The single-celled ancestors of animals: a history of hypotheses

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Abstract

Animals, with their complex and obligate multicellularity, evolved from microbial eukaryotes that were likely obligately or facultatively unicellular. The nature of the unicellular progenitors of animals has intrigued biologists since the late 19th century, coinciding with the parallel spread of the cell theory and the theory of evolution. However, views on the ancestry of animals have been extremely varied. The huge diversity of single-celled organisms, the tremendous plasticity of animal cellular phenotypes, and the difficulties of organizing both into clear phylogenies in the pre-molecular era allowed a wide range of hypotheses to flourish, with nearly every major single-celled lineage, at one time or another, having been proposed as the precursor of animals (Table 13.1). Most of these hypotheses never gained followers beyond their originator (such as the ideas that animals evolved directly from either bacteria, *Volvox* or brown algae) and will not be discussed further (see Table 13.1). Three concepts, however, have been enduring and influential: (1) the amoeboid hypothesis; (2) the flagellate hypothesis; and the (3) the ciliate hypothesis – to which a fourth category can now be added: (4) a mixed model, in which the ancestor was phenotypically plastic. We will discuss their origin, history, and current relevance.

13.1 Origin of the question: the cell theory and the concept of common descent

The question of the single-celled ancestor of animals only makes sense in the light of two concepts that are now central to biology and that emerged in parallel in the second half of the 19th century: (1) the cell theory, which posits that all living beings are composed of cells (some of many, some of only one) (Schleiden, 1839; Schwann, 1839); and (2) the theory of common descent, which posits that all living species – unicellular or multicellular – descended from a single common ancestor (Darwin and Wallace, 1858).

That all living beings are made of cells is the first fact many of us learned about biology, and is so familiar that we sometimes take it for granted. But the cellular organization of all life forms was not initially obvious, and it took a full 250 years after the invention of the microscope for this idea to gain general acceptance. Two of the first people to observe microorganisms (van Leeuwenhoek, 1677; Müller, 1786) indiscriminately used the terms "infusorians" or "animalcules" to describe what we now think was a *mélange* of unicellular

Table 13.1. Timeline of hypotheses on the single-celled precursor of animals. Hypotheses are organized in chronological order. The four most influential hypotheses (the amoeboid hypothesis, flagellate hypothesis, ciliate hypothesis and amoeboflagellate hypothesis) are underlined in the table and discussed in specific sections in the text (sections 13.2, 13.3/13.4, 13.3/13.4 and 13.7/13.8 respectively). Note that proponents of the ciliate hypothesis usually thought that animals were polyphyletic, with sponges having evolved from flagellates and all other animals from ciliates.

Nature of the hypothesized ancestor	Proposed as early as	References
Amoeba	1876	(Haeckel, 1876, 1914), (Reutterer, 1969), (Hanson, 1977)
Ciliate	1882	(Kent, 1882), (Sedgwick, 1895), (Hadzi, 1953, 1963), (Steinböck, 1963), (Hanson, 1963, 1977)
Flagellate	1884	(Bütschli, 1884), (Metchnikoff, 1886), (Nielsen and Norrevang, 1985), (King, 2004), (Nielsen, 2008), (Cavalier-Smith, 2017)
Fucus-like syncytial brown alga	1924	(Franz, 1924)
Amoeboflagellate or complex ancestor	1949	(Zakhvatkin, 1949), (Sachwatkin, 1956), (Willmer, 1971), (Mikhailov *et al.*, 2009), (Arendt *et al.*, 2015), (Sebé-Pedrós, Degnan and Ruiz-Trillo, 2017), (Brunet *et al.*, 2021)
Volvox-like alga	1953	(Hardy, 1953)
Prokaryote	1974	(Pflug, 1974)

protists (e.g. ciliates, heliozoans, amoebae, and flagellates) and small multicellular animals (e.g. rotifers and flatworms). Multicellular organization was first described in 1665 by Robert Hooke based on his observations of dead plant tissue in the form of a bottle cork. Hooke was intrigued by the structures he was later to name "cells," but had no idea he had discovered a general phenomenon, and considered them a structural peculiarity of cork. An additional 170 years of research and many additional observations were needed before the official "birth date" of the cell theory, often attributed to Schwann (1838) and Schleiden (1839) (reviewed in (Morange, 2016). Once the cell theory was accepted, several early cell biologists (including Meyen (1839), Dujardin (1841), Barry (1843) and von Siebold (1845)) took the leap to posit that the simplest life forms might consist of only one cell (reviewed in Leadbeater and McCready, 2002).

The theory of evolution emerged in parallel with the cell theory. The first elaborate theory of evolution, proposed in 1809 by the French biologist Jean-Baptiste de Lamarck (1744-1829), assumed that life started with the spontaneous generation of "infusorians"– including both protists and small animals (Lamarck, 1809). Infusorians were then inferred to have gradually evolved into all other organisms through a progressive increase in size and complexity, with no individual step that would have clearly paralleled our modern concept of a transition to multicellularity. Lamarck's ideas attracted attention and criticism, but the concept of common descent did not become widely accepted until after the debate spurred by the theory of evolution through natural selection proposed by Charles Darwin (1809-1882) and Alfred Russel Wallace (1823-1913) (Darwin and Wallace, 1858; Darwin, 1859). Their theory was the first to propose a plausible mechanism for descent with modification, and thus brought new credibility to the concept of evolution.

By the end of the 19th century, the scientific stage was set for considering the origin of animals: both evolution and cell theory had gained widespread acceptance, and three of the most abundant and charismatic groups of single-celled organisms had been identified – flagellates, ciliates and amoebae (Table 13.1). Quickly, all three were considered as potential ancestors of animals.

13.2 Haeckel's hypothesis: amoebae as ancestors

The first researcher to attempt to reconstruct the unicellular progenitor of animals was the German biologist Ernst Haeckel (1834-1919), arguably one of Darwin's most high-profile supporters in continental Europe (Richards, 2008). While Haeckel's name is most often mentioned today in the context of his now-obsolete theory of recapitulation (according to which development directly recapitulated evolution (Gould, 1977)) or for his controversial drawings of vertebrate embryos (Pennisi, 1997; Richards, 2009), his contributions to biology were much broader, and one can get an idea of their scope by considering that he coined the words "ecology", "ontogeny", "phylogeny", and "gastrulation" among many others.

Haeckel had an exceptionally ambitious research program: organizing all of life's diversity into a phylogenetic framework and – if that was not enough – reconstituting the extinct ancestors that occupied the most important nodes of that tree. In his attempt to reconstitute the single-celled progenitor of animals, he inferred it was an amoeba based on two independent sources of evidence: (*1*) his theory of recapitulation; and (*2*) *Magosphaera planula*, a mysterious organism that he considered the "missing link" between protists and animals.

13.2.1 Haeckel's embryological arguments for an amoeboid animal ancestor

Haeckel's case for an amoeboid ancestor started with embryology (Haeckel, 1874, 1876, 1914). He noted that the egg cells of animals lack a flagellum but are often contractile. Moreover, he observed that in sponges, the unfertilized eggs are *bona fide* crawling amoeboid cells (later confirmed by Franzen, 1988; Ereskovsky, 2010). After fertilization, the sponge zygote divided to give rise to a ball of non-ciliated cells (the morula) that only later acquired cilia and collectively formed an internal space (thus becoming a blastula). According to Haeckel, future feeding cavities then formed during gastrulation. Seen through a recapitulationist lens, these developmental facts told a compelling evolutionary story: animals had evolved from free-

living amoebae that had first formed balls of cells before acquiring ciliation, an internal cavity and then, eventually, evolving a gut (Haeckel 1874, 1914; Fig. 13.1; Box 13.1). Embryology might have been enough to convince Haeckel of the amoeboid origin of animals. But he thought he had another critical piece of evidence: a "missing link."[1]

