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The Unicellular Ancestry of Animal Development

Review

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The transition to multicellularity that launched the evolution of animals from protozoa marks one of the most pivotal, and poorly understood, events in life's history. Advances in phylogenetics and comparative genomics, and particularly the study of choanoflagellates, are yielding new insights into the biology of the unicellular progenitors of animals. Signaling and adhesion gene families critical for animal development (including receptor tyrosine kinases and cadherins) evolved in protozoa before the origin of animals. Innovations in transcriptional regulation and expansions of certain gene families may have allowed the integration of cell behavior during the earliest experiments with multicellularity. The protozoan perspective on animal origins promises to provide a valuable window into the distant past and into the cellular bases of animal development.

Over 600 million years ago (MYA), the multicellular progenitor of modern animals evolved from a unicellular flagellate. From such modest beginnings evolved the entire diversity of Metazoa: from deep sea sponges to beetles, frogs, and humans. Trumping even the origins of gastrulation, segmentation, and the germline, the transition to multicellularity stands as a pivotal event in metazoan history. It is also the least understood.

The origin of animals from a protozoan ancestor was shaped by a convergence of environmental forces, genomic innovation, contingency, and natural selection (Table 1) (Carroll, 2001; Knoll and Carroll, 1999). The emerging picture of environmental and geochemical events surrounding animal origins has revealed a time of increasing atmospheric and oceanic oxygen concentrations (reviewed in Knoll, 2003). Limited oxygen availability prior to the late Proterozoic is thought to have prevented the evolution of large three-dimensional multicellular eukaryotes (Table 1). (In contrast, filamentous prokaryotes decorate the fossil record beginning far earlier, perhaps 3200 MYA [Knoll, 2003].) The niche of multicellularity, left vacant by the dominance of the unicellular lifestyle, was apparently colonized by only a subset of lineages following the lifting of environmental barriers. In concert with ecological influences, preadaptations in certain lineages (e.g., possession of a minimal molecular machinery for cellular interactions) helped determine in which lines the transition to multicellularity occurred.

Although the question of how animals evolved from their protozoan ancestors has at times seemed intractable, recent developments in phylogenetics and the emergence of comparative genomics have paved the way for new insights. Long-standing hypotheses regarding the identity of our protozoan relatives and the cellular foundations of development are now topics of active inquiry. While comparative embryology and genomics within animals have been fruitful for inferring early events in the radiation of Metazoa, I argue here that meaningful insights into animal origins will require greater focus on protozoan biology, diversity, and genomics.

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The Benefits of Staying Together

Upon completion of each round of mitosis, daughter cells have two options: they can migrate apart, dedicating themselves to a unicellular existence, or stay together, taking the first step toward integrated multicellularity (Figure 1A). Alternatively, cells can aggregate and develop coordinated structure and behavior (Kaiser, 2001). Nonetheless, for over 1500 million years after the origin of eukaryotes, not one known eukaryotic foray into multicellularity stuck, leaving the early fossil record devoid of multicellular eukaryotes (Knoll, 2003). Thus, the organisms to which animals and other macroscopic groups owe their origins were shaped over millennia by the demands of unicellular life. This historical predisposition of eukaryotes to the unicellular lifestyle begs the question of what selective advantages might have been conferred by the transition to multicellularity.

Escape from Predation

For reasons not entirely clear, possibly dependent upon environmental conditions and the simplicity of the food web, new species evolved and expired at a relatively slow rate until ~1500 MYA. Then, in an accelerating wave of evolution, the fossil record depicts a series of morphological experiments by eukaryotes, including filamentous forms of red algae (1200 MYA), green algae (ancestors to the green plants; 750 MYA), and early evidence of animal embryos (598 MYA; Knoll, 2003). Furthermore, after a long history of autotrophy (e.g., photosynthesis) among eukaryotes, the finding of testate amoeba fossils in 750 million-year-old sediments documents early evidence of heterotrophy (Porter et al., 2003).

In addition to environmental factors, what cell biological changes might have contributed to this increase in morphological evolution? One explanation for the sudden evolution and radiation of multicellular eukaryotes posits that with the origin of predation, and particularly the ability to engulf prey through phagocytosis, came selection for multicellularity among normally unicellular prey (Stanley, 1973). The first heterotrophs probably targeted small bacteria and particles of detritus, with the ability to ingest larger cells (e.g., algae and other heterotrophic protozoa) evolving later (Table 1). By assembling as a multicelled organism (either through aggregation or failure to separate following mitosis), prey could escape the upper limits of a predator's capacity to ingest foreign objects.

The potential effectiveness of phagotrophy as a selective agent for multicellularity has been demonstrated

Table 1. Glossary

heterotrophic	in the context of protozoa, the ability of cells to capture and feed upon other living cells, typically through phagocytosis
multicellular	possessing stably adherent cells whose activities are coordinated or integrated
protozoa	a diverse, polyphyletic group of mainly single-celled non-photosynthetic eukaryotes
Urmetazoan	the first multicellular animal; the progenitor of animal diversity

for laboratory cultures of unicellular eukaryotes. In an experimental predator-prey system, predation by the phagotrophic predator *Ochromonas vallescia* reproducibly selected for multicellularity within a population of the unicellular alga *Chlorella vulgaris* (Boraas et al., 1998). Whereas some predators secrete pheromones that can induce colony formation in their prey, the transition to multicellularity in this example was heritable and stable in the absence of the predator. The rapid evolution of multicellularity demonstrates a latent and normally untapped genetic potential within populations of *C. vulgaris*. Furthermore, it lends credence to the idea that predation may have selected for fixation of multicellularity in the unicellular progenitors of animals.

The Flagellar Synthesis Constraint

Given their unicellular lifestyle, the versatility and adaptability of protozoa is remarkable. Protozoa have evolved systems for locomotion, food capture, predator avoidance, and response to environmental perturbations, all while maintaining the capacity to divide and reproduce (Buss, 1983; Wolpert, 1992). Some of this juggling act comes at a cost, with cells specializing for one need at a time through a constant reorganization of cell morphology and behavior.

Two cellular activities in particular—motility and mitosis—compete for the same cellular machinery. Depending on its location in the cell and the phase of the cell cycle, the microtubule organizing center (MTOC) can serve either as a basal body supporting flagellar synthesis or a mitotic spindle supporting chromosome segregation. Hence, the challenge for those protozoa with limited numbers of MTOCs is to balance the requirements of motility against those of mitosis (Buss, 1987; Margulis, 1981). In organisms that have not resolved the competition between cell division and flagellar synthesis, the flagellum retracts and motility ceases prior to formation of the mitotic spindle (Buss, 1987; Margulis, 1981). This conflict seems to hold as well for animals. No flagellated or ciliated animal cell—including sperm, epithelial cells, nerve cells, and statocysts—ever divides (Buss, 1987; Cavalier-Smith, 1991; Margulis, 1981)

The flagellation constraint may have had important consequences for animal origins. Leo Buss has argued that the trade-off between locomotion and mitosis may have granted a selective advantage to multicellular variants in which these dueling functions were allocated to different sets of cells (Buss, 1983). Whereas the MTOCs of the unicellular ancestors of animals alternated between flagellar synthesis and mitotic spindle formation, thus sacrificing motility during cell division, colony formation would potentially circumvent the constraint. Initially, the division of labor between cell fission and motility may have been temporal, with colonies composed

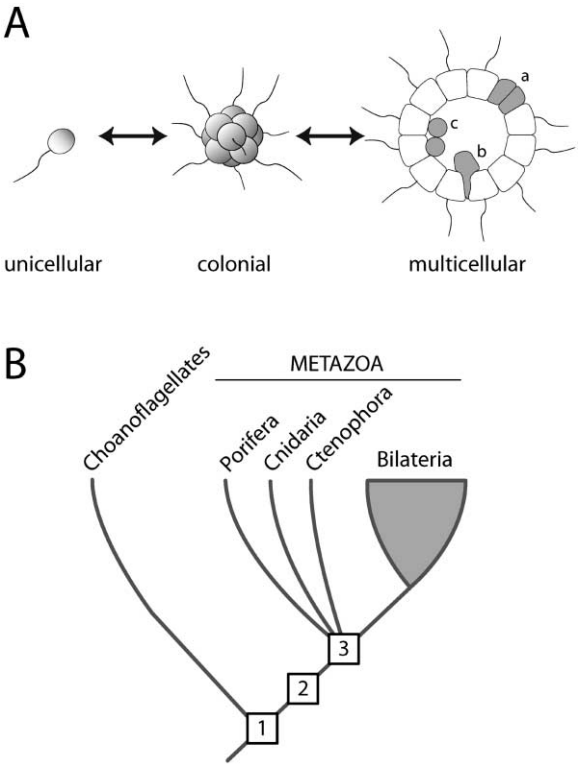

Figure 1. Stages in the Transition to Multicellularity

(A) From unicellular flagellates evolved motile colonies of multipotent cells. Genetic variants of colonial flagellates may have produced differentiated cells, and eventually given rise to multicellular, integrated individuals with subsets of cells dedicated to proliferation and others dedicated to preserving colony motility. Cells may divide on the colony surface (a) or introgress (b) and divide in the interior of the colony (c).

(B) Metazoa are monophyletic, and their last common ancestor, the Urmetazoan, was multicellular (3). The multicellular Urmetazoan evolved from a colonial flagellate (2), whose last common ancestor with choanoflagellates was a unicellular flagellate (1).

of multipotent cells each of which took turns either dividing or providing flagellar activity. For each cell in a colony, a benefit of cooperation would be the maintenance of motility during cell division.

