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SPOTLIGHT

Towards understanding the origin of animal development

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ABSTRACT

Almost all animals undergo embryonic development, going from a single-celled zygote to a complex multicellular adult. We know that the patterning and morphogenetic processes involved in development are deeply conserved within the animal kingdom. However, the origins of these developmental processes are just beginning to be unveiled. Here, we focus on how the protist lineages sister to animals are reshaping our view of animal development. Most intriguingly, many of these protistan lineages display transient multicellular structures, which are governed by similar morphogenetic and gene regulatory processes as animal development. We discuss here two potential alternative scenarios to explain the origin of animal embryonic development: either it originated concomitantly at the onset of animals or it evolved from morphogenetic processes already present in their unicellular ancestors. We propose that an integrative study of several unicellular taxa closely related to animals will allow a more refined picture of how the last common ancestor of animals underwent embryonic development.

KEY WORDS: Choanoflagellates, Evolution, Filasterea, Ichthyosporea, Multicellularity

Introduction

How a complex multicellular organism (plant or animal) can develop from a single cell, the zygote, is one of the most amazing questions in biology. Plants and most other eukaryotic multicellular lineages have hard cell walls, which help to keep cells together and maintain their relative positioning. In contrast, animals are mostly composed of 'naked' cells (Meyerowitz, 2002). This places particular constraints on animal developmental processes that are only paralleled by dictyostelids (social amoebae also known as slime moulds). Nevertheless, the lack of cell walls provides several advantages: cells are able to communicate with each other via direct transmembrane protein contacts, tissues are more flexible, anchored to extracellular matrices, and the cytoskeleton has a major effect on collective shape formation. Such a tissue-level organisation leaves cells less constrained by position, and cellular migration is common. This flexibility underlies one of the distinguishing characteristics of the animal kingdom, their incredible motile capacity.

We have learnt a lot about animal development and the diversity of developmental processes across different animal phyla. Complex gene regulation is key to the development of animals, which is orchestrated by a precise combination of genes that are activated and inactivated at the right time and place, giving rise to cell patterning and morphogenesis (Gilbert, 2000). We know many of the specific genes that play a crucial role in the control of animal development and understand how their absence impacts embryonic development. However, a long-standing question that has evaded experimental investigation for years remains to be answered: how did animal development arise? There are two potential scenarios. One is that developmental innovations specific to the animal lineage sparked the evolutionary success and incredible diversification of animal body plans. Another scenario is that animal development evolved using morphogenetic processes already present in their ancestors and later recruited for animal development.

The evolutionary framework of animal development

There can be a tendency to consider animals as an evolutionarily unique lineage, but they share a not-so-ancient common ancestor with several unicellular lineages, and the consensus is that animals evolved from a unicellular (protist) ancestor (Torruella et al., 2015). Thus, if we want to understand the origins of animal development, we need first to unravel the phylogenetic relationships of these closest living unicellular relatives and then analyse their cellular capacities.

The closest living relatives of animals

Molecular phylogenies show that animals and fungi share a common ancestor with several unicellular lineages, forming what is known as the Opisthokonta clade. Opisthokonta means 'posterior flagellum' [from the Greek *opisthen* (posterior) and *kontos* (oar or pole)], since this group is defined by an ancestral state with a single posterior flagellum (Adl et al., 2018; Cavalier-Smith, 1987). The opisthokonts are divided into two main clades or groups: the Holozoa, which includes animals and their closest unicellular relatives, and the Holomycota, which includes fungi and their unicellular relatives (Torruella et al., 2015) (Fig. 1). The most relevant species for studying the origins of animal development are therefore the unicellular lineages of the Holozoa, which include the choanoflagellates, the filastereans, the ichthyosporeans and the corallochytreans (Fig. 1) (Grau-Bové et al., 2017; Hehenberger et al., 2017; Lang et al., 2002; Ruiz-Trillo et al., 2008; Shalchian-Tabrizi et al., 2008; Torruella et al., 2015).

