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Choanoflagellate models — *Monosiga brevicollis* and *Salpingoeca rosetta*

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Choanoflagellates are the closest single-celled relatives of animals and provide fascinating insights into developmental processes in animals. Two species, the choanoflagellates *Monosiga brevicollis* and *Salpingoeca rosetta* are emerging as promising model organisms to reveal the evolutionary origin of key animal innovations. In this review, we highlight how choanoflagellates are used to study the origin of multicellularity in animals. The newly available genomic resources and functional techniques provide important insights into the function of choanoflagellate pre- and postsynaptic proteins, cell–cell adhesion and signaling molecules and the evolution of animal filopodia and thus underscore the relevance of choanoflagellate models for evolutionary biology, neurobiology and cell biology research.

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Introduction

'To understand how animals evolved, we must study choanoflagellates' Peter Holland, University of Oxford, UK [1••].

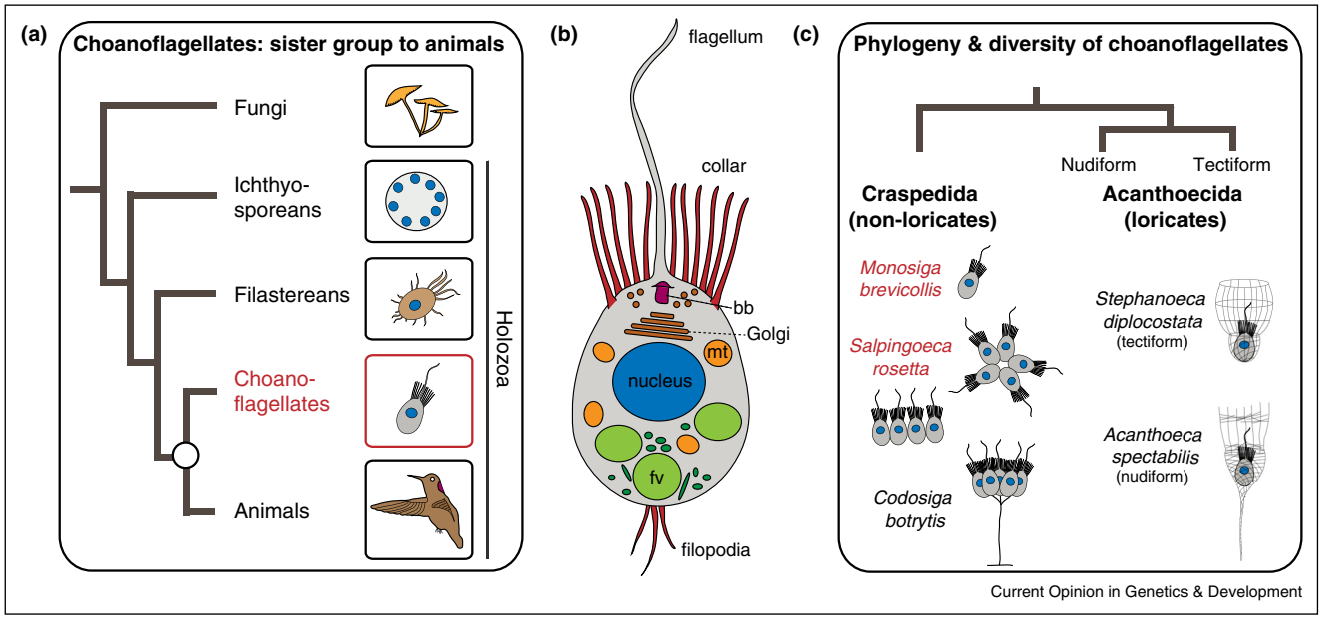
How multicellular animals evolved from their single cell progenitors is an important question in biology. In the last years the study of choanoflagellates has started to answer this question. It is now well accepted that choanoflagellates are the closest single-celled relatives of animals, meaning that they form the sister group to animals (Figure 1a) [2–4]. This important position makes choanoflagellates fascinating models to study the evolutionary origin of key animal innovations [5]. Choanoflagellate morphology is well characterized and their organelle arrangement shows that choanoflagellate cells are highly polarized [1••,6]. Choanoflagellates possess a single posterior flagellum that is enclosed by a collar composed of microvilli (Figure 1b). The movement of the flagellum serves two main functions: to allow motile cells to swim and to create water currents which trap bacteria to the collar to allow phagocytosis [7,8]. Phagocytosed bacteria are digested in anterior localized food vacuoles (Figure 1b). The Golgi apparatus with many associated vesicles is positioned posterior to the prominent nucleus. Moreover, many choanoflagellates possess anterior filopodia (Figure 1b), which allow for substratum attachment of the cells [1••,9].

