in *D. purpureum*, it is functionally conserved in other dictyostelids such as *Polysphondylium pallidum*. The recent discovery of novel factors that induce stalk cell fate suggests we still have a lot to learn about the mechanisms of differentiation and multicellular developmental transitions.

4 Culmination and Fruiting Body Formation

Environmental cues dictate whether slugs continue migrating or culminate to form fruiting bodies. Temperature influences the rate of migration, and light increases the amount of ammonia produced by cells (Bonner et al. 1989, 1988; Poff and Skokut 1977). Slugs stop migrating to begin culmination when they sense a drop in ammonia concentration (Schindler and Sussman 1977). The tip of migratory slugs is thought to mediate these decisions and thus the area is called tip-organizer (Smith and Williams 1980). As culmination initiates, pstA and pstO cells enter the elongating stalk tube, which ultimately embeds in the basal disc. Stalk cells become vacuolated and eventually die (Jermyn et al. 1996). Concurrently, elevated intracellular cAMP mediates spore encapsulation, a process induced by at least three signal peptides known as spore differentiation factors (SDFs) (Anjard et al. 1998, 2009). Several transcription factors are involved in regulating the decision to begin culmination and others in the spore differentiation and maturation processes.

Over three decades ago, *D. discoideum* mutants were generated by chemical mutagenesis that fail to leave the slug stage and culminate, known as the "slugger" phenotype (for example, see Smith and Williams 1980). Fukuzawa and colleagues (1997) sought to identify these genes by REMI mutagenesis. Their culmination-defective mutant, *cudA*, encodes a putative TF that localizes to the nucleus, and is expressed in two separated regions in the slug: the tip-organizer and psp regions. The *cudA* slugger forms gnarled structures failing to specify both stalk cells and spores, and can be rescued by the expression of *cudA* in pst cells. While the expression of pst specific genes *ecmA* and *ecmB* appears to be normal, *cudA*$^-$ cells show reduced expression of *expl7* (expansin-like 7) in tip-organizer cells, and psp marker genes including *spiA*, *cotC,* and *pspA* (Fukuzawa et al. 1997; Wang and Williams 2010). The distinct spatial separation of *expl7* and *cotC* expression appears to be driven by combinatorial effects of CudA with GbfA. Interestingly, the *dstA*$^-$ mutant has reduced *cudA* expression in its tip-organizer cells, suggesting these TFs may interact as well (Fukuzawa and Williams 2000; Ogasawara et al. 2009; Shimada et al. 2004; Wang and Williams 2010).

A similar REMI screen for slugger mutants yielded the *mybC* gene. MybC is also essential for the transition from slug to culminant (Guo et al. 1999). Peak *mybC* expression is observed around 16 h, and its mRNA is enriched in the pst area in slugs, the basal disc and stalk-tube entry region in culminants, and the

stalk-tube proper (Guo et al. 1999; Parikh et al. 2010b). Over the course of *mybC*$^{-}$ development, *ecmA* expression was delayed and levels of *ecmB* were dramatically reduced. These results suggest that MybC plays a regulatory role in various pst subtypes. The levels of the psp genes *cotA* and *cotB* (but not *pspA* and *cotD*) were significantly reduced in the mutant as well. The *mybC*$^{-}$ defect proved to be non-cell autonomous, rescued by co-development with as few as 10% wild-type cells, which suggests a defect in some aspects of intercellular signaling. Indeed, the mutant failed to produce key signal peptides SDF-1 and SDF-2. Further, the *mybC*$^{-}$ morphological defect could be rescued by overexpression of PKA catalytic domain or by induction of PKA via 8-Br-cAMP treatment, though production of SDF-1 and SDF-2 were not restored. These results suggest that MybC regulates the production of SDF-1 and SDF-2, which in turn signal the activation of the PKA pathway leading to developmental maturation.

The putative TF PadA (pst a differentiation) was identified in a DIF-1 monolayer assay for mutants lacking *ecmB* induction (Núñez-Corcuera et al. 2008). PadA belongs to the Nitrogen Metabolic Regulation Protein family and is thought to be essential for growth. In vegetative cells, *padA* expression remains low, then mRNA begins to accumulate around 14–16 h and peaks around 20–24 h of development (Núñez-Corcuera et al. 2008; Parikh et al. 2010b). A temperature-sensitive mutant *padA*^{ts} exhibited slow vegetative growth, delayed development, hypersensitivity to ammonia, and compromised spore viability, even at the permissive temperature. When *padA*^{ts} develops at the restrictive temperature, it exhibits severe phenotypes: no growth in axenic cultures and permanent developmental arrest around culmination without making any stalk cells or spores. The mutant failed to express *ecmA* in the pstA region and *ecmB* in pstAB cells, at least partly explaining the developmental arrest. PadA might also regulate the slug-to-culminant switch via the repression of two candidate targets, ammonium transporter genes *amtA* and *amtC* (Núñez-Corcuera et al. 2008).

Whereas slugger mutants fail to culminate, often due to misregulated or abolished stalk differentiation, the late developmental phenotype "stalky" describes mutants that form fruiting bodies with greatly reduced or absent sori. StkA, isolated as a REMI mutant that fails to produce sori, is a putative GATA family TF with two distinct zinc-finger domains (Chang et al. 1996). Transcript levels of *stkA* show a monotonic increase over development and are enriched in psp cells (Chang et al. 1996; Parikh et al. 2010b). Consistent with its phenotype, the *stkA*$^{-}$ mutant showed a delay in the expression of psp genes *pspA* and *cotC*, total abrogation of spore differentiation marker *spiA*, and late, persistent overexpression of pst genes *ecmA* and *ecmB*. These results suggest that StkA plays roles as a negative regulator of stalk-related pathways and an activator of spore formation. The stalky phenotype was not reversible by activation of either the Gsk3 or PKA pathway. Further, four activated targets of StkA, culmination-specific proteins A and B (*cspA*, *cspB*), RNA binding protein *rnpA,* and SrfA-induced gene *sigB* (see below), were identified by differential mRNA display (Loughran et al. 2000).

Similar to StkA, the MADS-box transcription factor SrfA mediates processes involved in spore maturation (Escalante and Sastre 1998). In fact, SrfA and StkA

have been shown to share some downstream transactivation targets (Escalante et al. 2004, 2003; Loughran et al. 2000). Degenerate PCR against the conserved mammalian MADS-box domain led to *srfA* cloning. The *srfA*$^-$ mutant exhibits delayed culmination and this defect is exacerbated under slug-migratory conditions. Its irregular fruiting body contains severely compromised spores, characterized by an abnormal round shape, reduced phase-birefringence, and dramatically low viability. Loss of viability is due at least in part to incomplete cellulose deposition indicative of partial spore coat formation (Escalante and Sastre 1998). The *srfA* locus includes several alternative promoters that drive spatially and temporally variable expression, with transcripts gradually accumulating throughout development and spiking around 16–20 h (Escalante et al. 2001; Escalante and Sastre 1998; Parikh et al. 2010b). The proximal promoter is responsible for induction in pst cells, while a middle promoter induces general slug-wide expression. Upon culmination, the distal promoter strongly upregulates *srfA* expression in psp cells and maturing spores, likely induced by the cAMP-PKA pathway. Downstream transcriptional defects in the *srfA*$^-$ background include dramatic reductions of *ecmB* expression in pstAB cells and of the late spore gene *spiA* in the sorus. In addition to spore-maturation, SrfA may be important for regulating *ecmB* expression at the slug tip, a region critical for the slug-to-culminant switch (Escalante et al. 2001; Escalante and Sastre 2002, 1998). More than 20 SrfA-induced genes (*sig*) were identified by microarray analysis, expanding on earlier cDNA subtraction results (Escalante et al. 2004, 2003).

BzpF, one of 19 *D. discoideum* bZIP TFs, plays a similarly important role in spore maturation and viability as SrfA does, yet acts in a largely independent pathway (Huang et al. 2011). Expression is detected from 16 h, peaking at 24 h and enriched in spores. Transcriptional network and bioinformatics analyses implicated BzpF as a cAMP response element binding protein (CREB)-like protein (Parikh et al. 2010a). Development of *bzpF*$^-$ resulted in fruiting bodies with irregular, thick stalks, and translucent sori containing compromised spores. Ectopic PKA activation did not rescue the phenotype. Specific binding motifs almost identical to the canonical mammalian element CRE were identified *in vitro*, while bioinformatics analyses using protein-binding DNA microarray and RNA-seq data identified more than 30 putative downstream targets including *sigD*, *sigK,* and *rnpA*, which are also induced by SrfA. Fifteen of these were induced by 8-Br-cAMP in wildtype but not in *bzpF*$^-$, indicating BzpF is necessary for their expression via the PKA pathway. Two of these genes were upregulated during vegetative growth when *bzpF* was overexpressed, indicating that BzpF alone is sufficient to drive their expression (Huang et al. 2011). In sum, BzpF is important for at least two developmental processes, earlier pst differentiation and later spore maturation. It is sufficient for the expression of a subset of its targets, but likely requires interacting partners to activate many others.

Transcription factor activated by calcineurin (TacA) directly integrates cell signaling and physiology with changes in gene expression. Recognizing that cytosolic calcium levels affect stalk cell differentiation, and that Ca^{2+} responsive phosphatase calcineurin (CN) is developmentally regulated, Thewes and

colleagues (2012) sought to identify an effector molecule that relays these signals into a transcriptional response. *TacA* was identified in a whole genome search to identify proteins similar to *Saccharomyces cerevisiae* Crz1, a well-characterized TF that translocates to the nucleus upon dephosphorylation by CN. TacA expression is developmentally regulated and RNAi knockdown mutants show delayed development resulting in smaller fruiting bodies with less ascended sori. TacA translocates to the nucleus in response to ectopic addition of Ca^{2+}. RT-PCR analysis revealed misregulation of pst markers *ecmA* and *ecmB*, as well as misexpression of Ca^{2+}-regulated *cupC* and *patA*. The authors propose that TacA negatively regulates these genes as an effecter of the Ca^{2+}/CN signaling pathway (Thewes et al. 2012).

5 Open Questions and Future Directions

Painstaking work over the last several decades has established the involvement of many transcription factors in *Dictyostelium* development. These have been identified by genetic screens, molecular and biochemical analyses, and increasingly, bioinformatics predictions. As the *Dictyostelium* community adopts and adapts methods for increasing throughput, collecting 'omics scale data, and performing systems level analyses, we will gain a more complete picture of the independent and interconnected gene networks that govern development.

Such changes will make it possible to more rapidly discover novel key genes to assess and assign their functions. In the last decade, the community has sequenced the genomes of several dictyostelid species, enabling comparative genomics analyses (Eichinger et al. 2005; Heidel et al. 2011; Sucgang et al. 2011). Further, improvements in methods for mutagenesis and genetic engineering have made it easier to study genetic interactions (Faix et al. 2004; Kuwayama et al. 2002). Ultra-deep sequencing of RNA including the ability to multiplex up to 228 samples (Miranda et al. 2012) is changing the depth and breadth of transcriptomics studies in *Dictyostelium*, and will play an integral role in determining signaling network topologies (reviewed in Loomis and Shaulsky 2011). Publicly available data analysis platforms such as dictyExpress, PIPA (http://pipa.biolab.si), and Orange (http://orange.biolab.si/) make such a goal realistic (Curk et al. 2005; Rot et al. 2009). We believe that *in silico* promoter analysis and *in vivo* reporter assays might aid in the discovery of functional cis-regulatory elements. Protein binding microarrays and ChIP-seq analyses agnostically evaluate the binding preferences of TFs (Weirauch et al. 2013). Yet biology, not technology, must be the driving force behind movement in the field.

In this chapter we have discussed the TFs known to regulate development, but in many cases, we can only speculate as to their precise roles. Identifying direct and indirect targets and identifying new master regulators could help gain a comprehensive understanding of the regulatory events that mark the transition from vegetative growth to aggregation, cell differentiation and development into

fruiting body formation. One approach to identify downstream targets would be to assess the changes in the transcriptional profiles of TF mutants. Orthologs that display developmental co-regulation in *D. discoideum* and *D. purpureum* might be prioritized for transcriptional phenotyping, and many of these mutants already exist from previous efforts. In fact, a number of TFs discussed here including *cbfA, gbfA, dimA, dimB, dstA, dstC, cudA, padA, stkA, mybC, srfA,* and *bzpF* have almost identical expression patterns between *D. discoideum* and *D. purpureum* (Pearson's correlation > 0.5) (Parikh et al. 2010b). Uncovering the molecular details of spatial and temporal organization, pattern formation, and cell fate specification would not only help understand the complexity of *Dictyostelium* development but also provide insights into evolutionarily conserved cellular and developmental processes.

We recognize that transcriptional regulators are only one class of participants in regulating gene expression. The roles of mediators, chromatin remodelers, and histone modifiers during development are missing from our conversation. We hope that through the course of constructing more robust models of gene networks, dependencies on other types of regulators become more apparent. Meanwhile, direct analysis of mutant phenotypes of these other regulatory genes is warranted and welcome. As illustrated in Fig. 2, some of these groups are even more evolutionarily conserved than the regulators, and thus may teach us general principles of eukaryotic development achieved through universal transcriptional regulation.

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Non-coding RNAs in *Dictyostelium discoideum* and Other Dictyostelid Social Amoebae

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Abstract Non-coding (nc)RNAs have recently emerged as ubiquitous and important regulators of a multitude of different processes, such as stress response, cell differentiation, infection, and cell death. The means by which ncRNAs affect these processes are numerous and diverse, ranging from protein localization to regulation of gene expression. ncRNA-mediated gene expression control has been the subject of especially intense study in recent years and has shown to occur through several mechanisms. Different ncRNAs can regulate gene expression transcriptionally by inducing modification of DNA or chromatin, or post-transcriptionally by directing cleavage, degradation, or translational inhibition of messenger (m)RNAs. ncRNAs come in a broad spectrum of sizes, from ~20 nucleotides (nt) to several thousand nt, and function in complexes with various proteins that usually exert a catalytic function while the RNAs act as guides. In Dictyostelia, we have only started to

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understand the extent of ncRNA regulation, mostly from studies in *Dictyostelium discoideum*, which is the focus of this chapter.

1 Introduction

RNA was long considered simply as an intermediate in the transfer of information from genes to proteins, in the case of messenger RNA (mRNA), or as a structural component of RNA–protein complexes that function in mRNA maturation and protein synthesis, e.g., small nuclear RNAs (snRNAs), transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs). When other types of RNA molecules were discovered that did not belong to any of these classes, these molecules were generally considered as by-products, i.e., junk. However, today it is clear that RNA molecules can come in many different forms and act via a multitude of mechanisms (Mattick 2009; Wilusz et al. 2009). There is now a wide array of known RNAs that commonly do not have protein-coding capacity, together referred to as non-coding (nc)RNAs.

ncRNAs are often grouped based on their size, ranging from small RNAs as short as 20 nt to long non-coding (lnc)RNAs which can be several thousand nt long. The majority of ncRNAs function in ribonucleoprotein (RNP) complexes, and each class of ncRNA associates with a specific set of proteins (Hogg and Collins 2008). Within these complexes, the RNAs usually function as guides for the protein complex, interacting with specific target nucleic acids by complementary base pairing, allowing the proteins to exert their specific functions such as chemical modification or cleavage. Thus, the RNA gives specificity and the protein(s) execute the function, meaning that a single set of proteins can regulate a number of different targets by using different ncRNAs as guides. Small nucleolar RNAs (snoRNAs) are examples of such guide RNAs. They bind other RNA molecules, such as rRNAs and snRNAs, enabling the snoRNA associated proteins to catalyze chemical modifications of the target RNAs (Reichow et al. 2007).

Other examples of small ncRNAs are 20–30 nt small interfering (si)RNAs, micro (mi)RNAs, and Piwi-interacting (pi)RNAs. The discovery of these numerous and widespread small RNAs has radically changed our understanding of gene regulation in recent years. These RNAs can guide protein complexes to fully or partially complementary mRNA targets and thereby play critically important roles in gene expression and genome defense in eukaryotes (Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009; Voinnet 2009). The regulation of gene expression mediated by small RNAs can occur on many different levels, from modification of DNA and histones to RNA stability and translation. It has been suggested that at least 60 % of all human protein-coding genes are conserved miRNA targets, and aberrant expression of specific miRNAs can lead to severe diseases such as cancer (Esteller 2011; Friedman et al. 2009). piRNAs have so far only been found in animals and will not be discussed further here.

Although much recent research effort has been focused on short ncRNAs, lncRNAs are attracting increasing interest. The functions of the majority of these long RNAs, often many kb in length, so far have not been characterized. However, recent studies have revealed important regulatory roles for some (Mercer et al. 2009; Clark and Mattick 2011). These roles include regulation of transcription by chromatin remodeling or inhibition of recruitment of RNA polymerase II (pol II), sense–antisense hybridization of transcripts leading to RNA degradation or blocking of splice sites, and induction of altered activity or localization of proteins. Possibly the most famous, and best studied, lncRNA is the mammalian Xist (and its antisense transcript Tsix), which is involved in X chromosome inactivation (Lee 2010).

Many ncRNAs belong to classes that share similar structure and/or sequence elements while others seem to be unique. Furthermore, certain ncRNAs are well conserved throughout all kingdoms of life while others have only been found in one phylum or a single isolated species (Mattick 2009). An example of the former is the 7SL RNA, which is part of the signal recognition particle (SRP), involved in protein translocation, and is found in species as diverse as *Escherichia coli* and human (Pool 2005 and see below).

The ncRNA population of Dictyostelia has long been an unexplored area of research, despite the fact that the first regulatory antisense RNA in eukaryotes was discovered in *D. discoideum* (Hildebrandt and Nellen 1992). However, computational mining of genomes combined with experimental approaches, mainly focusing on *D. discoideum,* during the last decade have led to the discovery of many ncRNAs. These include ncRNAs expected to be present in any eukaryotic cell but also surprises such as an entirely new class of ncRNA. In this chapter we focus largely on newly discovered ncRNAs relevant to *D. discoideum* and other dictyostelids rather than on well known ncRNAs such as rRNAs or tRNAs. We will also discuss regulatory RNAs in the context of what is known about these RNAs from research on other organisms. The ncRNAs present in different dictyostelids and discussed in this chapter are presented in Table 1.

2 lncRNAs as Developmental Regulators

lncRNAs are usually several kb long and can be derived from most types of genomic regions. For example, they can originate from intergenic regions, from introns, or they can occur as antisense transcripts of other RNAs such as mRNAs (Clark and Mattick 2011; Mercer et al. 2009).

The first example of antisense RNA-mediated regulation in eukaryotic cells was reported 20 years ago by Nellen's group (Hildebrandt and Nellen 1992). They showed that the *D. discoideum* prespore gene *PsvA/EB4* was regulated by a 1.8 kb complementary antisense transcript. Although transcription of the mRNA was basically the same in growing and developing cells, the mRNA only accumulated in the latter. This was explained by the observation that, in contrast to the mRNA,

Table 1 Non-coding RNAs (nc)RNAs identified in *Dictyostelium discoideum* and in other Dictyostelia

ncRNA or class of ncRNA	DUSE	Length (nt)	Function: assigned or likely	References
Specific to *D. discoideum* (Dictyostelia)?				
Antisense RNA	n.d.	1,800	Post-transcriptional gene regulation/late development	Hildebrandt and Nellen (1992)
dutA RNA	–	1,322	Regulates transcription factor	Yoshida et al. (1994); Shimada and Kawata (2007)
D1/D8	+	188	Unknown	Kaneda et al. (1983)
Class I RNAs[a]	+	45–90	Role in early development	Aspegren et al. (2004); Avesson et al. (2011); Sucgang et al. (2011)
Present in other organisms				
miRNA	–	21	Post-transcriptional gene regulation	Hinas et al. (2007); Avesson et al. (2012)
siRNA	–	21	Transcriptional and post-transcriptional gene regulation	Hinas et al. (2007); Kuhlmann et al. (2005)
D2/D9 (U3) snoRNA	+	210[b]	rRNA processing	Takeishi and Kaneda (1981); Wise and Weiner (1981); http://rfam.sanger.ac.uk/
Box C/D snoRNA	–	66–113	(r)RNA methylation	Aspegren et al. (2004)
Box H/ACA snoRNA	+	147	(r)RNA pseudouridylation	Aspegren et al. (2004)
snRNAs[a]	+	110–268	Splicing	Aspegren et al. (2004); Hinas et al. (2006); Sucgang et al. (2011)
RNase MRP RNA	+	307/367[b]	rRNA processing	Piccinelli et al. (2005)
RNase P RNA	+	390 and 370	tRNA processing	Piccinelli et al. (2005); Marquez et al. (2005); Stamatopoulou et al. (2010)
7SL RNA	+	280	Protein localization	Aspegren et al. (2004)
msRNA (5S rRNA?)[a]	–	129	Translation/mitochondria	Pi et al. (1998); Bullerwell et al. (2010)

DUSE: *Dictyostelium* upstream sequence element

[a] Also identified in other dictyostelids

[b] Computationally predicted size. n.d, not determined

the antisense RNA was under transcriptional control and was expressed only in growing cells. Thus, it appeared that the antisense RNA was post-transcriptionally down regulating the mRNA. There are also a few other reports in *D. discoideum* indicating similar *cis*-antisense mediated regulation, or at least cases where antisense transcripts have been identified, but very little is known about their putative regulatory functions (Okafuji et al. 1997; Maruo et al. 2004; Hinas et al. 2007).

Another lncRNA present in *D. discoideum* is the dutA RNA (development-specific but untranslatable RNA) (Yoshida et al. 1994). The expression of this 1322 nt ncRNA is induced early in development, during aggregation, and shows distinct expression patterns in the two pre-stalk cell types, PstA and PstO (Maeda et al. 2003; Yoshida et al. 1994; Kumimoto et al. 1995). Although the dutA RNA is mainly localized in the cytoplasm and contains a short ORF that possibly could give rise to a 42 amino acid protein, the RNA was shown not to be associated with ribosomes and hence denoted as an untranslatable RNA (Yoshida et al. 1994). This is further supported by a recent transcriptome analysis utilizing serial analysis of gene expression (SAGE) in high-density cultures of *D. discoideum* cells. In this study, aimed at examining changes in gene expression during the transition from growing cells to multicellular development, *dutA* turned out to be the most highly expressed gene (Whitney et al. 2010).

What is the function of the dutA RNA? Disruption of *dutA* did not yield any clues to its function since no obvious phenotype was observed (Yoshida et al. 1994). However, in a screen for suppressors of a partially active mutant of Dd-STATa, a functional homolog of metazoan STAT transcription factors, cDNAs expressing dutA RNA fragments could rescue the culmination defect of the mutant Dd-STATa (Shimada and Kawata 2007). It was further reported that the dutA RNA is localized to the same cell types, PstA cells, where Dd-STATa is activated and that expression of several Dd-STATa target genes is restored upon expression of dutA fragments in the Dd-STATa mutant background. In addition, overexpressed dutA fragments increased the tyrosine phosphorylation of the partially active Dd-STATa. Although the mechanism is still unknown, the authors suggest that dutA RNA acts upstream of Dd-STATa to control its tyrosine phosphorylation. Another interesting observation is that dutA RNA is down-regulated during *Legionella pneumophila* infection (Farbrother et al. 2006). One intriguing possibility is that *L. pneumophila* down regulates dutA RNA to affect the transcription of specific genes that affect its ability to establish infection. Infection of the *dutA* knockout strain is one way to test this hypothesis, which at this point is pure speculation.

3 Medium-Sized ncRNAs

In this chapter we define ncRNAs between 40 and 500 nt as medium-sized ncRNAs. Many RNAs of this size have been identified by computational and/or experimental methods and will be described below. These RNAs include classes of

ncRNA also present in other eukaryotes while others seem to be unique to Dictyostelia and yet others even *D. discoideum*-specific (Table 1).

3.1 Class I RNAs are Involved in Development and Conserved Throughout Dictyostelia

The most abundant new RNAs identified by shot-gun cloning represent a group of RNAs with no obvious homology to any other class of ncRNA (Aspegren et al. 2004). These RNAs, initially termed Class I and Class II RNAs but recently redefined as just one class, Class I, are ~40–65 nt long and share specific structure and sequence motifs (Aspegren et al. 2004; Avesson et al. 2011). Altogether, 37 Class I RNA genes (and some putative pseudogenes) have been computationally identified in *D. discoideum* and at least 29 of these seem to be expressed (Aspegren et al. 2004; Avesson et al. 2011). The genes are found on all six chromosomes but heavily enriched on chromosome 4. These RNAs are predicted to form a short stem structure where the 5′ and 3′ ends base-pair. Immediately following the 5′ part of the stem is a conserved 11 nt sequence motif while the rest of the RNA varies in sequence (Fig. 1). The overall structure of these RNAs was confirmed by in vitro probing of one member of the class (Avesson et al. 2011). Class I RNAs are down-regulated during development and are preferentially located in the cytoplasm (Aspegren et al. 2004).

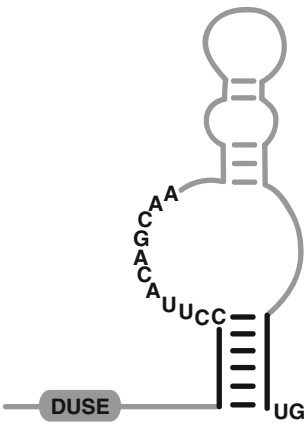

Fig. 1 Schematic drawing of Class I RNAs in Dictyostelia. The 11 nucleotide conserved sequence motif and the two highly conserved nucleotides in the 3′ end is shown. Base pairs confirmed by experimental data and presence of compensatory base substitutions are depicted in *black*. The *gray* parts represent the region which varies both in primary sequence, secondary structure, and length between different Class I RNAs. The upstream element DUSE which is present in *front* of all Class I RNA genes is shown

To get a handle on their function, two Class I genes were deleted by homologous recombination (Avesson et al. 2011). One of these knockout strains showed aberrant development, forming more and smaller fruiting bodies, indicating a role in early development. Furthermore, proteins interacting specifically with one of the Class I RNAs were identified. One of these proteins contains two RNA recognition motifs (RRM) but otherwise this protein has no function assigned to it. In other organisms, proteins containing RRM motifs are common and bind both single and double-stranded RNA but can also bind DNA and protein (Clery et al. 2008).

Class I RNAs in *D. discoideum* are not substantially associated with ribosomes or polysomes instead they seem to form smaller RNA–protein complexes (Avesson et al. 2011). Computational and experimental analyses reveal that expressed Class I RNA genes are present in species belonging to all four groups of Dictyostelia, but no obvious homologs could be identified in non-dictyostelids (Sucgang et al. 2011; Avesson et al., manuscript in preparation).

3.2 Spliceosomal RNAs with Unexpected Features

Splicing to remove intron sequences is an important step in the maturation of mRNAs in eukaryotes. This multi-step process is performed by the spliceosome, a large complex of proteins and RNAs. The ncRNAs involved, named spliceosomal or small nuclear (sn)RNAs, are essential for this process (Wahl et al. 2009). Genes for all the snRNAs involved, i.e., U1, U2, U4, U5, and U6, as well many of the associated proteins have been computationally predicted in *D. discoideum* (Hinas et al. 2006; Yu et al. 2011). Recently, a computational search also identified snRNA genes in another group 4 dictyostelid, *D. purpureum* (Sucgang et al. 2011). In *D. discoideum*, 18 snRNA genes were predicted, of which 17 were shown to be expressed. Only U6 RNA is expressed from a single gene whereas the other snRNAs originate from multiple genes (Hinas et al. 2006). The snRNA genes in *D. discoideum* are located on four different chromosomes and mostly cluster in pairs of homologous genes, transcribed divergently, convergently, or in tandem. The identified snRNAs are predicted to fold into conserved functional structures and the sequence motifs important for splicing are present. Also the presence of a canonical trimethylated 5′-cap structure was verified for all the snRNAs except for U6, which is not expected to carry this structure.

Although the snRNAs of *D. discoideum* have the required features to act as functional ncRNAs in the spliceosome, there are also some unusual features worth mentioning. For example, among the seven U2 RNAs found, four carry a 5′ extension predicted to form a stem-loop structure (Hinas et al. 2006). In contrast to the other snRNAs (which, as expected, are located in the nucleus), these extended U2 variants are enriched in the cytoplasm and down-regulated during development. This could indicate that this subset of U2 RNAs have evolved other functions. Another interesting finding is that a fraction of the transcripts for each spliceosomal RNA is polyadenylated and that, at least for U1 RNA, the

polyadenylated version is present in the cytoplasm. In other organisms polyadenylation of ncRNAs has been suggested to act as a surveillance mechanism whereby the A-tail marks aberrant ncRNAs for degradation (Win et al. 2006; Watanabe et al. 2002; Anderson 2005). The snRNA components of the minor spliceosome that catalyzes the excision of an atypical class of introns, is present in, e.g., plants and some metazoans but have not been identified in *D. discoideum* (Hinas and Soderbom 2007; Yu et al. 2011; Rodriguez-Trelles et al. 2006).

3.3 snoRNAs: Modifiers of RNA

snoRNAs are ncRNAs involved in modification of other RNAs in Eukarya and Archaea (Reichow et al. 2007). These ncRNAs are ∼60–300 nt long and bind to specific sets of proteins, forming small nucleolar ribonucleoprotein particles (snoRNPs). The snoRNAs are divided into two main families, box C/D snoRNAs, and box H/ACA snoRNAs, which use sequence complementarity to guide 2′-O-methylation and pseudouridylation, respectively, of their specific RNA targets. Commonly the target is an rRNA but snoRNPs can also modify other RNAs such as snRNAs and, in archaea, tRNAs. Among the subset of snoRNAs involved in pre-rRNA processing, some have been demonstrated to act as miRNA precursors (see below). snoRNAs have also been implicated in other processes such as alternative splicing and editing (Kishore et al. 2010; Kishore and Stamm 2006; Vitali et al. 2005). Still other snoRNAs are orphans, i.e., no complementary sequences have been found that could indicate their targets and/or functions.

In *D. discoideum,* the U3 RNA snoRNA (originally named D2/D9), shown to play a role in pre-rRNA processing in other organisms, was discovered some 30 years ago (Takeishi and Kaneda 1981; Wise and Weiner 1981). We isolated an additional 17 box C/D and one box H/ACA snoRNAs using a shot-gun cloning approach (Aspegren et al. 2004). The *D. discoideum* snoRNAs contain conserved structures and sequences important for their function. However, their box C/D snoRNAs appear to lack the inverted repeats, which are common in other organisms and bring the 5′ and 3′ ends together to form a typical stem. Absence of this stem structure has also been reported for box C/D snoRNAs in organisms such as *Asparagillus fumigatus*, *Euglena gracilis*, *Trypanosoma brucei*, and *Tetrahymena thermophila* (Jochl et al. 2008; Russell et al. 2006; Darzacq and Kiss 2000; Liang et al. 2005). In addition, rRNA target sites for most of the snoRNAs in *D. discoideum* have been predicted and methylation of one target nucleotide has also been verified experimentally, indicating that these snoRNAs conform to their canonical functions (Aspegren et al. 2004). However, several snoRNAs are orphans, i.e., no targets could be predicted. In addition, several of the snoRNAs are developmentally regulated, possibly reflecting specific functions during growth or development.

The *D. discoideum* snoRNA genes are organized as separate genes or in clusters where they are transcribed as polycistronic transcripts, resembling the situation of most snoRNAs in, for example plants and yeast (Dieci et al. 2009; Aspegren et al.

2004). In addition to the experimentally identified snoRNAs, several *D. discoideum* snoRNAs have also been predicted computationally. The *D. discoideum* snoRNAs, verified as well as predicted, can be found, together with predicted associated proteins, in the snoRNP database: http://evolveathome.com/snoRNA/snoRNA.php (Ellis et al. 2010).

3.4 Non-coding RNAs with Functions in Protein Delivery and RNA Processing

Medium-sized ncRNAs can also play important roles in processing of specific RNAs into mature functional molecules by endonucleolytic cleavage. Two of these ncRNAs, RNase P RNA and the related RNase MRP RNA, have been identified in *D. discoideum* and shown to be expressed during growth and development (Table 1) (Marquez et al. 2005; Piccinelli et al. 2005; Stamatopoulou et al. 2010; Avesson and Söderbom, unpublished). Both RNAs associate with specific proteins, many of which are shared between the two RNAs, to form RNA–protein complexes. RNase MRP is an essential universal eukaryotic ribonuclease and involved in pre-rRNA processing. In *Saccharomyces cerevisiae*, RNase MRP has been shown to specifically cleave the internal transcribed spacer 1 (ITS1) in pre-rRNA leading to maturation of the 5.8S rRNA (Esakova and Krasilnikov 2010 and references within). An analogous cleavage intermediate has also been demonstrated in *D. discoideum*, indicating a similar function of RNase MRP in this organism (Boesler et al. 2011).

More is known about the RNase P in *D. discoideum*. RNase P is an endoribonuclease found in all domains of life whose function is mostly attributed to cleavage/maturation of tRNAs (Kalavrizioti et al. 2007; Stamatopoulou et al. 2010; Stathopoulos et al. 1995; Vourekas et al. 2007). The gene for *D. discoideum* RNase P RNA is probably transcribed as a ~390 nt precursor which is subsequently processed through an unknown mechanism to its mature form of ~370 nt (Marquez et al. 2005; Piccinelli et al. 2005; Stamatopoulou et al. 2010). Sequence analysis and structural probing showed that the RNA contains all the conserved features expected of a eukaryotic RNase P RNA (Stamatopoulou et al. 2010). Furthermore, homologs to the majority of the proteins forming functional RNase P in other organisms have been demonstrated to be part of this RNP in *D. discoideum* (Kalavrizioti et al. 2007; Stamatopoulou et al. 2010; Stathopoulos et al. 1995; Vourekas et al. 2007). At least in vitro, RNase P RNAs have also been shown to be catalytically active in the absence of protein in some prokaryotic and eukaryote species, including human (Guerrier-Takada et al. 1983; Kikovska et al. 2007; Pannucci et al. 1999; Esakova and Krasilnikov 2010). Whether this is also true for *D. discoideum* RNase P RNA is still an open question.

