A twelve-step program for evolving multicellularity and a division of labor

David L. Kirk

Summary

The volvocine algae provide an unrivalled opportunity to explore details of an evolutionary pathway leading from a unicellular ancestor to multicellular organisms with a division of labor between different cell types. Members of this monophyletic group of green flagellates range in complexity from unicellular *Chlamydomonas* through a series of extant organisms of intermediate size and complexity to *Volvox*, a genus of spherical organisms that have thousands of cells and a germ–soma division of labor. It is estimated that these organisms all shared a common ancestor about 50 ± 20 MYA. Here we outline twelve important ways in which the developmental repertoire of an ancestral unicell similar to modern *C. reinhardtii* was modified to produce first a small colonial organism like *Gonium* that was capable of swimming directionally, then a sequence of larger organisms (such as *Pandorina*, *Eudorina* and *Pleodorina*) in which there was an increasing tendency to differentiate two cell types, and eventually *Volvox carteri* with its complete germ–soma division of labor. *BioEssays* 27:299–310, 2005.

Introduction

The evolution of multicellular eukaryotes was one of the most-profound developmental transitions in the history of life. Although most of the individual organisms living on Earth today are still unicellular, if all multicellular eukaryotes suddenly vanished from Earth, our planet would appear as barren as Mars.

The origin of multicellular organisms with a division of labor is also one of the most interesting and complex problems in the field of evolution of development, because it presumably involved—at a minimum—a transition from cellular autonomy to cellular cooperation, the invention of novel morphogenetic mechanisms, and the elaboration of novel spatial patterns of differential gene expression.

Such a transition from unicellularity to multicellularity occurred not just once, of course, but repeatedly.[1,2] It is now widely accepted that—except for animals and fungi[3,4]—the major lineages of large, multicellular "crown" eukaryotes (namely, plants, animals, fungi, red algae and brown algae) had independent origins, being derived from different unicellular ancestors more than 1,000 million years ago (MYA).[5,6] Moreover, multicellularity evolved independently in two different groups of cellular slime molds, in diatoms, in ciliates and in several other minor eukaryotic groups, as well as in several groups of prokaryotes.[1,2] However, the Guinness record for the most-repetitive invention of multicellularity goes to the green algae in the class Chlorophyceae. Most chlorophyceans are unicellular, but multicellular forms are found in 9 of the 11 chlorophycean orders, and it appears that multicellularity has arisen independently in each of those orders at least once, and sometimes more than once.[7] One of the best-known and most-studied examples of chlorophycean multicellularity occurs in *Volvox*, a spherical green alga with a division of labor between somatic and germline cells (Fig. 1).

Department of Biology, Washington University, Campus box 1229, St. Louis, MO 63130, USA.
E-mail: kirk@biology.wustl.edu
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Abbreviations: AP, anterior-to-posterior; BAC, bacterial artificial chromosome; BBs, basal bodies; ECM, extracellular matrix; HRGP, hydroxyproline-rich glycoprotein; ISG, inversion specific (or initial-scaffold) glycoprotein; MYA, million years ago; *n*, the number of divisions that occur in one round of multiple fission in a volvocine alga; VSP-3, vegetative serine/proline-rich protein-3.

The volvocine algae as a model system for studying the evolution of multicellularity

Volvox and its closest relatives ("the volvocine algae") provide a particularly promising model system for exploring the details of an evolutionary pathway leading from a unicellular ancestor to multicellular organisms with a division of labor.

Recency

Molecular–phylogenetic studies indicate that, whereas multicellularity evolved in the various eukaryotic crown-groups more than 1,000 MYA, it evolved much more recently in the volvocine algae (Fig. 2A): it has been estimated that multicellular *Volvox carteri* and unicellular *Chlamydomonas reinhardtii* shared a common ancestor as recently as 50 ± 20 MYA.[2,8] Thus, the winds of time have had only about 1/20th as long to obscure details of the pathway leading from

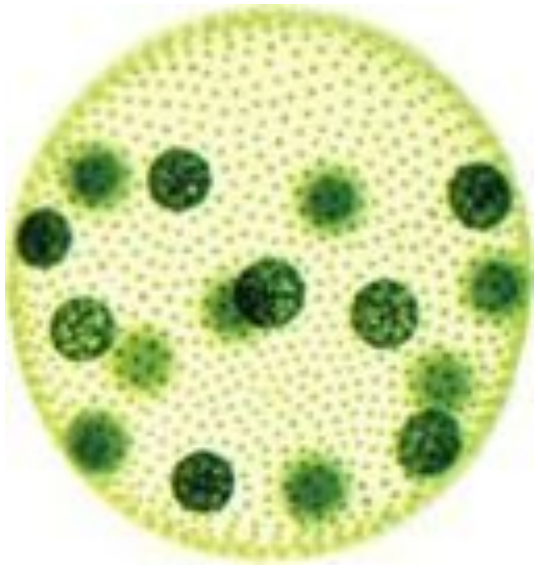

Figure 1. A young asexual adult of *Volvox carteri* consists of a monolayer of several thousand small, biflagellate, "*Chlamydomonas*-like" somatic cells at the surface of a transparent sphere of extracellular matrix, and ~16 large, non-motile, asexual reproductive cells (called "gonidia") just internal to the somatic cell monolayer.

unicellularity to multicellularity in these algae as in the various eukaryotic crown groups.

Extant intermediates

A distinctive advantage of the volvocine system is the availability of several genera of green flagellates in the family Volvocaceae (as traditionally defined) that are intermediate in size and complexity between *Chlamydomonas* and *Volvox*. Textbook authors frequently present a conceptual sequence in which *Chlamydomonas* is placed first, *Volvox* last, and the other volvocacean genera in between, in order of increasing size and complexity (as in Fig. 2B). It then seems a trivial extrapolation to suggest that this is how *Volvox* may have evolved: by a simple, progressive increase in size and complexity. Contemporary studies indicate that, although this scheme is too simplistic, it is a reasonable first approximation of the apparent history of the group.