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[1] The concept of the extant "missing link" that represents the ancestral condition is no longer considered valid, but it accurately captures Haeckel's views, which often envisioned some living groups as identical to the ancestors of other living groups.
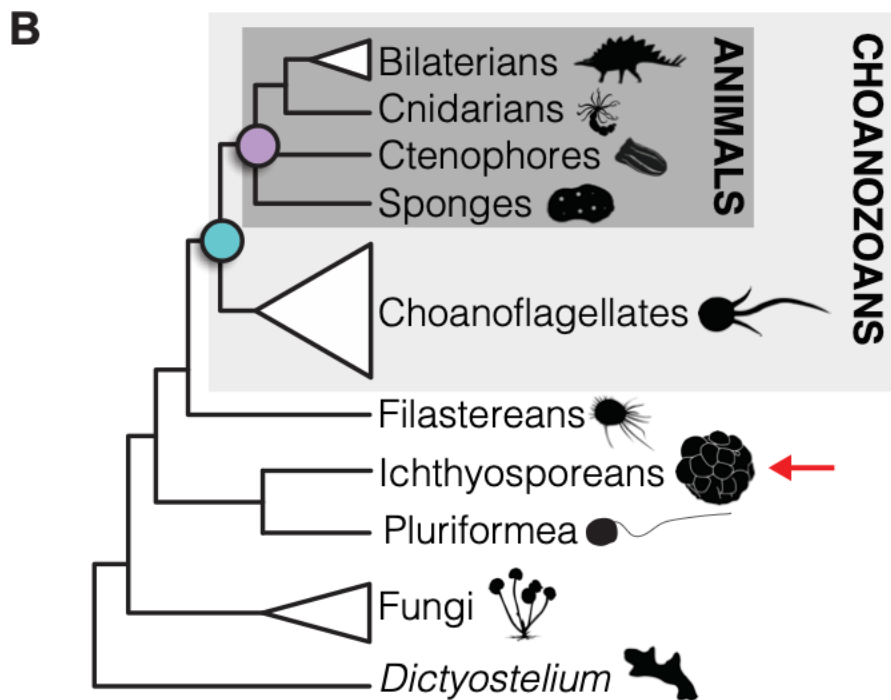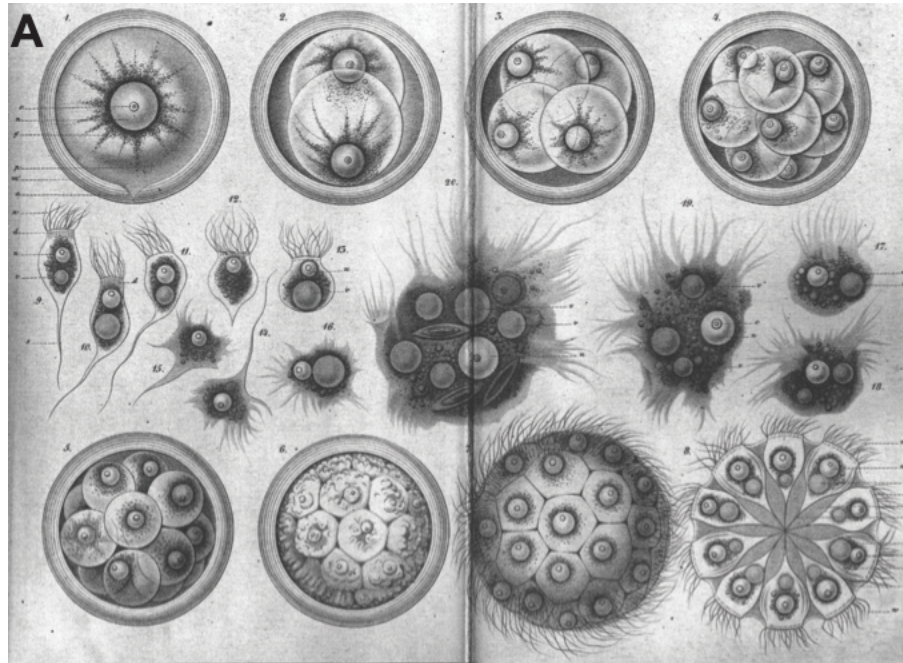
Figure 13.1. Haeckel's amoeboid hypothesis of animal origins. (A) The amoeboid egg cell of a sponge (from *Sycon raphanus* (Franzen, 1988)). (B) A free-living amoeba, *Chaos carolinense* (Creative Commons license; https://commons.wikimedia.org/wiki/File:Chaos_carolinense.jpg). (C) Haeckel's model of animal origins, in which ontogeny (development) parallels phylogeny (evolution). In both courses, the starting point was depicted as an amoeboid cell, similar to the sponge oocyte and free-living amoebae. Proliferation of such amoeboid cells was inferred to first result in the formation of a non-ciliated sphere, the Moraea/morula. Ciliation in this scenario only arose after the evolution/development of multicellularity, at the Blastaea/blastula stage. The

sketch of the cytula stage is a sponge egg cell (Haeckel, 1872) and sketches of the following stages are coral embryos (Haeckel, 1914).

13.2.2 Magosphera planula: *Haeckel's "missing link" between amoebae and animals*

Haeckel's purported encounter with *M. planula* (Haeckel, 1870, reviewed in Reynolds and Hülsmann 2008; see also Levit *et al.*, 2020) occurred in 1869 off the coast of Bergen, Norway. In a seaweed sample, Haeckel observed tiny round capsules (~ 70 μm large in diameter) that resembled egg cells, with a single central nucleus. These egg-like structures then started dividing at constant cell volume – like the cleavage of an early animal embryo – and gave rise to spheres of cells, each of which then acquired a covering of motile cilia. These ciliated spheres started swimming around, but did not develop further; instead, they fell to the bottom and dissociated into individual amoeboid cells that crawled around. Haeckel did not observe the further development of these amoebae but assumed they would eventually increase in volume to give rise to another spherical cell, thus completing the cycle (Haeckel, 1870) (Fig. 13.2A).

Insert Box 13.1 here

In Haeckel's view, *Magosphaera* provided an important window into animal origins. Its amoeboid single-celled form matched his recapitulation-inspired view of the animal ancestor. It had facultative multicellularity, which it reached by a cleavage process similar to animal embryos. It was a concrete, living embodiment of the Moraea stage of his evolutionary timeline.

Yet, *M. planula* is shrouded in mystery. Haeckel saw it only once and modern efforts to re-isolate it have failed (Reynolds and Hülsmann, 2008). It is an interesting exercise to take Haeckel's description at face value and wonder what he might have seen. The closest parallel to *Magosphaera* may be found among the ichthyosporeans, a lineage of unicellular opisthokonts closely related to animals and choanoflagellates (Fig. 13.2B). Ichthyosporeans are free-living amoebae or flagellates that, like *Magosphaera*, can grow into large round cysts that divide at constant cell volume and finally dissociate back into single cells (Mendoza, Taylor and Ajello, 2002; Glockling, Marshall and Gleason, 2013; Suga and Ruiz-Trillo, 2013). They differ from *Magosphaera* in three main ways: *(1)* the spherical multicellular form of known ichthyosporeans is never swimming or multiciliated; *(2)* almost all known ichthyosporeans are commensal or parasitic, not free-living; *(3)* the large ichthyosporean cysts are multinucleated and divide by simultaneous global cellularization around pre-existing nuclei (Dudin *et al.*, 2019), rather by serial binary division of a large, initially mononucleated cell (as in animal zygotes). However, and interestingly, an environmental metabarcoding study has suggested the existence of undescribed free-living ichthyosporeans (Del Campo and Ruiz-Trillo, 2013) and three apparently free-living species have since been isolated (belonging to the genera *Chromosphaera* (Grau-Bové *et al.*, 2017) and *Sphaeroforma* (Hassett, López and Gradinger, 2015)).
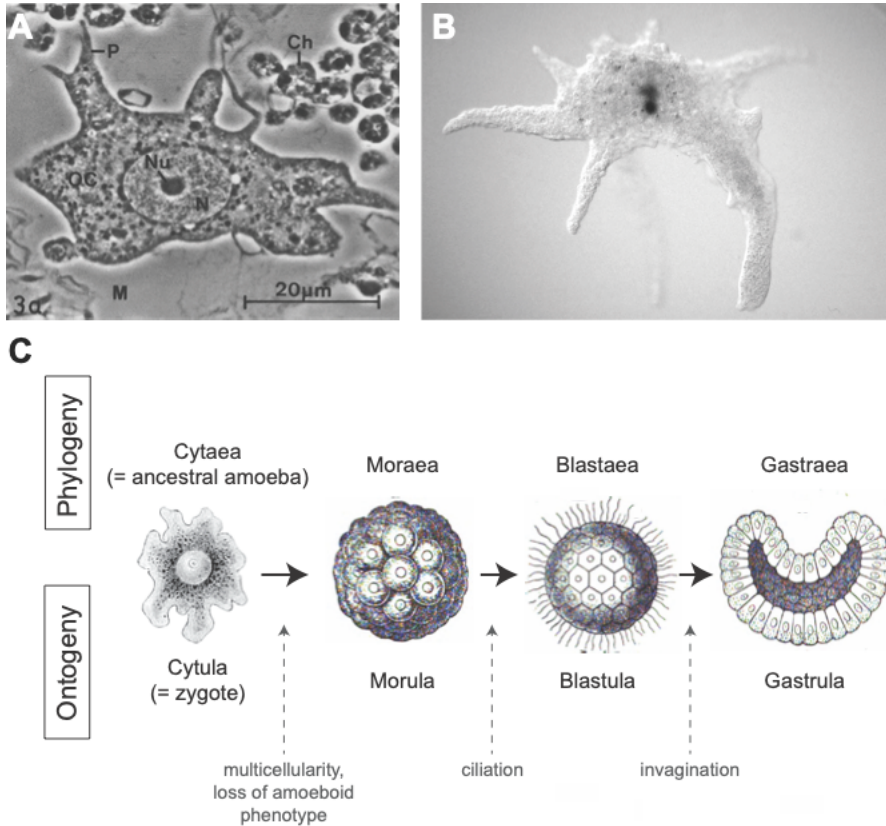
Figure 13.2. Mystery Organism 1: *Magosphaera planula*, a facultatively multicellular amoeba described by Haeckel but never re-observed, which he thought resembled the ancestor of animals. (A) Haeckel's depiction of the life history of *M. planula* (Haeckel, 1870). The first and third row depict the cleavage of the large, spherical cell initially observed by Haeckel, resulting in a swimming sphere of multiciliated cells. The second row shows the cells produced by dissociation of that sphere, which are first multiciliated and then become amoeboid. (B) Modern phylogenetic tree showing interrelationships between animals and their closest relatives (modified from (Nichols, Dayel and King, 2009). Ichthyosporeans (red arrow) belong to a lineage considered among the closest relatives of animals and form spherical masses of spores by cleavage at constant volume, which evokes *M. planula*.