Among the unicellular relatives of animals, there is a tremendous morphological disparity, each lineage showing vastly different life cycles and strategies (Sebé-Pedrós et al., 2017). Choanoflagellates are free-living and have a flagellum surrounded by a collar of microvilli. They eat bacteria and some species have a colonial state formed by clonal division. Choanoflagellates have been known since the 19th century and there are more than a hundred described species to date (King, 2005). In contrast, filastereans are relatively novel to science, with only four described species, three of them free-living phagotrophs and one potential parasite. All display a filopodial amoeba morphology, and some have flagellate states (Torruella et al., 2015; Tikhonenkov et al., 2020). *Capsaspora*

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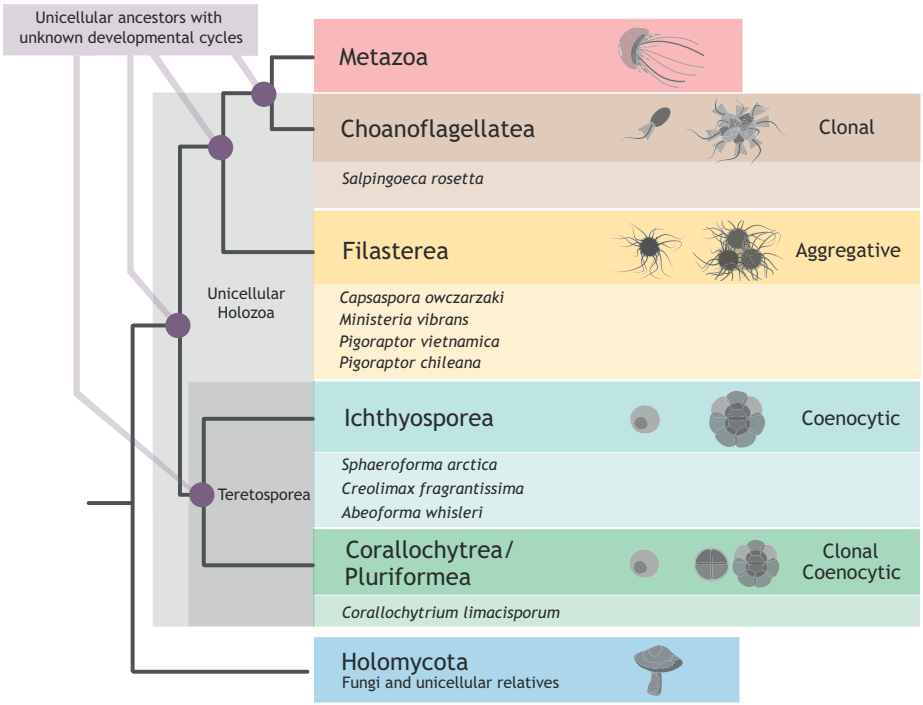

Fig. 1. Schematic tree of the Holozoa clade, comprising Metazoa (animals) and their closest unicellular relatives. The most characteristic life stages, including the temporal multicellular stage are depicted on the right of each lineage.

owczarzaki is the best known filasterean and has the capacity to develop a multicellular structure by cellular aggregation. The ichthyosporeans represent a more diverse group in terms of described species. Most are parasites or commensals but there is evidence of free-living species (Hassett et al., 2015). The two known corallochytreans are free-living (Raghu-kumar, 1987; Hehenberger et al., 2017). Most of the ichthyosporeans and corallochytreans have a thick cell wall reminiscent of fungi, and develop through a multinucleate coenocyte that cellularises forming a transient multicellular stage (Glockling et al., 2013). After cellularisation, amoebae or cell-walled cysts detach and disperse.