A recent phylogenetic reconstruction based on the sequences of five genes in 59 volvocine taxa[9] leads to two important conclusions. (i) The family Volvocaceae, as traditionally defined, constitutes a robust monophyletic group, whose members have shared a common ancestor with *Chlamydomonas reinhardtii*. (ii) In general, the position of various genera within the molecular phylogeny corresponds fairly well to the sequence predicted by the traditional volvocine lineage hypothesis depicted in Fig. 2B, with *Gonium* basal,

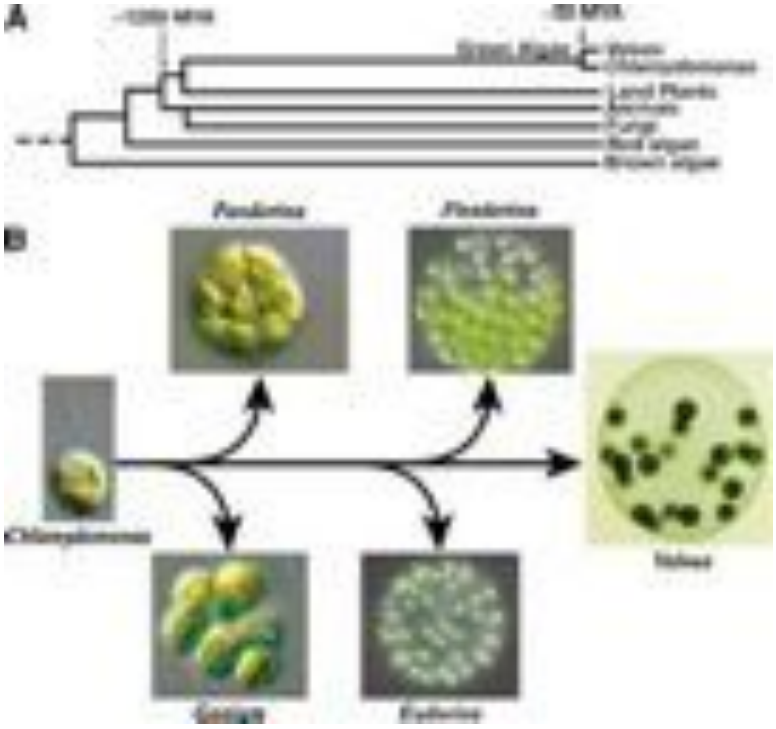

Figure 2. Volvocine relationships. **A:** The branching patterns of the crown-group multicellular eukaryotes—plus Chlamydomonas and *Volvox*—as deduced from various molecular–phylogenetic data.[5,6,8] **B:** The classical "volvocine lineage hypothesis" found in many textbooks. Six genera of green flagellates are arranged in a conceptual series such that there is a progressive increase from left to right in cell number, organismic size, ratio of ECM volume to cellular volume, and the tendency to develop sterile somatic cells—all of which culminate in *Volvox*. It has frequently been suggested that this may resemble the way that *Volvox* evolved: by a simple progressive increase in' size and complexity.

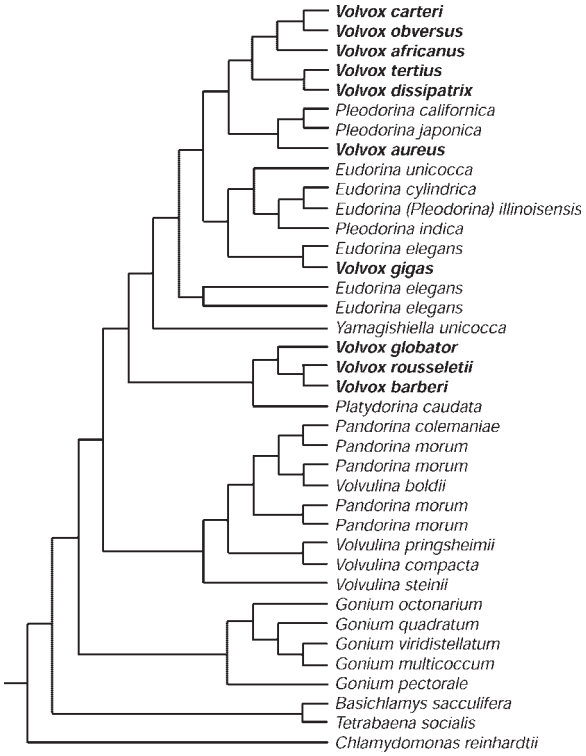

Figure 3. A volvocine family tree. Adapted, with substantial simplification (and with the approval of the author) from a molecular–phylogenetic reconstruction that is based on the sequences of five chloroplast genes in 59 taxa.[9] Different species of *Volvox* are found on four different branches, indicating that the genus is polyphyletic, and that the course of volvocine evolution was not quite as simple as diagrams like the one in Figure 2B suggest.

Volvox distal, and *Pandorina, Eudorina* and *Pleodorina* in intermediate positions (Fig. 3).[9] However, the phylogenetic pattern is a bit more complex than Fig. 2B suggests.

Parallel evolutionary lineages

The volvocine family tree is highly branched (Fig. 3),[9] with several taxa found on more than one branch, indicating that several of the genus and species names used in this family identify morphological grades, rather than monophyletic clades. *Volvox*, for example, is clearly polyphyletic, with different species found on four different branches of the family tree. This suggests that the evolution of organisms with the defining features of *Volvox* (large spheroids containing thousands of somatic cells and a few reproductive cells) must not have required a great many genetic changes, and that these features must have provided some significant ecological advantage. Ultimately it will be of particular interest to determine how similar or dissimilar the genetic pathways leading to different *Volvox* species were. But the present discussion must be restricted to *V. carteri*, because it is the only *Volvox* species for which any genetic information is available.[2]

Molecular–genetic resources

A recent molecular phylogeny of *Chlamydomonas* and its relatives indicates that *C. reinhardtii* shared a common ancestor with *V. carteri* more recently than it has shared a common ancestor with any of the scores of other *Chlamydomonas* species that were included in that study.[10][1] Since there are hundreds of named species of *Chlamydomonas*, it is quite astonishing that *C. reinhardtii*—the species that was selected more-or-less at random 75 years ago to serve as a genetic model system—happens to be the closest living unicellular relative of *Volvox*. The practical significance of this is that *C. reinhardtii* brings to the study of volvocine evolution a rich endowment of genetic resources[11,12] including a fully sequenced genome (http://www.biology.duke.edu/chlamy_genome/cgp.html). This endowment has now been complemented by the production of *V. carteri* BAC libraries (Dina Mandoli, pers. commun.) and sequencing of the *V. carteri* genome (Daniel Rokhsar pers. commun.).