If Haeckel's description was accurate, he could be the first person to have seen a free-living ichthyosporean, that would have (unlike other described ichthyosporeans) undergone serial binary cleavage from a mononucleated cyst. Alternatively, he might have misinterpreted – or exaggerated – what he saw.

For all of Haeckel's fame, his amoeboid hypothesis never seems to have gained followers. The reasons for this are unclear, but his hypothesis may have suffered from the rise of a worthy competitor: the flagellate hypothesis of animal origins.

13.3 Metchnikoff's hypothesis: choanoflagellates as ancestors

Colonies of flagellates have been known since van Leeuwenhoek first observed *Volvox* (van Leeuwenhoek, 1677). The similarity of such colonies to the blastula stage of animal development (which impressed even Haeckel (Haeckel, 1914)) seemed to suggest a plausible evolutionary path from flagellates to animals – an idea that emerged shortly after Haeckel's amoeboid hypothesis of animal origins.

A first piece of evidence was the striking similarity between choanoflagellates and the feeding cells of sponges, the choanocytes. Both have a near-identical appearance with a flagellum surrounded by a ring of microvilli, together forming a "collar complex" (Brunet and King, 2017) (Fig. 13.3A, B). This resemblance was already evident to some of the earliest choanoflagellate observers, Henry James-Clark (1826-1873) and William Saville-Kent (1845-1908) (James-Clark, 1867; Kent, 1882). Both authors erroneously concluded that sponges were specialized choanoflagellates and not animals at all. In support of his hypothesis, Saville-Kent described facultative multicellular colonies in several choanoflagellates (Kent, 1882), suggesting a possible path to complex multicellularity. This idea was extended by Otto Bütschli (1848-1920), who suggested that sponges had evolved from choanoflagellates, while other animals had evolved from another (unidentified) flagellate group (Bütschli, 1884).

The Russian biologist Elie Metchnikoff[2] (1845-1916; better known for having later discovered macrophages), inspired by Haeckel's inference that sponges were *bona fide* animals, took seriously the similarity of sponges to both choanoflagellates and to other animals. On this basis, he suggested that all animals, including sponges, might have evolved from a choanoflagellate-like ancestor (Metchnikoff, 1886)[3] (Fig. 13.3C-D; Box 13.2). To explain the apparent absence of collar cells in animals other than sponges, Metchnikoff suggested that the microvillous collar had been lost in these lineages, and pointed out that it is retracted in some phases of the choanoflagellate life cycle (Leadbeater, 2015). (In the 20th century, it would be discovered that collar cells are in fact widespread in the animal kingdom and not restricted to sponges – see section 13.5 below).

The flagellate hypothesis was easy to combine with Haeckel's Blastaea theory: one just had to replace Haeckel's amoeboid ancestor with a flagellate. This made the resulting hypothesis more parsimonious, as it no longer required convergent evolution of flagella in protists and in animals. Perhaps because this synthesis appeared so intuitive, the concept of a flagellate ancestor has often been erroneously attributed to Haeckel himself in textbooks and in review papers of the 20th and 21st centuries, including by ourselves (Hyman, 1940; Willmer, 1990; Wainright *et al.*, 1993; Brunet and King, 2017; Sogabe *et al.*, 2019).

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[2] Alternatively spelled Metschnikoff or Mechnikov.
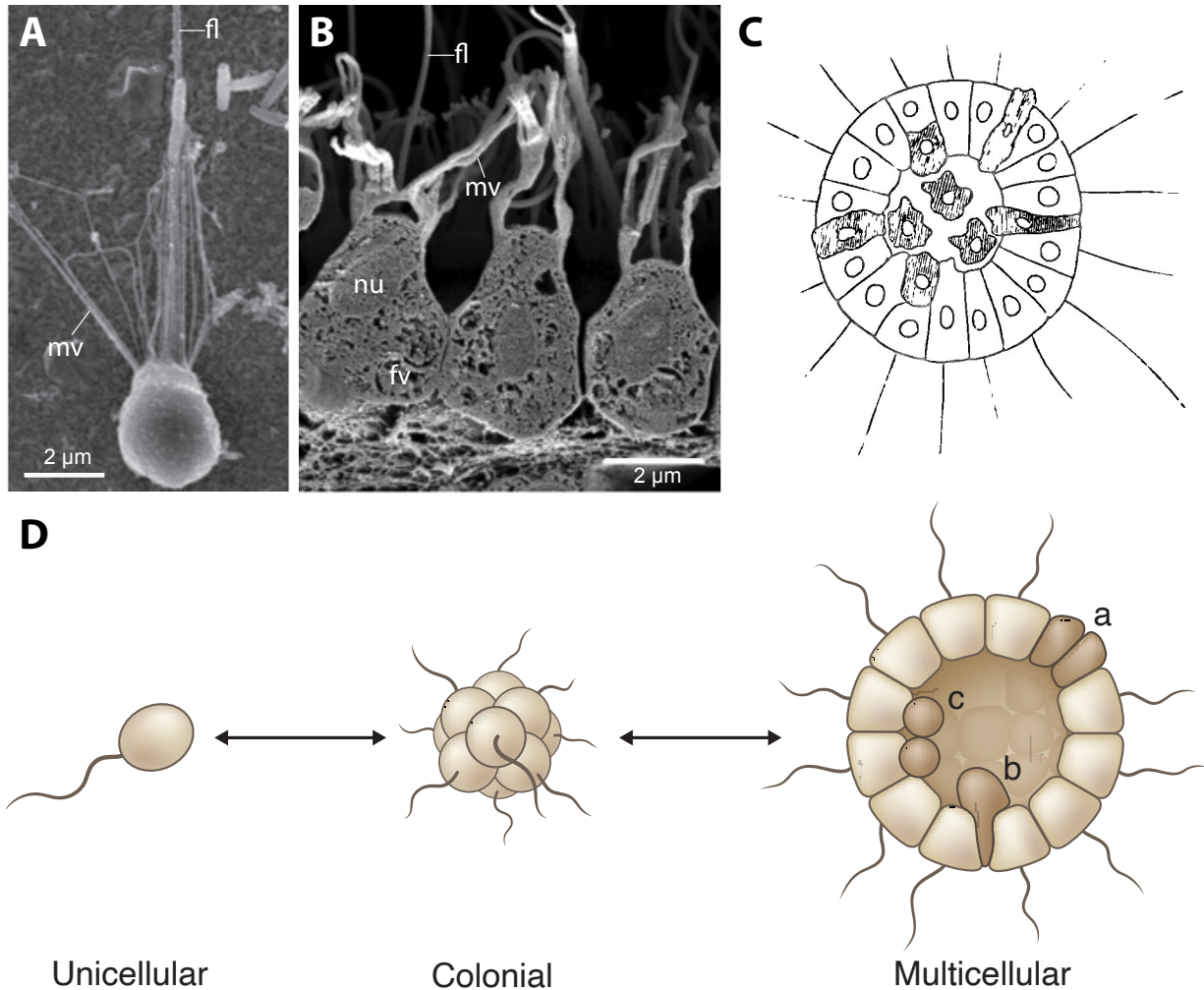[3] See (Wilson, 1887) for an English translation.