This disparity of life stages, ecologies and morphologies among unicellular relatives of animals highlights why none of the single lineages could be considered as a proxy to reconstruct the unicellular ancestor of animals. Each of these lineages is the result of millions of years of evolution since they last diverged from our common ancestor. What we see today is their extant morphology in their current ecological niche, which could be quite different from the ancestral state. Choanoflagellates are phylogenetically the sister group to animals and are structurally very similar, although contentiously homologous (Mah et al., 2014; Sogabe et al., 2019), to sponge choanocytes. Consequently, choanoflagellates are a key system for understanding animal origins (King, 2005). However, there are issues with relying uniquely on choanoflagellates. For example, gene loss has been shown to be a major evolutionary force in eukaryotes (Albalat and Cañestro, 2016; O'Malley et al., 2016), and has also been quite prevalent in some choanoflagellate species, potentially obscuring comparisons to animals (Richter et al., 2018; Sebe-Pedros et al., 2010; Sebé-Pedrós et al., 2011). More generally, given that only some of the characteristics of any species alive today might resemble the last common ancestor, it is only the set of features gathered from a broader comparative approach across the Holozoa clade that will allow reconstruction of the unicellular ancestor of animals and understanding of the different evolutionary steps towards animals and animal development.

Multicellularity in animal relatives

An intriguing feature of some protists that are closely related to animals is that they display a 'multicellular' life stage. This strongly suggests that the ancestors of animals had the capacity for one (or more) multicellular life stages. It is worth noting that multicellularity can be achieved through three different strategies. First, multicellularity can be achieved by clonal division, such as in animals or plants. Another option is to obtain a multicellular organism by cell aggregation, such as in social amoebas. Although cellular aggregation is widespread in eukaryotes, it tends to give rise only to transient and reversible multicellular states. Finally, multicellularity can be achieved by undergoing a multinucleate coenocyte development with later cellularisation. Strikingly, we find an example of each of these modes in each of these protistan lineages (Sebé-Pedrós et al., 2017) (Fig. 1). Therefore, it is conceivable that the unicellular ancestor of animals could develop temporary multicellular structures via any or all of these routes depending on the environmental conditions. Importantly, there are few indications of cellular differentiation occurring across the cells forming any of these multicellular stages. Division of labour across cell types is one of the criteria of 'complex multicellularity', so far restricted to few lineages (plants, animals, fungi and various algae).

The genetic developmental toolkit

One way to test whether these multicellular behaviours are related to animal development is to assess the origins of genetic developmental toolkits. Many of the important, highly conserved, developmental genes were previously hypothesised to be specific to animals, an exclusive patrimony of the 'metazoan genetic toolkit'. For example, many transcription factors are considered the master regulators of embryonic development (Spitz and Furlong, 2012); therefore, one could argue that their origin should be concomitant with the origin of embryonic development. The same argument could be made with genes involved in cell signalling or cell adhesion, all functions essential for multicellularity. But are those metazoan developmental genes specific to animals? If so, this

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would support the uniqueness of animal development, setting a clear boundary between them and the simpler forms of multicellularity that we observe in protistan lineages.

During the last decade, genome sequencing of several of the closest unicellular relatives of animals allowed a systematic reassessment of the uniqueness of the metazoan genetic toolkit. So far, the genome of four choanoflagellates, one filasterean, six ichthyosporeans and one corallochytrean have been sequenced, providing a taxon-rich evolutionary perspective (King et al., 2008; Fairclough et al., 2013; Suga et al., 2013; Torruella et al., 2015; de Mendoza et al., 2015; Grau-Bové et al., 2017; Paps and Holland, 2018; Dudin et al., 2019). What came as a surprise was to discover that these species already had a considerable repertoire of genes and gene families involved in cell adhesion, cell signalling, and transcriptional regulation shared with animals, including several genes that had previously been thought to be animal specific (Sebé-Pedrós et al., 2017). These genomic resources demonstrated that the last common ancestor of animals must have inherited many of these genes, as they evolved in a 'unicellular' context.

Although some major transcription factor gene families are specific to Metazoa, many originated before the onset of animals and were later on expanded during animal evolution (de Mendoza et al., 2013; Sebé-Pedrós et al., 2011). Among these are iconic genes of the metazoan genetic toolkit, such as nuclear factor-kappa B (a major regulator of the immune response), P53 (a regulator of apoptosis and a tumour suppressor) and Myc (associated with cell growth and oncogenic transformation). Moreover, the most important cell-extracellular matrix and cell-cell adhesion systems (integrins and cadherins, respectively) were also shown to have been present in the unicellular ancestor of animals, together with protein tyrosine kinases and some signalling pathways (de Mendoza et al., 2014; Nichols et al., 2012; Sebe-Pedros et al., 2010; Suga et al., 2012). The question therefore arises: are these conserved genes performing comparable functions in protists?