An overview of *Volvox carteri* development

Appreciation of the steps required to evolve multicellular *V. carteri* from a *C. reinhardtii*-like unicellular ancestor will be facilitated by an introductory review of the steps by which a multicellular adult of modern *V. carteri* develops from a single cell. It is important to note that green algae, including *Chlamydomonas* and *Volvox*, are normally haploid and reproduce asexually. In nature they use sex not for reproduction, but to produce dormant zygotes capable of surviving adverse conditions. The return of favorable conditions causes zygotes to undergo meiosis and produce haploid offspring that then proliferate asexually. Hence, the rest of this review deals exclusively with the asexual portion of the volvocine life cycle.

Each *V. carteri* young adult contains ~16 large asexual reproductive cells called gonidia (Fig. 1), each of which normally divides to produce a new individual (Fig. 4A).

Cleavage (Fig. 4B, a–d)[2]

Each mature gonidium initiates a stereotyped sequence of synchronous cleavage divisions that produce all of the cells that will be present in an adult of the next generation. The first five divisions are symmetrical, so that all cells of a 32-cell embryo are similar in size. In the sixth cycle, however, ~16 cells divide asymmetrically, producing large–small sister-cell pairs. Each large cell becomes a gonidial initial that will produce one

[1]It should also be noted that since all such studies indicate that *C. reinhardtii* is more closely related to other species of *Chlamydomonas* than any of the volvocaceans are, there is no support for the hypothesis that *C. reinhardtii* is a unicellular derivative of one of the multicellular volvocaceans.

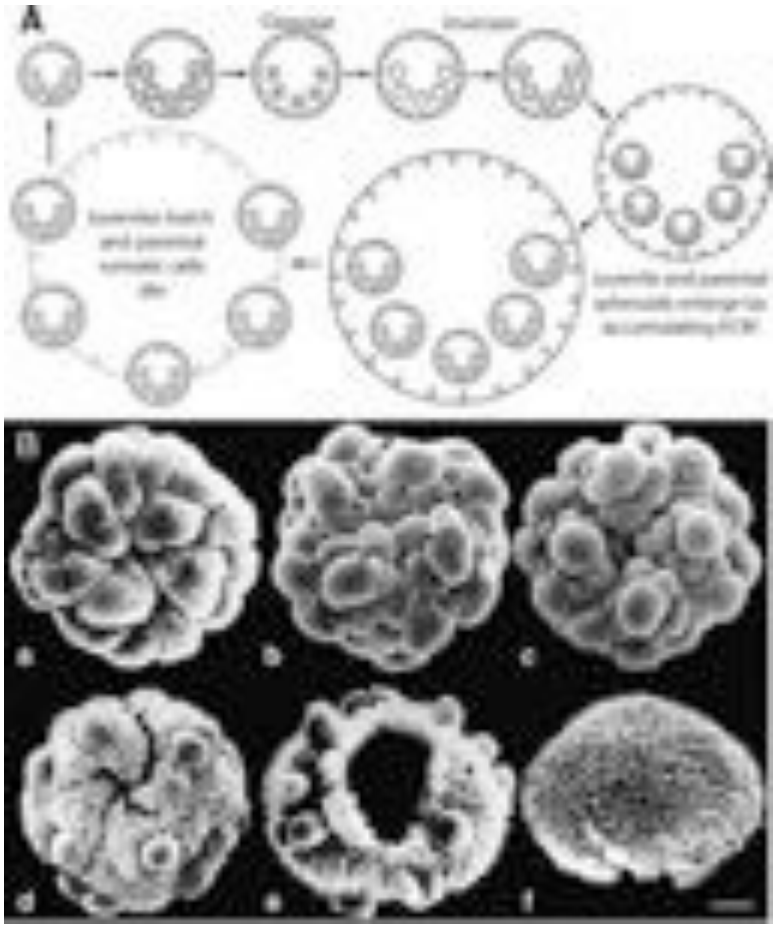

Figure 4. Development of *Volvox* carteri. **A:** The asexual life cycle. The embryonic phase begins when mature gonidia initiate cleavage, and it ends when the fully cleaved embryo inverts (turns inside-out) and becomes a juvenile (a miniature adult). Following embryogenesis, both the juvenile and parental spheroids enlarge (without further cell division) by deposition of large quantities of ECM. Part way through their enlargement phase the juveniles digest their way out of the parental ECM to become free-swimming young adults. The parental somatic cells then undergo programmed death while the gonidia of the new generation mature and initiate another round of embryogenesis. **B:** Embryogenesis. **a.** The first five cleavage divisions are symmetrical, so all cells of the 32-cell embryo are similar in size. **b.** In the sixth division cycle, ~16 cells divide asymmetrically to produce large-small sister-cell pairs (connected here by arrowheads). **c.** The large gonidial initials divide asymmetrically two more times and then withdraw from the division cycle while the small somatic initials continue dividing. **d.** At the end of cleavage, the embryo contains all the cells that will be present in an adult of the next generation, but in an inside-out configuration, with gonidia on the outside and the flagellar ends of somatic cells on the inside. The embryo will now invert through its "phialopore"—the swastika-shaped slit seen here. **e.** By a combination of cell-shape changes and movements, the lips of cells flanking the phialopore bend outward and backward over the rest of the embryo. As cells progressively further from the phialopore execute similar movements, the region of maximum curvature moves toward the opposite pole, until the embryo has turned completely right-side-out. **f.** Inversion has brought the flagellar ends of the somatic cells to the exterior and sequestered the gonidia on the interior of the juvenile spheroid.

gonidium, while each small cell becomes a somatic initial that will produce a clone of somatic cells. Gonidial initials divide asymmetrically two more times, producing more somatic initials; but then they stop dividing while the somatic initials continue dividing synchronously three more times. By the end of cleavage each gonidial initial is ~30 times the volume of a somatic initial (Fig. 4B,d), and it has been shown that this difference in size, and not a difference in cytoplasmic quality, determines whether cells will develop as germ or soma.[13]

The cytoplasmic bridge system (Fig. 5A)

Throughout embryogenesis, the embryo is syncytial, because all of its cells are linked by a network of cytoplasmic bridges that

