4 Protists and Multiple Routes to the Evolution of Multicellularity

Vidyanand Nanjundiah, Iñaki Ruiz-Trillo, and David Kirk

CONTENTS

Complex multicellular eukaryotic forms independently evolved at different moments in life's history, most of them around approximately 800 Myr ago (Sebé-Pedrós et al. 2017).

4.1 INTRODUCTION

The aim of this chapter is to ask what aspects pertaining to the evolutionary origin of multicellular life might be inferred from a survey of otherwise dissimilar protists that display one or both of two features: a unicellular-to-multicellular transition as part of their normal life cycle, or membership of a closely related group that contains both unicellular and multicellular members. Accordingly, we highlight aspects of multicellular development in three different "supergroups" of eukaryote protists: Dictyostelid or cellular slime molds (CSMs) (supergroup *Amoebozoa*, Mycetozoa; Section 4.2); Choanoflagellates, Filastareans, and Icthyosporeans (*Opisthokonta*, unicellular Holozoa; Section 4.3); and Volvocine green algae (*Archaeplastida*, Chlorophyceae; Section 4.4); their last common ancestor is believed to lie at the very root of the evolutionary tree of eukaryotes (Burki 2014). In the first two, multicellularity is achieved by the aggregation of single cells, in the third, by the products of cell division staying together. A striking difference from metazoan multicellularity is that none of the life cycles contain an obligatory sexual phase. Because not many species have been studied in detail, the information to be presented comes from a very small number of (what one hopes are) representative cases. As it happens, the most interesting evolutionary implications pertain to discordances between studied features, which means that they are likely to be robust. A brief summary concludes each of the three sections that follow. A general discussion comes at the end of this chapter.

4.2 EVOLUTION OF MULTICELLULARITY VIA AGGREGATION: THE CELLULAR SLIME MOLDS

This section is restricted to the CSMs and some other amoeboid organisms that display facultative multicellularity with similar life cycles. It is organized around four themes:

1. Aggregative multicellularity followed by differentiation into a fruiting body is seen in six of the seven "supergroups" of eukaryotic life
2. In the best-studied forms, the CSMs, (which belong to the supergroup Amoebozoa), there is a disconnection between morphological similarity and phylogenetic relationship
3. In *Dictyostelium discoideum*, the CSM about which we know the most, much of the genetic repertoire required for multicellular development appears to have preexisted in unicellular ancestors
4. A small number of elementary cell behaviors and self-organization may be sufficient to account for the varied multicellular morphologies that are observed

Themes 1 through 3 are based on published findings whereas (4) has a strong speculative element. All four themes have been discussed recently from a developmental perspective (Nanjundiah 2016); the emphasis here is on evolution. This contributes the speculative element to what we say, especially in the context of theme (4). The focus is on how multicellularity may have originated. The evolution of traits *within* the CSMs has been discussed by several writers (Bonner 1982, 2003, 2013a; Loomis 2014, 2015; Schaap 2011; Schilde et al. 2014) and is not our main concern.

The evolutionary transition from unicellularity-to-multicellularity is believed to have taken place independently on at least 25 occasions, in both prokaryotes and eukaryotes (Grosberg and Strathman 2007; Sebé-Pedros et al. 2017). Phylogenetic reconstruction shows that in six of the seven supergroups of eukaryotes in which the transition occurred, it did so via the aggregation of free-living cells (Figure 4.1). The Archaeplastida, which includes the red algae, green algae, and land plants, is the only eukaryotic supergroup in which no evidence of aggregative multicellularity has been found so far (though multicellularity did evolve, as discussed toward the end).

Going by the extant species that have been studied, five of the six independent origins of aggregative multicellularity involve amoeboid forms and a sorocarpic life cycle. The sixth is found in the Alveolata, where *Sorogena*, a ciliate, forms multicellular groups by aggregation. In the other five cases, the descendants are free-living amoebae that aggregate when they run out of food and organize themselves into an integrated multicellular mass. The mass goes through changes in morphology and the cells comprising it differentiate, which results in a sorocarp or fruiting body, containing stress-resistant spores, supported by an upright stalk. As we shall see, there are variations within the broad contour just sketched. Still, as Brown (2010) said, "… in all major lineages of amoeboid protists there appears to be at least a single example of the cellular slime mold habit." A brief description of that "habit" follows.

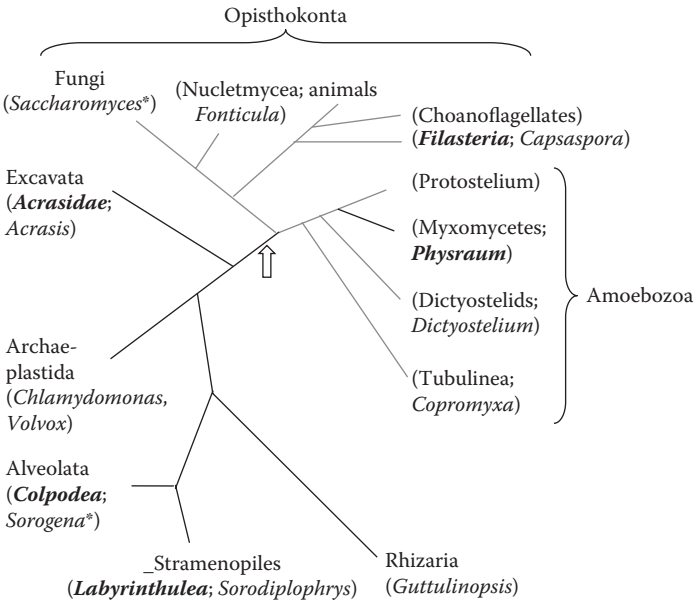

FIGURE 4.1 Independent origins of multicellularity and fruiting body formation via aggregation (indicated by italics and bold lettering) in the major eukaryotic supergroups. Except for *Sorogena*, all begin as amoeboid single cells. The large arrow indicates the putative root of the tree, which has been dated to between 1866 and 1679 Myr ago. (From Parfrey et al. 2011; Brown, M.W. and Silberman, J.D., The non-dictyostelid sorocarpic amoebae, in *Dictyostelids. Evolution, Genomics and Cell Biology*, Romeralo, M. et al. (Eds.), Springer, Heidelberg, Germany, pp. 219–242, 2013; Romeralo, M. and Fiz-Palacios, O., Evolution of dictyostelid social amoebas inferred from the use of molecular tools, in *Dictyostelids. Evolution, Genomics and Cell Biology* Romeralo, M. et al. (Eds.), Springer, Heidelberg, Germany, pp. 167–182, 2013; Burki, F., *Cold Spring Harb. Perspect. Biol.*, 6, a016147, 2014; Modified from Nanjundiah, V., Cellular slime mold development as a paradigm for the transition from unicellular to multicellular life, in *Multicellularity: Origins and Evolution,* Niklas, K.J. and Newman, S.A., (Ed.), MIT Press, Cambridge, MA, pp. 105–130, 2016. With permission.)

A The Cellular Slime Mold Life Cycle

CSMs are predatory amoebae found worldwide in a variety of soils, in bird droppings, and animal dung—namely wherever their prey, bacteria, and yeasts, are found (for details of CSM development, including references to the original literature, see Bonner 1967, Raper 1984 and Kessin 2001). As long as food is available, single amoebae keep going through vegetative cycles of feeding, growing, and doubling by mitosis, followed by cytokinesis. In a few cases that have been examined, postmitotic amoebae are haploid. When the food supply is exhausted, they adopt one of four strategies to mitigate the stress of starvation. (1) Some amoebae

remain *solitary* in an apparent attempt to wait it out until they encounter food once more (Dubravcic et al. 2014). (2) An amoeba *encysts* itself and forms a dormant *microcyst*. (3) A large number of amoebae *aggregate* and develop into a fruiting body in which some differentiate into dormant *spores*. (4) If the aggregate contains cells of opposite mating (sexual) types, a pair of them can fuse to form a diploid cell. The diploid is cannibalistic and feeds on the remaining cells, eventually forming a dormant giant cell, a protozygote known as the *macrocyst*. The return of food supply, either in the same location or wherever the dormant form has (passively) dispersed, induces the release of an amoeba from the microcyst or spore. In the case of the macrocyst, there is a meiotic division, following the release of products as haploid amoebae. A released amoeba proceeds to feed, grow, and divide; and the life cycle is reinitiated. Once starvation has set in, the number of cells can remain unchanged (in 1, 2 and 3), or decrease, whether slightly, on account of cell death (in 1 and 3), or drastically, following cannibalism (in 4). A characteristic of the CSM life cycle, which the other sorocarpic amoebae appear to share, is that growth (which includes an increase in cell number) and development are clearly separated in time.

We have listed the consequences of starvation that have been observed in one or more CSM species. A form of responding to starvation may not be seen under laboratory conditions (e.g., microcysts have not been found in some species), but because we are largely ignorant of CSM ecology, we cannot say whether or not it is found in the wild. Similarly, even though conditions that favor microcyst formation, or favor the macrocyst pathway, are known, we do not know precisely how a cell fixes the relative probabilities of responses 1 to 4. By sampling spores from the same fruiting body, we can infer whether the preceding aggregate, and possibly the feeding group that gave rise to it, was clonal or polyclonal (one has to verify that a polyclonal aggregate has not split into two or more clonal fruiting bodies). Amoebae and spores of different species undoubtedly co-occur frequently, but either interspecies aggregation does not occur, or, if it does, cells sort out and form "almost species-pure" groups (Raper and Thom 1941, Bonner and Adams 1958, Jack et al. 2008, Sathe et al. 2014). For this reason, we will not consider multispecies groups any further. Therefore, we ignore the fascinating case of interspecies predation, to date known only in *D. caveatum*: its amoebae coaggregate with those of other species, form a chimeric multicellular mass, and then start to feed on the others. Instead, we will concentrate on developmental pathway (3). It is the one that involves aggregative multicellularity, a form of collective self-protection against the threat posed by starvation, and is found across supergroups.

B Similarities with Life Cycles of Other Sorocarpic Amoebae

Life cycles resembling those of the CSMs are found in the Amoebozoa (*Copromyxa* in Tubulinea, and Dictyostelia), the Opisthokonta (*Fonticula*), the Excavata (*Acrasis/Pocheina* in Heterolobosea), the Rhizaria (*Guttulinopsis*, Cercozoa), and the Stramenopila (the amoeboid labyrinthulid *Sorodiplophrys*) in

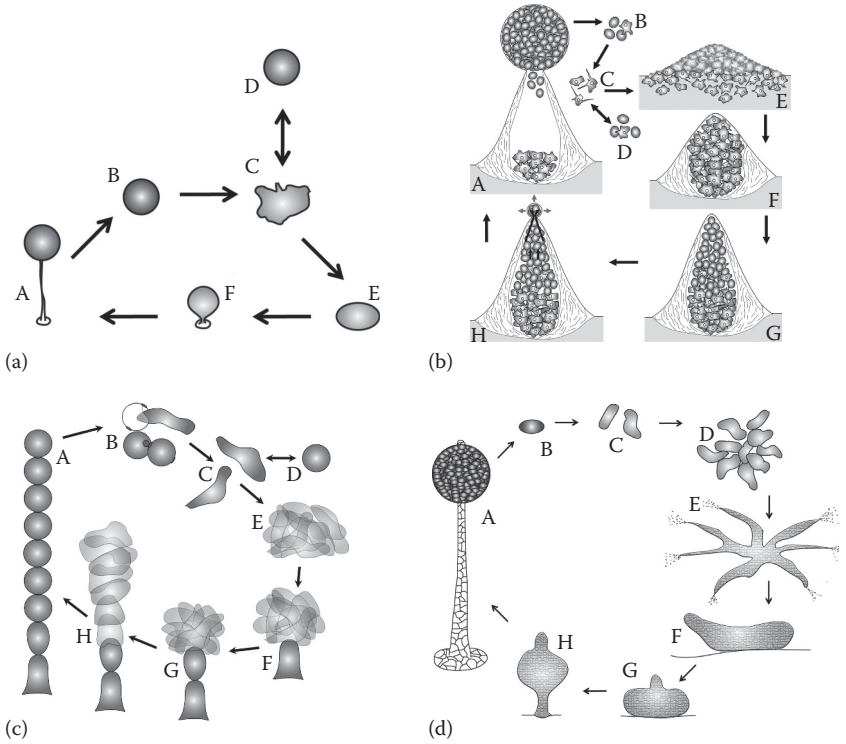

FIGURE 4.2 Schematic representation of post-starvation development in four groups of sorocarpic amoebae (name of supergroup within parentheses). (a) *Protostelium mycophaga* (Amoebozoa); (b) *Fonticula alba* (Opisthokonta); (c) *Acrasis helenhemmesae* (Excavata); (d) *Dictyostelium discoideum* (Amoebozoa). In (a) a single amoeba differentiates into a fruiting body; in the others, many amoebae do so following aggregation. See text for details. (From Brown, M.W., Placing the Forgotten Slime Molds (*Sappinia*, *Copromyxa*, *Fonticula*, *Acrasis*, and *Pocheina*), using molecular phylogenetics, 258p. PhD thesis dissertation, University of Arkansas, Fayetteville, AR, 2010. With permission.)

the Chromalveolata; as discussed in the next section, aggregative multicellularity per se is also seen in unicellular holozoans. Consider the developmental cycles illustrated in Figure 4.2 (reproduced from Brown 2010 with the kind permission from the author).

a. *Protostelium mycophaga* (Amoebozoa; Shadwick et al. 2009 and Figure 4.2, p. 41 in Brown 2010) provides a striking example of the same stereotypic morphological transitions, as in the CSMs but without any multicellularity at all. The free-living amoeba (C) can either transform reversibly into a dormant cyst (D), or it can become a spore (B) that is on top of a fruiting body (E→F→A) with an extracellular stalk.
b. In *Fonticula alba* (Opisthokonta) (Ophisthokonta; Brown et al. 2009 and Figure 4.1, p. 134), the sequence of events is similar except that free-living

amoebae (C) can either form solitary cysts (D) or aggregate (E) into groups. Eventually, a slime sheath (F) surrounds the aggregate mound, and the cells inside begin secreting an extracellular stalk. The amoebae that are towards the top of the stalk become spores (G). Some are pressed upwards into a bulge (H) as the stalk matures; others remain outside the sorus (A), and yet other cells stay at the bottom of the fruiting body as undifferentiated amoebae.

c. Newly germinated limax amoebae of *Acrasis helenhemmesae* (Excavata; Brown et al. 2010 and Figure 4.1 on p. 168 of Brown 2010) emerge from spores (B); they encyst reversibly (D) or aggregate (E); one cell within the mound encysts itself to form a stalk cell (F), a second cell follows to become another stalk cell (G). The amoebae remaining in the mound stay on top, become aligned (H) and differentiate into spores, thereby, completing the fruiting body (A). Stalk cells and spores can both germinate and release amoebae.
d. Within the same broad sequence, the aggregative life cycle of *Dictyostelium discoideum* (Amoebozoa; Bonner 1967 and Figure 4.4, p. 43 of Brown 2010) displays more elaborations: spores (B) germinate, release amoebae (C) and begin to aggregate in pulsating waves formed by inwardly streaming cells (D→E). The finished aggregate forms a motile worm-like structure, the slug (F), which migrates to the top of the soil or dung substratum and erects itself (G→H) to form a fruiting body (A) that consists of a spore mass on top of a stalk of dead cells. (Note the similarity in appearance between the *D. discoideum* and *P. mycophaga* fruiting bodies.) Other Dictyostelid species display modifications on this theme: the stalk may be laid down concomitantly with the formation of the slug; the fruiting body may be branched and contain secondary stalks, each with its own spore mass; aggregate size may decide whether the stalk is made up of dead cells or is an extracellular exudate.

Three inferences can be drawn from the striking example of convergent morphogenesis illustrated in Figure 4.2 (the fraction of cells that die during development varies a lot, and we will comment on it at the end). First, *Protostelium* shows that a fruiting body can develop in the absence of aggregation; a single cell can construct one too, and it resembles that formed by an aggregate of cells. Tice et al. (2016) report that *Acanthamoeba pyriformis* (earlier known as *Protostelium pyriformis*), another unicellular relative of the CSMs, can also do so (and they propose that at least one "*Protostelium*," namely *P. pyriformis*, should be classified as an *Acanthamoeba*). Bonner (1967) points out that there is a complementary situation: amoebae of *Hartmannella astronyxis* form multicellular aggregates, but thereafter, the cells encyst without making a fruiting body (Ray and Hayes 1964; according to Brown 2010, *Copromyxa*, not *Hartmannella*, is the more appropriate genus name). Second, the developmental trajectories in the four cases show an extraordinary degree of convergence. This makes it likely that the unicellular ancestors of each of the supergroups in question, and perhaps a common unicellular ancestor of all of them, already possessed much of the genetic and behavioral repertoire needed to form a fruiting body. Third, the fact that the sequence of intermediate stages in the four cases is so similar suggests that they are consequences of a "generic" morphogenetic mechanism (Newman and Comper 1990) that can be implemented

in the same way by cells whose ancestries are very different. A perspective on the evolution of multicellularity in myxobacteria (Arias del Angel et al. 2017) contains interesting speculations along similar lines. Molecular phylogenetic studies on the CSMs indirectly support the last two inferences, and we turn to them next.

C Poor Correlation between Phylogeny and Morphology in the CSMs

Scattered observations in the literature indicate that the fruiting body of one species can sometimes resemble that of another (Bonner 1967, Olive 1975, Raper 1984). For example, Acytostelium forms an extracellular stalk whereas in *Dictyostelium lacteum* the stalk is made up of dead cells; but in very small fruiting bodies of *D. lacteum* the stalk can be partly acellular (Bonner and Dodd 1962). Shaffer found that sparse cultures of *Acytostelium* sometimes contained single-celled fruiting bodies similar to those of *Protostelium* (cited in Bonner 1967). Raper noted the mirror-image case in *Protostelium*: fruiting bodies are mostly unicellular but occasionally consist of two to four cells (Raper 1984). In working with *D. discoideum*, now and then one comes across a fruiting body that mimics a *Polysphondylium* fruiting body by having a lateral stalk in addition to the main stalk. Thus, CSM fruiting body phenotypes are plastic, but the fact becomes evident only rarely or when there is a significant alteration in size (Romeralo et al. 2013). Indeed, based on observations of developmental compatibility in intra- and interspecies mixes, Bonner and Adams (1958) went so far to conclude that "specific differences between strains of one species are as great, or even greater, than those between different species" (p. 352, referring to *D. mucoroides, D. discoideum, D. purpureum, and P. violaceum*).

