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The origin of animal multicellularity and cell differentiation

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Abstract

How animals evolved from their single-celled ancestors over 600 million years ago is poorly understood. Comparisons of genomes from animals and their closest relatives – choanoflagellates, filastereans and ichthyosporeans – have recently revealed the genomic landscape of animal origins. However, the cell and developmental biology of the first animals have been less well examined. Using principles from evolutionary cell biology, we reason that the last common ancestor of animals and choanoflagellates (the 'Urchoanozoan') used a collar complex - a flagellum surrounded by a microvillar collar – to capture bacterial prey. The origin of animal multicellularity likely occurred through the modification of pre-existing mechanisms for extracellular matrix synthesis and regulation of cytokinesis. The progenitors of animals likely developed clonally through serial division of flagellated cells, giving rise to sheets of cells that folded into spheres by a morphogenetic process comparable to that seen in modern choanoflagellate rosettes and calcareous sponge embryos. Finally, we infer that cell differentiation evolved in the animal stem-lineage by a combination of three mechanisms: division of labor from ancient plurifunctional cell types, conversion of temporally segregated phenotypes into spatially segregated cell types, and functional innovation.

Keywords: multicellularity, choanoflagellates, Choanozoa, metazoan origins

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Introduction

Every aspect of animal life – from morphology to physiology and behavior – requires the cooperation of thousands to billions of cells. This multicellular state is established in each generation by serial divisions of a single cell, the zygote. Under joint control by the genome and the environment, daughter cells produced by these divisions change shape, migrate, and selectively attach or detach to give rise to the adult body form through a process known as morphogenesis. In parallel, a process of cell differentiation under fine spatiotemporal control delineates the division of labor between each of the final cell types. The correct execution of this cellular choreography, repeated anew in every generation, is fundamental to the life of every animal on the planet.

Yet, this type of complex development did not always exist. The discontinuous phylogenetic distribution of multicellularity and fundamental differences in cellular mechanisms argue that multicellularity evolved independently in at least 16 different eukaryotic lineages, including in animals, plants, and fungi (Gould 1996; Bonner 1998; King 2004; Rokas 2008; Knoll 2011). Thus, the mechanisms underpinning animal multicellularity and spatially controlled cell differentiation were likely elaborated in the stem lineage of animals, building upon pathways present in their single-celled ancestors (Richter and King 2013).

Despite the centrality of multicellularity and cell differentiation to animal biology, their origins are relatively poorly understood. What did the single-celled ancestors of animals look like? How and when did multicellularity and cell differentiation evolve, and in what ecological context? What were the underlying molecular mechanisms? Did features of the single-celled progenitors of animals facilitate the early evolution of multicellularity? Conversely, did this single-celled ancestry exert constraints upon the form and function assumed by early animal ancestors?

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While the gene complements of animal ancestors have been discussed in depth elsewhere and will not be the focus of this review (see e.g. (King 2004; King et al. 2008; Larroux et al. 2008; Richter and King 2013; Suga et al. 2013; de Mendoza et al. 2013; Sebé-Pedrós et al. 2017)), it is notable that many genes required for animal multicellularity (e.g. tyrosine kinases (King and Carroll 2001; King et al. 2003), cadherins (Abedin and King 2008; Nichols et al. 2012), integrins (Sebé-Pedrós et al. 2010; Suga et al. 2013), and animal-like extracellular matrix domains (King et al. 2008; Williams et al. 2014)) evolved before animal origins. Building upon these ancient proteins, the stem-animal lineage was notably marked by an explosive diversification of transcription factor families and signaling molecules, fueled both by the emergence of new families (e.g. the Antennapedia and Pax families of homeodomain transcription factors and the secreted signaling proteins Wnt and BMP) and by expansion of existing families (Larroux et al. 2008; Srivastava et al. 2010; de Mendoza et al. 2013). The cell biology and morphology implemented by these ancestral genomes, however, have been less thoroughly explored (Nielsen 2008; Richter and King 2013; Arendt et al. 2015; Cavalier-Smith 2017). In this review, we will consider how the evolution of cellular phenotype shaped the origin of animals.

Although the first animals evolved over 600 million years ago, meaningful insights into their origin may be gained through the comparison of extant lineages. This approach has revealed a number of features that were likely present in the last common ancestor of animals, the "Urmetazoan" (Figure 1). For example, nearly all extant animals have obligate multicellularity (see (Metzger et al. 2015; Chang et al. 2015) for exceptions in some parasitic forms) with adult stages typically displaying a specialized morphology and at least five morphologically distinguishable cell types (Valentine 2006; Smith et al. 2014). This suggests that the Urmetazoan evolved from a lineage with a long prior history of obligate multicellularity (Nielsen 2008; Richter

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and King 2013; Budd and Jensen 2017). Likewise, multicellularity in animals is invariably the result of a complex embryogenesis initiated by sperm/egg fusion, followed by serial rounds of cell divisions (cleavage). This suggests the same was true of the first animals. Finally, in every major animal lineage from sponges and ctenophores to bilaterians, the cells of the future feeding cavity move inside the embryo, morphogenesis establishes the adult body shape, and cell differentiation takes place (Leys and Degnan 2002; Arendt 2004; Leys and Eerkes-Medrano 2005; Ereskovsky 2010; Nielsen 2012). A form of this elaborate developmental process presumably already existed in the Urmetazoan. Rather than evolving in one step in a single-celled ancestor, it more plausibly resulted from a long and gradual evolution. Therefore, to more fully reconstruct the origin of animal development, we must extend our comparisons beyond animals to include their closest living relatives.

The sister-group of animals has unambiguously been shown to be the choanoflagellates (King et al. 2008; Ruiz-Trillo et al. 2008; Shalchian-Tabrizi et al. 2008; Torruella et al. 2015), a globally distributed group of marine and freshwater protists (Leadbeater 2014) with a highly distinctive morphology (Figure 2A,B,E). Choanoflagellates are characterized by an apical flagellum surrounded by a collar of microvilli, which together form a "collar complex" (Leadbeater 2014). The beating of the flagellum generates forces that can both propel the cell through the water column and produce a flow that allows the choanoflagellate to collect bacterial prey on the outer surface of the microvillar collar. The morphological similarity between choanoflagellates and certain animal cells, particularly sponge choanocytes, was evident even to the first choanoflagellate observers (James-Clark 1867) and inspired the hypothesis of a close relationship between choanoflagellates and animals. This hypothesis, first proposed in the 19th century on morphological grounds, remained otherwise untested for more than a century, as both

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the affinities of choanoflagellates to sponges and of sponges to other animals were the subject of a variety of alternative hypotheses (see historical summary in (Leadbeater 2014)). The issue was only settled with the application of comparative genomics and molecular phylogenetics, which provided conclusive evidence both that sponges belong to the animal kingdom (Wainright et al. 1993; Srivastava et al. 2010; Telford et al. 2015) and that animals and choanoflagellates are sister-groups (King et al. 2008; Ruiz-Trillo et al. 2008; Shalchian-Tabrizi et al. 2008; Torruella et al. 2015). Together, choanoflagellates and animals thus form a monophyletic clade, which we refer to as "Choanozoa" (see box). Furthermore, phylogenomic studies of previously enigmatic taxa have revealed the closest living relatives of choanozoans (Ruiz-Trillo et al. 2008; Torruella et al. 2015) to be Filasterea (a group comprising filopodiated amoebae and recently discovered flagellated protozoans (Hehenberger et al. 2017)) and Ichthyosporea (which alternate between large syncytial spores and individual amoebae). Together, Choanozoa, Ichthyosporea, and Filasterea make up the clade Holozoa, which is the sister-group of Fungi (Figure 1).

Box 1: Choanozoa: the clade composed of choanoflagellates and animals.

We define Choanozoa as the clade containing the most recent common ancestor of animals (represented by *Homo sapiens* Linnaeus 1758) and choanoflagellates (represented by *Monosiga brevicollis* Ruinen 1938), along with all of its descendants. The Greek root "*choanē*" (or funnel) refers to the collar, which in the current state of knowledge is a synapomorphy of the clade. Although "Choanozoa" was used previously to refer to an assemblage of protists (Cavalier-Smith, 1991) that later proved paraphyletic (Shalchian-Tabrizi et al., 2008); this usage was not adopted and the name is more appropriately applied as defined here. The informal term "choanimal" (Fairclough et al., 2013) and the formal term Apoikozoa (Budd and Jensen, 2017) have both been previously proposed for the clade containing choanoflagellates and animals, but neither has been formally described nor fully adopted. In particular, the term "Apoikozoa" is less fitting, as the root "*apoiko-*" refers to colony formation, which is neither universally present in choanozoans, nor exclusive to them.

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I. Choanoflagellates reveal the cellular foundations of animal origins

In light of the sister-group relationship between choanoflagellates and animals, a fundamental question is whether shared cellular features – such as the collar complex – were already present in the last common choanozoan ancestor, the "Urchoanozoan" (Figure 1). Electron microscopy has revealed that the similarities between choanoflagellates and choanocytes extend beyond morphology to include a shared underlying ultrastructure (Richter and King 2013). In both choanoflagellates and sponge choanocytes, the flagellum is supported by microtubules (Karpov and Leadbeater 1998; Gonobobleva and Maldonado 2009) and often displays a characteristic "vane" – a pair of bilateral wing-like filamentous extensions of the flagellum that have only been reported in choanoflagellates and in sponge choanocytes (Figure 2C,D) (Petersen 1929; Vlk 1938; Hibberd 1975; Mehl and Reiswig 1991; Leadbeater 2006; Mah et al. 2014). The ovoid cell body is encased in parallel arrays of sub-membranous microtubules that emerge from the basal body and span the cell from the apical to the basal side. Underneath the flagellum, the basal body is supported by a basal foot surrounded by an elaborate "crown" of transverse microtubules. Like the flagellar vane, this organization appears unique to sponge choanocytes and to choanoflagellates (Figure 2F,G) (Garrone 1969; Woollacott and Pinto 1995; Leadbeater 2014). Finally, in both choanoflagellates and choanocytes, the microvilli are supported by bundled actin microfilaments maintained at a constant length within a given cell (Karpov and Leadbeater 1998; Rivera et al. 2011). Choanoflagellate genomes encode homologs of most animal microvillar proteins, among which two families appear choanozoan-specific: Ezrin/Radixin/Moesin (ERM) and Whirlin, both

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of which are involved in controlling microvillar length (Sebé-Pedrós et al. 2013a; Peña et al. 2016). This further supports the notion that the collar is a choanozoan synapomorphy[1].

Besides its conserved ultrastructure, the stem choanozoan ancestry of the collar complex is further supported by its broad distribution in animals. Beyond sponges, collar complexes featuring a flagellum surrounded by a ring of microvilli are found in most animal phyla (Figure 1, Supplementary Table 1) – e.g. in epidermal cells (often sensory (Supplementary Figure 1A) (Fritzsch et al. 2007; Fritzsch and Straka 2014)), terminal cells of protonephridia ensuring excretion (Supplementary Figure 1B) (Ruppert and Smith 1988), or as part of diverse inner epithelia (Supplementary Figure 1C) (Nerrevang and Wingstrand 1970; Goldberg and Taylor 1989). Collar cells often function in food absorption: choanoflagellates and sponge choanocytes phagocytose live bacteria, and the collar cells lining the gastrodermis of some cnidarians endocytose food particles produced by extracellular digestion (Goldberg and Taylor 1989). In bilaterians and ctenophores, nutrient acquisition through endocytosis is performed by enterocytes lining the midgut that frequently display a motile flagellum and microvilli (although the microvilli are packed into a dense brush border rather than forming a simple ring) (Hernandez-Nicaise 1991; Takashima et al. 2013), consistent with a possible derivation from ancestral collar cells (Arendt et al. 2015).

Finally, the homology of the collar complex in animals and choanoflagellates is supported by its phylogenetic distribution. Its absence from all other eukaryotic lineages[2] suggests

[1] Although the collar complex appears choanozoan-specific, microvilli in general may be of more ancient origin. The protozoan *Ministeria vibrans*, which belongs to Filasterea (the sister-group of choanozoans), displays microvilli-like radiating tentacles that are distributed over the whole cell cortex and are of constant length within a given cell at a given time (Cavalier-Smith and Chao 2006). Neither the ultrastructure of the radiating tentacles nor presence of ERM and Whirlin homologs in the *Ministeria* genome have yet been reported.

[2] Although morphologically analogous forms exist in a few distantly related species (Mah et al. 2014), in all cases the underlying ultrastructure differs. For example, in the amoebozoan *Phalansterium* (Cavalier-Smith et al. 2004), the flagellum is surrounded by a "collar" formed by a continuous fold of cytoplasm rather than by independent microvilli (Hibberd 1983). In the pedinellales (a group of heterokonts (Cavalier-Smith and Chao 2006)) *Actinomonas* and

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143 that it is unlikely to evolve easily through convergence. Although there are subtle differences in
144 the form and function of the collar of choanoflagellates and sponge choanocytes (Mah et al. 2014)
145 (unsurprisingly, given that the two lineages diverged more than 600 million years ago), these
146 differences are likelier to be the result of derivation from an ancestral collar cell than the
147 similarities arising through convergent evolution. Therefore, bacterivorous collar cells likely trace
148 their ancestry back to the choanozoan stem lineage, and the study of modern choanoflagellates
149 holds the promise of illuminating the cellular foundations of animal origins.
150

151 ## II. Division or aggregation? The two paths to multicellularity

152

153 How and when did the ancestors of animals become multicellular? Each of the multiple
154 independent evolutionary origins of multicellularity (Figure 3) took place through one of two
155 mutually exclusive mechanisms: clonal development, in which multicellularity arises through
156 serial cell division without separation of sister cells, and aggregation, in which separate cells
157 converge and adhere to each other. Clonal and aggregative multicellularity both have broad,
158 scattered distributions in the phylogenetic tree of eukaryotes, suggesting that they both evolved a
159 number of times independently (Figure 3A). Interestingly, the two pathways to multicellularity
160 result in different types of multicellular forms, and arguably evolved in response to different
161 selective pressures.
162

Pteridomonas, the flagellum is surrounded by a double ring of tentacles which are supported by microtubule triads rather than actin microfilaments, and are thus not microvilli (Larsen 1985; Patterson and Fenchel 1985). Finally, the golden alga *Chrysosphaerella multispina* (Bradley 1964) has a superficially collar cell-like appearance, with a flagellum flanked by two spines of acellular silicate (Kristiansen 1969) instead of microvilli.

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II.1. Independent origins of aggregative multicellularity

Although all extant animals display clonal development, could early animal ancestors have developed by aggregation? Though this hypothesis is hard to formally rule out, it appears unlikely in the current state of knowledge. The few cases of aggregation-like processes reported in animals are responses to artificial perturbations: for example, experimentally dissociated sponge cells can re-aggregate *in vitro* (Curtis 1962) but sponge embryonic development is strictly clonal *in vivo* (Ereskovsky 2010). We argue below that aggregative multicellularity is rarer than clonal multicellularity, and represents a distinct adaptive niche with a more limited evolutionary potential.

Aggregative multicellularity evolved at least seven times independently in eukaryotes (Figure 3A) as well as in some bacterial lineages such as Myxobacteria (Bonner 1998; Brown et al. 2012; Sebé-Pedrós et al. 2013b; Du et al. 2015). In nearly all well-characterized cases of aggregative multicellularity, cells respond to adverse conditions (e.g. starvation (Dworkin 1963; Stratford 1992; Souza et al. 1999)) by migrating toward each other and aggregating into a resistant mass of propagules (spores or cysts) – called a sorocarp, sporangium or (if a stalk is present) a fruiting body (Figure 3B)[3]. The resulting structure is formed of quiescent cells that neither feed nor divide. Motility, if present (as in *Dictyostelium* slugs), is a transient step toward the formation of the sorocarp. The multicellular form eventually dissociates and the propagules disperse. Aggregation is thus a specialized "emergency response" in which cells transiently assemble under

[3] The few exceptions represent an infrequently observed type of aggregative multicellularity, sexual agglutination, that has been reported in yeasts (Wickerham 1958), choanoflagellates (Woznica et al. 2017) and *Chlamydomonas* (Bergman et al. 1975). Like other cases of aggregation, it results in transient multicellular forms without coordinated motility, feeding or proliferation, and which dissociate after karyogamy has been accomplished. In some species (such as *Chlamydomonas*) environmental stress (e.g. starvation) is a precondition to sexual agglutination.