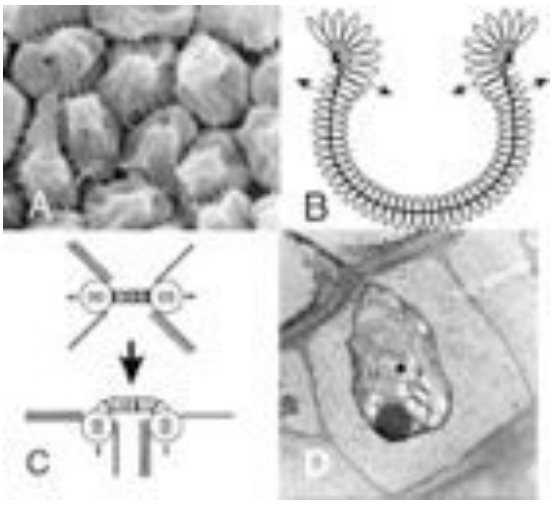

Figure 5. Some developmental details. **A:** Cytoplasmic bridges. Throughout embryogenesis all cells of the embryo are linked by numerous cytoplasmic bridges that form as a result of incomplete cytokinesis. The cytoplasmic bridges of all cells are aligned and interconnected, forming a coherent "bridge system" that runs through the whole embryo.**B:** This diagrammatic saggital section of an embryo in early inversion illustrates the role played in inversion by the bridge system (the heavy line running through all of the cells). Cells about to enter the region of outward curvature form thin, microtubule-lined extensions at their outer ends (outward -directed arrows). Then the cells move inward individually (inward-directed arrows) relative to the cytoplasmic bridges that link them to their neighbors. As they do this, they go from being linked to their neighbors at their widest points to being linked to their neighbors at their thin, outermost tips, which forces the cell sheet to curl outward.[15] **C:** Rotation of the basal bodies (BBs) in developing somatic cells. In *C. reinhardtii* and other unicellular green flagellates the pair of BBs (and the flagella that they template) are arranged with 180° rotational symmetry, so that their flagella beat in opposite directions. *V. carteri* gonidia and embryonic cells do not have functional flagella, but they do each have a pair of BBs arranged with 180° rotational symmetry, as in *Chlamydomonas*. (Upper diagram) However, during somatic cell differentiation the BBs (and certain cytoskeletal elements attached to them) rotate 90° in opposite directions, to assume a parallel orientation (Lower diagram). Consequently, the two flagella on each *V. carteri* somatic cell beat in parallel, as indicated by the arrows on the BBs.[17] **D:** ECM Organization. The ECM consists principally of a complex assortment of HRGPs[21] that are organized into a variety of distinctive fibrous elements that form a boundary layer over the surface of the spheroid, a honeycomb of compartments that surround individual cells, and a layer that encloses the voluminous central region of the spheroid that—like each of the cellular compartments—is filled with a loose feltwork of other HRGP fibers and filaments.[20]

are formed as a result of incomplete cytokinesis. In a fully cleaved embryo, each cell is linked to its neighbors by an average of 25 bridges that are organized into a single band girdling that cell at its widest point. Moreover, the bridge bands of all the cells in an embryo are aligned and linked into a coherent "bridge system" that extends throughout the embryo, and that plays a centrally important role in the next phase of embryogenesis.

Inversion (Fig. 4B,d–f)

At the end of cleavage, each embryo contains all of the cells that will be present in an adult of the next generation, but it is inside-out with respect to the adult configuration: its gonidia are on the outside and the flagellar ends of its somatic cells face the interior. But inversion turns the embryo right-side-out, bringing the flagellar ends of the somatic cells to the surface, and tucking the gonidia away on the interior. Inversion is known to be driven by a change in cell shape, coupled with movement of individual cells, as diagrammed in Fig. 5B.[14]

Cytodifferentiation

By the end of inversion, the presumptive somatic and gonidial cells differ in little but size. But soon the cytoplasmic bridges break down, whereupon the two cell types initiate very different patterns of protein synthesis and cytodifferentiation.[15,16]

Rotation of the basal bodies

An early step in somatic cell differentiation involves a striking rotation of the basal bodies (BBs) that underlie the flagella and determine their orientations. In *Chlamydomonas* and other unicellular green flagellates, the BBs, the fibers attached to them and the flagella are all arranged with 180° rotational symmetry (that is, they "face" in opposite directions; Fig. 5C, top). As a result, the flagella beat in opposite directions, and the cell swims with an algal version of the breast stroke. Although this kind of flagellar beat is adaptive for a unicell, it would be maladaptive for a spherical multicellular flagellate like *Volvox*. If the members of each flagellar pair in *Volvox* beat in opposite directions, they would have no effect other than to push a bit of water toward the surface of the spheroid. Locomotion would be impossible.

This potential predicament is solved by rotation of the BBs at an early stage in somatic cell differentiation. Gonidia and embryonic cells do not have functional flagella, but they have paired BBs that are arranged with 180° rotational symmetry (Fig. 5C, top). However, during early somatic cell differentiation the paired BBs (and certain of the fibers attached to them) rotate 90° in opposite directions, so that they end up facing in nearly the same direction (Fig. 5C, bottom).[17] This remarkable reorganization assures that the flagella of each somatic cell will beat nearly in parallel, toward the posterior of the spheroid, propelling the spheroid forward.

Anterior–posterior (AP) polarity

The fact that the BBs of all cells rotate in the same direction, so that the effective strokes of all the flagella are directed toward the posterior pole of the spheroid, is just one indicator of the AP polarity of development. Other indicators include: (i) gonidia are preferentially located toward the posterior end, (ii) somatic cells are graded in size, from largest in the anterior to smallest in the posterior, (iii) eyespots of the somatic cells exhibit a similar size gradient, and (iv) the eyespots are also graded in position, from lateral in anterior cells to more nearly apical (closer to the flagella) in more posterior cells.

Synthesis and assembly of the ECM

Another critical step in early postembryonic development is the synthesis and assembly of an extracellular hydroxyproline-rich glycoprotein (HRGP) called ISG[18] that plays a crucial role as the "initial scaffolding glycoprotein" upon which the rest of the extracellular matrix (ECM) assembles.[19] Shortly after inversion, and while the cells are still linked by cytoplasmic bridges, ISG assembles into a thin layer that is continuous over the entire surface of the juvenile except where it is perforated by the flagella. When self-assembly of ISG is inhibited in either of two ways, normal assembly of the rest of the ECM fails to occur, and therefore the juvenile falls apart into a single-cell suspension as soon as the cytoplasmic bridges have broken down.[18,19]

Provided that the ISG scaffold is permitted to assemble in the normal manner, however, a very complex ECM is eventually produced that includes individual compartments for each of the cells (Fig. 5D) and an array of other distinctive structural features,[20] all of which are composed predominantly of other kinds of HRGPs.[21] Accumulation of ECM components continues over the next three days, causing the spheroid to "expand" ~10,000-fold in volume, so that the ECM comes to constitute >99% of the volume of a fully mature adult spheroid.