Figure 13.3. The flagellate hypothesis of animal origins. (A) The choanoflagellate *S. rosetta* (Dayel *et al.*, 2011). (B) Sponge choanocytes (Leys and Hill, 2012). Note the similarity of the apical collar complex between choanoflagellates and choanocytes, composed of a flagellum (fl) surrounded by microvilli (mv). nu: nucleus, fv: food vacuole. (C) Metchnikoff's postulated flagellate colony, with some cells internalizing to balance the flagellation/motility constraint (Metchnikoff, 1886). (D) A contemporary illustration of the flagellate model, redrawn after (King, 2004). Cells can either divide on the surface of the colony (*a*) or ingress inside the colony (*b*) and divide internally (*c*). (Drawing: Debbie Maizels)

Box 13.2: Metchnikoff's flagellate hypothesis in his own words

"*The hypothesis which supposes that colonies of flagellate Infusoria were transformed into primitive Metazoa explains very clearly the most important phenomena of metazoan development. On this view, the segmentation of the egg, and especially the more primitive total segmentation, has been derived from the division which the Flagellata undergoes in building up a colony. In like manner the fact that the cells of so many blastospheres are ciliated is probably due to inheritance from the Flagellata. This hypothesis (…) enables us,*

as Bütschli first pointed out, to comprehend the origin of sexual multiplication. As a fact most embryologists, Ray Lankester and Balfour among others, have adopted this (...) hypothesis, and after a prolonged trial it has become a basis for further speculations. Having progressed this far, we should ask ourselves whether it is not possible, with the help of our present knowledge, to determine more or less exactly the nature of those Flagellate colonies from which the Metazoa are descended. Bütschli believes the Metazoa have had a double origin: the Sponges he derives from colonies of the Choano-Flagellata, the rest of the Metazoa from colonies of true Flagellata. Aside from the fact that there is very little ground for such a venturesome assumption, we must remember that the two groups (of Flagellata) are not sharply separated, and that the collar, which constitutes the main point of difference, is in some cases entirely retracted." ((Metchnikoff, 1886) translated in (Wilson, 1887))

13.4 Saville-Kent's polyphyletic hypothesis of animal origins: sponges from flagellates and bilaterians from ciliates

William Saville-Kent and Henry James-Clark – two of the first choanoflagellate experts – agreed with Metchnikoff on the evolution of sponges from choanoflagellate-like ancestors. But they disagreed (collegially) with Metchnikoff and (much more passionately) with Haeckel on the connection of sponges to animals (reviewed in (Leadbeater, 2015)). This led Saville-Kent to conclude that animals had a dual origin: sponges had evolved from choanoflagellates, while all other animals had evolved from ciliates.

Haeckel initially thought of sponges as protists rather than animals (Haeckel, 1876) but changed his mind after he discovered that they went through a gastrula stage (Haeckel, 1872) – an observation that was doubted for more than a century but was confirmed in 2005 (Leys and Eerkes-Medrano, 2005). Saville-Kent, on the other hand, strongly objected to the concept of sponges as animals, apparently because he thought that it conflicted with their connection to choanoflagellates. From the modern perspective, it seems clear that sponges can be related both to other animals (through exclusive common ancestry) and to the sister group of animals, the choanoflagellates (as was evident to Metchnikoff). However, both Haeckel and Saville-Kent seem to have strongly felt that sponges could either be related to one or the other, not both.

Saville-Kent's hostility toward Haeckel often got personal, and his comments on Haeckel's work contained a surprising density of personal attacks (e.g. Kent, 1878; reviewed in Leadbeater, 2015). Saville-Kent thought the strongest blow to Haeckel's views was the discovery of his own "missing link" (see Footnote 1)– a living species that he felt was the perfect intermediate between choanoflagellates and sponges. Out of sheer spite[4], Saville-Kent named that organism after his nemesis: *Proterospongia haeckelii*. *P. haeckelii* occupied a similar place in Saville-Kent's mind as *Magosphaera* did in Haeckel's: it was the keystone – and the concrete proof – of his hypothesis. It was also similar in another way: no one else ever managed to observe it, and to this day, we still don't know if it was real (Figure 13.4).

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[4] Naming species out of spite is a venerable tradition that dates back to Linnaeus, who named an especially smelly weed, *Siegesbeckia*, after one of his detractors. For a list, see https://www.science-shenanigans.com/species-named-out-of-spite/
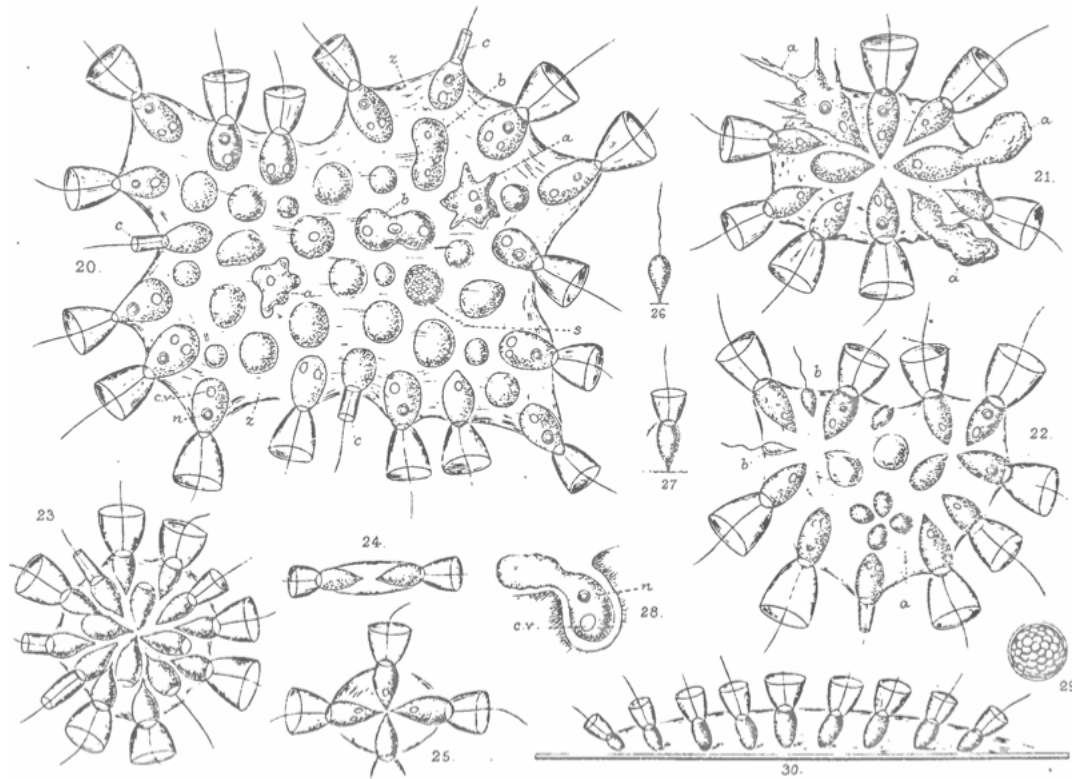
Figure 13.4. Mystery Organism 2: *Proterospongia haeckelii* (from (Kent, 1882)). This purported colonial choanoflagellate was reported to contain both flagellated collar cells and amoeboid cells. All panels shown depict different developmental stages – arranged by Saville-Kent to minimize space on the page, and thus not in developmental order, which is as follows: 24, 25, 23, 21, 22, and 20. 30 is a side-view of a mature colony (same stage as 20). 28 is a close-up of a cell in the process of becoming amoeboid. 26/27 and 29 are respectively the thecate and spore forms of *P. haeckelii*.

    P. haeckelii was a flat colony of choanoflagellates with a unique feature: just like a sponge, it had spatially differentiated cells. Collar cells positioned on the outside of the colony (similar to sponge choanocytes) coexisted with amoeboid cells on the inside (similar to sponge archeocytes). All cells were embedded in a shared flat layer of extracellular matrix. The classification of these two cell types within the same species of choanoflagellate was supported by observed interconversions between both. Saville-Kent's drawing of a mature colony with differentiated cells (Fig. 13.4, panel 20) found its way into textbooks and has been widely reproduced since (Buss, 1987; Brusca and Brusca, 2003), but he also produced many lesser-known illustrations covering the complete developmental trajectory of *P. haeckelii*, including a single collar cell serially dividing into 2, 4, and 8 cells, after which amoeboid cells started differentiating (Fig. 13.4).

    Saville-Kent was a thorough and careful microscopist, with his meticulous sketches anticipating structures that have been consistently detected and verified using modern techniques in microscopy. It is therefore unlikely that he would have simply misunderstood or misobserved an isolated specimen (such as a sponge larva). Instead, his description of the life history of *P. haeckelii* implies a detailed and extensive familiarity with multiple specimens, followed over an

extended period of time. He was also a generally reliable observer, and his descriptions of other protists have been largely confirmed. Even though choanoflagellates have recently been shown to switch to an amoeboid form under confinement (Brunet *et al.*, 2021), it is unlikely that Saville-Kent would have accidentally confined his samples: indeed, in the same book in which he described *P. haeckelii* (Kent, 1882), he reported the retraction of the choanoflagellate collar complex under confinement and its regeneration after confinement release.