As motile colonies grew, the balance between flagellated and dividing cells would have been critical for the maintenance of motility (Figure 1A; Buss, 1983). Too many dividing (and therefore unflagellated) cells would create an overpowering drag on the colony. Buss suggests that this constraint may have played into the evolution of gastrulation if selection acted against colonies with unflagellated cells on the surface. If cells primed for mitosis first migrated into the hollow center of the colony, they would have little impact on overall colony motility, thus balancing the flagellation constraint against environmental pressures (Figure 1A). Furthermore, this migration would have led to an early pattern of spatial differentiation, with flagellated cells on the periphery and unflagellated proliferating cells (precursors to the germline) in the interior (Buss, 1983). Therefore, a simple feature of protozoan cell biology (that is, the participation of the MTOC in both mitosis and motility) may have

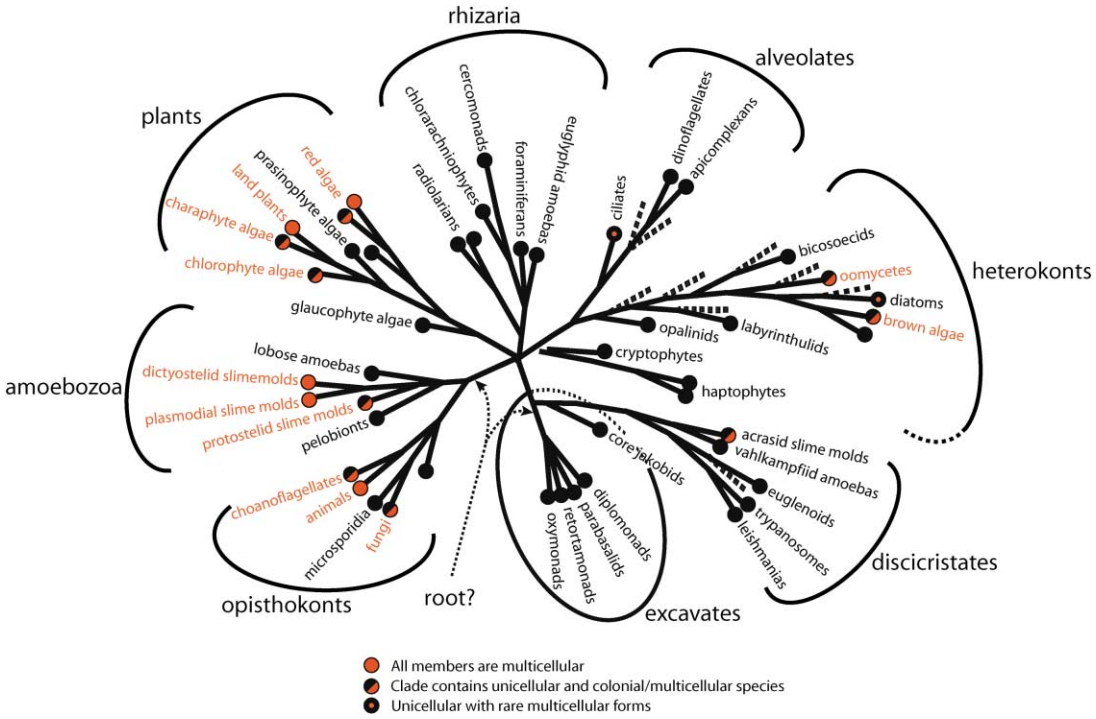

Figure 2. Diverse Origins of Multicellularity

Multicellular and colonial species are found throughout the diversity of eukaryotic phyla (Bonner, 1998; Buss, 1987). Although some phyla are strictly multicellular (e.g., land plants and animals), many more contain a mix of unicellular and multicellular forms. The apparent clustering of multicellularity among related branches of the tree suggests the existence of heritable genomic features that facilitate the evolution of higher order cellular interactions. With regard to animal origins, it is worth noting that the closest relatives, the choanoflagellates, are thought to be primitively unicellular and have evolved the ability to form colonies in some species. Modified from Baldauf, 2003.

profoundly impacted early events in animal evolution and development.

The Ties that Bind

Although studies of the transition to multicellularity were once hindered by uncertainty regarding the evolutionary relationships among extant taxa, progress on three phylogenetic issues has rekindled interest and opened up new avenues of research. Here I briefly discuss recent findings regarding the phylogenetics of multicellular organisms, the common ancestry of all Metazoa, and the close relationship between Metazoa and a special group of protozoa, the choanoflagellates.

Multiple Transitions to Multicellularity

To place the origin of animals from protozoa in context, it is valuable to consider the relationships among multicellular eukaryotes. Despite the challenges of inferring the evolutionary relationships from among long-diverged taxa, a consensus picture of eukaryotic phylogeny has emerged (Figure 2; Baldauf, 2003). Armed with a new understanding of the eukaryotic tree, we are now equipped to ask if all multicellular eukaryotes are related, reflecting a single transition to multicellularity, or if their evolutionary histories imply multiple independent origins of multicellularity. Mapping all known examples of multicellularity onto this phylogenetic framework reveals its roots throughout eukaryotic diversity (Bonner, 1998; Buss, 1987). Including the better-known multicellular groups (animals, land plants, fungi, and green, brown, and red algae), multicellularity appears in at least 16 independent eukaryotic lineages. In some of these lineages (e.g., Fungi) the relationships among diverse multicellular and unicellular members suggest that multicellularity evolved repeatedly after the initial radiation of the lineage and was subsequently lost in select taxa (Medina et al., 2001). In contrast, land plants and animals are entirely multicellular, suggesting that the transition from unicellularity occurred early in their evolutionary histories.

Three groups, the plants, amoebozoa, and opisthokonts, are particularly enriched for multicellularity, whereas others (e.g., the excavates, rhizaria, and alveolates) are notably deficient. The clustering of multicellular origins within closely related groups may indicate that some genomes and some cell biologies have been better building blocks for multicellularity than others. Additionally, the natural histories of some groups (e.g., their susceptibility to predation) may have generated greater or lesser selective advantages for colonial forms over solitary cells. The key to understanding the foundations of multicellularity and development in each multicellular lineage is to have a clearer picture of its unicellular prehistory.

Monophyly of Animals

A central question regarding animal origins, then, is whether animals are monophyletic and owe their history to a single transition to multicellularity, or polyphyletic, meaning Porifera (i.e., sponges) and the remaining animal phyla derive from two or more separate protozoan

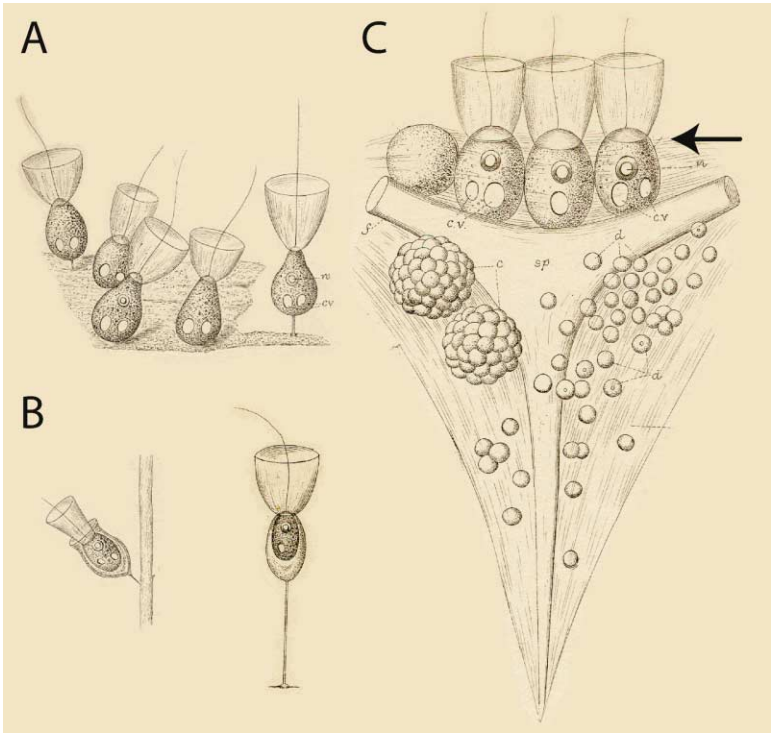

Figure 3. Resemblance between Choanoflagellates and Poriferan Choanocytes

In historical sketches by William Saville-Kent (*A Manual of the Infusoria* [London: David Brogue], 1880–1882), choanoflagellates (A and B) and choanocytes (C) are shown to display similar cellular architectures: a spherical or ovoid cell body and an apical flagellum subtended by a collar of tentacles. Both choanoflagellates and choanocytes use the flagellum to create water currents that propel bacterial food onto the collar for capture. (A) *Monosiga consociata* (as modified from plate IV-19; Saville-Kent); (B) (left) *Salpingoeca convallaria* (as modified from plate IV-13; Saville-Kent), (right) *Salpingoeca infusionum* (as modified from plate VI-8; Saville-Kent); (C) *Leucosolenia coriacea*. Triradiate spicule (sp) and three associated choanocytes (arrow) (as modified from plate X-2; Saville-Kent).

ancestors. Morphological analyses have been ambiguous, alternately supporting or rejecting the homology of early development in all animals, and highlighting morphological and cell ultrastructural similarities and differences between Porifera and the tissue-level Metazoa (reviewed in Leys, 2003; Maldonado, 2004; Morris, 1993; Nielsen, 1995). Further confusion has arisen from the observation that the unique and specialized feeding cells or "choanocytes" of Porifera bear a striking resemblance to a class of protozoa, the choanoflagellates (Figure 3; reviewed in Leadbeater and Kelly, 2001). Importantly, this cell type has been observed only in choanoflagellates and Metazoa, suggesting that the two groups share recent common ancestry.

