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Regulation of Form in Multicellular Choanoflagellates and the Evolutionary Cell Biology of Morphogenesis

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Regulation of Form in Multicellular 
Choanoflagellates and the Evolutionary Cell Biology 
of Morphogenesis 

by 

Benjamin Timothy Larson

A dissertation submitted in partial satisfaction of the 
requirements for the degree of 
Doctor of Philosophy 
in 
Biophysics 
and the Designated Emphasis 
in 
Computational and Genomic Biology 
in the 
Graduate Division 
of the 
University of California, Berkeley

Committee in charge: 
Professor Nicole King, Co-Chair 
Professor Oskar Hallatschek, Co-Chair 
Professor Daniel Fletcher 
Professor Nipam Patel

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Abstract

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Doctor of Philosophy in Biophysics

And the Designated Emphasis in Computational and Genomic Biology

University of California, Berkeley

Professor Nicole King, Co-Chair

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Proper morphogenesis requires the regulated interplay between cellular behaviors and physical constraints. Studies of physiology and morphogenesis in protist and animal systems with respect to this interplay have led to important mechanistic insights. Comparatively little work, however, has studied how morphogenetic processes evolve in the context of physical constraints. Choanoflagellates, the closest living relatives of animals, can form multicellular colonies of various shapes and sizes. This diversity and the simplicity of multicellular forms in conjunction with their important phylogenetic position makes choanoflagellates an ideal system for studying the evolution of morphogenesis. Because most work has focused on genetics and genomics, little is known about the cellular and biophysical mechanisms underlying the regulation of multicellular form in choanoflagellates.

Here, I quantify the biophysical processes underlying the morphogenesis of rosette colonies in the choanoflagellate Salpingoeca rosetta. I find that rosettes reproducibly transition from an early stage of 2D growth to a later stage of 3D growth, despite the underlying stochasticity of the cell lineages. I postulate that the extracellular matrix (ECM) exerts a physical constraint on the packing of proliferating cells, thereby sculpting rosette morphogenesis. Perturbative experiments coupled with biophysical simulations demonstrate the fundamental importance of a basally-secreted ECM for rosette morphogenesis. In addition, this yields a morphospace for the shapes of multicellular colonies, consistent with observations across a range of choanoflagellates. Overall, this biophysical perspective on rosette development complements previous genetic perspectives and thus helps illuminate the interplay between cell biology and physics in regulating morphogenesis.
I also present the previously undescribed species *Choanoeca flexa*, a splash pool choanoflagellate that forms cup-shaped colonies. The colonies rapidly invert their curvature in response to changing light levels, which they detect through a rhodopsin-cGMP pathway. Inversion is mediated by cell shape changes requiring actomyosin-mediated apical contractility and allows alternation between feeding and swimming behavior. *C. flexa* thus rapidly converts sensory inputs directly into multicellular contractions. In this respect, it may inform reconstructions of hypothesized animal ancestors that existed before the evolution of specialized sensory and contractile cells.
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Chapter 1: The interplay between cellular behavior and physical constraints in evolutionary cell biology

1.1 Introduction

Life is fundamentally cellular. In nearly every context, proper biological function relies on the regulated interplay between active cellular processes, cellular behaviors\(^1\), and physical constraints. The centrality of cells has long been an emphasis of biological investigation, with the idea that all cells arise from other cells, i.e. “cell theory”, first emerging in the 1800s (1, 2). The term “cell” was coined by Robert Hooke in 1665 based on observations of plant material (3), but the most detailed and extensive early observations of cells were of microbial eukaryotes, “protists”, described by Antonie van Leeuwenhoek as wondrous “animalcules” in the late 1600s to the early 1700s (4–6). Eukaryotic cells are defined in part by their complex cellular architecture, including a nucleus, diverse other organelles, and a complex cytoskeleton. In part due to their subcellular complexity, protists also display an incredible diversity of form and function (Fig. 1.1, 2).

A key challenge in biology is to understand the mechanistic basis and evolutionary origins of cellular diversity. In fact, all aspects of biological diversification across the tree of life can be traced to cellular modifications (7). While revolutions in molecular biology, genomic sequencing, and computation have begun to provide insight into the evolution of eukaryotes, we have only scratched the surface in terms of understanding the diversity of eukaryotic cell biology (8). Although plants and animals display the most familiar examples of form and function among the eukaryotes, the vast majority of eukaryotic diversity lies in the protists, a paraphyletic group defined by the absence of the complex multicellularity that characterizes plants, animals, and fungi (Fig. 1.2).