The SRP RNA, 7SL RNA, has also been isolated from *D. discoideum* (Aspegren et al. 2004). The SRP RNA–protein complex is present in all kingdoms of life and functions in co-translational delivery of proteins to cell membranes. In eukaryotes, this complex functions in delivering ribosomes translating proteins with an amino terminal signal peptide to the endoplasmic reticulum (ER). Most eukaryote SRP RNAs can be divided into two functional domains, the Alu- and the S-domain. The S-domain interacts with the signal peptide of the protein as it emerges from the ribosome, while the Alu-domain is involved in delay of translation. This allows the SRP to guide the ribosome to the ER membrane where translation can resume, allowing the protein to be processed for transport to its correct cellular location (Pool 2005). The *D. discoideum* ~280 nt long 7SL RNA contains both conserved sequence motifs, and SRP protein homologs have also been identified (Andersen et al. 2006; Aspegren et al. 2004; Rosenblad et al. 2004 and http://dictybase.org/). In addition, there is a second copy of 7SL RNA in *D. discoideum* with 88 % sequence identity but its expression has so far not been analyzed.

3.5 Medium-Sized ncRNAs with Unknown Functions:Specific to D. discoideum?

In a computational *de novo* search for ncRNA genes in *D. discoideum*, a large number of ncRNA genes were predicted. Many of these are similar in sequence and structure, indicating amplification of ncRNA genes to generate classes with similar functions. The search algorithm was verified by searching for known *D. discoideum* ncRNAs of which 94 % were recovered. Furthermore, 6 out of 10 predicted ncRNAs were shown to be expressed, at least in growing cells. Notably, the ncRNAs which are preceded by the putative promoter element (see below) were all expressed in this analysis) (Larsson et al. 2008). These results further demonstrate the wealth of ncRNAs in *D. discoideum*, many of which are still awaiting functional assignment.

A few other studies have revealed *D. discoideum* ncRNAs with unknown functions. The nuclear 188 nt RNA D1/D8 was identified and sequenced some 30 years ago and a 129 nt long mitochondrial small (ms)RNA with 5S rRNA-like features has also been identified (Table 1). However, the latter was reported not to be associated with the mitochondrial ribosomes (mitoribosomes) (Pi et al. 1998; Kaneda et al. 1983). Although the function of the *D. discoideum* msRNA remains elusive, in a recent report, the msRNA and related sequences from other members of Amoebozoa, including Dicyostelia, were suggested to be divergent 5S rRNA homologs and components of the mitoribosome after all (Bullerwell et al. 2010).

4 Small RNAs: Important Regulators of Gene Expression

The discovery of small RNAs, only 20–30 nt long, has truly revolutionized our view on how gene expression is controlled in most eukaryotes. Silencing of genes by small RNAs, RNA interference (RNAi), has also become an important experimental tool to down regulate specific genes in order to study their function. Besides the Piwi-interacting (pi)RNAs, which have only been found in animals, there are two main classes of small RNAs involved in RNA silencing—small interfering (si)RNAs and micro (mi)RNAs (Ghildiyal and Zamore 2009). siRNAs are derived from long double-stranded (ds)RNA precursors which can originate from either exogenous or endogenous sources such as viruses and transposable elements. These RNAs act as a defense system protecting the cell from virus infection and helping to maintain genome integrity. In contrast to siRNAs, miRNAs are generated from endogenous hairpin precursors, derived from intergenic regions or introns. However, other ncRNAs, e.g., snoRNAs, can also function as precursors for miRNAs (Ender et al. 2008; Saraiya and Wang 2008). In humans, 60 % of all mRNAs have been suggested to contain conserved miRNA targets and miRNAs are therefore thought to regulate basically every cellular process (Friedman et al. 2009).

Besides the small RNAs, there are three key players in the RNAi machinery present in *D. discoideum*, namely Dicer-like proteins, Argonautes, and RNA-dependent RNA polymerases (RdRPs). Dicer proteins generate small RNAs by processing double-stranded precursors into ~21 bp dsRNAs (MacRae and Doudna 2007). One of the strands of the dsRNA is then incorporated into the RNA-induced silencing complex (RISC), which has an Argonaute protein at its core. Argonaute proteins belong to a large family of proteins and the number of members varies widely among organisms. These proteins bind to the small RNA and can exert different functions at the target RNA, such as cleavage, mRNA destabilization, or inhibition of translation via different mechanisms (Czech and Hannon 2011). Hence, the function of a small RNA depends on which Argonaute it interacts with. RNA-dependent RNA polymerases (RdRPs) are the third major class of proteins involved in small RNA silencing. RdRPs can amplify silencing signals by generating secondary siRNAs. The amplifying effect can be achieved by using single stranded RNA as templates, either by making a longer dsRNA that can be processed into small RNAs by Dicers or, as demonstrated in *Caenorhabditis elegans,* by direct *de novo* synthesis of small complementary RNAs. RdRPs are present in most eukaryotes with functional RNAi systems, although canonical RdRPs have not been found in, e.g., mammals and insects (Maida and Masutomi 2011).

RNAi can be used to knock down genes in *D. discoideum* and the organism has all the functional components required for silencing of endogenous genes by small RNAs (Martens et al. 2002; Cerutti and Casas-Mollano 2006). Thus, the *D. discoideum* genome encodes two Dicer proteins (DrnA and DrnB), five Argonautes (AgnA–E), and three RdRPs (RrpA–C) (Cerutti and Casas-Mollano 2006; Martens

et al. 2002). In addition, the putative RNA helicase HelF is involved in suppression of RNAi in *D. discoideum* (Popova et al. 2006).

4.1 Abundant Small Interfering RNAs Derived from the Transposon DIRS-1

In *D. discoideum*, the great majority of siRNAs (~75%) are derived from DIRS-1, a retrotransposon that also constitutes centromeres (Table 1) (Glockner and Heidel 2009; Hinas et al. 2007; Kuhlmann et al. 2005; Dubin et al. 2010; Avesson et al. 2012). The large number of small RNAs, which are derived from both strands of this element indicates that DIRS-1 is under stringent control by the RNAi machinery and may also infer an amplification mechanism, perhaps involving one or more of the three RdRPs. Furthermore, at least one of the small RNAs, antisense to the DIRS-1 mRNA, is up-regulated during development (Hinas et al. 2007). How DIRS-1 may be regulated by siRNAs is still unknown. Other siRNAs present in *D. discoideum* are derived from, e.g., additional transposons/repetitive elements or the complementary strand (asRNA) of mRNAs (Hinas et al. 2007, Avesson et al. 2012).

4.2 MicroRNAs in D. discoideum

One of the key questions concerning small RNAs in *D. discoideum* has been whether miRNAs are present or not. Large numbers of miRNAs have been found in plants and animals and more recently in a few unicellular organisms (Kozomara and Griffiths-Jones 2011; Molnar et al. 2007; Zhao et al. 2007; Braun et al. 2010; Saraiya and Wang 2008). These RNAs seem to regulate most biological processes, mainly by post-transcriptional gene regulation involving translational repression and/or deadenylation and decay (animals) or by inducing specific cleavage of their target mRNA leading to degrardation of the target RNA (plants) (Ghildiyal and Zamore 2009; Krol et al. 2010). The number of miRNAs (and siRNAs) discovered in different organisms has increased dramatically during the past few years due to the advent of high-throughput sequencing methods.

In 2007, we reported a few miRNA candidates identified in a cDNA library of small RNAs from *D. discoideum* (Hinas et al. 2007). More recently, using a high-throughput sequencing approach, we identified 17 *bona fide* miRNAs in *D. discoideum* (Table 1) (Avesson et al. 2012). In order to find the miRNAs, stringent computational search criteria (Kozomara and Griffiths-Jones 2011) were used to screen millions of sequenced small RNAs and, based on these data, we expect the number of miRNAs to increase in the future. The currently identified miRNAs in *D. discoideum* are mostly derived from intergenic regions but also repetitive elements and, in one case, an intron seems to be the source of an miRNA.

Biogenesis of miRNAs in *D. discoideum* is strictly dependent on DrnB, one of the two Dicer-like proteins in *D. discoideum*. Furthermore, miRNAs are up-regulated in strains where the gene encoding one of the three RdRPs, RrpC, has been disrupted. As discussed above, RdRPs normally amplify the siRNA signal but for miRNAs in *D. discoideum* the effect seems to be the reverse. It will be of great interest to understand the mechanism behind this unusual phenomenon.

What are the functions of miRNAs in *D. discoideum*? Although disruption of the gene for DrnB eliminates the production of miRNAs, the cells grow and develop normally. This indicates that miRNAs do not have any major functions during these processes but may fine tune gene expression or have a role during conditions not yet tested. Identification of target (m)RNAs for the miRNAs will be important to reveal their function. Computational prediction of targets has been successful in, e.g., plants, where the miRNA binds to its target RNA with full complementarity (Rhoades et al. 2002). We have predicted a few such binding sites in *D. discoideum*, with almost full complementarity to mRNAs, but have so far been unable to validate them experimentally (Reimegård et al., unpublished). Instead, *D. discoideum* miRNAs may interact with their targets in a fashion similar to that found in animals, where short target sites, often consisting of ~6–7 contiguous nucleotides, are present mostly in the 3′ untranslated region (3′ UTRs) of mRNAs (Lewis et al. 2003). However, in this case, target predictions become much more difficult and as expected, each miRNA in *D. discoideum* could potentially target numerous mRNAs (Reimegård et al., unpublished). Therefore, we are presently using a high-throughput sequencing approach in combination with bioinformatics to reveal the true targets for the *D. discoideum* miRNAs.

5 Conserved ncRNA Gene Promoter Element?

The great majority of ncRNA genes in *D. discoideum* are preceded by a short upstream sequence motif that most likely functions as a promoter element (Fig. 1). This eight nucleotide long sequence, named DUSE for *Dictyostelium* upstream sequence element, has a highly conserved sequence: [A/T]CCCA[C/T]AA (Aspegren et al. 2004; Hinas et al. 2006). The motif is located ~64 nt upstream of transcription start sites for the *D. discoideum* ncRNA genes encoding 7SL, RNase P, RNase MRP, and U6 RNAs, as well as of snRNA genes encoding U1, U2, U4, and U5, which are transcribed by RNA polymerase III and II, respectively, in other organisms (Aspegren et al. 2004; Hinas et al. 2006; Hinas and Soderbom 2007; Stamatopoulou et al. 2010; Dieci et al. 2007; Fischer et al. 2011). In addition, the DUSE motif is found in front of all the Class I RNA, D1/D8, U3 (D2/D3), and box H/ACA RNA genes and also many of the ncRNA genes predicted by bioinformatics (Aspegren et al. 2004; Avesson et al. 2011; Larsson et al. 2008; Söderbom, unpublished). Although many ncRNA genes in other eukaryotes are preceded by similar sequence elements, these elements tend to vary in distance from the start site of transcription depending on which class of genes they regulate. Taken

together, this strongly suggests that additional, hitherto unidentified, sequence elements are associated with ncRNA genes in *D. discoideum* which can direct the different RNA polymerases to the correct class of genes.

Is DUSE, including its conserved distance to the start of transcription, unique to *D. discoideum* ncRNA genes or also present in other species of Dictyostelia? In a recent analysis of another group 4 species, *D. purpureum*, DUSE was identified in front of all the snRNA and Class I RNA genes (Sucgang et al. 2011). Furthermore, we have identified this element upstream of all Class I RNA genes in species from the remaining three major groups of Dictyostelia. Hence, this putative promoter motif has been highly conserved throughout the evolution of Dictyostelia (Avesson et al., manuscript in preparation).

6 Conclusions and Future Perspectives

For a long time, it was thought that the majority of eukaryotic genomic DNA was not transcribed. In cases where transcripts were detected that were not derived from protein-coding genes or ncRNA involved in proteins synthesis, e.g., tRNA and rRNA, these transcripts were believed to be junk RNA without any function. However, it is now clear that only a minor fraction of the human genome encodes mRNA while an estimated 90 % is transcribed into RNA (Birney et al. 2007; International Human Genome Sequencing Consortium, 2004). Recent developments in high-throughput methodologies have further advanced our understanding of the transcriptional landscape, including ncRNAs, in a wide variety of organisms. However, most of the ncRNAs have not been functionally defined and the question as to whether these transcripts play biological roles or are merely by-products still remains mostly unanswered. Nevertheless, evidence is rapidly building that many of these RNAs play important roles in most, if not all, biological processes, acting as regulators at many different levels of gene expression (Mattick 2009 and references therein).

The role of ncRNAs in Dictyostelia is still largely unexplored although recent computational and experimental surveys of *D. discoideum* RNAs have revealed both expected and unexpected findings. As anticipated, conserved ncRNAs involved in important biological processes are present, such as 7SL RNA, RNase P RNA, MRP RNAs, snoRNAs, and snRNAs (Hinas and Soderbom 2007; Piccinelli et al. 2005; Stamatopoulou et al. 2010; Aspegren et al. 2004; Hinas et al. 2006; Marquez et al. 2005). Although it is reasonable to believe that these RNAs fulfill the same roles in *D. discoideum* as in other organisms, their functions have, at best, only been briefly studied (Aspegren et al. 2004; Stamatopoulou et al. 2010). One curious feature of the *D. discoideum* U2 snRNA is the presence of 5′ extended U2 variants in the cytoplasm (Hinas et al. 2006). It will be interesting to investigate whether these developmentally regulated U2 RNAs have adopted an alternative function in place of or in addition to their traditional role in splicing.

Small interfering RNAs have also been identified in *D. discoideum* with a surprisingly large proportion being derived from the retrotransposon DIRS-1, which seems to double as centromeres in *D. discoideum* (Dubin et al. 2010; Glockner and Heidel 2009; Hinas et al. 2007; Kuhlmann et al. 2005). Hence, regulation of this element appears to be a major undertaking for the RNAi machinery in this social amoeba. Notably, *Polysphondylium pallidum*, a member of the distantly related dictyostelid major group 2, does not have DIRS-1 sequences (Heidel et al. 2011). It will be interesting to see if *P. pallidum* has a functional RNAi machinery and in that case, whether it is involved in regulation of any other major elements and/or pathways.

The other class of small RNAs present in *D. discoideum* is miRNAs (Hinas et al. 2007; Avesson et al. 2012). This class of RNA has mostly been studied in multicellular organisms, i.e., plants and animals. However, they have also recently been identified in some unicellular organisms, although their function in these taxa has been only scarcely investigated (Kozomara and Griffiths-Jones 2011; Molnar et al. 2007; Zhao et al. 2007; Braun et al. 2010; Saraiya and Wang 2008). The function of miRNAs in *D. discoideum* is still unknown but the developmental regulation of miRNAs, some present only in growing cells while others are exclusive for cells undergoing development, indicates a role during both life stages (Avesson et al. 2012). Recently completed and ongoing genome projects for other Dictyostelia will aid in our understanding of the conservation of the RNAi machinery within this apparently ancient group of organisms (Heidel et al. 2011). This, in combination with high-throughput sequencing of transcriptomes, will give us insights into the evolution of small RNA-based silencing mechanisms. This will allow us to address questions such as (i) have any of the components been lost during evolution and, (ii) can we distinguish any species-specific features, and if so, do these rely on species-specific RNAi components?

Certain ncRNAs seem to be specific to Dictyostelia, such as Class I RNAs (Fig. 1), or even to *D. discoideum*, e.g. dutA RNA (Aspegren et al. 2004; Avesson et al. 2011; Sucgang et al. 2011; Yoshida et al. 1994). The latter may be involved in regulation of the STATa transcription factor via upstream elements and could thereby influence expression of several different genes and/or processes (Shimada and Kawata 2007). However, these effects would most likely be subtle and involved in processes not yet investigated, since depletion of the RNA does not result in any obvious phenotype. On the other hand, deletion of one of the Class I RNA genes caused a subtle but distinct phenotype, i.e., more and smaller fruiting bodies (Avesson et al. 2011). This indicates a function in early development, maybe in the processes determining how many cells that aggregate to form multicellular structures. Interestingly, Class I RNA is conserved throughout the evolution of Dictyostelia but we have failed to find this class of RNAs outside this group of organisms (Sucgang et al. 2011; Avesson et al., manuscript in preparation). Hence, the RNAs may play a general role in cell counting/aggregation in social amoebae.

D. discoideum has proven to be a useful and pertinent model to study biogenesis and function of known ncRNAs as well as a model to discover new and sometimes abundant regulatory RNAs. The intricate life cycle of social amoebae, including

features of unicellular as well as multicellular organisms, allows for a broad spectrum of studies with the added advantage of the large molecular tool box available for this well-studied model organism. The sequencing of other dictyostelid social amoebae and the development of molecular methods to study their genes will open entirely new avenues for functional studies of ncRNA specific for this group of organisms but also for ncRNA research in general.

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Sex in Dictyostelia

Gareth Bloomfield

Abstract Dictyostelid social amoebae possess both sexual and parasexual cycles. In the former, diploid zygotes attract surrounding haploids and then cannibalise them, forming large immobile structures known as macrocysts. In the parasexual cycle, amoebae of the same sex fuse to form diploids that can continue to grow and multiply. Species with more than two sexes (or mating types) are not unusual among dictyostelia, and recently the genetic basis for sex determination was described in the model species *Dictyostelium discoideum* . Macrocysts have so far only been observed in a minority of the known species, and their ecological context and significance is still not understood. Important questions regarding altruism, genetics and the basic cell biology of both the sexual and parasexual cycles remain to be addressed experimentally, so there remains tremendous scope for future research.

1 Introduction

The social amoebae are well known for their unusual aggregative version of multicellularity: upon starvation, separate amoebae can collect together to form a multicellular, asexual fruiting body. This phenomenon has interested developmental biologists, since near identical and often clonal groups of cells undergo an exquisitely coordinated differentiation programme resulting in two very different major cell types, stalk and spore. It has also fascinated sociobiologists, since unrelated amoebae can join together into the same multicellular organism giving rise to potential conflict if some cells preferentially form spores, which survive, rather than stalk cells, which do not. It is much less well known that these organisms possess a sexual cycle that is just as complex and interesting as its

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asexual counterpart. This chapter will describe the biology of sex in the dictyostelid social amoebae, drawing attention to similarities between the sexual and asexual cycle, as well as such peculiarities as parasexuality, cannibalism and the presence of more than two sexes within species. The key events of both the sexual and parasexual cycles are shown diagrammatically in Fig. 1. Given such interesting features, and the possibility of harnessing sexual genetics for experimental purposes in the widely used model organism *Dictyostelium discoideum*, the dictyostelid sexual cycle remains surprisingly under investigated (Raper 1984). The following sections will introduce its key features, review recent discoveries and attempt to highlight some of the more important questions yet to be resolved or even addressed.

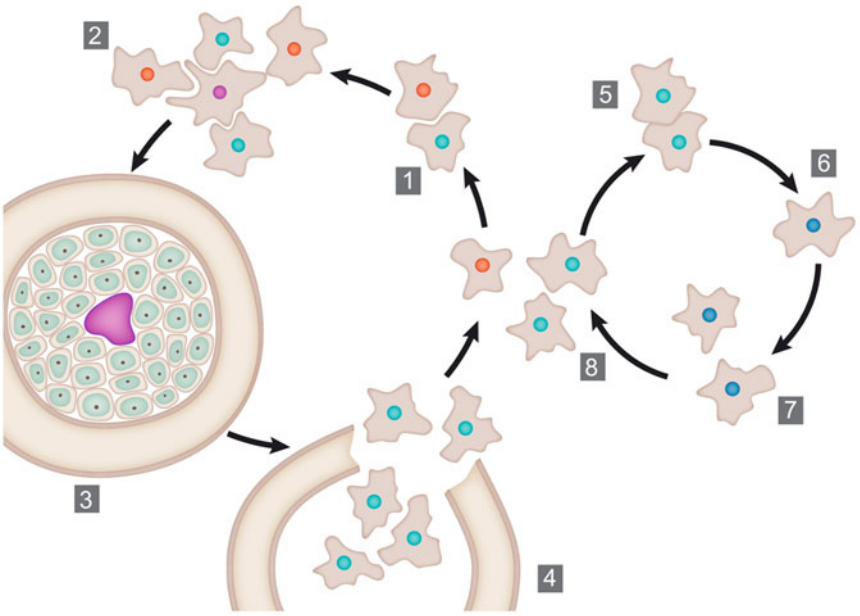

Fig. 1 Overview of the dictyostelid sexual and parasexual cycles. In dark, wet conditions and in the presence of calcium, mixed sex groups of social amoebae can pass through the sexual cycle. Pairs of cells of different sex (or mating-type, indicated here by the colour of the *cartoon* nuclei) can mate (*1*), fusing to form a diploid zygote (*purple* nucleus) that attracts surrounding cells using chemical signals. As the zygote or giant cell is surrounded by other cells, it begins to ingest them (*2*). The group of cells, with a cytophagic giant cell at its centre, secretes cellulose containing walls around itself, forming a macrocyst (*3*) that ultimately consists of a single greatly enlarged zygote, by now containing all of the peripheral cells held with vacuoles in its cytoplasm. The single viable macrocyst nucleus undergoes meiosis followed by several rounds of mitosis before dividing into haploid amoebae. These can then escape the cyst by breaking open its walls (*4*). Cells of the same mating-type can undergo fusion (*5*) to form parasexual diploids (*6*), shown here with *darker* nuclei. These can continue to grow and divide through meiosis (*7*) and can pass through the asexual developmental cycle just like haploids. Parasexual diploids revert to haploids (*8*) through random loss of chromosomes during cell division

2 Historical Background

Dictyostelids normally grow and divide as haploids (Sussman and Sussman 1962). As interest grew in their biology, attempts were made to identify sexual cell fusion (syngamy) and meiosis during their developmental cycle (reviewed in Olive 1963). First, Skupienski reported that *Dictyostelium mucoroides* cells fused during aggregation, and that meiosis occurred prior to sporulation, each diploid nucleus giving rise to four progeny (Skupienski 1920). Later, (Wilson 1952, 1953; Wilson and Ross 1957) and (Ross 1960), also reported diploid formation during aggregation, followed by meiosis and two rounds of mitosis before sporulation. In this case, engulfment of one haploid by another was reported to precede admixture of the cells' contents (Wilson and Ross 1957). Other investigators failed to confirm these observations, and early experiments involving the mixture of morphological mutants prior to aggregation did not provide evidence of high-frequency recombination between strains (Sussman 1954). More extensive work on mutants showed the diploids, and afterwards recombinant haploid progeny could be formed during asexual development, but that this occurs at very low frequency (Sussman 1961); this reflects parasexual, not truly sexual events. Cell engulfment, and cytoplasmic contact through anastomosis and complete fusion do occur fair regularly during aggregation (Huffman et al. 1962; Huffman and Olive 1964), but nuclear fusion resulting in stable diploids is rare (see the section on parasexual genetics below).

Earlier, Raper had reported an alternative developmental structure in some isolates of *D. mucoroides* and *Dictyostelium minutum*, which he termed the macrocyst (Raper 1951, 1956), and tentatively also identified them among drawings in Brefeld's original description of dictyostelids (Brefeld 1869; Raper 1984). These were seen to be formed after an initial aggregation of amoebae, similar to that seen in the asexual fruiting bodies, and also involved the production of a cellulose containing sheath. However, in this case the entire cell mass was walled in, or encysted, and typically remained dormant for several weeks. The cycle of macrocyst formation, quiescence and germination was described in 1957 (Blaskovics and Raper 1957). Subsequent work in the same laboratory, clarified the conditions that favoured their production over the better known fruiting bodies: macrocyst formation was favoured in the dark and in very humid or submerged conditions (Nickerson and Raper 1973a). Several other isolates of other species were also identified as capable of producing these structures. Their biological significance remained a matter of speculation, as later recounted by (Raper 1984), until two meticulous studies of macrocyst ultrastructure were published in the early 1970s (Filosa and Dengler 1972; Erdos et al. 1972). These demonstrated that the macrocyst forms through the action of a single specialised cell that ingests amoebae that aggregate around it, thereby growing considerably in size as the surrounding walls are laid down around it. This 'cytophagic' or 'giant' cell was observed to have an enlarged nucleus, and in the second study evidence of synaptonemal complexes, and therefore meiosis, was found. This indicated that the

macrocyst was in fact diploid, and the long sought sexual phase of the dictyostelid life cycle. Soon after, other isolates not previously known to produce macrocysts were found to form them when cultured in pairs (Clark et al. 1973; Erdos et al. 1973b). These self-infertile strains are called heterothallic, while the self-fertile strains that had been studied earlier are homothallic.

In parallel to these early studies on macrocysts, other investigators were characterising the parasexual cycle in the model organism *D. discoideum* (Katz and Sussman 1972). Parasexuality involves the fusion of haploid cells to form diploids that grow and divide like haploids and that do not revert back to the haploid state through meiosis but rather by random loss of chromosomes via a transient aneuploid state (Brody and Williams 1974). These features led to the use of the parasexual cycle for experimental genetics. Genetic experiments making use of the sexual cycle were also attempted, much less successfully mainly because of difficulties in germinating macrocysts.

3 The Biology of Macrocysts and Macrocyst Formation

Several environmental factors influence cells ability to enter the developmental programme that leads to macrocyst formation (Nickerson and Raper 1973a), in addition to the genetic factor determining whether cells are homothallic or heterothallic, and if the latter which sex (more often called 'mating type') the cell belongs to. The first is nutrient availability: macrocysts were first observed in starved cultures alongside asexual fruiting bodies, and just like in the asexual cycle aggregation of amoebae occurs only after cells sense that their nutrient supplies are becoming exhausted (Blaskovics and Raper 1957). Starvation is not necessary for fusion of compatible haploids, and indeed the presence of bacteria is necessary for high-efficiency fusion (Suzuki and Yanagisawa 1989). Heterothallic diploids can form among populations of cells that are still growing, but homothallic diploids tend to occur later, arising in the centre of already-formed aggregates (Wallace 1977).

The second important factor is the level of light. Very few homothallic strains will produce macrocysts when illuminated with visible light; in most strains the asexual cycle is strongly favoured in the light (Nickerson and Raper 1973a). The critical phase is during the late stages in growth since suitable cells grown in darkness then transferred into the light upon starvation will form macrocysts efficiently (Erdos et al. 1976). Blue light at around a wavelength of 425 μm is most effective in blocking the sexual cycle, and the effect diminishes completely at infrared wavelengths (Chang et al. 1983). The loss of fusion competence in dark-grown cells transferred into the light is cycloheximide sensitive, indicating that the synthesis of one or more proteins is necessary for this effect (Habata et al. 1991). However, the molecular basis of light inhibition, and indeed the photoreception involved remains unknown. Here, it is worth noting that although Skupienski was clearly mistaken in some of his interpretations of the *Dictyostelium* life cycle, it is

not unlikely that he correctly identified syngamy in *D. mucoroides*. He described his putative diploids with some care, and crucially noted that their formation was inhibited by light ("*la moindre lumière l'empêche*") (Skupienski 1920). Since *D. mucoroides* is mostly homothallic (Filosa and Chan 1972), it is possible that he observed syngamy among cells that subsequently switched into the asexual cycle due to the culture conditions he employed.

Thirdly, macrocyst formation is favoured in very humid or moist conditions, and occurs efficiently when cells are submerged in water (Nickerson and Raper 1973a). Asexual fruiting bodies absolutely require an air–water interface, and asexual aggregates undergo a developmental arrest if completely submerged (Sternfeld and Bonner 1977; Bonner et al. 1998). Thus, the sexual cycle would be favoured even in the case of an inefficient progression through these conditions, if it allows some cells to survive. It is believed that the ability to form macrocysts in submerged conditions is positively adaptive, giving cells the ability to survive periods without food in conditions when dispersal via fruiting bodies is impossible (Bonner 1967). However, macrocysts have never been incontrovertibly observed in the wild, so we do not know with any certainty the predominant conditions under which they form in nature, and their adaptive significance therefore remains a matter of speculation.

The fourth critical factor is the presence of extracellular calcium. Early experiments noted a strong inhibitory influence of phosphate ions on macrocyst formation (Nickerson and Raper 1973a). Later experiments showing an absolute requirement for calcium during cell fusion imply that phosphate might act principally by precipitating and thereby sequestering extracellular calcium, so that an excess of calcium is required (Chagla et al. 1980). The events of membrane fusion are clearly stimulated in the presence of calcium, but it is still not certain whether protein–protein interactions, as cells bind to and recognise each other are the most important or whether the apparatus of fusion itself represents the critical step. Recent evidence of calcium-mediated chemotaxis in *Dictyostelium* (Scherer et al. 2010) also raises the possibility that earlier steps prior to cell contact could depend to some extent on calcium. Intracellular calcium signals may also be important during the sexual cycle, with calmodulin-dependent processes implicated in stimulating cell and nuclear fusion but inhibiting the formation of gametes (Lydan and O'Day 1988). Since these effects have only been defined pharmacologically so far, further molecular genetic studies of these phenomena would be illuminating.

Another factor affecting the attainment of fusion competence is temperature. Macrocyst formation in several homothallic isolates is favoured at higher temperatures (20–25 °C), while asexual development is favoured at lower temperatures (Nickerson and Raper 1973a). Cell fusion is delayed at temperatures below 15° and its frequency is greatly reduced above 25 °C (Suzuki and Yanagisawa 1989). It is not known if specific sexual phase proteins are affected directly by temperature or whether it acts indirectly by alterations upon broad metabolism.

The abundance of mRNA of a large set of genes is altered as cells attain fusion competence (Muramoto et al. 2003). In addition to the proteins directly required for membrane fusion, intra- and intercellular signalling components may also be

induced or repressed along with proteins necessary for the early stages of aggregation (Muramoto et al. 2005; Urushihara and Muramoto 2006). Some of these gene expression changes must be dependent on intrinsic genetic influences (the sex determination locus as well as any other variant loci that quantitatively affect mating ability) and the nutritive status of each cell, while many must be controlled by extrinsic factors including the environmental factors described above and perhaps also pheromone signals between cells (O'Day and Lewis 1975). However, it should be noted that some early reports of pheromone signalling could not be independently verified (Wallace 1977). It is not clear whether large-scale changes of cellular form are required prior to fusion, since presumably in amoebae the presence in the plasma membrane of proteins that promote fusion could be sufficient. However, there are reports of an accumulation of distinctly compact presumptive gametes in cultures undergoing syngamy (O'Day et al. 1987).

Cells produce several molecules that influence the frequency of cell fusion. Several unidentified secreted factors in addition to the putative pheromones mentioned above have been reported to induce or repress fusion (O'Day et al. 1981; Saga and Yanagisawa 1983; Urushihara et al. 1990). However, none has been fully identified and so their significance is unclear. Two small secreted molecules do have clear effects. Ethylene is released by fusion competent cells and promotes fusion (Amagai 2011). Its production is dependent on 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase. In *D. mucoroides* ethylene appears to function in part through induction of the *zyg1* gene (Amagai et al. 2007), but since no homologues of this gene are detectable in the other dictyostelid genomes that have been sequenced, the broader context remains uncertain. Any further similarities with ethylene signalling in plants would be highly interesting. Ammonia on the other hand inhibits cell fusion above a critical concentration (Fang et al. 1992). It is produced continually by cells and so may have a role in limiting the number of cells that undergo fusion in a given volume, as well as switching cells into the asexual developmental programme in certain circumstances.

Wherever cell fusion occurs across the eukaryotes, it depends on specific proteins that couple apposing membranes and allows the spatial reorganisation that connects the two previously separate bilayers into one (Shinn-Thomas and Mohler 2011). In *Dictyostelium*, candidate membrane recognition and fusion proteins have been identified as surface antigens that differentiate between mating types. When these are blocked with specific antibodies fusion cannot occur (Aiba et al. 1992). One mutant in which fusion appears to be specifically blocked was identified genetically (Urushihara and Muramoto 2006; Araki et al. 2012), however, it remains possible that the affected gene, *macA*, is necessary for an earlier critical recognition event preceding fusion. More recently, homologues of a widely conserved putative gamete fusogen, HAP2-GCS1 (Wong and Johnson 2010), were identified in *Dictyostelium* (Bloomfield et al. 2010). The expression patterns and molecular functions of these proteins would be of great interest.

Cell fusion at the cellular level has been studied in some detail, thanks to techniques that enable cells of different mating types to be grown separately in conditions that promote very high levels of fusion competence (Saga et al. 1983).

When mixed, very high frequency fusion is possible within in very short time. In these conditions, many cells can fuse together sequentially, forming syncytia with up to several dozen nuclei (Saga and Yanagisawa 1982). This is quite unlike the case in many other eukaryotes, in which fusion is inhibited after the initial fertilisation event (Liu et al. 2010), and reminiscent of the multinucleate plasmodia formed by the myxogastrid slime moulds during their sexual cycle (Bailey 1995). In *Dictyostelium*, when more than two haploid cells fuse, syncytia separate themselves again sequentially by cytokinesis until only two nuclei remain (Ishida et al. 2005), which can then undergo karyogamy to form a diploid that is able to progress through the rest of the sexual cycle. Beyond a broad description of the timing of its occurrence (Szabo et al. 1982; Okada et al. 1986), nuclear fusion has not been studied in *Dictyostelium*.

There is also evidence of inhibition of cell fusion by certain proteins at the cell surface. Limited protease treatment of cells of one of the *D. discoideum* mating types (now designated type III) renders them able to fuse among themselves (Urushihara and Aiba 1996). Similar treatment of the other mating types did not have the same result, and trypsin-treated type III cells were not able to progress and produce macrocysts. The recognition and binding events leading up to fusion between mating types seem likely to be complex. Recently, in the *D. discoideum* asexual cycle polymorphic membrane proteins were found to enable kin discrimination between cells of the same mating type, with related amoebae more like to co-aggregate (Hirose et al. 2011). It is possible that cells also examine their relatedness during the sexual cycle.

After nuclear fusion, giant cells begin to attract surrounding haploid amoebae by secreting a chemoattractant, cyclic adenosine monophosphate (cAMP) (O'Day 1979; Abe et al. 1984). This is the same molecule used by mutually aggregating haploid amoebae during the asexual cycle of group 4 dictyostelids (Schaap et al. 2006). There are similarities and differences between these two aggregation processes: some of the genes required for asexual aggregation are also required during the sexual process, but others are not (Urushihara and Muramoto 2006). Cells translocating towards sexual aggregates can show the extremely polarised morphology typical of migrating cells in the asexual cycle (O'Day 1979), and can also form large, distinct streams (Raper 1962) suggesting that signal relay may also occur. However, during sexual aggregation the secreted form of cAMP phosphodiesterase (PDE) is not released by giant cells, while a PDE inhibitor is secreted in large quantities (Abe et al. 1984), suggesting that relay might not be necessary.