If an honest mistake is ruled out, then *P. haeckelii* might have been real – and close to Saville-Kent's description. However, efforts to re-isolate *P. haeckelii* from the source location in Kew Gardens by one of us (T.B., together with Barry Leadbeater) have failed so far[5]. Given the personal rivalry between Saville-Kent and Haeckel, an alternative interpretation is that the description of *P. haeckelii* by Saville-Kent was either partly or entirely fabricated, possibly to get back at Haeckel. As with *Magosphaera*, the existence of *P. haeckelii* remains a mystery.

As significant as Saville-Kent thought *P. haeckelii* was, he only considered it relevant to the origin of sponges, but not of other animals. Instead, he proposed that (most) animals had evolved from ciliates – not just once, but many times, with different ciliates giving rise to different animal lineages (Kent, 1882). Saville-Kent was struck by the similarity in size, shape, and behavior between ciliates and small animals (both meiofaunal species – like rotifers or flatworms – and planktonic larvae; Fig. 13.5A). His idea initially drew skepticism (Lankester, 1883) but had a few early supporters (Sedgwick, 1895). It would, however, make a spectacular comeback and then recede again in the 20th century.

13.5 20th century: the rise and fall of the ciliate hypothesis

13.5.1 Similarities between acoels and ciliates and the rise of the ciliate hypothesis

Saville-Kent was correct on one point: the similarities between ciliates and small animals of the interstitial fauna are striking (reviewed in Leander, 2008; Rundell and Leander, 2010). At first sight, one could easily mistake *Paramecium* for an acoel worm (Fig. 13.5B,C). Both are elongated, bilaterally symmetrical, nearly half a millimeter long, and densely covered in motile cilia. Acoel worms are minute animals of extreme simplicity (long believed to be flatworms, but now known to belong to a separate bilaterian lineage (Ruiz-Trillo *et al.*, 1999; Cannon *et al.*, 2016; Marlétaz, 2019; Philippe *et al.*, 2019). They lack excretory organs, an anus, and even a proper gut. Early histological studies emphasized that simplicity, and many observers went so far as to erroneously conclude that acoels lacked separate cells (except perhaps in the epidermis) and instead represented a single large syncytium containing floating nuclei. Uncertainty around this point persisted from the 1880s to the 1960s, when electron microscopy

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[5] As a caveat, the reisolation of even a well-studied choanoflagellate species can be challenging. For example, the laboratory model species *Salpingoeca rosetta* (Dayel *et al.*, 2011) has been isolated only once and we have been unable to re-isolate it from its source location despite repeated attempts.

finally demonstrated that acoels were actually almost entirely cellular (to the exception of their digestive cellular mass, which is genuinely syncytial; see Delage, 1886; Pedersen, 1964 for reviews).
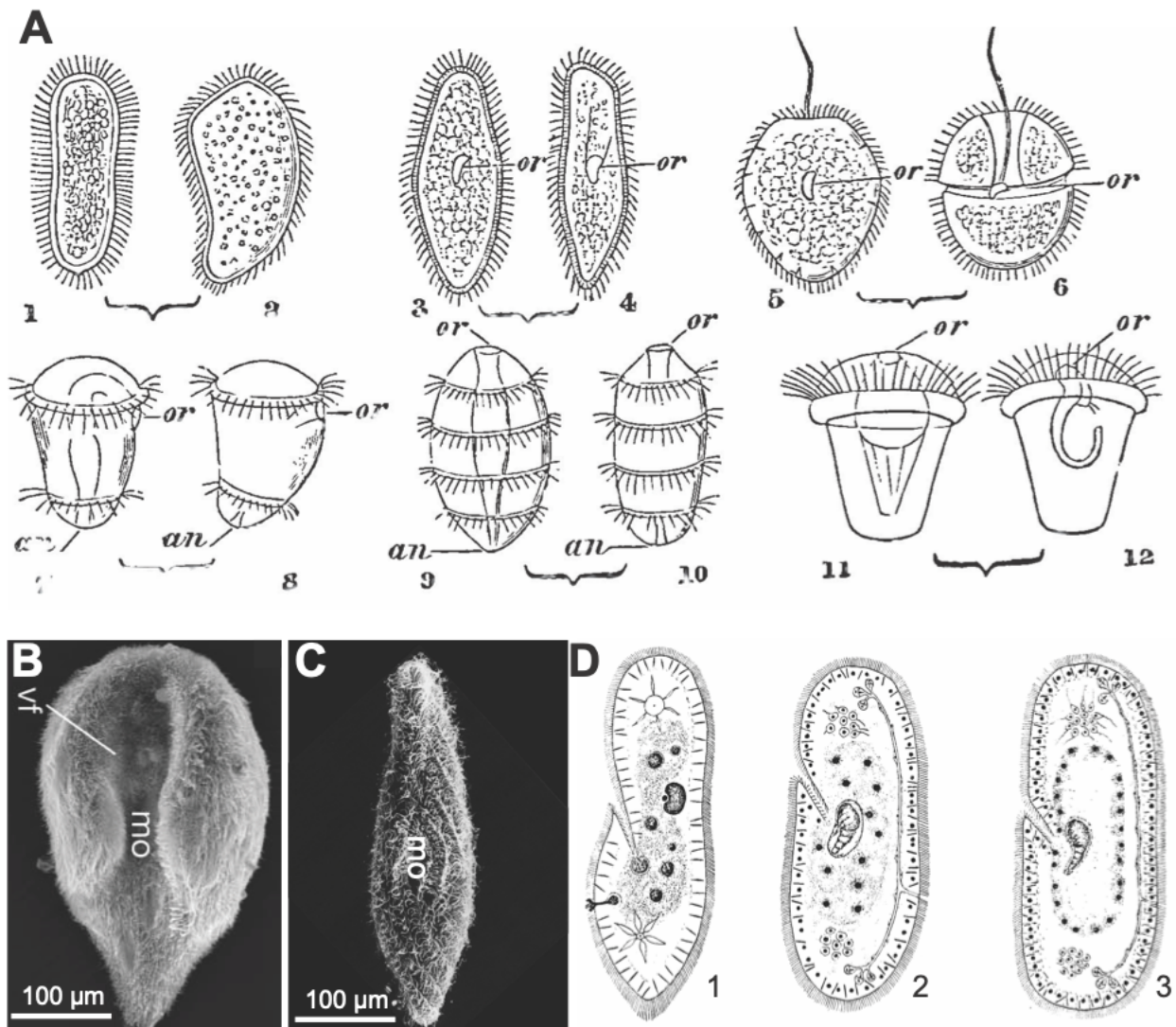
Figure 13.5. The ciliate hypothesis of animal origins. (A) Saville-Kent's depicted parallels between small animals (left) and ciliates (right). 1: Cnidarian planula larva; 2: *Opalina*; 3: flatworm; 4: *Paramecium*; 5: nemertean larva; 6: *Melodinium*; 7: annelid trochophore larva; 8: *Telotrochidium*; 9: echinoderm larva; 10: *Didinium*; 11: bryozoan larva; 12: *Vorticella*. From (Kent, 1882). (B) The acoel *Convolutriloba longifissura*. *mo*: mouth, *vf*: ventral folds. From (Hejnol and Martindale, 2008). (C) The ciliate *Paramecium sonneborni*, from (Aufderheide, Daggett and Nerad, 1983). *mo*: cytostome ("cellular mouth"). (D) Hadzi's cellularization hypothesis, from (Willmer, 1990) after (Hadzi, 1963). (1) A *Paramecium*-like ciliate hypothesized to be ancestral to animals, with multiple nuclei, pulsatile vacuoles, a cytostome ("cellular mouth") and periodic invaginations of the plasma membrane. (2) Nuclei migrate to the periphery of the cell to be lodged underneath the plasma membrane and between the invaginations. The cytostome becomes more elaborate and acquires features of a pharynx. (3) An acoel-like hypothetical ancestor of animals, with different body parts (epidermis, gut, nephridia) having evolved by partial or complete cellularization from ciliate structures.

In the meantime, however, the supposedly syncytial organization of acoels, together with their overall similarity to ciliates prompted a revival of Saville-Kent's ciliate hypothesis of bilaterian origins. The idea was proposed independently by Jovan Hadži (1884-1972) and Otto Steinböck (1893-1969), and further elaborated by Earl D. Hanson (1927-1993) (Hadzi, 1953, 1963; Hanson, 1963, 1977; Steinböck, 1963). These authors identified many purported homologies among ciliates and acoels: ciliary arrays of the former were homologized to the ciliated epidermis of the latter; the contractile infraciliary lattice of ciliates was inferred to represent an antecedent of acoel musculature; the digestive vacuoles were proposed to be equivalent to the acoel digestive mass; and pulsatile vacuoles in ciliates were considered homologous to nephridia (excretory organs that are absent in acoels but found in flatworms). The fact that ciliates only have two nuclei (a micronucleus and a macronucleus) and do not display a multicellular or even syncytial organization was countered by pointing to *Opalina*, a protist then considered to be a ciliate which possessed many nuclei underneath its cell membrane (and which is now known to be a heterokont that only convergently resembles ciliates (Cavalier-Smith and Chao, 2006)). Like Saville-Kent, supporters of the ciliate hypothesis explained the similarity between choanoflagellates and choanocytes by hypothesizing that sponges were specialized choanoflagellates, and thus unrelated to other animals. Animals were thus assumed to have had at least two independent origins in the protistan world, and maybe even three (with cnidarians possibly descending from amoebae (Hanson, 1977)).