An unexpected discovery from recent studies of molecular phylogeny in the CSMs makes us see the observations cited above in a new light (Schaap et al. 2006). The CSMs fall into four major clades ("groups"), with their most recent common ancestor dated to ~600 Myr ago (Figure 4.1 in Schilde et al. 2016; the most recent common ancestor with *Physarum polycephalum*, a myxogastrid in which amoebae fuse to form syncytia, is dated to ~650 Myr ago; and that with the unicellular *Acanthamoeba castellani* to ~850 Myr ago). An examination of the constructed phylogeny shows that developmental similarities, especially about fruiting body morphology, are poor indicators of recent common ancestry. There are many illustrations of this. The genus *Acytostelium* used to be characterized by the secretion of an extracellular stalk (Raper and Quinlan 1958), but in fact, the trait is polyphyletic and extends across species that were believed to belong to different genera. An extracellular stalk is found in *Acytostelium* species from two different clades, one of which also contains species with cellular stalks (Schaap et al. 2006; Romeralo et al. 2013). Species in the same clade (Group 4) can form fruiting bodies with an unbranched stalk and spores at the apex (e.g., *Dictyostelium mucoroides* and *D. discoideum*), or with spore masses distributed along the stalk (e.g., *Dictyostelium rosarium*), or with more than one transverse branch of the stalk at the same horizontal level and a spore mass at the tip of each (*Polysphondylium violaceum*, though it has been placed on a small side clade in between the clades formed by Group 3 and Group 4). Among members of the same clade, a cellular stalk can be released by the slug during migration and rise directly

from the substrate (*D. giganteum*), or it can form after migration is completed and rest on a disc-shaped base (*D. discoideum*); in the clade comprising Group 2, the stalk can be cellular (*D. oculare*) or acellular (*Acytostelium leptosomum*). One member of the Group 3 clade preys on other CSM species (*D. caveatum*); two others, not known to be predatory on CSMs, form stalk that is cellular (*D. tenue*) or occasionally acellular (*D. lacteum*). Romeralo et al. (2013) reinforce the point in a study whose main finding is that across 99 CSM species, only a weak correlation exists between phenotype and molecular phylogeny. According to them, there is "a fairly scattered distribution of character states … with many states reappearing multiple times in different clades" (by character state they mean mainly the size and shape of multicellular structures; Discussion, p. 7 in Romeralo et al. 2013). They further observe that the morphology and distribution of fruiting body types (unbranched versus branched, solitary versus clustered) changes as a function of the density at which starved amoebae are plated prior to the onset of aggregation.

Schilde et al. (2016) found CSM nuclear genome sizes ranging between 31 and 35 Mb (*D. discoideum, D. fasciculatum and P. pallidum*); at 23 Mb, *D. lacteum* was the smallest of the four. The same study estimated 12,319 protein-coding genes in *D. discoideum* (10,232 in *D. lacteum*). There are significant overlaps with "metazoan" gene products and regulatory pathways, for example, the ones involving beta integrin (Cornillon et al. 2006), btg, and retinoblastoma (RB) (Conte et al. 2010), and Wnt and STAT (Sun and Kim 2011). Attempts have been made to use sequence comparisons and temporal gene expression profiles to identify novel protein-coding genes, or novel patterns of gene expression, that might be linked to the origin of multicellularity in the CSMs. Glöckner et al. (2016) compared 385 "developmentally essential genes" (DEGs) in *D. discoideum* with potentially similar genes in other CSMs. The genes were chosen on the basis that when their function was disrupted, multicellular development was affected, but feeding, growth, or cell division were not, or at least not grossly (the comparison rested on reports from studies carried out by different groups, and the original data did not refer to regulatory genes or noncoding DNA sequences, etc.; for instance, it was not based on saturation mutagenesis). They found that homologues of a significant proportion of the DEGs were present in all four major CSM subclades (i.e., besides *D. discoideum* itself, in *D. fasciculatum*, group 1; *P. pallidum*, group 2; *D. lacteum*, group 3). Significantly, 80% of protein-coding genes essential for (multicellular) CSM development were predicted to exist in their unicellular relatives. The overlaps were 76% with *Physarum polycephalum*, 46% with *Acanthamoeba castellani*, and 19% with *Entamoeba histolytica*. The authors point out that the low values of the latter two proportions are misleading and could be the consequence of secondary gene losses. Also, homologs of 72% of DEGs are found in non-Amoebozoan species—including in the Opisthokonta, other eukaryotes and prokaryotes. Conceivably the prokaryote links could originate from horizontal gene transfer, which is believed to have been common in prokaryotes (Thomas and Nielsen 2005) and rare but not unknown in eukaryotes (Danchin 2016).

The 305 DEGs that were also found in members of the Amoebozoa are predicted to encode protein kinases, nucleotide binding proteins, and a range of cytosolic and nuclear proteins. The remaining 80 DEGs did not yield Amoebozoan

homologs (in this study) and are predicted to encode secreted proteins and proteins with an extracellular exposure, both being classes that would be expected to have roles in sensing the environment (including other cells) and mutual recognition. Interestingly, 37 DEGs are not shared by all CSMs, and among them, 26 appear to encode proteins that are secreted or likely to have an extracellular face, once again suggestive of a role in intercellular recognition and signaling, also possibly in mediating interspecies recognition. Evidently, "the cellular slime mould habit" does not depend on the presence of at least these 37, since one or the other is missing in some CSM; but the absence of any among them results in the failure of multicellular development in *D. discoideum*. The functions of the remaining DEGs, which seem to be exclusive to one or more CSMs but not all four, are known or conjectured to be related to the regulation of group size, cell-type proportioning, the timing of aggregation and, in one case, normal fruiting body formation.

The study by Schilde et al. (2016) compared (in the same 4 species) 186 genes of *D. discoideum* that were identified as vital for multicellular development based on similar but slightly different criteria. Their expression was upregulated by at least threefold during normal development. Additionally, they were known (from the published literature) to induce aberrant development when manipulated in some manner. It transpired that 33 of the 186 lacked an ortholog in representatives of at least one of the other three CSM groups and 20 lacked an ortholog in all three (the same three species mentioned above were used as representatives). Four hundred and sixty-six other genes, known to induce aberrant development when modified, were not upregulated significantly in development. In other words, they functioned quasi-constitutively, as would be expected of putative "housekeeping" genes. Tellingly, among the 2,352 to 2,395 genes that were developmentally upregulated in the four species taken together, roughly one half of the number in any one species had no ortholog in any of the remaining three.

D Possible Routes for the Unicellular-to-Multicellular Transition

Having prepared the ground with that sketch of comparative morphologies and genetic similarities, we proceed to speculate on how multicellularity could have evolved from an ancestor whose entire life was spent as a single cell to a descendant with the "CSM habit." From what we have seen, much of the required genetic repertoire would have been present in the unicellular ancestor, though the gene product in question may have played some other role. Further, *D. discoideum* shares not only genes, but regulatory mechanisms and morphogenetic pathways with the "higher" Metazoa (Kawata 2011; Loomis 2014, 2015; Santhanam et al. 2015), and very likely so do other sorocarpic amoebae. With regard to *D. discoideum*, and the genes homologous to those coding for beta integrin, btg, and RB, Wnt and STAT mentioned earlier, one can add the following gene products, genes, or regulatory pathways for which a homology with metazoans has been claimed: beta catenin (Coates et al. 2002), presenilin/γ-secretase (McMains et al. 2010), fused kinase (Tang et al. 2008), Src homology 2 (SH2) domain

proteins (Sugden et al. 2011), tyrosine kinase phosphorylation (Sun and Kim 2011), and homeobox genes (Mishra and Saran 2015). Besides, noncoding RNAs have been found to regulate the development of *D. discoideum* (Avesson et al. 2011). No doubt more instances will crop up over time. Therefore, irrespective of how the transition may have taken place, it would have been facilitated by preadaptations.

Multicellularity through step-by-step adaptations via the acquisition of new genes. The conventional way of thinking about the evolutionary origin of multicellularity is to view it in terms of a series of cumulative steps, each being the consequence of a random genetic mutation that spread in the population because it happened to be adaptive. The mutation could be in the coding or regulatory portion of an existing gene, which, thereby, acquired an additional function. Alternatively, a functional role could have followed the duplication of a preexisting gene or the acquisition of a foreign gene by horizontal transfer.

In parallel with the evolution of multicellularity, sophisticated communication systems for the coordination of cellular activities must have come into play (Bonner 2001). Cell–cell adhesion would be of obvious advantage, as would contact-dependent signaling and communication via diffusible, long-range signals, and signal transduction mechanisms. Intercellular signaling may have preceded the evolution of a multicellular fruiting body. Starvation-induced encystment occurs near-simultaneously in clusters of *Hartmannella astronyxis* (Ray and Hayes 1954) and is said to begin with a single amoeba in *Acanthamoeba castellani*, from where it moves outward in the form of a wave-like propagated signal (Jahn and Bovee 1967, Pickup et al. 2007).

We do not know what modes of intercellular signaling operated during the evolution of multicellularity from a unicellular ancestor, but many have been identified in *D. discoideum* and some other CSM species. Released quorum-sensing factors enable a starved cell to decide whether there are enough other cells in its neighborhood for aggregation to be initiated. Cyclic AMP (or another chemoattractant) fosters aggregation. Calcium-independent cell–cell adhesion is present in vegetative amoebae, and other adhesion mechanisms come into play during and following aggregation. Later in development, cAMP and other signals induce cell type differentiation and the coordination of fruiting, also in species that do not use cAMP as the aggregation pheromone (reviews in Coates and Harwood 2001; Schaap 2011; Loomis 2014).

In addition to intercellular communication, one can speculate on the adaptive steps that may have accompanied the evolution of aggregative multicellularity as displayed by sorocarpic amoebae: (1) encystation, (2) coming together by clumping, (3) the development of adhesiveness, (4) aggregation via chemotaxis, (5) morphogenesis leading to an erect fruiting body, (6) differentiation within the fruiting body (with the constituent cells possibly differing in viability depending on their locations), (7) the secretion of an extracellular stalk as the fruiting body is being formed and the death of a subset of cells, leading to a fruiting body in which viable cells are supported by a dead cellular stalk (Nanjundiah 1985; Bonner 1998). These steps are conjectured based on what is known from studies on CSMs. However, as we have seen, molecular

phylogenies show that steps 1 to 6 do not reflect evolutionary relationships—rather, there is no single "true" sequence in which morphology and genealogy go in parallel. All the same, it can be argued that taken individually, each of steps 1 to 8 is adaptive.

The adaptive value of step 1 seems obvious (survival in a dormant state the face of starvation, as frequently observed in unicellular organisms too), and so does that of Steps 5–7 (improved chances of dispersal because of elevation above the substrate). Steps 2–4 would be beneficial if being situated close to other cells and later, forming a cohesive group, enhanced the probability of long-term survival for each cell. For instance, coming together may improve the chance of surviving predatory attacks simply because of an increase in size. Boraas et al. found that when the unicellular green alga *Chlorella vulgaris* was cultured for many generations with the (predatory) flagellated protist *Ochromonas vallescia*, it evolved to a ~8-celled multicellular stably propagating form in which cells were protected against predation (Boraas et al. 1998). Similarly, Sathe and Durand (2015) found that in the presence of their natural predator *Peranema trichophorum*, single cells of *Chlamydomonas reinhardtii* protected themselves against predation by aggregating (as an interesting aside, the aggregates tended to be chimeric, not necessarily clonal). Kapsetaki (2015) has summarized the case of size increase in response to predation as a plausible adaptation in the evolution of multicellularity. However, group encystment may also be an automatic consequence of starvation setting in after cells have foraged collectively. (After coming together, cooperative foraging can improve the range of accessible prey and feeding efficiency, as seen in myxobacteria and myxomycetes; see Bonner 1998. But that is unlikely to be relevant in the situation we are considering.) If a single cell can form a fruiting body (as in *Protostelium*), the mechanical stability of the stalk increases, and the energetic cost per cell decreases, if more than one cell makes a fruiting body in the same place (Kaushik and Nanjundiah 2003).

Step 6, which has to do with spatiotemporal patterning and division of labor, and more so step 8, which involves cell death, have attracted so much attention in the CSM literature that they demand an extended discussion (also see Nanjundiah 2016). It has been argued that differential reproductive success, particularly, the death of some cells within the group, can be a stable evolutionary outcome if and only if the cells' genetic interests overlap substantially—for instance, via the formation of clonal groups. Thus the evolution of "altruistic" death in cells that form the stalk is accounted for on the hypothesis of kin selection, the reasoning being that the contribution by a stalk cell to spore fitness more than makes up for abandoning the possibility of its own reproduction (Kaushik and Nanjundiah 2003). The supporting evidence comes from three sorts of observations on *D. discoideum*. First, high relatedness is found to be a safeguard against "cheating," meaning the exploitation of the group by an amoeba that—in the extreme case—invariably differentiates into a spore and never contributes to the stalk (Gilbert et al. 2007). Second, polymorphism at the *lag* locus is correlated with the propensity for one clone to sort out from another, and this could be a form of implementing kin selection by excluding non-kin from a group (Benabentos et al. 2009). Third, *D. discoideum* can be found in large clonal patches in nature (Gilbert et al. 2009).

On the other hand, one can make a case for the hypothesis that reproductive division of labor is a concomitant of inter-individual competition for maximizing relative

fitness within the multicellular group and has nothing to do with kinship as such (Atzmony et al. 1997). To begin with, as illustrated in Figure 4.1, cell death is indeed an obligatory part of the pathway leading to fruiting body formation in some sorocarpic amoebae but not in all. In *Fonticula alba*, which belongs to the Opisthokonta, a sister group to the Amoebozoa, all amoebae differentiate into spores and produce an extracellular stalk (Worley et al. 1979). The Acrasids belong to the Excavata, yet another supergroup. Their fruiting bodies have the appearance of amoebae piled on top of each other, possibly with secondary branches; again, all amoebae are viable (e.g., *Acrasis rosea*; Olive and Stoianovitch 1960). But cell death is present in *Guttulinopsis vulgaris* (Olive 1965), classified under a more distant sister group, the Rhizaria. Evidently, developmental cell death is not essential for spore dispersal. When it does occur, its extent varies across species. Under standard laboratory conditions, the proportion of aggregated amoebae contributing to the stalk is ~50% in *D. giganteum* and ~20% in *D. discoideum*. In considering what these numbers might imply, one should bear two things in mind. First, the same CSM clade can contain some species that have a cellular stalk and some that do not: evolutionarily, the trait of stalk cell death can be a gain or a loss. Second, CSM species live in the same microenvironment, co-occur on the same speck of soil, can feed on the same bacteria, possibly have similar chances of being preyed upon and very probably their spores are dispersed over similar distances. Under these circumstances, it is difficult to think of differences in the fraction of cells that die while forming a stalk as different specific adaptive outcomes at all, let alone as underpinned by kin selection—unless there are unknown factors behind fruiting body viability and spore dispersal, and for reasons that remain to be discovered, some CSMs are more likely to form clonal aggregations than others.

Besides, there is the finding, also involving *D. discoideum*, that unlike what one might expect, successful social exploitation and relatedness within the group do not show a straightforward correlation (Saxer et al. 2010). Other studies, involving *D. giganteum* and *D. purpureum*, show that naturally occurring CSM social groups can be genetically heterogeneous (Filosa 1962), with up to 9 clones in a single spore mass (Kaushik and Nanjundiah 2003, Sathe et al. 2010). It has been proposed that rather than kinship, nonlinear interactions (for want of a better term) and the social context decide who ends up "cheating" whom. In the long term, this could enable genetically heterogeneous "guilds" of a species to coexist (Kaushik et al. 2006, Nanjundiah and Sathe 2011, Sathe et al. 2014). Mathematical models have explored the consequences of a starved cell being allowed to choose whether to join a group or remain solitary. The results show that for cooperative group behavior to persist stably across generations, it is sufficient if some cells that participate in group living (i.e., in forming an aggregate and fruiting body) in one generation give rise to some cells that form a group in the next generation (De Monte and Rainey 2014). Also, fitness trade-offs that are built on the basis of intercellular differences and depend on the ecological context (Tarnita et al. 2015; Wolf et al. 2015), or variations in the kinetic parameters that underlie components of intercellular signalling, can leave cell-type proportions unchanged (Uchinomiya and Iwasa 2013). They can give rise to the illusion that one genotype is "cheating" another, or that a "cheater" is predisposed to allocate cells to the stalk and spore pathways in proportions that

are different from that of the wild type. To sum up, there are two ways to look at the evolution of differential reproductive success among the cells that make up an aggregate, one in terms of shared genes and the other in terms of cellular phenotypes and intercellular interactions. Future work should clarify the situation.

Multicellularity through self-organization. As we have seen, 72% of the 385 "developmentally essential" genes tested in *D. discoideum* are present in non-amoebozoan unicellular relatives (Glöckner et al. (2016), and ~50% of 2352-plus genes that are upregulated during development in at least one of four CSM species, each belonging to a different group, have no homolog in any of the others (Schilde et al. 2016). The proportions and numbers are no doubt provisional and subject to change. However, as more gene sequences and gene expression profiles are analyzed, two sets of figures seem likely to remain either as they are, or go up: concordances between a "developmental gene" in a CSM species and in at least one constitutively unicellular organism, and discordances between "developmental genes" among different CSM species. But even as they stand, some inferences are inescapable: (a) DNA sequences and gene expression profiles do not display substantial differences among CSMs, (b) intergroup differences within CSMs are blurred by the variations in developmental pattern that can be elicited from a single species, and (c) a substantial proportion of CSM "developmental genes" are likely to have homologs in unicellular organisms. The inferences are based on experiments carried out in the laboratory under controlled environmental conditions. It is on the cards that a similar investigation, conducted under diverse ecological circumstances, will show up an even weaker correlation between common ancestry and developmental signposts—not just in the CSMs, but also among other protists that show aggregative sorocarpic development.

That being the case, it may be worth thinking of features of the life cycles we have been discussing, in particular, fruiting body morphologies that look very different, as examples of what Bonner has termed neutral phenotypes (Bonner 2013b, 2015). If so, they could be viewed as different outcomes of self-organization. The same underlying system of physicochemical interactions among identical cells (apart from stochastic variations) could lead to alternative stable spatiotemporal patterns that arise spontaneously (Newman and Bhat 2009). There is a precedent in *D. discoideum* itself: clonal suspensions of starved amoebae spontaneously form two subgroups of cells with high and low calcium content, respectively; the groups go on to exhibit presumptive spore (low calcium cells) and stalk (high calcium cells) tendencies (Saran et al. 1994). Which of the alternatives is chosen in any given instance can depend on variations in a small number of parameters that specify cell behavior. The parameters can be thought of as reflecting preadaptations that were already present in a unicellular ancestor. Mathematical models and simulations show how groups of cells can aggregate, achieve the correct spatial distribution of cell types and turn into a fruiting body (Savill and Hogeweg 1997; Marée and Hogeweg 2001; see especially the film by A. F. M. Mareé, P. Hogeweg and N. J. Savill [https://www.youtube.com/watch?v=GyAQepksJLU] along with the description in the Ph.D. thesis of A. F. M. Marée [http://theory.bio.uu.nl/stan/Thesis/]). Other models show how interactions between oscillating units can lead to mutual dependence and organization in the form of "phase clusters" (Kaneko 2016).