In this review, we highlight recent advances in choanoflagellate phylogeny and critically discuss the latest progresses made on the establishment of choanoflagellates as model organisms to understand the origin of multicellularity in animals. In particular, we emphasize how newly available genomic resources [3,10], the identification of a sexual life cycle [11], novel functional techniques like the development of a forward genetic screen [12••], and the first successful immunoprecipitation experiments [13•] have allowed getting insights into the function of choanoflagellate pre- and postsynaptic proteins, cell-cell adhesion and signaling molecules, and the evolution of animal filopodia. In the future, these newly available resources will also form the basis for targeted gene activity disruptions.

Phylogeny and morphological diversity of choanoflagellates

Choanoflagellates comprise a group of aquatic protists that can be divided into two different taxonomic orders: Craspedida and Acanthoecida (Figure 1c) [2,14,15]. While the general cell morphology is highly conserved in all choanoflagellate species (Figure 1b) [1••], striking differences exist in their extracellular coverings and their life history stages (Figure 1c). The Craspedida are characterized by an organic extracellular covering, either in form of a rigid theca or of a glycocalyx [2,15]. Among the Craspedida, there are unicellular species which can swim or attach to the substratum (e.g. *Monosiga brevicollis*). Many species have additionally the ability to form free swimming colonies (e.g. *Salpingoeca rosetta*) or substratum attached colonies (e.g. *Codosiga botrytis*) [1••,2]. The Acanthoecida, on the other hand, are characterized by an inorganic extracellular covering (basket-shaped lorica made of silica strips) (Figure 1c) [1••,14]. This order comprises two families, the nudiform Acanthoecidae (nudiform: after cell division one of the daughter cells leaves the lorica naked, e.g. *Acanthoeca spectabilis*) and the tectiform Stephanoecidae (tectiform: after cell division

Figure 1



Phylogenetic position, morphology and diversity of choanoflagellates. **(a)** Choanoflagellates are the closest single-celled relatives of animals [2–4]. White circle: last common ancestor of choanoflagellates and animals. **(b)** Choanoflagellate morphology and disposition of organelles [1••]. bb, basal body; mt, mitochondria; fv, food vacuole. **(c)** Choanoflagellates can be divided into two different taxonomic orders: Craspedida and Acanthoecida [2,14]. Among the Craspedida, unicellular species, and species with the ability to form free swimming colonies (e.g. *Salpingoeca rosetta*) or substratum attached colonies (e.g. *Codosiga botrytis*) exist. The Acanthoecida are characterized by a basket shaped inorganic covering (lorica). This order comprises two families, the nudiform Acanthoecidae (e.g. *Acanthoeca spectabilis*) and the tectiform Stephanoecidae (e.g. *Stephanoeca diplocostata*). Choanoflagellate species with a sequenced genome are highlighted in red.