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183 adverse conditions that compromise cell division, thus preventing clonal multicellularity. The
184 potential advantages of aggregation include enhanced resistance to environmental stressors (with
185 the outer cells shielding the inner cells from harmful agents such as UV and toxic chemicals
186 (Stratford 1992)) and, in terrestrial species, formation of a stalk allowing elevation from the
187 substrate and wind dispersal of the propagules (Bonner 1998).

188 Aggregation presents an impediment to the evolution of division of labor between cell
189 types (Buss 1988): the multicellular mass is composed of cells that are not necessarily genetically
190 related, making it especially vulnerable to invasion by "cheater" mutants that benefit from their
191 presence in the aggregate without sharing resources or labor with the other cells (Strassmann et al.
192 2000; Santorelli et al. 2008). Indeed, theoretical arguments and experimental data suggest that
193 aggregation can only be evolutionarily stable if it is restricted to closely related individuals (Gilbert
194 et al. 2007; Kuzdzal-Fick et al. 2011). For example, the aggregative multicellularity of
195 dictyostelids has been evolutionarily stable for more than 400 million years (Sucgang et al. 2011),
196 and sophisticated ways to recognize kin have accordingly evolved (Benabentos et al. 2009; Hirose
197 et al. 2011). Perhaps due to the difficulty of overcoming such conflicts, aggregative forms have a
198 transient existence and little or no division of labor, with at most two to three coexisting cell types
199 – usually germ cells (the inner spores or cysts) and somatic cells (e.g. the cells of stalk in the
200 *Dictyostelium* fruiting body (Town et al. 1976; Dickinson et al. 2011)).

201 In all its forms, aggregative multicellularity involves an elaborate series of coordinated
202 events: mutual cell attraction by chemotaxis, migration, adhesion, and, finally, differentiation into
203 propagules (Du et al. 2015). How this cascade originated in each lineage is unknown in detail, but
204 a likely evolutionary prerequisite was the presence of an inducible pathway for the
205 sporulation/encystment of individual cells. Supporting this notion, comparison of dictyostelids and

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206 single-celled amoebozoans have shown that the cAMP/PKA pathway controlling the switch to
207 multicellularity in dicytostelids evolved from an ancient encystment stress response found in
208 diverse single-celled amoebae (Ritchie et al. 2008; Du et al. 2014; Kawabe et al. 2015). As it is
209 contingent on specific prior, permissive evolutionary steps and vulnerable to cheaters, aggregative
210 multicellularity might be both more difficult to evolve and less conducive to the evolution of
211 differentiated cell types than is clonal multicellularity.
212
213 *II.2. Independent origins of clonal multicellularity*
214
215 As clonal multicellularity can in principle result from a simple failure to complete cell
216 division, it might evolve relatively easily through loss-of-function mutations (Bonner 1998). All
217 three documented examples of multicellular forms that have evolved *de novo* in the laboratory
218 developed clonally by incomplete cytokinesis within a small number of generations under
219 selection (100 to 315; (Boraas et al. 1998; Ratcliff et al. 2012, 2013). The relative ease of evolving
220 clonal multicellularity could explain why clonal forms are both phylogenetically more widespread
221 (Figure 3A) and morphologically more diverse than aggregative forms. Indeed, clonal
222 development underlies all known multicellular forms with active metabolism and proliferation (but
223 also some sorocarps or fruiting bodies) (Figure 3C). Moreover, clonal multicellularity is likely less
224 vulnerable to cheating than aggregation, as all cells share an identical genome (Buss 1988). These
225 two factors together may account for the fact that all five known independently evolved instances
226 of "complex multicellularity" (defined as obligate multicellularity with different cell types and
227 elaborate organismal morphology (Knoll 2011)) involve clonal development: animals, fungi, green
228 algae/land plants, red algae, and brown algae. The selective advantages that favored the evolution

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and maintenance of clonal multicellularity are unknown, but may include resistance to predators (Boraas et al. 1998), cooperative feeding (Koschwanez et al. 2011; Roper et al. 2013), simple division of labor (e.g. between flagellated cells and dividing cells) (Margulis 1981; Buss 1988; Michod 2007) and formation of a *milieu intérieur* (i.e. a controlled "internal environment" of stable composition). Finally, one cannot rule out the possibility that the initial evolution of animal multicellularity was a neutral event, that would have conferred no immediate fitness benefit and have first reached fixation by genetic drift.

III. The origin of animal multicellularity

III.1. How ancient is animal multicellularity?

The last common ancestor of animals was clearly multicellular, but might multicellularity extend back to the last common ancestor of choanozoans? Tantalizingly, many choanoflagellates form facultative multicellular forms, including swimming spherical (rosette), linear or flat colonies, and sessile branching colonies (Leadbeater 2014). These multicellular forms are all thought to develop clonally (Hibberd 1975; Karpov and Coupe 1998; Fairclough et al. 2010; Dayel et al. 2011; Leadbeater 2014). Although the phylogenetic distribution of known instances of multicellularity in choanoflagellates remains patchy, the life cycles of most species are incompletely known, precluding robust parsimony-based inference of the ancestral state. In favor of an ancient origin, the rosette colonies of three distantly related species (*Salpingoeca rosetta* (Leadbeater 1983; Fairclough et al. 2013), *Codosiga botrytis* (Hibberd 1975) and *Desmarella* Kent (Karpov and Coupe 1998)) are composed of cells joined by cytoplasmic bridges with a common

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252 distinctive ultrastructure featuring two parallel electron-dense plates flanking an electron-dense
253 cytoplasmic core. The last common ancestor of these three species was also the last common
254 ancestor of one of the two main choanoflagellate clades (Carr et al. 2008, 2017), suggesting that
255 clonal multicellularity might already have evolved before the first split in the choanoflagellate
256 phylogenetic tree. Characterization of the life cycles and multicellular development of more
257 choanoflagellate species will further test this hypothesis. Whether clonal multicellularity arose
258 once or several times in choanozoans, choanoflagellate rosettes offer a tantalizing proxy for the
259 first stages of animal evolution, due to their close phylogenetic affinity to animals and to
260 similarities in both cell ultrastructure and developmental mode.

261 Multicellularity might thus be as ancient as stem choanozoans, but could it be even older?
262 The two closest relatives of choanozoans are filastereans and ichthyosporeans (Figure 1).
263 Intriguingly, both form facultative multicellular forms, but in different ways from choanozoans –
264 aggregation in filastereans (Sebé-Pedrós et al. 2013b) and fragmentation of a syncytium in
265 ichthyosporeans (Suga and Ruiz-Trillo 2013). If holozoan multicellular forms are homologous to
266 each other, this would imply that interconversions took place between aggregative and clonal
267 multicellularity in stem-filastereans or stem-choanozoans[4]. Perhaps more likely is the possibility
268 that multicellularity evolved independently in the three holozoan clades, although this remains to
269 be tested.

270

[4] There is no known definitive example of such a transition. The most plausible candidates are slime molds, in which similar fruiting bodies form clonally in Myxogastria and by aggregation in Dicytostelia (Stephenson and Schnittler 2016) which might be sister-groups (Fiore-Donno et al. 2010). However, no individual species is known to be able to develop multicellularity alternatively by either route.

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III.2. The genetic basis for the origin of animal multicellularity

What molecular mechanisms first supported the evolution of animal multicellularity? This problem cannot be studied solely in animals, as there is no known animal mutant where cleavage of the zygote would directly produce separate free-living cells rather than an embryo, thus reverting to unicellularity. To answer this question, it is therefore necessary to study phylogenetically relevant groups with facultative multicellularity. Thus far, a dozen genes have been found to be either necessary or sufficient for multicellularity in the four groups investigated: green algae, fungi, slime molds and choanoflagellates[5]. All known multicellularity genes encode proteins that belong to one of two major functional categories: extracellular matrix (ECM) proteins and, in the case of clonal multicellularity, cytokinesis regulators (Table 1). This suggests that the initial evolution of multicellularity on different branches of the tree of life repeatedly converged on similar mechanisms (Abedin and King 2010).

Among the taxa studied, choanoflagellates occupy a privileged position as the sister-group of animals. The recent establishment of forward genetics in the model choanoflagellate *Salpingoeca rosetta* has revealed the first gene known to be required for multicellular development, named *rosetteless* for its mutant phenotype (Levin et al. 2014). While wild-type *S. rosetta* reliably develops into spherical colonies (called "rosettes") upon induction with bacterial signals (Dayel et al. 2011; Alegado et al. 2012; Woznica et al. 2016), *rosetteless* mutants are unable to form rosettes under all studied conditions (although the ability to develop into another clonal multicellular form, linear chains, is unaffected). The *rosetteless* gene encodes a C-type lectin that

[5] Some pleiotropic genes are necessary for multicellularity as an indirect effect of them being involved in a more general cell function, such as transcription or cell motility. For example, across the 123 *Dictyostelium* mutants with aberrant or abolished aggregation (Glöckner et al. 2016), most are deficient in transcription, cell movement or cAMP synthesis. These genes are not discussed here as their role is indirect.

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Table 1. Cell cycle and ECM genes from diverse eukaryotes regulate the unicellularity/multicellularity switch.

Species	Type of multicellularity	Gene required for the unicellular/multicellular switch	Gene function
Gonium pectorale (green alga)	Clonal	*retinoblastoma*	**Cell cycle** (transcription inhibitor) (1)
Saccharomyces cerevisiae (fungus)	Aggregative (flocculation)	*flo1*, *flo5*, *flo8*, *flo9*, *flo10*, *flo11*, *sta1*	**ECM** (*flo1*, *flo5*, *flo9*, *flo10*, *flo11*: lectins, *sta1*: endoamylase, *flo8*: transcriptional activator of the formers) (2-6)
Saccharomyces cerevisiae (fungus)	Clonal (chain and snowflake mutants)	*cts1* *ACE2*	**ECM** (chitinase) (7) **Cell cycle** (transcription factor) (8,9)
Dictyostelium discoideum (slime mold)	Aggregative	*cbp-26*	**ECM** (lectin) (11,12)
Salpingoeca rosetta (choanoflagellate)	Clonal	*rosetteless*	**ECM** (lectin) (10)

References: 1. (Hanschen et al. 2016); 2. (Douglas et al. 2007); 3. (Lo and Dranginis 1996); 4. (Soares 2011); 5. (Stratford 1992); 6. (Fidalgo et al. 2006); 7. (Kuranda and Robbins 1991); 8. (Oud et al. 2013); 9. (Ratcliff et al. 2015); 10. (Shinnick and Lerner 1980); 11. (Ray et al. 1979); 12. (Levin et al. 2014)

is secreted into the core of the rosette as an ECM component (Figure 4A). In *S. rosetta*, the integrity of the colonies is thus possibly ensured by the basal extracellular matrix, to which cells appear to anchor by filopodia (Dayel et al. 2011). Both ECM and filopodia also contribute to the cohesion of animal blastulae. Blastomeres are held together by an abundant ECM rich in lectins (Fraser and Zalik 1977; Roberson and Barondes 1983; Harris and Zalik 1985; Outenreath et al. 1988; Lee et al. 1997) and appear linked by filopodia in sponges (Ereskovsky 2010), cnidarians (Benayahu et al. 1989), echinoderms (Vacquier 1968), amphioxus (Hirakow and Kajita 1994), and mouse (Salas-Vidal and Lomelı 2004). In mice, laser ablation of the filopodia results in loss of blastomere cohesion (Fierro-González et al. 2013). Thus, the multicellular state of choanoflagellate rosettes and animal embryos is established and maintained by comparable mechanisms.

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303 An intriguing open question is whether cadherins, which contribute to adhesion between
304 animal embryonic and adult cells (Abedin and King 2010), are also involved in rosette formation.
305 Choanoflagellate genomes encode a large number of cadherins (23 in *Monosiga brevicollis* and 29
306 in *S. rosetta*) (Nichols et al. 2012) but their localization is so far only known in the solitary species
307 *Monosiga brevicollis*, in which the cadherins MBCDH1 and MBCDH2 were found localized to
308 the collar and might contribute either to collar integrity or to bacterial capture (Abedin and King
309 2008).
310
311 *III.3. Choanoflagellate multicellularity and the origin of animal embryogenesis*
312
313 The similarity of choanoflagellate rosettes to the blastula/morula stage of animal
314 development is consistent with a modern version of Haeckel's Blastaea hypothesis – which
315 proposes that early animal ancestors were motile spheres of flagellated cells that formed clonally
316 (Haeckel 1874, 1892; Nielsen 2008; Arendt et al. 2015). Indeed, the first developmental stage of
317 marine invertebrates is often a ciliated free-swimming blastula that resembles choanoflagellate
318 rosettes (minus the microvillar collar) and, given its widespread taxonomic distribution, was
319 plausibly part of development in the Urmetazoan (Nielsen 2012).
320 How did serial cell divisions give rise to these early multicellular forms? In most
321 eukaryotes, including choanoflagellates, cell division has to accommodate a constraint: the two
322 centrioles that form the flagellar basal body during interphase also organize the mitotic spindle
323 during division. Moreover, in choanoflagellates, microvilli are directly transmitted to daughter
324 cells. As a consequence, the plane of cell division in choanoflagellates must comprise the apical
325 pole, where centrioles and microvilli are located, and symmetric cell divisions necessarily take

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place along the apico-basal axis (Figure 4A). This constraint on the direction of cell division seems universal in choanoflagellates (Leadbeater 2014; Anderson et al. 2016). As a consequence of this fixed division orientation, in the absence of cell reorientation and/or rearrangements, cell proliferation alone can only produce cells linked together in linear chains (e.g. in *S. rosetta* (Dayel et al. 2011)) or planar sheets (e.g. in *Choanoeca perplexa* – formerly *Proterospongia choanojuncta* (Leadbeater 1983)). Any more complex shape – such as a spherical rosette – must be achieved by cell rearrangements after division to allow bending and, ultimately, "closure" of the sheet at the point where non-sister cells meet.

These cell rearrangements are apparent during rosette formation in *S. rosetta* (Fairclough et al. 2010) and the final "closure point" can be identified in mature rosettes as an ECM-free spot visible in immunostainings for the Rosetteless protein (Woznica et al. 2016). This constraint seems to apply to any spherical colony formed by clonal division of motile flagellated cells: in flagellated volvocale green algae, a similar folding process takes place (Höhn and Hallmann 2016). It plausibly applied to the first animal embryos as well: indeed, a similar series of steps takes place during the early development of calcareous sponges, which first form as concave sheets and secondarily fold into spherical embryos called "amphiblastulae" (Figure 4B) (Franzen 1988; Ereskovsky 2010; Arendt et al. 2015). In both volvocales and calcareans, the hole resorbed at the closure point is called the phialopore. In other animals (eumetazoans and non-calcareous sponges), after the evolution of non-flagellated zygotes, this constraint was eventually overcome: early cell division can take place along all cellular axes and cleavage directly produces a spherical blastula (Arendt and Nübler-Jung 1997).

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IV. The ancestry of animal cell differentiation

IV.1. Alternative hypotheses for the origin of animal cell types: temporal-to-spatial transition and division of labor

Choanoflagellate rosettes and the multicellular forms of ichthyosporeans and filastereans are not known to undergo cell differentiation. This contrasts with the "complex multicellularity" (Knoll 2011) of animals, which are mosaics of different cell types with controlled spatial distribution. The origin of cell types thus represents one of the key steps in the evolution of animals from their microeukaryote ancestors. Two main hypotheses have been put forward to explain the evolution of animal cell differentiation: the temporal-to-spatial transition (TST) hypothesis (Zakhvatkin 1949; Mikhailov et al. 2009; Sebé-Pedrós et al. 2017) and the division of labor (DOL) hypothesis (Mackie 1970; Arendt 2008). The TST hypothesis proposes that cell differentiation predated multicellularity, building upon the observation that many modern microbial eukaryotes can switch between different cell phenotypes over their life history (Figure 5). In this scenario, some of these temporally alternating phenotypes were converted into spatially segregated cell types in animal ancestors. The DOL hypothesis starts from the observation that individual microbial eukaryotes combine in a single cell multiple functions that are accomplished by different cell types in animals (Figure 6), including the perception and transduction of environmental signals, movement, feeding, and division. In the DOL hypothesis, the bulk of cell differentiation evolved after multicellularity, by differential loss of function from multifunctional ancestral cell types. The TST and the DOL hypotheses are complementary rather than mutually exclusive, and it is plausible that both processes contributed to the early evolution of animal cell types.