To the extent that they are good models of the biological situation, such approaches lead to a different conceptual picture of the evolution of multicellularity. In this picture aggregation and the various morphogenetic alternatives displayed by sorocarpic amoebae are canalized outcomes analogous to the canalized phenotypes that develop in metazoans (Waddington 1942), with the cells that take part possessing preadaptations that equip them for multicellular life. Subsequent genetic changes would stabilize one or the other multicellular morphology once it has come into being (Newman 2002). Studies on prokaryotes and eukaryotes have shown that once cells are forced to live in close proximity, multicellularity and division of labor follow more or less automatically under laboratory conditions (see for example Ďťovíček et al. 2012; Włoch-Salamon 2013; Hammerschmidt et al. 2014). Also, complementary metabolic pathways could potentiate multicellularity (Wintermute and Silver 2010). The nature of the participating units would be less important than the capacities that they manifest. In ending, we point out that aggregative multicellularity may be a more plausible route to the origin of multicellularity of the metazoan type than is usually thought (Newman 2011, Dickinson et al. 2012). Finally, the view here expressed lends itself to the further extension that preexisting developmental control systems can be adapted to serve as functional control systems (Robertson and Cohen 1972).

E Summing Up

The life cycles of sorocarpic amoebae share broad features in common: aggregation in response to the stress of starvation, differentiation along with the construction of a fruiting body, long-lasting dormancy and, in the CSMs and some others, reproductive division of labor (i.e., a germ line-soma separation). Each life cycle involves a shift from unicellularity-to-multicellularity, and it is tempting to view it as mirroring the evolutionary transition from constitutive unicellularity-to-facultative multicellularity. "Higher" metazoans and *D. discoideum* have many genes and regulatory pathways in common, and of the genes that appear to be essential for multicellularity in the CSMs, a substantial proportion were already present in unicellular ancestors. To the extent that they are reflected in molecular phylogenies, genetic changes are poorly correlated with conventional taxonomic assignments. The discordance may be since the same set of cell signals and responses has the potential to lead to a number of developmental outcomes, one or the other of which gets frozen by secondary genetic changes.

4.3 THE ROUTE TO ANIMAL MULTICELLULARITY: THE UNICELLULAR HOLOZOANS PAVED THE WAY

Among all acquisitions of multicellularity that had occurred within eukaryotes, the animal one is probably one of the most relevant to understand the evolution of complex life forms (Ruiz-Trillo et al. 2007; Rokas 2008; King 2004; Knoll 2011). Indeed, the emergence of multicellularity in the animal branch of life gave rise to the whole diversity of animals that we see today with their unique, complex, and coordinated embryonic development. This transition to such complex body plans is even more surprising when we consider that the ancestors were unicellular protists.

To understand such transition, we first need to understand how the unicellular ancestor of animals was. Given that we do not have that ancestor among us anymore, we can only infer how it was by comparing animals with their extant closest unicellular relatives. However, which of all extant protists are the closest unicellular relatives of animals is something that only became clear a few years ago with the advent of phylogenomic (multigene phylogenetic) analyses. Based on morphology, a group of flagellate protists, known as choanoflagellates, had already been proposed on the nineteenth century to be the closest unicellular relatives to animals (King 2005; Leadbeater 2015). The reason for uniting choanoflagellates with animals was a suggested homology of choanoflagellates with a specific cell type of sponges (the choanocyte) (although the homology has been recently disputed; see Mah et al. 2014). Given that sponges were thought to be the earliest animal lineage, the homology was easily explained if choanoflagellates were the sister-group to animals. Indeed, the first ribosomal phylogenies (Medina et al. 2003), and subsequent multigene or phylogenomic analyses (Lang et al. 2002; Steenkamp et al. 2006; Ruiz-Trillo et al. 2004, 2006, 2008; Shalchian-Tabrizi et al. 2008; Carr et al. 2008), supported this view, so that it is now clear that choanoflagellates are the sister-group to animals. These trees showed that animals and choanoflagellates, together with fungi, shared a closer ancestor than with plants or algae, forming a clade known as the opisthokonts.

Further molecular data from other potential opisthokont protists demonstrated that the tree of opisthokonts had additional lineages (Torruella et al. 2012, 2015; Ruiz-Trillo et al. 2004; Shalchian-Tabrizi et al. 2008; Steenkamp et al. 2006). Three of those lineages, the Filasterea, the Ichthyosporea, and the Corallochytrea, were subsequently shown to be close relatives to Metazoa. The clade composed of animals and their closest unicellular relatives is known as the Holozoa (Lang et al. 2002) (Figure 4.3). The most taxon-rich and gene-rich phylogenetic analysis of the Holozoa

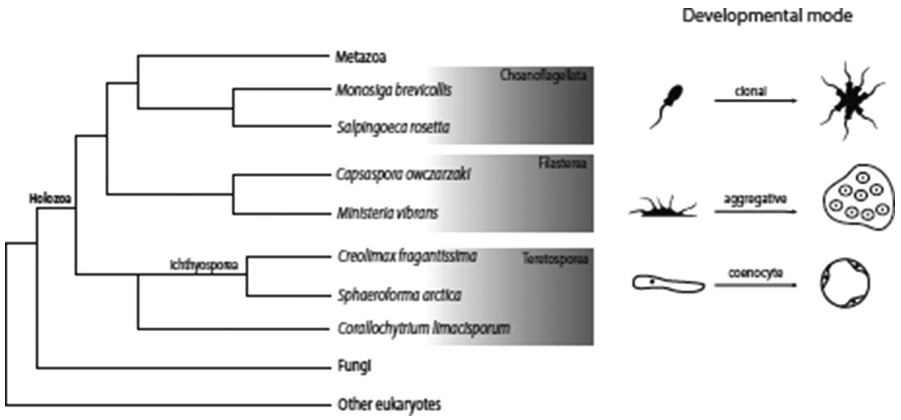

FIGURE 4.3 Schematic tree of the Holozoa with some known taxa and showing the three main lineages. On the right shows a scheme of their developmental modes. (Adapted from Figure 4.1 de Mendoza, A. et al., *Elife*, e08904, 2015. With permission.)

shows that Teretosporea (Ichthyosporea + Corallochytrea) represents the earliest branching lineage, followed by Filasterea, and then Metazoa and Choanoflagellata (Torruella et al. 2015; Figure 4.3).

A The Three Closest Unicellular Relatives of Animals

Choanoflagellata, Filasterea, and Teretosporea are the key lineages to understand animal origins. Interestingly, these three lineages are very different morphologically and have distinct developmental modes (Figure 4.3). Choanoflagellates, with dozens of species described, are free-living unicellular flagellates that predate bacteria and leave in marine and fresh-water environments (King 2005; Leadbeater 2015). Some species can also form colonies by clonal division. Interestingly, bacteria seem to be responsible for the formation of the choanoflagellate colonies, at least in *Salpingoeca rosetta* (Alegado et al. 2012), which also have additional life stages, such as a sessile form and slow and fast swimmer stages (Dayel et al. 2011).

Filasterea, on the other hand, has only two described species: (1) *Capsaspora owczarzaki* and (2) *Ministeria vibrans* (see Paps and Ruiz-Trillo 2010 *for a review*). Both are amoeboid protist with filopodia. *C. owczarzaki* was isolated from the hemolymph of the freshwater snail *Biomphalaria glabrata*, while *M. vibrans* is marine and free-living. The life cycle of *C. owczarzaki* has been described, under laboratory conditions, and has a "multicellular" stage that forms by cell aggregation (Sebé-Pedrós et al. 2013b). In particular, three life stages have been described: (1) An adhaerent/filopodial stage in which cells crawl; thanks to their filopodia (actin-based cell protrusions) (see video in https://www.youtube.com/watch?v=0Uyhor_nDts. The title of the video is Time-lapse video of the growth–maturation–dissemination stages of Creolimax fragrantissima (Ichthyosporea) by Hiroshi Suga and Iñaki Ruiz-Trillo). (2) A cystic stage in which there is no filopodia. (3) An aggregative cell stage in which cells actively come together and form an aggregative structure that seems to have some kind of extracellular matrix (ECM) in between the cells (see https://www.youtube.com/watch?v=OvI6BvBucrc).

Finally, most of the Teretosporea go through a syncytial (multinucleate) development, some species with an amoeboid stage, some without (Mendoza et al. 2002; Glockling et al. 2013; Suga and Ruiz-Trillo 2013). A good example is the ichthyosporean *Creolimax fragrantissima*, originally isolated from the gut of different marine invertebrates (Marshall et al. 2008). Its life cycle starts with one cell with one nucleus and an external cell wall. The cell will go through several nuclear divisions, given rise to a mature, multinucleated coenocyte. The nuclei will cellularize and create amoeboid cells that will be released from the mature coenocyte. Those amoebas will crawl and encyst starting the life cycle again (Marshall et al. 2008; Suga and Ruiz-Trillo 2013) (see https://www.youtube.com/watch?v=7Gvrg1I8jBA).

Thus, the three known lineages that are most closely related to animals have very different morphologies and developmental modes (Figure 4.3). Undoubtedly, if one aims to understand the emergence of metazoans from their unicellular ancestor, and given that ancestor is not present anymore, one should investigate those three extant lineages that are more closely related to animals. A few labs have investigated them from a genomic perspective providing important insights into the origin of animals.

B Genome Data from Unicellular Holozoans Draws a Complex Unicellular Ancestor of Animals

The first genome sequence to be obtained from a unicellular holozoan was that of the choanoflagellate *Monosiga brevicollis* (King et al. 2008). A comparison of the genome sequence of *M. brevicollis* with the genomes of different animals showed that choanoflagellates had less genes (8,700) than, for example, sponges, cnidarians, or placozoans. The genome analysis also showed that choanoflagellates already have some genes involved in multicellularity functions, such as protein tyrosine kinases, cadherins, or the Myc transcription factor, all of them previously thought to be animal-specific. Interestingly, the analysis revealed that some other important "multicellular" genes were animal innovations. That was the case, for example, of integrins, one of the most important adhesion systems in animals, as well as several developmental transcription factors such as NF-kappaB, ETS, Smad, T-box, Runx and Grainyhead (King et al. 2008).

This initial comparative genomic analysis between animals and choanoflagellates was further updated and improved with the addition of new genomes from other taxa. Thus, the genomes of another choanoflagellate (*Salpingoeca rosetta*) and one filasterean (*C. owczarzaki*) provided a more complete view of the evolutionary history of the gene families with key functions in multicellularity and animal development (Fairclough et al. 2013; Suga et al. 2013). What those new genomes showed is that many other components were already present in *C. owczarzaki* and, therefore, in the unicellular ancestor of animals, but were secondarily lost in choanoflagellates (Suga et al. 2013; Sebé-Pedrós et al. 2010, 2011). This is the case of the integrin adhesome, as well as NFkappa, T-box, Runx, and Grainyhead, all present in the genome of *C. owczarzaki* (Figure 4.4).

Additional work showed that not only the genes but also some transcription factor gene regulatory networks are conserved between animals and their unicellular relatives. A good example is *Brachyury*, a member of the T-box gene family that in bilaterian animals is involved in gastrulation. *C. owczarzaki* has a homolog of that gene that in *Xenopus* can rescue the endogenous function of Xenopus Brachyury (Sebe-Pedros et al. 2013a). Moreover, the downstream gene regulatory network seems to be conserved between *C. owczarzaki* and animals. An analysis of the Capsaspora-Bra downstream target network revealed genes involved in the establishment of cell polarity, phagocytosis, metabolism, transcription factors, and GPCR signaling genes (Sebé-Pedrós et al. 2016a). Moreover, the comparison of downstream orthologs of Capsaspora-Bra and mouse Brachyury targets shows that those orthologs common between the two taxa are significantly enriched in actin cytoskeleton and cell motility functions. That means the Brachyury downstream network was already present in the shared ancestor between animals and *C. owczarzaki* and likely regulating cell motility.

In general, what the genomes of unicellular holozoans (i.e., the closest unicellular relatives to animals) have told us is that the unicellular ancestor of animals already had a complex repertoire of genes involved in cell adhesion, cell signaling, and transcriptional regulation. Some of those genes were subsequently duplicated in the animal lineage, leading to extended gene families, but the complexity

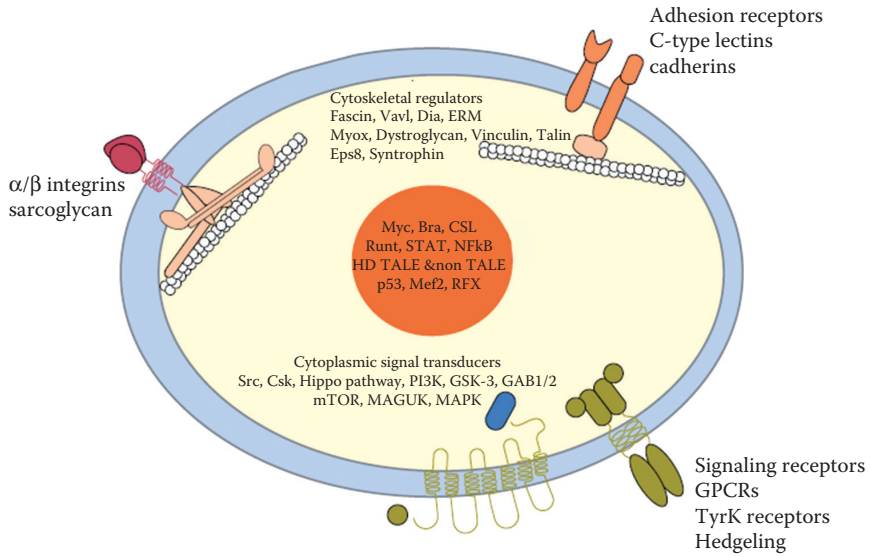

FIGURE 4.4 The potential cell of the unicellular ancestor that gave rise to animals, depicting some of the genes and pathways that were present. (Adapted from Suga, H. et al., *Nat. Commun.*, 4, 2325, 2013. With permission.)

at the gene level of the unicellular ancestor cannot be put into question (Figure 4.4). Moreover, the data has shown that the downstream networks of some developmental transcription factors evolved long before the advent of animals. This points to an important role of co-option at the origins of animals, in which ancestral genes that were working within a unicellular context were recycled to work within a multicellular organism.

C Regulation of Gene Expression by Holozoans

The genome sequences from holozoans demonstrated that those taxa already had many genes involved in multicellularity. However, it remained unclear their capacity to regulate the expression of those genes. Recent transcriptomics analyses from one choanoflagellate (*S. rosetta*), one filasterean (*C. owczarzaki*), and one ichthyosporean (*C. fragrantissima*) have shown that those taxa tightly regulate gene expression to go from one life stage to another (Fairclough et al. 2013; Sebé-Pedrós et al. 2013b; de Mendoza et al. 2015).

In all cases, different cell types representing different life stages had different and specific transcriptomic profiles. In the case of *S. rosetta*, for example, septins and cadherins (which are involved in cell adhesion in animals) were found to be upregulated in the colonial stage (Fairclough et al. 2013). Similarly, the different life stages

of *C. owczarzaki* had specific transcriptomic profiles involving specific functions (Sebé-Pedrós et al. 2013b). The "multicellular" aggregative stage, for example, is upregulated in genes involved in the integrin adhesome, as well as in proteins with domains typical of ECM in animals. In contrast, genes related to filopodia formation and tyrosine kinase signaling were upregulated in the filopodial, adhaerent stage. Completely different and specific transcriptomic profiles were also found for the amoeba and the multinucleated stage of *C. fragrantissima* (de Mendoza et al. 2015). For example, genes involved in DNA replication, RNA and amino acid metabolism, as well as translation were significantly upregulated in the multinucleate coenocyte, while genes involved in protein kinase activity and cell–ECM adhesion were significantly upregulated in the amoebas. In the case of the latter two taxa (*C. owczarzaki* and *C. fragrantissima*), it was also found that alternative splicing was also contributing to the regulation of gene expression. Both *C. owczarzaki* and *C. fragrantissima* were shown to have both intron retention, as in most eukaryotes, and exon skipping in some genes (Sebe-Pedros et al. 2013b; de Mendoza et al. 2015). Intron retention in those taxa were found to be differentially regulated between the different life cycle stages, probably contributing to the control of transcript levels. Interestingly, in *C. owczarzaki*, genes with differential exon skipping were found to be significantly enriched in protein kinase activity, suggesting the presence in *C. owczarzaki* of a regulated exon network linked to cell signaling, a feature that was thought to be animal-specific. In *C. fragrantissima* genes involved in exon skipping were significantly enriched in several biological functions, such as channel activity and histone modifications.

D Cell Types in Holozoans

The data from transcriptomics point to the fact that premetazoan taxa have the capacity to differentiate into different cell types (each one with its own specific transcriptomic profile) in a temporal manner, within their life cycles. Two more recent analyses confirmed that the regulation of the different cell types (life stages) in *C. owczarzaki* is, in turn, regulated by a dynamic proteome and phosphoproteome remodeling, as animals do, as well as long noncoding RNAs and histone marks (Sebé-Pedrós et al. 2016a, b). The phospho-signaling regulation also affects some developmental transcription factors, such as Runx, P53, or CREB (Sebé-Pedrós et al. 2016b).

All these data suggest that the unicellular ancestor of animals not only had the gene repertoire needed for multicellular functions and animal development but also had most of the mechanisms to regulate cell-type differentiation in animals. In this case, those mechanisms were probably working in transitions from one life stage to another and were later co-opted to work spatially within a multicellular body plan.

E Conclusion

The study of unicellular relatives of animals has clearly improved our understanding of how unicellular protists became multicellular animals. Thanks to those analyses.

We now know that the unicellular ancestor of animals was genetically much more complex than previously thought. Not only had that ancestor a rich repertoire of genes involved in multicellular functions, but it also had the capacity to strongly regulate the expression of those genes and had the mechanisms to perform cell differentiation. That means that many genes and gene regulatory networks present in extant animals and key for their multicellularity and development appeared in the premetazoans, being later recycled to work within a multicellular body. This, together with the acquisition of some novel genes and an important expansion of some gene families provided the basic metazoan genetic toolkit, allowing the evolution of spatial cell differentiation as well.

4.4 THE EVOLUTION OF MULTICELLULARITY AND CELLULAR DIFFERENTIATION IN GREEN ALGAE

> "Few groups of organisms hold such a fascination for evolutionary biologists as the Volvocales. It is almost as if these algae were designed to exemplify the process of evolution...." (Bell 1985)

The chlorophytes (green algae in the class Chlorophyceae) are unrivaled champions at transitioning from unicellularity to multicellularity. Such a transition is said to have been made independently in more than two dozen different lineages (Bonner 1998; Grosberg and Strathman 2007); but nearly half of that many such transitions may have been made by the chlorophytes alone. Although most species of chlorophytes are unicellular flagellates, multicellular species are present in 9 of the 11 chlorophyte orders, and in each of those 9 orders multicellularity is believed to have arisen independently—and in some cases more than once (Melkonian 1990).