One particularly illustrative example is brachyury, a T-box transcription factor that in many animals plays a crucial role in morphogenetic developmental processes such as animal gastrulation (Marcellini et al., 2003). The genome of the filasterean amoeba *Capsaspora owczarzaki* contains an orthologue of brachyury, expression of which is capable of rescuing the endogenous function of *Xenopus brachyury* (also known as *tbxt*) in a knockdown background, suggesting that it has conserved molecular capacities (Sebé-Pedrós et al., 2013a). Interestingly, further analysis of the open chromatin landscape of *Capsaspora* revealed that brachyury likely acts upstream of a network of genes involved in cell motility (Sebé-Pedrós et al., 2016a). This potential downstream set of genes and their associated functions are very similar to the regulatory network of brachyury in mammals (associated with cell motility, e.g. myosins), which suggests that, at least partially, a gene regulatory network was present before animals or the origins of gastrulation (Sebé-Pedrós et al., 2016a). Thus, not only did at least some parts of the metazoan genetic toolkit originate before animals emerged, but they may also have played similar roles in protists.

Crucially linked to animal development is the capacity to differentiate cell types (Arendt et al., 2016). As development progresses, pluripotent cells become more restricted in their lineage and end up adopting specialised forms with unique functions. In animals, cellular differentiation is tightly linked to epigenomic regulation, including epigenetic marks such as histone modifications that retain cell identity across cell divisions (Amemiya et al., 2013). But was the capacity to differentiate into different cell types already present in the ancestor of animals? Work on *Capsaspora owczarzaki* has shown that this organism uses histone modifications dynamically to establish cell identities (Sebé-Pedrós et al., 2016a). Furthermore, differential cell type-specific phosphosignalling (Sebé-Pedrós et al., 2016b), alternative splicing, and long non-coding RNAs characterise the transition from one life stage to another in *Capsaspora* and ichthyosporeans (Sebé-Pedrós et al., 2013b; Sebé-Pedrós et al., 2016b; de Mendoza et al., 2015; Dudin et al., 2019). These are similar regulatory mechanisms to those that animals use to differentiate into diverse cell types. Thus, the unicellular ancestor of animals presumably not only had multicellular stages and a relatively abundant gene repertoire for multicellular-like functions, but also shared many mechanisms of cell differentiation with animals. Distal gene regulatory elements, also known as enhancers, appear to be an exception to this principle, as they seem to be specific to animals (and plants) (Sebé-Pedrós et al., 2016a). Overall, this suggests that the unicellular ancestors were pre-equipped to perform animal-like developmental processes, making even more important the study of the multicellular life stages of extant protistan holozoans.

This therefore raises the key question: if these unicellular relatives of animals are genetically pre-equipped for development, how does that translate into a unicellular lifestyle? There are two main models to explain the origin of animal development. One involves considering animal origins as a big jump only made possible by a specific combination of biological features coming together. The other is a more parsimonious explanation, in which the unicellular ancestor (and the unicellular relatives of animals that diverged from that ancestor) indeed underwent some sophisticated and coordinated morphogenetic processes that could potentially be homologous to animal developmental processes. To test these ideas, we need to deepen our understanding of unicellular holozoans. This idea challenges our perception of the uniqueness of animal development, perhaps providing a more plausible scenario for the origin of animal development.

Development in unicellular relatives of animals?