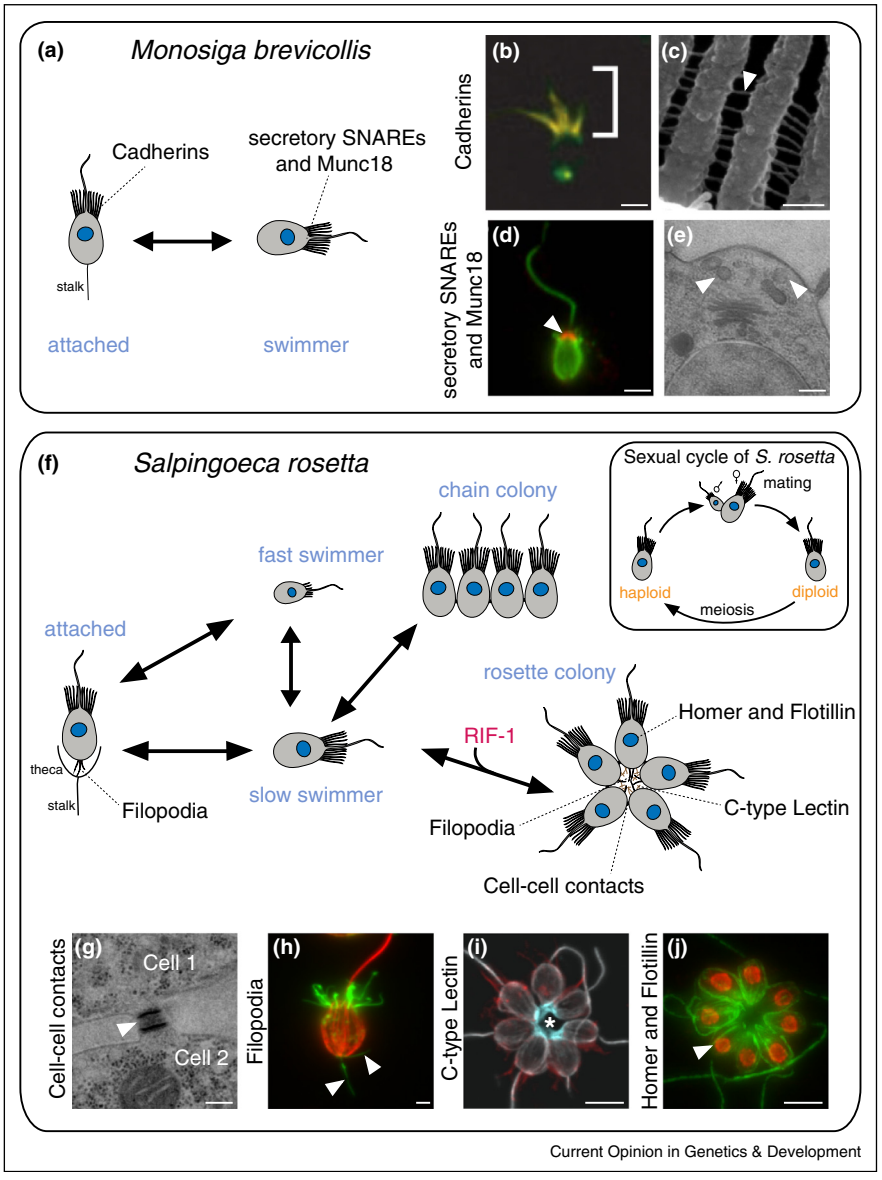
one of the daughter cells is turned upside down and pushed into already accumulated silica strips, e.g. *Stephanoeca diplocostata*) [1••,2,14]. Multicellular forms with cell-cell contacts have not been observed for Acanthoecida [14,16], although some species attach to each other with their lorica (e.g. *Polyoeca dichotoma* and *Parvicorbicula socialis*) [1••,17,18]. Here, we are focusing on *M. brevicollis* and *S. rosetta*, two species belonging to the Craspedida, that are emerging as choanoflagellate models.