As aggregates form, with haploid cells beginning to surround each giant cell at the aggregation centre, the giant cell begins to ingest by phagocytosis the cells clinging to it, retaining them in endosomes inside its cytoplasm (Filosa and Dengler 1972). Two such cells are visible in Fig. 2a. Concurrently, the cells around the outside of the aggregate begin to lay down an outer wall outside themselves, giving rise to precysts (Fig. 2b). As the giant cell eats its way outwards, a thicker secondary wall is produced inside the first, followed by a final tertiary wall (Fig. 2c) (Erdos et al. 1972). All of these structures contain cellulose

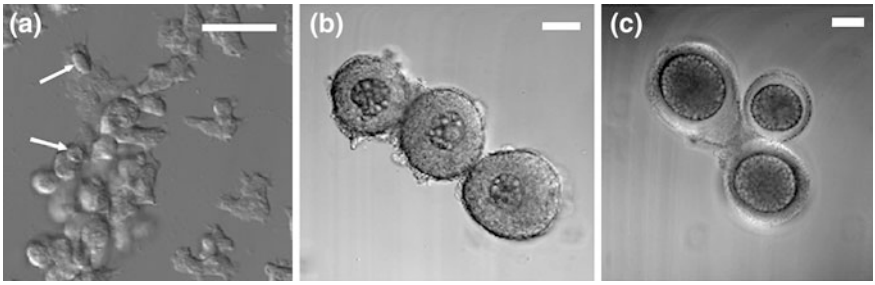

Fig. 2 Cells of the sexual cycle. Homothallic *D. discoideum* cells (strain AC4) were grown in shaken suspension on heat-killed *Klebsiella aerogenes* in MSS buffer. In late log phase they were harvested, washed free of bacteria and deposited in a chambered coverglass. After 4 h (**a**), cells begin to form aggregates, and a small number of cells visibly contain one or more ingested amoebae, or endocytes (*arrows*, two separate cells here contain one endocyte each). After 24 h (**b**), precysts were enveloped within primary walls; zygotes are clearly visible within as *darker* masses containing loosely packed endocytes; peripheral cells are very tightly packed around the zygote. After 48 h (**c**), macrocysts are mature, with thick walls and no peripheral cells remaining outside of the zygote. Scale = 20 μm

(Blaskovics and Raper 1957; Fukui 1976) and most likely multiple glycoproteins (West 2003). The wall also contain molecules with the properties of sporopollenin (Maeda 1985), as well as the sugar trehalose (Temesvari et al. 1996). The secondary wall has some similarity to the wall laid down around microcysts (Erdos et al. 1973a), single-celled structures that allow isolated dictyostelid amoebae to survive transient dry periods (Ritchie et al. 2008). The third wall has similarities with the coat that surrounds the spores produced during the asexual cycle (Erdos et al. 1973a). A mutant that is unable to produce the sorophore sheath that normally surrounds the mature stalk of asexual fruiting bodies, also produces abnormally walled macrocysts (Larson et al. 1994), indicating that production of both relies on the gene(s)—which have not been identified—that are affected in this strain.

Once the giant cell has ingested all the peripheral cells surrounding it within the macrocyst tertiary wall, it enters a period of inactivity, at least as observed from the outside (Blaskovics and Raper 1957). After a period of up to several weeks in which the ingested amoebae (described as endocytes in the early studies) remain relatively intact within their vacuoles trapped in the cyst's cytoplasm, start to be visibly diminished in size, ultimately taking on a fine granular appearance (Blaskovics and Raper 1957). Ultrastructural studies suggest that the endocytes are broken down gradually, presumably as lysosomal enzymes are added to the vacuoles and nutrients are extracted and utilised by the developing macrocyst (Erdos et al. 1973a). At the same time, nuclear events take place, but have not been studied in any detail. Electron micrographs show evidence of meiosis in the diploid macrocyst nucleus within a few days of its formation (Erdos et al. 1972; Okada et al. 1986), but it is not known whether all of the meiotic stages occur without interruption or if it is arrested for any period during the cyst's apparent

dormancy. At the stage when endocytes have been reduced to granules, multiple nuclei become visible within the cyst (Filosa and Dengler 1972), which are assumed to be formed by multiple mitoses following after the meiotic divisions. Next, this coenocyte divides into uninucleate amoebae, initially larger than normal free-living amoebae (Erdos et al. 1973a). Finally, the cyst walls are broken and the haploid progeny escape, free to resume growth if conditions permit them to.

The later stages of macrocyst development are not well understood, since the long quiescent period discourages intensive cell-biological studies. Germination is also poorly understood, and with the exception of a small number of isolates only occurs at very low frequencies (Wallace and Raper 1979). The conditions that trigger germination are not known, though in some cases exposing the mature cysts to light was stimulatory (Nickerson and Raper 1973b). Since cycloheximide inhibits germination (Erdos et al. 1973a) it is clear that the amoebae within the germinating cyst play an active role. Whether some unknown environmental change or signal is required to trigger the stubbornly dormant majority of cysts, or whether they are in some way defective (perhaps because of unfavourable culture conditions in the laboratory) is not known.

4 Sexual and Parasexual Genetics

The earliest studies noted the presence of homothallic and heterothallic isolates, sometimes within the same species; notably some heterothallic species have more than two sexes (documented in Bloomfield 2011). Mating orientation is stably inherited and is not known to be influenced by environmental conditions. The first attempt at experimental sexual genetics involved a homothallic isolate of *D. mucoroides* (MacInnes and Francis 1974). Different mutants derived from the same starting clone were crossed and recombinant progeny produced, giving evidence supporting the hypothesis that meiosis occurs during the sexual cycle. Subsequent studies using heterothallic strains of *Dictyostelium giganteum* (Erdos et al. 1975), *D. discoideum*, (Wallace and Raper 1979), and *P. album* (Francis 1980) gave results following the same pattern: genetic exchange was found to occur, and mating orientation was inherited in manner consistent with determination by a single genetic locus. Note that here we follow Kawakami and Hagiwara (Kawakami and Hagiwara 2008) in reassigning some strains formerly described as *P. pallidum* to *P. album*; this includes the isolate whose genome sequence was recently published (Heidel et al. 2011). In most cases a single genotype was found for the progeny of each germinated cyst although across a population of cysts all possible phenotypes were observed, implying competition between progeny within cysts. These pioneering genetic studies demonstrated the potential value but also the limitations of the dictyostelid sexual cycle as an experimental tool: in most species the extended necessary periods of dormancy and low germination frequencies are a severe disadvantage.

In parallel to these studies, the parasexual cycle proved to be somewhat easier, if still limited tool to use (Newell 1978). It was found that cells passing through the asexual cycle would fuse to form stable diploids at a frequency of about 10^{-5}–10^{-6} (Katz and Sussman 1972). Diploids could be identified by the volume of their spores (Sussman and Sussman 1962) as well as by karyotyping, but most conveniently by complementation of two different mutants (Newell 1978). Haploid recombinants occurred spontaneously after random loss of one chromosome followed by a rapid reduction to the haploid karyotype (Sinha and Ashworth 1969; Brody and Williams 1974), presumably due to strong selection against aneuploids. Haploids could be selected by drug treatment, either if one parent possessed a recessive resistant marker (Newell 1978), or by use of a microtubule-destabilising agent to interfere with mitosis causing non-disjunction events (Williams and Barrand 1978). Markers were introduced that allowed selection of diploids, most often mutants that are able to grow only below a given restrictive temperature (Loomis 1969), and parasexual crosses became routine and relatively amenable. Care was always needed to quantify results accurately and to properly distinguish recombinant haploids from diploids and parental cells. As more mutations were identified, complementation tests and linkage analysis became straightforward, and some progress was made in analysing synthetic phenotypes (Rothman and Alexander 1975). However, because of the relatively low frequency of mitotic recombination, mapping could be carried out only at low resolution (Welker and Williams 1982). Evidence of frequent translocation mutations added complication to analyses of linkage (Welker and Williams 1985).

Another practical limitation of parasexual genetics in *D. discoideum* proved to be informative. All of the early crosses were among strains descending from one clone, the type isolate of the species, NC4. It was later found to be much more difficult to obtain diploids in crosses between strains of different mating types (Robson and Williams 1979). When diploids resulting from such crosses were obtained, they were all found to have lost heterozygosity at the mating-type locus. This indicates strong selection against the growth of diploids carrying two different versions of this locus, and the phenomenon was called ‘vegetative incompatibility’ by analogy with a similar process in filamentous fungi (Robson and Williams 1979). No other loci were found to contribute to the effect, implying a role for the products of the sex-determining locus in controlling the growth of sexual diploids. This is not surprising given the nature of the dictyostelid sexual cycle: such diploids will exit the mitotic cell cycle and ultimately progress instead through meiosis, unlike parasexual diploids which continue to grow and divide mitotically exactly like haploids. All three *D. discoideum* mating types, as well as homothallic isolates of this species were found to be vegetatively incompatible (Robson and Williams 1980).

The genetics of sex dertermination in dictyostelids was recently clarified by the identification of the mating-type locus of *D. discoideum* (Bloomfield et al. 2010). A candidate sex-determining sequence, *matA*, was identified in mating type I strains in comparative genomic hybridisation experiments. The same locus in the two other mating types was different in each case. The type II version of the locus

contains a gene homologous to the type I gene, *matB*, which is flanked by two genes, *matC* and *matD*, not present in the type I genome. The third mating type contains homologues of these last two genes, *matS* and *matT*, but no homologue of *matA* and *matB*. Type I mating is dependent on *matA*, and type III only on the presence of *matS*, while in type II cells *matB* is required only for mating with type III cells, and *matC* is necessary for mating with type I. Type II is therefore effectively a composite of type I and type III, and it should be remembered that it is not self-compatible, indicating an incompatibility of some kind between the *matB* and *matC* genes. The other genes encoded by the locus, *matD* and *matT*, are not required for mating to occur, but may have quantitative effects, since in some experiments yields of macrocysts are higher in their presence.

Although the three pairs of homologous genes reside at the same genetic locus, they are not formally characterised as alleles. They have diverged considerably (up to 40 % in amino acid sequence), have clearly distinct functions and are flanked by different genes in each case (Bloomfield et al. 2010). This last consideration is perhaps the most important since correct co-inheritance with the other *mat* genes contained at the locus appears to be critical. We therefore describe the three pairs of genes as gametologues, and call the three different versions of the entire locus idiomorphs. The genetic basis of homothallism is still not precisely understood, but appears to involve more than one locus (unpublished data).

It is believed that MatA, MatB, MatC and MatS are regulatory proteins, activating and repressing genes in the haploid state, in order to determine their mating orientation with respect to other cells, and also acting in diploids to repress haploid-specific genes and mostly likely to activate sexual-diploid genes, accounting for the vegetative incompatibility phenotype (unpublished data). Their precise molecular function remains to be determined; they are cytoplasmic in location, and biochemical evidence suggests that MatA and MatB are nucleic acid binding proteins (unpublished data), like many other key regulators encoded by sex determining loci.

5 Evolutionary, Taxonomic and Ecological Considerations

The dictyostelids have been divided into four main groups on the basis of their molecular phylogeny (Schaap et al. 2006), although more recent work incorporating more species demonstrates that eight groups in total can be supported (Romeralo et al. 2011). Macrocysts have been reported across all main groups, suggesting that the last common dictyostelid ancestor possessed a sexual cycle resembling that of the best-characterised examples of extant species. Indeed, apparently sexual cysts bearing some resemblance to macrocysts are present in other Amoebozoa, for instance *Copromyxa*, although these are structures formed only by the two gametes without a contribution by surrounding cells (Spiegel and Olive 1978). The sexual cycle of the myxomycetes also has some parallels with

that of the dictyostelids: it also involves a syncytial stage, but the final structure is differentiated and multicellular (Bailey et al. 1990).

Homothallism and heterothallism are both found widely among dictyostelia (Bloomfield 2011), and it is not known which is the ancestral pattern. The relationship of heterothallic and homothallic isolates contained within the same species is also not clear. Until evidence is presented of mating incompatibility between them (or some other significant reproducible difference in phenotype is discovered) we consider it parsimonious to retain them within the same species. In contrast, where syngens of heterothallic strains exist within the same morphologically defined species, a division should clearly be made and new species descriptions formally introduced, and sequence data made use of wherever possible.

The genomes of three dictyostelids other than *D. discoideum* have been published up until the time of writing. Two of these species, *Dictyostelium purpureum* and *P. album* were previously known to be heterothallic. The sequenced *D. purpureum* strain (Sucgang et al. 2011) contains homologues of *matS* and *matT* (Bloomfield et al. 2010), suggesting it is the equivalent of the *D. discoideum* type III. In contrast, the *P. album* isolate (Heidel et al. 2011) contains no discernible homologues of the *mat* proteins; since it is heterothallic and it is possible that a distant homologue of *matA* is present but not identifiable due to its small size (*matA* is 107 amino acids in length) and the extent of sequence divergence. It is also possible that this species uses a completely different sex determination mechanism. Similarly, the sequenced strain of *Dictyostelium fasciculatum* (Heidel et al. 2011) possesses no recognisable *mat* homologues; this species has not been observed to produce macrocysts, so as well as the possibilities mentioned for *P. album*, it could be that this species is asexual. *Acytostelium subglobosum*, a member of *Dictyostelium* Group 2A, has not been shown to produce macrocysts but contains a clear homologue of *matT*, and adjacent to it a possible, but extremely distantly related, *matS* sequence (Bloomfield et al. 2010).

The mating type genes identified so far appear to be evolving rapidly causing large divergence in sequence. This is common in sex determining genes (Swanson and Vacquier 2002), and may reflect positive selection with changes in sequence of these regulatory genes accompanying speciation events. Similar sequence changes very likely also occur in the cell–cell recognition proteins and perhaps also cell fusion proteins. As these become better understood comparative evolutionary analyses ought to become possible.

The evolution of the third *D. discoideum* mating type poses an interesting puzzle. Structurally, it clearly resembles an interleaving of the other two idiomorphs of the mating type locus, but containing altered homologues of *matA* and *matS* that render it compatible with type I and type III partners but not with other type II cells (Bloomfield 2011). One model for the formation of this third sex involves a cell fusion followed by an unlikely recombination event that exchanged *matA* from one chromosome to the other leaving it placed in between *matS* and *matT*, and perhaps as a result, self-fertile. Subsequent selection for cells that became self-infertile could have resulted in the third sex. However, taking into

account the implications of vegetative incompatibility, which suggests that cells containing both *matA* and *matS* should be unable to multiply mitotically, an alternative would be a cell fusion during which a *matA* variant is introduced together with a *matS* variant that it is in some way incompatible with, so that the diploid thus formed is unable to progress through the sexual cycle. A recombination event could then take place, and subsequent mutations to the nascent *matB* and *matC* sequences might then increase their compatibility with *matS* and *matA,* respectively, if this function were deficient, provided that the co-evolving *matB* and *matC* remain incompatible. Other mutations in cell recognition and fusion proteins might also be necessary.

A similar model can be proposed to account for extensions of the number of sexes beyond three. A cell might acquire mutations in *matB* and *matC* (making *matB'* and *matC'*) that make each of them compatible with 'normal' *matC* and *matB* but not with each other. If fusion with 'normal' type II cells were possible, this could then constitute a new mating-type, provided it were still able to fuse with the other mating types but not with itself. Again, mutation (and perhaps amplification followed by mutation) of cell–cell recognition factors might also be necessary. Population genetic evidence suggests that sex and recombination occur commonly in wild *D. discoideum* populations (Flowers et al. 2010), presumably reflecting a selective advantage conferred by them. The presence of more than two sexes most likely reflects ecological conditions that favour it: with each increasing number of sexes any random pairing of two cells becomes more likely to be sexually compatible (assuming approximately equal numbers of individuals of each sex, which is very likely (Edwards 1998)). Perhaps encounters between different clonal groups are rare enough to make this favourable, more than balancing out disadvantageous factors such as the possibility of conflict between cytoplasmic endosymbionts (Hurst 1996).

Based on our knowledge of the conditions that favour macrocyst production in the laboratory, it is possible to speculate as to when and where they are formed in nature. The necessity of an air–water interface for asexual development and the ease with which macrocysts can be generated in flooded cultures, has encouraged the idea that they may be important for survival, when cells are starved and submerged in water far from air (Bonner 1967). Their prolonged dormancy might even enable the organism to wait out an entire wet season or a temperate cold winter during which water tends to accumulate; the double walled cysts of the Archamoeba *Pelomyxa palustris* remain viable for up to 7 years (Chapman-Andresen 1978). Macrocysts could also be favoured when cells are dispersed to sites in which nutrients may become limited and sorocarp formation is not possible. For instance cells can be washed into relatively large bodies of water (O'Dell 1979), or deep into the soil. Dispersal out of these sites is presumably much less frequent than from among the topsoil or leaf litter (or from upon living plants), so again dormancy could be adaptive. Finally, since some other amoebae form asexual cysts inside host organisms (Ma et al. 1990), and distantly related protozoa pass through their sexual cycle only while infecting one of their hosts (Paul et al. 2002), one might speculate that the dark and wet conditions favourable to

macrocyst production might be satisfied when dictyostelids find themselves inside some larger organism. Very few dictyostelids are able to tolerate temperatures above 30 °C (Raper 1984), so they are not likely to infect mammals or birds, although they are able to pass through the digestive systems of warm-blooded animals (Suthers 1985; Stephenson and Landolt 1992). Many invertebrate animals that share the favoured habitats of social amoebae could be potential hosts for heat-intolerant amoebae. In this context it is worth noting, a recent report of a relatively heat tolerant isolate of *Dictyostelium polycephalum* isolated from the cornea of a keratitis patient, where it apparently formed walled cysts (Reddy et al. 2010).

6 Outlook

Since the dictyostelid sexual cycle remains so little studied, it is not surprising that there are many possible avenues for future research. The recent discovery of the sex-determining locus of *D. discoideum* makes possible molecular approaches capable of elucidating the higher level regulatory events that govern cellular behaviour, and also should enable evolutionary analyses that will gain depth as more species' genomes are sequenced. Progress is being made on the molecular and cell biology of sexual cell fusion and many more cell-biological questions can be addressed. The nature and control of the cannibalistic phagocytosis carried out by the zygote has hardly been studied at the molecular level, and the structural organisation and metabolism of the maturing and mature macrocyst will undoubtedly lead to valuable insights. Likewise the nuclear events from karyogamy (and earlier the selection of two nuclei within a syncytium) onwards remain very unclear, and cell cycle regulation in this kind of shift from haploid to dormant diploid has not yet been studied in detail.

Dictyostelid genomes are also notable for their lack of a homologue of *Spo11*, which is responsible for forming the double strand breaks during meiosis in most eukaryotes (Malik et al. 2007). Is an alternative enzyme used by the social amoebae or does synapsis arise in a completely different way? Finally of course germination and the events immediately preceding it remain mysterious, and here especially the development of molecular tools in species other than *D. discoideum* would be invaluable. Most work on macrocyst germination has been done on one isolate of *D. mucoroides* that germinates at relatively high frequencies after a short dormant period (Raper 1984), and it is likely that other species, heterothallic and homothallic, could be identified that equal or surpass its utility. For most of the other cell-biological problems mentioned, tools such as cytoskeletal and organelle markers are already available for *D. discoideum*, all that remains is the effort required to employ them and modify existing experimental protocols.

Given the advances in the study of altruism and conflict in the dictyostelid asexual cycle (Strassmann and Queller 2011), it seems certain that aspects of the

sexual cycle will also become interesting to sociobiologists. Initial studies in this regard gave disappointing results because of difficulties in obtaining recombinant progeny in crosses of *D. discoideum* (Shaulsky and Kessin 2007). However, several approaches are possible: fusion events can be monitored directly if cells are suitably marked with fluorescent proteins or dyes, as can biases in phagocytic uptake. Additionally, the potential for conflict between endosymbionts is of great interest in the context of a prominent explanation of the typical limit of two sexes only per species; how do dictyostelids overcome this potential hazard? Mitochondrial inheritance appears to be uniparental in *P. album*, (Mirfakhrai et al. 1990), which has two sexes; is this also true in *D. discoideum, D. purpureum,* and *D. giganteum*? Relatedly, one can ask whether sexual cell fusion and later events are affected by the presence of other microorganisms inside amoebae. Again, if multiple cells fuse together, each one presumably adds some risk of bacterial, viral, or other contagion that would be detrimental to the eventual progeny. Since amoebae in the soil must continually be exposed to potential pathogens, mechanisms to mitigate these risks surely must exist.

Finally, any effort to study the sexual cycle in its natural context would be extremely valuable. Our knowledge of the ecological context of macrocyst formation is still effectively nil; this is understandable since finding macrocysts in the wild will mostly likely involve identifying these small brownish structures among many other small brownish particles in soil. Perhaps molecular investigations will find evidence of dictyostelids in unexpected places, or perhaps the use of marked strains, or an informed guess about possible locations might yield a breakthrough.

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A Global Overview of Dictyostelid Ecology with Special Emphasis in North American Forest

James C. Cavender

Abstract Development in 1965 of a quantitative method for dictyostelid isolation from soil samples, made possible ecological studies based on frequency and density of occurrence. Information has subsequently been obtained on a number of aspects of dictyostelid ecology. I discuss some of them in this chapter, especially those aspects studied after Raper's publication in 1984 of his famous book "*The Dictyostelids*". These include dispersal, relative abundance, optimum conditions for maximum diversity, comparison of temperate and tropical populations, ecological individuality of species (especially *Dictyostelium discoideum*), possible decline in species numbers, and global distribution of Dictyostelia.

1 Introduction

Development of a quantitative method of isolation (Cavender and Raper 1965a) provided a necessary tool for exploring the ecology of dictyostelids. The first ecological survey was done in forests of southern Wisconsin by sampling six stands along a continuum based on a moisture gradient (Cavender and Raper 1965b). Nine species were isolated. Although most of the species were present across the continuum, the peak frequencies of five species separated nicely. The peak relative densities usually matched the peak frequencies. Four species, *Dictyostelium discoideum, D. minutum, D. lacteum*, and *Acytostelium leptosomum* peaked in the drier end of the continuum. One species, *D. polycephalum* peaked in the wet end. The frequency of *Polysphondylium pallidum* was high over most of the continuum but the relative density was highest in west-mesic forest.

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The experiment showed that dictyostelids are differentially responsive to environmental factors especially moisture.

By relating the dictyostelid densities to nine different physiographic regimes in Ohio (Cavender and Hopka 1986), it was shown that *Dictyostelium polycephalum* and *Acytostelium leptosomum* have the greatest niche specificity; the former is associated with depressional forests and flood plains and the latter with sandstone ravines. *Dictyostelium mucoroides*, *P. pallidum* and *P. violaceum* have the broadest environmental spectra but *P. violaceum* and *D. purpureum* did show a statistically positive relationship with soil pH, occurring more abundantly in forests on soils derived from limestone. In a DCA ordination of species in 21 sites in West Virginia (Landolt and Stephenson 1990), good separation (niche specificity) was shown for *D. polycephalum* and *A. leptosomum* as was found in Ohio as well as good separation of *D. giganteum.*

2 Dispersal

In his book *The Dictyostelids* (1984), Dr. Ken Raper's two chapters on dictyostelid occurrence and ecology summed up well most of the material known at the time. There was in 1984, however, no available work on dispersal. Since dictyostelid spores are imbedded in slime which dries and hardens in time, there is little chance for airborne dispersal except when the soil dries and is blown by wind which is not likely to occur often in a humid forest environment. Then Suthers (1985) working out of Dr. John Bonner's lab at Princeton showed how ground feeding birds such as thrushes, finches, sparrows, and warblers can carry spores in their guts and deposit them at considerable distances during migration. Suthers made collections in Mexico, Guatemala, and Costa Rica. Some otherwise hard to explain distributions can be explained by bird migrants depositing dictyostelid spores. For example a species, *Dictyostelium rosarium,* associated with dry, salty environments such as those in southern California (Benson and Mahoney 1977) and first isolated from deserts in Mexico (Raper and Cavender 1968) showed up at a salt marsh in Ohio created by the Morton Salt company. It was probably carried in by a migrating bird. This was the only isolation of this species in Ohio. Another example of probable bird dispersal is the occurrence of *D. rhizopodium*, a species associated with tropical and subtropical forest, from oak forest at Big Oak Tree State Park in the Mississippi River flood plain. It was again isolated from Arkansas upland forest (Landolt et al. 2009). These isolates may have been carried north from Mexican seasonal evergreen forest by migrating birds.

In 1989, some important information was published on the dispersal of dictyostelids in Kansas prairie soil by two soil invertebrates, earthworms and pill bugs (Huss 1989). He showed that dictyostelid propagules can be obtained from invertebrate guts. Soil invertebrates not only can distribute dictyostelids but some of them, nematodes in particular, are voracious predators and quickly remove dictyostelids from the soil. This partly explains why dictyostelids invest so much

energy into raising their spores into the air, temporarily out of the reach of nematodes, and can produce rather stout stalks with many cells. A morphological feature such as the digitate base helps the stalk stay hydrated and erect to avoid nematodes as much as possible (Vadell, personal communication).

Bats may also be carrying around dictyostelid spores. One hundred caves were studied in temperate North America and the subtropical Caribbean (Landolt et al. 2006b). Seventeen species of dictyostelid were isolated. Their occurrence was associated with proximity to the bat guano. Dictyostelids were found in the guts of brown bat as well as in cave crickets (Stephenson and Landolt 1992), other invertebrates (moths), amphibians (red-backed salamanders), small mammals (white-footed deer mouse), and birds. The occurrence of dictyostelids in canopy soil also implies their distribution by animal life in the forest (Stephenson and Landolt 1998).

Recently, a paper from Systematic Biology at Uppsala University (Perrigo et al. 2012) has shown how easily dictyostelids are dispersed by boots of human agents. Four species were found repeatedly from soil tracked around on the soles of boots.

3 Relative Abundance

In West Virginia deciduous forest, the very common and common species are similar to those in Ohio (Landolt and Stephenson 1990). However, as one progresses westward into drier climatic conditions with less diversity of deciduous trees and greater presence of conifers, the abundance of dictyostelid species change. The dictyostelids from eight physiographic regions in Ohio (85 sites and 677 samples) produced 23,884 total clones at an average of 700 clones/g. When these were ordered by number of clones and percent presence (Cavender and Vadell 2006), we find four very common species (>50 % presence): *D. mucoroides, D. minutum, P. pallidum*, and *P. violaceum*; five species are common approaching 50 % presence, e.g., occurring in almost half the sites: *D. aureostripes, D. lacteum, D. discoideum, D. giganteum and D. purpureum*; four are rare with 1–3 %presence: *A. leptosomum, D. sphaerocephalum, D. implicatum* and *D. polycephalum*. The remaining ten species are very rare, found in less than 1 % of the sites. In the central Rocky Mountains (Cavender 1983), *D. mucoroides* and *D. sphaerocephalum* become overwhelmingly abundant making up 68 and 29.5 % of the clones, respectively. One of the remaining seven other species isolated was rare, *D. aureo-stripes* and the others very rare, <1 %. *D. mucoroides* and *D. sphaerocephalum* become proportionately more abundant when conditions are less favorable for dictyostelids. This is supported by research of others, e.g., (Hagiwara 1992). In Alaska where the number of species is also less than ten and conditions are less favorable for much of the year, these two species are also overwhelming dominants (Cavender 1978; Stephenson et al. 1997; Romeralo et al. 2010). On the west coast of North America where there are dry hot summers and cool wet winters, there are few deciduous forests and deciduous trees are generally

subordinate to coniferous trees. In southern California, *D. mucoroides* dominated numbers except in drier sites where *D. rosarium* was more abundant and *D. sphaerocephalum* dominated at higher elevation (Benson and Mahoney 1977). Thirteen species were found. In northern California, *D. mucoroides* and *D. sphaerocephalum* had 43 and 24 % of the clones and were most widespread and dominant in 102 collection sites (Quinones 1989). There were 11 species and total clones were low, 361 from 180 soil samples. Coastal redwood forest was richest in species with six and the coastal range region with six forest types had ten.

Deciduous forest regions of temperate Europe were generally poorer in species compared to that of eastern North America (see Table 2). Germany and Switzerland have the most abundant and diverse forests and the most dictyostelid species with 19 reported from Germany (Cavender et al. 1995; Leitner 1987). Southern Europe with a Mediterranean climate had 14 species (Romeralo and Lado 2006), while northern Europe, e.g., Sweden had nine (Kawabe 1995; Perrigo et al. 2013). *D. mucoroides* is the most widespread and abundant species in Europe (Cavender 1969a). Temperate deciduous forests of Japan with 26 species are more favorable for dictyostelids than those of Europe and are equivalent to those of North America (Cavender and Kawabe 1989). Hagiwara (1989) has added other species. *D. mucoroides* had 1,881 of the total 4,371 clones or 43 %. Temperate India is less favorable with ten species while *P. pallidum* not *D. mucoroides* is the dominant species in terms of frequency, density, and presence (Cavender and Lakhanpal 1986). In fact five other species are more important than *D. mucoroides.* However, above 2,600 m it is dominant as was also reported for alpine and subalpine communities in Nepal (Hagiwara 1982). The dictyostelid distribution in India recalls that of Arkansas (Landolt et al. 2009) where *P. pallidum* and four other species were more important than *D. mucoroides* in terms of number of clones. In drier and cooler environments, it appears that *D. mucoroides* and *D. sphaerocephalum* have a distinct advantage (Hagiwara 1982) (see Table 1).

4 Diversity and Numbers in Tropical and Subtropical Environments

In tropical and subtropical environments, dictyostelids have their greatest diversity (>30 species) although one temperate area was found which has a dictyostelid diversity that approaches some of those found in the tropics (see Table 2). In the Smoky Mountains National Park, 30 species were found including ten new ones (Landolt et al. 2006a; Cavender et al. 2005). This is higher than Ohio and is probably the result of greater elevation differences and perhaps the mountains acting as a refugium during the last glaciation. Samples were collected at three elevation zones 465–615, 730–915, and 1,470–1,920 m. In Ohio, new species were found by sampling marginal environments such as a salt marsh border and acid bog margins. This was also found to be true in the Smoky Mountains where seven

Table 1 Examples of dictyostelids with different types of distribution

Primarily tropical[a]	Disjunct sps[b]	Widespread sps (50 % presence)	Rare species[c] (<1 % presence)	Extreme habitats
D. purpureum	*D. discoideum*	*D. mucoroides*	*D. deminutivum*	*D. mucoroides*
D. rhizopodium	*D. fasciculatum*	*P. pallidum*	*D. boreale*	*D. giganteum*
D. vinaceo-fuscum	*D. microsporum*	*D. minutum*	*D. arcisporum*	*D. sphaerocephalum*
D. lavandulum	*D. capitatum*	*P. violaceum*	*A. ellipticum*	
D. tenue	*D. deminutivum*	*D. sphaerocephalum*		
	D. parvisporum	*D. giganteum*		
		D. purpureum		
		D. aureostipes		

[a] Primarily tropical because *D. purpureum, D. rhizopodium,* and *D. vinaceo-fuscum* are also found in temperate zones

[b] Disjunct species: *D. fasciculatum* was reported in Swanson et al. (1999) as a restricted species (only found in Europe). Now it is also known from North America Smoky Mountains National Park; *D. deminutivum*: found in Mexico and Madagascar; *D. microsporum*: in Japan and Europe; *D. capitatum*: Japan, Europe; *D. discoideum*: Japan, N. America, Central America, S. America, India–Pakistan Nepal, Japan but not Europe; *D. parvisporum* was reported as restricted in Swanson et al. (1999) (only found in Japan). It has recently been extensively found in Norway (Romeralo, personal communication)

[c] Rare species: most of the small species belonging to Groups 1, 2, and 3

new species were found from the highest elevations, especially from habitats such as bog margins where bacterial numbers would be low. In these habitats, there may be less competition from other more common dictyostelids as well as from soil protozoans. Although diversity is high in the Smoky Mountains, the numbers are low (143 clones/g/site). The same relationship is true for the subtropical environments. Tikal, Guatemala with 35+ species of dictyostelids had an average of 182 clones/g from 91 samples from four sites (Cavender 2005). Mexico had an average of 167 clones/g from 286 samples and 39 sites although a small percentage of these were temperate sites (Cavender et al. 2012). Costa Rica produced 188 clones/g from 21 sites and 187 samples (Cavender 2005, 1963). Densities at Iguazu Falls, Argentina were very low at <100/g yet produced 17 species (Vadell and Cavender 2007). Colombia, with 15 species, 415 clones/g from 11 sites, and 102 samples (Cavender 2005) and the Peruvian Amazon with 27 species, 353 clones/g had higher counts, (Cavender 1996). Some of the lowest numbers/g have been from the Southern Hemisphere e.g., New Zealand 112 clones/g from 33 sites and 225 samples (Cavender et al. 2002) and Australia 78 clones/g from 30 sites and 223 samples (Landolt et al. 2008a), Iguazu <100 g from 4 sites and 80 samples (Vadell and Cavender 2007) and South Africa 210 clones/g from 27 sites and 70 samples (unpublished data). Means for four temperate Southern Hemisphere sites were 235 clones/g, eight temperate northern Hemisphere sites 517 clones/g and seven tropical-subtropical sites, 281 clones/g (Table 2). In the island environment of the Caribbean, collections were made on three islands of

Table 2 Number of species and absolute densities isolated across the world

Australia, NZ	America	Europe	Asia	Africa
NZ, 13 (95 clones/g)	Smoky Mountains, 30 (143 clones/g)	Germany, 19 (462 clones/g)	India, 12 (580 clones/g)	South Africa, 14 (210 clones/g)
Australia, 12 (78 clones/g)	Butterfly Woods (Ohio), 19 (350 clones/g)	Spain, 14 (570 clones/g, 4 sites)	Japan, 26 (244 clones/g)	Cent. African Republic, 13 (536 clones/g)
	Southern Appalachians, 13 (909 clones/g)	Switzerland, 14 (548 clones/g)	SE Asia (5) 16 (400 clones/g)	East Africa, 10 (595 clones/g)
	Eastern Canada, 9 (1462 clones/g)			
	Mexico, 33 (167 clones/g)			
	Tikal, Guatemala, 35 (182 clones/g)			
	Costa Rica, 14 (188 clones/g)			
	Colombia, 15 (415 clones/g)			
	Peruvian Amazon, 27 (353 clones/g)			
	Iguazu, Argentina, 17 (<100 clones/g)			
	Patagonia and Tierra del Fuego (Argentina), 12 (383 clones/g)			

decreasing area: Puerto Rico, St John's Virgin Islands and San Salvador, Bahamas (Cavender et al. 2004). A direct positive relationship was found between plant diversity and number of cellular slime mold species, which in turn reflected island area, however, numbers per gram were not compared.