Protists play important roles in nearly every ecosystem and also hold key phylogenetic positions for elucidating major evolutionary events including the origin and evolution of various organelles (9–12), the origin of eukaryotes (13–17), and the origin of animals (18–22). Despite their ecological and evolutionary importance, the biology of the vast majority of protists is poorly understood, particularly at mechanistic, functional levels (8, 23). Unlike in bacteria, where detailed understanding of metabolic pathways allows for inference of cell biology and metabolism from genome sequences alone, it is difficult, if not impossible, to predict form, function, and behavior of eukaryotic cells from their genomes (8). If we are to determine whether general principles underlying the evolution of form and function in eukaryotes, protists represent a vast, largely untapped resource.

1.2 Protist behavior in the context of physical constraints

Cellular behavior takes place in the context of physical constraints, and this context can often be mechanistically important. Constraints are limiting and can act not only as barriers, but can also yield robustness (24). In this way, constraints can provide an important mechanistic component of the regulation of biological function.

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\(^1\) In this chapter, I use the term “behavior” in its broadest sense to refer to the range of actions of an individual such as movement, size or shape change, and secretion resulting from various internal and external inputs. The term behavior also is meant to emphasize an action or output as opposed to active internal processes such as changes in gene expression and vesicular trafficking, which have received more attention at the mechanistic level.
Approaches combining perspectives from physics and biology have been instrumental to addressing many mechanistic questions in biology, such as the regulation of cytokinesis (25–28) and cell crawling (29–32). However, much of this work has been confined to relatively few well-studied systems representing a small subset of eukaryotic diversity. While not receiving as much attention as traditional model systems, work on protists at the intersection of biology and physics can provide mechanistic insight into fundamental questions in biology.

Because most protists are free-living, their natural cellular behaviors are amenable to direct observation and targeted manipulation in controlled environments. Indeed, a substantial amount of the history of cell biology from the development of microscopy to the rise of molecular biology has been dominated by careful observation of protist behavior and structure (33, 34). More recently with the increasing interest in non-model systems, descriptive and mechanistic characterization of protists are becoming more prevalent (23, 35, 36). Work on protists illustrates how investigating function at the intersection of biology and physics can yield mechanistic insight. Furthermore, as we will illustrate by detailing a few examples, this perspective can lead to more general insights.

1.2.1 Volvox and Dictyostelium: Morphogenesis

The interplay between cellular behavior and physical constraints has perhaps been most extensively studied in the context of morphogenesis. D’Arcy Thompson’s “On Growth and Form” (37) is often identified as the seminal work bringing ideas from mathematics and physics to bear on problems of biological morphogenesis. This idea is perhaps best summed up in the following quote:

“Cell and tissue, shell and bone, leaf and flower, are so many portions of matter, and it is in obedience to the laws of physics that their particles have been moved, moulded and conformed...Their problems of form are in the first instance mathematical problems, their problems of growth are essentially physical problems, and the morphologist is, ipso facto, a student of physical science.” (37)

The green alga Volvox, with its large, swimming colonies, has long captivated microscopists (38) (Fig. 1.3A). Like embryogenesis in many animals, Volvox colony development begins with an early phase of synchronous cleavage divisions, during which cells divide without growth (39). This early phase is followed by a growth and expansion phase mediated by ECM secretion (39, 40). Strikingly, after the growth phase, nascent colonies undergo an inversion process, in which the entire colony flips inside out (39, 41) (Fig. 1.3B, C). This inversion process is reminiscent of gastrulation in many animals in that such processes involve the bending of sheets of cells through coordinated cellular deformations. In Volvox, inversion allows cells to reorient their flagella from pointing toward the inside of the spherical colony to the outside in order for the colony to swim.