The twelve-step program leading from a *Chlamydomonas reinhardtii*-like ancestor to *Volvox carteri*

Having reviewed key aspects of *V. carteri* asexual reproduction and development, we can now consider what processes had to be added to the ancestral developmental repertoire of *Chlamydomonas* in order to evolve a developmental repertoire like that of modern *V. carteri*. Twelve such processes are mapped onto a simplified volvocine cladogram in Fig. 6. The first six steps are all mapped onto the *Chlamydomonas*-to-*Gonium* interval because, as will be discussed below, all of the traits that they led to are present in *Gonium pectorale*, the basal member of the genus *Gonium*, as recently redefined by Nozaki and his coworkers (Fig. 3).[9,22]

Step 1. Incomplete cytokinesis

The volvocine algae share with thousands of other green flagellates a distinctive form of cellular reproduction called multiple fission, in which cells grow 2^n fold without dividing, and then divide rapidly n times to produce 2^n progeny cells. The asexual reproductive cycles of *C. reinhardtii* and *G. pectorale* both involve multiple fission, but they differ in one obvious and

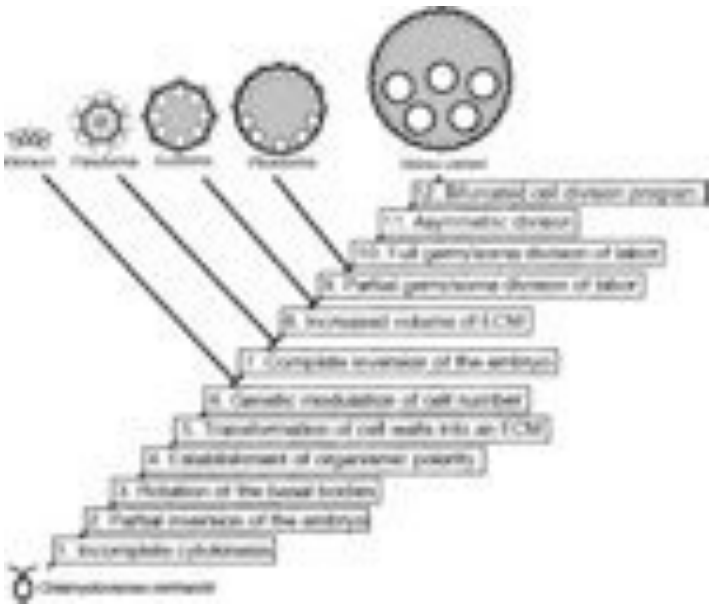

Figure 6. Twelve major steps in the evolution of *Volvox carteri* from a *C. reinhardtii*-like ancestor. See text for discussion.

important way: whereas the daughter cells produced by multiple fission in *C. reinhardtii* separate from one another and behave as independent organisms, the daughter cells produced by multiple fission in *G. pectorale* cohere, to form an embryo that becomes a multicellular colony of distinctive size and shape.[23] The reason that *G. pectorale* cells maintain such a stable spatial relationship during cleavage is that they, like cells of the *V. carteri* embryo shown in Fig. 5A, are linked to one another by cytoplasmic bridges that form as a result of incomplete cytokinesis.[24] Similar cytoplasmic bridges have been seen in the embryos of every volvocacean species that has been examined carefully.[25,26]

Ultrastructural studies of cleaving *V. carteri* embryos revealed that, although the anterior end of each cell is partitioned by an ingressive furrow (as in animal cells), the furrow in the bridge-forming internuclear region is formed by alignment and fusion of vesicles along the prospective cleavage plane (as in plant cells), and it was postulated that the cytoplasmic bridges are produced in those regions where such vesicles fail to fuse completely.[14] Similar vesicles are seen in the internuclear region of a dividing *C. reinhardtii* cell[27] raising the possibility that it took only a relatively modest evolutionary modification of the *C. reinhardtii* cytokinetic mechanism to generate a system of cytoplasmic bridges in the internuclear region that would hold the cells of a cleaving organism together. Such a possibility was reinforced by the description of *Gonium dispersum*, an alga in which some cells within a clone divide completely to produce unicellular (*Chlamydomonas*-like) progeny and others divide incompletely to produce colonial (*Gonium*-like) progeny.[28] Furthermore, Annette Coleman (pers. commun.) reports that occasional cells divide incompletely and produce small *Gonium*-like colonies in a number of different *Chlamydomonas* species. The difference is that, in *G. pectorale* and the larger volvocaceans, incomplete cytokinesis is the rule, not the exception.

Steps 2 and 7. Incomplete—and then complete—inversion of the embryo

At the end of cleavage, a *G. pectorale* embryo has the shape of a shallow bowl, with the flagellar ends of all 16 cells on the inner, concave surface. A modest form of inversion then occurs to reverse the curvature of the bowl and bring the flagellar ends of the cells to the outer, convex surface.[24] Fully cleaved *Pandorina morum* embryos, like *G. pectorale* embryos, also contain 16 cells in a hollow bowl configuration. But when they invert, they do not stop at the convex-plate stage as *G. pectorale* does; they go all the way and form little spheroids of tightly packed cells.[25] All other volvocaceans invert as fully as *Pandorina* does, and the available evidence is consistent with the idea that cytoplasmic bridges play a role in the inversion of *Pandorina, Eudorina* and *Pleodorina* embryos similar to the role that they have been shown to play in *V. carteri* inversion.[25,26,29]

It was recently found that inversion in *V. carteri* requires the action of the *invA* gene, which encodes InvA, a novel type of kinesin that is located in the cytoplasmic bridges.[30] It is thought that InvA drives inversion by pushing on the cortical microtubules that line the inverting cells, thereby forcing those cells to move relative to the cytoplasmic bridge system (see the model in Fig. 5B). *Chlamydomonas, Gonium, Pandorina, Eudorina* and *Pleodorina* all possess a gene that is an apparent orthologue of *invA*, and the cloned *Chlamydomonas* orthologue, *IAR1*,[30] has now been used to cure the inversionless phenotype of a *Volvox invA* mutant (I. Nishii and D. Kirk, unpublished data). It is not yet known what role the IAR1 kinesin plays in *Chlamydomonas*, but it now appears clear that it was adopted, without any significant change, for use in inversion, probably very early in volvocacean evolution. A search for additional genes and gene products that are required for inversion of the embryo is now underway, using methods similar to the ones that were used to clone and characterize the *invA* gene and its product (I. Nishii, pers. commun.).