The apparent homology between choanoflagellates and poriferan choanocytes originally prompted Henry James-Clark to regard sponges as highly specialized choanoflagellate colonies, and therefore separate from the animal lineage (James-Clark, 1868). With the subsequent discovery of collar cells in diverse non-poriferan animals (e.g., Cnidaria and Echinodermata), Porifera tentatively gained re-entry into Metazoa, but the controversy did not end (Lyons, 1973; Norrevang and Wingstrand, 1970). Both the homology of choanoflagellates and choanocytes, and the utility of morphological characters for assessing animal monophyly have been called into question (Ax, 1996; Karpov and Leadbeater, 1998; Mehl and Reiswig, 1991; Woollacott and Pinto, 1995).

As a complement to morphological data, comparisons of sequences from conserved genes may allow the inference of evolutionary relationships from among long-diverged and morphologically dissimilar taxa. In contrast with the uncertainty derived from morphological studies, analyses of ribosomal RNA sequences and sequences from low copy number nuclear genes provide independent, robust, and consistent support for the monophyly of Metazoa, including Porifera (Baldauf, 1999; Borchiellini et al., 1998; Cavalier-Smith et al., 1996; Schutze et al., 1999; Wainright et al., 1993).

Unicellular Relatives of Animals

The key phylogenetic question regarding animal origins concerns the identity of the closest protozoan relatives, particularly those that might inform considerations of the transition to multicellularity. The similarities between choanoflagellates, poriferan choanocytes, and the collar cells of Cnidaria and echinoderms prompted early speculation that animals might have evolved from a choanoflagellate-like ancestor. In fact, choanoflagellates are the only known protozoa whose cell biology uniquely allies them with Metazoa. Nonetheless, as mentioned earlier, there remains uncertainty about the validity of uniting choanoflagellates with Metazoa based upon the collar cell structure. To evaluate the evolutionary history of animals relative to choanoflagellates and other protozoa, several research groups have performed independent phylogenetic analyses of multiple nuclear and mitochondrial genes from diverse taxa. Although analyses of rRNA sequences tend not to provide sufficient resolution for the question of choanoflagellate relationships with Metazoa, those emphasizing protein sequences have consistently revealed strong statistical support for the grouping of choanoflagellates with animals (Atkins et al., 2000; Burger et al., 2003; Cavalier-Smith et al., 1996; King and Carroll, 2001; Kumar and Rzhetsky, 1996; Philippe et al., 2004; Ragan et al., 1996; Snell et al., 2001; Wainright et al., 1993; Zettler et al., 2001).

Given the potential importance of choanoflagellates as a window on animal origins, the question of choanoflagellate monophyly warrants further examination. In the small number of studies for which SSU rRNA sequences from multiple choanoflagellate species were

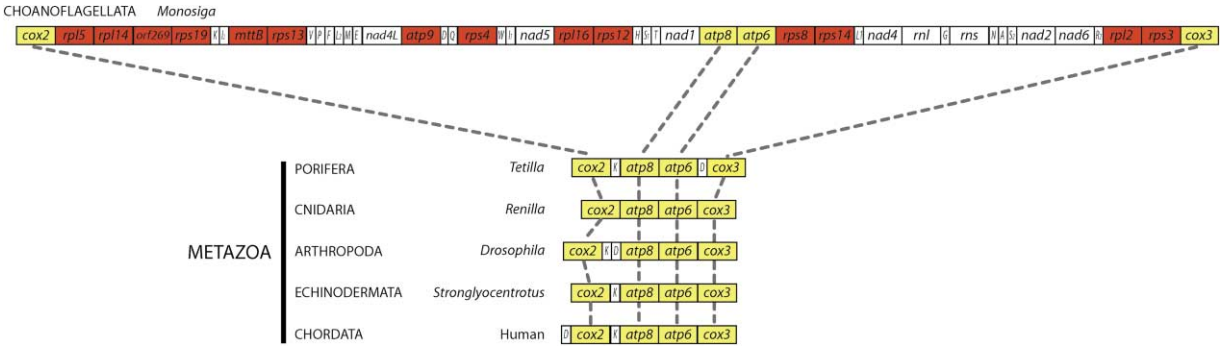

Figure 4. Choanoflagellates Are an Outgroup of Metazoa

The cox2-ATP8-ATP6-cox3 gene cluster (yellow) is conserved in the mtDNAs of diverse animals, including Porifera, Cnidaria, arthropods, echinoderms, and chordates (Boore, 1999; Watkins and Beckenbach, 1999). In contrast, the orthologous set of genes from a choanoflagellate mtDNA contains 22 additional protein-coding genes, 14 of which (red) are absent from animal mtDNAs (Burger et al., 2003). Modified from the supplement to King et al. (2003).

included, choanoflagellates have emerged as a monophyletic group (e.g., Medina et al., 2003). The analysis of multiple independent molecular markers (including protein coding genes) from a greater diversity of species will improve our understanding of the relationships among choanoflagellates. Regardless, the relationship between animals and the choanoflagellates thus far sampled has proven to be much closer than that between animals and their nearest multicellular neighbors, the Fungi.

A small number of nonchoanoflagellate protozoa of previously uncertain affinities, and with no obvious structural similarities to animal cells, have also emerged from SSU rRNA studies as possible members of the internode between Metazoa and Fungi (Mendoza et al., 2002). These taxa suffer from ambiguity regarding their exact phylogenetic placement relative to choanoflagellates and animals and, in some cases, have evolved parasitic lifestyles that may mask their common ancestry with animals. For example, although members of Class Mesomycetozoea appear monophyletic with choanoflagellates in some analyses of SSU rRNA, analyses of EF-1α failed to resolve their placement relative to animals and Fungi (Cavalier-Smith and Allsopp, 1996; Herr et al., 1999; Mendoza et al., 2002; Ragan et al., 1996, 2003; Zettler et al., 2001). In contrast, a recent study using 11 mitochondrial genes strongly supports their placement as an outgroup to the choanoflagellates + Metazoa (Burger et al., 2003). Inferences about the phylogenetic positions of other potential outgroups, e.g., *Corallochytrium limacisporum*, the nucleariid amoebae, and two *Ministeria* species, remain somewhat uncertain; future studies with larger numbers of independent molecular markers will help clarify their evolutionary relationships to animals (Cavalier-Smith and Chao, 2003; Medina et al., 2003; Zettler et al., 2001). As we learn more about the biology and phylogenetic relationships of these groups, they may offer important insights into protozoan evolution preceding the origin of Metazoa. In the meantime, the aggregate of morphological similarities between choanoflagellates and animals, and our relative confidence about their phylogenetic affinity, suggest that choanoflagellates are the most appropriate protozoan reference group for near-term studies of animals origins.

Are Choanoflagellates an Outgroup of Metazoa?

As confidence that choanoflagellates cluster closely with animals has increased, so has concern that they might, in fact, be degenerate Porifera (Maldonado, 2004; Rieger and Weyrer, 1998). Unfortunately, poor sampling of phylogenetically informative genes from Porifera has limited proper testing of the "Choanoflagellates from Porifera" hypothesis. To evaluate the finer-scale relationships between choanoflagellates and sponges and examine whether choanoflagellates diverged before the origin of animals or, instead, evolved from sponges, multiple protein sequences from diverse sponges, two choanoflagellates, and a variety of diploblasts and triploblasts have been collected (Rokas et al., 2003a). Analyses of the data set, the largest of its kind at the time, failed to resolve either the relationships between previously well-defined metazoan taxa (e.g., Bilateria) or those between animals and choanoflagellates. These findings indicate that data sets with what we now consider small numbers of genes are insufficient for resolving the relationships of early-branching Metazoa. Instead, the problem calls for much more sequence data than is commonly used (Rokas et al., 2003b). One such source of data has recently become available with the sequencing of a choanoflagellate mitochondrial genome (Burger et al., 2003; Lang et al., 2002).