In most cases, such "multicellular" chlorophytes are multicellular in only the simplest sense; each individual is composed of more than one cell. Indeed, they usually consist of 2^n sister cells of identical type that have remained in association after being produced by n rounds of cell division (Figure 4.5). Such organisms are usually referred to by those who study them as "colonies" or "colonial organisms," and are characterized by the fact that each of their cells is capable of dividing n times to produce a new colony comprising 2^n sister cells (Starr 1980).

There can be little doubt that the propensity of the chlorophytes to form such colonies of sister cells is due in large measure to their shared capacity to produce and secrete two kinds of extracellular materials: (1) a glycoprotein-rich (cellulose-free) cell wall and (2) "mucilage," an amorphous sticky mixture of glycoproteins, acid mucopolysaccharides and other carbohydrates. In family after family of colonial chlorophytes, one finds that sister cells are held together by glycoprotein-rich walls that have either fused or have formed some sort of specialized junction between cells. And in case after case it is also found that any spaces between the cell walls are filled with mucilage (see Figure 4.5).

However, in addition to its use to refer to simple clusters of similar cells, the term "multicellular organism" has a second meaning. When the term "multicellular organism"

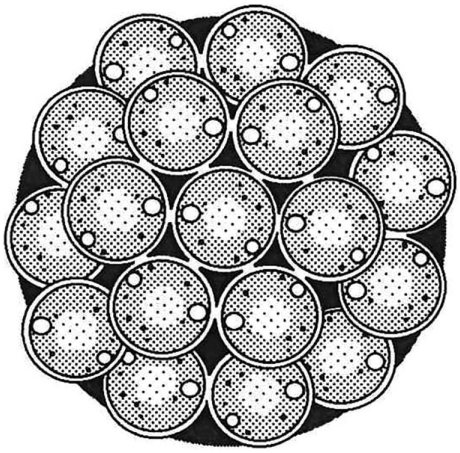

FIGURE 4.5 A drawing of a 32-cell colony of *Coelastrum microsporum*, a representative small, green Chlorophyte alga. Chlorophyte colonies may consist of 4, 8, 16, 32, 64, or 128 sister cells, depending on how many times the mother cell divided. Each cell is surrounded by a glycoprotein-rich cell wall that is shown here as a dark-light-dark tripartite ring surrounding each cell. The walls of neighboring cells are connected by specializations that were formed at points of contact. The cellular monolayer surrounds a central space filled with mucilaginous extracellular matrix (indicated in solid black). In such colonies, every cell is capable of dividing to produce a new colony of similar type. (Reprinted from Kirk, D.L., *Volvox: Molecular Genetic Origins of Multicellularity and Cellular Differentiation*, Cambridge University Press, Cambridge, UK, 1998. With permission.)

is used in the abstract, it most often conjures up images of more complicated beings—such as plants, animals, or fungi—consisting of multiple cell types that differ in both structure and function, and that must cooperate to survive and produce offspring. Elegant examples of this type of multicellular organism also have evolved in the Chlorophyceae.

A The Genus *Volvox*: Multicellular Algae with a Germ-Soma Division of Labor

In a subset of chlorophytes known as the volvocine algae, the evolution of multicellularity in the simpler sense was followed by the evolution of cellular differentiation, resulting in algae such as *Volvox carteri* (Figure 4.6) that exhibit a germ-soma division of labor rather similar to that seen in most animals. The developmental biology of *V. carteri* has been reviewed frequently (Kirk 1998, Nishii and Miller 2010, Matt and Umen 2016). Here it will suffice to summarize key aspects of that biology.

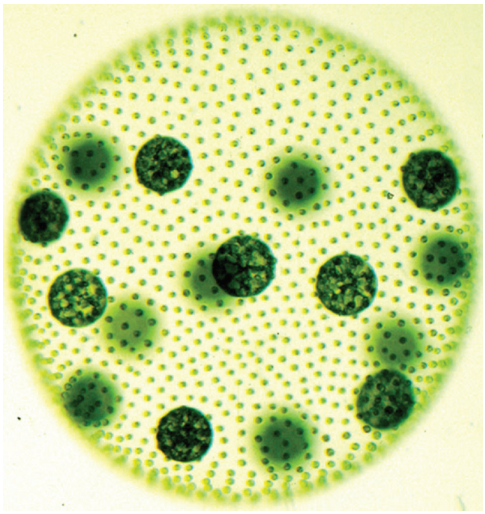

FIGURE 4.6 *Volvox carteri*, an alga with a striking germ-soma division of labor. Each individual (called a "spheroid") contains more than 2,000 small, biflagellate somatic cells embedded at the surface of a transparent sphere of glycoprotein- and mucopolysaccharide-rich extracellular matrix. 16 much larger cells, called gonidia, are located just internal to the somatic cells. The flagella of the somatic cells protrude from the surface and provide the spheroid with motility. The gonidia never have functional flagella; they serve as germ cells in the asexual reproductive cycle, by dividing to produce a new generation of spheroids with a similar cellular composition.

A *V. carteri* individual (called a "spheroid," because of its shape) contains two fully differentiated cell types: more than 2,000 tiny, biflagellate somatic cells, and about 16 large asexual reproductive cells called "gonidia." The somatic cells lie in a monolayer at the surface of a transparent sphere of ECM, with their flagella projecting from the surface and oriented so that their beating propels the spheroid through the water with a highly characteristic rolling motion. The gonidia lie just internal to the somatic cells in the ECM.

Gonidia lack flagella, are as much as 1,000 times the volume of somatic cells and are specialized for cell division. Once mature, each gonidium will divide rapidly 11 or 12 times, to produce all the cells that will be present in an adult of the next generation. In marked contrast, once the somatic cells have begun to differentiate, they have become postmitotic; they never divide again. The asexual life cycle of *V. carteri*, by which a new generation of spheroids is produced every two days, is diagrammed in Figure 4.7 and is described in the accompanying caption.

V. carteri also has a very interesting sexual reproductive cycle (Kirk 1998, Umen 2011), but space constraints prevent discussing it here.

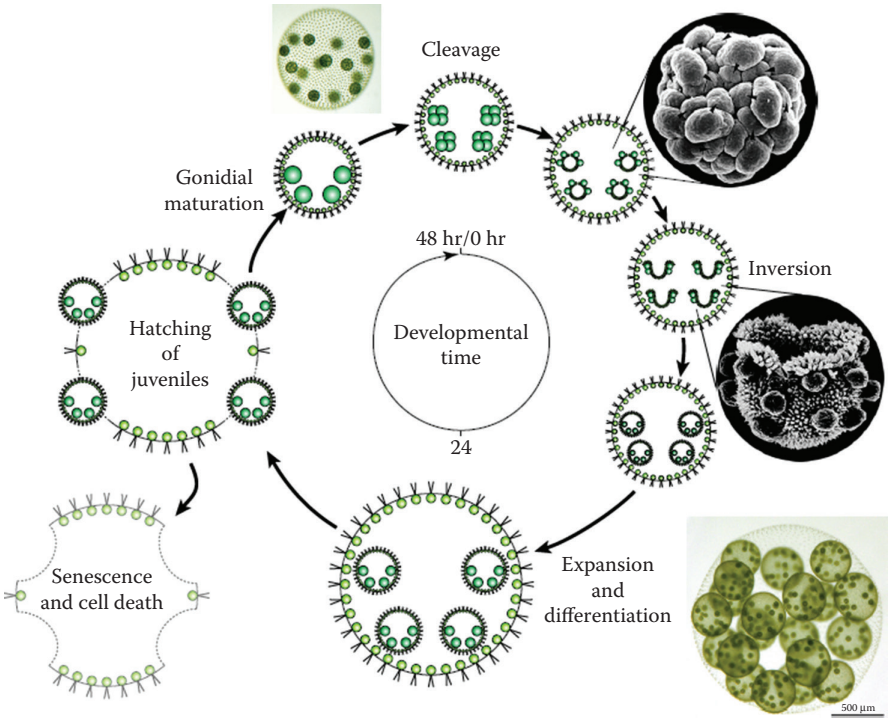

FIGURE 4.7 A diagram of the asexual reproductive cycle of *Volvox carteri*. As indicated by the inner circle, one asexual life cycle takes precisely two days, if a synchronizing light-dark cycle (16L:8D) is used. Each life cycle comprises 5 phases of development that are labeled on the circumference, and in several cases illustrated with photomicrographs. These phases are as follows: (i) Cleavage. A mature gonidium executes a series of synchronous cell divisions. The first five divisions are symmetrical, producing 32 cells of similar size, arranged in a hollow sphere. In the sixth division cycle, the 16 cells in one hemisphere divide symmetrically again, but the 16 cells in the other hemisphere divide asymmetrically, producing 16 large-small sister-cell pairs (as shown in the micrograph, where arrowheads point from each large cell to its small sister cell). The large cell in each such pair becomes a gonidial initial, while its smaller sister becomes a somatic initial, as do all the cells of the other hemisphere that divided symmetrically. After being formed by asymmetric division in cycle six, the gonidial initials divide asymmetrically one or two more times, producing another small somatic initial in each division. Then the gonidial initials withdraw from the division cycles, while all the somatic initials go on dividing symmetrically, until they have completed a total of 11 or 12 divisions. (ii) Inversion. At the end of cleavage, the embryo contains all the cells that will be present in the adult. But they are arranged in a maladaptive orientation: all the somatic initials are oriented with their flagellar ends directed toward the interior space, and all the gonidial initials are on the outside of the sphere. This predicament, which would preclude spheroid motility, is resolved as the embryo turns itself completely inside-out, bringing the flagellar ends of the somatic initials to the exterior and moving the gonidia to the interior in the process known as inversion. Inversion occurs by a stereotyped series of changes in cell shape and cellular movement that have been analyzed extensively with regards to mechanical, cytological and genetic parameters (Viamontes et al. 1979; Nishii et al. 2003). (*Continued*)

B The Volvocine Lineage: A Simple, Linear Progression in Size and Complexity?

Volvox is the eponymous ("name-giving") member of the "volvocine algae," a group that encompasses the unicellular green flagellate, *Chlamydomonas reinhardtii*, a variety of multicellular green flagellates in the family Volvocaceae (including about 20 species of *Volvox*) and a few other small, green flagellates in two families, closely related to the Volvocaceae. A unifying feature is that they all have cells morphologically similar to *Chlamydomonas*. The notion that these algae constitute a monophyletic group of organisms that are related by what Darwin called "descent with modification" was accepted by many biologists for decades, in the absence of any supporting evidence beyond their common cytological features. A number of textbook authors went as far as to line up the various volvocine algae in order of increasing size and complexity, from *Chlamydomonas* to *Volvox*, and then imply that this probably resembled the pathway by which members of the group evolved: by a simple progressive increase in organismic size and complexity. The latter notion has been called "the volvocine lineage hypothesis" (Figure 4.8).

About a century ago, however, a markedly different hypothesis about aspects of volvocine evolution was proposed, based on the presence or absence of cytoplasmic connections in adults of various species of *Volvox* (Crow 1918). During their embryonic development, all members of the family Volvocaceae have cytoplasmic bridges that form between sister cells as a result of incomplete cytokinesis, thereby, linking all cells of the embryo into a syncytium. In many species of *Volvox* (including *V. carteri*, shown in Figure 4.6), and in all species of the other volvocacean genera, these cytoplasmic bridges break down at the

FIGURE 4.7 (*Continued*) At the end of inversion, the embryo has taken on the adult configuration and is called a juvenile spheroid. But at that stage, its presumptive somatic and gonidial cells differ in little but size. (iii) Cytodifferentiation and expansion. Under synchronizing growth conditions, the last stages of cleavage and inversion occur in the dark, after which nothing changes visibly until the lights come back on. As soon as the lights come on, both cell types begin actively translating mRNAs that had accumulated in the dark (Kirk and Kirk 1985), flagella grow outward from the somatic cells, the gonidia begin to enlarge, and the two cell types diverge progressively in their patterns of gene expression and morphology. Now the principal activity of the gonidia is growth, in preparation for the next round of embryogenesis, whereas the major activity of the somatic cells is synthesis and secretion of glycoproteins, mucopolysaccharides and other ECM components that will self-assemble and cause the spheroid to increase greatly in diameter. (iv) Hatching. A day and a half after their formation began with cleavage of the maternal gonidia, each juvenile spheroid digests an opening in the overlying parental ECM, creating a birth canal through which it then swims to the exterior, to become a free-living young adult. By this time, the somatic cells of the parent have become senescent, and they and the parental ECM soon undergo dissolution and disappear. (v) Gonidial maturation. The gonidia of the free-swimming young adults now do whatever is required to initiate a new round of embryogenesis. As those gonidia begin to divide, one asexual life cycle has been completed, and another begins. (Reprinted from Prochnik, S.E. et al., *Science*, 329, 223–226, 2010. With permission.)

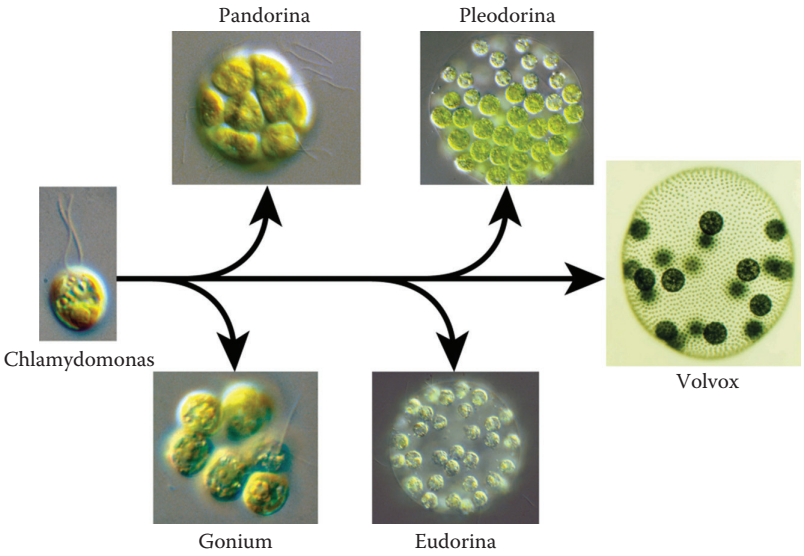

FIGURE 4.8 The volvocine lineage hypothesis. It is possible to line up *Chlamydomonas* and selected volvocacean genera in a conceptual series (as in this diagram) in which there is a progressive increase in cell number, the ratio of ECM to cellular volume, and the number of cells that are set aside as nondividing, sterile somatic cells. A typical colony of Gonium, one of the smallest volvocine algae, consists of a convex disc of 8 or 16 *Chlamydomonas*-like cells, each of which will eventually divide to produce a new colony of similar form. A colony of *Pandorina* consists of 16 or 32 cells that are initially packed together closely to form an elliptical ball: later however, the cells separate from each other somewhat as ECM accumulates. As in *Gonium*, every *Pandorina* cell is capable of dividing to produce a new colony. *Eudorina* colonies are spheres of 16, 32, or 64 cells (depending on the species and environmental conditions) in which cells are more widely separated than in the previous two genera. In a 64-cell *Eudorina* colony 4 cells at the anterior pole of the sphere may function as nonreproducing somatic cells and continue beating their flagella to keep the colony afloat while all the other cells lose their flagella, enlarge and divide to produce progeny. In *Pleodorina* there are usually 64 or 128 cells per individual. They are all initially biflagellate cells contributing to motility; but then cells in the posterior hemisphere resorb their flagella, grow and function as gonidia to produce progeny, while the remainder of the cells functions as terminally differentiated somatic cells. The more total cells there are in *Pleodorina*, the higher will be the ratio of somatic cells to gonidia. That trend continues in the genus *Volvox*. In species of *Volvox* having as few as 1,000 cells per spheroid, there may be as many as 75 gonidia. But in species with as many as 10,000 cells, there may be fewer than ten very large gonidia, with all the rest being terminally differentiated somatic cells (Kirk 1998). So, the following relationships can be generalized for *Eudorin*a, *Pleodorina* and *Volvox*: as the total number of cells per colony or spheroid increases, the gonidia decrease in number but increase in size, while the somatic cells increase in number but decrease in size (Bell 1985, Koufopanou 1994). The Volvocine Lineage Hypothesis postulates that by lining up these algae in order of increasing size and complexity we get a good approximation of the way in which the group evolved: by a simple progressive increase in organismic size and complexity, from *Chlamydomonas* to *Gonium*, to *Pandorina*, to *Eudorina*, and so forth.

end of embryogenesis. As a result, adult cells in all those species lack intercellular connections and have a smooth, round profile when viewed in optical cross section. However, in another large section containing about half of all the recognized species of *Volvox*, the cytoplasmic bridges are retained and thickened in the adult, so that each adult cell is connected to all its neighbors by stout cytoplasmic bridges, which makes the cells appear stellate in optical cross section. Crow postulated that *Volvox* species, lacking cytoplasmic connections in the adult might have evolved from a *Chlamydomonas*-like ancestor—as others had previously suggested—but that those species of *Volvox* in which the adult cells are connected by bridges, had evolved independently from a very different unicellular green flagellate genus, *Haematococcus*—cells of which appear somewhat stellate in optical cross section.

C Comparative DNA Sequencing Falsifies the Volvocine Lineage Hypotheses

The first published comparison of the sequences of nuclear-encoded rRNAs of *Volvox* and *Chlamydomonas* (Rausch et al. 1989) reinforced the inference drawn from morphology that these two genera were closely related. A more extensive comparison of volvocine nucleic acid sequences (Kirk et al. 1990, later amplified in Larson et al. 1992) produced several interesting conclusions that have been corroborated by subsequent work: First, it falsified Crow's hypothesis, by showing that both the *Volvox* species that had adult cytoplasmic bridges and the species that lacked such bridges had rDNA sequences that were much more closely related to the rDNA sequences of *Chlamydomonas reinhardtii* than they were to those of *Haematococcus*. Second, it justified the choice of *C. reinhardtii* as a proxy for the unicellular ancestor of the Volvocaceae by showing that *C. reinhardtii* rDNA is much more closely related to that of all volvocaceans than it is to the rDNA sequence of another species of *Chlamydomonas*. Third, it produced results consistent with the hypothesis that the family Volvocaceae is a monophyletic assemblage of closely related organisms; they all share a common unicellular ancestor. Fourth, it falsified the volvocine lineage hypothesis by indicating clearly that the phylogenetic relationships among the genera represented in Figure 4.8 are much more complicated than the volvocine lineage hypothesis suggests. Fifth, it indicated that, although the volvocine algae exist as a group are monophyletic, the genus *Volvox* is not monophyletic, since the two species of *Volvox* that were studied were placed on two separate branches of the preliminary family tree that was produced in the study. Several of these conclusions were quickly supported by the results of a similar study by Buchheim and Chapman (1991).