13.5.2 The fall of the ciliate hypothesis

The hypothesis of the syncytial nature of acoels was finally disproved by electron microscopy in the mid-1960s (Pedersen, 1964), but the ciliate hypothesis of animal origins had by then taken a life of its own and survived the loss of its former central argument (Hanson, 1977). As late as the 1980s-1990s, the ciliate hypothesis and the polyphyletic origin of animals were still often presented as the likeliest hypotheses of animal origins in popular texts and textbooks. In his best-seller *Wonderful Life*, Stephen Jay Gould wrote: "*The vernacular term* animal *itself probably denotes a polyphyletic group, since sponges (almost surely), and probably corals and their allies as well, arose separately from unicellular ancestors – while all other animals of our ordinary definition belong to a third distinct group.*" (Gould, 1989). Similar statements could be found in many contemporary zoology textbooks (Mitchell, Mulmor and Dolphin, 1988; Willmer, 1990; Miller and Harley, 1999), although a few were critical (Brusca and Brusca, 1990). Surprisingly, the ciliate hypothesis survived the first molecular phylogenies as well: early studies included only a few genes analyzed with simple, similarity-based algorithms and often failed to recover the monophyly of the animal kingdom, thus apparently lending credence to multiple independent origins of animals from several protist groups (Field *et al.*, 1988; Lake, 1990; Christen *et al.*, 1991). It was only with larger datasets and better models of sequence evolution that a consistent picture of monophyletic animals closely related to choanoflagellates finally emerged, with ciliates relegated to a very distant branch (Wainright *et al.*, 1993), making the ciliate hypothesis untenable. Unsurprisingly, the hypothesized homologies also eventually failed to withstand molecular scrutiny. For example, the infraciliary contractile lattice of *Paramecium* was found to be made of centrins, a family of contractile proteins unrelated to actin and myosin, the contractile proteins of animal musculature (Levy *et al.*, 1996).

With the benefit of hindsight, many of the arguments underlying the ciliate hypothesis appear contrived. Yet, it convinced many – if not most – experts for nearly 30 years. We now

know that its proponents were misled by an impressive suite of morphological convergences between metazoans, ciliates, and additional protists like *Opalina*. While the ciliate hypothesis has now been dismissed as inconsistent with the modern eukaryotic phylogeny, it serves as a reminder of how much complexity – in morphology, patterning, and behavior – can be achieved by a single cell (Marshall, 2020). The animal-like behaviors of ciliates, which fascinated scientists and philosophers at the turn of the 20th century (Schloegel and Schmidgen, 2002), are currently undergoing a renaissance as a research topic (Coyle *et al.*, 2019; Dexter, Prabakaran and Gunawardena, 2019; Mathijssen *et al.*, 2019; Wan and Jékely, 2020), as are the mechanisms of their patterning and morphogenesis (Marshall, 2020). Properly understood as an independent and unique evolutionary experiment in achieving levels of size and morphological complexity that rival those of small animals, ciliates remain as fascinating as ever.

13.6 20th century: the collared flagellate/Choanoblastaea model

Although it had to compete with the ciliate hypothesis for part of the 20th century, Metchnikoff's concept of a choanoflagellate-like ancestor for all animals – and not just for sponges – was continuously supported by some authors (Hyman, 1940; Rieger, 1976; Salvini-Plawen, 1978; Nielsen and Norrevang, 1985). These researchers were each convinced about the monophyly of animals based on shared features such as sperm and eggs, epithelia, and gastrulation. This implied that all animals had evolved from a single lineage of protist, of which choanoflagellates were considered the most plausible living representative as their similarity to choanocytes was so strong. This view received further support from the discovery of choanocyte-like collar cells by electron microscopy in diverse animal phyla other than sponges (Nerrevang and Wingstrand, 1970; Lyons, 1973; Rieger, 1976; Brunet and King, 2017). Claus Nielsen named this revised Blastaea model – starting from a collared ancestor – the "Choanoblastaea" (Nielsen, 2008) (Fig. 13.6).

13.6.1 Molecular phylogenies and the rise of the Choanoblastaea model

While early molecular studies initially contradicted the Choanoblastaea hypothesis and suggested animal polyphyly (see section 13.4 above), improved analyses with more data and better statistical models of sequence evolution ended up consistently supporting the monophyly of animals and their sister-group relationship to choanoflagellates (Wainright *et al.*, 1993; King and Carroll, 2001; Lang *et al.*, 2002; King, Hittinger and Carroll, 2003; Rokas *et al.*, 2003; King *et al.*, 2008; Ruiz-Trillo *et al.*, 2008). Unlike hypothesized homologies between ciliates and animals, the inferred homology of the collar complex in animals and choanoflagellates survived molecular and biochemical analyses, which confirmed that the collar is composed of homologous cytoskeletal filaments in both choanoflagellates, sponges, and other animals (reviewed in Leadbeater, 2015; Brunet and King, 2017). The hypothesis of the homology of the collar complex – proposed on morphological grounds in the 19th century – thus appears to have been predictive (Colgren and Nichols, 2020) and is now accepted by many authors (but see Mah, Christensen-Dalsgaard and Leys, 2014; Sogabe *et al.*, 2019 for exceptions and Brunet and King, 2017; Myers, 2019; Colgren and Nichols, 2020 for responses).

13.6.2 The limits of the Choanoblastaea model

Despite its support from the data, the Choanoblastaea model leaves some questions unresolved. One is the similarity of crawling amoeboid cells, widespread in animals, to the amoeboid motility of diverse protists. While some authors explicitly ascribed that similarity to evolutionary convergence (Cavalier-Smith, 2017), few directly recognized or addressed the issue. While one solution could have been to revive Haeckel's amoeboid hypothesis, a strict interpretation of his hypothesis had clearly become incompatible with structural information that had emerged in the 20th century showing the homology of flagella in animals and diverse protists (reviewed in Margulis, 1981). Instead, one parsimonious way to account for all the data has been to reconstruct the progenitor of animals as a shape-shifter: sometimes flagellate, sometimes amoeba, and maybe more.

13.7 20th century: the amoeboflagellate model and the synzoospore model

Complex life cycles in protists have been known since the 19th century. In 1898, the British medical doctor Ronald Ross (1857-1932) described the different life stages of the unicellular parasite that causes malaria, *Plasmodium falciparum* (reviewed in Cox, 2002). A year later, the Austrian biologist Franz Schardinger (1853-1920) discovered *Naegleria gruberi* (then named *Amoeba gruberi*), a free-living amoeba that had the unusual ability to transdifferentiate into a flagellate form (Schardinger, 1899; Fulton, 1977, 1993).

The transition between the amoeboid and the flagellate forms of *Naegleria* is reminiscent of the reversible transdifferentiation between the flagellated choanocytes and the amoeboid archeocytes of sponges (Fig. 13.7) that was already known to Saville-Kent (Kent, 1882) and later confirmed by modern studies (Nakanishi, Sogabe and Degnan, 2014; Sogabe *et al.*, 2019)).

In spite of this parallel, shape-shifting protists such as *Naegleria* were apparently never considered relevant to animal origins before the mid-20th century, when the Soviet biologist Alexey Zakhvatkin (1906-1950, alternatively spelled Sachwatkin) and the British biologist E. N. Willmer (1902-2001) independently hypothesized that the elaborate life cycles of animals might have their roots in the unicellular world. In his treatise *Comparative embryology of the low invertebrates* (Zakhvatkin, 1949 for the Russian original and Sachwatkin, 1956 for the German translation, which we consulted), Zakhvatkin explicitly compared the complex life cycles of protists and animals. By comparing animal cell differentiation with the reversible amoeboid/flagellate switches of *Naegleria gruberi*[6] and of *Polytomella citri* (a parasitic green alga[7]; Kater, 1925), Zakhvatkin suggested animals evolved from an amoeboflagellate. He also noted that cleavage at constant volume of the animal zygote (a process called "palintomy") had parallels in several protists, including dinoflagellates and green algae, in which it resulted in a mass of flagellated "zoospores" that eventually dissociated and underwent dispersal. Zakhvatkin suggested that the morula stage of animal development might have evolved from zoospores that failed to separate – a "synzoospore."

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[6] Which he referred to as *Vahlkampfia gruberi*.