While qualitative descriptions of the dynamics of inversion have existed for decades (41), recent advances in microscopy have facilitated detailed, quantitative analysis (42, 43). Dynamics alone, however, only provide the starting point for an explanation. As in any multicellular morphogenetic process, correlations between local cellular behavior and global deformations can be observed (Fig. 1.3A, B), but determining precisely how those cellular behaviors are related to the global deformations requires further investigation. For example, whether cellular deformations are the cause or the result of tissue bending cannot be determined purely by observation. An understanding of mechanics is necessary to answer such questions. In conjunction with mathematical modeling, quantitative analysis of Volvox inversion has shown how coordinated
shape changes (Fig. 1.3B) in conjunction with geometrical and mechanical constraints inherent to elastic bending drive the inversion process (42, 43). Cells in colonies are connected by cytoplasmic bridges that constitute a stable, structural framework that physically couples all cells (39, 41) (Fig. 1.3B). The details of the different phases of inversion, which differ between species, have been described in depth elsewhere (39, 41, 42, 44). Briefly, inversion involves the formation of cells that are constricted on the side facing into the colony, called flask cells (Fig. 1.3B), which are analogous in form and function to bottle cells of animal epithelia that form during gastrulation (39, 41). In a coordinated wave, cells begin as spindle cells before transitioning into flask cell morphology, followed by resolution to columnar form (Fig. 1.3B). As the flask cells constrict, bridges are displaced toward the narrow distal end of cells (39, 41) (Fig. 1.3B). Stresses in the connected sheet of cells induced by the cell shape changes lead to colony-wide deformation (43, 44). It had been hypothesized that the cellular deformations eventually lead to a mechanical snap-through that passively carries the colony through the final stages of inversion (41), and recent work has supported and refined this hypothesis based on a more detailed mechanical treatment (42, 43). Indeed, this interplay between coordinated changes in cell shape in the face of global geometrical constraints gives rise to overall robustness in inversion in the face of variability between embryos (42).

Unlike multicellular sheet bending in many animals, which may involve many cellular behaviors such as growth, division, migration, and intercalation, the relative simplicity of Volvox inversion facilitates mathematical analysis (42, 43). As such, studies of the mechanics of Volvox inversion stand to clarify geometrical and mechanical aspects of cell sheet bending, which is ubiquitous in morphogenesis and movement in multicellular systems. In some cases of sheet bending, such as gastrulation (45–49), neurulation (49–51), placode formation (52), and primitive streak formation (53) in various animals, polarized cell shape changes analogous to those in Volvox in conjunction with geometrical constraints also drive sheet bending. Although, in these cases, the mechanics are not as well understood as in Volvox. Therefore, mechanistic studies of the physics of Volvox embryo inversion stand to illuminate morphogenetic processes involving sheet bending more generally.

Another protist system that has yielded insights into morphogenetic processes is the social amoeba Dictyostelium. Under starvation conditions, the normally solitary amoebae undergo chemotaxis to aggregate and form a multicellular structure called a slug (54, 55) (Fig 1.3D, i-iii). Slugs contain up to hundreds of thousands of individual cells and undergo coordinated motility, directed by light and temperature, to search for a suitable soil surface where the slug transforms into a stalked fruiting body that ultimately produces and releases spores (54, 56) (Fig. 1.3D, iv). This process is arises from cellular behaviors such as directed motility, shape change, and intercalation in conjunction with physical mechanisms (54).

In terms of physical mechanisms, reaction-diffusion based pattern formation is key to directing many of the cell behaviors that ultimately underlie all aspects of Dictyostelium aggregation and morphogenesis. Specifically, periodic cAMP signals resulting from the cellular production, secretion, sensing, and degradation of cAMP drive the cellular behavior underlying aggregation as well as spatial patterning and morphogenesis at later steps of the starvation response (54, 57) (Fig 1.3D). These physico-chemical patterns (Fig. 1.3D) orchestrate the directed crawling of cells that constitutes aggregation (Fig 1.3Dii, ii). They also play an important role in organizing the timing and spatial organization of crawling, adhesion, and cell shape change that constitute slug formation and motility (Fig. 1.3Diii) and the differentiation, adhesion, secretion of extracellular matrix, cell shape change, and migration that constitute fruiting body formation (54,
The entire morphogenetic cycle unfolds in a self-organized manner in which cells secrete and take in signals subject to the physical process of diffusion and subsequently modulate their movement and shape as well as adhesion to one another in order to undergo coordinated movement and morphogenesis (54, 59–61).