Surprising insights from *M. brevicollis* — a unicellular choanoflagellate

M. brevicollis, a unicellular choanoflagellate can switch between two different life cycle stages: a sedentary stage (attached to the substratum) and a motile stage (swimmer) (Figure 2a) [2]. The sedentary stage is attached via a microfibrillar stalk (Figure 2a) [2]. It was the first choanoflagellate species with a fully sequenced genome [3] (link to the genome browser: http://genome.jgi.doe.gov/Monbr1/Monbr1.home.html). The genome of *M. brevicollis* revealed many surprising findings and highlighted the importance of studying choanoflagellates. For example, despite the inability of *M. brevicollis* to form colonies, its genome encodes homologs of many proteins critical for animal cell adhesion and signaling. Two very prominent examples are the presence of cadherins [3,19] and tyrosine kinases [3,20,21] in *M. brevicollis*, proteins that were previously thought to be animal specific. In particular, the presence of cadherins in a unicellular organism generated much excitement, as these proteins are key components required for animal cells to stick to each other. The *M. brevicollis* genome encodes 23 different cadherins [19], a very similar number of cadherins can be found in diverse animal genomes (16 in *Amphimedon queenslandica*, 16 in *Nematostella vectensis*, 17 in *Drosophila melanogaster* and 15 in *Ciona intestinalis*) [22]. While the exact function of many *M. brevicollis* cadherins remains to be investigated, two *M. brevicollis* cadherins (MBCDH1 and 2) localize to the collar of the choanoflagellate (Figure 2b) and may mediate the direct interaction with bacterial prey [19] or intra-collar adhesion (Figure 2c) [9,23].

Tyrosine kinases (TKs) are critical components of signaling cascades that are involved in cell-cell-communication and other processes important for the establishment of multicellularity in animals [24]. The *M. brevicollis* genome revealed the presence of 128 TKs [3,21], opposed to 90 in the human genome [25]. Only 7 *M. brevicollis* TKs are homologous to animal TKs [21], one of which (Abl2) has been shown to phosphorylate cellular proteins when expressed in mammalian cells [26]. The other *M. brevicollis* TKs show domain combinations distinct from animal TKs [3,21], revealing individual diversification of TKs in choanoflagellates and animals [21,27]. Subsequent genome

Figure 2



The choanoflagellates *Monosiga brevicollis* and *Salpingoeca rosetta* emerge as model organisms. **(a)** *M. brevicollis* can switch between two different life cycle stages: a sedentary stage (attached) and a motile stage (swimmer). **(b)** Cadherin MBCDH1 and 2 localize to the collar of *M. brevicollis* (modified from [19]). **(c)** Scanning electron micrograph of collar links between microvilli (modified from [23]). **(d)** Secretory SNARES and Munc18 localize to the apical membrane of *M. brevicollis* (modified from [52]). **(e)** Transmission electron micrograph shows vesicles at the apical membrane of *M. brevicollis* (modified from [52]). **(f)** *S. rosetta* can switch between five different life cycle stages: a sedentary stage (attached), two different motile stages: fast swimmers and slow swimmers and two different colonial stages: chain and rosette colonies (modified from [9]). Cells in colonies are held together by cytoplasmic bridges and extracellular matrix (not shown), and rosette colonies are additionally stabilized by filopodia (modified from [9]). **(g)** Transmission electron micrograph reveals a cytoplasmic bridge between two cells in *S. rosetta* colonies (modified from [9]). **(h)** Attached *S. rosetta* cells possess long basally positioned filopodia (modified from [41]). **(i)** C-type lectin (asterisk) localizes to the centre of *S. rosetta* colonies (modified from [12••]). **(j)** The postsynaptic protein homolog Homer localizes to the nucleus of *S. rosetta* (modified from [13•]). Scale bars: 2 μm in B, 100 nm in C; 2 μm in D; 200 nm in E; 200 nm in G; 1 μm in H; 5 μm in I and J. RIF-1, rosette inducing factor 1.

surveys of other holozoans (group including animals, choanoflagellates, filastereans, ichthyosporeans and corallochytreans (Figure 1a)) and closely related organisms, revealed that choanoflagellates are not the only phylum besides animals with homologs of tyrosine kinases and cadherins [27,28•]. Two Cadherins have been identified in the filasterean *Capsaspora owczarzaki*, and two more in *Thecamonas trahens* — a non-holozoan flagellate [28•]. TKs are present across holozoans [27] and were also identified in *T. trahens* and in the amoeba *Acanthamoeba castellanii* [29].