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371

372 *IV.2. Testing the TST hypothesis*

373

374 Many single-celled eukaryotes have temporally alternating cell phenotypes (Figure 5).
375 The distinct morphologies, transcriptomes (Fairclough et al. 2013; Sebé-Pedrós et al. 2013b; de
376 Mendoza et al. 2015), proteomes (Sebé-Pedrós et al. 2016b), and chromatin states (Sebé-Pedrós et
377 al. 2016a) of these cell phenotypes in single-celled holozoans suggest that they represent stable
378 cell types, like those existing in animals, rather than instances of short-term phenotypic plasticity.
379 Are some of these cell types homologous to those of animals, as the TST hypothesis postulates?
380 The simplest example of temporal-to-spatial transition is probably meiosis, which clearly predated
381 animals and dates back to the last common eukaryotic ancestor (Ramesh et al. 2005). In
382 choanoflagellates, gametes competent to undergo cell-cell fusion directly transdifferentiate from
383 haploid solitary cells (Figure 5B) (Levin and King 2013; Woznica et al. 2017), while in all extant
384 animals meiosis and gametogenesis start from diploid cells integrated within the adult organism
385 (Nielsen 2012).

386 Another temporal switch frequently found in protozoa that has been proposed to have
387 been co-opted in animal cell differentiation is the alternation between a flagellated phenotype
388 (allowing locomotion in an aqueous environment by flagellar beating) and a deformable
389 "amoeboid" crawling phenotype that navigates solid environments by actin-mediated
390 deformations of the cell body[6] (Figure 5A). These two phenotypes and associated genes are broadly

[6] In the past, shape-shifting and crawling cells have often been encompassed within the general adjective "amoeboid" (Taylor and Condeelis 1979). More recently, there has been a tendency to distinguish different types of deformable crawlers based on the mechanisms of movement – restricting the term "amoeboid migration" to fast-migrating cells moving by actomyosin contraction and blebbing independently of adhesion (Liu et al. 2015; Ruprecht et al. 2015). Other categories include "mesenchymal migration" for slow-migrating animal cells with a lamellipodium propelled by actin polymerization and integrin adhesion (Panková et al. 2010) and more recently "α-motility" for fast migration with a pseudopod propelled by actin polymerization without specific adhesion (Fritz-

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391 distributed in the eukaryotic tree of life (Supplementary Figure 2), suggesting that the last common
392 eukaryotic ancestor might have been able to switch between both (Fritz-Laylin et al. 2010, 2017).
393 If so, this switch could have existed in the last single-celled ancestors of animals[7]. Suggestively,
394 virtually all animals combine static epithelial cells (often bearing a flagellum and lining the body
395 surface) with deformable crawling cells located in the primary body cavity that patrol the tissues
396 by actin-mediated locomotion. These migratory interstitial cells usually function as phagocytes
397 (and sometimes also as stem cells) and quickly accumulate upon infection, wound repair or
398 allograft rejection. They are called archaeocytes in sponges (Cheng et al. 1968; Alié et al. 2015),
399 amoebocytes in cnidarians (Patterson and Landolt 1979; Olano and Bigger 2000; Couch et al.
400 2013) and hemocytes or macrophages in bilaterians (Hartenstein 2006; Schmidt-Rhaesa 2007). If
401 the interstitial phagocytes of these large animal clades are homologous (which remains to be
402 tested), they could have evolved from the crawling phase of the Urchoanozoan or one of its
403 descendants (Mendoza et al. 2002; Arendt et al. 2015). In parallel, the flagellated phase of this
404 organism would have provided the genetic program for the ciliated epithelium that putatively
405 covered the surface of the Blastaea and subsequently diversified into several epithelial cell types
406 and into other cell types thought to have evolved from epithelia (epidermal cells, enterocytes,
407 sensory cells, neurons and muscle cells (see section IV.3 and Figure 6B)).

Laylin et al. 2017). A common feature of nearly all deformable crawlers is their reliance on actin microfilaments (the only known exception is nematode sperm (Bottino et al. 2002)). Here, in line with recent studies (Fritz-Laylin et al. 2017), we use the general term "crawling cells" to refer globally to these cells regardless of the precise underlying mechanism.

[7] Notably, no deformable crawling phase is known in extant choanoflagellates (despite one early unconfirmed report (Saville-Kent 1880)), suggesting they might have lost it – or that it does not occur under standard laboratory conditions. Choanoflagellates do show the ability to crawl on solid substrates by dynamic extension and retraction of adhesive filopodia prior to settlement, but without apparent deformations of the cell body outside filopodia (Figure 5B) (Dayel et al. 2011).

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How could one test the homology between animal and single-celled holozoan cell types? In the past few years, the comparison of transcriptomes has emerged as a promising approach to investigate cell type homology (Arendt 2005, 2008; Lauri et al. 2014) and to build cell type trees (Liang et al. 2015; Kin et al. 2015; Musser and Wagner 2015). Central to these comparisons are the "terminal selector" transcription factors that directly implement cell phenotypes by sitting directly above large batteries of differentiation genes (Arendt et al. 2016; Hobert 2016). This approach can potentially be extended to single-celled holozoans: do terminal selector transcription factors play a role in establishing and maintaining their cell types? If so, how do they compare to their animal counterparts? These questions are still open, but intriguing potential case studies can already be identified. For deformable crawlers, an interesting candidate is the Runx family of transcription factors (Coffman 2003), which were likely present as a single copy in urmetazoan and urholozoan progenitors (Rennert et al. 2003; Sullivan et al. 2008; de Mendoza et al. 2013). Runx transcription factors specify circulating cells that move by actin-mediated crawling (Pancer et al. 1999; Otto et al. 2003; Waltzer et al. 2003; Burns et al. 2005) and directly promote cell motility (Leong et al. 2010; Zusso et al. 2012; Lie-A-Ling et al. 2014; VanOudenhove et al. 2016). High levels of Runx transcription have been detected by RNA-seq in the archaeocytes of the sponge *Ephydatia fluviatilis* (Alié et al. 2015), suggesting that the link between this transcription factor family and the crawling cell phenotype might be ancient in animals. Runx is also present in the genomes of ichthyosporeans and filastereans (de Mendoza et al. 2013), which both have a crawling ("amoeboid") phase. The *runx* gene is significantly upregulated during the crawling phase of the ichthyosporean *Creolimax* (de Mendoza et al. 2015) and, in *Capsaspora*, computationally predicted Runx targets show a significant enrichment for genes encoding actin cytoskeleton and other proteins involved in crawling motility (Sebé-Pedrós et al. 2016a).

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Another potentially relevant transcription factor with stem-holozoan ancestry is Brachyury, which is upregulated in *Capsaspora* amoebae and is predicted to regulate homologs of genes controlled by Brachyury in mouse, including those required for cell motility (Sebé-Pedrós et al. 2016a). In animals, Brachyury is often involved in the motility of embryonic (but not adult) cells (Yanagisawa et al. 1981; Gross and McClay 2001). Both Runx and Brachyury appear to have been lost from sequenced choanoflagellate genomes, in line with the possibility that choanoflagellates might have lost crawling by whole-cell deformation. Direct mechanistic studies of these and other transcription factors in both single-celled holozoans and non-bilaterian animals, as well as more extensive molecular characterization of the relevant cell types in a broad sampling of phylogenetically relevant species, will help in testing these hypotheses and in revealing other potential instances of TST.

IV.3. Testing the DOL hypothesis

The phenotypic comparison of animal cells with free-living microeukaryotes offers some direct observations in support of the DOL hypothesis. Several cellular features that are constitutively present in choanoflagellates are restricted to a subset of animal cell types. These include a motile flagellum, filopodia, contractile stress fibers, polarized secretion (Burkhardt et al. 2011), and mitotic machinery – respectively restricted in animals to ciliated cells (in humans: vestibular hair cells, spermatozoa, tracheal lining, fallopian tubes), migratory cells, muscle cells (and other contractile cells), neurons (and other secretory cells), and stem/progenitor cells. In support of the antiquity of these cellular modules, many of the effector genes that implement these phenotypes in animals are conserved in choanoflagellates and other holozoans (Figure 6A).

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Intriguingly, the same is true of the selector transcription factors that control expression of these modules in animals (Figure 6A) – for example FoxJ1 and RFX for flagella (see Figure 6 for other examples and the legend for references). It is currently unknown whether the targets of most of these transcription factors are the same in choanoflagellates as in animals. In the case of Myc, bioinformatic data suggest that the choanoflagellate ortholog preferentially controls ribosomal biogenesis genes, as it does in animals (in line with the general requirement for intense protein synthesis during proliferation) (Brown et al. 2008), and that RFX controls at least a few flagellar genes in *Monosiga brevicollis* (Piasecki et al. 2010).

Detailed comparisons of these cellular modules and of their upstream transcriptional circuits with the cell type-specific modules and circuits of animals will be crucial to further testing the DOL hypothesis. The targets of the cell type-specific transcription factors (Figure 6) could be determined experimentally by ChIP-seq and/or loss-of-function experiments in choanoflagellates and other single-celled holozoans. If they are at the top of the same regulatory networks as in animals, this would further support the DOL hypothesis, and would suggest a mechanistic basis for division of labor. Indeed, it would imply that the transcriptional networks of the single-celled ancestors of choanozoans or holozoans were modular, with different transcription factors sitting upstream of functionally distinct gene modules. Such an architecture would imply that animal ancestors were 'pre-adapted' to evolving division of labor once multicellularity evolved, as a module could be selectively 'shut down' in a given cell by inhibiting its upstream transcription factor with minimal impact on the expression of other modules. Intriguingly, and consistent with this hypothesis, a modular architecture of gene regulatory networks, with functionally related genes or operons being controlled by the same transcription factors, has repeatedly been found in single-celled organisms including *E. coli*, *B. subtilis* (Shen-Orr et al. 2002; Madan Babu and

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Teichmann 2003; Fadda et al. 2009) and yeast (Tavazoie et al. 1999; Tanay et al. 2004). The advantage of such a modular architecture in single-celled organisms is not generally known, but possibilities include modulating the total synthesized quantity of a module (depending for example on environmental signals or on the phase of the cell cycle), ensuring stoichiometry between its components, or allowing regeneration of a given part of the cell. For example, choanoflagellates regenerate their flagellum after each division and can also do so when recovering from microtubule-depolymerizing treatment (Froesler and Leadbeater 2009). In the unicellular alga *Chlamydomonas*, flagellum regeneration involves a coordinated rise in flagellar gene transcription (Keller et al. 1984) and investigation of promoter motifs suggests shared regulation of these flagellar genes by yet unidentified, specific transcription factors (Stolc et al. 2005).

Currently, the evidence for the TST and DOL scenarios remains primarily descriptive and restricted to a few candidate genes. It will be crucial to ground future comparative studies in a firm mechanistic basis to more fully test these two hypotheses and investigate their respective contributions to the origin of animal cell types. This endeavor should benefit from ongoing efforts to obtain an unbiased transcriptomic, proteomic and phenotypic characterization of animal cell types, notably by single-cell approaches (Achim et al. 2015; Wagner et al. 2016; Villani et al. 2017; Regev et al. 2017). Analyzed with refined methods of phylogenetic reconstruction and ancestral state inference (Liang et al. 2015; Kin et al. 2015), such datasets should help illuminate our understanding of the origin and evolution of animal cell differentiation.

Conclusion and outlooks

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In the pre-molecular era, the reliance on morphology to reconstruct the origin of animals posed a risk of circular reasoning, as ancestral state reconstructions relied on phylogenetic trees that were themselves built from hypotheses of morphological evolution. This circularity was broken with the application of molecular phylogenetics, which has resolved the relationships among most animal phyla to each other and to protozoan relatives, with only a few outstanding controversies – such as whether the sponge or ctenophore lineage branched first (Whelan et al. 2015; Simion et al. 2017). By mapping morphological features onto molecular phylogenies, it has become possible to re-approach the morphology, cell biology and development of the first animals from a modern perspective.

The first animals likely developed through serial division of flagellated, bacterivorous cells that sported a microvillar collar. Diverse cell types then evolved through a combination of innovation, division of labor, and spatiotemporal regulation of cell types that were ancestrally separated temporally in life histories of the progenitors of animals. Determining the relative contributions of these different mechanisms, and the interrelationships among different cell types in diverse animals will not only help us understand our evolutionary origins, but help to deepen our understanding of the structure and function of animal cells themselves. Indeed, insights into evolutionary relationships have already led to unexpected discoveries in cell biology – e.g., the discovery that parasitic apicomplexans such as *Toxoplasma* and *Plasmodium* harbored modified plastids provided the rationale for targeting them with drugs known to affect cyanobacteria (Fichera and Roos 1997); and comparative characterizations of ciliary proteomes have, by identifying an evolutionarily conserved core, revealed central players of clinical relevance (Li et al. 2004).

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521 In the future, it will be valuable to develop functional molecular and cellular techniques in
522 a broader range of early-branching animals and single-celled holozoans, and to expand
523 observational research to clarify uncertainties concerning, for example, the life cycles of additional
524 choanoflagellates or the embryology of sponges and placozoans. Transcriptome sequencing of
525 diverse cell types from choanoflagellates and early branching animals may also help to reveal the
526 relative importance of division of labor as opposed to temporal-to-spatial transitions in the
527 evolution of animal cell differentiation. By integrating these lines of research, we hope to gain a
528 clearer picture of how our protozoan ancestors emancipated from the microbial world and founded
529 the animal kingdom.
530
531
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533
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538

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Figure legends

Figure 1. Phylogenetic distribution of traits inferred in the Urmetazoan. The presence of epithelia (Leys et al. 2009), sperm, eggs and multicellularity (Nielsen 2012) and collar complex (see Supplementary Table 1 for details and references) are mapped onto a eukaryotic phylogeny. The collar complex is inferred to have been present in the Urchoanozoan, and to be a choanozoan synapomorphy. The eukaryotic phylogenetic tree follows (Torruella et al. 2015). The animal phylogenetic tree is a consensus based on (Struck et al. 2014; Laumer et al. 2015) for Spiralia, (Borner et al. 2014) for Ecdysozoa, (Cannon et al. 2016) for Xenacoelomorpha and (Telford et al. 2015) for other animal phyla. Species silhouettes are from PhyloPic (http://phylopic.org).

Figure 2. Morphology and ultrastructure of choanoflagellates and sponge choanocytes. (A) Scanning Electron Micrograph (SEM) of the choanoflagellate *Salpingoeca rosetta*, reproduced from (Dayel et al. 2011). In this panel and all others: (fl): flagellum, (mv): microvilli. (B) SEM of choancoytes from the sponge *Sycon coactum*, from (Leys and Hill 2012). In this panel and others: (nu): nucleus, (fv): food vacuole. (C) Transmission Electron Micrograph (TEM) of the flagellar vane of the choanoflagellate *Salpingoeca amphoridium*, from (Leadbeater 2014). (D) SEM of the flagellar vane of a choanocyte from the sponge *Spongilla lacustris*, from (Mah et al. 2014). Arrow shows the fibrous structure of the vane and lateral contact with the collar. (E) Comparative ultrastructural schematics of a choanoflagellate and a sponge choanocyte, modified from (Maldonado 2004) following (Woollacott and Pinto 1995) for the microtubule cytoskeleton and (Karpov and Leadbeater 1998) for the actin cytoskeleton. The presence of filopodia in choanocytes is indicated following (Grassé 1973). (mt): mitochondria. (F,G) basal microtubular foot supporting the flagellum in choanoflagellates and choanocytes, following (Garrone 1969; Woollacott and Pinto 1995; Leadbeater 2014).