5 Optimum Habitat for Dictyostelids

While searching for dictyostelids in Ohio, one woods in particular was found which produced more species from the initial collection (13) than any of the other 119 forest sites, which were sampled. This was a small 6 ha. Wood (Butterfly Woods) in Muskingum County, which is now part of The Wilds conservation area, an area of 14,000 acres that was salvaged from strip-mined land. Of the 25 species of dictyostelids found in Ohio, this site subsequently produced 19 of them over a period of 7 years and 17 collections. There were an average of 11.2 species per collection (usually five samples) and 351 clones/g. Individual soil samples produced up to ten species (Cavender and Cavender, in press). This suggests dictyostelid species may occupy microniches as reported by Eisenberg (1976). Butterfly Woods is therefore considered the optimum habitat for dictyostelids in Ohio. The biological and geological features of Butterfly Woods have been described (Cavender and Cavender 2005) to help pinpoint the characteristics of the optimal habitat for dictyostelid diversity. This is a mixed mesophytic (Braun 1950) wood with a diverse tree and herb composition (26 tree species, 59 herb species). The area has not been glaciated or subjected to postglacial flooding. There is a substantial surface humus (0) layer with a continual cover of decaying leaf litter. The soil is derived from thinly bedded sandstone, shale, and limestone, which weather into a mixture of soil microhabitats ranging from pH 6.2–7.0. There is an east/northeast slope exposure in an environment of rolling hills. The slope provides generally favorable moisture conditions, which may vary somewhat from top to bottom. There is also a small gorge with an intermittent stream which gives protection from rapid temperature and moisture changes and the erosion of the bedrock helps renew soil fertility. The wood is small (6 ha) but apparently large enough to provide a stable environment for dictyostelids. Other remnant woods nearby may be a factor in the conservation of these species. Surrounding disturbance and some possible internal disturbance (selective cutting) may be a contributing factor to dictyostelid diversity (Cavender et al. 1993).

Butterfly Woods in Ohio together with the seasonal evergreen forest of Tikal in Guatemala (Table 2), are two of the optimal places for dictyostelids diversity found so far. Both forests have: high pH, limestone bedrock, seasonality-wet and dry or warm and cold, high plant diversity (especially Tikal), animal spore dispersers (especially Tikal), varied topography, and some human disturbance. The tropical environment gives Tikal the edge for dictyostelids diversity.

6 Dictyostelid Decline

There is some evidence for the decline in dictyostelid numbers in forest soils since records began being kept in 1970. For example, the results of six collections from 1970 to 2007–2008 show decline in numbers. Those collections cover 185 sites in 3 different areas with 4 of the 6 collections from Ohio. The highest average number per gram of soil was 1,462/g from 23 sites in eastern Canada (Cavender 1972). Then an average of 909 clones/g from 23 sites was reported for the southern Appalachians (Cavender 1980). Finally, four successive collections from Ohio: 1970 (Hopka 1972), 1981, 1998 (Cavender and Vadell 2006), and 2007–2008 (1970, 65 sites, 909/g; 1981, 35 sites 570/g; 1998, 26 sites, 418/g; and 2007–2008, 13 sites, 391/g) show a progressive decrease in numbers and a total drop of 44 %. The rate of decline seems to have slowed after 1998. Why dictyostelid numbers appear to have declined is not known but may have something to do with the invasion of the earthworm, *Lumbricus terrestris,* which was invading North America during this period (Frelich et al. 2006). The soils in Canada in 1971 were the least likely to be invaded. That earthworms may be a factor in the dictyostelid decline is indicated by an experiment carried out by the author at Porcupine Mountain State Park in the Upper Peninsula of Michigan, which was being invaded by earthworms at the time (month of August 2009). The no-worm site was in the highest area of the park in elevation where worms had not yet invaded. This area produced 380 clones/g and 5 species, while the average for three sites invaded by earthworms was 60/g with four species. The higher numbers found in the forest areas more remote than Ohio, eastern Canada, and the southern Appalachians, may indicate that numbers had already been dropping in Ohio before 1970 due to earthworms or anthropogenic disturbance. These more remote forests also tend to have more litter than in Ohio. The amount of litter that feeds the bacteria upon which the dictyostelids feed may be a critical factor. The humus in more northern latitudes and at higher elevations is mostly of the mor type which decomposes more slowly than the mull type. Mull humus results in part from the mixing and digesting activities of earthworms. The highest densities of dictyostelids in individual soil samples are mostly from forests with mor humus and considerable litter. Of the 37 highest counts recorded by the author for individual samples over a 30-year period, ranging from 3,158/g to 20,000/g, 26 had the mor type humus. Decline in dictyostelid numbers in Ohio may reflect the more rapid decomposition of litter due to warming climate as well as the conversion of mor to mull humus as a consequence of earthworm invasions.

7 Ecology of *Dictyostelium discoideum*

The author collected data from 144 sites in the eastern deciduous forest of the United States, Mexico, and Canada. Data included total clones, clones of *D. discoideum* per sample, frequency, relative density, and absolute density. 62 of the

144 sites had *D. discoideum*. Of the highest number of *D. discoideum* clones per sample, which varied from 13 to 75, 8 were northern hardwood forest. Of the 6 highest sites with 56–75 clones/sample, 3 were northern hardwood. Of the 25 sites with highest frequencies for *D. discoideum*, varying from 60 to 100 %, 7 were northern hardwood. The conditions in this forest type are: abundant humus mostly of the mor type, acid pH 4–6, and cool temperate climate.

In Ohio, the highest frequencies for *D. discoideum* were in sandstone ravines, which supported hemlock—northern hardwoods. Regionally in eastern North America, this forest is found in New England, Great Lakes region, eastern Canada, and at elevations of 3,500–5,000 feet in the Middle and Southern Appalachians where *D. discoideum* was first discovered (Raper 1984). It is also abundant in cloud forests of Mexico (Cavender and Raper 1968; Cavender et al. 2012) and to some extent in Costa Rica (Cavender 2005, 1963), but is only rarely found in lowland tropical forests. The occurrence of *D. discoideum* in deciduous forest of the northern island of Hokkaido, Japan duplicates that of the northern hardwood forest of eastern North America (Cavender and Kawabe 1989, Kanda, personal com.). *D. discoideum*, however, was not found in the Rocky Mountains (Cavender 1983), California (Quinones 1989), Africa (Cavender 1969b, Cavender et al. 2010), Southeast Asia (Cavender 1976), Australia (Landolt et al. 2008a), New Zealand (Cavender et al. 2002), Patagonia (Vadell et al. 2011), or Europe (Cavender 1969a, b; Romeralo and Lado 2006). There is one report from South America (Vadell and Cavender 2007) and the Cascades of Washington State (Mishou and Haskins 1971), Nepal and Pakistan (Hagiwara 1990, 1996) as well as two reports from India (Agniothruda 1956; Sathe et al. 2010).

A temperature study by the author of 7 isolates from decreasing latitudes of 50, 45, 42, 30, 20, 19, and 12 showed differences in temperature optima for growth and fruiting as well as differences in temperature range at which they could grow. For example, the northern most isolates could grow at 8–10 °C, while the southernmost isolates could not grow below 14–16 °C but could grow at higher temperatures than the others, up to 35 °C. This suggests that there are ecotypes in *D. discoideum*.

D. discoideum is one of the species that develops well with relatively high concentrations of bacteria which may explain why it is one of the species most frequently found on animal dung in a forest environment (Stephenson and Landolt 1992). One more interesting feature is the basal disc, which appears to be an adaptation to hold the fruiting body well above the substratum as it performs as a holdfast securing the fruiting body to projections such as fungal strands.

8 Ecological Individuality

Research shows that dictyostelids, even though they are very primitive in terms of their evolutionary history, do have an ecological individuality with an interesting spectrum of environmental responses and adaptations. The species differ

considerably from one another; *D. mucoroides* is the most prominent species, competing well in a variety of environmental conditions. It may be the only species in very cool environments, e.g., Tierra del Fuego (Vadell et al. 2011), MacQuarie Island (Stephenson et al. 1998), or Alaskan north slope tundra (Cavender 1978). Surprisingly *D. mucoroides* gives way to *D. rosarium* in the drier regions of southern California (Benson and Mahoney 1977) even though in other regions of California *D. mucoroides* is dominant (Quinones 1989). A sister species, *D. sphaerocephalum* often shares extreme cool, dry, or disturbed environments with *D. mucoroides* (Cavender 1983) and sometimes is the more prominent species (Hagiwara 1992) (see Table 1).

When the dictyostelid population is diverse as in Butterfly Woods (Cavender and Cavender, in press), *Polysphondylium violaceum, P. pallidum, D. minutum* and even *D. aureo-stripes* may be more prominent. *Polysphondylium violaceum* is one of the largest species and is always the one that appears first in the isolation plates indicating its rapid growth and development. Although whorled, it is not in the same phylogenetic linkage as the light-spored Polysphondylia such as *P. pallidum* (Schaap et al. 2006; Romeralo et al. 2011) which grow more slowly and can use "killer" substances to compete with other dictyostelids (Mizutani et al. 1990). There are other morphologically close Polysphondylia, e.g., *P. candidum*, *P. pseudocandidum* and *P. album* (Kawakami and Hagiwara 2008) which make group 2B taxonomically difficult. Often *Dictyostelium minutum* (Raper 1941), a group 3 species, usually produces the most propagules germinating in isolation plates when it is present and will have counts in the hundreds in soils with abundant humus (Cavender 1972). This is one of the species that shows diminishing numbers in Ohio (Cavender, unpublished data).*D. discoideum* has some of the most ecologically interesting adaptations. It prefers a cool temperate deciduous forest environment (North America, Japan) except in Europe where it does not occur (DictyStock has a collection by D. Francis from Germany, the only one ever collected). As suggested in Swanson et al. (1999), this disjunct distribution may indicate it is slowly disappearing. It invests heavily in a thick stalk, as does another cool temperate species *D. septentrionalis* (Cavender 1978). The thicker stalk may be an adaptation for fruiting at a cool temperature (20 °C or lower) as it would help keep the stalk erect for a longer time. The basal disc, as it appears on agar, is probably not disc-like in nature but may appear more like a holdfast, enabling the fruiting structure to form on projections above the soil surface such as fungal hyphae. The stalkless slug migration enables it to climb vertically. *Dictyostelium polycephalum*, on the other hand migrates without phototaxis. In Ohio, it prefers litter-free surface of flood plain or depressional forest that has mull humus worked over by earthworms. The stalkless migration may give it an advantage avoiding worms in this type of environment. *Dictyostelium polycephalum* is much more common in the tropics (Cavender et al. 2012; Cavender 1973) where there is less surface litter. The tropics stimulate other species such as *D. purpureum* as well as *D. rhizopodium* and other crampon-based species and the small group 3 dictyostelids (Cavender et al. 2013). *Dictyostelium purpureum* increases proportionately as latitude decreases (Cavender 1973). It also occurs in strand habitat close to the

ocean and may be more tolerant of salt concentration similar to *D. rosarium*. The crampon-based species are subtropical and tropical. The superficially root-like basal crampons increase in size with the size of the species from *D. vinaceo-fuscum* to *D. rhizopodium.* It may help the sorocarp stay erect longer serving as a water-absorbing organism to keep the stalk cells turgid. The small size of the Acytostelia is probably an adaptation associated with their narrow niche requirements. For example, in Ohio *A. leptosomum* is most abundant in sandstone gorges, while *A. magnuphorum* was only found in forest around an acid bog.

The soil pH is important in dictyostelid ecology. Not the highest densities but the highest diversities are associated with neutral pH or slightly alkaline soils. Examples are Butterfly woods in Ohio (Cavender and Cavender, in press) and Tikal seasonal evergreen forest (Vadell 1993). There is also a response to moisture. The wetland forest environments are poor, e.g., true rainforest while the seasonal rainforests appear far better. The oak forests of California are poorer for dictyostelids than the more mesic oak of Southern Wisconsin (Cavender 1963). Of course forest is not the only vegetation with dictyostelids. Dictyostelids have also been recovered from grassland soils (Rollins et al. 2010), canopy soils (Stephenson and Landolt 1998), and agricultural fields (Stephenson and Rajguru 2010) among other habitats.

9 Notes on Global Distribution

The study of global distribution of dictyostelids was given a big boost by the award of a National Science Foundation Plant Biodiversity Inventory grant (NSF PBI DB0316284) for the further inventory of Eumycetozoans. Steve Stephenson at The University of Arkansas was the principal investigator and Cavender (Ohio), Lado (Madrid), and Schnittler (Greifswald) were coinvestigators. The inventory of dictyostelids had been underway for some time resulting in the publication of "global distribution of forest soil dictyostelids" (Swanson et al. 1999). This paper show some big gaps in collecting dictyostelids particularly in the Southern Hemisphere. Funds from PBI were used for inventories in New Zealand (Cavender et al. 2002); Australia (Landolt et al. 2008a), Patagonia (Vadell et al. 2011), Madagascar (unpublished), and South Africa (unpublished). In addition to the PBI-sponsored studies, major forest dictyostelid inventories since 1999 were done in Smoky Mountains National Park (Landolt et al. 2006a, b; Cavender et al. 2005), Alaska (Romeralo et al. 2010), Iguazu Falls, Argentina (Vadell and Cavender 2007), Ohio (Cavender and Vadell 2006; Cavender and Cavender, in press), and Arkansas (Landolt et al. 2009). Additional collecting was done in Mexico at Colima Nevada (Cavender et al. 2012) and in the Aberdare Mountains of Kenya (Landolt et al. 2011). Initial collections were made in northern Thailand (Landolt et al. 2008b), Laos, and Vietnam (Stephenson, unpublished) and in Crimea, Ukraine (Liu and Li 2011).

We now know considerably more about dictyostelid distribution in the Southern Hemisphere. Populations tend to be smaller with fewer species than in the Northern Hemisphere. This was particularly true for forests in South Africa and Patagonia. There were a considerable number of new species in phylogenetic groups 1, 2, and 3, particularly from Australia, e.g., *D. boomeransporum* and *D. myxobasis* (group 1), *D. flexuosum* and *D. granulosum* (2B), *D. radiculatum* (2A) (Romeralo et al. 2011) but only a few new group 4 isolates, e.g., *D. gargantuan*, *D. valdivianum* and *D. austroandinum* from Patagonia. *Dictyostelium mucoroides* dominated populations in southern beech forest (Nothofagus) and in Tierra del Fuego it was the only species although often in high numbers. Numbers per gram of soil were mostly low in South Africa, Australia, New Zealand, and Iguazu Falls National Park, Argentina.

The temperate deciduous forest is the best-studied biome for dictyostelids. There have been intensive studies in Ohio (Cavender and Hopka 1986; Cavender and Vadell 2006; Cavender and Cavender, in press),Virginia and West Virginia (Landolt and Stephenson 1986, 1990), Arkansas (Landolt et al. 2009), Smoky Mountains National Park, Tennesee, and North Carolina (Landolt et al. 2006a, b; Cavender et al. 2005), several countries in Europe (Cavender 1969a, b), Germany (Cavender et al. 1995), Switzerland (Traub et al. 1981a, b), Spain and Portugal (Romeralo and Lado 2006), Nepal (Hagiwara 1982), India (Cavender and Lakhanpal 1986), and Japan (Hagiwara 1989, Cavender and Kawabe 1989). Diversity is greatest in North America and Japan and less in Nepal, India, and Europe. Disjunct species have appeared, e.g., *D. discoideum* is in Japan, Nepal, and Eastern North America but is missing from Europe, *D. fasciculatum* is in Europe and North America but is missing in Japan and Nepal, while *D. microsporum* and *D. capitatum* are in Japan and Europe but are not found in North America at this time. These disjunct species may indicate a wider distribution at one time that is now contracting.

The greatest species richness was in the Smoky Mountains at 30+ species, which comes close to the 32+ species from the country of Mexico, which however will certainly increase as more collecting is done. For distribution in Mexico, the data from four investigators was pooled together which now gives us a good idea of the dictyostelid distribution for the country as a whole (Cavender et al. 2012). There were almost 600 soil samples from 68 sites in desert, thorn forest, tropical deciduous forest, tropical seasonal evergreen forest, and montane and cloud forest. Using relative densities, as a basis of comparison and out of a total of 33 species *D. mucoroides* was the most prominent followed by *Polysphondylium violaceum, P. pallidum, D. purpureum* and *D. minutum*. These are the same species most prominent in eastern North America although in a different order. *Polysphondylium violaceum* was more prominent at the drier end of the vegetational spectrum, e.g., desert and thorn forest (higher soil pH may have also been a factor), while *D. minutum* along with *D. discoideum* are mostly confined to more temperate montane and cloud forest. The most species (24) but not the highest numbers (291/g) were in seasonal evergreen forest, while highest numbers were in tropical deciduous forest (315/g). Montane and cloud forest sites had the next highest numbers

per gram (221/g) and number of species (19). Thirteen species had a relative density of <0.001, which means they are very rare. The number of species per site was 6.4 lower than in Ohio at 8.7 (26 species, 437/g). There is also pooled data for the continent of Africa but much of the continent is unexplored for dictyostelids. East Africa had 10 species (Cavender 1969b), Central African Republic 13 (Cavender et al. 1993), Aberdare Mountains, Kenya 10, South Africa, 7 described, 7 undescribed, and Madagascar, 13 described, 13 undescribed (Cavender et al. 2010). There is a total of 25 species so far for Africa and about the same number for Madagascar. The total species for Africa is low compared to North and South America as well as Asia.

The distribution of dictyostelids in western United States is much different than in the eastern deciduous forest. Conditions are generally drier and conifer tree species become much more abundant, while deciduous broadleaf species are less so. The change begins even in Arkansas (Landolt et al. 2009) where diversity and density diminish and surprisingly *P. pallidum* is the most prominent species. The trend continues in The Rocky Mountains where diversity is even less. However, *P. pallidum* is not a prominent species and populations are dominated overwhelmingly by *D. mucoroides* and *D. sphaerocephalum*. In southern California plant communities (Benson and Mahoney 1977), *D. mucoroides* and *D. sphaerocephalum* were most frequent although another species *D. rosarium* was the most frequently found. This species was first found in the desert of Mexico and the dry environment of southern Texas (Cavender and Raper 1968; Raper and Cavender 1968). Densities were generally very low because of the dry conditions. In northern California, densities were also low; *D. mucoroides* and *D. sphaerocephalum* were again dominants while *D. rosarium* was much less frequent (Quinones 1989). The redwoods of the California coast, a remnant of the Arctotertiary circumglobal forest, harbor quite a different assemblage than the eastern forest. *D. mucoroides* and *D. implicatum* are dominant. There appears to be *D. aureo-stipes var helvetium* in abundance but there is very little *P. pallidum* and *D. minutum* and no *D. discoideum, D. purpureum* or *P. violaceum* were found. Instead there is a different species of violet-pigmented *Polysphondylium* (Cavender, unpublished).

The far north has produced some interesting new dictyostelids. First there is *D. septentrionalis* from the coastal spruce–fir rain forest which is adapted to cooler temperatures than most dictyostelids and will not develop and fruit above 20 °C (Cavender 1978). It is relatively large with a thick stalk, which may be an adaptation to keep the sorocarp erect during the slow development at cool temperatures. Then two species were described from subalpine Alaska (Romeralo et al. 2010). One *D. ammophilum* was found on sand dunes at Kobuk Valley National Park, an area never glaciated. This species has polar spore granules but is a group 4 species. It has morphological similarities to *D. valdivianum* from Patagonia in the southern hemisphere, another rare group 4 species with polar spore granules. It is paired with *D. valdivianum* in the subtree (Romeralo et al. 2011) The second isolate *D. boreale*, from burnt boreal forest near Fairbanks, is situated between the species *D. oculare* and the pale polysphondylids in group 2B (Romeralo et al. 2010). The

boreal and tundra habitats as a whole are poor in species (Cavender 1972, 1978; Landolt et al. 1992a, b; Stephenson et al. 1997). Cavender collected in tundra at Point Barrow and at Mckinley (Denali) National Park. Mckinley Park was especially favorable for dictyostelid growth. *Dictyostelium mucoroides* had 60 % frequency and an average 1,150 clones/g while *D. sphaerocephalum* had a 70 % frequency and an average of 4,100 clones/g from 25 samples. These are very high counts for any habitat and could result from fertility augmentation due to marmot activity. Nine species have been reported for boreal forest in Alaska and along the southern border of Canada Cavender (1972) reported ten species from nine boreal forest sites. The species *D. discoideum, D. lacteum,* and *Acytostelium leptosomum*, were not in Alaska. *Dictyostelium purpureum* and *D. polycephalum* were found in northern hardwood deciduous forest. Densities were high at an average of 1,863 clones/site and *D. mucoroides* was the dominant species in terms of frequency and density followed closely by *D. minutum.* Cavender lumped *D. sphaerocephalum, D. aureo-stipes,* and *D. giganteum* together with *D. mucoroides* because the taxonomy was deficient at the time but states that *D. mucoroides* was the most abundant.

We now have a good idea of the distribution of dictyostelids in most parts of the globe and which species we are likely to find. More collecting is needed in Russia, China, and Indonesia in particular. Many of the species are rare, e.g., in Ohio 40 % of the species would occur once out of a hundred samples. In Mexico, 21 out of 33 species were considered very rare or extremely rare. One of the rare species from Mexico, *D. deminutivum,* which was not found elsewhere, has since turned up from Madagascar indicating a wider distribution or distributional link. Among the very rare isolates are many small dictyostelids. Recent work (Cavender et al. 2013) on ten of these small isolates from Central America shows that they are different species but all belong in phylogenetic group 3. There are many other of these small group 3 isolates from around the globe which have been isolated but not described. Probably most of the species still undiscovered belong in this group.

10 Conclusions

As ecological information is acquired, it becomes possible to use the data in other fields. For example, the NSF-supported global inventory doubled the number of described species. With at least 100 species in hand, investigators were able to construct phylogenetic trees and begin to more accurately assess questions of evolution. It has also become possible to more accurately define a dictyostelid species. There will undoubtedly be other offshoots from these ecological endeavors.

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Evolution of Dictyostelid Social Amoebas Inferred from the Use of Molecular Tools

Maria Romeralo and Omar Fiz-Palacios

Abstract Dictyostelid social amoebas are eukaryotic microbes distributed all around the globe. As with many other protist groups, one fundamental and revolutionary event in the study of dictyostelid (Amoebozoa) systematics has been the use of molecular tools. This has radically changed our understanding of evolution across the group and has greatly expanded the potential use of dictyostelids as model organisms for a wide range of areas including biomedicine, development, evolutionary biology, and molecular ecology. This is further supported by genome sequencing that has been carried out for at least one species in each of the major groups. Phylogenomic data are also essential to pinpointing the origin of diversification of dictyostelids in terrestrial ecosystems, which is basic for understanding the evolutionary history across eukaryotic amoeboid lineages.

Keywords Taxonomy · Protists · Amoebozoa · Soils · Molecular sequences · Molecular clocks

1 Introduction

Protist amoeboid species are difficult to differentiate based only on the morphology and to do this requires a high degree of taxonomic expertise (Caron et al. 2009). Therefore the advent of molecular tools has been a fundamental and revolutionary event in the study of protist systematics as a whole. It started with sequencing

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independent nuclear and mitochondrial molecular markers and continues now with the sequencing of whole genomes. Molecular data and phylogenetic analyses have been crucial in disentangling the phylogenetic position of different amoeboid species across the eukaryotic tree of life (Baldauf et al. 2000; Brown et al. 2009).

Strong convergent evolution is visible among morphological characters of several protist groups that are now known to belong to completely different groups in the eukaryotic tree of life (reviewed in Foissner 2006; Leander 2008). Among amoeboid protists, there are several groups that form multicellular sorocarps, called sorocarpic amoebas. These are scattered among the eukaryotic tree in six major supergroups (see Table 1). Historically, these organisms have been grouped together due to the similarity of their life cycles, but recently molecular phylogenetic analyses of sorocarpic amoebae have shown that these organisms have multiple independent origins (Adl et al. 2012; Brown et al. 2009, 2011). Important changes in the understanding of sorocarpic protist systematics can be illustrated by examples shown in Table 1, which are further described in Chap. 10 of this book.

Within Mycetozoa (Myxogastrea, Dictyostelia, Protostelia), a subgroup of the eukaryotic supergroup Amoebozoa, molecular tools, and more accurate phylogenetic analyses have been recently applied. This started with the phylogeny based on 18S rDNA and EF1a in Myxogastrids (Fiore-Donno et al. 2005, 2008) and was followed by molecular phylogenies of dictyostelid social amoeba based on 18S rDNA, alpha tubulin, and Internal Transcribed Spacer (nrITS) (Schaap et al. 2006; Romeralo et al. 2010a, 2011). Subsequently, phylogenetic analyses were also applied to the study of protostelids (Shadwick et al. 2009). These molecular phylogenies radically changed our previous vision of Mycetozoa systematics. Molecular phylogenies have confirmed that Myxogastrea and Dictyostelia form a monophyletic clade in Amoebozoa (Pawlowski and Burki 2009) while protostelids are highly polyphyletic. Within Mycetozoa, Myxogastrea are the most species-rich group (920 sps), followed by Dictyostelia (150 sps) and Protostelia (35 sps) (numbers from www.eumycetozoa.com).

Myxogastrea, or plasmodial slime molds, can be found in all terrestrial ecosystems and also some resistant stages have been found in aquatic habitats where they persist, but cannot form fruiting bodies (Stephenson et al. 2011). They present a complex life cycle with two vegetative stages: unicellular amoebas alternating with myxoflagellates and multinucleate plasmodia, culminating in the formation of fruiting bodies dispersing multiple spores. Myxogastrea have three dormant stages: microcysts, sclerotia, and spores and their life cycle can be sexually or asexually completed (Schnittler et al. 2012). On the other hand, Protostelia form very simple fruiting bodies. A single amoeba differentiates to form a single, stalked, sporocarp that is 5–500 μm tall and supports one or a few spores. Molecular studies (Fiore-Donno et al. 2010a; Lahr et al. 2011) have shown that protostelids are found in nearly every major branch of Amoebozoa, except in Tubulinea (Shadwick et al. 2009; Fiore-Donno et al. 2010a; Lahr et al. 2011).

Dictyostelid social amoebas, the subject of this book, were discovered in the nineteenth century and, because of their morphological similarity with the genus *Mucor*, were considered to be fungi and named *Dictyostelium mucoroides*.

Table 1 Species complexes across Dictyostelia

Species name	Group according to morphology	Molecular markers	Eukaryotic supergroup according to molecular data	References
Acrasis rosea	Acrasids	GAPDH, SSU + Protein coding genes	Excavate	Roger et al. (1996); Keeling and Doolittle (1996); Andersson and Roger (2002); Archibald et al. (2002); Nikolaev et al. (2004); Stechmann et al. (2006)
Guttulinopsis vulgaris	Acrasids	159 Protein dataset	Rhizaria	Brown et al. (2012)
Fonticula alba	Fungi	SSU, Actin, beta-tubulin, EF1-alpha, the cytosolic isoform of heat shock protein 70	Opisthokonta	Brown et al. (2009)
Sorogena stoianovitchae	Ciliates	Nuclear and mitochondrial SSU	Alveolate	Lasek-Nesselquist and Katz (2001); Dunthorn et al. (2008, 2011)
Sorodiplophrys stercorea	Ciliates	SSU	Stramenopiles	Baldauf et al. (2000)
Dictyostelium discoideum	Mycetozoa	SSU, ITS, Alpha tubulin	Amoebozoa	Schaap et al. (2006); Romeralo et al. (2010a, b)
Copromyxa protea	Acrasids	SSU	Amoebozoa	Brown et al. (2011)

Subsequently, some studies have demonstrated that the absence of hyphae, the presence of a cellulose wall and their aggregative behavior separates them from fungi or other groups of protists such as acrasids. Finally, the use of molecular tools such as 18S rDNA has helped to confidently place them within the eukaryotic supergroup Amoebozoa (Baldauf et al. 2000).

With phylogenetic relationships among eukaryotic groups becoming more and more resolved, analyses using molecular data are now possible to be performed. This will be of benefit for future studies on biogeography, comparative analysis, diversification rates, and reconstruction of divergence times. In fact, different approaches to reconstruct molecular clocks have been carried out recently, placing the origin of diversification of dictyostelids several hundred million years ago. Molecular tools have been key in developing this kind of analysis in groups like Dictyostelids where there is a complete absence of a fossil record.

2 Morphology Versus Molecular Data

Traditionally dictyostelid species were divided into three genera based on morphological characters: *Acytostelium* (acellular stalks), *Dictyostelium* (cellular stalks), and *Polysphondylium* (cellular stalks with whorls of branches) (Raper 1984). The first molecular phylogeny of Dictyostelia, based on two independent molecular markers (18S rDNA and alpha tubulin) divided the known species into four major groups, which do not correspond to any of the three traditional genera. Moreover, it showed a tremendous molecular depth roughly equivalent to metazoa. This phylogeny radically changed previous beliefs about evolution of form and function across the group (Schaap et al. 2006).

In 2007, Romeralo et al. demonstrated the suitability of the nrITS for assessing dictyostelid phylogenetics. Three years later a joint 18S rDNA–nrITS molecular phylogeny was produced for all species from the 2006 phylogeny, which resolved relationships to a finer level and revealed the presence of species complexes within all major morphotypes (*Acytostelium*, *Dictyostelium,* and *Polysphondylium*), (see Table 2, Romeralo et al. 2010a, b).

In 2009 molecular data (18S rDNA and nrITS sequences) were applied to confirmed species based on morphological characters. Since then, it has become standard for dictyostelid species to be described based on both morphological and molecular data (Romeralo et al. 2009, 2010a, b; Vadell et al. 2011; Cavender et al. 2013; Perrigo et al. 2013). This is helping enormously in the difficult task of resolving morphological species complex (cryptic species), across the group (e.g., *D. mucoroides*, *Polysphondylium pallidum*) (see Table 2).

In just the last 10 years the number of described species of Dictyostelia has been doubled. This is mainly due to an intensive sampling effort ("The Eumycetozoa project") to sample mycetozoa from different biomes across the world. In 2011 an updated molecular phylogeny based on 18S rDNA sequences was released (Romeralo et al. 2011). The new phylogeny continues to show the four previously

Table 2 Species of sorocarpic protists distributed among five major eukaryotic groups

Species name	Dictyostelid group	References	Molecular markers	Genome sequenced
Dictyostelium aureostipes	Group 1	Romeralo et al. (2010a, b)	18S rDNA, nrITS	Not
Dictyostelium fasciculatum	Group 1	Romeralo et al. (2010a, b)	18S rDNA, nrITS	Yes
Acytostelium leptosomum	Group 2A	Romeralo et al. (2011)	18S rDNA	Not
Acytostelium amazonicum	Group 2A	Romeralo et al. (2011)	18S rDNA	Not
Polysphondylium pallidum	Group 2B	Schaap et al. (2006; Romeralo et al. (2010a, b)	18S rDNA, nrITS	Yes
Dictyostelium tenue	Group 3	Romeralo et al. (2010a, b)	18S rDNA, nrITS	Yes
Dictyostelium sphaerocephalum	Group 4	Romeralo et al. (2007)	nrITS	Not
Dictyostelium discoideum	Group 4	Schaap et al. (2006); Romeralo et al. (2010a, b)	18S rDNA, nrITS	Yes
Dictyostelium mucoroides	Group 4	Schaap et al. (2006; Romeralo et al. (2010a, b)	18S rDNA, nrITS	Not
Dictyostelium polycarpum	Polycarpum Complex Group	Romeralo et al. (2011)	18S rDNA	Not
Dictyostelium polycephalum	Polycephalum Complex Group	Romeralo et al. (2011)	18S rDNA	Not
Polysphondylium violaceum	Violaceum Complex Group	Romeralo et al. (2011)	18S rDNA	Not

identified major groups. In addition, three previously isolated branches are now seen to form major divisions in their own right. They are formed by a lower number of isolates and provisionally named after the main species. We refer to these new groups as the "polycarpum", "polycephalum", and "violaceum" complexes in order to retain the original numbering scheme until formal names can be assigned. In addition the new species further emphasize the deep split in Group 2 recognizing two separate major groups, Group 2A and Group 2B.

Many of the species described in the last 10 years contradict the few morphological patterns tentatively identified within the first molecular phylogeny (Schaap et al. 2006) particularly for Groups 1 and 4 (e.g., some of the new species in Group 4 have clustered and coremiform sorocarps, presence of branches and polar granules inside their spores). Furthermore there is a high level of species complexes found throughout the Dictyostelia, and most morphotypes have not been examined from multiple isolates. However, since morphological patterns can be identified for more limited subgroups, morphological evolution seems to be at least moderately conservative. The lack of deeper morphological patterns should perhaps not be entirely unexpected given the small numbers of characters, the essentially simple nature of many of them, and the apparent antiquity of the group. The future discovery of additional species, together with extensive genome sequencing, should lead to a better understanding of the mechanisms and evolutionary forces shaping them.

In brief, Dictyostelids Group 1 is formed only by *Dictyostelium* morphotypes. Group 2A includes all acytostelid species except *A. ellipticum*, which belongs to Group 2B. Group 2B is a much more heterogeneous group with representatives of the three morphotypes. Group 3 is exclusively made up of *Dictyostelium* morphotypes as is Group 4, which is the richest species group and includes the model organism, *Dictyostelium discoideum.* The Polycarpum Complex Group, formed by two isolates of the morphotypes *Dictyostelium polycarpum,* whose 18S sequences are as different as any other two species in Group 4. The Polycephalum Complex Group includes five different isolates of the morphotypes *Dictyostelium polycephalum* and their 18S sequences are also extremely different. Finally the "Violaceum Complex" Group includes representatives from the *Dictyostelium* and the *Polysphondylium* morphotypes (Fig. 1). The new species revealed the presence of species complexes across the group (Table 2). Because of this inconsistency between phylogenetic groupings and the systematic naming of species in the group a major taxonomic revision of Dictyostelia is urgently needed.