Another big surprise to emerge *M. brevicollis* genome analyses was the identification of synaptic proteins [13•,30–34]. *M. brevicollis* clearly lacks synapses and neurons, but possesses many critical components for synapse function known from animals. For example, the *M. brevicollis* genome encodes for voltage gated calcium channels that are very similar to the ones found in animal nerve cells [34], for voltage gated sodium channels important for generating action potentials in animal nerve cells [31,35] and also several proteins that animal nerve cells use to anchor neurotransmitter receptors at the postsynaptic membrane, so-called postsynaptic scaffolding proteins like PSD-95, Homer and Shank [32]. In addition, a neurosecretory apparatus was identified in *M. brevicollis* [36]. A conserved set of presynaptic neurosecretory proteins, which are called SNARE and Munc18 proteins and are key components of the synaptic vesicle release machinery in neurons, interact in *M. brevicollis* in the same way as they do in vertebrates [36]. These proteins localize to the apical membrane in *M. brevicollis* and may mediate the release of molecules from vesicles (Figure 2d, e).

S. rosetta — a colony-forming choanoflagellate enters the stage

A major step in choanoflagellate research was the introduction of *S. rosetta* as a model organism. This species is able to form beautiful colonies, and the complex life cycle of *S. rosetta* has now been investigated in detail (Figure 2f) [9]. *S. rosetta* possesses a sedentary stage (attached to the substratum) and at least two different motile stages: fast swimmers and slow swimmers [9]. Slow swimmers can differentiate into fast swimmers or attached cells (Figure 2f). Importantly, slow swimmers also have the unique ability to form rosette or chain colonies by cell division. [9,16]. Cells in both types of colonies are held together by cytoplasmic bridges and extracellular matrix (ECM) (Figure 2g); whereas rosette colonies are additionally stabilized by filopodia (Figure 2f) [9]. Rosette colony formation can be induced by the addition of the bacterium *Algoriphagus machipongonensis* to *S. rosetta* cultures (Figure 2f) [37,38••]. Analysis of the lipid extract of A. *machipongonensis* revealed that a specific molecule, a sulfonolipid called RIF-1 (rosette inducing factor), is responsible for the induction of colony formation (Figure 2f) [38••]. RIF-1 synthesized *in vitro* also induces colony formation [39], and additional molecules, synergizing with RIF-1 in its colony inducing effect have been identified [40]. The sequencing of the genome and transcriptome of *S. rosetta* has further contributed to our understanding of the putative genome composition of animal ancestors [10]. Homologs of proteins involved in cell adhesion and signaling (29 cadherins) and neuropeptide signaling were identified [41] (link to the genome browser: http://www.ncbi.nlm.nih.gov/bioproject/37927: link to the transcriptome browser http://www.ncbi.nlm.nih.gov/bioproject/62005). The transcriptome has assisted in attributing specific genes to the different life cycle stages of *S. rosetta* [10]. For example, specific sets of cadherins and tyrosine kinases are upregulated in colonies, whereas other sets are upregulated in attached cells only [10].

Moreover, the study of *S. rosetta* has led to insights into the origin of animal filopodia, which are thin actin-based projections and essential organelles for animal cells [41]. Filopodia are present in *S. rosetta* attached cells and rosette colonies (Figure 2f) [9,41] and homologs of many animal filopodial components are encoded in its genome, for example fascin and myosin X [41]. Fascin in animal filopodia is known as a critical actin crosslinking protein [42,43]. In *S. rosetta* attached cells Fascin localizes to long basally positioned filopodia and microvilli (Figure 2h) [41]. Interestingly, the *S. rosetta* genome encodes for two Fascin paralogs. Fascin 1 is upregulated in attached cells, whereas Fascin 2 is upregulated in rosette colonies [41]. This has important implications, as Fascin 1 could function in filopodia formation for cell-attachment to substratum, Fascin 2 on the other hand might function in filopodia formation for rosette colony formation (Figure 2f) and thus could directly be involved in colony formation and/or stabilization in *S. rosetta*.