Figure 3. Clonal and aggregative multicellularity. (A) Phylogenetic distribution of clonal and aggregative multicellularity. Eukaryotic phylogeny is modified from (Keeling et al. 2014). Instances of multicellularity are mapped following (Bonner 1998; King 2004; Raven 2005; Ott et al. 2015; Sebé-Pedrós et al. 2017). (B and C) Examples of aggregative and clonal multicellularity. The organism indicated is shown in bold, and other organisms with similar forms of multicellularity are listed below. (B) Aggregative multicellularity gives rise to spherical masses of spores or cysts, sometimes atop a stalk. From (Bonner 1998; Brown et al. 2012; Du et al. 2015). (C) Clonal multicellularity gives rise to diverse multicellular forms. Drawn from (Bonner 1998; Fairclough et al. 2010).

Figure 4. Morphogenesis in choanoflagellate rosettes, calcareous sponge embryos and volvocale embryos. (A) Morphogenesis during rosette formation in the choanoflagellate *Salpingoeca rosetta,* following (Fairclough et al. 2010). (B) Early embryonic development of the calcareous sponge *Sycon ciliatum*, including amphiblastula inversion, from (Franzen 1988). (C) Early embryonic development of the volvocale *Pleodorina californica* (Höhn and Hallmann 2016). In other volvocales such as *Volvox*, an additional developmental stage is intercalated, in which the embryo first forms a sphere with flagella pointing inward, which later opens up into a sheet before inversion.

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583 **Figure 5. Temporally alternating cell types in protozoans.** (A) The heterolobosean excavate
584 *Naegleria gruberi* can switch between a flagellated swimmer phenotype and a deformable crawler
585 ("amoeboid") phenotype. Redrawn from (Fritz-Laylin et al. 2010). (B) Cell types and life history
586 transitions in the choanoflagellate *S. rosetta*, from (Dayel et al. 2011; Levin and King 2013). Main
587 panel depicts the dynamic asexual life history of *S. rosetta* whereas the inset indicates its sexual
588 cycle. Dotted lines indicated inferred transitions that have not been directly observed.
589
590 **Figure 6. The division of labor hypothesis.** (A) Cellular modules present in the choanoflagellate
591 *Salpingoeca rosetta*. On the right: choanoflagellate orthologs of the selector transcription factors
592 that control these modules in animals, on top of a list of choanoflagellate orthologs of their animal
593 targets (Supplementary Figure 3 and references in the text). The transcription factors are FoxJ1
594 and RFX for flagella (Stubbs et al. 2008; Yu et al. 2008; Thomas et al. 2010; Vincensini et al.
595 2011; Vij et al. 2012), Mef2 for contractility (Black and Olson 1998; Sebé-Pedrós et al. 2011;
596 Brunet et al. 2016), SRF for filopodia (Knöll and Nordheim 2009; Gervais and Casanova 2011;
597 Weinl et al. 2013; Franco et al. 2013), and the Myc::Max complex for stem cells (Young et al.
598 2011). No terminal selector is indicated for the secretion apparatus, as there seems to be no known
599 neural terminal selector with a choanoflagellate ortholog. Dotted lines indicate that it is unknown
600 whether the choanoflagellate transcription factors control the same genes as their animal orthologs,
601 except for the Myc::Max complex for which regulation is indicated by computational predictions
602 (Brown et al. 2008) and electrophoretic mobility shift assays (Young et al. 2011). (B) Cellular
603 modules shown in A are segregated into distinct cell types in animals (here, the putative cell type
604 complement of stem-eumetazoans is illustrated based on the cell types shared by cnidarians and
605 bilaterians (Fautin and Mariscal 1991; Schmidt-Rhaesa 2007; Arendt et al. 2015)) and terminal
606 selector transcription factors specify distinct cell types.
607
608 **Supplementary Figure 1. Collar cells of cnidarians and bilaterians.** All panels are TEM.
609 (A) Epidermal collar cell from the ciliary bands of the brachiolaria larva of the starfish *Asterina*
610 *rubens*, from (Nerrevang and Wingstrand 1970). (B) Protonephridial terminal cell from the
611 trochophore larva of the annelid *Glycera* (Ruppert and Smith 1988). (C) Gastrodermal collar cell
612 from the cnidarian *Polypodium hydriforme* (Raikova 1995). (fl): flagellum, (mv): microvilli, (bb):
613 basal body, (vc): vacuole, (mu): mucus.
614
615 **Supplementary Figure 2. Phylogenetic distribution of flagellated swimmers and of**
616 **deformable crawling ("amoeboid") cells.** Eukaryotic phylogeny is modified from (Keeling et al.
617 2014). Distribution of crawling cells follows (Fritz-Laylin et al. 2017). Cells with pseudopodia
618 used for prey capture and phagocytosis but not for motility are not classified as deformable
619 crawlers (e.g. choanoflagellates (Leadbeater 2014), parabasalids (Brugerolle 2005)).
620
621 **Supplementary Figure 3. SRF and FoxJ phylogenetic trees.** (A) FoxJ family phylogenetic tree.
622 The split between the FoxJ1 clade and the FoxJ2/3 clades is inferred to have preceded
623 choanozoans, in accordance with previous reports (Larroux et al. 2008). Two Forkhead-box
624 proteins which are mutual best BLAST hits with *Homo sapiens* FoxJ1 cluster in a clade together
625 with animal FoxJ1. (B) SRF family phylogenetic tree. The tree is rooted with the Mef2 family, the
626 most closely related family of MADS-box transcription factors (including a previously identified
627 ortholog in *Monosiga brevicollis* (Sebé-Pedrós et al. 2011) and a newly predicted ortholog in
628 *Salpingoeca rosetta* on genomic scaffold supercontig 1.36 positions 82482-84459). A *Monosiga*

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629 *brevicollis* predicted protein annotated as SRF and the *Salpingoeca rosetta* protein PTSG_11095
630 (both mutual best BLAST hits with *Homo sapiens* SRF) cluster in a clade with animal SRF. Both
631 trees have been reconstructed with MrBayes 3.2.3, with 10,000 generations under the GTR
632 substitution model with default assumptions and convergence assessed by standard deviation <
633 0.01. Support values are displayed on nodes.
634
635

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636 **References**

637

638 Abedin M, King N (2008) The premetazoan ancestry of cadherins. Science 319:946–948. doi:
639 10.1126/science.1151084

640 Abedin M, King N (2010) Diverse evolutionary paths to cell adhesion. Trends Cell Biol 20:734–742. doi:
641 10.1016/j.tcb.2010.08.002

642 Achim K, Pettit J-B, Saraiva LR, et al (2015) High-throughput spatial mapping of single-cell RNA-seq data to tissue
643 of origin. Nat Biotechnol 33:503–509. doi: 10.1038/nbt.3209

644 Alegado RA, Brown LW, Cao S, et al (2012) A bacterial sulfonolipid triggers multicellular development in the
645 closest living relatives of animals. eLife 1:e00013. doi: 10.7554/eLife.00013

646 Alié A, Hayashi T, Sugimura I, et al (2015) The ancestral gene repertoire of animal stem cells. Proc Natl Acad Sci U
647 S A 112:E7093-7100. doi: 10.1073/pnas.1514789112

648 Anderson DP, Whitney DS, Hanson-Smith V, et al (2016) Evolution of an ancient protein function involved in
649 organized multicellularity in animals. eLife 5:e10147. doi: 10.7554/eLife.10147

650 Arendt D (2004) Comparative aspects of gastrulation. Gastrulation Cells Embryos 679–693.

651 Arendt D (2008) The evolution of cell types in animals: emerging principles from molecular studies. Nat Rev Genet
652 9:868–882. doi: 10.1038/nrg2416

653 Arendt D (2005) Genes and homology in nervous system evolution: comparing gene functions, expression patterns,
654 and cell type molecular fingerprints. Theory Biosci Theor Den Biowissenschaften 124:185–197. doi:
655 10.1016/j.thbio.2005.08.002

656 Arendt D, Benito-Gutierrez E, Brunet T, Marlow H (2015) Gastric pouches and the mucociliary sole: setting the
657 stage for nervous system evolution. Philos Trans R Soc Lond B Biol Sci. doi: 10.1098/rstb.2015.0286

658 Arendt D, Musser JM, Baker CVH, et al (2016) The origin and evolution of cell types. Nat Rev Genet 17:744–757.
659 doi: 10.1038/nrg.2016.127

660 Arendt D, Nübler-Jung K (1997) Dorsal or ventral: similarities in fate maps and gastrulation patterns in annelids,
661 arthropods and chrodates. Mech Dev 61:7–21.

662 Benabentos R, Hirose S, Sucgang R, et al (2009) Polymorphic members of the lag gene family mediate kin
663 discrimination in Dictyostelium. Curr Biol CB 19:567–572. doi: 10.1016/j.cub.2009.02.037

664 Benayahu Y, Berner T, Achituv Y (1989) Development of planulae within a mesogleal coat in the soft coral
665 Heteroxenia fuscescens. Mar Biol 100:203–210.

666 Bergman K, Goodenough UW, Goodenough DA, et al (1975) Gametic differentiation in Chlamydomonas
667 reinhardtii. II. Flagellar membranes and the agglutination reaction. J Cell Biol 67:606–622.

668 Black BL, Olson EN (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2)
669 proteins. Annu Rev Cell Dev Biol 14:167–196. doi: 10.1146/annurev.cellbio.14.1.167

670 Bonner JT (1998) The origins of multicellularity. Integr Biol Issues News Rev 1:27–36.

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671 Boraas ME, Seale DB, Boxhorn JE (1998) Phagotrophy by a flagellate selects for colonial prey: a possible origin of
672 multicellularity. Evol Ecol 12:153–164.

673 Borner J, Rehm P, Schill RO, et al (2014) A transcriptome approach to ecdysozoan phylogeny. Mol Phylogenet Evol
674 80:79–87. doi: 10.1016/j.ympev.2014.08.001

675 Bottino D, Mogilner A, Roberts T, et al (2002) How nematode sperm crawl. J Cell Sci 115:367–384.

676 Bradley DE (1964) A study of the Mallomonas, Synura and Chrysosphaerella of Northern Iceland. Microbiology
677 37:321–333.

678 Brown MW, Kolisko M, Silberman JD, Roger AJ (2012) Aggregative multicellularity evolved independently in the
679 eukaryotic supergroup Rhizaria. Curr Biol CB 22:1123–1127. doi: 10.1016/j.cub.2012.04.021

680 Brown SJ, Cole MD, Erives AJ (2008) Evolution of the holozoan ribosome biogenesis regulon. BMC Genomics
681 9:442. doi: 10.1186/1471-2164-9-442

682 Brugerolle G (2005) The Amoeboid Parabasalid Flagellate Gigantomonas herculeaof the African Termite
683 Hodotermes mossambicusReinvestigated Using Immunological and Ultrastructural Techniques. Acta
684 Protozool 44:189–199.

685 Brunet T, Fischer AH, Steinmetz PR, et al (2016) The evolutionary origin of bilaterian smooth and striated
686 myocytes. eLife. doi: 10.7554/eLife.19607

687 Budd GE, Jensen S (2017) The origin of the animals and a "Savannah" hypothesis for early bilaterian evolution.
688 Biol Rev Camb Philos Soc 92:446–473. doi: 10.1111/brv.12239

689 Burkhardt P, Stegmann CM, Cooper B, et al (2011) Primordial neurosecretory apparatus identified in the
690 choanoflagellate Monosiga brevicollis. Proc Natl Acad Sci U S A 108:15264–15269. doi:
691 10.1073/pnas.1106189108

692 Burns CE, Traver D, Mayhall E, et al (2005) Hematopoietic stem cell fate is established by the Notch–Runx
693 pathway. Genes Dev 19:2331–2342. doi: 10.1101/gad.1337005

694 Buss LW (1988) The Evolution of Individuality, 1St Edition edition. Princeton University Press, Princeton, N.J

695 Cannon JT, Vellutini BC, Smith J, et al (2016) Xenacoelomorpha is the sister group to Nephrozoa. Nature 530:89–
696 93. doi: 10.1038/nature16520

697 Carr M, Leadbeater BSC, Hassan R, et al (2008) Molecular phylogeny of choanoflagellates, the sister group to
698 Metazoa. Proc Natl Acad Sci U S A 105:16641–16646. doi: 10.1073/pnas.0801667105

699 Carr M, Richter DJ, Fozouni P, et al (2017) A six-gene phylogeny provides new insights into choanoflagellate
700 evolution. Mol Phylogenet Evol 107:166–178. doi: 10.1016/j.ympev.2016.10.011

701 Cavalier-Smith T (2017) Origin of animal multicellularity: precursors, causes, consequences-the
702 choanoflagellate/sponge transition, neurogenesis and the Cambrian explosion. Philos Trans R Soc Lond B
703 Biol Sci. doi: 10.1098/rstb.2015.0476

704 Cavalier-Smith T, Chao EE-Y (2006) Phylogeny and megasystematics of phagotrophic heterokonts (kingdom
705 Chromista). J Mol Evol 62:388–420. doi: 10.1007/s00239-004-0353-8

706 Cavalier-Smith T, Chao EE-Y, Oates B (2004) Molecular phylogeny of Amoebozoa and the evolutionary
707 significance of the unikont Phalansterium. Eur J Protistol 40:21–48.

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708 Chang ES, Neuhof M, Rubinstein ND, et al (2015) Genomic insights into the evolutionary origin of Myxozoa within
709 Cnidaria. Proc Natl Acad Sci U S A 112:14912–14917. doi: 10.1073/pnas.1511468112

710 Cheng TC, Yee HW, Rifkin E, Kramer MD (1968) Studies on the internal defense mechanisms of sponges: III.
711 Cellular reactions in Terpios zeteki to implanted heterologous biological materials. J Invertebr Pathol
712 12:29–35.

713 Coffman JA (2003) Runx transcription factors and the developmental balance between cell proliferation and
714 differentiation. Cell Biol Int 27:315–324. doi: 10.1016/S1065-6995(03)00018-0

715 Couch CS, Weil E, Harvell CD (2013) Temporal dynamics and plasticity in the cellular immune response of the sea
716 fan coral, Gorgonia ventalina. Mar Biol 160:2449–2460.