[7] *P. citri* is a secondarily non-photosynthetic, parasitic green alga, and thus belongs to a lineage whose sequenced representatives have lost regulators of cell crawling such as SCAR/WAVE (Fritz-Laylin, Lord and Mullins, 2017) and myosin II (Sebé-Pedrós *et al.*, 2014). While the genome of *P. citri* itself has not been sequenced, it is interesting to wonder how amoeboid mobility could function in this species if it also lacks those genes.
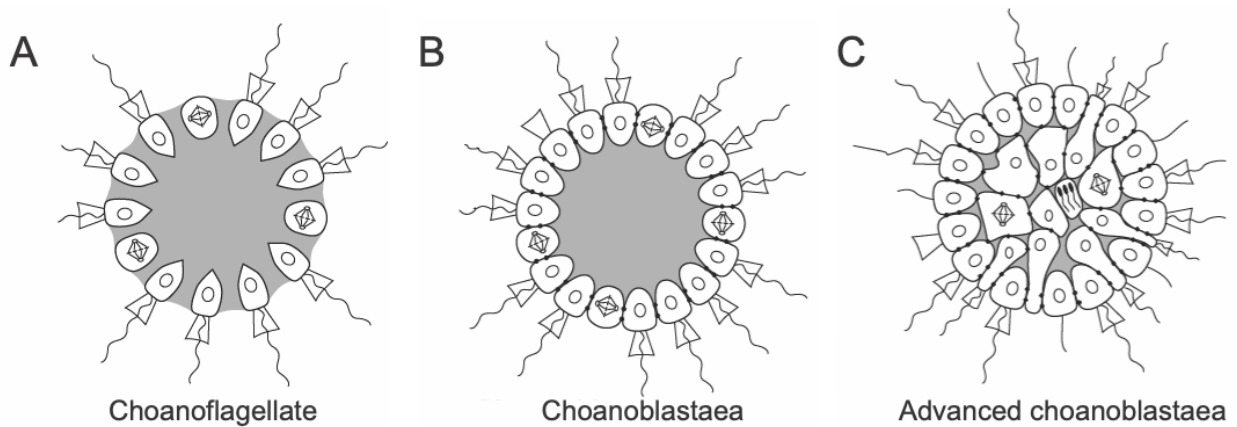
Figure 13.6. The Choanoblastaea model of animal origins (Nielsen, 2008)**.** (A) A modern choanoflagellate rosette colony, proposed to resemble early stem-animals. Cells are arranged as a sphere surrounding a shared core of extracellular matrix (dark grey). (B) A hypothetical later stem-animal ("Choanoblastaea"), in which cells have become adjacent and have evolved intercellular junctions and now form a sealed epithelial sphere. (C) A later hypothetical stem-animal ("Advanced choanoblastaea") in which some cells have become amoeboid and populated the inner space of the colony (compare *P. haeckelii*, Fig. 13.4). Note that cell division is now restricted to those inner cells.

Because Zakhvatkin's work was only available in Russian and in German, it did not immediately reach the English-speaking world. It is thus independently of Zakhvatkin, and based on his own studies of *Naegleria*, that Willmer came to remarkably similar conclusions and proposed an amoeboflagellate ancestry for animals in his 1971 book *Cytology and Evolution* (Willmer, 1971). While he did not believe that *Naegleria* was directly related to animals, he thought it gave an idea of what animal ancestors might have looked like.

Zakhvatkin's and Willmer's ideas seem to have gone mostly unnoticed in their time, and debates regarding animal origins remained dominated by the ciliate hypothesis and the flagellate hypothesis. It is only in the last decade – the 2010s – that the concept of a protist ancestor with a complex life history has undergone a revival.

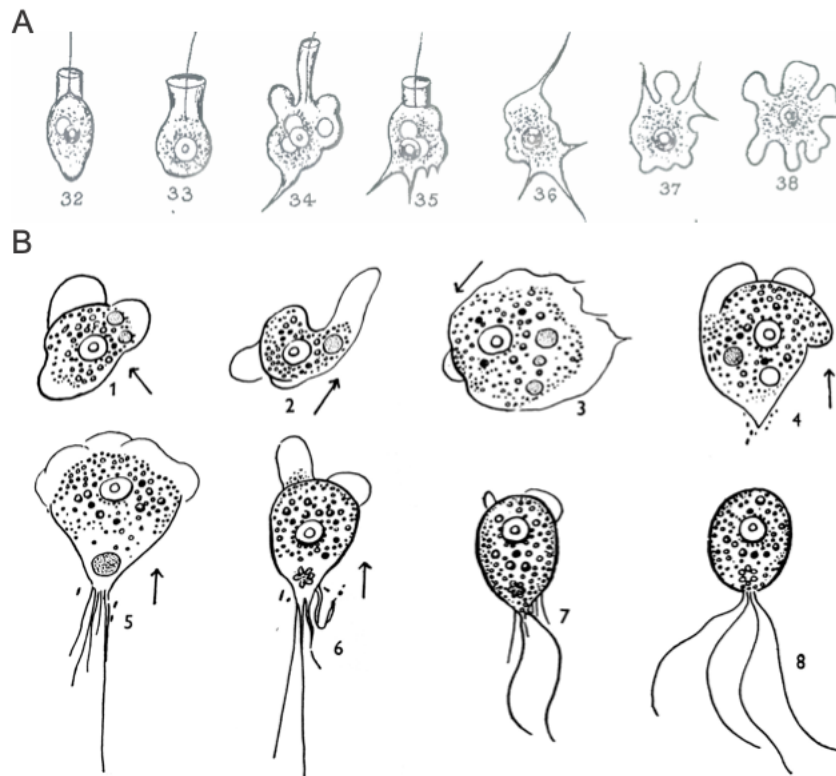13.8 21st century: how complex was the metazoan precursor?

Figure 13.7. Interconversions between flagellate and amoeboid phenotypes in sponge cell transdifferentiation. (A) (Kent, 1882) and in *Naegleria* development (B) (Willmer, 1971).

In 2009, Zahkvatkin's ideas were shared with a broader audience thanks to a review paper which presented his hypothesis in English and named it the "temporal-to-spatial transition" model of animal origins (Mikhailov *et al.*, 2009). Nearly at the same time, molecular phylogenies revealed that the previously enigmatic filasterans and ichthyosporeans (Ruiz-Trillo *et al.*, 2008) are the closest known living relatives of choanozoans (the clade formed by choanoflagellates and animals; Fig. 13.2). Together, choanozoans, ichthyosporeans and filasterans form the clade Holozoa[8]. Interestingly, single-celled holozoans assume diverse cellular forms (including flagellates, amoebae, and cystic forms), and many of them have complex life histories with multiple phenotypes (as do choanoflagellates, which have sessile, swimming and colonial flagellate forms, and often spores as well; Leadbeater, 2015).

Several studies have investigated the cellular and molecular basis for the complex life histories of unicellular holozoans (Fairclough, Dayel and King, 2010; Dayel *et al.*, 2011; Sebé-Pedrós *et al.*, 2013; Suga and Ruiz-Trillo, 2013). Remarkably, many of these cell type transitions correlate with chromatin remodelling and pre- and post-transcriptional regulation (Fairclough *et al.*, 2013; Sebé-Pedrós, Ballaré, *et al.*, 2016; Sebé-Pedrós, Peña, *et al.*, 2016; Dudin *et al.*, 2019), suggesting the existence of shared mechanisms with animal cell differentiation. Adding to this picture of generally dynamic cell phenotypes, novel amoeboflagellate species were recently discovered among holozoans (Hehenberger *et al.*, 2017; Tikhonenkov, Hehenberger, *et al.*,

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[8] A few additional lineages (such as corallochytrids) have since been added (Figure 2B).