Despite these advances, until recently we were still missing a very important point in the phylogenetic reconstructions: the position of the root. This is the oldest point in a phylogeny and therefore essential to infer evolutionary patterns over time. This issue has been recently addressed with the use of genomic data (Romeralo et al. 2013). Orthologs for 32 genes were retrieved from the six dictyostelid (Heidel et al. 2011) and three amoebozoan outgroup genomes and consensus alignments for the 32 encoded protein sequences were performed. The concatenated alignment of about 181,80 amino acids robustly placed the root between the branches of Groups 1–2 and 3–4 (Romeralo et al. 2013). In agreement

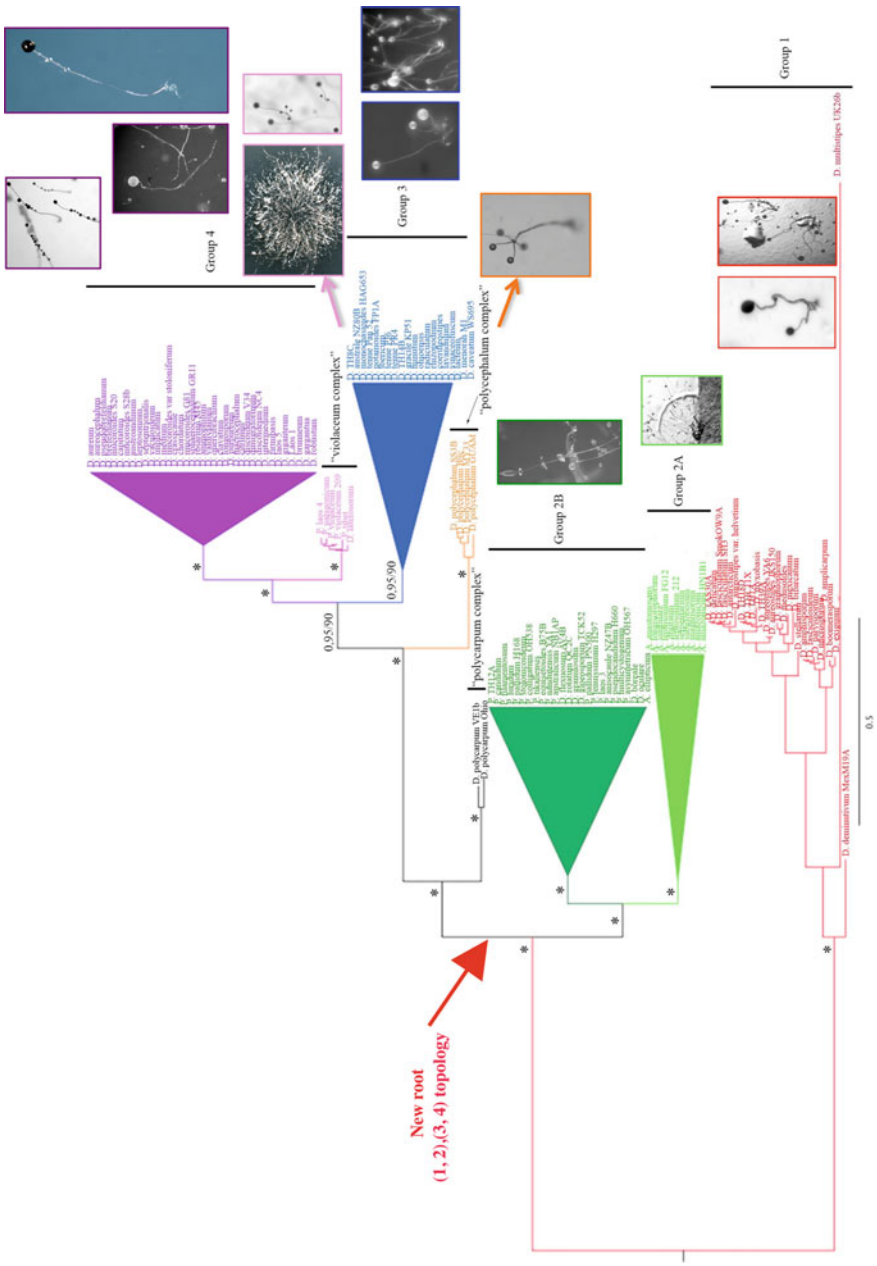

Fig. 1 Molecular phylogeny showing the major groups. (Modified from Romeralo et al. 2011)

with the previous SSU rDNA phylogenies, the inferred phylogeny largely retains the same ordering of species within the four major groups (Romeralo et al. 2011; Schaap et al. 2006), but instead of the (1), (2, 3, 4) topology shown then, now displays the (1, 2), (3, 4) topology.

Molecular tools are also becoming relevant in the study of population genetics in Dictyostelia but so far, such analyses have been carried out exclusively in Dictyostelid Group 4 species and the "Violaceum Complex" Group and show different results depending upon the species used. The species *Dictyostelium rosarium* seems to be a well-define species with all studied isolates forming a monophyletic group (Romeralo et al. 2010b) while *Dictyostelium purpureum* shows extensive genetic variation between populations and clear evidence of phylogenetic structure (Mehdiabadi et al. 2009). On the other hand, the species *Dictyostelium giganteum*, displays little genetic variation, phylogeographic structure or genetic differentiation among populations, relative to the cryptic species observed within *D. purpureum* (Mehdiabadi et al. 2010). Within the model organism *D. discoideum,* the different isolates examined form a monophyletic group, but there are several subclades and pronounced genetic differentiation among locations, suggesting the presence of geographic or other barriers between populations. These results reveal the need for further investigation into potential cryptic species (Douglas et al. 2011). Finally *Polysphondylium violaceum* phylogenetic analyses also suggest the possibility of cryptic species. The level of divergence found is comparable to the divergence between sibling species in other dictyostelids (Kalla et al. 2011). These results have important implications for our understanding of speciation and social evolution in dictyostelids in particular and eukaryotic microbes in general.

The existence of molecular tools has also allowed us to study the diversity of Dictyostelia by culture-independent techniques such as culture-independent PCR (ciPCR) (Romeralo, unpublished data). Development of ciPCR has been particularly challenging for soils, which are complex ecosystems dominated by fungi. Preliminary results show a big diversity of unknown phylotypes distributed across the molecular tree. Here again, the recent development of modern technologies of massive sequencing such as 454 or Illumina and the availability of dictyostelid-specific primers will probably change our current vision of Dictyostelia systematics and biogeography in ways that are intriguing to contemplate.

3 Diversification of Dictyostelia in the Proterozoic

Fossil calibration, the assignment of a fossil's age to its corresponding node in a phylogeny, is an essential tool used to reconstruct divergence times. Also, a fully resolve phylogeny is required for an accurate reconstruction of divergence times and this notably depends on the number of taxa and the number of genes. Unfortunately, Dictyostelia lacks a fossil record due to the absence of hard "fossilizable" structures and only two molecular markers (18S rDNA and nrITS)

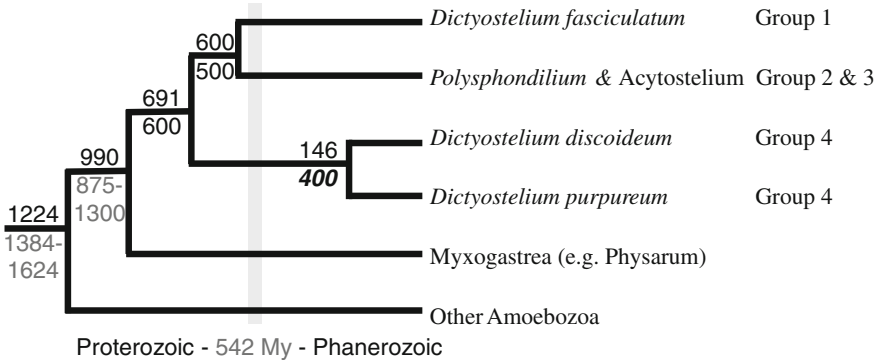

Fig. 2 Reconstructions of divergence times for Dictyostelia from different molecular studies. *Vertical gray bar* indicates the placing of the Proterozoic-Phanerozoic transition. Numbers above branches are divergence time after Fiz et al. (2013); (*bold*), below branches after Parfrey et al. (2011) (*grey*), Sucgang et al. (2011) (*kursive*) and Heidel et al. (2011). Note that branch lengths are not scaled to time

are currently sequenced for most known dictyostelids species. The nrITS marker is largely unalignable among the major groups of dictyostelids. In fact recent phylogenies use alignments of the individual groups of dictyostelids (Romeralo et al. 2010a, b). Therefore no sister group with a fossil record can be incorporated into a nrITS alignment. Since no internal calibration is possible in dictyostelids the only option is the use of a secondary calibration derived from other reconstruction, which would incorporate a huge uncertainty on reconstructed molecular ages. On the other hand, using a 18S rDNA phylogeny allows for the incorporation of other amoebozoans which do have a fossil record. However, the use of 18S rDNA for divergence time reconstruction of two closely related species may not be very robust since it may rely on few (3–4) nucleotides difference between the species. Therefore tracing a fine scale chronology within dictyostelids species becomes highly difficult with the data currently available. Alternatively, different approaches can be taken at higher taxonomic levels, such as, the use of a universal rate of substitution or the use of fossils from outside Amoebozoa.

The interest in molecular dating of protist is growing thanks to the advent of new and abundant genomic data (Groussin et al. 2011). Two recent genomic studies (Heidel et al. 2011; Sucgang et al. 2011) sought to reconstruct divergence times for major groups of dictyostelids. These two studies applied a universal rate of nucleotide substitution and then re-scaled the branch lengths for their phylogenetic tree (known as a "strict molecular clock"). In the case of Heidel et al. (2011) the tree included three dictyostelids and six representatives of fungi, animals, and plants. Heidel et al. (2011) retrieved an age for the dictyostelids' crown node of 600 My (*D. discoideum* from *Dictyostelium fasciculatum* plus *P. pallidum* split; Fig. 2) while Sucgang et al. (2011) obtained an age of 400 My for the crown age of Group 4 (*D. discoideum–D. purpureu*m split; Fig. 2). One of the risks associated with the use of strict molecular clocks (universal rate of substitution) is

the overestimation of reconstructed ages (Benton and Ayala 2003). For instance, using this method Heidel et al. (2011) estimated an age of 560 My for the crown age of land plants despite the fact that the oldest evidence of a land plant is almost 100 My younger (Rubinstein et al. 2010). On the other hand if the rate of substitution used by Sucgang et al. (2011) for Group 4 is applied in the eukaryote tree of life then the split between prokaryotes and eukaryotes would probably predate the age of any kind of eukaryote fossil including the oldest which are around 2,000 My and are very disputable (Berney and Pawlowski 2006).

Fossil calibration from outside Amoebozoa can be used for temporal reconstructions of major clades in Dictyostelids. When including only sister groups of Dictyostelids for which fossils are present (e.g., Arcellinids; Schmidt et al. 2004) the problem is the lack of resolution of the phylogenetic tree of Amoebozoa (Lahr et al. 2011). This has led to the reconstruction of divergence times using large-scale phylogenies. Different fossils from sister clades (e.g., Fungi, Metazoa) can be assigned to their corresponding nodes and the ages for major clades of Amoebozoa can be recovered. Parfrey et al. (2011) reconstructed divergence times for the eukaryote tree of life in this way using up to 15 genes. They also used multiple fossil calibration points and found ages of more than 1,000 My for the dictyostelids (Fig. 2). Unfortunately, in the calibration scheme of Parfrey et al. (2011) the oldest amoebozoan fossil (742–770 My; Porter and Knoll 2000) was assigned to the Arcellinids, which is highly debated (Cavalier-Smith 2009). A conservative approach would be to assign this fossil to Lobosa (as in Berney and Pawlowski 2006) or even more conservatively to assign it to the crown node of Amoebozoa. Interestingly, one of the alternative analyses of Parfrey et al. (2011), where Amoebozoa is sister to Opisthokonts, retrieved a crown age of these amoebas (ca. 1150 My; Fig. S5 on Parfrey et al. 2011) which is more consistent with other studies (Berney and Pawlowski 2006).

The most recent studies dating dictyostelids diversification include a wide sampling of dictyostelid species (Fiz-Palacios et al. 2013) and a high number of genes (Heidel et al. 2011). As mentioned above, Heidel et al. (2011) did not use fossil calibration but rate extrapolation while Fiz-Palacios et al. (2013) used relaxed-clock methods (that allows for branch substitution rates to be independent across the tree) together with different fossil calibration schemes from outside Amoebozoa. These two studies widely agree in placing the origin of diversification of dictyostelids (crown age). While Heidel et al. (2011) placed the origin around 600 My, Fiz-palacios et al. (2013) estimated slightly older ages within the Proterozoic (Fig. 2). Therefore both studies suggest a land colonization of dictyostelids predating the land plants' "terrestrialization" in the Ordovician. Different evolutionary perspectives can support the early colonization of land by dictyostelids. On one hand, Heidel et al. (2011) argue that dictyostelids have (1) non-aquatic fruiting bodies and (2) high resistance to DNA damage by UV due to absence of plant canopy. On the other hand, Fiz-Palacios et al. (2013), in line with other authors (Kenrick and Crane 1997 and Porter and Knoll 2000), suggest that dictyostelids diversification happens thanks to the rise of terrestrial ecosystems. A synchrony of diversification among different eukaryotes can be inferred when

considering the fossil record of land plants (470 My; Rubinstein et al. 2010), arbuscular mycorrhizal fungi (ca. 460 My; Taylor and Barbee 2006) and different soils arthropods (see Rehm et al. 2011). Then the question remains regarding the dictyostelids' sister group, Myxogastrea, which is a species-rich clade (ca. 920 species) of terrestrial organisms. Did both sister clades (Dictyostelids and Myxogastrids) colonize land independently? Or did the last common ancestor colonized land with a later specialization on coarse woody debris, ground litter, and the bark surface of living trees in Myxogastrea and forests soil/litter microhabitat in Dictyostelia? We hope that the coming genomic data of Dictyostelida and sister groups, as well as the improvement of molecular clocks techniques, will help resolving these and other evolutionary questions.

4 Ecology and Biogeography of Protists?

An important outcome of the use of molecular tools is their relevance in the fields of ecology and biogeography. Since as little as 1–10 % of all microbial organisms can be cultured (Handelsman and Smalla 2003; Pace 1997; Foissner 2006), environmental surveys based on the amplification, cloning, and sequencing of small-subunit ribosomal RNA genes (18S rDNA) directly from the environment, are a powerful tool to study the diversity of microorganisms (Lara et al. 2007; Caron et al. 2012). This is especially true with protists, where the extent of total diversity is unknown and of considerable debate (review in Foissner 2006). Environmental DNA surveys can also be used to compare several taxonomical groups within a locality (Fierer 2007; Moreira and Lopez-Garcia 2002) or to test biogeographical hypothesis such as the "everything is everywhere (EiE), but the environment selects" hypothesis (Bass et al. 2007). However, there is still no common species concept for protists as a group and it remains difficult to evaluate to which extent morphological, ecological, and/or ultrastructural variation is associated with genetic variation (Boenigk et al. 2012).

These taxonomical uncertainties together with the ancient origin of the protist groups may have led to the establishment of hypothesis such as the EiE (Fenchel and Finlay 2004). This controversy over microbial biogeography (Foissner 2006) presents two main hypotheses. On the one side of the EiE debate it is argued that free-living microbial eukaryotes can reach any geographic location due to their small size, large number of progeny, and great dispersal capabilities, and as a result they will establish at any favorable environment (Fenchel and Finlay 2004; Finlay 2002). On the other side it is argued that dispersal is restricted in some protists and this is reflected in the geographic distribution patterns of different species (i.e., not any habitat favorable for a micro-eukaryote species will be occupied due to dispersal limitations). This is commonly referred to as the moderate endemicity model (Foissner 2006). Biogeography is a quite well-developed field for animals and plants and a hot topic for protist nowadays (Fierer 2008). Protist species with restricted distributions have already been found (Foissner

2006, 2008; Smith and Wilkinson 2007). Furthermore, the use of molecular data and phylogenetic analyses is helping enormously to resolve the common problem of geographically restricted cryptic species across groups (Foissner 2006; Epstein and Lopez-Garcia 2008). Molecular clocks can further help in untangling species delimitations by providing a temporal framework: i.e., a clade of 50 myr is likely to harbor more cryptic species than a clade of 1 myr. This highlights the critical importance of molecular data in protist species delimitations and thus in differentiating cosmopolitan versus geographically restricted species.

Within the soil, Dictyostelia are major consumers of bacteria and play an important role in nutrient cycling and soil health (Raper 1984; Hagiwara 1989). However, information about the role of dictyostelid amoebas in soils remains limited, mostly because they can only be differentiated by their multicellular structures, which are microscopic and therefore only observable in laboratory-based cultures. As a consequence, we know relatively little about the behavior of dictyostelids in their natural habitats. Their ecological relationships with other taxonomic groups are also unknown, other than the fact that they are bacterivores. Some general trends have been suggested over the years, but these have been difficult to test by traditional methods. The culture-independent approach allows us to examine these questions on a broad scale for the first time and lays the necessary groundwork for future quantitative studies. These will allow us to study soil samples worldwide and get insights into the ecological role dictyostelid amoebas play in soil ecosystems. At the same time, this will allow us to have a better understanding of dictyostelid diversity and distribution and therefore be in a better position to answer questions such as: is everything really everywhere?

The genomic era has brought about a great variety of techniques for measuring biodiversity. The latest advance in the field is the single-cell genome sequencing which could be a highly beneficial approach to understand the microbial diversity and evolution of different ecosystems worldwide (Kalinsky and Quake 2011; Kalinsky et al. 2011). This is especially important in protists where the known diversity is very small and only 1–10 % of the species are cultivable. Therefore, the application of this technique is an exciting and promising approach for the field, as shown by some examples from uncultured bacteria from the human mouth (Marcy et al. 2007) and the first single-cell archaeal genome (Blainey et al. 2011).

5 Concluding Remarks

The study of dictyostelids has enormously benefited from the arrival of the genomic era. Thanks to molecular data we know that the genetic diversity of the group is enormous and equivalent to that of metazoan, however, only ca. 150 species are known at the time of writing. Multiple comparisons can be made across dictyostelid social amoeba (Amoebozoa) and Metazoa, especially considering that many genes have been conserved in this group and lost in fungi, metazoa's sister group (for more details see Chap. 1 in this book). In the near future the availability

of genomes within Amoebozoa and other protists groups will allow us to extend these comparisons to other major groups across the tree of life. Especially interesting will be the sequencing of genomes among the sorocarpic protist groups, which are distributed in five major eukaryotic groups (Amoebozoa, Alveolates, Excavata, Opisthokonta, and Rhizaria), in order to gain insights into the evolution of multicellularity across the tree of life. Finally, molecular clocks assessments will also play a key role in the genomic era, thus making it possible to date big evolutionary events such as the origin(s) of multicellularity.

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The Evolution of the Cellular Slime Molds

John Tyler Bonner

Abstract The cellular slime molds have existed for a very long time; they have an ancient history. It is surprising that the early morphological species have not been supplanted and gone extinct, as is the case for larger organisms; a sizable number of ancient cellular slime molds still exist today—they are living fossils. This unusual phenomenon can be explained if one assumes that their morphology is only weakly affected by natural selection leading to a modest variation of morphology among the different species. I argue that the reason for their apparent relative immunity to the effects of natural selection can be explained by their small size; this is a general rule among all microorganisms.

A few years ago when working on a book on the biology of the cellular slime molds, I asked myself when did they first appear on the surface of the earth? The main function of their fruiting bodies, as they stick up into the air, is to facilitate dispersal, to spread their spores so that they might reach a distant patch of bacterial food. They do this by lifting the sticky spore masses up into the air and the spores become attached to insects and other motile invertebrates passing by. This led to the question of when did soil first appear on the surface of the earth and when might that soil be inhabited by small, motile animals? For answers to these questions I turned to some paleontologists who were most helpful: a colleague, Gerta Keller whose office is further down my hallway at Princeton University, and Gregg Retallack at the University of Oregon, an authority on the origin of soils in the early history of our globe.

Their answer was that certainly the required conditions of soil with small crawling beasties were present in the Ordovician, over 400 million years ago, but I was cautioned that new discoveries might push the time even further back. More recently, there have been two molecular studies that estimate the time of divergence of the ancestor of two modern species and the span they find is from 400

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million years ago, to 600 (Sucgang et al. 2011; Heidel et al. 2008). All of these approaches to the problem are consistent; cellular slime molds are indeed very ancient.

These latter studies are founded on the pioneer work establishing molecular phylogenetic trees among the living species of cellular slime molds of Schaap et al. (2006) and Romeralo et al. (2011). Their work made clear that cellular slime molds have existed for a long span of time Some species are ancient and the age of other species arose later over a great time span. This means one can trace the molecular changes that have taken place over an enormous period of time. This has been done to great advantage by Alvarez-Curto et al. (2005) for the chemotaxis biochemistry in the dictyostelids. They built on the discovery of Parent and Devreotes (1996) that in *Dictyostelium discoideum* there are four related proteins that are receptors for cyclic AMP and they are produced at different times during development. Each one, once triggered by cyclic AMP, has a specific effect on a development step. The first appears at aggregation where it is turned on by cyclic AMP, with the result that the stimulated cell produces and emits additional cyclic AMP—it is autocatalytic. Alvarez-Curto et al. found that in an ancestral slime mold, *Dictyostelium minutum*, only one such protein existed and it was one that appeared later in development to activate some other step that requires the stimulus of cyclic AMP. It has long been known that *D. minutum* uses folic acid as its acrasin, the chemoattractant responsible for aggregation (Pan et al. 1972). It might be presumed that since vegetative amoebae are attracted to bacteria that give off folic acid, this folic acid feeding chemotaxis was commandeered to bring the amoebae together in aggregation in early cellular slime mold history. And later it was supplanted by cyclic AMP that became the aggregation attractant. A similar example has been revealed by T. Winkler and his group (Asghar et al. 2012). They find that the dipeptide glorin, which is known to be the aggregation chemoattractant for *Polysphondylium,* also exists in other more ancient species where it plays a different role and activates developmental genes.

To me the most interesting aspect of these molecular phylogenies is that we can follow a sequence of biochemical change, which may become increasingly elaborate as we have just seen, yet the morphological changes are few and the fruiting bodies remain remarkably similar over the ages. There has been a conspicuous biochemical evolution which is not necessarily accompanied by an equivalent dramatic morphological evolution. Furthermore, many of the primitive ancestors continue to thrive today. At least in many cases the old were not supplanted by the new; they both seem to be present today and exist side by side. This seems counter intuitive. Schaap (2007) provides an excellent summary of the molecular evolution and how it relates to the morphological evolution.

We are all brought up with the notion that evolution consists of the new supplanting the old. This certainly is the case for most large organisms such as various dinosaurs, and other groups of reptiles that finally went extinct, and were eventually replaced by mammals that went through the same process so the mammals living today little resemble their early ancestors. As a good example take the evolution of the horse where, starting with the small dawn horse, there are

numerous fossils of extinct ancestors some of which led to the modern horse. In all these cases, we do not find the ancestors still present today.

There are a few notable exceptions: horseshoe crabs exist today pretty much the same as they were 250 million years ago; coelacanth fish were found not too long ago in inaccessible depths in the ocean where they have existed relatively unchanged for around 100 million years. There are also good examples among plants such as the primitive Ginko tree, which has recently been found in the wild, and has existed relatively unchanged for 270 million years (until recently it was thought to be extinct and only existing as a cultivated plant in ancient Buddhist monasteries in China, but now wild trees have been found). There is a similar story for metasequoia, which was only known as a fossil until the 1940s where it was found thriving in a remote part of China. These are all considered to fit Darwin's concept of "living fossils," and are most interesting exceptions to the rule of the old being supplanted by the new.

This picture of the grand scheme of evolution is the conventional one, and the exceptions that have just been described are explained away in a number of fashions, all of which fit in with conventional wisdom. They are adapted to a niche that has not changed over geological time; their formula has, for eons, remained stable; they are isolated from competition because they had unbeatable qualities that kept them thriving. These are not very satisfactory explanations because they are so general, but it is hard to be certain of anything more explicit.

Let us now look at the evolution of cellular slime molds with this background of the case for larger, more complex organisms. Here are some of the differences. There are no known fossil cellular slime molds. This means we have no way of knowing how much extinction has occurred over the last many million of years. We know there are numerous species that exist today, and with confidence we can say which ones are ancient and which ones are relatively modern, as we have learned from the molecular phylogenetic trees. They would seem to indicate that the species that live today are in fact "living fossils." They have not all gone extinct, but exist in considerable numbers compared to the small number of large, complex organisms that have not done so—is a surprising difference. Let us ask the question why?

In recent years, I have been possessed with the idea that small organisms are affected differently by natural selection than large ones and have presented the case in a book (Bonner 2013). The main point is that small organisms (which include cellular slime molds) are more likely to have morphologies that are relatively unaffected by natural selection than large ones; their morphology is more likely to be the result of a random change, a random mutation that has been to some degree ignored by natural selection. I argue that such an explanation might be the best one for small organisms, such as radiolaria and diatoms. There are approximately 50,000 known species of radiolaria, and some 100,000 species of diatoms. It is hard to imagine that such an extraordinary variation on the shape of their skeletons is the result of thousands of selection events. It is easier to argue that many of them might be the result of random mutation and that some of the

shapes produced are barren of features that can be seen and culled by natural selection. Their morphology is relatively neutral.

Furthermore they have left a fossil record for they have inorganic skeletons, usually made of silica. While some show clear evolutionary changes in the successive layers of rock, it is a well known fact that we find ancient rocks that have species of radiolaria, foraminifera, and diatoms that appear to be identical, or very similar, to ones that exist live today. In a fashion this makes up for the fact that we lack cellular slime mold fossils; other microorganisms that have fossils support, the idea that small organisms can remain relatively unchanged morphologically over great spans of geological time despite the internal biochemical changes that may have occurred.

It has been pointed out to me by V. Nanjundiah that in the third edition of *The Origin of Species,* there is a statement by Darwin that very much seems to be saying the same thing. He makes the point that: "If it were no advantage, these (simple) forms would be left by natural selection unimproved or but little improved; and might remain for indefinite ages in their present little advanced condition. And geology tells us that some of the lowest forms, as the infusoria and rhizopods, have remained for an enormous period in nearly their present state."

Now I shall look to cellular slime molds to see if there is any evidence that the morphology of these microorganisms is also only modestly affected by natural selection. Let me begin my argument by discussing their morphology in a general way and contrasting to what is found in larger organisms, something that is familiar to us all.

Perhaps one of the best known and most striking examples of adaptive radiation is that of the Hawaiian honeycreepers. It is estimated that their ancestor arrived on the islands somewhere between 3 and 7 million years ago. The first honeycreeper arrived in a complex tropical environment laden with a great richness of possible niches and they radically diversified into 54 different species. And they are indeed very different: with an impressive variety of beak shapes to reach nectar in different shaped flowers and to reach other foods, such as larval insects under bark. Besides such niche filling, like most birds they have developed sexual selection, producing among other things, tremendous variety in their plumage and its coloration. There already have been some extinctions, and in this modern world, we can expect many more to follow.

Now contrast this to the cellular slime molds, which have probably been on earth for many millions of years. The biggest difference is how little variety there is in their morphology, despite having been around for so long. It is true there is some variety: there can be single stalks with terminal spore masses (sori), single stalks with a series of sori along the stalk, branching that come in more than one form, and clusters of stalks each with a terminal sorus (a coremiform fruiting body), and numerous minor variations to these shapes (see Schaap 2007 for details). But this is nothing compared to the honeycreepers that achieved so much more in a much shorter time. How can we explain this difference?

First there is a big difference in the two environments. Hawaii is packed with a great variety of niches, especially ones connected with food. There is not only

nectar from many different shaped flowers, but there are a variety of insects and their larvae, which the birds can tug and tweak out of their holes. The upper layer of earth, where we find the cellular slime molds, is of a much simpler composition. While it does hold many small invertebrates, they are not food for slime molds. If anything it is the reverse and the slime mold amoebae are their prey. For instance, it is known that their amoebae are devoured by nematodes, a common inhabitant in the soil (Kessin et al. 1996), and no doubt other animals as well. The important point is that the small amoebae can only feed on bacteria (and other little cells, such as small yeasts). They are known to have food preferences, and Horn (1971) showed they like some species of bacteria better than others and will eat them with greater vigor. But it is also known from the earlier work of Raper (1937) that cellular slime molds will consume most species of bacteria regardless of their preferences. This means that it is unlikely that there are food niches for them in their soil habitat; any old bacterium will do. As far as the richness of niches is concerned, honeycreepers are surrounded by innumerable opportunities, while slime mold's only opportunity is the presence or absence of bacterial food. They are limited by chance—where the food is located—and by the size of the food—only very small size will do.

The key trick is to find those virgin patches of bacteria. After cleaning an area of food their only road to survival is to find more food. This is the same problem facing many animals: a hungry lion must find another antelope; a vulture must find another carcass, and so forth. Each of these large animal examples involves searching, and the searching involves locomotion, and usually orientated locomotion. In the case of cellular slime molds, as we shall see, some species also make use of locomotion, and even oriented locomotion. However, their main method of finding food involves the locomotion of others—of small animals in the soil, that brush by their fruiting bodies and carry away the sticky spores, and where, by chance, the animal might pass by a virgin clump of bacteria and in the process shed a few—or just one—of those spores, thereby giving rise to a new generation of slime molds. It should be added that the spores can also be spread by rain. Furthermore, by sticking the spore mass up into the air it might help preserve the viability of the spores in inclement conditions such as freezing weather.

These thoughts lead to a number of topics that I would like to examine in more detail. They are (1) the great power of natural selection for dispersal; (2) the rise of the ability of slime molds to move and orient to facilitate dispersal; (3) the paucity of fruiting body shapes; and (4) evidence for selection to specific environments.

1 The Great Power of Natural Selection for Dispersal

Since finding a fresh source of food is of such paramount importance to so many organisms, any change that favors dispersal will be favored by natural selection. Were there no mechanisms to permit and enhance dispersal, extinction would be

the common result. The ability to disperse will increase the chances of survival, which is the very foundation of natural selection.

If one looks to microorganisms that inhabit the soil one finds a great abundance of organisms, not only cellular slime molds, but innumerable other molds that are fungi. The number of species of such molds in the soil is quite remarkable; there are many thousand worldwide. To see their ubiquitous presence one needs only to take some soil into the laboratory, keep it moist and covered in a glass chamber, and it will soon sprout some small fungal fruiting bodies at its surface. If one is lucky one might see a small spider, or a mite, or some other small invertebrate, brushing by the fruiting bodies and picking up the spores. Some species of fungi favor their spores being dispersed by wind, but here also the stalk assists the dispersal.

There are a very large number of molds that have independently evolved the same mechanism for dispersal. It is possibly the biggest example of convergent evolution that is known, as I have argued elsewhere (Bonner 2009, p. 38 ff). The key is that in all these examples their spore masses are lifted up into the air above the soil: that is the feature for which there is such strong selection and appears in so many soil organisms.

2 The Ability of Slime Molds to Move and Orient to Facilitate Dispersal

One major trend in the evolution of cellular slime molds as revealed in the phylogenetic trees has been an increase in size of the fruiting bodies; the modern species tend to be larger. This fact is of special interest in the light of the effect of size on the behavior of the multicellular stage. Only larger species can, in a mass, migrate from the feeding zone below the surface of the earth to the optimal place to fruit, namely the soil surface. The smaller species cannot migrate at all. This is true even for a very small aggregate of a large species: it fruits where it aggregates.

The migrating slugs (with or without a stalk) can migrate great distances if the conditions are suitably moist. Furthermore they orient toward the surface of the soil, and how they do this has been the object of a great many experimental studies over the years (for a review, see Bonner 2009, Chap. 5). In brief, they will orient toward light, in a sophisticated way in heat gradients, and in gas gradients. And all these sensitive orientations seemed to direct the cell masses to the optimal location for fruiting, for dispersing the spores. What I find puzzling is that all those ancestral, small species completely lack these advantages, yet they thrive (and have for even longer than the more modern, larger species). Clearly, the modern species have not outcompeted the older ones for they co-exist happily. This would seem to say that the selection for larger, orienting slime molds is mild compared to that of just having something that sticks up in the air—a fruiting body—for which there is strong selection as I have just emphasized.

There are exceptions; some of the older species are large, and of great interest. I have a particular fondness for *Dictyostelium polycephalum,* having spent some time studying it (Bonner 2006). It produces a long, thin migrating slug with remarkable qualities. It can migrate great distances through dense soil, perhaps farther than any other species, and it always goes to the surface. And it is rugged: it can even make some headway migrating through agar. It lacks the ability to orient toward light, so its mechanism of orientation to the soil surface is not known. However, it is reasonable to assume that its slugs are oriented by gas gradients in the soil. As we showed for other species, since slugs go toward high concentrations of oxygen and away from high concentrations of ammonia, they will be oriented toward the surface of the soil by these gas gradients (Bonner and Lamont 2005).

In sum, the larger slime molds can move in a cell mass and facilitate dispersal by moving from the feeding area to the soil surface. This is the initial step toward spreading the spores. So like lions and vultures they can both move and orient, at least in their first steps toward dispersal.

3 The Paucity of Fruiting Body Shapes

As we saw, compared to Hawaiian honeycreepers, cellular slime mold fruiting bodies show a far more modest variety of shapes (Schaap 2007). There are two possible explanations, both of which may well be true. One is simply that slime molds have comparatively modest building materials compared to a vertebrate and this puts a severe limitation on the variety of shapes. This is kind of answer that would suit D'Arcy Thompson (1917 et seq.) who saw the form of living organisms in terms of the substance, the materials that make up an organism and the physical forces that act upon them. Clearly, a vertebrate has far greater richness of building materials upon which physical forces can act. The other possible explanation is that the shape of slime mold fruiting bodies is only weakly affected by natural selection. It is what Darwin suggests in the quote given above: "If it were no advantage, these (simple) forms would be left by natural selection unimproved or but little improved; and might remain for indefinite ages in their present little advanced condition." As I have pointed out there is very strong selection for small fruiting bodies sticking up into the air, but perhaps very weak selection for how they are shaped. One of the biggest differences in their form is that some are branched and others not. One might ask if this difference is significant for the dispersal of spores. In my view it is not, and to support this, two of the most common and cosmopolitan species are *Dictyostelium mucoroides* and *Polysphondylium pallidum*, unbranched and branched, respectively, are found side by side all over the globe from the tropics up to the far north and far south. And they must have done so for eons. It is very hard not to argue that they are equally successful. There are a number of other variations in the shape of fruiting bodies

such as stalks bunched together, but again there is no compelling evidence for their selective advantage. All that is required is to stick the spores up into the air for effective dispersal, the different shapes of the fruiting bodies might be relatively neutral as far as selection is concerned.

4 Evidence for Selection to Specific Environments

One of the obvious characteristics of larger organisms is that they are adapted to their environment. Desert animals and plants may be designed to preserve water one way or another; tropical rainforest animals and plants have devised special mechanisms to cope with the over abundance of water. And the list could go on and on: special adaptations for the cold, and for excessive heat; adaptation for living in a cave without light; adaptations for a nocturnal existence; adaptations for living at high altitude; adaptations for living in water or in air, and so forth, many times over.