717 Curtis ASG (1962) Pattern and mechanism in the reaggregation of sponges. Nature 196:245–248.

718 Dayel MJ, Alegado RA, Fairclough SR, et al (2011) Cell differentiation and morphogenesis in the colony-forming
719 choanoflagellate Salpingoeca rosetta. Dev Biol 357:73–82. doi: 10.1016/j.ydbio.2011.06.003

720 de Mendoza A, Sebé-Pedrós A, Šestak MS, et al (2013) Transcription factor evolution in eukaryotes and the
721 assembly of the regulatory toolkit in multicellular lineages. Proc Natl Acad Sci U S A 110:E4858-4866.
722 doi: 10.1073/pnas.1311818110

723 de Mendoza A, Suga H, Permanyer J, et al (2015) Complex transcriptional regulation and independent evolution of
724 fungal-like traits in a relative of animals. eLife 4:e08904. doi: 10.7554/eLife.08904

725 Dickinson DJ, Nelson WJ, Weis WI (2011) A Polarized Epithelium Organized by β- and α-Catenin Predates
726 Cadherin and Metazoan Origins. Science 331:1336–1339. doi: 10.1126/science.1199633

727 Douglas LM, Li L, Yang Y, Dranginis AM (2007) Expression and characterization of the flocculin Flo11/Muc1, a
728 Saccharomyces cerevisiae mannoprotein with homotypic properties of adhesion. Eukaryot Cell 6:2214–
729 2221. doi: 10.1128/EC.00284-06

730 Du Q, Kawabe Y, Schilde C, et al (2015) The Evolution of Aggregative Multicellularity and Cell-Cell
731 Communication in the Dictyostelia. J Mol Biol 427:3722–3733. doi: 10.1016/j.jmb.2015.08.008

732 Du Q, Schilde C, Birgersson E, et al (2014) The cyclic AMP phosphodiesterase RegA critically regulates
733 encystation in social and pathogenic amoebas. Cell Signal 26:453–459. doi: 10.1016/j.cellsig.2013.10.008

734 Dworkin M (1963) Nutritional regulation of morphogenesis in Myxococcus xanthus. J Bacteriol 86:67–72.

735 Ereskovsky AV (2010) The comparative embryology of sponges. Springer Science & Business Media

736 Fadda A, Fierro AC, Lemmens K, et al (2009) Inferring the transcriptional network of Bacillus subtilis. Mol Biosyst
737 5:1840–1852. doi: 10.1039/B907310H

738 Fairclough SR, Chen Z, Kramer E, et al (2013) Premetazoan genome evolution and the regulation of cell
739 differentiation in the choanoflagellate Salpingoeca rosetta. Genome Biol 14:R15. doi: 10.1186/gb-2013-14-
740 2-r15

741 Fairclough SR, Dayel MJ, King N (2010) Multicellular development in a choanoflagellate. Curr Biol CB 20:R875-
742 876. doi: 10.1016/j.cub.2010.09.014

743 Fautin DG, Mariscal RN (1991) Cnidaria: anthozoa. In: Microscopic Anatomy of Invertebrates Volume 2: Placozoa,
744 Porifera, Cnidaria, and Ctenophora. Wiley-Liss,

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745 Fichera ME, Roos DS (1997) A plastid organelle as a drug target in apicomplexan parasites. Nature 390:407–409.
746 doi: 10.1038/37132

747 Fidalgo M, Barrales RR, Ibeas JI, Jimenez J (2006) Adaptive evolution by mutations in the FLO11 gene. Proc Natl
748 Acad Sci U S A 103:11228–11233. doi: 10.1073/pnas.0601713103

749 Fierro-González JC, White MD, Silva JC, Plachta N (2013) Cadherin-dependent filopodia control preimplantation
750 embryo compaction. Nat Cell Biol 15:1424–1433. doi: 10.1038/ncb2875

751 Fiore-Donno AM, Nikolaev SI, Nelson M, et al (2010) Deep Phylogeny and Evolution of Slime Moulds
752 (Mycetozoa). Protist 161:55–70. doi: 10.1016/j.protis.2009.05.002

753 Franco CA, Blanc J, Parlakian A, et al (2013) SRF selectively controls tip cell invasive behavior in angiogenesis.
754 Dev Camb Engl 140:2321–2333. doi: 10.1242/dev.091074

755 Franzen W (1988) Oogenesis and larval development of Scypha ciliata (Porifera, Calcarea). Zoomorphology
756 107:349–357.

757 Fraser BR, Zalik SE (1977) Lectin-mediated agglutination of amphibian embryonic cells. J Cell Sci 27:227–243.

758 Fritz-Laylin LK, Lord SJ, Mullins RD (2017) WASP and SCAR are evolutionarily conserved in actin-filled
759 pseudopod-based motility. J Cell Biol. doi: 10.1083/jcb.201701074

760 Fritz-Laylin LK, Prochnik SE, Ginger ML, et al (2010) The genome of Naegleria gruberi illuminates early
761 eukaryotic versatility. Cell 140:631–642. doi: 10.1016/j.cell.2010.01.032

762 Fritzsch B, Beisel KW, Pauley S, Soukup G (2007) Molecular evolution of the vertebrate mechanosensory cell and
763 ear. Int J Dev Biol 51:663–678. doi: 10.1387/ijdb.072367bf

764 Fritzsch B, Straka H (2014) Evolution of vertebrate mechanosensory hair cells and inner ears: toward identifying
765 stimuli that select mutation driven altered morphologies. J Comp Physiol A Neuroethol Sens Neural Behav
766 Physiol 200:5–18. doi: 10.1007/s00359-013-0865-z

767 Froesler J, Leadbeater BS (2009) Role of the cytoskeleton in choanoflagellate lorica assembly. J Eukaryot Microbiol
768 56:167–173.

769 Garrone R (1969) Une formation paracristalline d'ARN intranucléaire dans les choanocytes de l'éponge Haliclona
770 rosea O.S. (Démosponge, Haplosclérides). Comptes-Rendus Académie Sci Paris 269:2219–2221.

771 Gervais L, Casanova J (2011) The Drosophila homologue of SRF acts as a boosting mechanism to sustain FGF-
772 induced terminal branching in the tracheal system. Dev Camb Engl 138:1269–1274. doi:
773 10.1242/dev.059188

774 Gilbert OM, Foster KR, Mehdiabadi NJ, et al (2007) High relatedness maintains multicellular cooperation in a social
775 amoeba by controlling cheater mutants. Proc Natl Acad Sci U S A 104:8913–8917. doi:
776 10.1073/pnas.0702723104

777 Glöckner G, Lawal HM, Felder M, et al (2016) The multicellularity genes of dictyostelid social amoebas. Nat
778 Commun 7:12085. doi: 10.1038/ncomms12085

779 Goldberg WM, Taylor GT (1989) Cellular structure and ultrastructure of the black coral Antipathes aperta: 2. The
780 gastrodermis and its collar cells. J Morphol 202:255–269.

781 Gonobobleva E, Maldonado M (2009) Choanocyte ultrastructure in Halisarca dujardini (Demospongiae,
782 Halisarcida). J Morphol 270:615–627. doi: 10.1002/jmor.10709

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783 Gould SJ (1996) Full House, F edition. New York Harmony 1996.

784 Grassé P-P (1973) Traité de zoologie: Anatomie, Systématique, Biologie. Spongiaires: anatomie, physiologie,
785 systématique, écologie. Masson

786 Gross JM, McClay DR (2001) The Role of Brachyury (T) during Gastrulation Movements in the Sea Urchin
787 Lytechinus variegatus. Dev Biol 239:132–147. doi: 10.1006/dbio.2001.0426

788 Haeckel E (1874) Memoirs: the Gastraea-Theory, the phylogenetic classification of the animal kingdom and the
789 homology of the germ-lamellæ. J Cell Sci 2:142–165.

790 Haeckel E (1892) The history of creation, 2 vols.

791 Hanschen ER, Marriage TN, Ferris PJ, et al (2016) The Gonium pectorale genome demonstrates co-option of cell
792 cycle regulation during the evolution of multicellularity. Nat Commun 7:11370. doi:
793 10.1038/ncomms11370

794 Harris H, Zalik SE (1985) Studies on the endogenous galactose-binding lectin during early development of the
795 embryo of Xenopus laevis. J Cell Sci 79:105–117.

796 Hartenstein V (2006) Blood Cells and Blood Cell Development in the Animal Kingdom. Annu Rev Cell Dev Biol
797 22:677–712. doi: 10.1146/annurev.cellbio.22.010605.093317

798 Hehenberger E, Tikhonenkov DV, Kolisko M, et al (2017) Novel Predators Reshape Holozoan Phylogeny and
799 Reveal the Presence of a Two-Component Signaling System in the Ancestor of Animals. Curr Biol. doi:
800 10.1016/j.cub.2017.06.006

801 Hernandez-Nicaise M-L (1991) Ctenophora. Microsc Anat Invertebr 2:359–418.

802 Hibberd DJ (1983) Ultrastructure of the colonial colourless zooflagellates Phalansterium digitatum Stein
803 (Phalansteriida ord. nov.) and Spongomonas uvella Stein (Spongomonadida ord. nov.). Protistologica
804 19:523–535.

805 Hibberd Dj (1975) Observations on the ultrastructure of the choanoflagellate Codosiga botrytis (Ehr.) Saville-Kent
806 with special reference to the flagellar apparatus. J Cell Sci 17:191–219.

807 Hirakow R, Kajita N (1994) Electron microscopic study of the development of amphioxus, Branchiostoma belcheri
808 tsingtauense: the neurula and larva. Kaibogaku Zasshi 69:1–13.

809 Hirose S, Benabentos R, Ho H-I, et al (2011) Self-recognition in social amoebae is mediated by allelic pairs of tiger
810 genes. Science 333:467–470. doi: 10.1126/science.1203903

811 Hobert O (2016) Terminal Selectors of Neuronal Identity. Curr Top Dev Biol 116:455–475. doi:
812 10.1016/bs.ctdb.2015.12.007

813 Höhn S, Hallmann A (2016) Distinct shape-shifting regimes of bowl-shaped cell sheets - embryonic inversion in the
814 multicellular green alga Pleodorina. BMC Dev Biol 16:35. doi: 10.1186/s12861-016-0134-9

815 James-Clark H (1867) IV.—Conclusive proofs of the animality of the ciliate sponges, and of their affinities with the
816 Infusoria flagellata. J Nat Hist 19:13–18.

817 Karpov SA, Coupe SJ (1998) A revision of choanoflagellate genera Kentrosiga Schiller, 1953 and Desmarella Kent,
818 1880. Acta Protozool 37:23–28.

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819 Karpov SA, Leadbeater BS (1998) Cytoskeleton structure and composition in choanoflagellates. J Eukaryot
820 Microbiol 45:361–367.

821 Kawabe Y, Schilde C, Du Q, Schaap P (2015) A conserved signalling pathway for amoebozoan encystation that was
822 co-opted for multicellular development. Sci Rep 5:9644. doi: 10.1038/srep09644

823 Keeling PJ, Burki F, Wilcox HM, et al (2014) The Marine Microbial Eukaryote Transcriptome Sequencing Project
824 (MMETSP): Illuminating the Functional Diversity of Eukaryotic Life in the Oceans through Transcriptome
825 Sequencing. PLOS Biol 12:e1001889. doi: 10.1371/journal.pbio.1001889

826 Keller LR, Schloss JA, Silflow CD, Rosenbaum JL (1984) Transcription of alpha- and beta-tubulin genes in vitro in
827 isolated Chlamydomonas reinhardi nuclei. J Cell Biol 98:1138–1143.

828 Kin K, Nnamani MC, Lynch VJ, et al (2015) Cell-type phylogenetics and the origin of endometrial stromal cells.
829 Cell Rep 10:1398–1409. doi: 10.1016/j.celrep.2015.01.062

830 King N (2004) The unicellular ancestry of animal development. Dev Cell 7:313–325. doi:
831 10.1016/j.devcel.2004.08.010

832 King N, Carroll SB (2001) A receptor tyrosine kinase from choanoflagellates: molecular insights into early animal
833 evolution. Proc Natl Acad Sci U S A 98:15032–15037. doi: 10.1073/pnas.261477698

834 King N, Hittinger CT, Carroll SB (2003) Evolution of Key Cell Signaling and Adhesion Protein Families Predates
835 Animal Origins. Science 301:361–363. doi: 10.1126/science.1083853

836 King N, Westbrook MJ, Young SL, et al (2008) The genome of the choanoflagellate Monosiga brevicollis and the
837 origin of metazoans. Nature 451:783–788. doi: 10.1038/nature06617

838 Knoll AH (2011) The multiple origins of complex multicellularity. Annu Rev Earth Planet Sci 39:217–239.

839 Knöll B, Nordheim A (2009) Functional versatility of transcription factors in the nervous system: the SRF paradigm.
840 Trends Neurosci 32:432–442. doi: 10.1016/j.tins.2009.05.004

841 Koschwanez JH, Foster KR, Murray AW (2011) Sucrose utilization in budding yeast as a model for the origin of
842 undifferentiated multicellularity. PLoS Biol 9:e1001122. doi: 10.1371/journal.pbio.1001122

843 Kristiansen J (1969) Chrysosphaerella multispina Bradley and some other remarkable Chrysophyceae from Lake
844 Stråken, Aneboda, Sweden. Plant Syst Evol 116:70–84.

845 Kuranda MJ, Robbins PW (1991) Chitinase is required for cell separation during growth of Saccharomyces
846 cerevisiae. J Biol Chem 266:19758–19767.

847 Kuzdzal-Fick JJ, Fox SA, Strassmann JE, Queller DC (2011) High relatedness is necessary and sufficient to
848 maintain multicellularity in Dictyostelium. Science 334:1548–1551. doi: 10.1126/science.1213272

849 Larroux C, Luke GN, Koopman P, et al (2008) Genesis and expansion of metazoan transcription factor gene classes.
850 Mol Biol Evol 25:980–996. doi: 10.1093/molbev/msn047

851 Larsen J (1985) Ultrastructure and taxonomy of Actinomonas pusilla, a heterotrophic member of the Pedinellales
852 (Chrysophyceae). Br Phycol J 20:341–355.

853 Laumer CE, Bekkouche N, Kerbl A, et al (2015) Spiralian phylogeny informs the evolution of microscopic lineages.
854 Curr Biol CB 25:2000–2006. doi: 10.1016/j.cub.2015.06.068

bioRxiv preprint doi: https://doi.org/10.1101/161695; this version posted July 12, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

855 Lauri A, Brunet T, Handberg-Thorsager M, et al (2014) Development of the annelid axochord: insights into
856 notochord evolution. Science 345:1365–1368. doi: 10.1126/science.1253396

857 Leadbeater BS (2006) The "mystery" of the flagellar vane in choanoflagellates. Nova Hedwig Beih 130:213.

858 Leadbeater BS (1983) Life-history and ultrastructure of a new marine species of Proterospongia (Choanoflagellida).
859 J Mar Biol Assoc U K 63:135–160.

860 Leadbeater BSC (2014) The Choanoflagellates: Evolution, Biology and Ecology, 1 edition. Cambridge University
861 Press

862 Lee JK, Buckhaults P, Wilkes C, et al (1997) Cloning and expression of a Xenopus laevis oocyte lectin and
863 characterization of its mRNA levels during early development. Glycobiology 7:367–372. doi:
864 10.1093/glycob/7.3.367

865 Leong DT, Lim J, Goh X, et al (2010) Cancer-related ectopic expression of the bone-related transcription factor
866 RUNX2 in non-osseous metastatic tumor cells is linked to cell proliferation and motility. Breast Cancer
867 Res 12:R89. doi: 10.1186/bcr2762

868 Levin TC, Greaney AJ, Wetzel L, King N (2014) The Rosetteless gene controls development in the choanoflagellate
869 S. rosetta. eLife. doi: 10.7554/eLife.04070

870 Levin TC, King N (2013) Evidence for sex and recombination in the choanoflagellate Salpingoeca rosetta. Curr Biol
871 CB 23:2176–2180. doi: 10.1016/j.cub.2013.08.061

872 Leys SP, Degnan BM (2002) Embryogenesis and metamorphosis in a haplosclerid demosponge: gastrulation and
873 transdifferentiation of larval ciliated cells to choanocytes. Invertebr Biol 121:171–189.

874 Leys SP, Eerkes-Medrano D (2005) Gastrulation in Calcareous Sponges: In Search of Haeckel's Gastraea. Integr
875 Comp Biol 45:342–351. doi: 10.1093/icb/45.2.342

876 Leys SP, Hill A (2012) 1 The Physiology and Molecular Biology of Sponge Tissues. Adv Mar Biol 62:1.

877 Leys SP, Nichols SA, Adams EDM (2009) Epithelia and integration in sponges. Integr Comp Biol 49:167–177. doi:
878 10.1093/icb/icp038

879 Li JB, Gerdes JM, Haycraft CJ, et al (2004) Comparative Genomics Identifies a Flagellar and Basal Body Proteome
880 that Includes the BBS5 Human Disease Gene. Cell 117:541–552. doi: 10.1016/S0092-8674(04)00450-7

881 Liang C, FANTOM Consortium, Forrest ARR, Wagner GP (2015) The statistical geometry of transcriptome
882 divergence in cell-type evolution and cancer. Nat Commun 6:6066. doi: 10.1038/ncomms7066

883 Lie-A-Ling M, Marinopoulou E, Li Y, et al (2014) RUNX1 positively regulates a cell adhesion and migration
884 program in murine hemogenic endothelium prior to blood emergence. Blood 124:e11–e20. doi:
885 10.1182/blood-2014-04-572958

886 Liu Y-J, Le Berre M, Lautenschlaeger F, et al (2015) Confinement and low adhesion induce fast amoeboid
887 migration of slow mesenchymal cells. Cell 160:659–672. doi: 10.1016/j.cell.2015.01.007

888 Lo WS, Dranginis AM (1996) FLO11, a yeast gene related to the STA genes, encodes a novel cell surface flocculin.
889 J Bacteriol 178:7144–7151.