The mitochondrial genomes of animals are highly reduced and compact relative to those of diverse protists, usually containing far fewer genes and little to no intergenic DNA (Boore, 1999; Gray et al., 1999; Lang et al., 1999). Sequences of the highly conserved cox2-ATP8-ATP6-cox3 gene cassette from two sponge species show that the compacted state of animal mtDNA evolved before the divergence of Porifera and Cnidaria from the lineage, giving rise to Bilateria (Figure 4; Watkins and Beckenbach, 1999). In contrast, the same quartet of genes from mtDNAs of diverse unicellular eukaryotes is embedded with many additional genes that, while common to protistan mtDNAs, are never found in animal mtDNAs. If choanoflagellates evolved from sponges, their mtDNAs should resemble animal mtDNA, lacking genes missing from animals, as well as introns and intergenic DNA. Instead, the region of choanoflagellate mtDNA containing cox2, ATP8, ATP6, and cox3 contains large numbers of extra genes found in protist mtDNAs

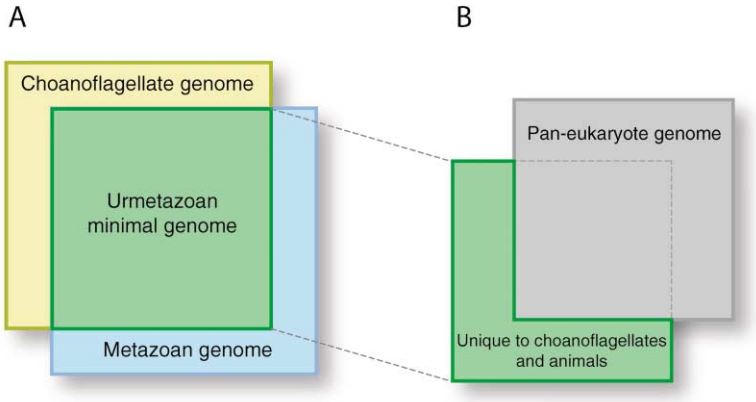

Figure 5. Inferring the Minimal Genomic Complexity of Common Ancestors

(A) Homologous genes found in choanoflagellates and animals represent the minimal genomic complexity of their most recent common ancestor (node 1, Figure 1), and of the Urmetazoan (node 3, Figure 1). Note that such comparisons reveal only the minimal genomic complexity of the common ancestor and that components of the ancestral genome may have been lost in one or more lineages.

(B) Genes unique to animals, choanoflagellates, and their most recent common ancestor may be identified by excluding genes (e.g., housekeeping genes) found in other eukaryotes (i.e., pan-eukaryote genes).

and the genome as a whole contains both intergenic DNA and introns (Figure 4; see supplement to King et al., 2003). Whereas the animal mtDNAs (including, apparently, those of sponges) evolved through early gene loss and compaction, the expanded state of choanoflagellate mtDNA suggests that choanoflagellates diverged before the origin and radiation of metazoan phyla (Boore, 1999; Gray et al., 1999; Lang et al., 1999). In other words, choanoflagellates are a bona fide outgroup of living animal phyla and provide an unprecedented opportunity to probe the origin and early evolution of Metazoa.

The Ancestral Genome

Ideally, one would like to learn how animals differ from their unicellular ancestors and identify the molecular genetic changes that made possible the origin of animals. Given the impossibility of directly examining the protozoan progenitor of animals, how might new insights on animal origins be derived? How might we learn which genes were important, and how novelties in transcriptional regulation might have contributed? How can we infer when the elements of animal multicellularity first evolved and what roles they originally played in the protozoan ancestors of animals?

By comparing the genomes of choanoflagellates with those of animals, we can infer the minimal genomic complexity of their most recent common ancestor (Figure 1B, node 1; Figure 5A) (Brooke and Holland, 2003; King et al., 2003). By broadening the comparison to include all other eukaryotes, we can identify genes that are shared uniquely between choanoflagellates and animals and those that have no known homologs outside of Metazoa (Figure 1B, node 3; Figure 5B). Within these subsets of the ancestral genome lie genes that played critical roles in the origin of animals.

The Parts List: Building the Urmetazoan Genome

While comparisons between the complete genomes of diverse animals and choanoflagellates promise to provide the most taxonomically informed perspective on animal origins, comparisons between animal and nonanimal genomes have already yielded valuable insights into the ground state of the Urmetazoan genome (Table 1). The shared ancestry of Metazoa predicts that all animals, including sponges, use a core set of genes to support the fundamentals of multicellularity: cell adhesion, signal transduction, and differentiation. Indeed, targeted investigations of candidate genes from sponges have uncovered numerous gene families required for cell interactions and the integrity of multicellular animals, including collagens, integrins, receptor tyrosine kinases (RTKs), and homeobox genes (Figure 6A) (Boute et al., 1996; Brower et al., 1997; Coutinho et al., 2003; Garrone, 1998; Suga et al., 2001., 1999; Wimmer et al., 1999). Functional analyses of sponge genes reveal similarities with the activities of homologs from other animals. For example, type IV collagen, which localizes to the basement membranes of bilaterians, also localizes to the basement membrane of a homoscleromorph sponge, suggesting that the two structures have a common origin (Boute et al., 1996). Similarities in function between poriferan and bilaterian genes have also been demonstrated for C-type lectins and β-integrin (Gundacker et al., 2001; Wimmer et al., 1999). As we learn more about the complement of gene families from sponges and can compare their functions with those in other animals, it may prove possible to trace the most basal aspects of animal development to the Urmetazoan (Muller et al., 2001).

A flurry of analyses accompanying the releases of the first genomes of eukaryotes revealed that many of the best-characterized protein domains involved in animal cell interactions (e.g., integrins, laminins, tyrosine kinases [TKs]) are apparently unique to animals (Arabidopsis Genome Initiative, 2000; Copley et al., 1999; Hutter et al., 2000; Hynes and Zhao, 2000; Rubin et al., 2000). In fact, protein domains involved in signal transduction, cell adhesion, and differentiation heavily populate lists of motifs with apparent restriction to animals (Table 2). Fundamental differences between the genome contents of animals and nonanimals, including a collection of over 800 protein domains found only in Metazoa, suggest a wealth of sequences for building unique facets of animal biology (Table 2). Considering the evolutionary distance between animals and the most closely related eukaryotes with sequenced genomes (Fungi), an unresolved question concerns whether "animal-specific" protein domains evolved before or after the origin of animals, and what their significance might be for the transition to multicellularity.

In contrast with the putatively "animal specific" protein domains, some components of animal signaling and

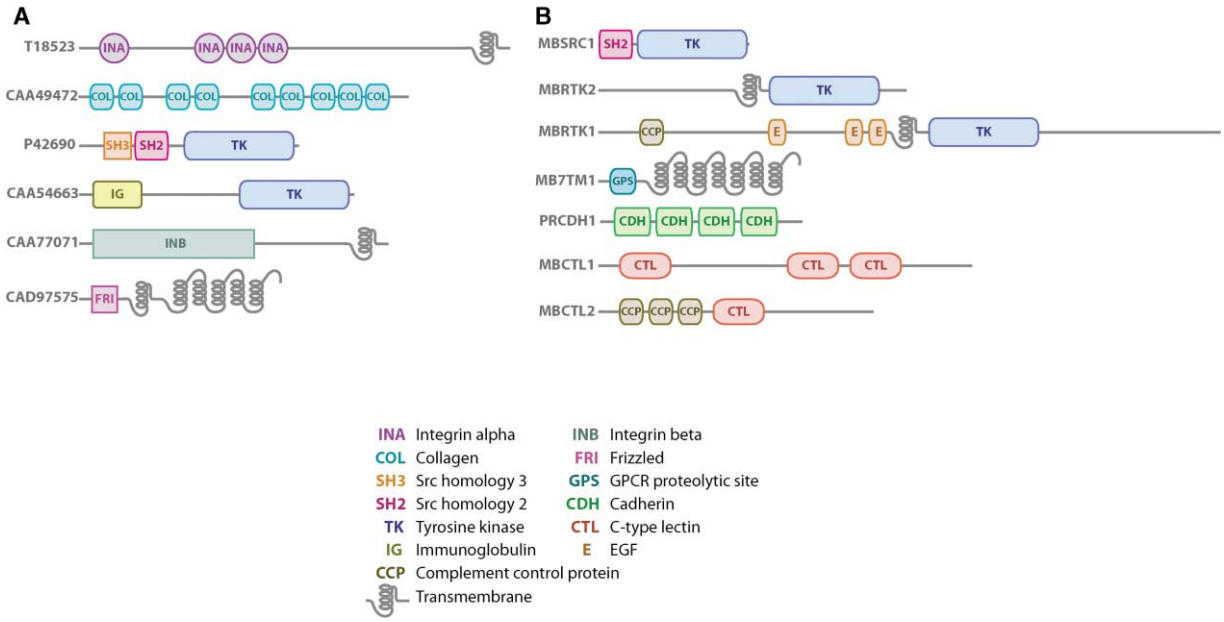

Figure 6. Multidomain Signaling and Adhesion Proteins from Porifera and Choanoflagellates

The domain architectures of representative genes from Porifera (A) and choanoflagellates (B) were predicted using PFAM (http://www.sanger.ac.uk/Software/Pfam/search.shtml) and SMART (http://smart.embl-heidelberg.de/). Poriferan sequences are labeled with their Genbank accession numbers. Note that some sequences (e.g., MBSRC1 and PRCDH1) are not full-length.

adhesion pathways have been identified in nonanimals. For example, the EGF-like domain, once thought to be diagnostic of animals, has now been found in diverse nonanimals including *Paramecium*, *Eimeria*, and *Dictyostelium* (Fey et al., 2002; Sperling et al., 2002; Tomley et al., 2001). Furthermore, homologs of animal β-catenin and STAT have been isolated from *Dictyostelium*, where they are used for signaling and adhesion (Grimson et al., 2000; Kawata et al., 1997). The finding in nonanimals of protein domains used for animal cell interactions hints that these domains, and the cellular activities they supported in ancient eukaryotes, may have served as preadaptations for the origin of animals. Alternatively, some components of the protein machinery that mediates animal cell interactions may have originally played other roles in ancestral unicellular eukaryotes before being co-opted to function in signaling and adhesion.