Do we find anything equivalent among the cellular slime molds? The answer is yes, but the degree of adaptation is very modest. James Cavender informs me that the only clear cut example is *Dictyostelium rosarium* which is only found in arid regions in southern California (however, recently it was found on animal dung in a moderately dry forest in India by Sathe et al. 2010). It is not known how it copes with the dryness, but that is the sole environment where it has been found in North America. There are some species that have only been isolated in the tropics, but it is not known to what degree they are restricted to that environment. In other words, evidence for slime mold adaptation to a particular environment is extremely limited which would lead one to conclude that there is very little selection for particular environments in the cellular slime molds.

Cellular slime molds are small and for this reason they are affected differently by natural selection than larger macroorganisms. There are a number of consequences of this that lead to unexpected differences that make the evolution of the slime molds appear to be heterodox. They have relatively modest variation in their morphology. Those morphologies they do have appeared to be stable and not in competition. They last over a great span of geological time, and indeed many are "living fossils." Of the few adaptations they posses one is overwhelming, and that is the formation of stalks to hold the spore masses up into the air to facilitate their all-important dispersal.

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Social Selection in the Cellular Slime Moulds

Vidyanand Nanjundiah and Santosh Sathe

Abstract Starvation triggers a complex series of intercellular interactions in the cellular slime mould amoebae. As a result the amoebae aggregate, form a coherent multicellular structure with division of labour and, eventually, differentiate into a fruiting body made up of a stalk and a spore mass. Whether an amoeba dies and forms part of the stalk or becomes a stress-resistant spore depends both on pre-existing biases and on post-starvation signalling between amoebae. Mutual communication permits one amoeba to influence the phenotype, and therefore affect the fitness, of another. The implication is that social selection has been a major factor in the evolution of cooperative behaviour in these amoebae.

1 Introduction

This article discusses the potential for social selection during the cellular slime mould (CSM) life cycle. Social selection is natural selection in the context of social behaviour. With two exceptions the term 'social behaviour' is used as commonly understood: it is "the suite of interactions that occur between two or more individual[s]…, usually of the same species, when they form…aggregations, cooperate… or simply communicate across space".[1] The exceptions pertain to

[1] http://www.britannica.com/EBchecked/topic/550897/social-behaviour-animal

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cell–cell repulsion during feeding, which may be considered anti-social behaviour, and inter-species interactions. Other than brief comments at the beginning ('null hypothesis') and the end (under 'Summing up') on possible evolutionary routes, we do not attempt to theorise on how social behaviour may have originated in the CSMs. The interested reader is referred to books and articles by (Bonner 1967, 1982, 2009) and speculations in our previous publications (Nanjundiah 1985; Kaushik and Nanjundiah 2003; Nanjundiah and Sathe 2011).

The characteristic feature of the Dictyostelid or CSMs is self-organisation of form and pattern. A large number of apparently identical amoebae come together via mutual attraction, work in coordination to build a motile multicellular structure, the slug and proceed to construct a terminally differentiated fruiting body made up of live spore cells and dead stalk cells.[2] Besides serving as the 'hydrogen atom' of eukaryotic multicellular development, the apparent altruism displayed by the amoebae that contribute to the stalk has made them an ideal system for studying the evolution of cooperation. The aim of this article is to point out that social selection must have played a major role in the process. In order to help the reader better to appreciate what this implies, and to illustrate how social selection differs from conventional natural selection, we begin by sketching a possible route for the evolution of post-starvation development in the CSMs that does *not* involve social selection.

What does multicellular development achieve? Starvation is the trigger for aggregation, the slug migrates to the soil surface, and the fruiting body contains amoebae that have differentiated into stress-resistant spores positioned along or at the tip of an erect stalk. Passing insects, worms and, indirectly, birds and mammals can all help to transport spores (Suthers 1985; Kessin et al. 1996; Sathe et al. 2010)—something that would be difficult in the case of a single spore on the soil surface. Therefore, by joining an aggregate, some amoebae improve their chances of (passive) dispersal from a nutrient-poor environment. More, on average, an amoeba that joins an aggregate must have a better chance of survival and reproduction than one that remains solitary and waits it out until food becomes available in the same place—or dies. Could a combination of standard physical and chemical processes and the properties of individual cells as they evolve via conventional natural selection be sufficient to ensure this?[3]

The first question we need to address is whether pre-existing traits of free-living amoebae might be sufficient to explain their coming together and building a complex differentiated structure? Motility and the ability to adhere to a surface (necessary in order to obtain traction) would be among the traits. That apart,

[2] The terms 'Dictyostelid' 'CSM' and 'social amoeba' are used interchangeably.

[3] By conventional natural selection we mean a process of selection that makes an amoeba adapted to its physical and biotic environments, with the added implicit assumption that the environment created by conspecifics plays at best a minor role. The approach is analogous to considering a physical property as resulting from the behaviour of independent particles or a chemical property as 'colligative'.

entirely chance factors[4] could introduce an element of variation between amoebae, variation in more than one respect. For example, some amoebae will be more likely to withstand starvation than others. Even genetically identical cells will exhibit such variations, and if a group of amoebae contains many genotypes, the range of variation will increase further. Thus both stochastic and heritable factors affect the likelihood that a starved amoeba survives until food reappears in the same place.[5]

Now consider the following hypothetical but plausible sequence of events. The sequence can be thought of as a null hypothesis for how CSM social behaviour could have evolved with a weak role at most for social selection. An amoeba is present in an environment—say the soil—on which bacteria are distributed at a uniform density. The amoeba and its descendents feed, grow and divide. As they do so, local clusters of high density form entirely due to random movements (Houchmandzadeh 2009). Cells that come into lose contact adhere to each other (by the same means through which a cell adheres to a surface), and loose clusters go on to become tight aggregates. The phenotypes of cells vary on account of stochastic factors, and the variations are correlated with differences in the cells' ability to survive, reproduce or both (Nanjundiah 2003). Cell to cell differences in properties such as surface tension and viscosity cause the tight aggregates to change shape. At the same time, cellular heterogeneity leads to spatial segregation, and cells with similar physical properties sort out from others that differ in respect of those properties (Gierer 1977; Newman and Comper 1990; Forgacs and Newman 2005). Lastly, by virtue of being in a compact mass, some cells are elevated above the surface; this improves their chances of dispersal. Overall, the consequence is that an amoeba that forms part of a group has a better chance of survival than one that does not. This is one version of the null hypothesis of how aggregation and differentiation could have originated by a combination of natural selection (acting on an amoeba whose life cycle was spent as a solitary individual, with intercellular interactions playing at most a minimal role) and self-organisation (via physical forces), but without social selection.[6] The null hypothesis that we have sketched is essentially 'a stochastic model of an elemental social system' (Cohen 1971).[7] Going by a recent report of astonishingly rapid evolution of multicellularity with division of labour in unicellular yeast (Ratcliff et al. 2012), it

[4] For example, minor differences in stored nutritional reserves.

[5] Soil microorganisms can take active steps to defend themselves against stress. Even in the CSMs, a starved amoeba can encyst itself (see later). This does not affect the argument.

[6] *Ipso facto*, morphological transition via self-organisation oes away with the requirement that intermediate stages be adaptive (see Newman and Forgacs 2005). The evolution of fruiting bodies with an extracellular stalk bundle starting from single-celled fruiting bodies also could have been favoured by the purely physical consideration that it is harder to bend or break a bundle of cylinders than a single cylinder (Kaushik and Nanjundiah 2003).

[7] Bonner (2013 and this book) advances the more extreme null hypothesis that morphological differences between CSMs are neutral—they are due to chance, not selection; also see Bonner and Lamont (2005).

may not be all that off the mark. Its main value is that it helps us to conjecture what features of CSM development were based on physical principles initially and in the course of time became reinforced—that is, were made more reliable—by natural selection, and what features may have arisen as de novo adaptations to social living. The extensive system of cell–cell interactions in the CSMs (to be discussed below) makes us confident that the form of natural selection that played a decisive role in this was social selection.

The rest of this article is organised as follows. We begin with a discussion of the conceptual issues that underlie social selection and point out that the CSMs are ideal for studying how it works. A brief sketch follows multicellular development in the CSMs, which can also be viewed as the development of division of labour and social behaviour. Next we list traits on which social selection can act. We go on to consider how social selection might work on a background of pre-existing differences of stochastic origin. The article ends with general remarks on the relevance of social selection for the evolution of CSM social behaviour. Most of the work on CSMs has been concentrated on a single species, *Dictyostelium discoideum*. It should be assumed that what we say comes from observations made on it; whenever another species is involved it is named explicitly. For a general background the reader is referred to an article and book by Bonner (1982, 2009) and earlier reviews (Kaushik and Nanjundiah 2003; Nanjundiah and Sathe 2011).

2 Social Selection

The simplest context in which natural selection can act is that of an asexual species of solitary individuals living in a spatially uniform and temporally unvarying environment.[8] Under these conditions, given genetic variation and a genotype-phenotype correlation, natural selection leads to one genotype getting fixed. Mutation and genetic drift will generate and maintain genetic variation in the population. But, apart from stochastic differences ('range variation'; Bonner 1965), every individual in the population will have the same phenotype.[9] Spatial or temporal heterogeneity in the environment can change the picture and lead to stable polymorphisms.[10] In all these situations fitness can be defined with respect to an environment that does not include conspecifics. Sexual reproduction makes the fitness of an individual depend on its ability to interact with another individual, but the interaction as such does not play an important role, at least in random-mating models.[11] Sexual selection brings in a qualitatively new feature: it requires

[8] Or in an environment that at any given time is the same for all individuals.

[9] Unless some phenotypes are neutral relative to one another (Bonner, 2013 and this book).

[10] And can lead to non-intuitive outcomes, for example stable polymorphisms in asexual populations of non-interacting individuals (Rainey et al. 2000; Dean 2005).

[11] In particular, if the loci in question are not sex-linked.

the fitness of one individual to depend on traits in another. Inter-individual interactions in social groups share the feature. Darwin's explanations of sexual dimorphism and cooperative behaviour introduced social selection into evolutionary theory, and he recognised that the explanations required significant modifications to be made to ordinary natural selection (Darwin 1859, 1871).

Social selection refers to natural selection when the fitness of an individual depends on the social context (Crook 1972; West-Eberhard 1979). Social selection can take place when the phenotype of one individual depends on the phenotype (and therefore on the genotype) of a second individual. It involves a consideration of extended phenotypes in the sense of Dawkins (1981), but the extension applies to conspecifics within the same group rather than to artefacts (such as nests). In social selection an individual contributes to the environment of another and at the same time, selection acts on it. Each individual is therefore both an agent of selection and its object (Moore et al. 1997; Wolf et al. 1999). Social selection blurs the distinction between organism and environment, and that can have unexpected consequences for quantitative models of evolution. For example, it is no longer a straightforward matter to partition phenotypic variation between 'genetic' (=heritable) and 'environmental' (=non-heritable) components, because the environmental component too can be inherited. Also, a built-in tendency of positive feedback when conspecifics interact means that social selection can potentiate more rapid evolutionary change than conventional natural selection.[12] Indeed mutual communication and feedbacks are common to all groups in which sociality involves more than the mere coming together of units that benefit from a mere increase in numbers (Nanjundiah and Sathe 2011). For an external signal to be capable of influencing the phenotype of a cell, the phenotype must be flexible or plastic. Phenotypic plasticity at the level of the individual can enlarge the scope for natural selection—in this context, social selection—to shape the evolution of group behaviour in terms of both direction and pace. This is because the capacity to adopt different phenotypes acts as a multiplier[13]—it can enhance the range of potential group states and thereby amplify phenotypic variation at the level of the group (Fig. 1).

A number of reasons make the CSMs an excellent system for studying the working of social selection. An extensive system of intercellular communication (Kessin 2001) includes some signals that enhance the chances of survival and others that lessen it, indicating that social selection is acting (Nanjundiah and Sathe 2011). Social selection in the CSMs involves social behaviour without obligatory sexual reproduction. There is a sexual or macrocyst phase of development as well. It requires the co-aggregation of amoebae of different mating types, nuclear fusion and extensive cell death (Raper 1984). There is one strain of *Polysphondylium*

[12] A signal from A to B contributes to the fitness of B, and via reciprocal communication, feeds back on A. The Darwin–Fisher model of sexual selection driven by female choice is a classic example.

[13] For example, if each individual in a group of three can exhibit any one of three phenotypes, the number of group phenotypes is at least 9.

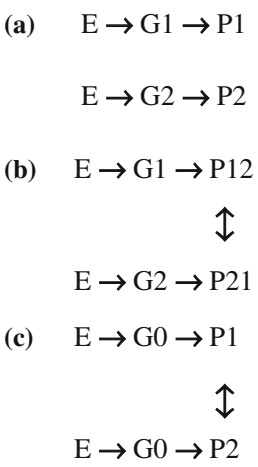

Fig. 1 A schematic depiction of how interactions and feedbacks form the background to social selection. E represents the common environment; G stands for genotype and P for phenotype. *Single arrows* indicate influences and *double-headed arrows* stand for interactions. In **a** P1 and P2 stand for phenotypes that result from genotypes G1 and G2; P1 and P2 do not interact, and their evolution is governed entirely by conventional natural selection. The genotypes of the individuals remain different. In **b**, because of interactions with P2, P1 is modified to P12 and similarly, P2 is modified to P21. In **c**, interactions between phenotypes can cause the same genotype G0 in the same environment E to give rise either to phenotype P1 or phenotype P2. One phenotype can modify another via direct signalling or indirectly. In the indirect route a phenotype in one individual can influence the genotype → phenotype link in the other individual. This sketch illustrates what is required for social selection to act. For it actually to act, the interactions must have a bearing on the reproductive fitness of the relevant individuals. Even so, social selection may not have evolutionary consequences; it can do so only when the genotypes in question differ. Situations **a**, **b** and **c** are all found in the CSMs

pallidum that produces a 'killer factor' that is lethal to all strains except itself and its opposite mating type (Mizutani et al. 1990). It is obvious that the sexual cycle offers ample scope for social selection (Nanjundiah and Sathe 2011). However, it is rarely observed under standard laboratory conditions, we do not know how frequent it is in nature and studies on it are few. This article is restricted to making a case for the prevalence of social selection during the asexual life cycle.

Individual amoebae can go through asexual life cycles in a physical environment that, for all practical purposes, is both uniform and unchanging. But their social environment is extremely heterogeneous through space and time because of signalling from other cells, and that is associated with behavioural heterogeneity.[14] Social groups can be generated easily in the laboratory, simply by throwing different cells together. This makes it possible for individuals of known genotypes to be allowed to interact and their fates followed over a large number of generations.

[14] The signal-receptor systems that are responsible for intercellular communication can evolve too, by a different variant of natural selection known as "signal selection": Zahavi 2006.

In other words traits that have consequences for fitness[15] can easily be monitored and measured under controlled conditions and it is easy to measure fitness over one or more life cycles.[16] The number of phenotypes exhibited by the CSMs depends on how one does the counting. If we restrict ourselves to gross cell morphology, there are four: amoeba, spore, stalk and basal disc (the last being absent in many species and the last two in some); if we take behaviour into account we need to add feeding cells, specifically chemotactic cells, 'sentinel' cells that phagocytose bacteria and clear toxins (Chen et al. 2007), and upper and lower cup cells that aid the spore mass to rise (Sternfeld 1998; Mujumdar et al. 2009); if we include gene expression patterns, the count goes up.[17] Still, cellular phenotypes are few in number. Therefore one outcome of social selection, namely the origin and maintenance of phenotypic differences within the group, should be much easier to study in the CSMs than, for example, in insect societies with their many morphological and behavioural castes (Wilson 1971).

3 Development of Sociality

The cellular slime moulds are characterised by an unusual life cycle made up of distinct phases of unicellular growth and multicellular development, the latter being the social phase. Free-living amoebae feed on bacteria and other microorganisms that are present in the soil or on animal dung, grow and increase in number via serial mitotic divisions as long as the food supply remains.[18] Once the food is exhausted a number of intracellular events including quorum sensing, the production and release of a chemical attractant, chemotaxis in response to an external gradient of the chemical, amplification and relay of the attractant and the development of intercellular adhesion systems make it possible for cells to communicate, attract each other via chemotaxis and form cohesive social groups consisting of anywhere from $\sim 10^2$ to $\sim 10^6$ amoebae depending on the species and food supply (Bonner 1967, 2009; Raper 1984; Kessin 2001). Division of labour appears soon after starvation sets in and one can detect presumptive cell types within the group, now a polarised mass called the slug. The slug moves

[15] For example growth rate, the time required to complete development, migration and oriented movement (taxis), and, reproduction or cell death.

[16] As of today most evolutionary experiments on the CSMs have been restricted to a single life cycle, but the situation is changing (Kuzdzal-Fick et al. 2011).

[17] Spatial and temporal gene expression patterns have been studied in detail only in *Dictyostelium discoideum* (Bonner 1967; Olive 1975; Jermyn et al. 1989; Kessin 2001; Chen et al. 2007).

[18] CSMs have also been found in water bodies and on trees (Olive 1975; O'Dell 2007; Sathe et al. 2010). In neither case have life cycles been properly studied. If they have an aquatic life cycle, it has not been studied. Recently a CSM was isolated from an infected human eye; the possibility that CSMs may be pathogens is new (Reddy et al. 2010).

towards the soil surface and undergoes a series of changes including tissue rearrangements. Some members of the group become encased in a stress-resistant polysaccharide coat and enter hibernation as spores; the others die and build a multicellular cellulosic stalk that provides a rigid support for the spore mass. The resulting structure, known as the fruiting body, is conventionally considered the terminal stage of the life cycle.[19] The sequential stages that cells go through during development—feeding and growth, aggregation, multicellular migration and terminal differentiation—exhibit their own characteristic pattern of gene expression (VanDriesche et al. 2002).

The entire course of events from the start of aggregation to the construction of the fruiting body involves complex and coordinated movements of cells and cell groups. The next life cycle begins after dispersal, a process in which spores are passive participants. Water, insects and worms can all act as dispersers over short distances; wind, large animals and birds can spread spores over much larger distances (Cavender 1973; Suthers 1985; Huss 1989; Stephenson and Landolt 1992; Kessin et al. 1996; O'Dell, 2007; Sathe et al. 2010). Amoebae can be consumed and digested by nematode worms and presumably other animals but spores emerge unscathed (Suthers 1985; Huss 1989; Kessin et al. 1996). If a spore happens to land in a food-rich habitat, it germinates: the hibernating amoeba emerges and starts to feed, grow and divide once again until the next bout of starvation sets in. Within this broad similarity in life cycles, there are significant differences between species.

4 Traits on Which Social Selection can Act

Any trait that involves behavioural modification of one cell by another is a candidate for social selection. Candidates can be found throughout the CSM life cycle. However, very few have been monitored with regard to their consequences for reproductive fitness. In fact most of the data pertains to essentially one trait, namely spore formation. Consequently most of the evidence we have pertains to the influence of different social environments on the probability that an amoeba becomes a spore. All the same, it is of interest to list other traits on which social selection can act. (a) Amoebae of some species repel each other while feeding (Keating and Bonner 1977; Kakebeeke et al. 1979). Thus feeding cells can influence each other, albeit in an anti-social fashion: strictly speaking, they cannot be treated as non-interacting individuals. One can conjecture that the strength of

[19] All Dictyostelids form fruiting bodies, but their forms are varied. The stalk can be branched or unbranched, cellular or extracellular, and when cellular, made up of live or dead cells. The arrangement of cells in the stalk can differ from species to species. See Bonner (1967) and Raper (1984) for details. In species where all cells form spores and each spore secretes an extracellular stalk, the stalk bundle may confer a group advantage via the collective behaviour of cells that act independently; see Kaushik and Nanjundiah (2003).

the repulsive signal is graded with the availability of food, repulsion being strong when food is abundant and weak when it is sparse (experimental evidence on the point is lacking). In the latter situation there would be a premium on getting together with other cells; indeed repulsion gives way to attraction after the food supply is exhausted. (b) Amoebae sense their numbers, or more correctly their density, in terms of the concentration of a released quorum sensing factor or factors (Clarke and Gomer 1995; Jain et al. 1992).[20] (c) Quorum sensing is a prelude to secreting and relaying the chemoattractant, which leads to long-range aggregation (Konijn et al. 1967; Shaffer 1975). Again, the amount and temporal profile of chemoattractant secreted and the sensitivity of the response would be subject to selection. (d) Aggregation depends on an elaborate coordination of sensory and motor behaviour among cells (Kessin 2001) besides the regulation of gene expression in anticipation of future requirements (VanDriessche et al. 2002). Aggregation territory sizes are tightly regulated (Bonner and Dodd 1962) and dependent on the production and degradation of the chemoattractant (Riedel et al. 1973; Nanjundiah and Malchow 1976); (e) Slug migration is a form of cooperative behaviour, and efficient and timely migration to the soil surface is necessary for efficient dispersal. In particular, the slug exhibits phototaxis and thermotaxis, and the sensory centres for both lie in the cells of the tip at the slug's anterior margin (Kessin 2001): this means that the polarised movement of the slug is coordinated by signals from the tip. Further evidence of the tip's guiding role comes from its resemblance to the classical embryonic organiser in the sense that a transplanted tip can direct the morphogenesis of the entire group (Bonner 1952; Rubin and Robertson 1975).[21] (f) Culmination, a process that involves differentiation and the ascent of the spore mass, requires elaborate signalling between cells and tissues. The presumptive spore mass is helped to rise by two subsets of cells that cradle it above and below, the upper and lower cups; the lower cup offers passive support whereas the upper cup actively lifts the mass (Sternfeld and David 1982; Sternfeld 1998; Mujumdar et al. 2009). The cells belonging to both cups die but remain amoeboid in appearance (Sternfeld and David 1982). Terminal differentiation into viable spore cells requires the active involvement of a secreted peptide from presumptive stalk cells (Anjard et al. 1997, 1998a, b). (g) In some species spores contain quorum sensing compounds that inhibit their own germination (Russell and Bonner 1960; Bacon et al. 1973). At least in a mutant of *D. discoideum*, they make an auto-activator of germination (Dahlberg and Cotter 1977).[22] Conceivably

[20] It has been suggested that quorum sensing may be a form of 'reproductive restraint', namely a prudent cessation of growth and cell division when the food supply becomes poor; see (Werfel and Bar-Yam 2004). However, there are sound arguments against this and similar models of group-level benefit; see Zahavi (2005).

[21] Work with 2-dimensional slugs suggests that the tip may be a dynamic entity whose cellular composition keeps changing, not a fixed group of cells (Bonner 1998).

[22] Curiously, the bacterium *Enterobacter* (*Aerobacter) aerogenes*, on which CSM amoebae feed, produces a substance that acts as an activator of spore germination (Hashimoto et al. 1976). Presumably the substance has been co-opted by amoebae to serve as an indicator of the

spore germination activators are also produced by spores of species that can germinate in the absence of food (for example *D. mucoroides* var. *stoloniferum*; Cavender and Raper 1968).

Under special environmental conditions that include starvation, high moisture, darkness, and low levels of phosphate, CSM amoebae belonging to opposite mating types aggregate and go through a series of events including cannibalistic feeding; the outcome is a dormant zygotic structure known as the macrocyst (Blaskovics and Raper 1957 Nickerson and Raper 1973; Erdös et al. 1975; Urushihara 1992). The sexual cycle offers special opportunities for social selection, because it requires the proximate co-occurrence of two cells that communicate and modify each other's phenotype, an extreme case of social selection. Mutzel (1991) has hypothesised that sexual predation and cannibalism may have been evolutionary forerunners of asexual multicellular development. In some strains of *P. pallidum* the 'sexes' also differ in morphology (Kawakami and Hagiwara 2002). It is tempting to think that as in many animals, here too sexual selection, if not in the classical Darwinian sense, in the form of signal selection, has been responsible for moulding the difference.[23] We have no evidence either way.

5 Stochastic Factors and Genetic or Environmental Differences can Make Pre-aggregation Cells Differ in Their Capacity to Withstand Starvation and Sporulate

There is ample evidence that differences in phenotypes can originate spontaneously among genetically identical amoebae—that is, on a stochastic basis and without cell–cell interactions.[24] This is true even when the amoebae have the same genotype and share the same environment. The evidence comes from different sorts of observations. (i) Certain differences between cells, e.g. in size, make them more likely to differentiate into one cell type than another (Takeuchi 1969; Bonner et al. 1971). (ii) Artificially provided pre-aggregation cues can bias the stalk-forming or spore-forming tendency of a cell. The cues can relate to nutrition

(Footnote 22 continued)
availability of food. This is an interaction between a predator and its prey and so does not form part of social behaviour within one species. It is mentioned here because of the unusual outcome, namely a 'closing of the loop' in the asexual life cycle: there is a smooth transition from the conventional end-point of the life cycle (terminal differentiation into spore and stalk cells) to its conventional beginning (feeding of bacteria by amoebae).

[23] See Zahavi (2006).

[24] Note that this is *not* the same as conventional phenotypic variation between the members of a species, which is usually thought to be based on genetic differences, environmental differences or genotype– environment interactions. Presumably something comparable could occur in other social organisms, e.g. the social insects, in which autonomous differences can be reinforced by inter-individual interactions.

(Leach et al. 1973), cell cycle phase at starvation (McDonald and Durston 1984; Weijer et al. 1984; Thompson and Kay 2000a) or cellular calcium content (Saran 1999; Azhar et al. 2001). (iii) When starved amoebae are plated at a density that is low enough to make intercellular communication unlikely and all of them exposed to an appropriate chemical stimulus, they can be induced to differentiate. However, not all cells respond in the same manner. The proportion that differentiates varies with the strength of the stimulus, indicating an element of randomness in cell to cell variability (Kay 1982).

What is finally perceived as an all-or-none distinction between two categories of cells, say spore and stalk, initially may be based on spontaneously occurring internal phenotypic differences (for example, differences in stored glycogen; Takeuchi et al. 1986. Heterogeneities in genotype or environment would be an additional source of variation that would amplify pre-existing phenotypic differences. The feature in question could vary continuously from cell to cell but be used to generate a qualitative difference between two categories—for example via a threshold mechanism or by some other means (Fig. 2).[25]

Even after an apparently qualitative distinction is possible—for example, between stalk and spore cells—an element of residual variation will remain. It will ensure that spore cells (or for that matter stalk cells) are not identical; indeed they may be functionally non-equivalent too (Bonner 1965).[26] To sum up, the intercellular interactions that lead to social selection act on a background of phenotypic variation that is stochastic in origin or, depending on the circumstances, is influenced by different genotypes or different environments.

6 Intercellular Interactions Reinforce Spontaneously Occurring Differences and Lead to Social Selection

A characteristic feature of CSM development is that the two cell types in the terminal structure, the fruiting body, are present in constant proportions over a range of 10^3–10^4 when proportions are assessed in terms of dry weights, volumes

[25] Intercellular interactions can cause phenotypic differences to arise spontaneously among two or more cells. A well-studied case involves the combination of stochastic fluctuations and negative cross-feedbacks that leads to the distinction between anchor and ventral uterine (AC/VU) cells in *Caenorhabditis elegans* (Wilkinson et al. 1994). Analogous negative feedbacks seem to exist in *D. discoideum*; see Fig. 3. A bimodal distribution of cell motility may arise in *D. discoideum* and other systems because mutual inhibition between two signal transduction pathways can lead to bistability (Goury-Sistla et al. 2012).

[26] In his Ph.D. thesis (submitted to the Indian Institute of Science, 1996), Baskar reports that he was able to stain spores differentially using the dye neutral red. The spores were allowed to germinate and the resulting amoebae were compelled to aggregate (by being deprived of food). Following aggregation, highly stained and poorly stained amoebae sorted out to the slug anterior and posterior respectively; that is, they exhibited presumptive spore or stalk tendencies. The effect disappeared if feeding and cell division were allowed to intervene.

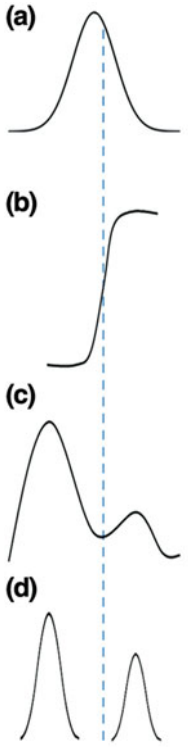

Fig. 2 Illustration of how a continuously varying unimodal phenotype can be transformed into a bimodally distributed phenotype or into two distinct phenotypes. The *curve* in (**a**) stands for the distribution of some quantity X that varies continuously from cell to cell. The distribution is symmetric around the mean and the value of X in most cells is close to the mean. The step function in (**b**) stands for a threshold filter (for example, a protein to which molecules of X bind cooperatively). One way in which the threshold filter could work is by the activation of a gene only in those cells in which the level of X is to the *right* of the *vertical dotted line*; the gene remains inactive in cells whose level of X is to the left of the line. Following this a second variable Y is distributed among cells either as a single bimodal distribution (**c**) or as two distinct distributions (**d**). Alternatively, **d** may reflect levels of a third variable Z. The distributions in **c** and **d** are supposed to reflect qualitative phenotypic differences relevant for reproductive fitness. In the CSM context, X would be a parameter that indicates a pre-aggregation cellular 'quality' related to fitness (e.g. nutritional state; see Atzmony et al. 1997) and Y and Z could stand for a parameter whose values are significantly different between prestalk and prespore cells

or total cell numbers (Bonner 1967; MacWilliams and Bonner 1979).[27] Therefore cells must be capable of actively adjusting their behaviour in response to the size

[27] Rafols et al. (2001) state that "the pattern of cell types is qualitatively the same for slugs of all different sizes, from 100 cells to more than ~100,000 cells". The actual proportions vary from species to species. In *D. discoideum* ~80 % of the amoebae form spores under standard laboratory conditions; in *D. giganteum* it is ~50 %; Raper (1940); Kaushik et al. (2006).

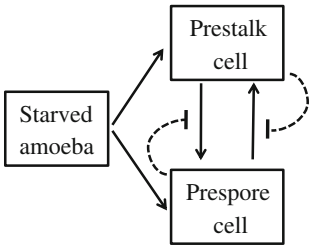

Fig. 3 An elementary scheme of intercellular interactions involved in cell-type differentiation in *D. discoideum* (adapted from Nanjundiah and Bhogle (1995); a scheme with some similar features was proposed by Loomis (1993) and Schaap et al. (1996)). Depending on the levels of one or more intracellular parameters, a starved amoeba has a certain probability of dying (forming a prestalk cell) and a certain probability of surviving (forming a prespore cell) even before the onset of aggregation.These probabilities vary from cell to cell (see Fig. 2). Prespore and prestalk cells can interconvert spontaneously (*straight arrows*), but interconversion is subject to inhibitory feedbacks (*dotted lines*). A prestalk cell differentiates terminally into a dead stalk cell and a prespore cell differentiates terminally into a live spore cell. A minimal model that accounts for the correct proportions of differentiated cell types requires, in addition to the processes shown here, a means whereby the strengths of the interactions are sensitive to overall size, i.e. to the total number of cells (presumptive stalk + presumptive spore). A complete developmental model would require the addition of a means for specifying other cell types (albeit within the broad categories indicated here), a consideration of cell and tissue movements and contacts

of the social group. Raper provided a direct demonstration when he showed that anterior or posterior fragments of *D. discoideum* slugs, i.e. fragments that contained a preponderance of one or the other cell type, could restore the minority component. This happened via trans-differentiation, that is, cell-type interconversion, on the part of some cells belonging to the majority (Raper 1940). A possible explanation would be that in the undisturbed condition any tendency to trans-differentiate from a presumptive stalk state to a presumptive spore state or vice versa is inhibited by negative feedbacks.[28] Inouye (1989) confirmed this in the case of trans-differentiation from the presumptive spore to the presumptive stalk state.

There is another reason for invoking negative feedbacks. When measured in groups of the same size, cell type proportions vary stochastically from group to group because of spontaneous fluctuations in the underlying physics and biochemistry—that is, because of an intrinsic lack of precision in the phenomenon. It so happens that even when different models predict the same mean proportions, the magnitude of the associated fluctuations that they predict depends on the precise model. When the experimentally observed fluctuations are compared with expectations based on various models, it turns out that one is required to invoke,

[28] Transdifferentiation, first indicated in Raper's observations on isolated prestalk and prespore fragments of the slug (Raper 1941), was shown explicitly later by Gregg (1965), and its kinetics was studied by Sakai (1973).

besides stochasticity, negative feedbacks and an ability to sense the size and the group (Nanjundiah and Bhogle 1995; see Fig. 3 below).

Direct evidence for the existence of intercellular interactions in social behaviour comes from a number of sources. First, identified diffusible chemical signals are known to pass from cell to cell and to lead to specific effects on the differentiation status of the recipient (Konijn et al. 1967; Bonner 1970; Shimomura et al. 1982; Van Haastert et al. 1982; Town et al. 1976; Morris et al. 1987; Thompson and Kay 2000b). Among the better-studied signals that mediate social behaviour, the lipid-soluble polyketides generically known as DIF (for differentiation-inducing factor) are made and released by *D. discoideum* cells (Kay 1997; for other social signals see Kaushik and Nanjundiah 2003). The DIFs can induce other cells to differentiate to a stalk-like state with varying degrees of efficiency. It was predicted (Atzmony et al. 1997) and subsequently verified (Kay and Thompson 2001) that the doubly chlorinated molecule DIF-1, the most potent known DIF, is made by amoebae that have a higher intrinsic predisposition to survive than the others. These are the presumptive spore-forming amoebae that are located in the slug's posterior; correspondingly, DIF-1 is broken down by presumptively stalk-forming amoebae in the slug's anterior (Kay et al. 1993). Second, the extracellular medium in which cells are shaken contains released factors that affect the development of other cells, of which only some of the active constituents have been identified (Oohata et al. 1997). At least two genes that are expressed in *D. discoideum* presumptive stalk tissue, *SDF-2* and *comD*, are required for spore differentiation from presumptive spore tissue (Anjard et al. 1998a, b; Kibler et al. 2003). The most compelling evidence for the existence of social selection comes from observations on synthetic social groups in which marked amoebae are combined soon after starvation and their fates monitored.[29] Generally, what is measured is the proportion of cells of a given type that contributes to forming spores, relative to the proportion in which the cells were mixed initially. Such experiments have been carried out with naturally occurring 'wild-type' or distinguishable variants and with mutants of known phenotype. In the most dramatic examples cells belonging to two strains that are unable to develop further after starvation do so when mixed and go on to form terminally differentiated fruiting bodies (Sussman 1952, 1954, 1955; Sussman and Lee 1955); in less dramatic cases an admixture of the wild-type rescues developmentally aberrant strains (Buss 1982; Filosa 1962).