890 Mackie GO (1970) Neuroid conduction and the evolution of conducting tissues. Q Rev Biol 45:319–332.

bioRxiv preprint doi: https://doi.org/10.1101/161695; this version posted July 12, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Madan Babu M, Teichmann SA (2003) Evolution of transcription factors and the gene regulatory network in Escherichia coli. Nucleic Acids Res 31:1234–1244. doi: 10.1093/nar/gkg210

Mah JL, Christensen-Dalsgaard KK, Leys SP (2014) Choanoflagellate and choanocyte collar-flagellar systems and the assumption of homology. Evol Dev 16:25–37. doi: 10.1111/ede.12060

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

Margulis L (1981) Symbiosis in Cell Evolution. W. H. Freeman, San Francisco

Mehl D, Reiswig HM (1991) The presence of flagellar vanes in choanomeres of Porifera and their possible phylogenetic implications. J Zool Syst Evol Res 29:312–319.

Mendoza L, Taylor JW, Ajello L (2002) The class mesomycetozoea: a heterogeneous group of microorganisms at the animal-fungal boundary. Annu Rev Microbiol 56:315–344. doi: 10.1146/annurev.micro.56.012302.160950

Metzger MJ, Reinisch C, Sherry J, Goff SP (2015) Horizontal transmission of clonal cancer cells causes leukemia in soft-shell clams. Cell 161:255–263. doi: 10.1016/j.cell.2015.02.042

Michod RE (2007) Evolution of individuality during the transition from unicellular to multicellular life. Proc Natl Acad Sci U S A 104 Suppl 1:8613–8618. doi: 10.1073/pnas.0701489104

Mikhailov KV, Konstantinova AV, Nikitin MA, et al (2009) The origin of Metazoa: a transition from temporal to spatial cell differentiation. BioEssays News Rev Mol Cell Dev Biol 31:758–768. doi: 10.1002/bies.200800214

Musser JM, Wagner GP (2015) Character trees from transcriptome data: Origin and individuation of morphological characters and the so-called “species signal.” J Exp Zoolog B Mol Dev Evol 324:588–604. doi: 10.1002/jez.b.22636

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

Nichols SA, Roberts BW, Richter DJ, et al (2012) Origin of metazoan cadherin diversity and the antiquity of the classical cadherin/β-catenin complex. Proc Natl Acad Sci 109:13046–13051. doi: 10.1073/pnas.1120685109

Nielsen C (2008) Six major steps in animal evolution: are we derived sponge larvae? Evol Dev 10:241–257. doi: 10.1111/j.1525-142X.2008.00231.x

Nielsen C (2012) Animal Evolution: Interrelationships of the Living Phyla, 3 edition. Oxford University Press, Oxford ; New York

Olano CT, Bigger CH (2000) Phagocytic activities of the gorgonian coral Swiftia exserta. J Invertebr Pathol 76:176–184. doi: 10.1006/jipa.2000.4974

Ott DW, Oldham-Ott CK, Rybalka N, Friedl T (2015) Xanthophyte, Eustigmatophyte, and Raphidophyte Algae. Freshw Algae N Am Ecol Classif Eds JD Wehr RG Sheath JP Kociolek 485–536.

Otto F, Lübbert M, Stock M (2003) Upstream and downstream targets of RUNX proteins. J Cell Biochem 89:9–18. doi: 10.1002/jcb.10491

bioRxiv preprint doi: https://doi.org/10.1101/161695; this version posted July 12, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

927 Oud B, Guadalupe-Medina V, Nijkamp JF, et al (2013) Genome duplication and mutations in ACE2 cause
928 multicellular, fast-sedimenting phenotypes in evolved Saccharomyces cerevisiae. Proc Natl Acad Sci U S A
929 110:E4223-4231. doi: 10.1073/pnas.1305949110

930 Outenreath RL, Roberson MM, Barondes SH (1988) Endogenous lectin secretion into the extracellular matrix of
931 early embryos of Xenopus laevis. Dev Biol 125:187–194.

932 Pancer Z, Rast JP, Davidson EH (1999) Origins of immunity: transcription factors and homologues of effector genes
933 of the vertebrate immune system expressed in sea urchin coelomocytes. Immunogenetics 49:773–786. doi:
934 10.1007/s002510050551

935 Panková K, Rösel D, Novotný M, Brábek J (2010) The molecular mechanisms of transition between mesenchymal
936 and amoeboid invasiveness in tumor cells. Cell Mol Life Sci CMLS 67:63–71. doi: 10.1007/s00018-009-
937 0132-1

938 Patterson DJ, Fenchel T (1985) Insights into the evolution of heliozoa (Protozoa, Sarcodina) as provided by
939 ultrastructural studies on a new species of flagellate from the genus Pteridomonas. Biol J Linn Soc 24:381–
940 403.

941 Patterson MJ, Landolt ML (1979) Cellular reaction to injury in the anthozoan Anthopleura elegantissima. J Invertebr
942 Pathol 33:189–196.

943 Peña JF, Alié A, Richter DJ, et al (2016) Conserved expression of vertebrate microvillar gene homologs in
944 choanocytes of freshwater sponges. EvoDevo 7:13. doi: 10.1186/s13227-016-0050-x

945 Petersen JB (1929) Beiträge zur Kenntnis der Flagellatengeisseln. Bot Tidsskr 40:373–389.

946 Piasecki BP, Burghoorn J, Swoboda P (2010) Regulatory Factor X (RFX)-mediated transcriptional rewiring of
947 ciliary genes in animals. Proc Natl Acad Sci U S A 107:12969–12974. doi: 10.1073/pnas.0914241107

948 Raikova EV (1995) Occurrence and ultrastructure of collar cells in the stomach gastrodermis of Polypodium
949 hydriforme Ussov (Cnidaria). Acta Zool 76:11–18.

950 Ramesh MA, Malik S-B, Logsdon Jr. JM (2005) A Phylogenomic Inventory of Meiotic Genes: Evidence for Sex in
951 Giardia and an Early Eukaryotic Origin of Meiosis. Curr Biol 15:185–191. doi: 10.1016/j.cub.2005.01.003

952 Ratcliff WC, Denison RF, Borrello M, Travisano M (2012) Experimental evolution of multicellularity. Proc Natl
953 Acad Sci U S A 109:1595–1600. doi: 10.1073/pnas.1115323109

954 Ratcliff WC, Fankhauser JD, Rogers DW, et al (2015) Origins of multicellular evolvability in snowflake yeast. Nat
955 Commun 6:6102. doi: 10.1038/ncomms7102

956 Ratcliff WC, Herron MD, Howell K, et al (2013) Experimental evolution of an alternating uni- and multicellular life
957 cycle in Chlamydomonas reinhardtii. Nat Commun 4:2742. doi: 10.1038/ncomms3742

958 Raven JA (2005) Evolution of Plasmodesmata. In: FRSE KJO (ed) Plasmodesmata. Blackwell Publishing Ltd, pp
959 33–52

960 Ray J, Shinnick T, Lerner R (1979) A mutation altering the function of a carbohydrate binding protein blocks cell-
961 cell cohesion in developing Dictyostelium discoideum. Nature 279:215–221.

962 Regev A, Teichmann S, Lander ES, et al (2017) The Human Cell Atlas. bioRxiv 121202. doi: 10.1101/121202

bioRxiv preprint doi: https://doi.org/10.1101/161695; this version posted July 12, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

963 Rennert J, Coffman JA, Mushegian AR, Robertson AJ (2003) The evolution of Runx genes I. A comparative study
964 of sequences from phylogenetically diverse model organisms. BMC Evol Biol 3:4. doi: 10.1186/1471-
965 2148-3-4

966 Richter DJ, King N (2013) The genomic and cellular foundations of animal origins. Annu Rev Genet 47:509–537.
967 doi: 10.1146/annurev-genet-111212-133456

968 Ritchie AV, van Es S, Fouquet C, Schaap P (2008) From drought sensing to developmental control: evolution of
969 cyclic AMP signaling in social amoebas. Mol Biol Evol 25:2109–2118. doi: 10.1093/molbev/msn156

970 Rivera AS, Hammel JU, Haen KM, et al (2011) RNA interference in marine and freshwater sponges: actin
971 knockdown in Tethya wilhelma and Ephydatia muelleri by ingested dsRNA expressing bacteria. BMC
972 Biotechnol 11:67. doi: 10.1186/1472-6750-11-67

973 Roberson MM, Barondes SH (1983) Xenopus laevis lectin is localized at several sites in Xenopus oocytes, eggs, and
974 embryos. J Cell Biol 97:1875–1881.

975 Rokas A (2008) The origins of multicellularity and the early history of the genetic toolkit for animal development.
976 Annu Rev Genet 42:235–251. doi: 10.1146/annurev.genet.42.110807.091513

977 Roper M, Dayel MJ, Pepper RE, Koehl M a. R (2013) Cooperatively generated stresslet flows supply fresh fluid to
978 multicellular choanoflagellate colonies. Phys Rev Lett 110:228104. doi: 10.1103/PhysRevLett.110.228104

979 Ruiz-Trillo I, Roger AJ, Burger G, et al (2008) A phylogenomic investigation into the origin of metazoa. Mol Biol
980 Evol 25:664–672. doi: 10.1093/molbev/msn006

981 Ruppert EE, Smith PR (1988) The Functional Organization of Filtration Nephridia. Biol Rev 63:231–258. doi:
982 10.1111/j.1469-185X.1988.tb00631.x

983 Ruprecht V, Wieser S, Callan-Jones A, et al (2015) Cortical contractility triggers a stochastic switch to fast
984 amoeboid cell motility. Cell 160:673–685. doi: 10.1016/j.cell.2015.01.008

985 Salas-Vidal E, Lomelı H (2004) Imaging filopodia dynamics in the mouse blastocyst. Dev Biol 265:75–89.

986 Santorelli LA, Thompson CRL, Villegas E, et al (2008) Facultative cheater mutants reveal the genetic complexity of
987 cooperation in social amoebae. Nature 451:1107–1110. doi: 10.1038/nature06558

988 Saville-Kent W (1880) A manual of the Infusoria: including a description of all known Flagellate, Ciliate, and
989 Tentaculiferous Protozoa, British and foreign, and an account of the organization and the affinities of the
990 sponges. D. Bogue

991 Schmidt-Rhaesa A (2007) The Evolution of Organ Systems, 1 edition. Oxford University Press, Oxford ; New
992 York

993 Sebé-Pedrós A, Ballaré C, Parra-Acero H, et al (2016a) The Dynamic Regulatory Genome of Capsaspora and the
994 Origin of Animal Multicellularity. Cell 165:1224–1237. doi: 10.1016/j.cell.2016.03.034

995 Sebé-Pedrós A, Burkhardt P, Sánchez-Pons N, et al (2013a) Insights into the origin of metazoan filopodia and
996 microvilli. Mol Biol Evol 30:2013–2023. doi: 10.1093/molbev/mst110

997 Sebé-Pedrós A, de Mendoza A, Lang BF, et al (2011) Unexpected Repertoire of Metazoan Transcription Factors in
998 the Unicellular Holozoan Capsaspora owczarzaki. Mol Biol Evol 28:1241–1254. doi:
999 10.1093/molbev/msq309

bioRxiv preprint doi: https://doi.org/10.1101/161695; this version posted July 12, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1000 Sebé-Pedrós A, Degnan BM, Ruiz-Trillo I (2017) The origin of Metazoa: a unicellular perspective. Nat Rev Genet.
1001 doi: 10.1038/nrg.2017.21

1002 Sebé-Pedrós A, Irimia M, Del Campo J, et al (2013b) Regulated aggregative multicellularity in a close unicellular
1003 relative of metazoa. eLife 2:e01287. doi: 10.7554/eLife.01287

1004 Sebé-Pedrós A, Peña MI, Capella-Gutiérrez S, et al (2016b) High-Throughput Proteomics Reveals the Unicellular
1005 Roots of Animal Phosphosignaling and Cell Differentiation. Dev Cell 39:186–197. doi:
1006 10.1016/j.devcel.2016.09.019

1007 Sebé-Pedrós A, Roger AJ, Lang FB, et al (2010) Ancient origin of the integrin-mediated adhesion and signaling
1008 machinery. Proc Natl Acad Sci 107:10142–10147. doi: 10.1073/pnas.1002257107

1009 Shalchian-Tabrizi K, Minge MA, Espelund M, et al (2008) Multigene phylogeny of choanozoa and the origin of
1010 animals. PloS One 3:e2098. doi: 10.1371/journal.pone.0002098

1011 Shen-Orr SS, Milo R, Mangan S, Alon U (2002) Network motifs in the transcriptional regulation network of
1012 Escherichia coli. Nat Genet 31:64–68. doi: 10.1038/ng881

1013 Shinnick TM, Lerner RA (1980) The cbpA gene: role of the 26,000-dalton carbohydrate-binding protein in
1014 intercellular cohesion of developing Dictyostelium discoideum cells. Proc Natl Acad Sci U S A 77:4788–
1015 4792.

1016 Simion P, Philippe H, Baurain D, et al (2017) A Large and Consistent Phylogenomic Dataset Supports Sponges as
1017 the Sister Group to All Other Animals. Curr Biol CB 27:958–967. doi: 10.1016/j.cub.2017.02.031

1018 Smith CL, Varoqueaux F, Kittelmann M, et al (2014) Novel cell types, neurosecretory cells, and body plan of the
1019 early-diverging metazoan Trichoplax adhaerens. Curr Biol CB 24:1565–1572. doi:
1020 10.1016/j.cub.2014.05.046

1021 Soares EV (2011) Flocculation in Saccharomyces cerevisiae: a review. J Appl Microbiol 110:1–18. doi:
1022 10.1111/j.1365-2672.2010.04897.x

1023 Souza GM, da Silva AM, Kuspa A (1999) Starvation promotes Dictyostelium development by relieving PufA
1024 inhibition of PKA translation through the YakA kinase pathway. Dev Camb Engl 126:3263–3274.

1025 Srivastava M, Simakov O, Chapman J, et al (2010) The Amphimedon queenslandica genome and the evolution of
1026 animal complexity. Nature 466:720–726. doi: 10.1038/nature09201

1027 Stephenson SL, Schnittler M (2016) Myxomycetes. In: Handbook of the Protists. Springer, 1-27,

1028 Stolc V, Samanta MP, Tongprasit W, Marshall WF (2005) Genome-wide transcriptional analysis of flagellar
1029 regeneration in Chlamydomonas reinhardtii identifies orthologs of ciliary disease genes. Proc Natl Acad
1030 Sci U S A 102:3703–3707. doi: 10.1073/pnas.0408358102

1031 Strassmann JE, Zhu Y, Queller DC (2000) Altruism and social cheating in the social amoeba Dictyostelium
1032 discoideum. Nature 408:965–967. doi: 10.1038/35050087

1033 Stratford M (1992) Lectin-mediated aggregation of yeasts--yeast flocculation. Biotechnol Genet Eng Rev 10:283–
1034 341.