Currently the best estimate is that *Chlamydomonas reinhardtii* and *Volvox carteri* last shared a common ancestor about 200 million years ago (Herron et al. 2009), which is several hundred million years more recent than animals, plants, or fungi, are thought to have shared a common ancestor with their unicellular forebears (Parfrey et al. 2011).

D The Family Volvocaceae Is Monophyletic, but Several of Its Genera Are Not

Since 1992 there have been a number of additional studies using comparative DNA sequencing of various nuclear and chloroplast genes from a growing set of volvocine algae, in an effort to establish a robust phylogeny for the group (e.g., Coleman 1999; Nozaki et al. 2000, 2014; Nozaki 2003; Nakada et al. 2008; Herron and Michod 2008; Herron et al. 2009, 2010). The number of taxa included in such studies has increased over the years, in part because new species—and even new genera—of volvocaceans are regularly being isolated from natural fresh water sources by Nozaki and his colleagues (examples: Nozaki and Coleman 2011; Isaka et al. 2012; Nozaki et al. 2014, 2015a,b, 2016.) Although various recent studies have differed in magnitude and methodology, they have generally supported three conclusions drawn from the earliest molecular–phylogenetic studies of the group: (1) The family Volvocaceae, taken as a whole, is monophyletic (i.e., its members share a common unicellular ancestor). (2) The phylogenetic relationships among the various genera and species of volvocaceans are more complex than the volvocine lineage hypothesis suggested. (3) The (so-called) genus *Volvox* is polyphyletic.[1] However, as such studies have included ever more taxonomic units and analyzed them with greater resolution, it has become increasingly apparent that *Volvox* is not the only polyphyletic genus in the family.

The latter point is clearly illustrated by a recent study, the results of which are summarized in a simplified form in Figure 4.9. There, in addition to finding species of *Volvox* on four different branches of the family tree, we find *Pleodorina* and *Eudorina* species on three branches each, and even different isolates of the so-called species *Eudorina elegans* are located on somewhat widely separated branches.

What conclusions should we draw from such results?

The first conclusion to be drawn is that several of the genus and species names that are currently used to classify volvocaceans identify *grades* of organizational complexity, *not clades* of closest relatives. This conclusion—that a single scientific name may sometimes group volvocine algae that are only somewhat distantly related at the genetic level is consistent with a number of earlier studies of natural populations. The clearest example is Annette Coleman's (1980) stunning analysis of *Pandorina morum* strains that had been isolated from ponds around the world. She found that various isolates of this morphologically monotypic "species" fell into at least 24 reproductively isolated mating groups, or syngens. Different syngens sometimes

[1] Spatial constraints here preclude full discussion of the range of developmental variations that occur among species currently assigned to the genus *Volvox* and the complex evolutionary relationships among those species. But for an extensive, authoritative, and enlightening discussion of such matters, see Herron et al. (2010).

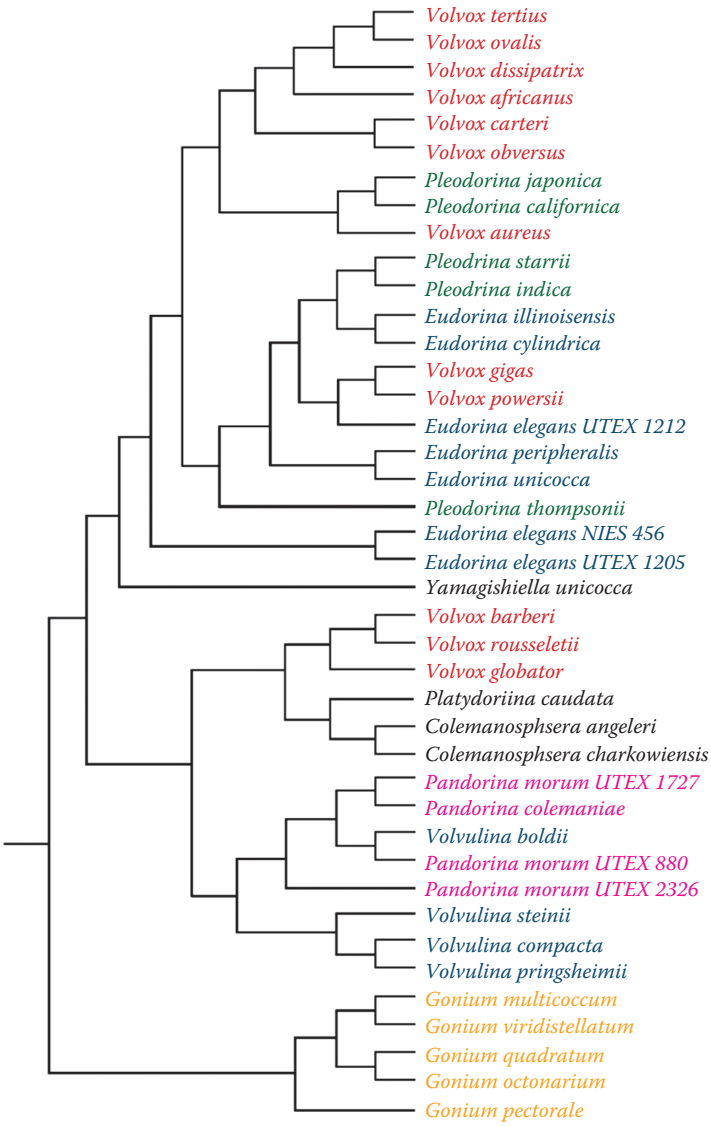

FIGURE 4.9 A dendrogram illustrating relationships among various volvocine genera and species. Adapted from a Bayesian inference tree that was based on the sequences of five chloroplast genes in 58 volvocine algae (Nozaki et al. 2014). In this adaptation, the tree has been greatly simplified, using lines of arbitrary length to emphasize the branching patterns between various taxa. For quantitative information, such as genetic distances along various branches, posterior probabilities and bootstrap values, see the original.

differed from one another by as much as threefold in chromosome number (from 4 to 12), in the absence of any visible distinguishing characteristics. She also showed that although two *P. morum* isolates from a single pond might be reproductively isolated from one another, each might be interfertile with a *P. morum* coming from some particular pond on the other side of the world. Clearly, *Pandorina morum* does not fit the "biological species concept" which asserts that: "species are groups of interbreeding natural populations that are reproductively isolated from other such groups." By this criterion, algae that are morphologically indistinguishable from the type specimen of *P. morum* constitute at least 24 species. Should they all be given different Latin binomials? And if so, how are field biologists going to decide which Latin binomial to write in their notebooks when they find an alga in a pond that looks like good old-fashioned *P. morum*? This is a taxonomic quandary that will probably never be resolved to the satisfaction of all biologists.

A second conclusion to be drawn from recent molecular–phylogenetic studies of the volvocine algae is that transitions have apparently been made repeatedly—and in both directions—between several organizational grades (aka genera) of volvocaceans in the past. A third conclusion follows close behind the second, which is, the genetic changes required to transition from one volvocacean organizational level to the next must be quite modest. That conclusion is consistent with earlier observations that (for example) a single mutation in *Volvox powersii* changes its morphology enough that it would be called *Pleodorina*, if it were found in the wild (Vande Berg and Starr 1971), or that a pair of point mutations is sufficient to change *Volvox carteri* into a form that would be called *Eudorina*, if it were found in nature (Tam and Kirk 1991).

It is important to note that there is no evidence indicating that the important transition from *Chlamydomonas*-like unicellularity to simple, colonial (*Gonium*-like) multicellularity has occurred more than once in this group of algae. So far, all the available evidence is consistent with the notion that this important transition has occurred only once and, therefore, the family Volvocaeae is monophyletic.

To understand how that transition occurred, and why it never occurred more than once, we need to step back to consider the unusual pattern of cell division seen in *Chlamydomonas.*

E Multiple Fission in *Chlamydomonas* Provided a Foundation for Multicellularity

Most eukaryotic cells have a cell division pattern called "binary fission." That is to say, after a period of growth in which they approximately double in size, they divide in two, and then they repeat the cycle of growth and division. But *Chlamydomonas* and certain other protists have a very different pattern, called "multiple fission," or "palintomy." In palintomic organisms, growth and cell division are uncoupled; cells grow 2^n-fold without dividing and then divide $\boldsymbol{n}$ times rapidly (in the absence of further growth) to produce 2^n progeny cells. In vegetatively reproducing *Chlamydomonas* cultures, the value of $\boldsymbol{n}$ is usually between 2 and 4 (depending on culture conditions), resulting in the production of 4, 8, or 16 progeny cells per cycle.

All colonial volvocine algae, including *Gonium*, *Pandorina*, *Eudorina*, and *Pleodorina* (plus several other genera not specifically named and discussed here)

exhibit the same multiple fission pattern as *Chlamydomonas* does, accounting for the fact that they typically contain 2^n cells per colony, with the value of ***n*** varying by genus. (In various *Volvox* species, however, the multiple-fission program becomes altered in various ways—or abandoned altogether—to arrange for production of gonidia of species-specific size and number; Herron et al. 2010.)

F Two Changes were Required in Concert to Convert Multiple Fission to Multicellularity

It has been proposed that the evolution of a *Volvox carteri*-like organism from a *Chlamydomonas*-like ancestor involve twelve substantial genetic and/or morphological changes (Kirk 2005). But two of those twelve changes were the *minimal* changes required to evolve a simple, multicellular colony from a *Chlamydomonas*-like unicell. Specifically, the minimal changes required for this critical transition were: (1) Incomplete cytokinesis, to form transient cytoplasmic bridges between sister cells that hold the cells in a fixed relationship during the division period. (2) Modification of the cell wall-forming process that follows cell division, to form some sort of attachment between adjacent cell walls, in order to hold the cells in the same fixed relationship after the cytoplasmic bridges have broken down.[1] Both of these important steps have been observed during *Gonium pectorale* development (Stein 1958, Iida et al. 2013); both of them occur, with some minor modifications, in all other colonial volvocaceans (Kirk 1998), and they also occur with certain additional modifications in all species of *Volvox* (Herron et al. 2010).

The importance of establishing firm cell wall, or ECM connections between neighboring cells before the cytoplasmic bridges break down, has been demonstrated most clearly with *V. carteri*. In normal development of *V. carteri* embryos, a glycoprotein called ISG self-assembles to form a layer over the surface of the newly inverted embryo. This ISG layer not only provides the first tenuous extracellular linkages between neighboring cells, but it also acts as a scaffold for assembling the other ECM components, including the fused cell walls that normally form solid connections between neighboring cells. So, when ISG assembly was prevented, the rest of the ECM never assembled properly, and as a result, as soon as the cytoplasmic bridges broke down, the embryo fell apart into a single-cell suspension (Hallmann and Kirk 2000). From such studies, we conclude that these two new features (incomplete cytokinesis and cell wall fusion) would need to have been achieved *in concert* to make the transition from a unicellular to a colonial body plan. And the improbability of two such morphological features evolving almost simultaneously may well account for the fact that the transition to multicellularity was made only once in this lineage.

In contrast, other volvocacean transitions (such as between the *Pandorina* and the *Eudorina* levels, or between the *Eudorina* and *Pleodorina* levels of size and

[1] *Gonium* exhibits other morphological differences from *Chlamydomonas* that are shared by all the other volvocaceans (Kirk 2005), but the two changes discussed here are the minimal changes that would be required to hold the post-division cells in a predictable spatial relationship.

complexity) appear to have involved only a single substantial change (Kirk 2005), which would have given them a much higher probability of occurring more than once over evolutionary time.

Next question: Can we discern what genetic changes underlie all the steps that were involved in the evolution of *Volvox carteri* from a *Chlamydomonas reinhardtii*-like ancestor?

G The *Chlamydomonas* and *Volvox* Genomes are Strikingly Similar

Three years after the sequence of the *C. reinhardtii* genome had been determined (Merchant et al. 2007), the *V. carteri* genome was also sequenced (Prochnik et al. 2010). A primary motivation for sequencing the *V. carteri* genome was, of course, the hope that a detailed comparison of the two genomes would reveal clearly which important genetic changes had accumulated in going from *Chlamydomonas* to *Volvox*.

In retrospect, two earlier observations probably should have alerted us to the realization that this might very well be a false hope, because it had been found that two genes that are essential for normal *V. carteri* development had undergone so little change in the ~200 million years since Chlamydomonas and *Volvox* lineages diverged that the *C. reinhardtii* genes could substitute for their *V. carteri* counterparts.

This finding came from a genetic study of two very interesting processes in *V. carteri* embryonic development that have no known parallel in *C. reinhardtii*, namely: asymmetric division, by which germ cell and somatic cell precursors are set apart during cleavage, and inversion, by which the fully cleaved *Volvox* embryo turns itself inside out (Figure 4.7). Transposon mutagenesis was used to establish that when a *Volvox* gene called *glsA* is inactivated, no asymmetric divisions occur (Miller and Kirk 1999). Strikingly, the *Chlamydomonas* ortholog of *glsA* is fully capable of rescuing the *glsA* mutant and restoring normal asymmetric division and germ cell specification (Cheng et al. 2003). Similarly, after a transposon insertion was used to mutagenize *invA* (a *Volvox* kinesin-encoding gene, Nishi et al. 2003), and show that its product is required for normal inversion of the *Volvox* embryo, the *Chlamydomonas* ortholog of *invA was* shown to be fully capable of rescuing the *invA* mutant and restoring perfectly normal inversion (Nishii and Miller 2010).

In the light of such observations, it is not surprising, perhaps, that the *C. reinhardtii* and *V. carteri* genomes turn out to be strikingly similar (Table 4.1) (Prochnik et al. 2010, Umen and Olson 2012). Although the *V. carteri* genome is ~17% larger than the *C. reinhardtii* genome, most of the difference is accounted for by the greater abundance of repetitive sequences in *Volvox*. The two genomes contain very similar numbers of protein-coding genes and encode similar numbers of largely overlapping protein families. More than 9,000 (~64%) of the protein-coding sequences in the two algae encode proteins in families that are shared with many other eukaryotes, but 1,835 of the coding sequences (~12%) are volvocine-specific, in the sense that they are found in both algal genomes, but not in other sequenced genomes.

The volvocine-specific genes are of potential long-term interest because many of them exhibit asymmetric expansion/contraction patterns between these two algae. In most cases, however, the functions of the encoded proteins are unknown,

TABLE 4.1
A Comparison of the *Chlamydomonas reinhardtii* and *Volvox carteri* Genomes

Species	*Chlamydomonas*	*Volvox*
Genome size (Mbp)	118	138
Number of chromosomes	17	14
Interspersed repeats (millions)	14.8	28.2
Protein-coding loci	14,516	14,520
PFAM (protein family domains)	2,354	2,431
% coding	16.3	18.0
Introns per gene	7.4	7.05
Median intron length (bp)	174	35
Volvocine-specific genes	1,835	1,835
Pherophorins	27	45
Matrix metalloproteinases	8	42
D1 cyclins	1	4
Histone gene clusters	35	14
Ankyrin repeat proteins	146	80

precisely because homologs have not been found elsewhere. But there are two volvocine-specific families where the significance is fairly obvious; genes encoding pherophorins and matrix metalloproteinases, which are significantly more abundant in the *V. carteri* than in the *C. reinhardtii* genome (Table 4.1, below the dotted line). The pherophorins are hydroxyproline-rich glycoproteins that are related to certain *Chlamydomonas* cell wall proteins, and are major building blocks of the *Volvox* ECM. Moreover, the matrix metalloproteinases are thought to be intimately involved in fashioning, refashioning, and dissolving the ECM at various stages of the life cycle (Hallmann 2006). In view of the fact that the ECM volume is nearly 100X the cellular volume in an adult *Volvox*, but less than 1% of the cellular volume in *Chlamydomonas*, it is hardly surprising that these two gene families that encode major ECM constituents are expanded in the *Volvox* genome relative to the *Chlamydomonas* genome.

It is slightly less obvious why the D1 cyclin gene family should be expanded 4-fold in *Volvox*. In animals and land plants, D-type cyclins play an important role in the regulation of the cell cycle, by activating cyclin-dependent kinases that then phosphorylate RB proteins, and both proteins have been shown to play a key role in regulating cell cycle progression in *Chlamydomonas* (Umen and Goodenough 2001). So, it has been hypothesized that the expansion of the D1 cyclin family in *Volvox* might be related to the fact that *Volvox* exhibits many more stage-specific and mating-type specific cell cycle variants than *Chlamydomonas* does (Umen and Olson 2012). We will return to that hypothesis later.

In contrast to the expansion of genes encoding major ECM components in *Volvox*, which are rather easy to rationalize, it is difficult to rationalize the greater abundance of genes encoding histones and ankyrin-repeat proteins

in *Chlamydomonas* (Table 4.1). The number of histone-encoding genes in *Chlamydomonas* (which is unusually high with respect to land plants and many other algae, as well as *Volvox*) might be rationalized if *Chlamydomonas* had much more rapid division cycles than *Volvox*; but it does not (Umen and Olson 2012).

The greater abundance of genes in *Chlamydomonas* is equally enigmatic. Ankyrin repeats are involved in protein–protein interactions in a wide variety of interesting proteins, such as transcriptional initiators, cell cycle regulators, cytoskeletal proteins, ion transporters, and signal transducers. But there is no obvious reason why any or all of those protein categories should be much more abundant in *Chlamydomonas* than in *Volvox*.

One important conclusion can safely be drawn from a comparison of these two genomic sequences: Major revision of the genome was not required to evolve from the *C. reinhardtii*-level to the *V. carteri*-level of size and developmental complexity.

H *Volvox reinhardtii* and *V. carteri* have Very Different Small-RNA Systems

The finding that *C. reinhardtii* and *V. carteri* do not have as many differences in protein-coding genes as some might have expected is not without precedent. When it was first realized in the 1970s that humans and apes were extremely similar at the DNA level, many considered this finding paradoxical, and the challenge became " to explain how species that have such substantially similar genes can differ so substantially…." (King and Wilson 1975). The situation had not changed significantly by the time the complete genome sequences of both apes became available, as the title of one review article made very clear ("…Searching for needles in a haystack." Varki and Altheide 2005). The conclusion drawn from many such studies by a substantial number of developmental biologists has been that "…changes in morphology generally result from changes in the spatiotemporal regulation of gene expression during development." (Carroll 2008).