Turing pattern formation, arising from reaction-diffusion systems, represents perhaps one of the most influential theories of physical mechanism in biology (62, 63). Pattern formation in reaction-diffusion system stems from the dynamic interplay between local excitation, in which chemical reactions transform substances into one another, and lateral inhibition, due to the spreading out of substances by diffusion. This dynamic of local activation or positive feedback and long-range inhibition or negative feedback is the essential feature of reaction-diffusion pattern formation (64). The resulting robust, stable patterns can come in a variety of forms, from spots and stripes to spirals and traveling waves. In Dictyostelium, cAMP reaction-diffusion patterns generally take the form of traveling linear and spiral waves (54, 57, 65, 66). These patterns are robust to differences in cell numbers and variability in the spatial distribution of cells (54).

Outside of Dictyostelium, reaction-diffusion systems have long been proposed to underlie many morphogenetic processes, particularly Turing pattern formation in animal development such as in vertebrate skin patterning and limb development (63, 67). Only recently, however, have such mechanisms become integrated with experimental validation an understanding of how they emerge based on cellular behaviors (such as motility and contraction) in the context of the mechanics of cells and tissues. Turing patterns in vertebrate skin along with their underlying cellular bases are especially well established. Work in various fish has shown that horizontal stripes of different colors stemming from different cell populations form according to a reaction-diffusion mechanism (68, 69). Additional examples of such pattern formation include hair follicle patterning in mice (70) and feather bud formation in chickens (71, 72). Tooth development has also been studied in terms of a similar convergence biological and physical mechanisms, in this case Turing mechanisms along with an additional physical mechanism of differential tissue growth, which drives the bending of tissues (73, 74).

The existence of a similar physical mechanisms in a number of different developmental contexts speaks to the generality of reaction-diffusion based pattern formation. Dictyostelium represents a system of intermediate complexity between non-living reaction-diffusion systems such as the Belousov-Zhabotinsky reaction (75–78) and animal systems (79). The relative simplicity and hence tractability of Dictyostelium in conjunction with shared physical mechanisms presents the opportunity for insight into reaction-diffusion based coordination of cellular behavior in morphogenesis.

1.2.2 Chlamydomonas: Motility and navigation

Another rich area of mechanistic research at the intersection of cell behavior and physical constraints is cellular motility and navigation. Nearly all cells live in a fluid environment and are therefore subject to the effects of a low Reynolds number local environment, where viscous effects dominate over inertial effects (80). Unlike when humans swim, if a cell ceases swimming, it will stop within a distance equivalent to about the width of an atom. One of the most influential treatments of physical constraints on biological systems has been E. M. Purcell’s essay “Life at low Reynolds number” (80). This work succinctly illustrates the constraints on motility of any micro-swimmer. The highly viscous fluid environment of the cell also means that at the scale of a cell, there is little mixing due to turbulence. Instead diffusion often dominates in terms of passive
dispersal, although the transport of molecular species by flow can often be more important relative to diffusion for larger protists compared to bacteria and small protists (81). Furthermore, turbulence can have marked effects on the population level spatial distribution of swimming microbes (82).

Chemotaxis of *Escherichia coli* bacteria, whereby cells sense and respond to chemical gradients in order to find nutrient sources is perhaps the most extensively studied example of cellular navigation (83–86). While swimming, *E. coli* cells undergo a series of linear “runs” and “tumbles” where the cell changes direction. By comparing past concentrations of a chemoattractant with current levels and then modulating the frequency of tumbles, *E. coli* cells are able to effectively follow gradients much too noisy or shallow to be sensed over the length of the cell (83, 87). This chemotactic process, called “run and tumble”, allows *E. coli* to perform directed swimming toward a target even in the face of Brownian motion that would otherwise stochastically reorient the cell.

Although *E. coli* chemotaxis has been studied most extensively, many other bacterial and eukaryotic cells also undergo various kinds of directed swimming. While the underlying mechanistic details vary, all modalities of directed swimming are robust to noise due to diffusion and tend to involve either modulation in frequency of direction change (as in *E. coli*) or changes in swimming speed. As far as protists go, the biflagellate green alga *Chlamydomonas* has been extensively studied in terms of swimming behavior (Fig. 1.3E). Interestingly, *Chlamydomonas* also swims with two phases similar to those of *E. coli*: one where flagella are synchronized leading to linear trajectories and the other where flagella beat asynchronously (Fig. 1.3E), leading to an abrupt change in direction (88). This behavior allows *Chlamydomonas* cells to diffuse rapidly in the dark and in conjunction with photosensing by an eyespot enables directed swimming toward light in a fashion analogous to *E. coli* chemotaxis (88, 89). Highlighting the generality of bimodal cellular navigation strategies, quantitative analysis of the ciliate *Tetrahymena* has revealed that a run and tumble model accurately captures swimming behavior and furthermore that tuning the length of runs and frequency of tumbles describes variability between cells (90).