1035 Struck TH, Wey-Fabrizius AR, Golombek A, et al (2014) Platyzoan paraphyly based on phylogenomic data
1036 supports a noncoelomate ancestry of spiralia. Mol Biol Evol 31:1833–1849. doi: 10.1093/molbev/msu143

bioRxiv preprint doi: https://doi.org/10.1101/161695; this version posted July 12, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1037 Stubbs JL, Oishi I, Izpisúa Belmonte JC, Kintner C (2008) The forkhead protein Foxj1 specifies node-like cilia in
1038 Xenopus and zebrafish embryos. Nat Genet 40:1454–1460. doi: 10.1038/ng.267

1039 Sucgang R, Kuo A, Tian X, et al (2011) Comparative genomics of the social amoebae Dictyostelium discoideum
1040 and Dictyostelium purpureum. Genome Biol 12:R20. doi: 10.1186/gb-2011-12-2-r20

1041 Suga H, Chen Z, de Mendoza A, et al (2013) The Capsaspora genome reveals a complex unicellular prehistory of
1042 animals. Nat Commun 4:2325. doi: 10.1038/ncomms3325

1043 Suga H, Ruiz-Trillo I (2013) Development of ichthyosporeans sheds light on the origin of metazoan multicellularity.
1044 Dev Biol 377:284–292. doi: 10.1016/j.ydbio.2013.01.009

1045 Sullivan JC, Sher D, Eisenstein M, et al (2008) The evolutionary origin of the Runx/CBFbeta transcription factors –
1046 Studies of the most basal metazoans. BMC Evol Biol 8:228. doi: 10.1186/1471-2148-8-228

1047 Takashima S, Gold D, Hartenstein V (2013) Stem cells and lineages of the intestine: a developmental and
1048 evolutionary perspective. Dev Genes Evol 223:85–102. doi: 10.1007/s00427-012-0422-8

1049 Tanay A, Sharan R, Kupiec M, Shamir R (2004) Revealing modularity and organization in the yeast molecular
1050 network by integrated analysis of highly heterogeneous genomewide data. Proc Natl Acad Sci U S A
1051 101:2981–2986. doi: 10.1073/pnas.0308661100

1052 Tavazoie S, Hughes JD, Campbell MJ, et al (1999) Systematic determination of genetic network architecture. Nat
1053 Genet 22:281–285. doi: 10.1038/10343

1054 Taylor DL, Condeelis JS (1979) Cytoplasmic structure and contractility in amoeboid cells. Int Rev Cytol 56:57–144.

1055 Telford MJ, Budd GE, Philippe H (2015) Phylogenomic Insights into Animal Evolution. Curr Biol CB 25:R876-
1056 887. doi: 10.1016/j.cub.2015.07.060

1057 Thomas J, Morlé L, Soulavie F, et al (2010) Transcriptional control of genes involved in ciliogenesis: a first step in
1058 making cilia. Biol Cell 102:499–513. doi: 10.1042/BC20100035

1059 Torruella G, de Mendoza A, Grau-Bové X, et al (2015) Phylogenomics Reveals Convergent Evolution of Lifestyles
1060 in Close Relatives of Animals and Fungi. Curr Biol CB 25:2404–2410. doi: 10.1016/j.cub.2015.07.053

1061 Town CD, Gross JD, Kay RR (1976) Cell differentiation without morphogenesis in Dictyostelium discoideum.
1062 Nature 262:717–719.

1063 Vacquier VD (1968) The connection of blastomeres of sea urchin embryos by filopodia. Exp Cell Res 52:571–581.

1064 Valentine JW (2006) On the Origin of Phyla, 1 edition. University Of Chicago Press, Chicago London

1065 VanOudenhove JJ, Medina R, Ghule PN, et al (2016) Transient RUNX1 Expression during Early Mesendodermal
1066 Differentiation of hESCs Promotes Epithelial to Mesenchymal Transition through TGFB2 Signaling. Stem
1067 Cell Rep 7:884–896. doi: 10.1016/j.stemcr.2016.09.006

1068 Vij S, Rink JC, Ho HK, et al (2012) Evolutionarily ancient association of the FoxJ1 transcription factor with the
1069 motile ciliogenic program. PLoS Genet 8:e1003019. doi: 10.1371/journal.pgen.1003019

1070 Villani A-C, Satija R, Reynolds G, et al (2017) Single-cell RNA-seq reveals new types of human blood dendritic
1071 cells, monocytes, and progenitors. Science. doi: 10.1126/science.aah4573

1072 Vincensini L, Blisnick T, Bastin P (2011) 1001 model organisms to study cilia and flagella. Biol Cell 103:109–130.
1073 doi: 10.1042/BC20100104

bioRxiv preprint doi: https://doi.org/10.1101/161695; this version posted July 12, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1074 Vlk W (1938) Über den bau der Geissel. Fischer

1075 Wagner A, Regev A, Yosef N (2016) Revealing the vectors of cellular identity with single-cell genomics. Nat
1076 Biotechnol 34:1145–1160. doi: 10.1038/nbt.3711

1077 Wainright PO, Hinkle G, Sogin ML, Stickel SK (1993) Monophyletic origins of the metazoa: an evolutionary link
1078 with fungi. Science 260:340–342.

1079 Waltzer L, Ferjoux G, Bataille L, Haenlin M (2003) Cooperation between the GATA and RUNX factors Serpent
1080 and Lozenge during Drosophila hematopoiesis. EMBO J 22:6516–6525. doi: 10.1093/emboj/cdg622

1081 Weinl C, Riehle H, Park D, et al (2013) Endothelial SRF/MRTF ablation causes vascular disease phenotypes in
1082 murine retinae. J Clin Invest 123:2193–2206. doi: 10.1172/JCI64201

1083 Whelan NV, Kocot KM, Moroz LL, Halanych KM (2015) Error, signal, and the placement of Ctenophora sister to
1084 all other animals. Proc Natl Acad Sci 112:5773–5778. doi: 10.1073/pnas.1503453112

1085 Wickerham LJ (1958) Sexual agglutination of heterothallic yeasts in diverse taxonomic areas. Science 128:1504–
1086 1505.

1087 Williams F, Tew HA, Paul CE, Adams JC (2014) The predicted secretomes of Monosiga brevicollis and Capsaspora
1088 owczarzaki, close unicellular relatives of metazoans, reveal new insights into the evolution of the metazoan
1089 extracellular matrix. Matrix Biol 37:60–68. doi: 10.1016/j.matbio.2014.02.002

1090 Woollacott RM, Pinto RL (1995) Flagellar basal apparatus and its utility in phylogenetic analyses of the Porifera. J
1091 Morphol 226:247–265.

1092 Woznica A, Cantley AM, Beemelmanns C, et al (2016) Bacterial lipids activate, synergize, and inhibit a
1093 developmental switch in choanoflagellates. Proc Natl Acad Sci U S A 113:7894–7899. doi:
1094 10.1073/pnas.1605015113

1095 Woznica A, Gerdt JP, Hulett R, et al (2017) An Aphrodisiac Produced By *Vibrio fischeri* Stimulates Mating In The
1096 Closest Living Relatives Of Animals. bioRxiv 139022. doi: 10.1101/139022

1097 Yanagisawa KO, Fujimoto H, Urushihara H (1981) Effects of the Brachyury (T) mutation on morphogenetic
1098 movement in the mouse embryo. Dev Biol 87:242–248. doi: 10.1016/0012-1606(81)90147-0

1099 Young SL, Diolaiti D, Conacci-Sorrell M, et al (2011) Premetazoan ancestry of the Myc-Max network. Mol Biol
1100 Evol 28:2961–2971. doi: 10.1093/molbev/msr132

1101 Yu X, Ng CP, Habacher H, Roy S (2008) Foxj1 transcription factors are master regulators of the motile ciliogenic
1102 program. Nat Genet 40:1445–1453. doi: 10.1038/ng.263

1103 Zakhvatkin AA (1949) The comparative embryology of the low invertebrates.

1104 Zusso M, Methot L, Lo R, et al (2012) Regulation of Postnatal Forebrain Amoeboid Microglial Cell Proliferation
1105 and Development by the Transcription Factor Runx1. J Neurosci 32:11285–11298. doi:
1106 10.1523/JNEUROSCI.6182-11.2012

1107

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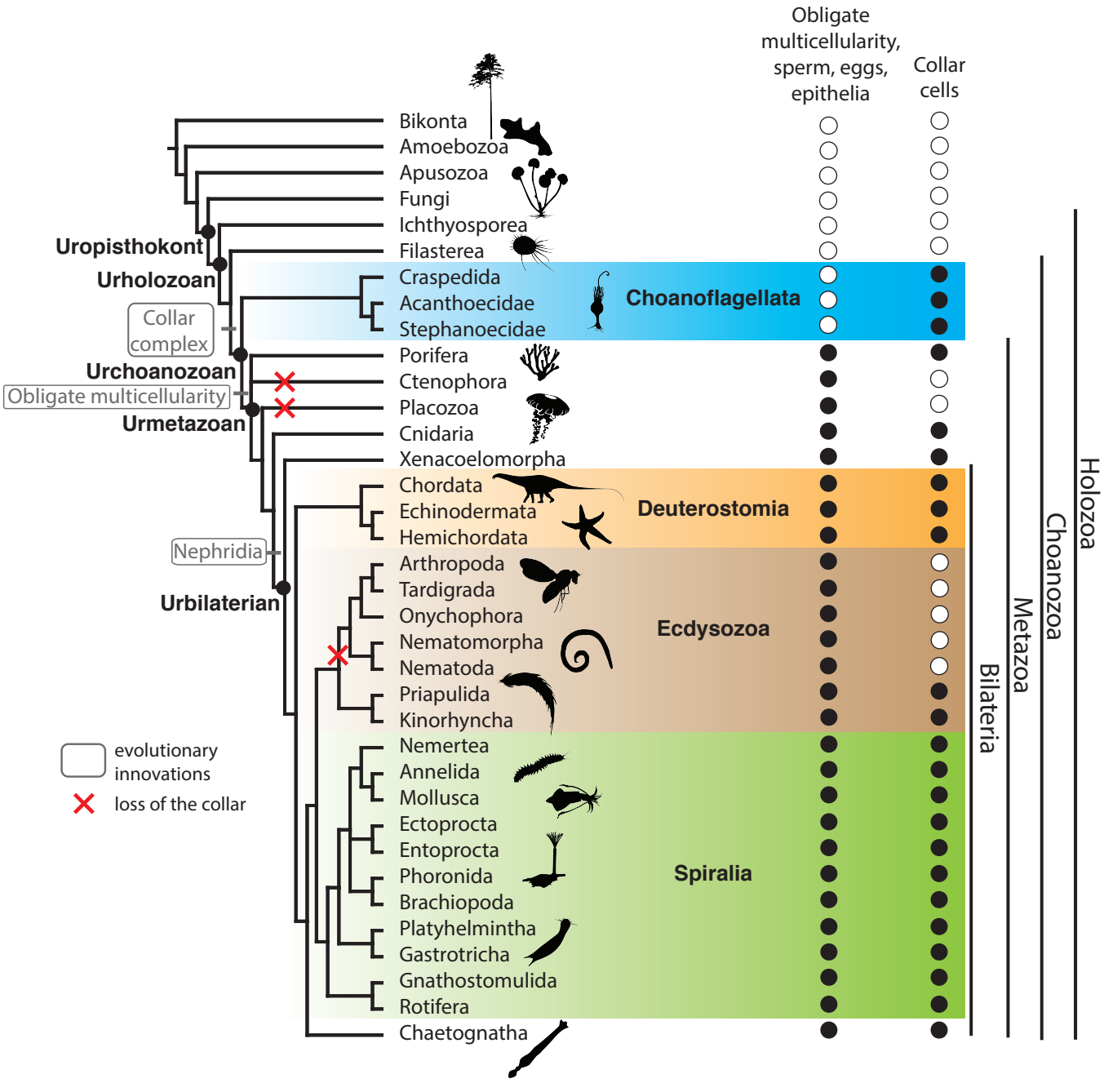

Figure 1

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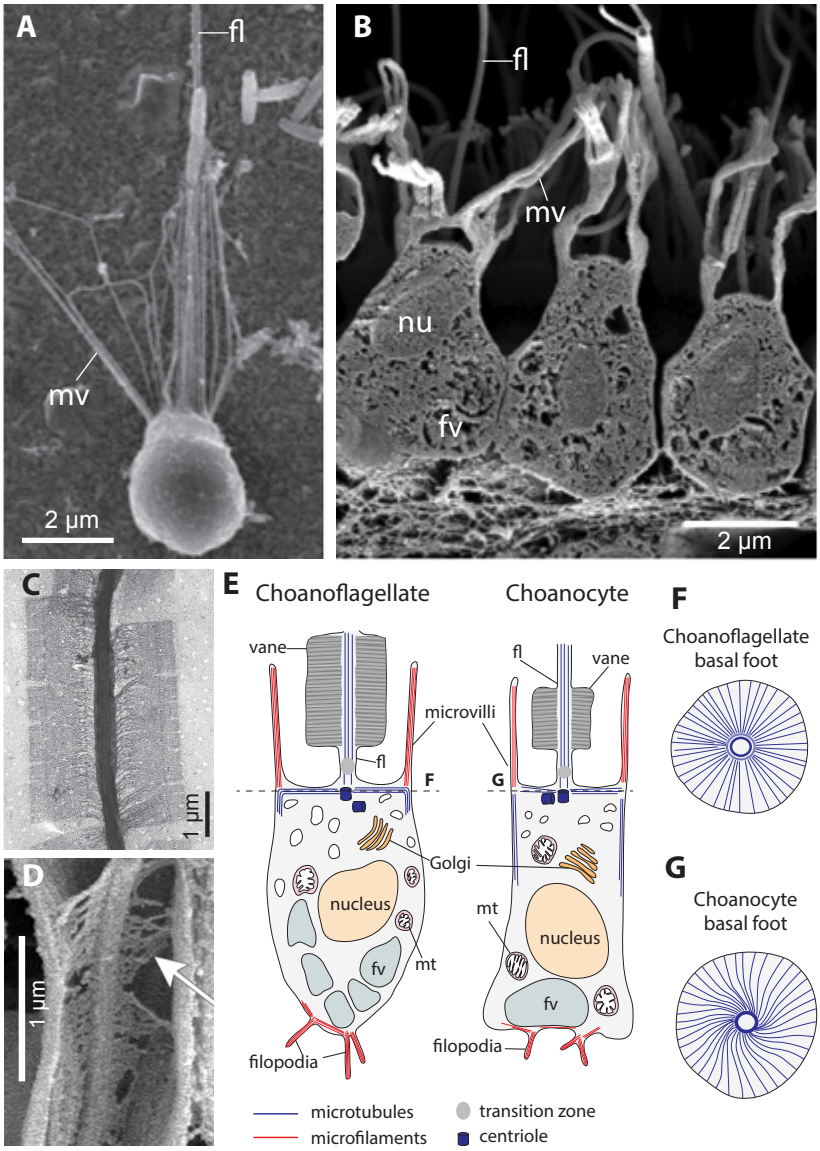

Figure 2

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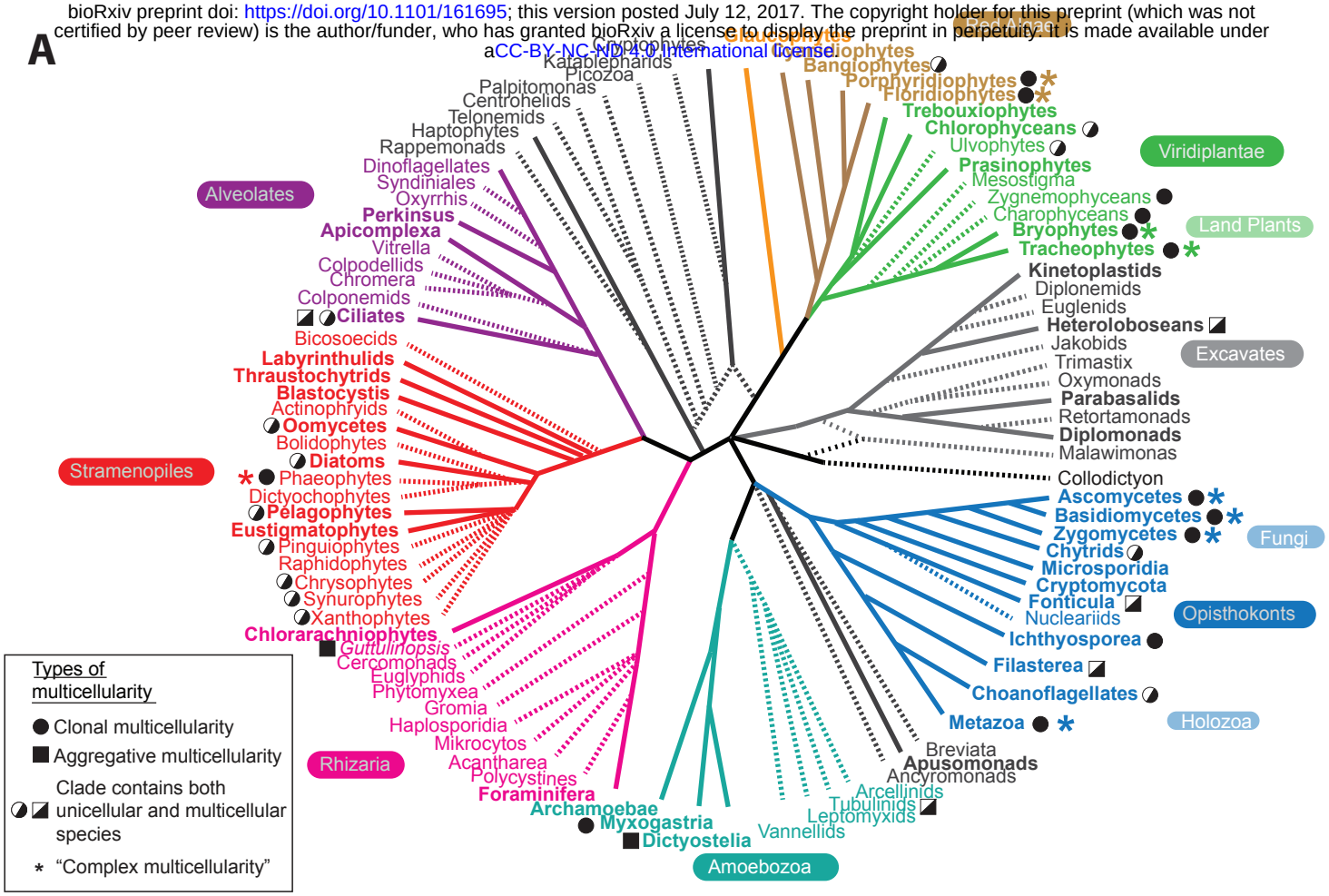