S. rosetta has also emerged as model to reconstruct the ancestral functions of synaptic proteins like Homer, PSD-95 and CamKII [13•,44,45]. The postsynaptic density protein Homer, which controls abundance and orientation of membrane receptors and regulates calcium signaling in neurons [46], unexpectedly localizes to the nucleus in *S. rosetta* (Figure 2j) [13•]. While the domain organization of *S. rosetta* Homer is well conserved, as is its ability to tetramerize, *S. rosetta* Homer does not interact with its well-known binding partner Shank, which is encoded in the *S. rosetta* genome and expressed throughout all life history stages [13•]. Instead, it was shown that *S. rosetta* Homer directly interacts with Flotillin, a component of lipid rafts [13•]. Thus, Homer might have originally functioned in the nucleus through its interaction with Flotillin and was only later co-opted to act as a scaffolding protein at the post synaptic density in neurons [13•].

The first genetic screen in choanoflagellates

The presence of sexual reproduction in choanoflagellates was suggested by earlier studies [9,47–49]. The first direct evidence of a choanoflagellate sexual life cycle was provided by Levin and King [11]. These researchers managed to induce and observe a mating process, and showed that upon changes in the availability of food bacteria, the ploidy of cells in the culture changes (Figure 2 inset) [11]. Under starving conditions haploid choanoflagellate cells form morphologically distinct gametes that fuse and produce diploid cells, while under conditions of constant food availability (high amount of food bacteria) the cells undergo meiosis and form haploid cells again (Figure 2 inset) [11].

This pioneering work laid the foundation for the first genetic screen in choanoflagellates. Levin and colleagues used forward genetics to identify essential genes for *S. rosetta* colony formation [12••]. In this approach mutations were generated by X-ray or EMS exposure, followed by a phenotypic screen for mutants with defects in rosette colony formation. The authors isolated different mutants, one of which they named Rosetteless. This mutant maps to a C-type lectin, and is absolutely required for rosette colony formation [12••]. Rosetteless protein localizes to the centre of *S. rosetta* colonies (Figure 2i) and may be important to stabilize connections between cells [12••]. The ability to outcross haploid mutants with haploid wild type individuals, is extremely helpful in order to show, if the wild type phenotype can be rescued, verifying its suggested function and providing evidence for the mutation being recessive or dominant [12••]. Establishing forward genetics in *S. rosetta* provides 'the first link between genotype and phenotype' for this group [12••] and was an important step forward in establishing choanoflagellates as model organisms.

Conclusions

Model organisms are widely used in order to understand key biological processes relevant to medicine, development and evolution [50]. Choanoflagellate models are key to reveal the ancestry of proteins required for animal multicellularity. The choanoflagellate species *M. brevicollis* and *S. rosetta* are easy to culture and have a short generation time of 6–8 h. Both species have a fully sequenced genome available [3,10], resources that led to the identification of gene homologs once considered to be animal specific. Both choanoflagellate genomes encode for many cadherins (cell–cell adhesion molecules) [3,10] and synaptic proteins [13•,32].

Cadherins are differentially expressed depending on the life cycle stages of *S. rosetta* [10]. In the future, it will be important to investigate the subcellular localization of additional choanoflagellate cadherins and the general ability of choanoflagellate cadherins to homo-dimerize (e.g. investigate if they bind each other and thus be able to mediate direct cell-cell contact). Moreover, the identification of intracellular binding partners would reveal downstream signaling functions of cadherins in choanoflagellates.

The identification of synaptic proteins in choanoflagellates and the presence of a conserved secretion apparatus raise intriguing questions: Is the choanoflagellate synaptic machinery involved in cell-cell communication? What is the function of choanoflagellate synaptic proteins and where do they localize in *S. rosetta* colonies? Do choanoflagellates produce action potentials [51]? It remains to be investigated if cell-cell communication is mediated by secretion of small molecules (Figure 2d) or if cells communicate via cytoplasmic bridges (Figure 2g).

The available genomic resources and novel functional techniques highlight the recent progresses made and showcase the suitability of choanoflagellate models to answer important questions in evolutionary biology, neurobiology and cell biology.

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- •• of outstanding interest

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