A good example of a coordinated morphogenetic process in unicellular holozoans is observed during the multinucleate coenocyte stage of ichthyosporeans. During coenocyte formation, an initial mononucleated cell divides its nuclei multiple times without cell division, forming a coenocyte (Suga and Ruiz-Trillo, 2013). When the coenocyte is mature, each nucleus is encased within an individual cell in the process of cellularisation (Fig. 2A), eventually creating a transient polarised epithelium-like layer. This process is very similar to the syncytial blastoderm stage in some arthropod embryos (such as *Drosophila*; Fig. 2B). However, it is very unlikely to be a homologous process between ichthyosporeans and animals, as coenocytic development is infrequent in animals and not likely to represent an ancestral state. Despite this, we can still find traces of homology in these processes. A detailed immunohistochemical and transcriptomic analysis of ichthyosporean development demonstrated that the cellularisation process involves a coordinated assembly of an actomyosin network with inward plasma membrane invaginations, as occurs in animals (Dudin et al., 2019). Moreover, after cellularisation, during the transitory stage of a clonally generated polarised cell layer resembling an animal epithelium, there is an upregulation of genes activated in cell adhesion in animals (Dudin et al., 2019). Many of the actomyosin network components or adhesion genes are older than holozoans (Arp 2/3 complex, myosin II or integrins) (Sebé-Pedrós et al., 2014, 2013b, 2010), thus these genes likely had ancestral roles in amoeboid cell movement later deployed in ichthyosporean cellularisation (Velle and Fritz-Laylin, 2019).

Another recent example comes from the colonial choanoflagellate *Choanoeca flexa* (Brunet et al., 2019). This species forms large

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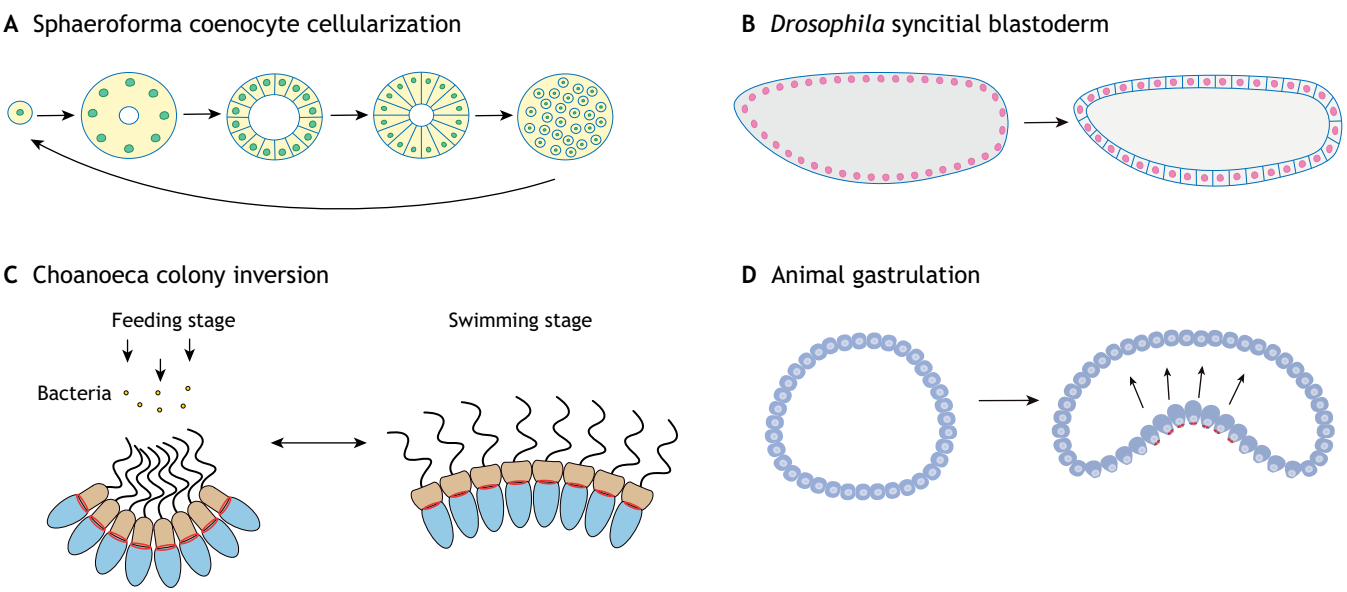