Step 3. Rotation of the basal bodies

A typical *G. pectorale* colony has 16 cells arranged as four central cells that are flanked on each side by three peripheral cells. At the end of cleavage, the BBs of all of these cells are arranged with 180° rotational symmetry, just as they are in *Chlamydomonas*.[31] Although no BB rotation occurs in the four central cells during flagellar development, the BBs of all 12 peripheral cells undergo a rotation similar to the one illustrated in Fig. 5C, so that the two flagella on each peripheral cell beat in parallel and toward the periphery of the colony.[29,31] All cells of every other volvocacean that has been carefully examined undergo such a BB rotation, but we have no clue how it occurs.

Step 4. Establishment of organismic polarity

The difference between central and peripheral cells of *G. pectorale* with respect to BB rotation provides clear evidence of a central-to-peripheral polarity in this organism. However, in all of the volvocaceans that undergo complete inversion to form spheroidal adults (such as *Pandorina, Eudorina, Pleodorina* and *Volvox*) the central-to-peripheral polarity of Gonium automatically becomes an anterior-to-posterior (AP) polarity. This AP polarity is usually apparent as a gradient in eyespot size and, in the larger volvocaceans, it is also expressed as a gradient in cell size.

In *Eudorina, Pleodorina* and *Volvox*, the tendency to form sterile somatic cells is also graded along the AP axis. Although normally all *Eudorina elegans* cells enlarge and divide, under certain circumstances, the four most-anterior cells remain small and in the biflagellate, somatic, state while the rest of the cells reproduce. In *Volvox* the gonidia are routinely located

toward the posterior and are always excluded from the extreme anterior. But this aspect of AP polarity is exhibited with greatest clarity by *Pleodorina californica*, in which all anterior cells function as sterile somatic cells and all posterior cells function as gonidia (see Figs. 2B and 6).[29]

Step 5. Transformation of cell walls into an ECM

In all volvocaceans except certain species of *Volvox* not otherwise discussed here, the cytoplasmic bridges that were present during embryogenesis break down as cellular differentiation begins. Then the job of holding the cells together falls to the ECM, which is a modified form of the *C. reinhardtii* cell wall. The *C. reinhardtii* cell wall (which is HRGP-based, and contains no cellulose) has two distinguishable concentric regions. The outer wall has a distinctive "tripartite" (dark, light, dark) appearance when viewed in cross section in conventional electron microscope images and an exceptionally regular, quasi-crystalline organization when examined in quick-freeze, deep-etched preparations; and it can be solubilized by certain chaotropic salts.[32] The inner wall, in contrast, is relatively amorphous and salt-insoluble. All *Gonium* cells are surrounded in a similar fashion by homologues of both the inner and outer walls of *Chlamydomonas*. However, the outer walls of adjacent *Gonium* cells are held together at their contact points by specializations that vary in structure from species to species (Fig. 7).[33] This constituted a first step toward converting cell walls into an ECM.

In *Pandorina* and all of the larger volvocaceans, in contrast, only the equivalent of the *C. reinhardtii* inner wall surrounds each cell, and the tripartite outer wall has become part of a "boundary layer" that is continuous over the surface of the spheroid, and that holds the organism together (Fig. 7).[26] Furthermore, as discussed below under step 6, as organismic size increased in the *Pandorina*-to-*Volvox* progression, the derivatives of the inner-wall homologues became much more voluminous and specialized, to the extent that, in *Volvox*, they eventually constitute >99% of the volume of the adult spheroid. (Various species-specific specializations of the boundary layer are also seen, however, both inside and outside the tripartite layer; see Fig. 5D.[20])

The fact that the outer wall of *C. reinhardtii* and the tripartite portion of the boundary layer of the *V. carteri* spheroid are truly homologous was demonstrated dramatically when the HRGPs constituting the tripartite layers of both species were extracted with salt, and then the salt-stripped organisms were used to nucleate reassembly of the salt-soluble HRGPs of the other species. That is to say, HRGPs derived from the *C. reinhardtii* outer cell wall were reassembled on stripped *V. carteri* spheroids, and vice versa, and in both combinations a quasi-crystalline array was reconstituted that was indistinguishable from the native pattern.[34]

Reconstitution of a crystalline layer of normal structure requires an inner layer as a scaffold.[34] As discussed previously, ISG appears to form such a scaffold in *V. carteri*. A cell wall HRGP of *C. reinhardtii* called VSP-3 is similar to ISG in structure, and possibly in function: both of these HRGPs have globular N-terminal domains of similar sequence attached to highly glycosylated, rod-like, hydroxyproline-rich C-terminal domains.[34,35] Such HRGPs, consisting of a rod-like hydroxyproline-rich module with a globular module attached to one or both of its ends are abundant in volvocine cell walls and ECM. Indeed, the *V. carteri* ECM contains dozens of such HRGPs,[21] and it has been postulated that much of this HRGP repertoire evolved through extensive gene duplication, divergence and domain swapping that produced many new combinations of fibrous and globular modules.[35]

Empirical studies suggest that the preceding five features probably evolved almost simultaneously, because abrogation of any one of these five traits in a modern volvocine alga results in a failure to produce a colony of defined shape that is capable of swimming directionally and maintaining its place in the sun.[2] The extreme improbability that any single unicell could produce an offspring with all five of these essential features may account for the fact that the only one of several hundred chlamydomonad lineages that ever produced a radiation of

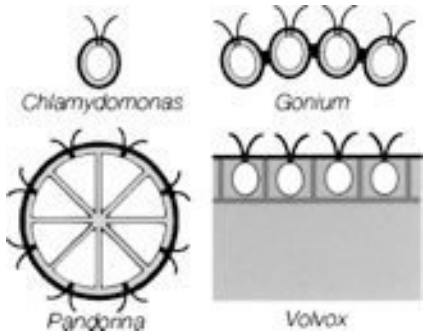

Figure 7. Evolution of the volvocacean ECM from the *Chlamydomonas* cell wall. The *C. reinhardtii* cell wall consists of two morphologically and chemically distinguishable concentric layers that are composed primarily of different HRGPs. The outer wall (here in black) has a highly regular, quasi-crystalline structure and appears "tripartite" (dark, light, dark) when viewed in cross section in the electron microscope. The inner wall (here in grey) appears relatively amorphous.[31] In *Gonium*, the homologues of both cell-wall layers surround each cell, and the cells are held together only by species-specific specializations that connect the outer walls of adjacent cells.[32] In *Pandorina*, in contrast, the homologue of the *C. reinhardtii* inner wall continues to surround each cell, but the homologue of the outer wall is continuous over the outer surface of the spheroid, forming a "tripartite boundary layer" that holds the cells together.[26] A tripartite boundary layer is seen in the remaining volvocaceans, but derivatives of the inner-wall homologues have become much more voluminous and specialized in larger volvocaceans, such as *Volvox*.

motile colonial offspring as successful as the volvocaceans was the *C. reinhardtii* lineage.