A first step in understanding the early evolutionary history (and prehistory) of the animal genome has been to catalog and characterize expressed genes of choanoflagellates. As might be predicted from the phylogenetic relationships between choanoflagellates and animals, the vast majority of choanoflagellate-expressed genes have homologs in animal genomes (King et al., 2003). Of particular interest is whether choanoflagellates express genes that are otherwise known only from animals; that is, genes of the type in Table 2. Surveys of expressed sequence tags (ESTs) and full-length cDNA sequences reveal choanoflagellates to express multiple members of gene families previously thought to be unique to animals. Despite the apparent simplicity of their lifestyle, choanoflagellates express a surprising diversity of animal signaling and adhesion gene homologs including TKs, G protein-coupled receptors, cadherins, and C-type lectins (Figure 6; King et al., 2003). In addition, several predicted polypeptides from choanoflagellates contain multiple protein-protein interaction domains (e.g., EGF, SH2, TNFR, and CCP) that typically function in animal signaling and adhesion proteins (Figure 6B).

These findings reveal that at least some gene families intimately linked to animal multicellularity and development evolved before the origin of animals, raising the possibility that they participated in the transition to multicellularity. A full recounting of the history of the animal proteome will require the comparison of complete genome sequences from diverse Porifera, Cnidaria, Ctenophora, choanoflagellates, and other unicellular relatives of animals. By identifying those genes shared with choanoflagellates and those found only in animals, it may prove possible to reconstruct early events in the assembly of the animal genome.

Evolving Novelty: Domain Shuffling, Gene Duplication, and Differential Gene Expression

In the face of apparently high levels of coding-sequence conservation among animals, and perhaps between animals and choanoflagellates, how might novel morphologies evolve? The increase in morphological complexity and the requirement for coordination of cellular activities during the transition to multicellularity would seem to have demanded radically new protein and cellular functions. Three complementary scenarios for the genomic bases of macroevolution have been proposed, each calling upon the modularity of the genome and its propensity for recombination: domain shuffling, gene duplication, and divergence, and the evolution of gene regulation (Bartel and Chen, 2004; Carrington and Ambros, 2003; Carroll, 2000; Levine and Tjian, 2002; Long, 2001; Lundin, 1999; Patthy, 1999). While there is evidence of

domain shuffling prior to the origin of animals, the extent to which the expansion of particular gene families and the evolution of novel modes of gene regulation coincided with and contributed to the transition to multicellularity is unknown (King et al., 2003).

(1) Domain Shuffling. Proteins with entirely new specificities and activities can evolve through domain shuffling, providing a potential route to rapid morphological evolution (Long, 2001; Patthy, 1999). The TK family, a diverse group of signal transduction proteins that regulates cell proliferation and differentiation in animals, beautifully demonstrates both the power and possibilities of domain shuffling. Particularly relevant to the discussion here, the central role of TKs in regulating morphogenesis and the expansion of the family in bilaterian animals have prompted the hypothesis that TK evolution was a key component of animal evolution (Darnell, 1997; Hunter and Cooper, 1985).

One family of TKs, the RTKs, no doubt owes its origin to domain shuffling. The canonical elements of RTKs, a cytoplasmic TK domain tethered through a transmembrane domain to an extracellular ligand binding domain, would have first been united through recombination (King and Carroll, 2001; Muller et al., 1999). Phylogenetic analyses of RTKs from diverse animals suggest the existence of approximately 30 distinct subfamilies, each with a different combination of extracellular ligand binding domains (Suga et al., 2001, 1999). Importantly, at least 15 (and probably more) of these subfamilies evolved before the divergence of Porifera from other Metazoa, suggesting that the Urmetazoan genome contained much of the TK diversity found in modern animals (Suga et al., 2001).

Although choanoflagellates have been less thoroughly sampled than Porifera, they are known to contain two RTKs and a Src kinase (Figure 6B; King et al., 2003). Importantly, the combination of extracellular domains found in one choanoflagellate TK, MBRTK1, does not directly match that of any known animal TK. A second choanoflagellate RTK, MBRTK2, lacks any identifiable ligand binding sequence. By analyzing the full diversity and abundance of TKs and members of other multidomain protein families from choanoflagellates, it may be possible to assess the extent and importance of domain shuffling during animal origins.

(2) Gene Duplication and Divergence. An enigma of protein evolution concerns an apparent constraint on protein function: how can new protein activities evolve without sacrificing the ancestral (and often essential) functions of the protein? A solution appears to have been rampant gene duplication and subsequent divergence (Lundin, 1999). Duplicate genes (paralogs) arise through local, regional, or total genome duplications, resulting in two genes of identical sequence and function (Sankoff, 2001). New paralogs can then diverge slowly, the sum of their activities including the ancestral role of their progenitor and new functions that evolve through selection and drift.

Gene duplication occurs frequently within extant populations, and large-scale fixations of duplicated genes have accompanied pivotal events in animal history: the origin of animals, the origin of Bilateria, and early vertebrate evolution (reviewed in Lundin, 1999; Lynch and Conery, 2000). The diversity and abundance of TKs, Gα proteins, protein tyrosine phosphatases, and collagens found in Porifera and Cnidaria approach those found in Bilateria, suggesting that the different protein families expanded through gene duplication prior to the initial radiation of the animal lineage (Exposito et al., 2002; Garrone, 1998; Ono et al., 1999; Suga et al., 1999). The extent to which these protein families expanded before or after the transition to multicellularity awaits further investigation of the genomes of choanoflagellates and related unicellular outgroups of animals.

In contrast with the early expansions of signaling and adhesion protein families, the Hox gene radiation seems to correlate more closely with the evolution of morphological complexity (Holland, 1999). With no Hox genes yet isolated from Porifera and two or fewer inferred for the common ancestor of Cnidaria, the Urmetazoan probably contained one or two Hox genes at most (Holland, 1999). In contrast, the progenitor of Bilateria probably had at least seven Hox genes and mice and humans have 39 (Holland, 1999; Prince, 2002). A thorough sampling of genomes from choanoflagellates and related protozoa will help resolve the question of whether Hox genes evolved before or after the origin of animals.

(3) Evolution of Gene Regulation. The real novelty in the transition to multicellularity is not simply that individual cells adhere or communicate, but that the functions of each component cell become integrated and interdependent (Buss, 1983; Michod, 2003; Szathmary and Maynard Smith, 1995). An important aspect of this integration in animals has been the differentiation of cells in time and space, a process largely directed by transcriptional and posttranscriptional regulation of gene activity. Whether novel modes of transcriptional regulation contributed to animal origins remains an unresolved issue, but one buoyed by comparisons of *cis*-regulatory DNA and transcription factor diversity in animal and fungal genomes (Levine and Tjian, 2002). Both the larger size and modular organization of metazoan *cis*-regulatory DNAs, which often contain multiple enhancers and fewer constraints on spacing, permit patterns of gene expression that are orders of magnitude more intricate than what is possible with the simple promoters of yeast (Levine and Tjian, 2002).

While the expanding complexity of transcriptional regulation in animals correlates with morphological diversification during metazoan evolution, it remains unclear whether it contributed to animal origins. Analyses of upstream sequences from a handful of poriferan genes hint at the existence of enhancers with combinations of transcription factor binding sites, and fusions of these sequences to reporters promotes expression in mammalian cell culture lines, but it has not been possible to test the endogenous activities of the purportedly *cis*-regulatory DNAs in vivo (Coutinho et al., 2003; Seack et al., 1999). Additionally, nothing is known about either the patterns or regulation of transcription in choanoflagellates. Until transcriptional regulation is better understood in Porifera and choanoflagellates, the connection between enhancer evolution and animal origins remains a mystery.

Likewise, little is known about how posttranscriptional phenomena, including alternative splicing of nascent transcripts and modulation of gene activity by small

Table 2. Diverse Protein Domains Found Solely in Metazoa

Accession	PFAM Domain	Function/Process
PF01064	Activin types I and II receptor domain	transforming growth factor β receptor activity
PF01586	Myogenic Basic domain	DNA binding transcription factor
PF01049	Cadherin cytoplasmic region	homophilic cell adhesion
PF01410	Fibrillar collagen C-terminal domain	extracellular matrix structural constituent
PF00812	Ephrin	membrane-attached ligand
PF01153	Glypican	extracellular matrix
PF01085	Hedgehog amino-terminal signaling domain	local and long-range signaling
PF00219	Insulin-like growth factor binding protein	insulin-like growth factor binding
PF05806	Noggin	BMP binding
PF00640	Phosphotyrosine interaction domain (PTB/PID)	phosphotyrosine binding
PF00907	T-box	transcription factor activity
PF00110	Wnt family	signal transducer activity

A list of 853 metazoan-specific domains was generated by the taxonomy search option at PFAM (http://pfam.wustl.edu/taxonomy.shtml). Select domains from the list are shown.

RNAs, may have contributed to the transition to multicellularity. The recent discovery of RNA-based gene regulation in diverse eukaryotes, including animals, plants, and the protozoan *Trypanosoma brucei*, suggests that the unicellular progenitor of animals was capable of regulating gene function and transcript abundance using small RNAs (Bartel and Chen, 2004; Carrington and Ambros, 2003; Ngo et al., 1998). An additional mode of regulating gene function posttranscriptionally, through alternative splicing of pre-mRNAs, contributes significantly to the complexity of metazoan transcriptomes and is gaining increasing attention within the plant community (Kazan, 2003; Maniatis and Tasic, 2002). However, with few complete genome sequences from nonmodel eukaryotes, it has been difficult to assess the relative frequency of alternative splicing either within or beyond the Metazoa. While our current understanding of posttranscriptional events in nonmodel organisms lags behind our knowledge of transcriptional regulation, genome-scale analyses of RNA-based gene regulation and alternative splicing in a broader diversity of taxa promise to provide clearer insights into their evolutionary histories and potential roles in the origin of animals.