Fig. 2. Schematics illustrating comparable developmental processes in protistan holozoans versus animals. (A,B) Ichthyosporean *Sphaeroforma arctica* multinucleate cellularisation, which is controlled by the coordinated assembly of an actomyosin network (A), as also occurs in the *Drosophila* syncytial blastoderm (B). (C,D) Apical actomyosin contractibility has a role in shaping the colony of the choanoflagellate *Choanoeca flexa* (C), a process that shows similarities to the apical constriction leading to invagination during animal gastrulation (D). Shape shifting in *Choanoeca flexa* reacts to light cues and is reversible, and one colony form favours feeding whereas the other favours swimming. The red ovals indicate the actomyosin rings responsible for constriction: unlike in animal epithelia, where apical constriction directly leads to inward folding, constriction of the apical actomyosin ring in *Choanoeca* leads to a change in collar morphology and outward bending.

colonies that can modify their shape in a very rapid, coordinated manner in response to light changes (Fig. 2C). The colonial cell sheet can invert, shifting the relative position of the flagellum from inward to outward pointing, in a coordinated movement reminiscent of that of animal gastrulation (Brunet et al., 2019; Fig. 2D). This is achieved through apical constriction mediated by actomyosin contractibility, using some of the same genes as ichthyosporean cellularisation (e.g. myosin II). *Choanoeca flexa* colonies are attached via direct contacts between microvilli collars of each cell, and changes in the collar morphology is what triggers colony inversion. This character is not shared with other choanoflagellates or animal epithelia. Thus, it is unlikely that this colony-shaping process is homologous to animal development; rather, it is likely to be the result of convergent evolution. Brunet et al. (2019) found similar actin-based apical contractibility in unicellular choanoflagellates, thus proposing an ancestral role in unicellular species (modification of collar hydrodynamics related to feeding or theca retraction) that was later co-opted in this type of colony formation. However, once again, a very similar set of effector genes involved in apical actomyosin contractibility are playing similar roles in animals and these choanoflagellates.

The examples described above demonstrate that these relatives of animals are capable of well-orchestrated and regulated morphogenetic changes. Thus, this collective set of cellular behaviours found in ‘unicellular’ protists can be considered developmental processes, at least in as much as they share molecular foundations with that of animal development. Therefore, we believe that the integration of such ancestral behaviours had a crucial role in the origin of animal development.

The question now rests on an important consideration: are these forms of pre-animal development directly related to animal development? As mentioned earlier, coenocytic development or large colonial inversion processes are unlikely to be fully conserved processes from common ancestors of these groups, but it could be that these are subtle convergent modifications of ancestral forms of development in the most recent ancestors. At least from a molecular point of view, it is clear that the same sets of genes are being used. One could argue that the genes involved in cytoskeleton configuration have an obvious impact on cellular morphology, so they are more likely to be re-deployed to perform multicellular processes. The question is: are the processes conserved? The answer is not simple, because the evolutionary timescale that divides animals and other holozoans blurs our capacity to discriminate between full homology (direct ancestry from an ancestor), homoplasy (i.e. convergent evolution) and a mixed model in which ancestral homologous genes are deployed in similar yet derived processes in lineage-specific ways. Whatever the case, the morphological similarities that we find across these lineages may also indicate that there are physical or developmental constraints that define an optimal way to perform those mechanical changes. So far, analyses of the closest unicellular relatives of animals have provided a more gradual perspective on the origin of animals, while also questioning the uniqueness of animal development.

Unicellular relatives of animals: an ideal target for evolutionary cell and developmental studies

How can we use these protistan relatives of animals to deepen our understanding of the origins of animal development? One way forward will be to gain greater understanding of these species from a cellular, behavioural, genetic and ecological point of view. This can be achieved by focusing on some model species for which molecular work is feasible, while also aiming to include as many representative species of each lineage as possible. Indeed, we would suggest this should be done for many other eukaryotes, as our knowledge of the cell biology of eukaryotes is limited to only a handful of well-studied organisms. For instance, we know about developmental processes of dictyostelids (social amoebae) (Roberge-White and Katoh-Kurasawa, 2011; Schaap, 2016), plants (Agustí and Blázquez, 2020), fungi (Krizsán et al., 2019),

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algae (Cock et al., 2014; Le Bail et al., 2008), animals (Gilbert, 2000) and now a few unicellular holozoans, but each of these is so divergent that assumptions about their relatedness are very challenging. Unlike gene evolution, in which we have models that tell us, for example, that is almost impossible to independently evolve a myosin II gene from random sequence, we lack such a framework for the evolution of morphogenetic processes. Therefore, aiming to explore the roots of development in the deep nodes of eukaryotic evolution is at this stage a rather quixotic quest, as we are just looking at very few pieces of an enormous puzzle. What we need is to gather, collectively, an integrative perspective that allows a more rigorous evaluation of the evolutionary processes that lead to multicellularity and developmental processes.