Step 6. Genetic modulation of cell number

As mentioned above under Step 1, all volvocine algae reproduce by multiple-fission: cells grow 2^n-fold without dividing, and then divide n times to produce 2^n progeny cells. Evolutionary increase in the number of cells present in a volvocacean adult would have required changing "*n*", the number of divisions that occur during the multiple-fission period.

In *Chlamydomonas*, the value of *n*, which can vary from 1 to 5, depends on the size of the mother cell, which depends in turn on the environmental conditions prevailing during its growth phase. Experimental dissection has led to a "timer-sizer" mode[36,37] to explain the regulation of multiple fission: an intrinsic timer triggers a sequence of check points at which a sizer mechanism determines whether the cell is above the threshold size that is required to make a commitment to divide once—or twice—or three times—etc. As a result of such sequential reevaluations, the daughter cells that are produced at the end of each multiple-fission cycle differ in size by less than a factor of two, even under environmental conditions that result in a 10-fold difference in growth rate.

Environmental conditions also influence the observed value of *n* in the multicellular volvocaceans, to the extent that suboptimal growth conditions may result in submaximal numbers of cells being present in members of the next generation. However, in all of the multicellular forms there is an additional layer of genetic regulation imposed that results in different maximal values of *n* in different species. For example, the maximum number of cells per colony that are seen in various species of *Gonium* indicates that the maximal value of *n* is 3 in *G. octonarium*, 4 in *G. compactum, G. pectorale, G. quadratum* and *G. viridistellatum*, and 5 in *G. discoideum* and *G. multicoccum*.[38,39] (Colonies in which *n* is 2 are not mentioned, because the 4-cell species that were traditionally called *Gonium* are now placed in different genera and a different family.[9,22])

Increasing the maximal value of *n* has been one of the hallmarks of volvocine evolution, reaching its acme in *Volvox*, where the maximal values of *n* range from 10 to 14 in various species.[40]

A recent study raises the possibility that control of this important aspect of volvocine evolution may be under the control of the volvocine homolog of the retinoblastoma (*Rb*) gene (a mammalian tumor-suppressor gene). Umen and Goodenough[41] found that the product of the *mat3* gene, which is the *C. reinhardtii* homologue of the mammalian RB protein, is involved in two "sizer" functions: (i) determining the minimum size at which mother cells are allowed to initiate division, and (ii) determining the size at which daughter cells are required to stop dividing. Umen proposes to test the hypothesis that increases in cell number during volvocacean evolution have involved changes in the *mat3* orthologue that increased the threshold size required to initiate division, with no concomitant increase in the size of daughter cells at which division would stop (James Umen, personal communication).

Step 8. Increased volume of ECM

The increase in organismic size that occurred during volvocine evolution was only partially due to increases in cell number; to a much greater extent, it was due to a huge increase in the ratio of ECM volume to cell volume as cell number increased. Whereas the ECM (cell wall) accounts for only about 1% of the volume of a *C. reinhardtii* cell, it accounts for >99% of the volume of a *Volvox* spheroid. Indeed, the amount of ECM per cell is approximately an exponential function of cell number, and thus *Volvox* has ~10,000 times more ECM per cell than *Chlamydomonas* does. This high ratio of ECM volume to cellular volume is apparent to even a casual observer of *Eudorina*, and of course it is even more striking in *Volvox* (Fig. 2B). It has been proposed that the ultimate cause (the selective advantage) of the evolutionary expansion of the ECM is that the more ECM these algae have, the more effectively they compete for growth-limiting nutrients such as inorganic phosphate.[43]

Steps 9 and 10. Partial and complete germ–soma division of labor

The smaller volvocine algae, from *C. reinhardtii* to *Pandorina*, possess only one cell type, and each of these cells first develops as a motile biflagellate cell that grows, and then later redifferentiates and enters a non-motile reproductive phase in which it executes multiple fission. This biphasic, "first biflagellate and then reproductive", pattern of development is shared with hundreds of other unicellular green flagellates, and thus it presumably is the ancestral developmental pathway of the group. In *V. carteri*, however, this pathway has become modified to generate a germ–soma dichotomy, in which two cell types are set apart during early embryogenesis, and then one set (the somatic cells) execute exclusively vegetative, non-reproductive functions while the other set (the gonidia) execute exclusively reproductive functions. An intermediate situation—a partial germ–soma division of labor—exists in *Pleodorina*, in which all cells differentiate first as biflagellate cells and contribute to motility, and then a subset of the cells redifferentiate as gonidia, while the rest remain sterile, biflagellate somatic cells.

What genetic changes may have been required to evolve a germ–soma dichotomy from the ancestral "first biflagellate and then reproductive" pattern of development? Mutational studies of *V. carteri* have defined the *regA* and *lag* genes that act to split the ancestral developmental program into two

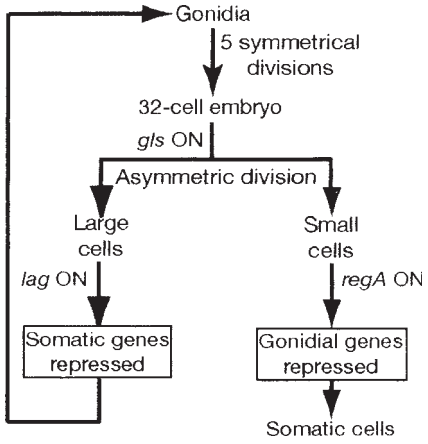

Figure 8. A minimal model for the genetic control of germ-soma specification in *V. carteri*. A mature gonidium divides symmetrically five times to generate a 32-cell embryo in which all cells are the same size. Then the *gls* (gonidialess) genes function to cause asymmetric division, producing large and small cells. In the large cells the *lag* (late gonidia) genes act to prevent development of somatic features, such as flagella and eyespots; therefore, these cells bypass the biflagellate phase of the ancestral developmental program and go directly to the reproductive phase. In the small cells, meanwhile, the *regA* (somatic regenerator A) gene acts to repress functions that are required for reproduction, so that these cells develop as terminally differentiated somatic cells.

mutually exclusive parts (Fig. 8).[44] The *regA* gene acts in developing somatic cells to prevent them from entering the reproductive phase of the ancestral pathway. Thus, when *regA* is inactivated by mutation, the somatic cells follow the ancestral "first biflagellate and then reproductive" developmental pathway: they first differentiate as biflagellate cells, but later redifferentiate as gonidia.[45] The *lag* genes, on the other hand, act in gonidia to prevent the development of somatic features, such flagella and eyespots. So, in a *lag* mutant, the presumptive gonidia follow the ancestral "first biflagellate and then reproductive" pathway, developing first as large biflagellate cells and later redifferentiating as gonidia.[44] The *lag* genes have not yet been cloned and characterized, but the *regA* gene has.