When *D. giganteum* wild-type isolates are mixed in pairs, some strains aggregate freely and complete development together; others co-aggregate but sort out and form separate fruiting bodies; and yet others inhibit the development of the other component, which remains as unaggregated amoebae (Kaushik et al. 2006). When pairs of strains co-aggregate and go on to build chimaeric fruiting bodies, the most common outcome is that one strain forms a disproportionate number of

[29] As the topic has been reviewed extensively recently (Nanjundiah and Sathe 2011), we restrict ourselves to listing the main points.

spores relative to the other (Strassmann et al. 2000; Kaushik et al. 2006). Whatever be the intercellular interactions responsible, they influence reproductive fitness. But there are other puzzling features. For example, there are cases in which the asymmetries between pairs of strains are transitive (A > B and B > C implies A > C, where the > sign means that a greater than expected proportion of the first member, and a smaller than expected number of the second number, forms spores). This hints at the existence of a dominance hierarchy. But the asymmetries weaken considerably or disappear when three strains are mixed (A = B = C; Kaushik, et al. 2006; Khare, et al. 2009). The implicit complexity or nonlinearity is suggestive of what is called the bystander effect by ethologists: the behaviour of one animal[30] towards another is affected by the presence of a third (Carlisle and Zahavi 1986). The specifics of how such an effect can work in a cellular context are a matter of much interest.[31] Variations in developmental phenotypes seen after mixing cells of *triA*$^-$, a morphological mutant, and its wild-type surrogate, Ax2, clearly show that phenotypes are partly autonomous and partly non-autonomous to the cells that exhibit them (Mujumdar et al. 2011)—again, a clear indication that intercellular signalling is involved.

7 Evolutionary Consequences of Social Selection

The manner in which the CSMs become multicellular (i.e., by aggregation of spatially separated amoebae) makes it possible, though not necessary, that the amoebae that come together belong to different genotypes. It turns out that fruiting bodies formed under natural conditions, or under laboratory conditions that may mimic what happens in nature, can be genetic chimaeras or clones (Gilbert et al. 2007; Sathe et al. 2010). Clonal as well as polyclonal groups of *D. discoideum* have been detected in nature (it is a straightforward matter to generate them by mixing in the laboratory); 77 % of the groups were clonal in one study (Gilbert et al. 2007). In contrast, among social groups of *D. giganteum* and *D. purpureum*, 15/17 sampled fruiting bodies were polyclonal and the estimated number of clones within a group ranged from 1 to 9 (Sathe et al. 2010). Social selection can lead to evolutionary change when social groups consist of more than one genotype (West-Eberhard 1989) and individuals belonging to the genotypes differ in fitness-related traits, as in fact they do (Strassmann et al. 2000; Fortunato et al. 2003; Kaushik et al. 2006). But if groups are genetically homogeneous, social selection can act but cannot affect genotype frequencies in the next generation. As mentioned earlier, genetically heterogeneous social groups are readily constituted in the laboratory. A number of observations have been carried out on such groups. Going

[30] In this case, of an 'animal' that, as Bonner (1994) has pointed out, is without nerves or muscles.

[31] Mesnil et al. (1996) have reported a bystander effect in cancer tissue.

by the outcomes, one thing is abundantly clear: social selection mediated via intercellular signalling can lead to either an increase or a decrease in the probability that a cell survives relative to the same probability as measured in a clonal group; in short, social selection can have evolutionary consequences (Fortunato et al. 2003; Kaushik et al. 2006; Nanjundiah and Sathe 2011).[32] However, past social selection in groups made up of related cells (i.e. kin selection) could have favoured the formation of genetically uniform social groups over heterogeneous groups. This could have been a means of guarding against the risk of being confronted by a cell belonging to a genotype that leads to its bearer deriving the benefits of group life without suffering the cost, i.e. without taking the risk of dying and forming part of the stalk (Mehdiabadi et al. 2006; Ostrowski et al. 2008).[33] On balance, it would appear that depending on the nature of their immediate neighbourhood at time starvation sets in, CSM amoebae can go through some life cycles entirely in the company of clone-mates and other life cycles as members of genetically heterogeneous groups (that conceivably include members of other species). Therefore, their traits must have evolved under social selection in social environments that were quite different from one generation to another (Kawli and Kaushik 2001). We see coordinated multicellular development in the CSMs as the evolutionary outcome of competition to sporulate. The competition is carried out between amoebae that differ in their intrinsic abilities to survive starvation and, on average, benefit by becoming part of a group along with other amoebae.

When aggregation involves cells of different mating types, it can lead to cell fusion, cannibalism and the formation of a stress-resistant zygote (the macrocyst). The sexual cycle is very different from the asexual cycle. But, considering the high level of mortality that accompanies macrocyst formation, it is evident that very strong social selection must be involved in it. Some CSMs mount a third response to starvation, which is to encyst themselves as single cells (the microcyst). According to Raper (1984) on the whole microcysts are found in species

[32] In these experiments the genetically heterogeneous nature of the group, which is engineered by the experimenter, is merely a tool of convenience. Genetic heterogeneity makes it easier to distinguish between two classes of cells and compare the efficiency with which each forms spores with the corresponding efficiency when either is in a clonal group. The assumption is that the experiments are telling us something about social behaviour in a group of interacting CSM amoebae that belonged to different phenotypes originally or acquired different phenotypes following intercellular interactions. Once the group forms and is stable, whether it is clonal or polyclonal is unimportant.

[33] A 'kin effect' can be present without kin selection. When an amoeba dies as a stalk cell, and no spore cell has the same genotype, it is strongly selected against—its genotype disappears. On the other hand, if genotype is also present in one or more spores, the amoeba is subject to equally strong negative selection but its genotype survives. However, by itself this is not evidence of kin selection. Kin selection requires that the death of a stalk cell be selected *because*—whenever different genotypes are found in the same social group—it enhances the probability that another cell of the same genotype forms a spore relative to the probability of an unrelated cell forming a spore.

that—when they go through the asexual cycle—form delicate fruiting bodies. As far as is known, microcyst formation, which is likely to have been an ancestral trait carried over from an asocial amoeba, is independent of intercellular interactions. If so, the evolution of the microcyst may not have involved social selection. It would be interesting to know what factors (in addition to cell density) decide on the relative likelihood that an amoeba will form a microcyst on its own or aggregate with others.

There are scattered but intriguing reports of cell behaviour being modified by intercellular interactions with the effect being carried over into the next life cycle. In separate experiments, and with two different species, *D. discoideum* and *D. purpureum* respectively, Sussman and Kahn mixed cells of an aggregateless mutant with wild-type cells. From the chimaeric fruiting bodies that were formed, they recovered spores of supposedly mutant background and discovered that amoebae derived from them had regained the ability to aggregate—the mutant phenotype had mysteriously been 'cured' (Sussman 1952; Kahn 1964). Kaushik found that after cells of three distinct strains of *D. giganteum* had been carried through 24 asexual life cycles in combination with one another, they had lost the ability to develop by themselves (Kaushik 2002). It is impossible to explain these findings on the basis of our present understanding. The experiments must be repeated, their findings verified and the possibility of selection (following spontaneous mutation) excluded. If the findings are confirmed and no genotypic change has occurred, they would point to phenotypic change triggered by social interactions (which is not a surprise by itself) combined with what seems to stable inheritance of the new phenotype (which falls outside what we know about CSMs but, as a recent survey shows, is prevalent in many other systems; Jablonka and Raz 2009). A less drastic inference would be that this experiments point to the occurrence of what one could call (by analogy to the animal context) social learning with epigenetic inheritance. Takeuchi has provided an instance. He raised fruiting bodies from isolated anterior or posterior fragments of *D. discoideum* slugs[34] and allowed their spores to germinate. The amoebae that emerged were not provided nutrition and so were unable to divide; instead they were made to aggregate immediately with reference cells. The slugs that ensued showed a clear pattern of spatial sorting: amoebae that were derived from spores generated from slug anteriors tended to sort out to slug anteriors once again, and those derived from spores that had been generated from slug posteriors tended to sort out to slug posteriors; the effect disappeared when a phase of growth and cell division was allowed to intervene (Takeuchi 1969; also see footnote 24).

Finally, CSM amoebae participate in a number of cross-species interactions whose evolutionary implications remain unexplored. The interactions may or may not be significant in the natural ecology of the CSMs (of which we know very little). CSM species can co-exist in close proximity (Raper 1984), sometimes on

[34] Raper (1940) had shown that this was possible. Presumptive stalk and spore cells can interconvert when inhibitory influences from the complementary tissue are removed (see Fig. 3).

the same speck of soil (Sathe et al. 2010), and the extracellular signals that they use overlap; indeed they can form combined (inter-species) groups (Raper and Thom 1941). Interference with intra-species signals is possible and, in principle, could be significant for the evolution of social behaviour.[35] On the other hand as far as social behaviour within a species goes, inter-species interactions could be a form of 'ecological noise'; at present we simply do not know.

Be that as it may, certain *P. pallidum* strains produce a killer factor that is lethal to other strains of the same species as well as to several *Dictyostelium* species (Mizutani et al. 1990). A different factor produced by *P. pallidum* causes *D. discoideum* amoebae to fuse and become multinucleated (Mizutani et al. 1991). Another instance of inter-species aggression in the CSMs is the behaviour of *D. caveatum*, which aggregates with amoebae of other species and proceeds to kill them and use them as food for increasing its own numbers (Waddell 1982; Nizak et al. 2007). We have already mentioned that *A. aerogenes* bacteria (for whom CSM amoebae are predators) produce a substance that activates spore germination in *D. discoideum* (Hashimoto et al. 1976; see footnote 20). Spores survive feeding by predatory soil nematodes (Kessin et al. 1996) and other soil invertebrates (Huss 1989), birds (Suthers 1985;) and mammals (Stephenson and Landolt 1992; Sathe et al. 2010), and in an environment containing them a CSM amoeba that can differentiate into a spore—which requires an intermediate social phase—would be at an advantage over an amoebal cell that cannot form a spore. Ellison and Buss (1983) found an intriguing case of cross-species communication and symbiosis (at least in one direction) involving a CSM. A soil isolate of *D. mucoroides* went through normal development only when exposed to a diffusible substance released by the fungus *Mucor hiemalis* or directly to the fungus itself. These cases point to selective pressures from the biotic environment that can affect social behaviour in the CSMs, but their prevalence and long-term consequences—if any—remain unknown.

8 Summing Up

Behavioural modification by means of intercellular communication occurs throughout the life cycle of the cellular slime moulds (CSMs). One cell can influence the likelihood that a second cell differentiates into a spore, or dies and forms part of the stalk. Thus the phenotype of a cell has both autonomous and non-autonomous aspects; both direct and indirect effects of natural selection have

[35] Cooperative communities of bacteria (Sachs and Hollowell 2012), nests founded by females of different species in social insects (Hunt 2009; especially the note there attributed to Snelling) and mixed-species foraging in bird flocks (Sridhar et al. 2009) all show that interactions between different species may be relevant for social evolution.

shaped traits in the cellular slime moulds. Indirect effects can work via communication among cells of a clonal or polyclonal social group of the same species. Communication can also take place between members of different species that happen to come together, or indeed between CSMs and other organisms in the environment. The possible impact of inter-organismal interactions on CSM development and behaviour is just beginning to be explored (Sonowal et al. 2013).

We offer three conjectures on what broad developments in understanding might lie ahead. Microarray analysis of temporal (Šášik et al. 2002) and spatial (Maeda et al. 2003) gene expression patterns during development in *D. discoideum* show a dynamic picture that—presumably—reflects constant interplay between cells. Temporal expression appears to occur in bursts within somewhat loosely defined time windows and later sequences of expression depend on earlier ones (Maeda et al. 2003). We know quite a bit about how the expression of individual genes is regulated and much of the knowledge has to do with gene products (or enzyme products) that are involved in intercellular communication. But a comprehensive view of the flow of information between cells and what it means for the coordination of multicellular behaviour is missing. Next, there is the prospect of insights from genome sequencing data. But, as with hopes raised by genome sequencing generally, expectations must be tempered with caution: Parikh et al. (2010) comment that the sequences of *D. discoideum* and *D. purpureum*, sister-species within the same clade, "are as divergent as those of man and jawed fish".[36] Finally, there is the fundamental question of non-selectionist (but evolutionary) explanations for aspects of CSM social behaviour. The range of phenotypic plasticity in the CSMs is such that occasionally one species exhibits an aspect of development that resembles a feature found in another. Sometimes one finds a branched stalk (normally seen in a different genus, *Polysphondylium*) in a fruiting body of *D. discoideum*; Bonner (2003) has pointed out that a CSM with a cellular stalk, *D. lacteum*, makes fruiting bodies that are partly acellular (an acellular talk characterises the genus *Acytostelium*) when the number of cells in the aggregate falls below a threshold. Clearly these variations fall within the repertoire of *D. discoideum*. Such being the case, we need seriously to examine to what extent the origin of social behaviour in the CSMs requires a specifically adaptationist explanation and to what extent it can be accounted for more parsimoniously as the outcome of self-organisation among a group of cells that led solitary lives but were equipped with traits that could be co-opted for social living (Newman and Forgacs 2005; Ratcliff et al. 2012). The observed phenotypic differences in morphology and behaviour could then be largely neutral (Bonner and Lamont 2005; Bonner 2013).

[36] Because, based on their DNA sequences the last common ancestor of the two lived 400 million years ago (Sucgang et al. 2011). But *D. purpureum* and *D. discoideum* have a number of orthologous genes whose expression patterns overlap considerably (Parikh et al. 2010), i.e., in terms of gene expression patterns the species look very similar.

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The Non-dictyostelid Sorocarpic Amoebae

Matthew W. Brown and Jeffrey D. Silberman

Abstract The social life cycle made famous through research on the dictyostelids is not an evolutionary innovation that is solely unique to the dictyostelids. Since 1873, other protistans with similar life styles have been recognized. Historically, they have been allied under various taxonomic classifications over the last 140 years; however, the recent influx of molecular data has proven that analogous methods through a social means to form a spore dispersal structure have independently arose in 7 different lineages of eukaryotic organisms. Here we provide a brief introduction to each of the amoeboid organisms that display this behavior focusing on their life histories and the history of the research on each taxon. These organisms represent one of the most striking examples of ultimate convergent evolution across the greatest possible evolutionary distances in eukaryotic evolution. Research into the molecular and developmental biology, that underlies the evolution of a social life cycle and formation of a fruiting body is still in its infancy when compared to the dictyostelids. However, the genomes from several non-dictyostelid sorocarpic amoebae are soon becoming available, and a new age of research into these fascinating organisms is beginning to gain traction.

1 Introduction

The evolution of multicellular organisms from simpler unicellular ancestors is one of the great outstanding mysteries of evolutionary biology. Specifically, how many times and where it has arisen over the course of life (for a brief review see (King

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2004; Bonner 1998)). Many fields of biology are often interested in examining the commonalities among complex and simple multicellular life and even unicellular life (Carroll 2001). Multicellularity has undoubtedly arisen numerous times over the course of both prokaryotic (i.e., Cyanobacteria, Actinomyces, and Myxobacteria), and eukaryote life (Bonner 1998). One of the great, unexploited, and all too often overlooked opportunities to examine multicellularity in a relatively simple and possibly primitive form, is the sorocarpic amoebae (historically referred to as cellular slime molds). Generally speaking, slime molds are eukaryotic amoeboid organisms that live their trophic lives as amoebae and at some point of their life cycle form a "fungus-like" fruiting body that serves as a dispersal structure of dormant propagules (Bonner 1967; Olive 1975; Raper 1984). Fruiting body formation in plasmodial slime molds is initiated by cleavage of a multinucleate solitary amoeba to form a stalk and a multicellular resistant resting structure atop. Dictyostelids and a handful of other, distantly related, amoebae form fruiting bodies through cooperation of many individual amoebae that work in concert, yet retain their individuality, to form a multicellular structure; these are the sorocarpic amoebae.

Sorocarpic amoebae are heterotrophic amoeboid organisms that live in freshwater and terrestrial environments as solitary amoebae feeding on various bacteria and fungi such as yeasts (Bonner 1967; Olive 1975; Raper 1984). After a period of vegetative growth, the amoebae aggregate with other amoebae as a result of a known or hypothesized chemotactic stimulus (Bonner 1947; Olive 1901; Raper 1984). What makes them unique among amoebae is that, they are capable of social cooperation with other amoebae of the same species (but also different species may cooperate to form chimerics in dictyostelids see (Jack et al. 2008)) to form a propagule-dispersal structure, the sorocarp (a fruiting body) (Bonner 1967). Organisms with this life history have historically been studied together and because fruiting by aggregates of amoebae was taxonomically important, they were often assumed closely related by experts and non-experts alike (see Table 1 of Brown et al. 2012b). Over the course of the last decade, in particular the last 5 years, many molecular data have come to light that unequivocally dismantle the notion that sorocarpic amoebae are closely related entities (Brown et al. 2012a).

For historical and for simplicity sake, here we consider two separate assemblages of sorocarpic amoebae. The first is the natural group of dictyostelid cellular slime molds (from here on referred to as the dictyostelids). The others are a group of unrelated assemblages, which were often referred to as the acrasid cellular slime molds (Brown et al. 2012b). The dictyostelids, discussed throughout this book, but not here, are a monophyletic group within Amoebozoa that is composed of the genera *Dictyostelium*, *Polysphondylium*, and *Acytostelium*. They form a clade in molecular reconstructions (Adl et al. 2012; Romeralo et al. 2010; Schaap et al. 2006). The other sorocarpic amoebae are composite lineages scattered across the eukaryote across the tree of Life, except for their notable absence within Archaeplastida (Fig. 1). Fruiting by cellular aggregation has arisen at least six times independently in the history of eukaryotes. The purpose of this chapter is to

Table 1 Details of each sorocarpic amoeba taxon

Taxon	Years	Author	Eukaryotic group	Assemblage	Rediscovered?	ATCC Stain	Sorocarp structure	Amoebae type	Mitochondrial cristae
Dictyostelium mucoroides	1869	Brefeld	Amoebozoa	Dictyostelia	Many times		Complex, stalk and spore cells (globose sorus), stalk tube	Fine subpseudopodia	Tubular
Pocheina (= Guttulina) rosea Loeblich and Tappan 1961	1873	Cienkowsky	Excavata	Acrasidae	Olive and Stoianovitch (1974)		Stalk and spore cells (globose sorus)	Eruptive limax	Discoid-Dykstra (1977)
Acrasis granulata	1880	van Teighem	Excavata	Acrasidae	NO		Crampon basal (stalk) cell and spore cells (chain)	?	?
Guttulina sessilis (Guttulinopsis?)	1880	van Teighem	?	?	NO		Stalk and spore cells (globose sorus)	?	?
Guttulina aurea (Guttulinopsis?)	1880	van Teighem	?	?	NO		Spore cells (sessile mass)	?	?
Copromyxa (= Guttulina) protea Zopf 1885	1883	Fayod	Amoebozoa	Tubulinea	Nesom and Olive (1972)	PRA-324	Single cell type (sorocysts) (column)	Limax lobose pseudopodia	Tubular-Dykstra (1977)
Polysphondylium violaceum	1884	Brefeld	Amoebozoa	Dictyostelia	Many times		Complex with whorls, stalk and spore cells (globose sorus), stalk tube	Fine subpseudopodia	Tubular-Hohl et al. (1970)
Guttulinopsis clavata	1901	Olive EW	Rhizaria?	Cercozoa?	NO		Stalk with mucus matrix and elongate cells, spore cells (globose sorus)	Irregular lobose pseudopodia	?

(continued)

Table 1 (continued)

Taxon	Years	Author	Eukaryotic group	Assemblage	Rediscovered?	ATCC Stain	Sorocarp structure	Amoebae type	Mitochondrial cristae
Guttulinopsis stipitata	1901	Olive EW	Rhizaria?	Cercozoa?	NO		Stalk with mucus matrix and elongate cells, spore cells (globose sorus)	Irregular lobose pseudopodia	?
Guttulinopsis vulgaris	1901	Olive EW	Rhizaria	Cercozoa	Olive (1965), Brown et al. (2012a)		Stalk with mucus matrix and elongate cells, spore cells (globose sorus)	Irregular lobose pseudopodia	Discoid-Dkystra (1977)
Acytostelium leptosomum	1958	Raper and Quinlan	Amoebozoa	Dictyostelia	Many times		Acellular stalks, spore cells.	Fine subpseudopodia	Tubular-Hohl et al. (1968)
Acrasis rosea	1960	Olive LS and Stoian.	Excavata	Acrasidae	Many times	PRA-321	Stalk and spore cells, arborescent	Eruptive limax	Discoid-Dykstra (1977)
Sorodiplophrys stercorea	1975	Olive LS and Dykstra	Stramenopiles	Labyrinthulomycetes	Not reported		Stalk with mucus matrix with embedded cells, spore cells (globose sorus)	Testate amoebae with filose pseudopodia	Tubular-Dykstra (1976a)
Guttulinopsis nivea	1977	Raper et al.	Rhizaria?	Cercozoa?	NO		Stalk with mucus matrix and elongate cells, Spore cells (globose sorus)	Irregular lobose psuedopodia	Discoid-Erdos and Raper (1978)
Copromyxella corralloides	1978	Raper et al.	Amoebozoa	Tubulinea	NO		Single cell type (sorocysts) (column)	Limax lobose pseudopodia	Tubular-Dkystra (1977)
Copromyxella filamentosa	1978	Raper et al.	Amoebozoa	Tubulinea	NO		Single cell type (sorocysts) (column)	Limax lobose pseudopodia	Tubular-Dkystra (1977)

(continued)

Table 1 (continued)

Taxon	Years	Author	Eukaryotic group	Assemblage	Rediscovered?	ATCC Stain	Sorocarp structure	Amoebae type	Mitochondrial cristae
Copromyxella silvatica	1978	Raper et al.	Amoebozoa	Tubulinea	NO		Single cell type (sorocysts) (column)	Limax lobose pseudopodia	?
Copromyxella spicata	1978	Raper et al.	Amoebozoa	Tubulinea	NO		Single cell type (sorocysts) (column)	Limax lobose pseudopodia	?
Fonticula alba	1979	Worley et al.	Opisthokonta	Nucletmycea	NO	38817	Complex, with extracellular matrix stalk, sorogenic amoebae and spore cells	Irregular filose pseudopodia	Discoid-Deasey (1982)
Pocheina flagellata	1983	Olive LS et al.	Excavata	Acrasidae	NO		Stalk and spore cells (globose sorus)	Eruptive limax and amoeboflagellates	?
Allovahlkampfia spelaea	2009	Walochnik and Mulec	Excavata	Acrasidae	Many times	PRA-288	Stalk and spore cells, simple column or globose sorus (Brown et al. 2012)	Eruptive limax	?
Acrasis helenhemmesae	2010	Brown et al.	Excavata	Acrasidae	Many times	PRA-322	Stalk and spore cells, simple column	Eruptive limax	?
Acrasis kona	2011	Brown et al.	Excavata	Acrasidae	Many times	MYA-3509	Stalk and spore cells, arborescent	Eruptive limax	?
Acrasis takarsan	2011	Brown et al.	Excavata	Acrasidae	NO	PRA-323	Stalk and spore cells, arborescent	Eruptive limax	?

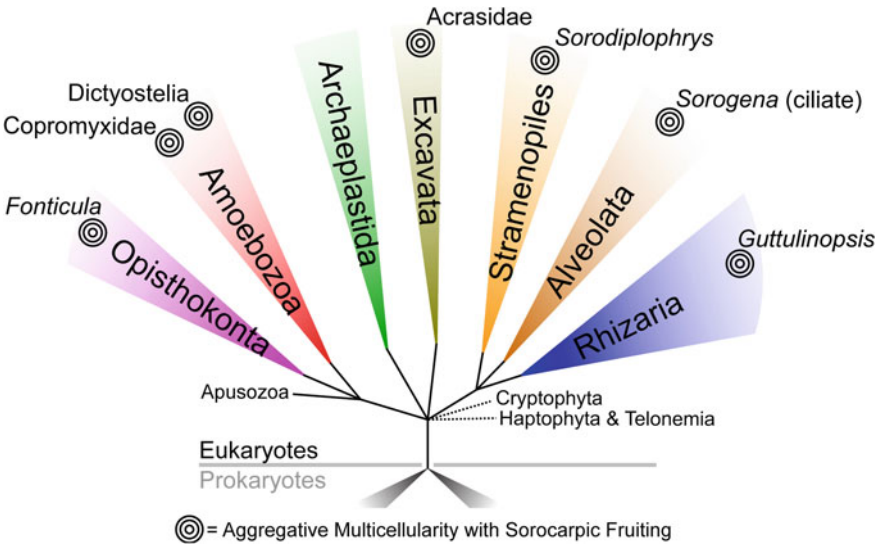

Fig. 1 Eukaryotic tree of Life edited from Brown et al. (2012a), with sorocarpic aggregative multicellular protists denoted with a bulls eye. Note that *Sorogena* is a ciliate and not an amoeboid organism

introduce these non-dicytostelid sorocarpic amoebae from a historical and evolutionary prospective.

Unlike the dictyostelids, the other sorocarpic amoebae have received little attention and have not become model organisms used to explore various aspects of biology. Aside from the species descriptions and subsequent molecular phylogenetic placements, we currently know very little about the physiological processes and genetic triggers that are responsible for cellular aggregation and differentiation of these cooperative multicellular structures in such a diverse set of independent lineages. Given their broad distribution across the eukaryotic tree, they offer some of the most compelling examples of convergent evolution onto a similar mode of cooperation. Perhaps with acquisition and analyses of comparative transcriptomic and genomic data, these now obscure cellular slime molds will shed light on basic biological processes such as cell–cell communication and environmental cues involved in cellular differentiation.

2 The History of Sorocarpic Amoeba Research

The sorocarpic amoebae in the broadest sense have been studied for around 140 years (summarized in Fig. 2); the first to be discovered was the dictyostelid, *Dictyostelium mucoroides* (Brefeld 1869). In the description of *Dictyostelium*, Brefeld mistakenly described the dung inhabiting organism as forming a

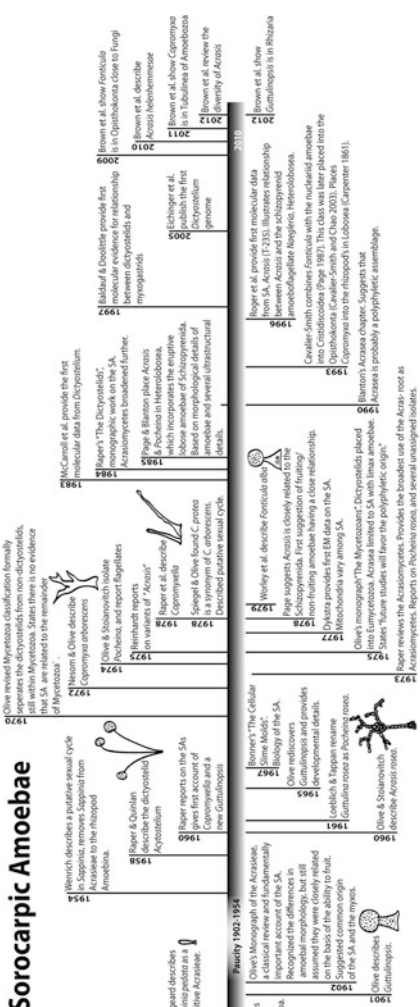

Fig. 2 Timeline of the 140 years of sorocarpic amoeba research, with a brief note of the most important works. Note not to scale

plasmodium that was the result of cell fusion. Plasmodia are large coenocytic (multinucleate) amoeboid cells and are common to the myxogastrid slime molds (also known as Myxomycetes). At the time of his discovery of *Dictyostelium,* Brefeld was an assistant of de Bary, a famed botanist noted particularly for his early work on the myxogastrids. Brefeld's knowledge of myxogastrids may have skewed his (mis)interpretation of the life cycle of his *Dictyostelium* that resulted in (Rostafinski 1875) placing *Dictyostelium* into de Bary's taxon Mycetozoa as a myxogastrid.

The first non-dictyostelid sorocarpic amoeba discovered was found on decaying wood collected from Poland and originally called *Guttulina rosea* (Cienkowsky 1873). In 1961, Loeblich and Tappan found that the name for the genus *Guttulina* was previously used for a foraminifera (d'Orbigny 1839) and proposed the genus *Pocheina*. The sorocarps are small and rose colored under reflected light. Each sorocarp consists of a stalk with wedge-shaped cells and a globose spore mass (sorus) atop the stalk. When placed in water, the amoebae that emerge from spores—stalk cells are limax in shape (slug or 'shoe' shaped) with rose/pink cytoplasm. Cienkowsky's description was published as an abstract of a presentation given to a meeting of Russian naturalists at Kazan, and is provided as an English translation in (Olive 1902). In this translation, Cienkowsky suggested that the organism was closely related to *Dictyostelium* but the amoebae pile up to form aggregates and form spores and stalk cells (cysts) without cellular fusion.

It was not until a pivotal study conducted in 1880 that the most important observation on sorocarpic amoebae was made from which our current concept of their life history stems (van Tieghem 1880). In van Tieghem's observations on *Dictyostelium* along with a new taxon, he described (isolated from spent beer yeast), *Acrasis granulata*, he discovered that, in water, the so-called plasmodium was readily separable into many amoeboid cells, although, Cienkowsky recognized this in his description of *Sorodiplophrys* (see below and Cienkowsky 1876). He observed amoebae aggregating to form an 'aggregative plasmodium' (*plasmode aggrégé*) comprised of many individual cells, now called a pseudoplasmodium. In this study, van Tieghem offered a novel taxon Acrasiées containg all of the cellular slime molds known at the time, *Acrasis*, *Guttulina* (*Pochenia*), and the dictyostelids. His name "*Acrasis*" was based upon an observation he stated in his manuscript (in French) "*C'est pour marquer l'absence de fusion entre les cellules nues qui sont appelées à former le fruit, que je propose de nommer cette plante* Acrasis." Translated into English as "to mark the absence of fusion between the naked cells, which form the fruiting body, I propose to name this plant *Acrasis*." He derived the name *Acrasis* from "*De α privatif et κρασις, fusion*" (footnote 1 on p. 320 of van Tieghem 1880). The Greek letter α can be understood as "signifying absence of" when used as a prefix and *κρασις* is transliterated from the Greek alphabet to Latin alphabet as *krasis* (or *crasis*). *Crasis* means a "mix or blend" in Greek. We interpret van Teighem's translation and reasoning of this term to mean "not mixed" or "an incomplete mix" (i.e., not fused), which is a logical translation, since the cells come together but their plasma membranes do not fuse with one another during the formation of fruiting bodies. Thus, his taxon Acrasiées

highlights the developmental nature of fruiting bodies in sorocarpic amoebae. Subsequently, from his root *Acras*—there have been multiple taxon names coined that have been used variously to refer to the sorocarpic amoebae as a whole or to refer to specific groups (see Table 1 of Brown et al. 2011).

Over the next 20 years from 1881 to 1901, a significant number of new taxa were described (Table 1). E. W. Olive, an influential author who was the primary scientist at the time working on sorocarpic amoebae, published a paper in 1901 in which he described three new coprophilous species in a novel genus, *Guttulinopsis*. From 1902 to 1960, there was a paucity of research on the diversity sorocarpic amoebae, although there were many works on the development of and diversity of the dictyostelids, primarily by Bonner and Raper. In the period of 1960–1979, a number of novel sorocarpic taxa were discovered, namely *Acrasis rosea* and a novel *Guttulinopsis* species, as well as the genera *Fonticula* and *Copromyxella*. In 1975, L. S. Olive's seminal book, "The Mycetozoans", presented the first classification of the cellular slime molds based on a morphological phylogenetic framework. In this classification, Olive removed the dictyostelids from other sorocarpic amoebae in his Acrasea, placing them into Eumycetozoa with myxogastrids and protosteloid amoebae. Olive further limited Acrasea to sorocarpic amoebae with limax amoebae.

The last 15 years have provided an enormous amount of molecular data on most known the sorocarpic amoebae, to generate a robust non-morphological phylogeny. These data show that they have independently evolved over the course of eukaryotic evolution. The first molecular data from a non-dictyostelid sorocarpic amoeba was from *Acrasis kona* in 1995, at that time referred to as *Acrasis rosea* (see Brown et al. 2012b). Subsequently, data from *Fonticula alba, Copromyxa*, several novel *Acrasis* species, *Allovahlkampfia*, *Pocheina*, and *Guttulinopsis vulgaris* are now available (Brown et al. 2009, 2010, 2011, 2012a, b) as well as from *Sorogena*, an analogous ciliate that makes a sorocarp (Lasek-Nesselquist and Katz 2001). Nonetheless, there are still many taxa that have yet to be examined at the molecular level, in particular *Copromyxella*, *Sorodiplophrys*, and other species of *Guttulinopsis* (Table 1).

3 Life Cycle

The sorocarpic life cycle can be divided into two separate stages, the trophic stage and fruiting stage, unlike in the myxogastrids slime molds where the plasmodium is both a trophic and fruiting stage. In the cellular slime molds, the trophic stage is an individual unicellular amoeba. The fruiting stage begins with amoebae aggregating to form a cluster of cells that are not fused. The details of how aggregation leads to the development of the mature fruiting body vary with respect to the particular sorocarpic taxon.

Although the details may vary, a generalized life cycle of these organisms can be constructed. Arbitrarily starting from the resistant-walled propagule (spore) in a

suitable environment it germinates as an amoeba. The amoeba feeds on various microorganisms and undergoes cellular divisions, and may reversibly encyst singly on the substratum. Through some signaling mechanism, the amoebae begin to gather with others to form an aggregate, in which the cells do not fuse with one another but are often covered with a common slime sheath. The aggregate, now called a sorogen, then begins to rise from the substratum by virtue of cells encysting (becoming walled) below. Sorogen is a general term for a mass of undifferentiated amoeboid cells that are in the process of fruiting and have not yet laid down cell walls. At sorocarp maturity all or most cells are walled (encysted).