Besides cell biology, ecology is also important and an extra challenge. We are currently restricted by the limitations of our understanding of the biology of many organisms in natural environments, including their full life cycles. We know that culture conditions, while allowing experimental tractability, usually restrict the full spectrum of cellular behaviours that can be observed, thus limiting the complete assessment of developmental modes. As an illustrative example, some close relatives of animals, such as the ichthyosporean *Abeoforma whisleri* (Glockling et al., 2013; Marshall and Berbee, 2011) or the recently described predatory holozoans *Syssomonas* and *Pigoraptor* (Tikhonenkov et al., 2020) show a huge diversity of life stages, yet many are rare and difficult to study. Genetic analysis of *Corallochytrium limacisporum* suggests it has a flagellum, yet this has not been characterised in culture (Torruella et al., 2015), implying it might require environmental cues to emerge. A recent experiment forcing spatial confinement on choanoflagellates (perhaps analogous to the ecological niches in which these organisms live), induces a flagellate-to-ameboid transition, revealing a cellular state previously unknown in choanoflagellates (Brunet et al., 2020 preprint). The lack of this ecological information hampers our full understanding of their biology. Thus, efforts to understand the full repertoire of cell biological capacities among the different animal relatives must involve maintaining those organisms in different conditions, and also allowing them to interact with other organisms. For instance, we know that choanoflagellates such as *Salpingoeca rosetta* form colonies or mate in response to distinct types of bacterial prey, so ecological interactions shape developmental processes in protists (Alegado et al., 2012; Woznica et al., 2017). This approach should be taken while maintaining efforts to discover and analyse the broadest diversity of species (del Campo et al., 2014), which, when combined, can provide important clues about the unicellular ancestry of animals as we have seen above.

Finally, efforts need to be invested in functional genetics. We now have a better understanding of the building blocks that were in place when the last common ancestor of animals transitioned to a permanent multicellular state. These building blocks were not only genes involved in animal development, but also the physical and genetic constraints that probably played an important role in the early steps of animal embryonic development. Thanks to recent advances in our ability to perform functional experiments in different unicellular relatives of animals (Booth et al., 2018; Suga and Ruiz-Trillo, 2013; Parra-Acero et al., 2018; Faktorová et al., 2020), we can now go beyond describing the presence or expression of these building blocks in unicellular holozoans. We will be able to manipulate the molecules that underpin these developmental processes and compare what happens with animal embryonic development, no longer requiring us to infer function based on sequence homology alone. Unravelling the degree to which the genetic mechanisms involved in morphogenetic changes in unicellular holozoans are the same as those used in animals will tell us to what extent the tools used in animal development were already assembled or gained new interactions along evolution. Even if protistan relatives of animals use tools that are not directly related to animals, such as the usage of bacterial-derived sphingolipids in colony formation in choanoflagellates (Alegado et al., 2012), that does not reduce their interest. In fact, the simpler modes of development in these lineages can inspire novel lines of research in animal development.

Conclusions

In summary, the insights that we have obtained from the relatives of animals have deconstructed the way we think about animal embryonic development. Protistan holozoans do not go through embryogenesis but nevertheless display developmental processes, using molecular tools and morphogenetic processes that resemble those of animals. Similarly, they have cell types without complex multicellularity. Furthermore, similar differentiation mechanisms are used to transition from one cell stage to another, and each of these life stages can have a specialised function. We therefore believe that by studying and thinking about such humble origins we can and should re-define concepts that were coined with an 'animal-centric' perspective. Recent discoveries and the potential to gain even further mechanistic insights into the development of unicellular species will surely revolutionise our understanding of the origins of animal development, and, most importantly, will expand the frontiers of the ever-changing field of developmental biology.

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Competing interests

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