Protozoan Antecedents to Multicellularity

The integrity of multicellular organisms requires stable adhesion between neighboring cells, coordination of cellular behavior (e.g., the cell cycle and cell migration) through cell-cell signaling, and fine-scale regulation of gene activity to control the identity and spatial distribution of differentiated cells. A paradox of the transition to multicellularity is that much of the requisite molecular machinery first evolved in unicellular protozoa and was later co-opted to support robust cellular interactions. Potential discomfort with the seeming unlikelihood of this scenario may be reduced with a broader appreciation of the diverse cellular activities of extant protozoa (Table 3; Wolpert, 1994). For example, cellular adhesion between animal cells may derive from protein families previously used by heterotrophic flagellates to recognize and capture select bacterial and protozoan prey. In fact, tethering of unicellular prey to the cell membranes of predatory protozoa may be considered the single-celled equivalent of animal cell adhesion. While remarkably little is known about the mechanisms underlying prey capture in protozoa, close examination of feeding in choanoflagellates might reveal unexpected commonalities with cell adhesion in animals (King et al., 2003).

Even more so than cell adhesion, the ability of protozoa to monitor and respond to cues from their changing environments means that metazoan signal transduction has many potential antecedents from the life histories of free-living protozoa. Critical aspects of protozoan cellular behavior are induced by chemical cues and secreted peptides, presumably through the activation of signaling cascades (Table 3; Gortz et al., 1999). In a lovely example, a small secreted peptide (A-factor) induces dramatically different cellular activities in a protozoan predator, *Amoeba proteus*, and its prey, ciliates of the genus *Euplotes* (Kusch, 1999). A-factor synthesized and secreted by *A. proteus* induces avoidance behavior in *E. octocarinatus,* allowing the prey to survive and coexist in the presence of an aggressive predator. In contrast, members of species *E. aediculatus* do not respond to A-factor and rapidly succumb to predation by *A. proteus*. Curiously, A-factor also induces a behavioral change in the predator, inhibiting phagocytosis of micrometer-sized beads by *A. proteus*. A related defense-inducing hormone from another protozoan, *Lembadion bullinum*, localizes to the cell surface, suggesting that a single peptide induces avoidance behavior in prey and acts as a self-recognition molecule to prevent cannibalism among conspecific predators (Kusch, 1999; Peters-Regehr et al., 1997).

A hallmark of animal multicellularity is the division of labor through highly regulated cell differentiation. Unicellular eukaryotes are also capable of differentiation; in their case, differentiation occurs temporally rather than spatially, often in response to environmental cues or biotic signals. The best-studied examples are of mating-type development and encystment/excystment (Table 3). Many protozoa capable of sexual reproduction favor asexual propagation given sufficient nutrient availability. For example, when nutrient levels fall, *Euplotes* cells transform into preconjugants capable of mating with cells of a complementary mating type (Ortenzi et al., 2000; Weiss et al., 1995). Multiple distinct mating types arise through cell differentiation, presumably the

Table 3. Signal Transduction and Adhesion Mediate Diverse Behaviors in Unicellular Protozoa

Behavior	Cellular Activity [a]	Species	Molecular Determinant	Reference(s)
Mating	Induction of conjugation (S)	*Blepharisma japonicum*	blepharmone glycoprotein	Sugiura and Harumoto, 2001
		Euplotes octocarinatus	Phr2b (membrane bound)	Mollenbeck and Heckmann, 2002
	Mating-type recognition (S)	*Euplotes raikovi*	Er-1mem	Weiss et al., 1995; Ortenzi et al., 2000
	Mating cell paring (A)	*Euplotes raikovi*	Er-1mem	Weiss et al., 1995; Ortenzi et al., 2000
Feeding	Food recognition (S)	*Actinophrys sol*	40-kDa glycoprotein	Sakaguchi, 2001
	Prey capture (A)	*Actinophrys sol*	40-kDa glycoprotein	Sakaguchi, 2001
Self-defense	Parasite avoidance (S)	*Alexandrium ostenfeldii*	unknown	Toth et al., 2004
	Predator avoidance (S)	*Euplotes octocarinatus*	A-factor polypeptide	Kusch, 1999
	Self-recognition (S)	*Amoeba proteus*	A-factor polypeptide	Kusch, 1999
		Actinophrys sol	40-kDa glycoprotein	Sakaguchi, 2001
	Excystment/Encystment (S)	*Sterkiella histriomuscorum*	cysteine proteases	Villalobo et al., 2003
Growth	Cell proliferation (S)	*Monosiga brevicollis*	tyrosine kinases	King, 2003
		Euplotes raikovi	Er-1	Weiss et al., 1995; Ortenzi et al., 2000

[a] S, Signal transduction; A, Adhesion

product of changes in gene expression and activity. Signaling between conjugants, either in the form of secreted pheromones or membrane-bound receptors, allows proper sorting of cells into pairs with complementary mating types. Finally, stable cell adhesion between each pair permits the exchange of genetic material.

Choanoflagellates may provide the most direct insights into the ancestral functions of animal signaling, adhesion, and transcription factor gene families. For example, with the finding of TKs in choanoflagellates, we can begin to ask what function TK signaling plays in a unicellular context. The presence of TKs in choanoflagellates argues that they evolved first in protozoa and were later co-opted into the multicellular lifestyle. By disrupting TK activity with specific pharmacological inhibitors, it has been possible to demonstrate a requirement for TK activity during choanoflagellate proliferation (King et al., 2003). Furthermore, choanoflagellates appear to interpret extracellular signals through a TK signaling pathway; changes in nutrient availability cause the profile of tyrosine-phosphorylated proteins to change rapidly (King et al., 2003). The development of techniques for targeting the functions of specific choanoflagellate genes in vivo will facilitate further investigations into the unicellular ancestry of animal signaling and adhesion. By examining signaling and adhesion gene homologs in diverse protozoa, we may identify previously unrecognized connections between the cell biology of animals and their protozoan relatives.

Back to the Future

This is an exciting time for research on animal origins, a time with great promise for new insights into a pivotal event in life's history. Meaningful progress in our understanding of animal origins rides on future developments in phylogenetics, poriferan embryology, comparative genomics, and the development of new protozoan model systems. First, to the extent possible, we need to remove all remaining ambiguity regarding the evolutionary relationships among early-branching Metazoa (Porifera, Cnidaria, and Ctenophora), choanoflagellates, and protozoan relatives of choanoflagellates (e.g., *Corallochytrium*, *Ministeria*, and *Amoebidium*). Second, comparative embryology within the Porifera and between Porifera and other Metazoa will aid inferences on the ground state of metazoan development, the fundamental elaboration of animal multicellularity. Third, comparisons between complete genomes of Porifera, choanoflagellates, and further removed protozoan outgroups will help depict the assembly of the genome during the transition to multicellularity. Finally, it will be crucial to develop a phylogenetically relevant protozoan model system (i.e., the choanoflagellates) in which to examine the activities of animal cell signaling, adhesion, and transcription factor gene homologs. By bringing functional approaches (e.g., RNAi) to bear on the problem of animal origins, we may make unprecedented inferences about the biology of the Urmetazoan and its spawning of animal diversity.

The starting point in our search to understand animal origins has been to identify which elements of animal genomes are shared with their protozoan relatives and which are unique. The holy grail will be to distinguish the genetic changes that laid the foundation for the transition to multicellularity. Let protozoa show the way.

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References

Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature *408*, 796–815.

Atkins, M.S., McArthur, A.G., and Teske, A.P. (2000). Ancyromonadida: A new phylogenetic lineage among the protozoa closely related to the common ancestor of metazoans, fungi, and choanoflagellates (Opisthokonta). J. Mol. Evol. *51*, 278–285.

Ax, P. (1996). Multicellular Animals. A New Approach to the Phylogenetic Order in Nature (New York: Springer).

Baldauf, S. (1999). A search for the origins of animals and fungi: comparing and combining molecular data. Am. Nat. *154*, S178–S188.

Baldauf, S.L. (2003). The deep roots of eukaryotes. Science *300*, 1703–1706.

Bartel, D.P., and Chen, C.Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat. Rev. Genet. *5*, 396–400.

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

Boore, J.L. (1999). Animal mitochondrial genomes. Nucleic Acids Res. *27*, 1767–1780.

Boraas, M., Seale, D., and Boxhorn, J. (1998). Phagotrophy by a flagellate selects for colonial prey: a possible origin of multicellularity. Evol. Ecol. *12*, 153–164.

Borchiellini, C., Boury-Esnault, N., Vacelet, J., and Le Parco, Y. (1998). Phylogenetic analysis of the Hsp70 sequences reveals the monophyly of Metazoa and specific phylogenetic relationships between animals and fungi. Mol. Biol. Evol. *15*, 647–655.

Boute, N., Exposito, J.Y., Boury-Esnault, N., Vacelet, J., Noro, N., Miyazaki, K., Yoshizato, K., and Garrone, R. (1996). Type IV collagen in sponges, the missing link in basement membrane ubiquity. Biol. Cell *88*, 37–44.

Brooke, N.M., and Holland, P.W.H. (2003). The evolution of multicellularity and early animal genomes. Curr. Opin. Genet. Dev. *13*, 599–603.