There are of course as many ways to control gene expression as there are steps in the conversion of a DNA coding sequence to a visible phenotype. But one of the most recently discovered categories of gene regulatory mechanisms, and the one that has been most studied by those interested in the regulation of volvocine gene expression is post-transcriptional regulation by two types of small, noncoding RNA molecules, namely: micro RNAs (mi-RNAs) and small interfering RNAs (siRNAs) (Carrington and Ambros 2003, Bartel 2004). Both types of small RNAs are usually 20–24 nucleotides in length, and both function to regulate gene expression at the post-transcriptional level by either interfering with the translation of mRNAs containing the complementary sequence or by triggering the destruction of such mRNAs by the Argonaute nuclease. But they differ in origin: miRNAs are derived from stem-loop regions of mRNAs, whereas siRNAs are derived from long double-stranded RNAs, but in both cases, they are released from their source molecules by a Dicer nuclease (Vaucheret 2006). It has been postulated that by fine-tuning gene expression, small RNAs have played a major role in macroevolution and the origin of morphological novelties (Peterson 2009). Therefore, they have attracted the interest of several groups interested in the evolution of the volvocine algae.

The first unicellular organism that was found to possess miRNAs, as well as Dicer and Argonaute nucleases was *C. reinhardtii* (Molnar et al. 2007). Prior to that time, it had been thought that the miRNA system was present in multicellular organisms only and that the system had evolved together with multicellularity, independently and convergently in the multicellular plant and animal lineages (Allen et al. 2004). Molnar et al. isolated more than 2,000 nonredundant sRNAs and identified many thousand candidate genes of origin. They then established that at least some of the miRNAs they had characterized were capable of directing site-specific cutting of target mRNAs encoding known proteins, with the cleavage being consistent with the action of an Argonaute nuclease.

Zhao et al. (2013) isolated several thousand small RNAs of unique sequences in *Chlamydomonas*, of which about twenty were judged to be miRNAs or candidate-miRNAs and were studied further. They were found to be capable of directing cleavage of their target sequences, and while some were more abundant, others were less abundant in gametes than in vegetative cells. None of these RNAs had sequence homologs in *Ostreococcus* (another unicellular green alga), in *V. carteri*, or in land plants or animals.

Subsequently, this same research group performed parallel studies with *V. carteri* RNAs (Li et al. 2014) and characterized 174 miRNAs in 160 different families. They then used methods similar to those used with *C. reinhardtii* to identify many potential target mRNAs that encode proteins involved in a variety of metabolic pathways and found evidence of miRNA-directed mRNA cleavage in 60% of the 243 potential target mRNAs that were studied. Only one *Volvox* miRNA exhibited a significant degree of sequence similarity to a *Chlamydomonas* miRNA.

Studies of the relative abundance of various miRNAs in somatic cells and gonidia yielded interesting results: Of the 99 miRNAs studied, 50 were more abundant in somatic cells, and 49 were more abundant in gonidia. In most cases, these distribution asymmetries were relatively modest, but nearly a dozen miRNAs were found to be 10–20 times more abundant in one cell type than in the other. Further study of such asymmetrically distributed miRNAs is likely to be rewarding.

A more recent study of *V. carteri* took a slightly different approach by cloning and sequencing small RNAs that coimmunoprecipitated with the *V. carteri* Argonaute-3 protein, which led to the identification of 490 members of 324 miRNA families (Dueck e al. 2016). The genomic sources of these RNAs were highly varied, including a number from known transposons (*Jordan* and *Kangaroo*), others from protein-coding genes (both sense and antisense strands), intergenic regions, repetitive elements, and so on. As in the preceding study, some of these miRNAs were found to be more abundant in somatic cells, while others were more abundant in gonidia or in eggs. A global comparison of *V. carteri and C. reinhardtii* miRNA sequences with multiple sequence alignments revealed essentially no conservation of sequences between the two species. Rather similar observations were made with respect to the other classes of functional small RNAs. In summary, the authors state, "Taken together, our data identify an extended small RNA system in *V. carteri*, which appears to be as complex as in higher plants."

What role these small RNAs play in *V. carteri* development remains to be determined. But the fact that the small RNAs of *Volvox* are almost entirely different

from those of *Chlamydomonas* raises the intriguing possibility that diversification of this category of gene-expression regulators may have played a crucial role in the evolution of volvocine multicellularity. It is hoped that such a hypothesis will be tested soon.

I A Third Volvocine Genome Sequence Provides as Many New Questions as Answers

The genome of a third volvocine alga, *Gonium pectorale* (one of the smallest colonial volvocine algae, Figure 4.8) has recently been sequenced (Hanschen et al. 2016). It is quite similar to the *Chlamydomonas* and *Volvox* genomes in terms of size, number of coding loci, number of introns per gene, and so forth (although all such numbers fluctuate to some extent as additional analyses of the genomes are performed with more sensitive or more stringent methods: Goodstein et al. 2011, Umen and Olson 2012, Hanschen et al. 2016).

The difference in the number of pherophorin-coding genes in these three genomes (31 in *Chlamydomonas*, 35 in *Gonium*, versus 78 in *Volvox*) is consistent with the fact that *Gonium* colonies produce a bit more ECM than *Chlamydomonas* does, but a great deal less than *Volvox*. A rather surprising finding was that *Gonium* and *Volvox* (the two multicellular forms) share far fewer volvocine-specific genes with one another (9) than either of them shares with *Chlamydomonas* (32 and 44, respectively; see Figure 4.10). This reinforces the notion that the evolution of multicellularity in this group appears not to have required a substantial number of new genes.

According to the title of the *Gonium* genome paper (Hanschen et al. 2016), as well as several statements throughout the text, what the evolution of multicellularity did require was "co-option of the RB cell cycle regulatory pathway." The RB cell-cycle pathway was analyzed in great detail in *C. reinhardtii* and shown to be in control of the unusual cell division pattern known as multiple fission, or palintomy (Olson et al. 2010). As noted above, multiple fission is also a hallmark of all the

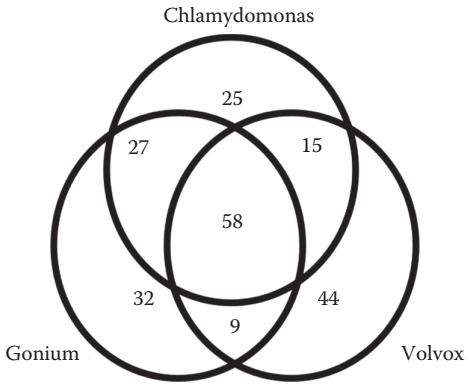

FIGURE 4.10 A Venn diagram indicating the numbers of volvocine-specific (i.e., "new") genes that are found in one, two or three of the sequenced volvocine genomes.

colonial volvocaceans, and all components of the RB pathway have been found to be present and functional in both *Gonium* and *Volvox* (Hanschen et al. 2016). So it appears that the volvocaceans have used the RB pathway that they inherited from their *C. reinhardtii*-like ancestor and continue to use it pretty much "as is," for its established function in controlling multiple fission. Whether that is an example of co-option, or simply of inheritance, is a moot point.

In any case, it is rather curious that in a paper that claims cell-cycle regulation plays such a centrally important evolutionary role, no mention is made of any attempt to evaluate cell-cycle parameters. Are the cell-cycle parameters of *Gonium* significantly different from those of *Chlamydomonas*? And when *Chlamydomonas* is transformed with the *Gonium* Rb gene (as will be discussed in the next section) do any of its cell-cycle parameters change? Such seemingly important questions are not addressed.

J Attempts to Experimentally Induce Volvocine Multicellularity

Three attempts to experimentally induce volvocine multicellularity have been reported in recent years, and are worthy of discussion here. However, we first need to discuss a phenomenon that is all too familiar to most *Chlamydomonas* investigators: the so-called palmelloid state. When an actively swimming population of *Chlamydomonas* cells in liquid culture is stressed in any one of a number of ways, the cells tend to resorb their flagella and secrete mucilage, which binds the cells together into amorphous clumps containing anywhere from few to several hundred immotile cells per clump (Schlösser 1976, Kirk 1998). This is called the palmelloid state because it resembles the normal growth form of a rather distantly related green alga, named *Palmella*. Some species of *Chlamydomonas* other than *C. reinhardtii*, alternate between the active-swimming phase and the palmelloid phase in every cell cycle (Schlösser 1976). But a few would assert that switching from the actively mobile to the palmelloid state is equivalent to having evolved multicellularity.

The assertion by Hanschen et al. (2016) that co-option of the RB pathway played a critical role in the evolution of volvocine multicellularity rests heavily on their observation that cell clusters (called "colonies") can be found in cultures of *C. reinhardtii* that had been transformed with the *Gonium* RB-encoding gene. The clusters varied in size from 2 to 16 cells, the four examples that were illustrated with very small, low-resolution photographs look different from one another and do not resemble any known colonial volvocacean. The authors in one sentence use the term "non-palmelloid colonies" for these cell clumps, but never indicate what criteria, if any were used to justify the use of the term "non-palmelloid." They do not indicate whether the clusters of transformed cells exhibited either of the two important differences that regularly distinguish dividing *Gonium* cells from dividing *Chlamydomonas* cells, namely: cytoplasmic bridges between sister cells that are the result of incomplete cytokinesis, and (somewhat later) attachments between neighboring cell walls (Stein 1958, Iida et al. 2013). Clumps of cells, which, as noted above, often develop in stressed *Chlamydomonas* cultures, do not necessarily constitute colonies.

Earlier, Ratcliff et al. (2013) had reported that multicellular variants of *C. reinhardtii* were generated by selecting for rapidly settling individuals in each transfer generation. To be more specific, after each three-day period of cultivation of *C. reinhardtii* in static

liquid medium, the investigators selected for transfer to fresh medium cells located at the bottom of a tube that had been centrifuged briefly. Twenty parallel cultures were subjected to this serial-transfer protocol for 219 days. By the end of that period, one of the twenty cultures had established a population of cell clusters that were so large that they would settle rapidly to the bottom of the tube under earth's gravitational field. These amorphous clusters contained hundreds of immotile cells trapped in a transparent matrix. They bear no resemblance to any of the recognized genera of colonial volvocaceans. But what they do resemble is the palmelloid phase described just above. Time will tell how significant the results of this centrifugal-selection protocol are.

Meanwhile, at a recent international *Volvox* conference, Herron (one of the coauthors of the preceeding study) reported successful production of multicell versions of *C. reinhardtii* with a different selection scheme, namely: cocultivation with the predatory ciliate, *Paramecium* (Herron 2016). Expanding on the published note, he told me in a personal note that:

> "…the evolved isolates from the predation experiment look quite different from the ones from the centrifugation experiment. Instead of large, amorphous clusters of up to a couple of hundred cells (as in the centrifugation experiment), we see smaller, more structured clusters of 4, 8, 16, or 32 cells. Probably they result from a simple failure of daughter cells to escape from the mother-cell wall, sometimes for two generations (i.e., we sometimes see 'superclusters' made up of four 4-celled clusters). Most look a lot like Pandorina or the like, but they can't swim and almost certainly don't invert." (M. D. Herron, pers. commun., quoted verbatim, with permission.)

Photographs that Herron provided with that note appear to confirm his interpretation that these individuals "result from a simple failure of daughter cells to escape from the mother-cell wall." Failure to escape from the mother cell wall (as *C. reinhardtii* daughter cells normally do right after they have completed their last division), presumably makes the clusters too large to be consumed by *Paramecium*. But it hardly qualifies them as newly evolved multicellular organisms.

One of the major selective advantages of the transition from unicellularity-to-multicellularity that occurred in the volvocine lineage some 200 million years ago may very well have resulted from an increase in organismic size, which greatly reduced predation pressures. Nevertheless, it will very likely take a bit more than cocultivation with a predator such as a *Paramecium* to duplicate that historic transition in a modern laboratory.

4.5 DISCUSSION

We have considered how multicellularity may have evolved in three major groups of life, the Amoebozoa (CSMs), Opisthokonta (unicellular holozoans and metazoans) and Archaeplastida (volvocine green algae), where it must be stressed that the studied examples come from very few species. A number of tentative inferences can be drawn from features that are common to the three cases. (1) Most important, perhaps, is this: naïve ideas of what is simple (="primitive") and what is complex (="evolved"), primarily based on morphology, bear no relation to what are categorized on the basis of DNA-based phylogeny as ancestral and derived states. To repeat, *grades* of organizational complexity need not necessarily reflect *clades* of closest relatives. The inference

hinges entirely on the assumption that deduced phylogenetic relationships reflect the true phylogeny, which is supposed to be based on descent with (possibly) modification. It would be invalid if lateral gene exchange is common among the taxa in question, something which does not appear to be the case as far as we know. (2) A related inference is of phenotypic plasticity. Cells with the same genome or similar genomes can become multicellular in more than one way, or go through multicellular phases differently, or display a variety of multicellular forms. In the CSMs, the same species occasionally mimics what was believed to be a different genus. The evidence from the unicellular holozoans is not as direct (though further studies may change the picture): choanoflagellates form clonal colonies, filastereans aggregate and teretosporeans form a coenocyte (therefore, strictly speaking, are unicellular but multinucleate).

In contrast, although cells of the prototypical unicellular volvocine alga, *Chlamydomonas*, can be caused to form loose aggregates under various experimental conditions, there is no evidence that such aggregates have ever played any role in the origins of true volvocine multicellularity. In every case that has been studied, multicellular volvocine algae arise by a failure of mitotic sister cells to separate fully at the end of the cell-division cycle, rather than by aggregation of free-living cells. Also, volvocine algae provide a dramatic example of temporal differentiation giving way to spatial differentiation beyond a critical size (=number of cells). The CSMs too show size-dependent morphologies and developmental patterns, though not as strikingly.

Given that single-celled ancestors seem to have possessed many of the protein-coding genes that were believed to be specific to metazoans, the evolutionary transitions to multicellularity may have been potentiated by minor changes in patterns of gene regulation. All that may have been required for a unicellular form to 'go multicellular' may have been an environmental trigger (e.g., an increase in atmospheric oxygen content) that permitted size increase that, among other things, was a defense against predation (Bonner 1998, 2001; Knoll 2011). Alternatively, environmental changes may have fostered multicellular forms arising on the basis of preexisting cellular interaction systems; genetic changes may have arisen secondarily by way of ensuring developmental reliability. In a subset of those cases in which embryonic development arose as well (i.e., embryophytes and metazoans), there could be a combination of all those causes, as well as the evolution of new major genomic regulatory capabilities, such as distal regulation (Sebé-Pedrós et al. 2016a). Clearly, additional data from more taxa, a better appreciation of the range of developmental forms consistent with a single genotype and an increased knowledge of the molecular basis of phenotypic plasticity will advance our understanding of how different unicellular organisms became multicellular.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance of many who read and commented on previous drafts of parts or all of this chapter, Pauline Schaap and Sunil Laxman. A special word of thanks to Matthew Brown for permitting the use of illustrations from his thesis, shown here as Figure 4.2. IRT acknowledges financial support from an European Research Council Consolidator grant (ERC-2012-Co -616960), and a grant (BFU2014-57779-P) from Ministerio de Economía y Competitividad (MINECO), the latter with FEDER funds.

REFERENCES

Alegado, R. A., Brown, L. W., Cao, S., Dermenjian, R. K., Zuzow, R., Fairclough, S. R., Clardy, J., and King, N. 2012. A bacterial sulfonolipid triggers multicellular development in the closest living relatives of animals. *Elife* 1: e00013. doi:10.7554/eLife.00013.

Allen, E., Xie, Z., Gustafson, A. M., and Carrington, J. C. 2004. microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121: 207–221.

Arias Del Angel, J. A., Escalante, A. E., Martínez-Castilla, L. P., and Beniítez, M. 2017. An evo-devo perspective on multicellular development of myxobacteria. *J Exp Zool (Mol Dev Evol)* 328B: 165–178.

Atzmony, D., Zahavi, A., and Nanjundiah, V. 1997. Altruistic behaviour in *Dictyostelium discoideum* explained on the basis of individual selection. *Curr Sci* 72(2): 142–145.

Avesson, L., Schumacher, H. T., Fechter, P., Romby, P., Hellman, U., and Söderbom, F. 2011. Abundant class of non-coding RNA regulates development in the social amoeba *Dictyostelium discoideum*. *RNA Biol.* 8: 1094–1104. doi:10.4161/rna.8.6.17214.

Bartel, D. P. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281–289.

Bell, G. 1985. The origin and early evolution of germ cells, as illustrated by the Volvocales. In *The Origin and Evolution of Sex* (Eds.) H. O. Halverson and A. Monroy, pp. 221–256. New York: Alan R. Liss.

Benabentos, R., Shigenori, H., Sucgang, R., Curk, T., Katoh, M., Ostrowski, E. A., Strassmann, J. E. et al. 2009. Polymorphic members of the lag gene family mediate kin discrimination in *Dictyostelium*. *Curr Biol* 19: 567–572.

Bonner, J. T. 1967. *The Cellular Slime Molds*, 2nd ed. Princeton, NJ: Princeton University Press.

Bonner, J. T. 1982. Evolutionary strategies and developmental constraints in the cellular slime molds. *Am Nat* 119: 530–552.

Bonner, J. T. 1998. The origins of multicellularity. *Integr Biol* 1: 27–36.

Bonner, J. T. 2001. *First Signals: The Evolution of Multicellular Development*. Princeton, NJ: Princeton University Press.

Bonner, J. T. 2003. Evolution of development in the cellular slime molds. *Evol Dev* 5: 305–313.

Bonner, J. T. 2013a. The evolution of the cellular slime molds. In *Dictyostelids: Evolution, Genomics, and Cell Biology* (Eds.) M. Romeralo, S. Baldauf, and R. Escalante, pp. 183–191. Berlin, Germany: Springer.

Bonner, J. T. 2013b. *Randomness in Evolution*. Princeton, NJ: Princeton University Press.

Bonner, J. T. 2015. The evolution of evolution: Seen through the eyes of a slime mold. *BioScience* 65: 1184–1187.

Bonner, J. T. and Adams, M. S. 1958. Cell mixtures of different species and strains of cellular slime moulds. *J Embryol Exp Morphol* 6: 346–356.

Bonner, J. T. and Dodd, M. R. 1962. Aggregation territories in the cellular slime molds. *Biol Bull* 122: 13–24.

Boraas, M. E., Seale, D. B., and Boxhorn, J. E. 1998. Phagotrophy by a flagellate selects for colonial prey: A possible origin of multicellularity. *Evol Ecol* 12: 153–164. doi:10.1023/A:1006527528063.

Brown, M. W. 2010. Placing the forgotten slime molds (*Sappinia*, *Copromyxa*, *Fonticula*, *Acrasis*, and *Pocheina*), using molecular phylogenetics. 258p. PhD thesis dissertation, University of Arkansas, Fayetteville, AR.

Brown, M. W. and Silberman, J. D. 2013. The non-dictyostelid sorocarpic amoebae. In *Dictyostelids. Evolution, Genomics and Cell Biology* (Eds.) M. Romeralo, S. Baldauf, and R. Escalante, pp. 219–242. Heidelberg, Germany: Springer.