RegA (the product of *regA*) is a nuclear protein with the features of an active transcriptional repressor[46] that is expressed in somatic cells under the control of two intronic enhancers and repressed in gonidia under the control of an intronic silencer.[47] Sequencing and analysis of 16 candidate targets of RegA revealed that they were all nuclear genes encoding important chloroplast proteins.[48] This finding led to the working hypothesis that the way that *regA* prevents somatic cells from engaging in reproduction is by repressing chloroplast biogenesis. *V. carteri* is an obligate photoautotroph with photosynthesis-limited cell growth and, although each somatic cell inherits a tiny bit of chloroplast from its maternal gonidium during cleavage, if it cannot make more chloroplast it cannot grow. And if it cannot grow, it cannot reproduce.

Step 11. Asymmetric division

Asymmetric division as a cell specification mechanism clearly was a late, lineage-specific step in volvocine evolution, because only two of the 18 recognized species of *Volvox* (*V. carteri* and its nearest relative, *V. obversus*) exhibit any asymmetric cleavage divisions. The others must have some other way(s) to specify gonidia. Nonetheless, asymmetric division plays a crucial role in *V. carteri* development, because *V. carteri* cells that are below the threshold diameter of 8 μm at the end of cleavage activate the somatic-cell program of differentiation, while cells above that threshold activate the gonidial program—even if all of the cytoplasm that they contain is cytoplasm that would normally have been found in somatic cells.[13]

V. carteri mutants that lack asymmetric division have a "gonidialess" (Gls) phenotype: they lack gonidia because all of their embryonic cells divide symmetrically until they are too small to undergo gonidial specification.[13] (Such mutants are recovered and maintained in the presence of a *regA* mutation that permits the somatic cells to take over the job of reproduction.) No cell-division abnormality other than an absence of asymmetric division has been detected in Gls mutants, so it appears that the function of the *gls* gene products is to shift the division plane in asymmetrically dividing cells.

The first *gls* gene to be cloned and characterized encodes a chaperone-like protein of the Hsp 40 class (GlsA) that is associated with the cell division apparatus during mitosis.[49] It is not yet known how GlsA affects the division plane, but it is of particular interest to note that the *C. reinhardtii* orthologue of *glsA* is fully capable of replacing *glsA* and restoring the capacity for asymmetric division in a *glsA* mutant.[50] Thus, in parallel with the case of the *invA* gene that was discussed earlier, it seems that a gene that must have some other, yet-to-be-determined function in *Chlamydomonas* was adopted without any significant change to play an entirely new role in *Volvox carteri* asymmetric division.

Step 12. Bifurcation of the cell division program

Asymmetric division is coupled to another derived trait: bifurcation of the cell-division program. As noted during discussion of *V. carteri* embryogenesis, the gonidial initials undergo three rounds of asymmetric division and then stop dividing, while the somatic initials go on dividing three or four additional times. The fact that gonidial initials withdraw from the division cycle while they are much larger than the somatic initials clearly indicates that they are no longer under the influence of the size-dependent division-control system that

operates in *Chlamydomonas*, in other volvocaceans, and even in the adjacent somatic initials. In all of those other volvocine cells, the number of division cycles are determined by the size of the mother cell. But if that were true of gonidial initials, they should divide more times, not fewer times than the somatic initials—which would abolish any effect of asymmetric division, of course. The nature of the novel mechanism controlling division of the gonidial initials remains to be determined. But whatever its nature, the co-existence of two different cell-division controls in the *V. carteri* embryo seems particularly amazing when we realize that the embryo is syncytial and that in every cleavage cycle all dividing cells divide in perfect synchrony—suggesting the existence of a pervasive cytoplasmic signaling system. It will be of particular interest to learn how the cell-division controls operating in the somatic initials are overridden in the gonidial initials of *V. carteri*.

Conclusion

The unrivalled advantages of the volvocine algae as a model system for analyzing details of an evolutionary pathway leading to multicellular organisms with different cell types have been noted repeatedly in the past. Recent studies have both reinforced and exploited certain of those advantages. Molecular–phylogenetic studies[9,51–53] indicate that the family Volvocaceae is monophyletic as a group (having shared a common ancestor fairly recently with *C. reinhardtii*), but that several of its constituent taxa are not monophyletic (Fig. 3). Of particular interest in this regard is the fact that species with the germ-soma division of labor that is the hallmark of the "genus" *Volvox* have apparently arisen independently at least four times during the relatively brief history of the group, suggesting that evolving such a cellular dichotomy must not have required a great many genetic changes, and probably provided a significant selective advantage under certain conditions.

Here we have first identified the most-salient features that distinguish the developmental repertoire of the best-studied species of *Volvox, V. carteri*, from that of the related unicell, *C. reinhardtii.*Then we have used data from the literature to map the appearance of each of those features on a simplified phylogeny of the group. What this exercise revealed was that most of the developmental features that distinguish the *Volvox* and *Chlamydomonas* developmental repertoires appear to be relatively ancient inventions that are shared by even the basal-most species considered (*Gonium pectorale*), that two of them clearly are recent inventions because they are specific to the *V. carteri* lineage, and that the remaining features were apparently added stepwise at intermediate stages of volvocacean phylogeny.

Interestingly, our molecular-genetic studies have revealed that one of the earliest and one of the most recent of these volvocacean inventions (inversion and asymmetric division, respectively) both rely upon the products of *Chlamydomonas* genes (*IAR1* and *GAR1*, respectively) that have been adopted, without any significant modification, to participate in novel morphogenetic processes.

What this review also reveals is that our ignorance concerning the details of the volvocacean pathway to multicellularity still greatly exceeds our understanding, but that the organisms appear to be willing to provide additional insights in response to properly designed experimental inquiries.

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