Brower, D.L., Brower, S.M., Hayward, D.C., and Ball, E.E. (1997). Molecular evolution of integrins: genes encoding integrin beta subunits from a coral and a sponge. Proc. Natl. Acad. Sci. USA *94*, 9182–9187.

Burger, G., Forget, L., Zhu, Y., Gray, M.W., and Lang, B.F. (2003). Unique mitochondrial genome architecture in unicellular relatives of animals. Proc. Natl. Acad. Sci. USA *100*, 892–897.

Buss, L.W. (1983). Evolution, development, and the units of selection. Proc. Natl. Acad. Sci. USA *80*, 1387–1391.

Buss, L.W. (1987). The Evolution of Individuality (Princeton, N.J.: Princeton University Press).

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

Carroll, S.B. (2000). Endless forms: the evolution of gene regulation and morphological diversity. Cell *101*, 577–580.

Carroll, S.B. (2001). Chance and necessity: the evolution of morphological complexity and diversity. Nature *409*, 1102–1109.

Cavalier-Smith, T. (1991). Cell diversification in heterotrophic flagellates. In The Biology of Free-living Heterotrophic Flagellates, D.J. Patterson and J. Larsen, eds. (Oxford: Clarendon Press), pp. 113–131.

Cavalier-Smith, T., and Allsopp, M.T. (1996). Corallochytrium, an enigmatic non-flagellate protozoan related to choanoflagellates. Eur. J. Protistol. *32*, 306–310.

Cavalier-Smith, T., and Chao, E.E. (2003). Phylogeny of choanozoa, apusozoa, and other protozoa and early eukaryote megaevolution. J. Mol. Evol. *56*, 540–563.

Cavalier-Smith, T., Allsopp, M.T.E.P., Chao, E.E., Boury-Esnault, N., and Vacelet, J. (1996). Sponge phylogeny, animal monophyly, and the origin of the nervous system: 18S rRNA evidence. Can. J. Zool. *74*, 2031–2045.

Copley, R.R., Schultz, J., Ponting, C.P., and Bork, P. (1999). Protein families in multicellular organisms. Curr. Opin. Struct. Biol. *9*, 408–415.

Coutinho, C.C., Fonseca, R.N., Mansure, J.J.C., and Borojevic, R. (2003). Early steps in the evolution of multicellularity: deep structural and functional homologies among homeobox genes in sponges and higher metazoans. Mech. Dev. *120*, 429–440.

Darnell, J.E., Jr. (1997). Phosphotyrosine signaling and the single cell:metazoan boundary. Proc. Natl. Acad. Sci. USA *94*, 11767–11769.

Exposito, J.Y., Cluzel, C., Garrone, R., and Lethias, C. (2002). Evolution of collagens. Anat. Rec. *268*, 302–316.

Fey, P., Stephens, S., Titus, M.A., and Chisholm, R.L. (2002). SadA, a novel adhesion receptor in Dictyostelium. J. Cell Biol. *159*, 1109–1119.

Garrone, R. (1998). Evolution of metazoan collagens. Prog. Mol. Subcell. Biol. *21*, 119–139.

Gortz, H.D., Kuhlmann, H.W., Mollenbeck, M., Tiedtke, A., Kusch, J., Schmidt, H.J., and Miyake, A. (1999). Intra- and intercellular communication systems in ciliates. Naturwissenschaften *86*, 422–434.

Gray, M.W., Burger, G., and Lang, B.F. (1999). Mitochondrial evolution. Science *283*, 1476–1481.

Grimson, M.J., Coates, J.C., Reynolds, J.P., Shipman, M., Blanton, R.L., and Harwood, A.J. (2000). Adherens junctions and beta-catenin-mediated cell signalling in a non-metazoan organism. Nature *408*, 727–731.

Gundacker, D., Leys, S.P., Schroder, H.C., Muller, I.M., and Muller, W.E. (2001). Isolation and cloning of a C-type lectin from the hexactinellid sponge *Aphrocallistes vastus*: a putative aggregation factor. Glycobiology *11*, 21–29.

Herr, R.A., Ajello, L., Taylor, J.W., Arseculeratne, S.N., and Mendoza, L. (1999). Phylogenetic analysis of Rhinosporidium seeberi's 18S small-subunit ribosomal DNA groups this pathogen among members of the protoctistan *Mesomycetozoa clade*. J. Clin. Microbiol. *37*, 2750–2754.

Holland, P.W. (1999). Gene duplication: past, present and future. Semin. Cell Dev. Biol. *10*, 541–547.

Hunter, T., and Cooper, J.A. (1985). Protein-tyrosine kinases. Annu. Rev. Biochem. *54*, 897–930.

Hutter, H., Vogel, B.E., Plenefisch, J.D., Norris, C.R., Proenca, R.B., Spieth, J., Guo, C., Mastwal, S., Zhu, X., Scheel, J., and Hedgecock, E.M. (2000). Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes. Science *287*, 989–994.

Hynes, R.O., and Zhao, Q. (2000). The evolution of cell adhesion. J. Cell Biol. *150*, F89–F96.

James-Clark, H. (1868). On the spongiae ciliatae as infusoria flagellata; or observations on the structure, animality, and relationship of *Leucosolenia botryoides*. Annals and Magazine of Natural History *1*, 133–142; 188–215; 250–264.

Kaiser, D. (2001). Building a multicellular organism. Annu. Rev. Genet. *35*, 103–123.

Karpov, S.A., and Leadbeater, B.S.C. (1998). Cytoskeleton structure and composition in choanoflagellates. J. Eukaryot. Microbiol. *45*, 361–367.

Kawata, T., Shevchenko, A., Fukuzawa, M., Jermyn, K.A., Totty, N.F., Zhukovskaya, N.V., Sterling, A.E., Mann, M., and Williams, J.G. (1997). SH2 signaling in a lower eukaryote: a STAT protein that regulates stalk cell differentiation in dictyostelium. Cell *89*, 909–916.

Kazan, K. (2003). Alternative splicing and proteome diversity in plants: the tip of the iceberg has just emerged. Trends Plant Sci. *8*, 468–471.

King, N., and Carroll, S.B. (2001). A receptor tyrosine kinase from choanoflagellates: molecular insights into early animal evolution. Proc. Natl. Acad. Sci. USA *98*, 15032–15037.

King, N., Hittinger, C.T., and Carroll, S.B. (2003). Evolution of key cell signaling and adhesion protein families predates animal origins. Science *301*, 361–363.

Knoll, A.H. (2003). Life on a Young Planet (Princeton, NJ: Princeton University Press).

Knoll, A.H., and Carroll, S.B. (1999). Early animal evolution: emerging views from comparative biology and geology. Science *284*, 2129–2137.

Kumar, S., and Rzhetsky, A. (1996). Evolutionary relationships of eukaryotic kingdoms. J. Mol. Evol. *42*, 183–193.

Kusch, J. (1999). Self-recognition as the original function of an amoeban defense-inducing kairomone. Ecology *80*, 715–720.

Lang, B.F., Gray, M.W., and Burger, G. (1999). Mitochondrial genome evolution and the origin of eukaryotes. Annu. Rev. Genet. *33*, 351–397.

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.

Leadbeater, B.S., and Kelly, M. (2001). Evolution of animals—choanoflagellates and sponges. Water and Atmosphere *9*, 9–11.

Levine, M., and Tjian, R. (2002). Transcription and the evolutionary diversification of the metazoa.

Leys, S. (2003). The significance of syncytial tissues for the position of the hexactinellida in the metazoa. Integr. Comp. Biol. *43*, 19–27.

Long, M. (2001). Evolution of novel genes. Curr. Opin. Genet. Dev. *11*, 673–680.

Lundin, L.G. (1999). Gene duplications in early metazoan evolution. Semin. Cell Dev. Biol. *10*, 523–530.

Lynch, M., and Conery, J.S. (2000). The evolutionary fate and consequences of duplicate genes. Science *290*, 1151–1155.

Lyons, K. (1973). Evolutionary implications of collar cell ectoderm in a coral planula. Nature *245*, 50–51.

Maldonado, M. (2004). Choanoflagellates, choanocytes, and animal multicellularity. Invertebr. Biol. *123*, 1–22.

Maniatis, T., and Tasic, B. (2002). Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature *418*, 236–243.

Margulis, L. (1981). Symbiosis in Cell Evolution: Microbial Communities in the Archean and Proterozoic Eons (New York, W.H. Freeman and Company).

Medina, M., Collins, A.G., Silberman, J.D., and Sogin, M.L. (2001). Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc. Natl. Acad. Sci. USA *98*, 9707–9712.

Medina, M., Collins, A., Taylor, J., Valentine, J., Lipps, J., Amaral-Zettler, L., and Sogin, M. (2003). Phylogeny of Opisthokonta and the evolution of multicellularity and complexity in Fungi and Metazoa. Int. J. Astrobiology *2*, 203–211.

Mehl, D., and Reiswig, H.M. (1991). The presence of flagellar vanes in choanomeres of porifera and their possible phylogenetic implications. Zeitschrift Fur Zoologische Systematik Und Evolutionsforschung *29*, 312–319.

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*, 314–344.

Michod, R.N., AM (2003). On the reorganization of fitness during evolutionary transitions in individuality. Integ Comp Biol *43*, 64–73.

Mollenbeck, M., and Heckmann, K. (2002). Identification and characterization of a pheromone 2 specific binding protein of *Euplotes octocarinatus*. Eur. J. Protistol. *37*, 391–403.