Brown, M. W., Silberman, J. D., and Spiegel, F. W. 2010. A morphologically simple species of Acrasis (Heterolobosea, Excavata), Acrasis helenhemmesae n. sp. *J Eukaryot Microbiol* 57: 346–353.

Brown, M. W., Spiegel, F. W., and Silberman, J. D. 2009. Phylogeny of the "forgotten" cellular slime mold, Fonticula alba, reveals a key evolutionary branch within Opisthokonta. *Mol Biol Evol* 26: 2699–2709.

Buchheim, M. A. and Chapman, R. L. 1991. Phylogeny of the colonial green flagellates: A study of 18S and 26S rRNA sequence data. *BioSystems* 25: 85–100.

Burki, F. 2014. The eukaryotic tree of life from a global phylogenomic perspective. *Cold Spring Harb Perspect Biol* 6: a016147.

Carr, M., Leadbeater, B. S. C., Hassan, R., Nelson, M., and Baldauf, S. L. 2008. Molecular phylogeny of choanoflagellates, the sister group to metazoa. *Proc Natl Acad Sci USA* 105: 16641–16646. doi:10.1073/pnas.0801667105.

Carrington, J. C. and Ambros, V. 2003. Role of microRNAs in plant and animal development. *Science* 301: 336–338.

Carroll, S. B. 2008. Evo-Devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. *Cell* 134: 25–36.

Cheng, Q., Fowler, R., Tam, L.-W., Edwards, L., and Miller, S. M. 2003. The role of GlsA in the evolution of asymmetric division in the green alga *Volvox carteri*. *Devel Genes & Evol* 213: 328–335.

Coates, J. C. and Harwood, A. H. 2001. Cell-cell adhesion and signal transduction during *Dictyostelium* development. *J Cell Sci* 114: 4349–4358.

Coates, J. C., Grimson, M. J., Williams, R. S., Bergman, W., Blanton, R. L., and Harwood, A. J. 2002. Loss of the beta-catenin homologue aardvark causes ectopic stalk formation in *Dictyostelium Mech Dev* 116: 117–127.

Coleman, A. W. 1980. The biological species concept: Its applicability to the taxonomy of freshwater algae. In *Proceedings of the 2nd International Symposium on Taxonomy of Algae* (Ed.) T. V. Desikachary, pp. 22–36. Chennai, India: University of Madras.

Coleman, A. W. 1999. Phylogenetic analysis of "Volvocaceae" for comparative genetic studies. *Proc Natl Acad Sci USA* 96: 13892–13897.

Conte, D., MacWilliams, H. K., and Ceccarelli, A. 2010. BTG Interacts with retinoblastoma to control cell fate in *Dictyostelium*. *PLoS One* 5(3): e9676. doi:10.1371/journal.pone.0009676.

Cornillon, C., Gebbie. L., Benghezal, M., Nair, P., Keller, S., Wehrle-Haller, B., Charette, S. J., Brückert, F., Letourneur, F., and Cosson, P. 2006. An adhesion molecule in free-living *Dictyostelium* amoebae with integrin β features. *EMBO Reps* 7: 617–621. doi:10.1038/sj.embor.7400701.

Crow, W. B. 1918. The classification of some colonial Chlamydomonads. *New Phytol* 17: 151–159.

Danchin, E. G. J. 2016. Lateral gene transfer in eukaryotes: Tip of the iceberg or of the ice cube? *BMC Biol* 14: 101. doi:10.1186/s12915-016-0315-9.

Dayel, M. J., Alegado, R. A., Fairclough, S. R., Levin, T. C., Nichols, S. A., McDonald, K., and King, N. 2011. Cell differentiation and morphogenesis in the colony forming *Choanoflagellate Salpingoeca* Rosetta. *Dev Biol* 35: 73–82. doi:10.1016/j.ydbio.2011.06.003.

de Mendoza, A., Suga, H., Permanyer, J., Irimia, M., and Ruiz-Trillo, I. 2015. Complex transcriptional regulation and independent evolution of fungal-like traits in a relative of animals. *Elife* e08904. doi:10.7554/eLife.08904.

De Monte, S. and Rainey, P. B. 2014. Nascent multicellular life and the emergence of individuality. *J Biosci* 39: 237–248.

Dickinson, D. J., Nelson, W. J., and Weis, W. I. 2012. An epithelial tissue in *Dictyostelium* and the origin of metazoan multicellularity. *Bioessays* 34: 833–840. doi:10.1002/bies.201100187.

Dubravcic, D., van Baalen, M., and Nizak, C. 2014. An evolutionarily significant unicellular strategy in response to starvation stress in Dictyostelium social amoebae [version 1; referees: 2 approved]. *F1000Research* 3: 133. doi:10.12688/f1000research.4218.1.

Dueck, A., Evers, M., Henz, S. R., Unger, K., Eichner, N., Merkl, R., Berezikov, E. et al. 2016. Gene silencing pathways found in the green alga *Volvox carteri* reveal insights into evolution and origins of small RNA systems in plants. *BMC Genomics* 17: 853–867.

Fairclough, S. R., Chen, Z., Kramer, E., Zeng, Q., Young, S., Robertson, H. M., Begovic, E. et al. 2013. Premetazoan genome evolution and the regulation of cell differentiation in the choanoflagellate *Salpingoeca rosetta*. *Genome Biol* 14: R15. doi:10.1186/gb-2013-14-2-r15.

Filosa, M. F. 1962. Hetercytosis in cellular slime molds. *Am Nat* 96: 79–91.

Gilbert, O. M., Foster, K. R., Mehdiabadi, N. J., Strassmann, J. E., and Queller, D. C. 2007. High relatedness maintains multicellular cooperation in a social amoeba by controlling cheater mutants. *Proc Natl Acad Sci USA* 104: 8913–8917.

Gilbert, O. M., Queller, D. C., and Strassmann, J. E. 2009. Discovery of a large clonal patch of a social amoeba: Implications for social evolution. *Mol Ecol* 18: 1273–1281.

Glockling, S. L., Marshall, W. L., and Gleason, F. H. 2013. Phylogenetic interpretations and ecological potentials of the Mesomycetozoea (Ichthyosporea). *Fungal Ecol* 6: 237–247. doi:10.1016/j.funeco.2013.03.005.

Glöckner, G., Lawal, H. M., Felder, M., Singh, R., Singer, G., Weijer, C. J., and Schaap, P. 2016. The multicellularity genes of dictyostelid social amoebas. *Nature Communications* 7: 12085, 1–11. doi:10.1038/ncomms12085.

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., Mitros T. et al. 2011. Phytozome: A comparative platform for green plant genomics. *Nucl Acid Res* 40: D1178–D1186.

Grosberg, R. K. and Strathman, R. R. 2007. The evolution of multicellularity: A minor major transition? *Annu Rev Ecol Evol Syst* 38: 621–654.

Hallmann, A. 2006. The pherophoriins: Common building blocks in the evolution of extracellular matrix architecture in volvocales. *Plant J* 45: 292–307.

Hallmann, A. and Kirk, D. L. 2000. The developmentally regulated ECM glycoprotein ISG plays an essential role in organizing the ECM and orienting the cells of Volvox. *J Cell Sci* 113: 4605–4617.

Hammerschmidt, K., Rose, C. J., Kerr, B., and Rainey, P. B. 2014. Life cycles, fitness decoupling and the evolution of multicellularity. *Nature* 515: 75–79. doi:10.1038/nature13884.

Hanschen, E. R., Marriage, T. N., Ferris, P. J., Hamaji, T., Toyoda, A., Fujiyama, A., Neme, R. et al. 2016. The *Gonium pectorale* genome demonstrates co-option of cell cycle regulation during the evolution of multicellularity. *Nature Commun* 7: 11370. doi:10:1038/ncomms11370.

Herron, M. D. 2016. Origins of multicellular complexity: *Volvox* and the volvocine algae. *Mol Ecol* 25: 1213–1223.

Herron, M. D. and Michod, R. E. 2008. Evolution of complexity in the volvocine algae: Transitions in individuality through Darwin's eye. *Evolution* 62: 436–451.

Herron, M. D., Desnitsky, A. G., and Michod, R. E. 2010. Evolution of developmental programs in *Volvox* (Chlorophyta). *J Phycol* 46: 316–324.

Herron, M. D., Hackett, J. D., Aylward, F. O., and Michod, R. E. 2009. Triassic origin and early radiation of multicellular volvocine algae. *Proc Natl Acad Sci USA* 106: 3254–3258.

Iida, H., Ota, S., and Inouye, I. 2013. Cleavage, incomplete inversion, and cytoplasmic bridges in *Gonium pectorale* (Volvocales, Chlorophyta). *J Plant Res* 126: 699–707.

Isaka, N., Kawai-Toyooka, H., Matuzaki, R., Nakada T., and Nozaki, H. 2012. Description of two new monoecious species of *Volvox* sect. *Volvox* (Volvocaceae, Chlorophyceae), based on comparative morphology and molecular phylogeny of cultured material. *J Phycol* 48: 759–767.

Jack, C. N., Ridgeway, J. G., Mehdiabadi, N. J., Jones, E. I., Edwards, T. A., Queller, D. C., and Strassmann, J. E. 2008. Segregate or cooperate—A study of the interaction between two species of *Dictyostelium*. *BMC Evol Biol* 8: 293. doi:10.1186/1471-2148-8-293.

Jahn, T. L. and Bovee, E. C. 1967. Motile behaviour of protozoa. In *Research in Protozoology* (Ed.) T.-T Chen. Oxford, UK: Pergamon Press.

Kawata, T. 2011. STAT signaling in *Dictyostelium* development. *Develop Growth Differ* 53: 548–557. doi:10.1111/j.1440-169X.2010.01243.

Kaneko, K. 2016. A scenario for the origin of multicellular organisms: Perspective from multilevel consistency dynamic. In *Multicellularity: Origins and Evolution* (Eds.) K. J. Niklas and S. A. Newman, pp. 201–223. Cambridge, MA: MIT Press.

Kapsetaki, S. E. 2015. Predation and the evolution of multicellularity. MSc thesis, University of Oxford. http://zoo-web02.zoo.ox.ac.uk/group/west/pdf/SteffiMastersThesis.pdf. http://zoo web02.zoo.ox.ac.uk/group/west/pdf/SteffiMastersThesis.pdf. Accessed March 3, 2018.

Kaushik, S. and Nanjundiah, V. 2003. Evolutionary questions raised by cellular slime mould development. *Proc Ind Natl Sci Acad* B69: 825–852.

Kaushik, S., Katoch, B., and Nanjundiah, V. 2006. Social behaviour in genetically heterogeneous groups of *Dictyostelium giganteum*. *Behav Ecol Sociobiol* 59: 521–530.

Kessin, R. H. 2001. *Dictyostelium—Evolution, Cell Biology, and the Development of Multicellularity.* Cambridge, UK: Cambridge University Press.

King, M. and Wilson, A. 1975. Evolution at to levels in humans and chimpanzees. *Science* 188: 107116.

King, N., Westbrook, M. J., Young, S. L., Kuo, A., Abedin, M., Chapman, J., Fairclough, S. et al. 2008. The genome of the Choanoflagellate *Monosiga brevicollis* and the origin of metazoans. *Nature* 451: 783–788. doi:10.1038/nature06617.

King, N. 2004. The unicellular ancestry of animal development. *Dev Cell* 7: 313–325.

King, N. 2005. Choanoflagellates. *Curr Biol* 15: R113–114.

Kirk, D. L. 1998. *Volvox: Molecular Genetic Origins of Multicellularity and Cellular Differentiation*. Cambridge, UK: Cambridge University Press.

Kirk, D. L. 2005. A twelve-step program for evolving multicellularity and a division of labor. *BioEssays* 27: 299–310.

Kirk, D. L., Kirk M. M., Stamer, K., and Larson, A. 1990. The genetic basis for the evolution of multicellularity and cellular differentiation in the volvocine green algae. In *The Unity of Evolutionary Biology* (Ed.) E. C. Dudley, pp. 568–581. Portland, OR: Discorides Press.

Kirk, M. M. and Kirk, D. L. 1985. Translational regulation of protein synthesis, in response to light, at a critical stage of development. *Cell* 41: 419–428.

Knoll, A. H. 2011. The multiple origins of complex multicellularity. *Ann Rev Earth Planet Sci* 39: 217–239.

Koufopanou, V. 1994. The evolution of soma in the volvocales. *Amer Nat* 143: 907–931.

Lang, B. F., O'Kelly, C., Nerad, T., Gray, M. W., and Burger, G. 2002. The closest unicellular relatives of animals. *Curr Biol* 12: 1773–1778.

Larson, A., Kirk, M. M., and Kirk, D. L. 1992. Molecular phylogeny of the volvocine flagellates. *Mol Biol Evol* 9: 85–105.

Leadbeater, B. S. C. 2015. *The Choanoflagellates.* Cambridge, UK: Cambridge University Press. doi:10.1017/CBO9781139051125.

Li, J. R., Wu, Y., and Qi, Y. 2014. MicroRNAs in a multicellular green alga *Volvox carteri*. *Chin Sci Life Sci* 57: 36–45.

Loomis, W. F. 2014. Cell signaling during development of *Dictyostelium*. *Dev Biol* 391: 1–16. doi:10.1016/j.ydbio.2014.04.001.

Loomis, W. F. 2015. Genetic control of morphogenesis in *Dictyostelium*. *Dev Biol* 402: 146–161. doi:10.1016/j.ydbio.2015.03.016.

Mah, J. L., Christensen-Dalsgaard, K. K., and Leys, S. P. 2014. Choanoflagellate and Choanocyte collar-flagellar systems and the assumption of homology. *Evol Dev* 16: 25–37. doi:10.1111/ede.12060.

Marée, A. F. M. and Hogeweg, P. 2001. How amoeboids self-organize into a fruiting body: Multicellular coordination in *Dictyostelium discoideum*. *Proc Natl Acad Sci USA* 98: 3879–3883. doi:10.1073/pnas.061535198.

Marshall, W. L., Celio, G., McLaughlin, D. J., and Berbee, M. L. 2008. Multiple isolations of a culturable, motile Ichthyosporean (Mesomycetozoa, Opisthokonta), *Creolimax fragrantissima* N. Gen., N. Sp., from marine invertebrate digestive tracts. *Protist* 159: 415–433.

Matt, G. and Umen, J. 2016. *Volvox*: A simple algal model for embryogenesis, morphogenesis and cellular differentiation. *Dev Biol* 419: 99–113.

McMains, V. C., Myre, M., Kreppel, L., and Kimmel, A. R. 2010. Dictyostelium possesses highly diverged presenilin/γ-secretase that regulates growth and cell-fate specification and can accurately process human APP: A system for functional studies of the presenilin/γ-secretase complex. *Dis Models Mech* 3: 581–594. doi:10.1242/dmm.004457.

Medina, M., Collins, A. G., Taylor, J. W., Valentine, J. W., Lipps, J. H., Amaral-Zettler, L., and Sogin, M. L. 2003. Phylogeny of opisthokonta and the evolution of multicellularity and complexity in fungi and metazoa. *Int J Astrobiol* 2: 203–211.

Melkonian, M. 1990. Phylum chlorophyta. In *Handbook of Protoctista* (Eds.) L. Margulis, J. O. Corliss, M. Melkonian, and D. J. Chapman, pp. 597–660. Boston, MA: Jones & Bartlett.

Mendoza, L., Taylor, J. W., and Ajello, L. 2002. The class mesomycetozoea: A Heterogeneous group of microorganisms at the animal-fungal boundary. *Annu Rev Microbiol* 56: 315–344.

Merchant, S. S., Prochnik, S. E., Vallon, O., Harris, E. H., Karpowicz, S. J., Witman, G. B., Terry, A. et al. 2007. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* 318: 245–250.

Miller, S. M. and Kirk, D. L. 1999. *glsA*, a *Volvox* gene required for asymmetric division and germ cell specification, encodes a chaperone-like protein. *Development* 126: 649–658.

Mishra, H. and Saran, S. 2015. Classification and expression analyses of homeobox genes from *Dictyostelium discoideum*. *J Biosci* 40: 241–255. doi:10.1007/s12038-015-9519-3.

Molnar, A., Schwach, F., Studholme, D. J., Thunemann, E. C., and Baulcombe, D. C. 2007. miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. *Nature* 447: 1126–1129.

Nakada, T., Misawa, K., and Nozaki H. 2008. Molecular systematics of Volvocales (Chlorophyceae, Chlorophyta) based on exhaustive 18S rRNA phylogenetic analysis. *Mol Phylogenet Evol* 48: 281–291.

Nanjundiah, V. 1985. The evolution of communication and social behaviour in Dictyostelium discoideum. *Proc Indian Acad Sci* 94: 639–653.

Nanjundiah, V. 2016. Cellular slime mold development as a paradigm for the transition from unicellular to multicellular life. In *Multicellularity: Origins and Evolution* (Eds.) K. J. Niklas and S. A. Newman, pp. 105–130. Cambridge, MA: MIT Press.

Nanjundiah, V. and Sathe, S. 2011. Social selection and the evolution of cooperative groups: The example of the cellular slime molds. *Integr Biol* 3: 329–342.

Newman, S. A. 2002. Putting genes in their place. *J Biosciences* 27: 97–104

Newman, S. A. 2011. Animal egg as evolutionary innovation: A solution to the "embryonic hourglass" puzzle. *J Exp Zool B Mol Dev Evol* 316: 467–483. doi:10.1002/jez.b.21417.

Newman, S. A. and Bhat, R. 2009. Dynamical patterning modules: A "pattern language" for development and evolution of multicellular form. *Int J Dev Biol* 53: 693–705.

Newman, S. A. and Comper, W. D. 1990. "Generic" physical mechanisms of morphogenesis and pattern formation. *Development* 110: 1–18.

Nishii, I. and Miller, S. M. 2010. *Volvox*: Simple steps to developmental complexity? *Curr Opin Plant Biol* 13: 646–653.

Nishii, I., Ogihara, S., and Kirk, D. L. 2003. A kinesin, invA, plays an essential role in *Volvox* morphogenesis. *Cell* 113: 743–753.

Nozaki, H. 2003. Origin and evolution of the genera *Pleodorina* and *Volvox* (Volvocales). *Biologia* (*Bratislava*) 58: 425–431.

Nozaki H. and Coleman, A. W. 2011. A new species of *Volvox* sect Merrillosphaera (Volvocales, Chlorophyceae) from Texas. *J Phycol* 47: 673–679.

Nozaki, H., Matsuzaki, R., Yamamoto, K., Kawachi, M., and Takahashi, F. 2015a. Delineating a new heterothallic species of Volvox (Volvocaceae, Chlorophyceae) using new strains of "*Volvox africanus*". *PLoS One* 10(11): e0142632. doi:10.1371/journal.pone.0142632.