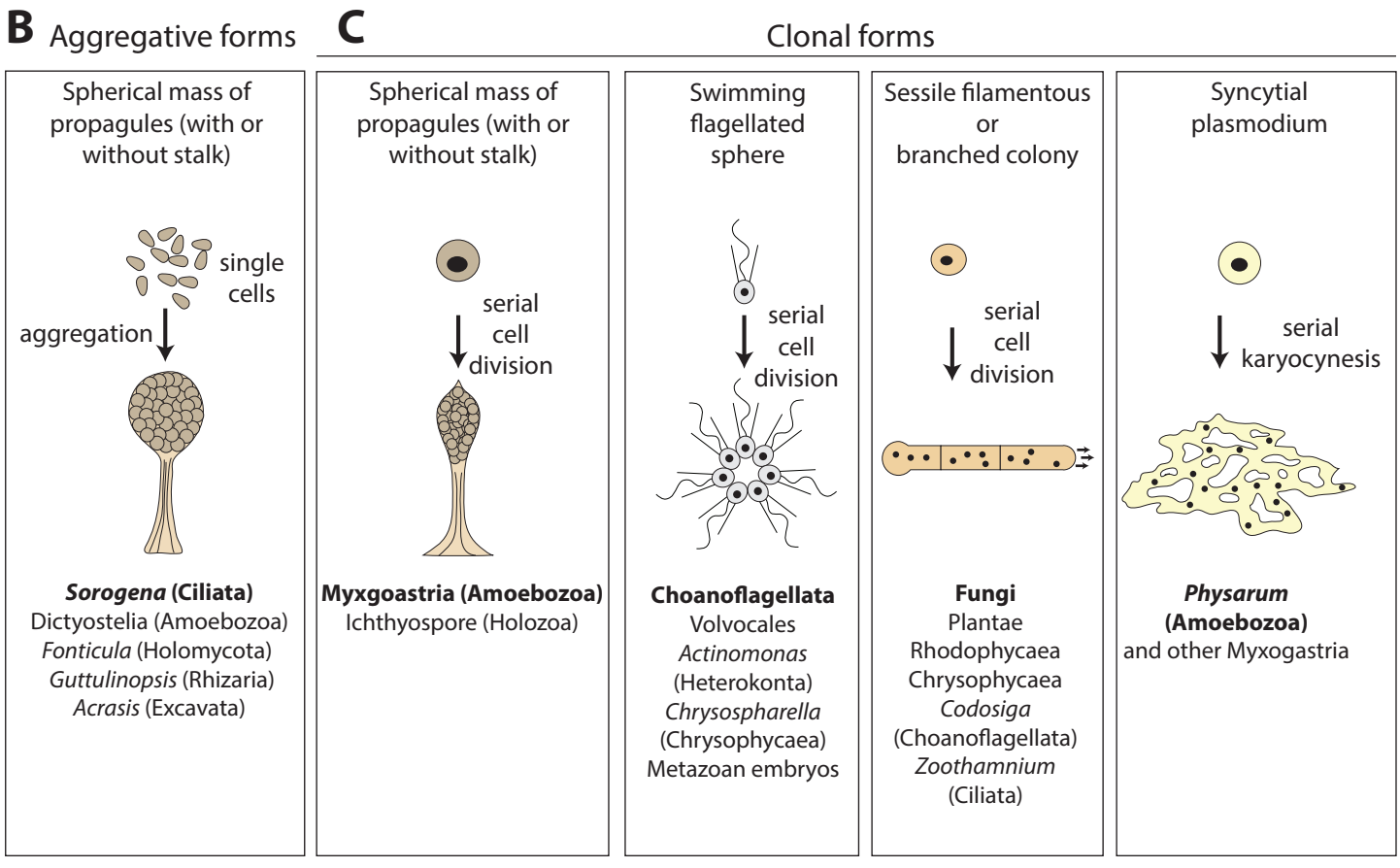

Figure 3

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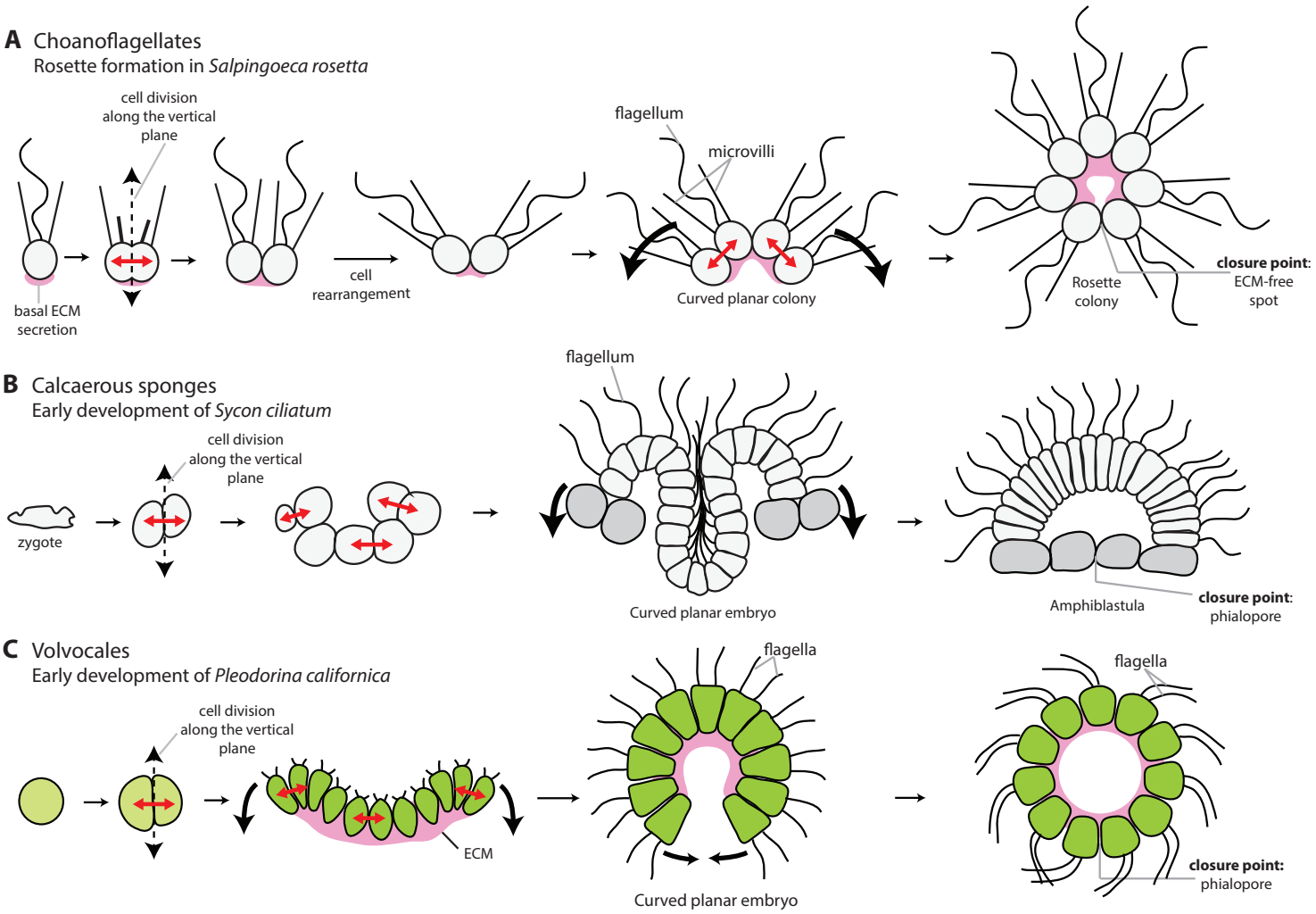

Figure 4

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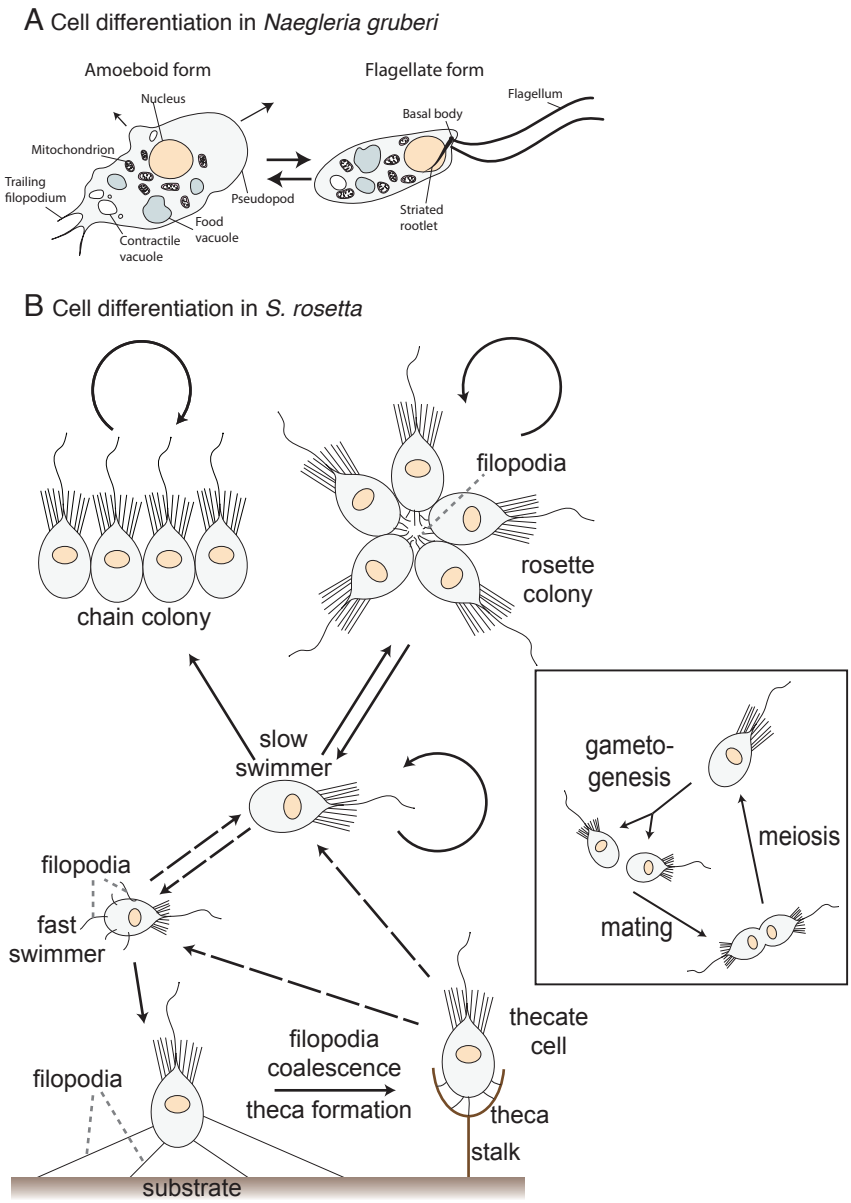

Figure 5

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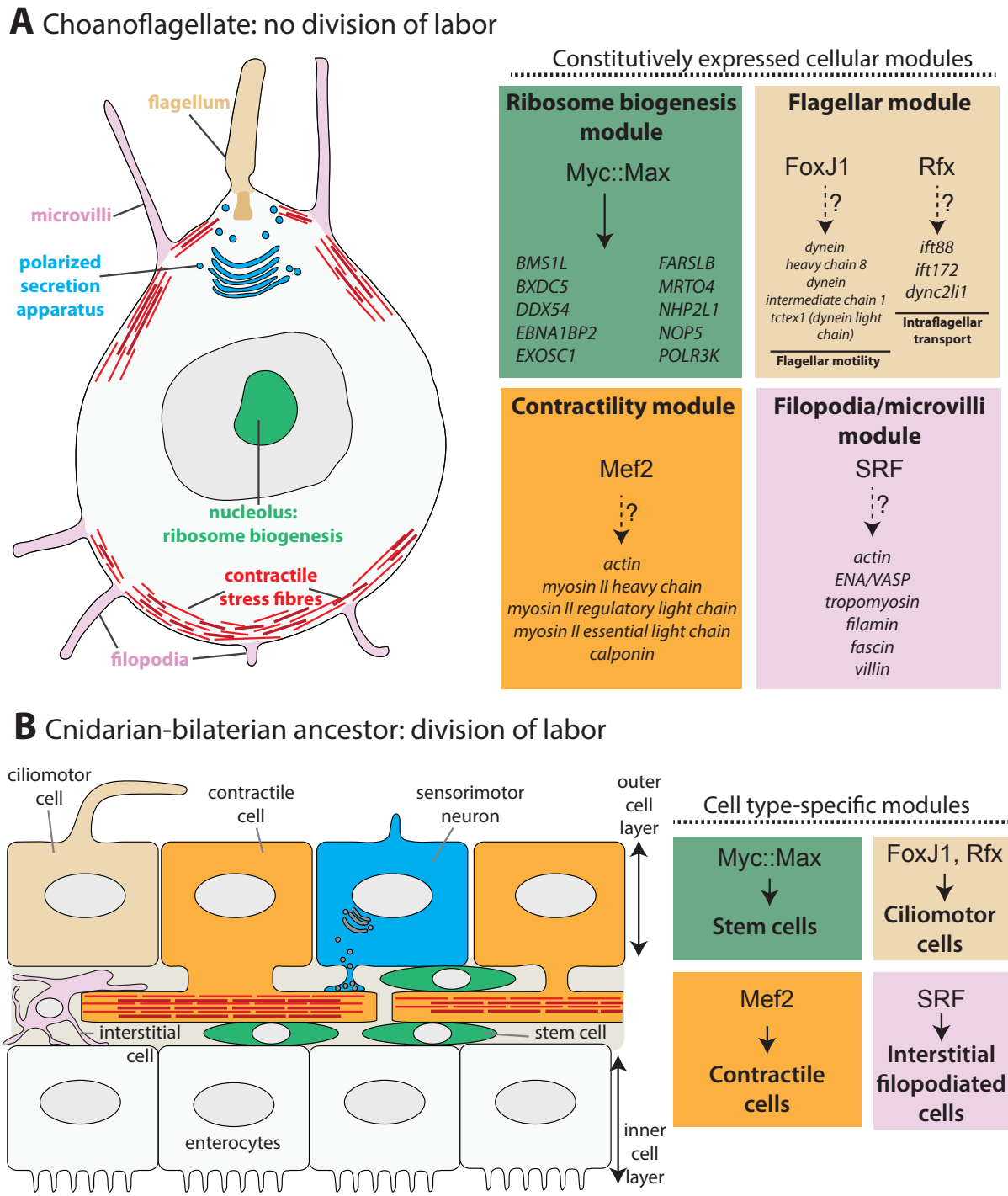

Figure 6