4 The Five Groups of Non-dictyostelid Sorocarpic Amoebae

4.1 Amoebozoa: Copromyxidae

The Amoebozoa contains the 'well known' slime molds—the Dictyostelia cellular slime molds as well as the myxogastrid and protosteloid slime molds (Adl et al. 2012). It turns out that it is also the home for at least one member of the Copromyxidae, *Copromxa* (Brown et al. 2011). The copromyxids form the simplest sorocarps of all sorocarpic amoebae and their fruiting development is unique. The sorocarps are little more than a cluster of cells that have encysted upon one another (Figs. 3 and 4). However, the gross structure of their sorocarps is often quite elaborate (Fig. 4).

Copromyxa protea was originally described as *Guttulina protea* in 1883 (Fayod 1883), found on herbivore dung worldwide (Olive 1901). Shortly afterward, Zopf (1885) renamed the species in a new genus because its sorocarps possessed only one cell type as opposed to the fruiting bodies of *G. rosea* (Cienkowsky 1873) (now known as *Pocheina rosea,* see below), with two dissimilar cell types. The genus *Copromyxa* currently consists of two species, *C. protea* and *C. cantabrigiensis*, although the latter is not known to form sorocarps (Brown et al. 2011). *Copromyxa protea* produces a round, thick-walled cyst (sphaerocyst) that was first observed by Fayod (1883) and latter suggested to be a zygote (Spiegel and Olive 1978). *Copromyxa arborescens* was described in 1972, because of the apparent absence of these sphaerocysts (Nesom and Olive 1972). However, Spiegel and Olive (1978) conducted mating experiments with different putative strains of *C. arborescens* and found that they represent individual mating types of heterothallic strains of *C. protea*.

Mature fruiting bodies of *C. protea* consist of irregularly shaped encysted cells, called sorocysts, all of which appear to be viable (Figs. 3a, b, and 4c) (Nesom and Olive 1972; Olive 1975; Spiegel and Olive 1978; Raper 1984). They germinate as a single, monopodial, limax-shaped amoeba (Fig. 3b). Amoebae can also encyst singly on the substrate (Figs. 3d and 4e). Aggregates of amoebae form when a cell

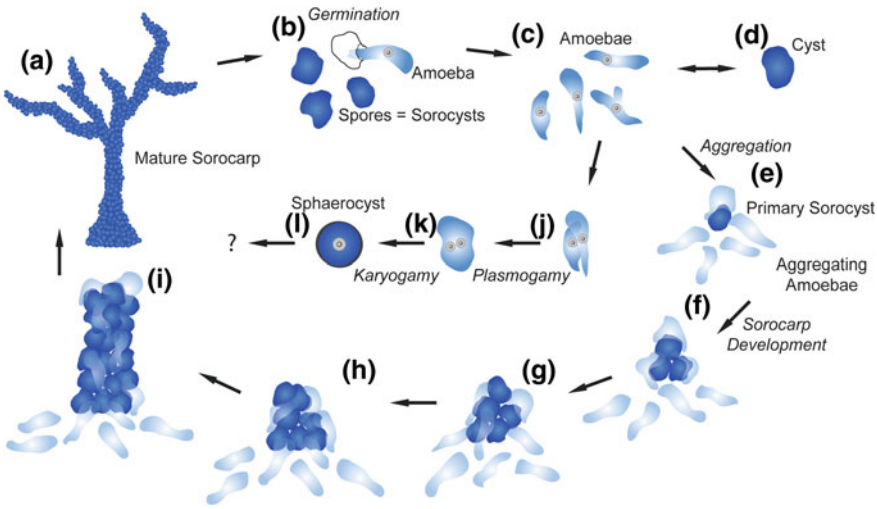

Fig. 3 Life cycle of *Copromyxa protea,* modified from Brown et al. (2011). **a** Mature sorocarp, which consist of a single cell type, sorocysts. **b** Sorocysts germinate as amoebae. **c** Trophic amoebae are limax shaped with a single nucleus. **d** Amoebae may encyst to form microcysts, which can germinate as amoebae. **e** Amoebae may form an aggregate, in which a founder cell of the sorocarp encysts and amoebae of the aggregate crawl on top of and encyst, to form sorocysts. **f–i** Surrounding amoebae continue to crawl up the column of sorocysts and encyst at the apex. **j** Trophic amoebae may come together. The two amoebae presumably undergo plasmogamy **k** then karyogamy, forming a thick walled sphaerocyst **l**. The resultant life stage that follows sphaerocyst germination is unknown. The lighter shaded cells represent trophic amoebae and the darker cells are encysted. Developmental process descriptions are italicized. Names of structures are in regular print

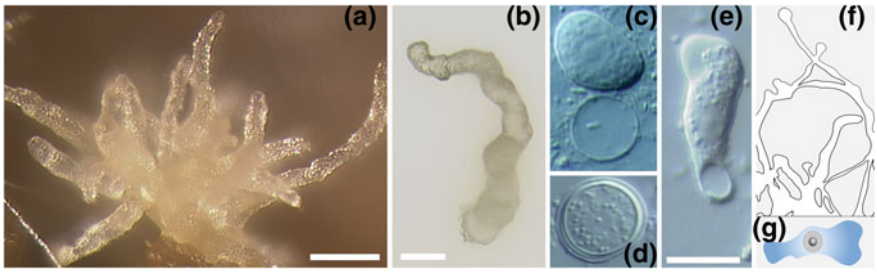

Fig. 4 Copromyxidae. **a–e** *Copromyxa protea.* **a** Complex branching sorocarp growing off of cow dung. **b** Simple columnar sorocarp fruiting on an agar culture dish. **c** Sorocysts (spores), with one empty sorocyst wall. **d** Limax-type monopodial amoeba. **e** Thick-walled sphaerocyst. **f–g** *Copromyxella spicata*, redrawn from Raper et al. (1977). **f** Delicate interlaced sorocarps. **g** Limax-type amoeba. Scale bars a = 100 μm, b = 50 μm, c–e = 10 μm (to scale)

encysts to become a founder cell of the sorocarp (Spiegel and Olive 1977) (Fig. 3e). These founder cells attract amoebae towards them by an unknown chemoattractant mechanism. Amoebae crawl on top of encysted founder cell and

subsequently encyst (Fig. 3e). Continuation of this process results in the apical growth of the fruiting body (Fig. 3f–i). The fruiting bodies may be columnar or branched (Fig. 4a, b). At sorocarp maturity, all cells are encysted (Fig. 3a). There is a presumed sexual cycle that is the result of two uninucleate amoebae undergoing plasmogamy (Fig. 3j) followed by karyogamy (Fig. 3k) with a zygote developing into uninucleate, thick-walled sphaerocyst (Figs. 3l and 4d) (Spiegel and Olive 1978). Although a life stage consistent with karyogamy has been observed and illustrated, fusion of cells has not been observed (Spiegel and Olive 1978). Sphaerocysts are easily recognizable since they are yellow-brown, have a double wall, and are usually larger and more uniform in shape (spherical) than sorocysts and microcysts (Fig. 4d). Sphaerocyst germination has not been observed and is a dead-end in the life cycle under the culture conditions utilized (Spiegel and Olive 1978, personal observation), but presumably the sphaerocysts germinate in the wild.

The presence of tubular mitochondrial cristae (Dykstra 1977) and broad, non-eruptive, lobose pseudopodia, rather than eruptive or filose pseudopodia, hinted that *Copromyxa* belongs in the eukaryotic supergroup Amoebozoa (Adl et al. 2005). Cavalier-Smith (1993, 2003) suggested that *Copromyxa* is a member of the amoebozoan taxon Lobosea (also referred to as Tubulinea, see Smirnov et al. 2005). Indeed, molecular phylogenetic data placed *Copromyxa* within the Tubulinea, branching separately from all other known amoebozoan "slime molds" (Brown et al. 2011) and is an additional innovation of multicellularity in the Amoebozoa.

In 1977, the genus *Copromyxella* Raper, Worley, and Kurzynski was formally described although the species in it were first discovered and briefly described by Raper in (1960) as *Guttulinopsis* sp. (Raper et al. 1978). The sorocarps are largely similar in form to those of *Copromyxa protea*, consisting of a single cell type, round sorocysts. However, the sorocarps are more delicate than those of *Copromyxa,* consisting of long spindly shaped columns (Fig. 4f). The amoebae are limax in form, but are apparently devoid of contractile vacuoles, unlike those of *Copromyxa* (Fig. 4g). Like *Copromyxa*, the mitochondria of *Copromyxella* have tubular cristae (Dykstra 1977). Another feature by which *Copromyxella* spp. can be distinguished from *Copromyxa*, is that in the former, aggregation results in a pseudoplasmodium from which the fruiting body develops while, in the latter, amoebae aggregate toward sorocarps in which sorocysts are already at least partially encysted (Blanton 1990; Fayod 1883; Nesom and Olive 1972; Raper et al. 1978).

Copromyxella is composed of four species, which differ from one another in their gross sorocarp morphology (Raper et al. 1978). *Copromyxella coralloides* with coral-like in form sorocarps was found on bald eagle dung collected in Wisconsin USA. *Copromyxella filamentosa* with delicate sorocarps was found from a sample forest soil collected in Costa Rica. *Copromyxella silvatica* with blunt ended interspersed sorocarps was found from forest soil collected in Illinois USA (Fig. 4f). *Copromyxella spicata* with "javelin-shaped" sorocarps was found originally on a rotting mushroom collected in Connecticut USA.

Only one additional report of *C. spicata* collected from soil humus from northern Georgia USA (Cavender 1980) has been published since the original species descriptions.

4.2 Excavata: Acrasidae

The Acrasidae are probably the best known and historically most important of the non-dictyostelid sorocarpic amoebae. In 1960, Olive and Stoianovitch discovered a sorocarpic amoeba growing on dried grass inflorescences that they equated with *Acrasis*, naming it *A. rosea*. Although they were unsure of the true identity of type species of the genus, *A. granulata*, the authors suggested that their organism was likely *Acrasis.* The doubt stemmed from the lack of morphological details available from *A. granulata* in van Tieghem's (1880) description, in which he offered no illustrations or details on the amoebae. Like *A. granulata*, *A. rosea* has two cell types and sorocarps consisting of chain(s) of spores born on a stalk of stalk cells. The life cycle of *A. rosea* is depicted in Fig. 5. The amoebae are limax in shape with a rose/pink cytoplasm and move by eruptive pseudopodia. This species was subsequently studied quite extensively at the morphological and ultrastructural level (Blanton 1981; Dykstra 1977; Fuller and Rakatansky 1966; Hohl and Hamamoto 1969) as well as at the molecular level (Roger et al. 1996). The taxon has discoid mitochondrial cristae with associated cisternae of rough endoplasmic reticulum (RER) (Dykstra 1977).

*P. rose*a was rediscovered in the 1960s from bark collected in the Netherlands, but it was recalcitrant to cultivation (Raper 1973). In 1974, several isolates of *P. rosea* were again rediscovered from bark, and they were amenable to culture. Among these isolates, several could differentiate into amoeboflagellates, while other isolates were strictly amoeboid (Olive and Stoianovitch 1974). Subsequently, the flagellated taxon was described as a distinct species, *Pocheina flagellata* (Olive et al. 1983). In (1977), Dykstra illustrated that the mitochondria of *Pocheina* are much like *Acrasis* with discoid cristae and associated RER and suggested that the two genera are closely related.

From the morphological data available from *Acrasis* and *Pocheina*, a new higher-level taxon Heterolobosea was suggested that combined these sorocarpic amoebae with the schizopyrenid amoebae (Page and Blanton 1985), All of these amoebae have characteristic eruptive pseudopodial locomotion and a RER-associated mitochondria with discoid cristae. The name Heterolobosea was used to refer to "other lobose amoebae" distinguishing them from the amoebae with tubular mitochondrial cristae and noneruptive pseudopodia now recognized as members of the Amoebozoa (Adl et al. 2012). The Heterolobosea represents the first major hypothesis in which both sorocarpic and non-fruiting amoebae were suggested to be each other's closest relatives. Page and Blanton (1985) further suggest limiting the Acrasidae as a taxon within Heterolobosea comprising *Acrasis*

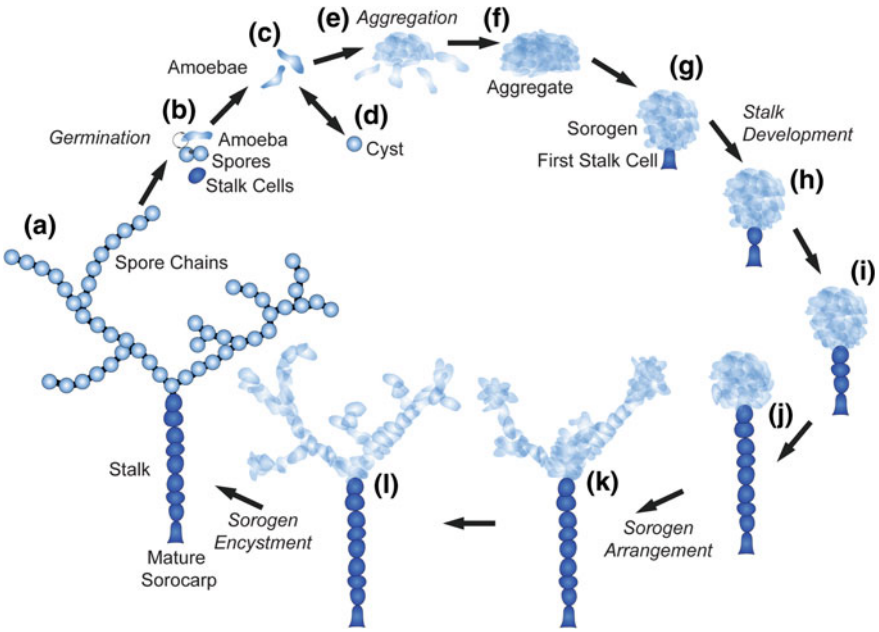

Fig. 5 Life cycle of *Acrasis rosea*. **a** Mature sorocarp arranged as a branched structure with several chain of spores. Sorocarp displays basal stalk cells (*dark gray*) with distal spore cells (*light gray*). **b** Spores germinate as limax-type amoebae. Spores have raised hila and the spore walls persist after germination. **c** Trophozoites are limax-type amoebae. **d** Amoebae may form microcysts, which can germinate as amoebae. **e** Amoebae aggregate to form small mounds. **f** Amoeba aggregation ceases. **g** A cell in the aggregate encysts to become the first stalk cell, with a mass of amoebae, the sorogen, remaining on top of the newly encysted stalk cell. **h–j** Cells in the sorogen encysts to become the next basal stalk cells, with the sorogen elevating by virtue of stalk cell encystment. **k–l** Cells of the sorogen align into chains of amoeboid cells and then encyst to become spores. Developmental process descriptions are italicized. Names of structures are in regular print

and *Pocheina* based on the unique combination of characteristics observed in their amoebae and mitochondria compared to those of all other sorocarpic amoebae.

The first molecular evidence for Heterolobosea was demonstrated by Roger et al. (1996), in which a close relationship between *Acrasis* and *Naegleria* was inferred based on phylogenies of glyceraldehyde 3-phosphate dehydrogenase (Roger et al. 1996). Subsequently, many authors have verified this relationship using other gene sequences (Baldauf et al. 2000).

Recently, a review of the Acrasidae has provided an insight into the evolution of the group, which is far more diverse than previously thought (Brown et al. 2010, 2012b). Until recently, *A. rosea* was thought to be an extremely plastic organism with the ability to form multiple sorocarp morphologies (Olive et al. 1961; Olive and Stoianovitch 1960; Reinhardt 1975). However, detailed molecular phylogenetic analyses coupled with careful morphological observations of cultures isolated from a wide set of globally sampled locales altered the species concept for

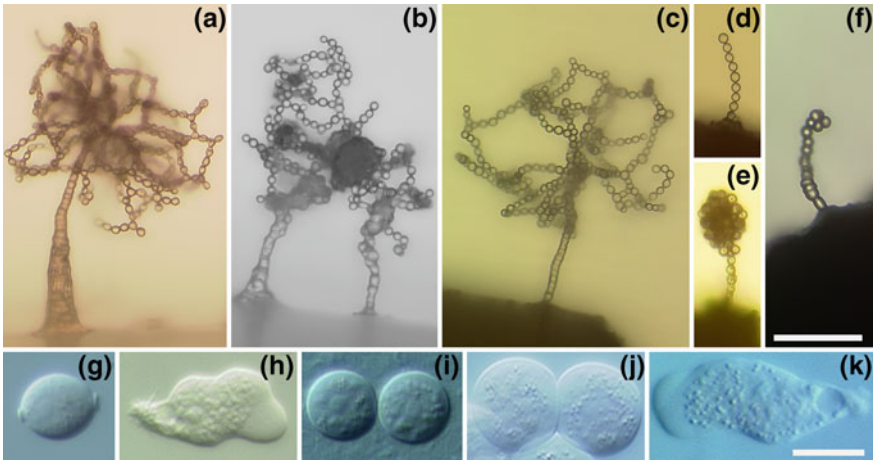

Fig. 6 Acrasidae. Complex sorocarp of *Acrasis kona* (**a**), *Acrasis rosea* (**b**), and *Acrasis takarsan* (**c**). **d** Simple sorocarp of *Acrasis helenhemmesae*. **e** Sorocarp with globose sorus of "*Pocheina*" *rosea*. **f** Simple sorocarp of *Allovahlkampfia spelaea*. **g** Spore of *Acrasis* sp. with characteristic areolate structures (hila). **h** Limax-type amoeba of *Acrasis* sp. **i** Cysts of *Acrasis* sp. **j** Spores of *Allovahlkampfia spelaea* with out the characteristic hila seen in *Acrasis* and *Pocheina*. **k** Limax-type amoeba of *Allovahlkampfia spelaea.* Scale bars a–f = 100 μm (to scale), g–k = 10 μm (to scale)

Acrasis and demonstrated that the diversity represents different species (Fig. 6a–d) (Brown et al. 2012b). Further, molecular data from *Pocheina* suggests that *P. rosea* (Fig. 6e) is actually a morphotype of *A. rosea* (Brown et al. 2012b). No molecular data are available from flagellated isolates of either *Acrasis* or *Pocheina* and it is unclear how they are related to the other acrasids. In addition to the known acrasids, a sorocarpic life style was also found to be present in the genus *Allovahlkampfia* (Fig. 6f) (Brown et al. 2012b). *Allovahlkampfia* has spores that lack aerolate structures at the junction of adjacent cysts (Fig. 6k), which are present in all described members of *Pocheina* and *Acrasis*. But the amoebae and cysts found in members of all three genera are indistinguishable (Fig. 6l).

Finally, the genome of *A. kona* is currently being sequenced (Chengjie Fu personal communication).

4.3 Opisthokonta: Fonticulaceae

Fonticula, a genus of unique sorocarpic amoeba found on canine dung was described in 1979, although it had been in culture since 1960 (Worley et al. 1979). The genus contains only a single species *F. alba*. The sorocarps are much different than those of any other sorocarpic organism; they are volcano-like, consisting of conical extracellular stalk from which spores from within are forced through the top to form a slimy mucoid spore mass (Figs. 7 and 8a). The life cycle is also

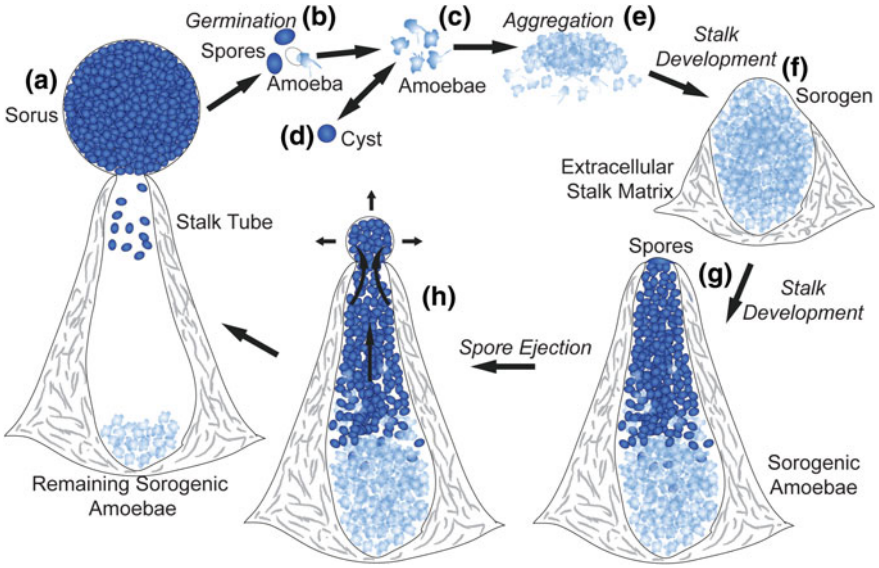

Fig. 7 Life cycle of *Fonticula alba*, modified from Brown et al.(2009). **a** Mature sorocarp with a mucoid spore mass (sorus) atop the stalk made of extracellular matrix material. Note that not all spores are incorporated into the spore mass at maturity. At the bottom of the sorocarp, some sorogenic amoebae remain, which do not become spores. **b** Spores, which are surrounded by a mucus sheath, germinate as amoebae. **c** Trophic amoebae with filopodia. **d** Amoebae can encyst to form cysts, which are morphologically similar to spores. Cysts germinate as amoebae. **e** Amoebae aggregate to form a mound. **f** The aggregate forms a common slime sheath, and sorogenic amoebae secrete an extracellular matrix of stalk material. **g** The upper two-thirds of amoebae within the stalk begin to encyst to form spores. **h** When the stalk reaches maturity, a bulge forms at the apex and spores are mechanically forced upward into the sorus, which expands as spores are forced upward. Developmental process descriptions are italicized. Names of structures are in regular print

unique (Fig. 7). Aggregates of amoebae form a common sheath and then secrete an extracellular matrix that encapsulates the amoeboid cells of the sorogen (Fig. 7f). The uppermost cells of the sorogen then encyst as spores (Fig. 8g) and are forcibly ejected from the top of the stalk (Fig. 8h). *Fonticula* has amoebae with filose pseudopodia that were considered similar to those of the dictyostelids (Worley et al. 1979). However, the mitochondria of *Fonticula* have discoid cristae (Deasey 1982), unlike those of dicytostelids. Molecular data placed this organism with the nucleariid amoebae of the Opisthokonta (Brown et al. 2009), which supports an earlier morphology-based suggested phylogenetic affiliation of Cavalier-Smith (1993). *Fonticula* along with the nucleariid amoebae and the Fungi compose the higher-level taxon Nucletmycea (Brown et al. 2009; Adl et al. 2012), this clade is also known as Holomycota (Liu et al. 2009).

F. alba appears to be exceedingly rare; it has only been found and cultured a once (Brown et al. 2009). Fortunately, the type culture is still available at the American Type Culture Collection (ATCC 38817). There are no sequences that

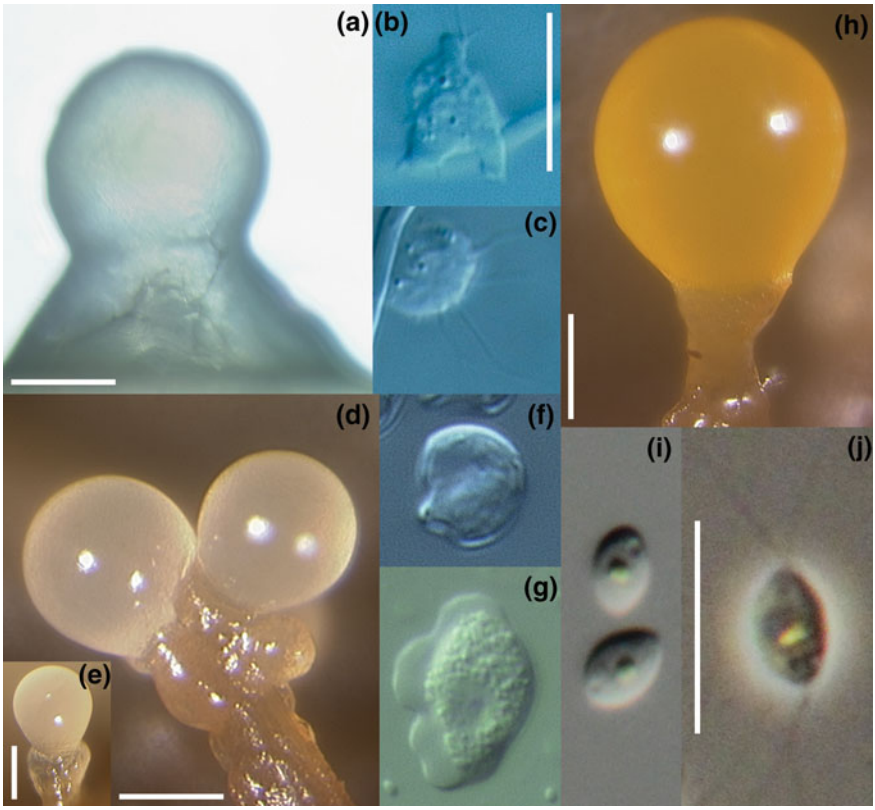

Fig. 8 Images of *Fonticula alba* (**a–c**), *Guttulinopis vulgaris* (**e–g**), and *Sorodiplophrys stercorea* (**h–j**). a. Sorocarp of *Fonticula alba*. **b–c** Amoebae of *Fonticula alba* with filose pseudopodia, amoebae are near a fungal hypha. **d** Two sorocarps of *Guttulinopsis vulgaris* fruiting near each other off of cow dung. **e** Single sorocarp fruiting on cow dung. **f** Spore of *Guttulinopsis* with a characteristic collapsed morphology. **g** Flabellate amoeba of *Guttulinopsis vulgaris*. **h** Sorocarp of *Sorodiplophrys stercorea* with a globose deep golden sorus. **i** Spores of *Sorodiplophrys stercorea.* **j** Amoeba of *Sorodiplophrys stercorea* with two filose pesudopodial extensions extending from either pole. Images **i–j** are from Alexander Tice at the University of Arkansas. Scale bars a = 100 μm, b,c,f,g = 10 μm (to scale), d = 50 μm, e = 50 μm, h = 100 μm, i,j = 15 μm (to scale)

can be unequivocally assigned to *F. alba* in the environmental SSU rDNA gene libraries available in GenBank. Although, in a recent phylogeny of environmental SSU rDNAs sequence from a marine anoxic environment (NCBI: EF219013) branches with high support as sister to *F. alba,* but it is significantly different with only 89 % sequence identity (del Campo and Ruiz-Trillo 2013).

Given the placement of *Fonticula* within Opisthokonta and its unique form of multicellularity, it represents a great opportunity to examine the evolution of multicellularity in an evolutionary microcosm. Currently, the genome is being sequenced.

4.4 Rhizaria: Guttulinopsidae

The genus *Guttulinopsis* was first described in 1901 (Olive 1901). It is composed of four species, that have been affiliated with a variety of taxa through the years (Olive 1902, 1975; Raper 1984). *G. vulgaris* is the by far most common species within the genus. It is found on the dung of various mammalian herbivores (primarily bovine) worldwide (Olive 1902, 1965, 1975; Raper et al. 1977). Sorocarps are macroscopic (200–500 μm) (Fig. 8d–e) and consist of upwards of 20,000 cells (Bonner 2003) with a distinct stalk and one to several spore masses (sorus(i)) atop the stalk (Fig. 8d–e). Mature sorocarps consist of distinct cell types; irregularly shaped walled spores (Fig. 8f) along with stalk compartments containing degenerated, amoeboid, and encysted stalk cells (Olive 1965). Like most dictyostelids (and unlike all other described cellular slime molds), altruism and the sacrifice of some cells is part of the developmental processes involved in the formation of sorocarps (Olive 1965).

Various morphological and ultrastructural studies of *G. vulgaris* amoebae and sorocarps, failed to accurately find its place among eukaryotes (Dykstra 1977; Raper 1984; Olive 1965, 1975). The amoebae are similar to the acrasidae with discoid mitochondria cristae, but no RER associated. These details lead many to believe that Guttulinopsidae is closely related to the Acrasidae. Recently, a multigene phylogeny placed this taxon into the Cercozoa of the Rhizaria (Brown et al. 2012a). These data along with morphological data suggest that is closely related to non-fruiting amoeba *Rosculus* (Brown et al. 2012a; Page 1988).

There are several other species of *Guttulinopsis* that have not been examined either ultrastructurally or molecularly. Morphological details of each organism suggest that they may be within the same genus, but molecular data are needed to examine this fully. Unfortunately, the morphological descriptions and illustrations of Olive(1901) for *G. stipitata* and *G. clavata* are inadequate for positive identification making the chance of their rediscovery slim (Raper et al. 1977). In 1977, *G. nivea* was discovered from Howler-monkey dung collected in Panama and tropical soils in Costa Rica and Java. The snow-white sorocarps are similar in size and habit to *G. vulgaris*, but have a broad expanded membrane covered base (Raper et al. 1977). The stalk of young sorocarps has a membranous covering encapsulating amoeba cells within the erect stalk. After about a day, the stalk becomes rigid with a sponge-like matrix of encapsulated cells.

4.5 Stramenopiles: Sordiplophrys

Sorodiplophrys stercorea was the second of all non-dictyostelid sorocarpic amoeba to be discovered in 1876 by Cienkowsky named *Diplophrys stercorea.* Due to its remarkable ability to aggregate and form sorocarps, which the type species of *Diplophrys* apparently does not, the species was later placed into a novel

genus *Sorodiplophrys* (Dykstra and Olive 1975). Researchers interested in the sorocarpic organisms have consistently overlooked the lone sorocarpic stramenopile, which can be attributed to the early opinion that they are probably not closely related to the other sorocarpic amoebae. At the time of its description it was placed with the labyrithulid amoebae, which at the time clearly demonstrated its uniqueness from the other known slime molds (Cienkowsky 1876; Olive 1901, 1902). Cienkowsky was the first to actually recognize that sorocarps are the result of aggregation. However, because they were not assumed closely related to the other sorocarpic amoebae, the life cycle was not equated to other sorocarpic amoebae. He found that when the cells within the sorocarp's mucoid sorus are collected they are individuals that are capable of aggregation, which in turn make new sorocarps (Cienkowsky 1876).

Sorodiplophrys is coprophilous. It is found on herbivore dung, in particular that of cow and horse (personal observation), but has also been observed on porcupine dung (Olive 1901). *Sorodiplophrys* produces large macroscopic sorocarps (400–600 μm) consisting of thousands of cells. These sorocarps are readily distinguishable from all other sorocarpic organisms due to their brilliant golden hue (Fig. 8h). Except for their color, they are grossly very similar in size and shape to the sorocarps of *G. vulgaris* (Fig. 8d). Sorocarps consist of a stalk made up of a core of dead cells and a matrix derived from ectoplasmic elements and degenerated cells (Dykstra and Olive 1975). Atop the stalk is a golden globose mucoid mass of encysted spores (Fig. 8i).

The amoebae of *S. stercorea* are small (6–17 μm) and covered with a thin round organic test (shell). They produce many filose pseudopodia that extend from opposite poles of the test (Dykstra and Olive 1975) (Fig. 8j). The amoebae have a golden-orange highly refractile lipid droplet within their cytoplasm, which gives the sorocarps their brilliant color. Two electron microscopic studies have been done on the amoebae, examining the formation of the organic test and the process of nuclear division (Dykstra 1976a, b).

Currently, there are no molecular data from *S. stercorea*. However, due to the nearly identical amoebae to *Diplophyrs* there is little doubt that it is closely related to it, which clearly branches within the Labyrinthulomycetes (Cavalier-Smith and Chao 2006; Gomaa et al. 2013; Leander and Porter 2001).

5 Outstanding Questions

From the data presented over the last 5 years a most striking realization emerges—in all major lineages where amoebae evolved, so has aggregative social multicellularity (i.e., sorocarpic amoebae). The five major lineages of amoebae are the Amoebozoa, Heterolobosea (Excavata), Labyrithulomycetes (Stramenopiles), Nuclearioid Amoebae (Opisthokonta), and Rhizaria. Given this observation, it is clear that the selective forces involved in the dispersal of spores via an aerially

directed sorocarp must be pervasive in terrestrial environments. The sorocarpic aggregative approach to elevate dispersal propagules is more ubiquitous than alternative methods of amoebal aerial spore dispersal structures, like that of the myxogastrid and protosteloid slime molds, which are limited to the Amoebozoa. This suggests that there are underlying mechanisms involved in aggregation, cell–cell communication, and cell adhesion that were coopted from ancestral traits, which were plesiomorphic to all protistan organisms. In addition to the aggregative multicellularity characteristic of sorocarpic organisms, aggregative mechanisms are widespread even in non-sorocarpic organisms. For example, during animal development there are aggregative processes involved in embryonic development, such as multipolar ingression and germ-line migration (Mergner 1971; Savage and Danilchik 1993). Aggregation very similar in form to that of sorocarpic amoebae is observed after physical cell separation of embryos of amphibians and sponge thalli (Townes and Holtfreter 1955; Wilson 1907). Even in the Archaeplastida, the supergroup containing plants and most algae, the Hydrodictyaceae algae make flagellated zoospores that display patterned aggregation inside a wall or vesicles then aggregate to form a new colony (discussed in Marchant and Pickett-Heaps 1972). Given this, it is very likely that chemoattractant and cell–cell recognition systems are ancestral traits of the eukaryotes.

With the phylogenomic framework, we have developed for the evolutionary histories of these fascinating organisms; we can begin to ask deep, novel comparative biological questions in evolutionary, cellular, and developmental biology. Further, we can also begin to understand the evolutionary forces that repeatedly gave rise to ‘sociality’ in diverse lineages of eukaryotic microbes. Each of the various independent lineages of sorocarpic protists has a life cycle that involves extensive complexity, more so than typical microbial eukaryotes. The various life cycles of these organisms share similarities such as unicellular feeding behavior, the aggregation of unicells by an extracellular signaling molecule, cell–cell communication, and fate determination. Therefore, the ability to aggregate and subsequently form a complex cooperative structure is likely an extreme example of analogous convergent evolution. However it is possible that, despite their independent origins, the various sorocarpic lineages may share homologous molecular mechanisms that underpin their distinctive life cycles. The established phylogenetic framework (Fig. 1) raises a number of important evolutionary questions. For example, are these groups the result of truly independent but analogous evolutionary events? To what extent were genes involved in aggregative multicellularity laterally transferred from one group of sorocarpic protists to another? Did gene transfers enable a non-fruiting organism to gain the ability to become multicellular? Or conversely, is there an ancestral predisposition for protists to aggregate and subsequently cooperate? Over the coming years with genomes of *Fonticula* and *Acrasis* becoming available, major insights into the biology of non-dictyostelids sorocarpic amoebae will be uncovered. From these data, we can begin to address these deep comparative biological questions.

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