Nozaki, H., Misawa, K., Kajita, T., Kato, M., Nohara, S., and Watanabe, M. M. 2000. Origin and evolution of the colonial Volvocales (Chlorophyceae) as inferred from multiple chloroplast gene sequences. *Mol Phylogenet Evol* 17: 256–268.

Nozaki, H., Ueki, N., Isaka, N., Saigo, T., Yamamoto, K., Matsuzaki, R., Takahashi, F., Wakabayashi, K. I. and Kawachi, M. 2015b. A new morphological type of *Volvox* from Japanese large lakes and recent divergence of this type and *V. ferrisii* in two different freshwater habitats. *PLoS One* 11(11): e0167148. doi:10.1371/journal.pone.0167148.

Nozaki, H., Ueki, N., Isaka, N., Saigo, T., Yamamoto, K., Matsuzaki, R. et al. 2016. A new morphological type of Volvox from Japanese large lakes and recent divergence of this type and V. ferrisii in two different freshwater habitats. *PLoS One* 11: e0167148. doi:10.1371/journal.pone.0167148.

Nozaki, H., Yamada, T. K., Takahashi, F., Matsuzaki, R., and Nakada, T. 2014. New "missing link" genus of the colonial volvocine green algae gives insights into the evolution of oogamy. *BMC Evol Biol* 14: 37–46.

Olive, L. S. 1965. A developmental study of Guttulinopsis vulgaris (Acrasiales). *Am J Bot* 52: 513–519. doi:10.2307/2440268.

Olive, L. S. and Stoianovitch, C. 1960. Two new members of the Acrasiales. *Bull Torrey Bota Club* 87: 1–20.

Olive, L. S. 1975. *The Mycetozoans*. New York: Academic Press.

Olson, B. J. S. C. and Nedelcu, A. 2016. Co-option during the evolution of multicellular and developmental complexity in the volvocine green algae *Curr Opin Genet Develop* 39: 107–115.

Olson, B. J. S. C., Oberholzer, M., Li, Y., Zones, J. M., Kohli, H. S., Bisova, K., Fang, S. C., Meisenhelder, J., Hunter, T., and Umen, J. G. 2010. Regulation of the *Chlamydomonas* cell cycle by a stable, chromatin-associated retinoablastoma tumor suppressor complex. *Plant Cell* 22: 3331–3347.

Paps, J. and Ruiz-Trillo, I. 2010. Animals and their unicellular ancestors. In *Encyclopedia of Life Sciences* (*ELS*). doi:10.1002/9780470015902.A0022853.

Parfrey, I. W., Lehr, D. J., Knoll, A. H., and Katz, L. A. 2011. Estimating the timing of early eukaryotic diversification with multigene molecular clocks. *Proc Natl Acad Sci USA* 108: 13624–13649.

Peterson, K. J., Dietrich, M. R., and McPeek, M. A. 2009. MicroRNAs and metazoan macroevolution: Insights into canalization, complexity, and the Cambrian explosion. *BioEssays* 31: 736–747.

Pickup, Z. L., Pickup, R., and Parry, J. D. 2007. A comparison of the growth and starvation responses of *Acanthamoeba castellanii* and *Hartmannella vermiformis* in the presence of suspended and attached *Escherichia coli K12*. *FEMS Microbiol Ecol* 59: 556–563.

Prochnik, S. E., Umen, J., Nedelcu, A. M., Hallmann, A., Miller, S. M., Nishii, I., Ferris, P. et al. 2010. Genomic analysis of organismal complexity in the multicellular green alga *Volvox carteri*. *Science* 329: 223–226.

Raper, K. B. 1984. *The Dictyostelids*. Princeton, NJ: Princeton University Press.

Raper, K. B. and Quinlan, M. S. 1958. Acytostelium leptosomum: A unique cellular slime—Mould with an acellular stalk. *J Gen Microbiol* 18: 16–32.

Raper, K. B. and Thom, C. 1941. Interspecific mixtures in *the Dictyosteliaceae*. *Am J Bot* 28: 69–78.

Ratcliff, W., Herron, M. D., Howell, K., Pentz, J. T., Rosenzweig, F., and Travisano, M. 2013. Experimental evolution of an alternating uni- and multicellular life cycle in *Chlamydomonas Reinhardtii*. *Nat Commun* 4: 2742. doi:10.1038/ncomms3742.

Rausch, H., Larsen, N., and Schmitt, R. 1989. Phylogenetic relationships of the green alga Volvox carteri deduced from small-subunit ribosomal RNA comparisons. *J Mol Evol* 29: 255–265.

Ray, D. L. and Hayes, R. E. 1954. *Hartmannella astronyxis*: A new species of free living amoeba. *J Morphol* 95: 159–188.

Robertson, A. and Cohen, M. H. 1972. Control of developing fields. *Ann Rev Biophys Bioengg* 1: 409–464.

Rokas, A. 2008. The origins of multicellularity and the early history of the genetic toolkit for animal development. *Annu Rev Genet* 42: 235–251.

Romeralo, M. and Fiz-Palacios, O. 2013. Evolution of dictyostelid social amoebas inferred from the use of molecular tools. In *Dictyostelids. Evolution, Genomics and Cell Biology* (Eds.) M. Romeralo, S. Baldauf, and R. Escalante, pp. 167–182. Heidelberg, Germany: Springer.

Romeralo, M., Skiba, A., Gonzalez-Voyer, A., Schilde, C., Lawal, H., Kedziora, S., Cavender, J. C., Glöckner, G., Urushihara, H., and Schaap, P. 2013. Analysis of phenotypic evolution in Dictyostelia highlights developmental plasticity as a likely consequence of colonial multicellularity. *Proc R Soc Lond B* 280(1764): 20130976.

Ruiz-Trillo, I., Roger, A. J., Burger, G., Gray, M. W., and Lang, B. F. 2008. A phylogenomic investigation into the origin of metazoa. *Mol Biol Evol* 25: 664–672.

Ruiz-Trillo, I., Lane, C. E., Archibald, J. M., and Roger, A. J. 2006. Insights into the evolutionary origin and genome architecture of the unicellular opisthokonts *Capsaspora owczarzaki* and Sphaeroforma arctica. *J Eukaryot Microbiol* 53: 1–6.

Ruiz-Trillo, I., Burger, G., Holland, P. W., King, N., Lang, B. F., Roger, A. J., and Gray, M. W. 2007. The origins of multicellularity: A multi-taxon genome initiative. *Trends Genet* 23: 113–118.

Ruiz-Trillo, I., Inagaki, Y., Davis, L. A., Sperstad, S., Landfald, B., and Roger, A. J. 2004. *Capsaspora owczarzaki* is an independent opisthokont lineage. *Curr Biol* 14: R946–R947. doi:10.1016/j.cub.2004.10.037.

Santhanam, B., Cai, H., Devreotes, P. N., Shaulsky, G., and Katoh-Kurasawa, M. 2015. The GATA transcription factor GtaC regulates early developmental gene expression dynamics in *Dictyostelium Nat Comm* 6: 7551. doi:10.1038/ncomms8551.

Saran, S., Azhar, M., Manogaran, P. S., Pande, G., and Nanjundiah, V. 1994. The level of sequestered calcium in vegetative amoebae of *Dictyostelium discoideum* can predict post-aggregative cell fate. *Differentiation* 57: 163–169.

Sathe, S. and Durand, P. 2015. Cellular aggregation in *Chlamydomonas* (Chlorophyceae) is chimaeric and depends on traits like cell size and motility. *Eur J Phycol* 51: 129–138.

Sathe, S., Kaushik, S., Lalremruata, A., Aggarwal, R. K., Cavender, J. C., and Nanjundiah, V. 2010. Genetic heterogeneity in wild isolates of cellular slime mold social groups. *Microb Ecol* 60: 137–148. doi:10.1007/s00248-010-9635-4.

Sathe, S., Khetan, N., and Nanjundiah, V. 2014. Interspecies and intraspecies interactions in social amoebae. *J Evol Biol* 27: 349–362. doi:10.1111/jeb.12298.

Savill, N. J. and Hogeweg, P. 1997. Modeling morphogenesis: From single cells to crawling slugs. *J Theor Biol* 184: 229–235.

Saxer, G., Brock, D. A., Queller, D. C., and Strassmann, J. E. 2010. Cheating does not explain selective differences at high and low relatedness in a social amoeba. *BMC Evol Biol* 10: 76.

Schaap, P. 2011. Evolution of developmental cyclic AMP signalling in the Dictyostelia from an amoebozoan stress response. *Dev Growth Differ*. 53: 452–462. doi:10.1111/j.1440-169X.2011.01263.x.

Schaap, P., Winckler, T., Nelson, M., Alvarez-Curto, E., Elgie, B., Hagiwara, H., Cavender, J. et al. 2006. Molecular phylogeny and evolution of morphology in the social amoebas. *Science* 314(5799): 661–663. doi:10.1126/science.1130670.

Schilde, C., Lawal, H. M., Noegel, A. A., Eichinger, L., Schaap, P., and Glöckner, G. 2016. A set of genes conserved in sequence and expression traces back the establishment of multicellularity in social amoebae. *BMC Genomics* 17: 871. doi:10.1186/s12864-016-3223-z.

Schilde, C., Skiba, A., and Schaap, P. 2014. Evolutionary reconstruction of pattern formation in 98 *Dictyostelium* species reveals that cell-type specialization by lateral inhibition is a derived trait. *EvoDevo* 5: 34.

Schlösser, U. W. 1976. Entwickslungsstadien-und sippenspezifische Zellwand-Autolysine bei der Freisetzung von Fortplanzungszellen in der Gattung Chlamydomonas. *Ber Deutsch Bot Ges* 89: 1–56.

Sebé-Pedrós, A., Ariza-Cosano, A., Weirauch, M. T., Leininger, S., Yang, A., Torruella, G., Adamski, M. et al. 2013a. Early evolution of the T-Box transcription factor family. *Proc Natl Acad Sci USA* 110: 16050–16055. doi:10.1073/pnas.1309748110.

Sebé-Pedrós, A., Roger, A. J., Lang, F. B., King, N., and Ruiz-Trillo, I. 2010. Ancient origin of the Integrin-mediated adhesion and signaling machinery. *Proc Natl Acad Sci USA* 107(22): 10142–10147. doi:10.1073/pnas.1002257107.

Sebé-Pedrós, A., Irimia, M., del Campo, J., Parra-Acero, H., Russ, C., Nusbaum, C., Blencowe, B. J., and Ruiz-Trillo, I. 2013b. Regulated aggregative multicellularity in a close unicellular relative of metazoa. *Elife* 2: e01287. doi:10.7554/eLife.01287.

Sebé-Pedrós, A., de Mendoza, A., Franz Lang, B., Degnan, B. M., and Ruiz-Trillo, I. 2011. Unexpected repertoire of metazoan transcription factors in the unicellular Holozoan *Capsaspora Owczarzaki*. *Mol Biol Evol* 28: 1241–1254. doi:10.1093/molbev/msq309.

Sebé-Pedrós, A., Ballaré, C., Parra-Acero, H., Chiva, C., Tena, J. J., Sabidó, E., Gómez-Skarmeta, J. L., Di Croce, L., and Ruiz-Trillo, I. 2016a. The dynamic regulatory genome of *Capsaspora* and the origin of animal multicellularity. *Cell* 165: 1224–1237. doi:10.1016/j.cell.2016.03.034.

Sebé-Pedrós, A., Peña, M. I., Capella-Gutiérrez, S., Antó, M., Gabaldón, T., Ruiz-Trillo, I., and Sabidó, E. 2016b. High-throughput proteomics reveals the unicellular roots of animal phosphosignaling and cell differentiation. *Dev Cell* 39: 186–197. doi:10.1016/j.devcel.2016.09.019.

Sebé-Pedrós, A., Degnan, B. M., and Ruiz-Trillo, I. 2017. The origin of Metazoa: A unicellular perspective. *Nature Rev Genet*. doi:10.1038/nrg.2017.21.

Shadwick, L. L., Spiegel, F. W., Shadwick, J. D. L., Brown, M. W., and Silberman, J. D. 2009. Eumycetozoa = Amoebozoa?: SSUrDNA phylogeny of protosteloid slime molds and its significance for the supergroup amoebozoa. *PLoS One* 4(8): e6754.

Shalchian-Tabrizi, K., Minge, M. A., Espelund, M., Orr, R., Ruden, T., Jakobsen, K. S., and Cavalier-Smith, T. 2008. Multigene phylogeny of Choanozoa and the origin of animals. *PLoS One* 3: e2098.

Starr, R. C. 1980. Colonial chlorophytes. In *Phytoflagellates* (Ed.) E. R. Cox, pp. 147–163. Amsterdam, the Netherlands: Elsevier.

Steenkamp, E. T., Wright, J., and Baldauf, S. L. 2006. The protistan origins of animals and fungi. *Mol Biol Evol* 23: 93–106.

Stein, J. 1958. A morphological and genetic study of *Gonium pectorale*. *Am J Bot* 45: 388–397.

Sťovíček, V., Váchová, L., and Palková, Z. 2012. Yeast biofilm colony as an orchestrated multicellular organism. *Commun Integr Biol* 5: 203–205. doi:10.4161/cib.18912.

Suga, H. and Ruiz-Trillo, I. 2013. Development of Ichthyosporeans sheds light on the origin of metazoan multicellularity. *Dev Biol* 377: 284–292. doi:10.1016/j.ydbio.2013.01.009.

Suga, H., Chen, Z., de Mendoza, A., Sebe-Pedros, A., Brown, M. W., Kramer, E., Carr, M. et al. 2013. The Capsaspora genome reveals a complex unicellular prehistory of animals. *Nat Commun* 4: 2325. doi:10.1038/ncomms3325.

Sugden, C., Ross, S., Annesley, S. J., Cole, C., Bloomfield, G., Ivens, A., Skelton, J., Fisher, P. R., Barton, G., and Williams, J. G. 2011. A Dictyostelium SH2 adaptor protein required for correct DIF-1 signaling and pattern formation. *Dev Biol* 353(2): 290–301. doi:10.1016/j.ydbio.2011.03.003.

Sun, T. and Kim, L. 2011. Tyrosine phosphorylation-mediated signaling pathways in *Dictyostelium*. *J Signal Transduct*. Article ID 894351. doi:10.1155/2011/894351.

Tam, L.-W. and Kirk, D. L. 1991. The program for cellular differentiation in *Volvox carteri* as revealed by molecular analysis of development in a gonidialess/somatic regenerator mutant. *Development* 112: 571–580.

Tang, L., Franca-Koh, J., Xiong, Y., Chen, M.-Y., Long, Y., Bickford, R. M., Knecht, D. A., Iglesias, P. A., and Devreotes, P. N. 2008. Tsunami, the *Dictyostelium* homolog of the fused kinase, is required for polarization and chemotaxis. *Genes Dev* 22: 2278–2290. doi:10.1101/gad.1694508.2278.

Tarnita, C. E., Washburne, A., Martinez-Garcia, R., Sgro, A. E., and Levin, S. A. 2015. Fitness tradeoffs between spores and nonaggregating cells can explain the coexistence of diverse genotypes in cellular slime molds. *Proc Natl Acad Sci USA* 112: 2776–2781.

Thomas, C. M. and Nielsen, K. M. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol* 3: 711–721.

Tice, A. K., Shadwick, L. L., Fiore-Donno, A. M., Geisen, S., Kang, S., Schuler, G. A., and Spiegel, F. W. 2016. Expansion of the molecular and morphological diversity of Acanthamoebidae (Centramoebida, Amoebozoa) and identification of a novel life cycle type within the group. *Biol Direct* 11: 69. doi:10.1186/s13062-016-0171-0.

Torruella, G., Derelle, R., Paps, J., Lang, B. F., Roger, A. J., Shalchian-Tabrizi, K., and Ruiz-Trillo, I. 2012. Phylogenetic relationships within the Opisthokonta based on phylogenomic analyses of conserved single-copy protein domains. *Mol Biol Evol* 29: 531–544. doi:10.1093/molbev/msr185.

Torruella, G., de Mendoza, A., Grau-Bové, X., Antó, M., Chaplin, M. A., del Campo, J., Eme, L. et al. 2015. Phylogenomics reveals convergent evolution of lifestyles in close relatives of animals and fungi. *Curr Biol* 25: 2404–2410. doi:10.1016/j.cub.2015.07.053.

Uchinomiya, K. and Iwasa, Y. 2013. Evolution of stalk/spore ratio in a social amoeba: Cell-to-cell interaction via a signaling chemical shaped by cheating risk. *J Theor Biol* 336: 110–118. doi:10.1016/j.jtbi.2013.07.024.

Umen, J. G. 2011. Evolution of sex and mating loci: An expanded view from Volvocine algae. *Curr Opin Microbiol* 14: 634–641.

Umen, J. G. and Goodenough, U. W. 2001. Control of cell division by a retinoblastoma protein homolog in Chlamydomonas. *Genes Dev* 15: 1652–1661.

Umen, J. G. and Olson, B. J. S. C. 2012. Genomics of volvocine algae. *Adv Bot Res* 6: 186–245.

Vande Berg, W. J. and Starr, R. C. 1971. Structure, reproduction and differentiation in *Volvox gigas* and *Volvox poswersii*. *Arch Protitenkd* 113: 195–219.

Varki, A. and Altheide, T. K. 2015. Comparing the human and chimpanzee genomes: Searching for needles in a haystack. *Genome Res* 15: 1746–1758.

Vaucheret, H. 2006. Post-transcriptional small RNA pathways in plants: Mechanisms and regulations. *Genes Dev* 20: 759–771.

Viamontes, G. I., Fochtmann, L. J., and Kirk, D. L. 1979. Morphogenesis in *Volvox*: Analysis of critical variables. *Cell* 75: 719–730.

Waddington, C. H. 1942. Canalization of development and the inheritance of acquired characters. *Nature* 150(3811): 563–565. doi:10.1038/150563a0.

Wintermute, E. H. and Silver, P. A. 2010. Emergent cooperation in microbial metabolism. *Mol Syst Biol* 6: 407. doi:10.1038/msb.2010.66.

Włoch-Salamon, D. M. 2013. Sociobiology of the budding yeast. *J Biosci* 38: 1–12. doi:10.1007/s12038-013-9344-5.

Wolf, J. B., Howie, J. A., Parkinson, K., Gruenheit, N., Melo, D., Rozen, D., and Thompson, C. R. 2015. Fitness trade-offs result in the illusion of social success. *Curr Biol* 25: 1086–1090.

Worley, A. C., Raper, K. B., and Hohl, M. 1979. *Fonticula alba:* A new cellular slime mold (Acrasiomycetes). *Mycologia* 71: 746–760.

Zhao, T., Li, G., Mi, S., Hannon, G. J., Wang, X. J., and Qi, Y. 2013. A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. *Genes Dev* 21: 1190–1203.