2020). Finally, choanoflagellates themselves turned out to be able to reversibly switch to an amoeboid phenotype in response to spatial confinement (Brunet *et al.*, 2021), thus reviving Saville-Kent's concept of amoeboid phenotypes in choanoflagellates. Overall, these data converged to suggest that our ancestors along the holozoan stem-line – including the choanozoan ancestor – almost certainly had the ability to generate more cell phenotypes than just a collared flagellate, potentially paving the way to animal cell differentiation; and modern variants of the Choanoblastaea hypothesis have started to incorporate that idea (Arendt *et al.*, 2015).
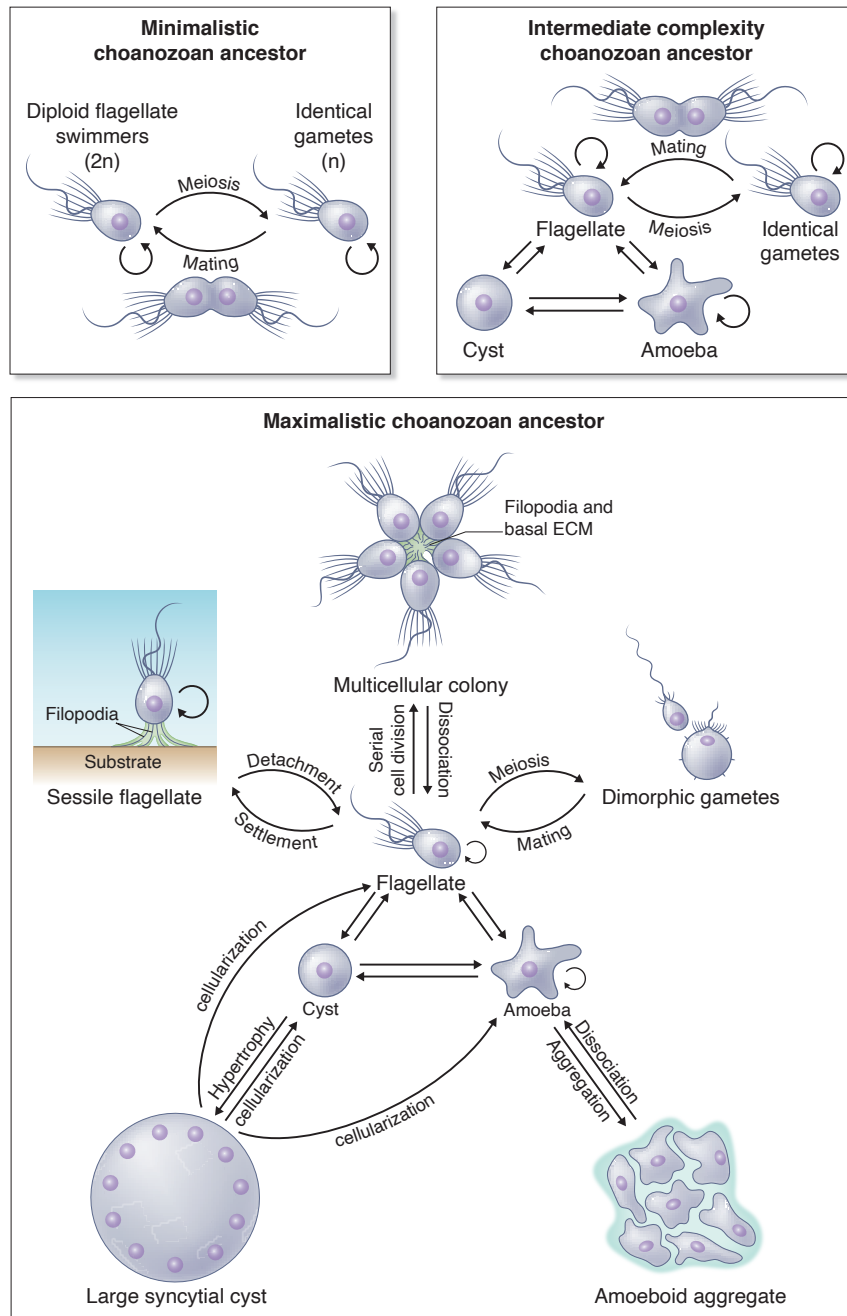
Figure 13.8. Current minimalistic, intermediate and maximalistic concepts of the last common ancestor of animals and choanoflagellates. The minimalistic ancestor (upper left) is reconstructed as a collared flagellate with a sexual cycle, but no multicellularity and no other cell phenotype. The intermediate ancestor (upper right) is also assumed to have been obligately unicellular, but can transdifferentiate into several forms, including a collared flagellate, an amoeba (similar to animal crawling cells such as macrophages), and a quiescent cyst (perhaps similar to animal quiescent stem cells or egg cells). The maximalistic ancestor (bottom) displays several forms of facultative multicellularity and combines several additional phenotypes known in single-celled relatives of animals, all of which have parallels among animal cell types and represent hypothetical evolutionary precursors of the latter. Spherical multicellular colonies of flagellates, similar to those of some choanoflagellates (Dayel *et al.*, 2011), resemble the Morula stage of animal embryos. Sessile flagellated cells adhere to the substrate by a combination of filopodia and secreted extracellular matrix (ECM, green), as in modern choanoflagellates (Dayel *et al.*, 2011) and in the filasteran *Capsaspora* (Parra-Acero *et al.*, 2018, 2020). This might have prefigured the adhesion of animal epithelial cells to the basal lamina. Amoeboid cells are proposed to undergo aggregative multicellularity, similar to *Capsaspora* (Sebé-Pedrós *et al.*, 2013) and to dissociated sponge cells (Dunham *et al.*, 1983). Note that aggregation of flagellated cells has been observed in *Syssomonas* (Tikhonenkov, Hehenberger et al. 2020) and might also have been present in the last choanozoan common ancestor, though it is not depicted here. Finally, cysts are proposed to undergo hypertrophy by nuclear proliferation without cytokinesis, resulting in a syncytium that can cleave at constant volume to revert to a uninucleated state, as in modern ichthyosporeans (Suga and Ruiz-Trillo, 2013; Dudin *et al.*, 2019) and chytrid fungi (Medina *et al.*, 2020). This process could have been the evolutionary precursor to the cleavage of animal zygotes. (Drawing: Debbie Maizels)

What did the choanozoan ancestor look like? Although we have made progress since Haeckel, Metchnikoff and Saville-Kent, many questions remain open. In Figure 13.8, we have illustrated two extreme options - a "minimalistic ancestor" (a simple collared flagellate without other phenotypes) and a "maximalistic ancestor" that combines several cell phenotypes frequently found in single-celled holozoans (most of which have an equivalent in animal biology) – along with an intermediate scenario that captures features we consider likely to have existed in the protistan ancestor of animals. The life cycle of this long extinct organism might have included "facultative features" such as amoeboid migration, encystment, clonal multicellularity (with or without palintomy) and aggregative multicellularity.

Interestingly, comparative genomics has revealed that many genes thought to be animal-specific are present in their single-celled relatives – but often with a patchy and mosaic distribution, indicating rampant gene loss in most lineages (Suga *et al.*, 2013; Richter *et al.*, 2018). This suggests that the last choanozoan common ancestor possessed a mosaic of features that is not fully realized in any of its living relatives or descendants. We think this lends credibility to the possibility of a "maximalistic ancestor." Future work will help to refine the "checklist" of ancestral choanozoan features – which will not necessarily include all those we depicted in Figure 13.8, nor will necessarily be restricted to them.

13.9 Conclusion

The past has only left incomplete traces, and our understanding of it is inevitably simplified. There is, however, another force that often pushes us to simplification: the urge to summarize history as a linear narrative that leads to the present. In this review, we have strived to embrace the complexity of the past – both of our scientific predecessors, and of our evolutionary ancestors. We hope the winding history of our field is worth appreciating for itself and for the many small gems it contains, before trying to extract an – inevitably simplified – global message.

Nonetheless, a few general themes emerge. The diversity of historical hypotheses simultaneously reflects the complexity of the problem itself, the limited information available at the time, and the personal assumptions and preferences of their authors. On the one hand, morphological data were clearly confounded by multiple events of evolutionary convergence (such as between ciliates and animals), parallelism, and rampant loss. Solving the problem from morphology only was genuinely challenging (even after the advent of electron microscopy), and involved some degree of subjective judgment. On the other hand, many authors seemed to have made the task unnecessarily more difficult by assuming that the last single-celled ancestors of animals necessarily had an exact equivalent within living protists – while this ancestor likely had its own, unique combination of features that is not necessarily represented today. This point has become increasingly salient in the past few years, and we expect it to remain central to future research. Consistently, several species of single-celled holozoans with novel phenotypes have been newly described in the past few years (Hehenberger *et al.*, 2017; Brunet *et al.*, 2019; Tikhonenkov, Hehenberger, *et al.*, 2020; Tikhonenkov, Mikhailov, *et al.*, 2020), and metagenomic surveys have provided evidence for the existence of additional undiscovered holozoan lineages (Del Campo and Ruiz-Trillo, 2013; Arroyo *et al.*, 2020). Further exploration of single-celled biodiversity thus holds the promise to enrich our reconstitution of animal ancestors – and eventually maybe even to clarify the mysteries of *Magosphaera planula* and

Proterospongia haeckelii. Depending on their phylogenetic position, these species – if they exist and if the original descriptions were accurate – might provide stronger evidence for a pre-metazoan origin of palintomy and special cell differentiation, respectively.

Another point of interest is the way in which past controversies were resolved. Many debates could only be settled after the invention of new techniques; yet, technical innovations alone were rarely sufficient. The first molecular phylogenies, for example, were rather inaccurate. Consensus was only reached after commonly accepted standards of evidence were agreed upon, and once multiple independent, technically solid studies converged toward the same answer. At a time where a new wealth of molecular data (notably from single-cell techniques) is promising to bring an unprecedented quantity of evidence to bear on the study of the evolution of cell phenotypes, we hope that our historical summary can be read both as a cautionary tale, and as a reason for optimism.

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