Morris, P.J. (1993). The developmental role of the extracellular matrix suggests a monophyletic origin of the kingdom Animalia. Evolution *47*, 152–165.

Muller, W.E., Kruse, M., Blumbach, B., Skorokhod, A., and Muller, I.M. (1999). Gene structure and function of tyrosine kinases in the marine sponge *Geodia cydonium*: autapomorphic characters in Metazoa. Gene *238*, 179–193.

Muller, W.E., Schroder, H.C., Skorokhod, A., Bunz, C., Muller, I.M., and Grebenjuk, V.A. (2001). Contribution of sponge genes to unravel the genome of the hypothetical ancestor of Metazoa (Urmetazoa). Gene *276*, 161–173.

Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA *95*, 14687–14692.

Nielsen, C. (1995). Animal evolution: interrelationships of the living phyla (Oxford: Oxford University Press).

Norrevang, A., and Wingstrand, K. (1970). On the occurrence and structure of choanocyte-like cells in some echinoderms. Acta Zool. *51*, 249–270.

Ono, K., Suga, H., Iwabe, N., Kuma, K., and Miyata, T. (1999). Multiple protein tyrosine phosphatases in sponges and explosive gene duplication in the early evolution of animals before the parazoan-eumetazoan split. J. Mol. Evol. *48*, 654–662.

Ortenzi, C., Alimenti, C., Vallesi, A., Di Pretoro, B., Terza, A.L., and Luporini, P. (2000). The autocrine mitogenic loop of the ciliate *Euplotes raikovi*: the pheromone membrane-bound forms are the cell binding sites and potential signaling receptors of soluble pheromones. Mol. Biol. Cell *11*, 1445–1455.

Patthy, L. (1999). Genome evolution and the evolution of exon-shuffling—a review. Gene *238*, 103–114.

Peters-Regehr, T., Kusch, J., and Heckmann, K. (1997). Primary structure and origin of a predator released protein that induces defensive morphological changes in Euplotes. Eur. J. Protistol. *33*, 389–395.

Philippe, H., Snell, E.A., Bapteste, E., Lopez, P., Holland, P.W., and Casane, D. (2004). Phylogenomics of eukaryotes: the impact of missing data on large alignments. Mol. Biol. Evol. *9*, 1740–1752.

Porter, S.M., Meisterfeld, R., and Knoll, A.H. (2003). Vase-shaped microfossils from the Neoproterozoic Chuar Group, Grand Canyon: a classification guided by modern testate amoebae. J. Paleontol. *77*, 409–429.

Prince, V. (2002). The Hox Paradox: more complex(es) than imagined. Dev. Biol. *249*, 1–15.

Ragan, M.A., Goggin, C.L., Cawthorn, R.J., Cerenius, L., Jamieson, A.V., Plourde, S.M., Rand, T.G., Soderhall, K., and Gutell, R.R. (1996). A novel clade of protistan parasites near the animal-fungal divergence. Proc. Natl. Acad. Sci. USA *93*, 11907–11912.

Ragan, M.A., Murphy, C.A., and Rand, T.G. (2003). Are Ichthyosporea animals or fungi? Bayesian phylogenetic analysis of elongation factor 1alpha of Ichthyophonus irregularis. Mol. Phylogenet. Evol. *29*, 550–562.

Rieger, R., and Weyrer, S. (1998). The evolution of the lower metazoa: evidence from the phenotype. In Molecular Evolution: Towards the Origin of Metazoa, W.E.G. Müller, ed. (Berlin, New York: Springer-Verlag), pp. 21–41.

Rokas, A., King, N., Finnerty, J., and Carroll, S.B. (2003a). Conflicting phylogenetic signals at the base of the metazoan tree. Evol. Dev. *5*, 346–359.

Rokas, A., Williams, B.L., King, N., and Carroll, S.B. (2003b). Genome-scale approaches to resolving incongruence in molecular phylogenies. Nature *425*, 798–804.

Rubin, G.M., Yandell, M.D., Wortman, J.R., Gabor Miklos, G.L., Nelson, C.R., Hariharan, I.K., Fortini, M.E., Li, P.W., Apweiler, R., Fleischmann, W., et al. (2000). Comparative genomics of the eukaryotes. Science *287*, 2204–2215.

Sakaguchi, M., Murakami, H., and Suzaki, T. (2001). Involvement of a 40-kDa glycoprotein in food recognition, prey capture, and induction of phagocytosis in the protozoon Actinophrys sol. Protist *152*, 33–41.

Sankoff, D. (2001). Gene and genome duplication. Curr. Opin. Genet. Dev. *11*, 681–684.

Schutze, J., Krasko, A., Custodio, M.R., Efremova, S.M., Muller, I.M., and Muller, W.E. (1999). Evolutionary relationships of Metazoa within the eukaryotes based on molecular data from Porifera. Proc. R. Soc. Lond. B. Biol. Sci. *266*, 63–73.

Seack, J., Kruse, M., Muller, I.M., and Muller, W.E.G. (1999). Promoter and exon-intron structure of the protein kinase C gene from the marine sponge *Geodia cydonium*: Evolutionary considerations and promoter activity. Biochim. Biophys. Acta *1444*, 241–253.

Snell, E.A., Furlong, R.F., and Holland, P.W.H. (2001). Hsp70 sequences indicate that choanoflagellates are closely related to animals. Curr. Biol. *11*, 967–970.

Sperling, L., Dessen, P., Zagulski, M., Pearlman, R.E., Migdalski, A., Gromadka, R., Froissard, M., Keller, A.M., and Cohen, J. (2002). Random sequencing of Paramecium somatic DNA. Eukaryot. Cell *1*, 341–352.

Stanley, S.M. (1973). Ecological theory for sudden origin of multicellular life in Late Precambrian. Proc. Natl. Acad. Sci. USA *70*, 1486–1489.

Suga, H., Koyanagi, M., Hoshiyama, D., Ono, K., Iwabe, N., Kuma, K., and Miyata, T. (1999). Extensive gene duplication in the early evolution of animals before the parazoan-eumetazoan split demonstrated by G proteins and protein tyrosine kinases from sponge and hydra. J. Mol. Evol. *48*, 646–653.

Suga, H., Katoh, K., and Miyata, T. (2001). Sponge homologs of

vertebrate protein tyrosine kinases and frequent domain shufflings in the early evolution of animals before the parazoan-eumetazoan split. Gene *280*, 195–201.

Sugiura, M., and Harumoto, T. (2001). Identification, characterization, and complete amino acid sequence of the conjugation-inducing glycoprotein (blepharmone) in the ciliate *Blepharisma japonicum*. Proc. Natl. Acad. Sci. USA *98*, 14446–14451.

Szathmary, E., and Maynard Smith, J. (1995). The major evolutionary transitions. Nature *374*, 227–232.

Tomley, F.M., Billington, K.J., Bumstead, J.M., Clark, J.D., and Monaghan, P. (2001). EtMIC4: a microneme protein from *Eimeria tenella* that contains tandem arrays of epidermal growth factor-like repeats and thrombospondin type-1 repeats. Int. J. Parasitol. *31*, 1303–1310.

Toth, G.B., Noren, F., Selander, E., and Pavia, H. (2004). Marine dinoflagellates show induced life-history shifts to escape parasite infection in response to water-borne signals. Proc. R. Soc. Lond. B. Biol. Sci. *271*, 733–738.

Villalobo, E., Moch, C., Fryd-Versavel, G., Fleury-Aubusson, A., and Morin, L. (2003). Cysteine proteases and cell differentiation: Excystment of the ciliated protist *Sterkiella histriomuscorum*. Eukaryot. Cell *2*, 1234–1245.

Wainright, P.O., Hinkle, G., Sogin, M.L., and Stickel, S.K. (1993). Monophyletic origins of the Metazoa: an evolutionary link with Fungi. Science *260*, 340–342.

Watkins, R.F., and Beckenbach, A.T. (1999). Partial sequence of a sponge mitochondrial genome reveals sequence similarity to Cnidaria in cytochrome oxidase subunit II and the large ribosomal RNA subunit. J. Mol. Evol. *48*, 542–554.

Weiss, M.S., Anderson, D.H., Raffioni, S., Bradshaw, R.A., Ortenzi, C., Luporini, P., and Eisenberg, D. (1995). A cooperative model for receptor recognition and cell adhesion: evidence from the molecular packing in the 1.6-A crystal structure of the pheromone Er-1 from the ciliated protozoan *Euplotes raikovi*. Proc. Natl. Acad. Sci. USA *92*, 10172–10176.

Wimmer, W., Perovic, S., Kruse, M., Schroder, H.C., Krasko, A., Batel, R., and Muller, W.E. (1999). Origin of the integrin-mediated signal transduction. Functional studies with cell cultures from the sponge *Suberites domuncula*. Eur. J. Biochem. *260*, 156–165.

Wolpert, L. (1992). Gastrulation and the evolution of development. Dev. Suppl., 7–13.

Wolpert, L. (1994). The evolutionary origin of development: cycles, patterning, privilege and continuity. Dev. Suppl., 79–84.

Woollacott, R., and Pinto, R. (1995). Flagellar basal apparatus and its utility in phylogenetic analyses of the porifera. J. Morphol. *226*, 247–265.

Zettler, L.A.A., Nerad, T.A., O'Kelly, C.J., and Sogin, M.L. (2001). The nucleariid amoebae: more protists at the animal-fungal boundary. J. Eukaryot. Microbiol. *48*, 293–297.