The mechanics and hydrodynamics of swimming have also yielded mechanistic insight. Hydrodynamics effects have also been implicated in the synchronization of flagella in various situations including motility of the green algae *Chlamydomonas* and *Volvox* (91–94). Flagellar synchrony is required for proper motility, and hydrodynamic coupling has been shown to be sufficient to produce synchrony (91–94). In contrast to *Volvox* where hydrodynamics effects alone appear to be sufficient for coordination during swimming, flagellar synchrony in swimming *Chlamydomonas* seems to be driven by competition between direct mechanical coupling of flagella through basal bodies in conjunction with hydrodynamic effects (91). Swimming of many ciliates is also mediated by synchronous flagellar activity. Presumably, this may be coordinated through hydrodynamic effects and basal body coupling, although mechanisms of flagellar coordination in ciliates has received less attention (95, 96). Outside of protists, flagellar synchrony mediated by hydrodynamics has been observed in spermatozoa (97–99) and ciliated epithelia of animals and can play an important role in human health and disease (100–103). Again, the relative simplicity of protists presents an opportunity for detailed investigation of physical mechanisms. Such fundamental understanding with respect to detailed investigation of physical mechanisms stands to illuminate more complex cases, for example, instances of flagellar synchrony in animal epithelia, where neural

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2 Here, we use flagellum and cilium interchangeably. Eukaryotic flagella and cilia are homologous and structurally identical, although some work has specified functional and/or morphological differences (in terms of length and beat frequency and form). These differences are not important for present purposes.
control an planar cell polarity are known to be important and add additional layers of complexity (102, 104–106).

The examples of swimming and navigation highlighted here represent only a few of the many different modalities of locomotion. Even restricted to flagellar-based swimming, the examples discussed here only begin to cover the spectrum represented by protists. For example, the rosette colonies of the choanoflagellate S. rosetta have been shown to undergo directed motility in response to oxygen gradients (107). Unlike directed motility in the previously described systems, in rosettes, there is no flagellar coordination. Instead, directed motility, which can be described by an aggregate random walker model, arises from the joint independent behavior of cells that change their swimming speed based on oxygen levels, leading to subtle changes in direction (107). However, all swimming protists are subject to the same physical constraints imposed by fluid mechanics. Are there more general principles unifying locomotion and decision making in sensing/responding and navigation? In nature, chemotaxis can play an important role in nutrient cycling at the ecosystem and even global scales (82). At a population level, the interplay between active swimming and shear due to natural turbulence in aquatic environments can lead to up to a 10-fold increase in local phytoplankton density (108). Therefore, comprehensive and potentially unified understanding of these mechanisms and dynamics in their environmental context will be important for understanding nutrient cycling in aquatic ecosystems where protists nearly always play important roles.

1.2.3 Physarum: Decision-making

Spanning elements of morphogenesis as well as environmental navigation, the slime mold Physarum polycephalum has become a model for studying how cells coordinate complex behaviors in terms of computation and decision-making (109–112). Physarum is a large (easily visible to the naked eye), plasmodial, or multinucleated, cell that grows as a dynamic network of interconnected tubes (Fig. 1.3F) through which cytoplasm rhythmically flows back and forth (113). In nature, Physarum crawls across damp forest environments in search of food and favorable environments (Fig. 1.3F). Upon finding food, the cell alters its structure to increase cytoplasmic flow and growth toward the food, which it then digests extracellularly (113–115). In the lab, Physarum has been shown to be able to solve mazes (112), to find the shortest distance between points of interest (109), a notoriously computationally difficult problem, and to organize its structure to optimize its diet (116). In order to accomplish this kind of sophisticated behavior, the undifferentiated cell needs to coordinate morphogenesis across centimeters. Diffusion alone would be prohibitively slow, taking even ions days to travel such distances assuming a cell size of 2 cm and diffusion on the order of 10-5 m²/s. However, cytoplasmic flow driven by rhythmic actomyosin mediated contractions of the gel-like tubular cell structure drive cytoplasmic flows, which can transport signals much more rapidly (113, 117). Flow increases Taylor dispersion, the effective dispersion of molecular substances beyond pure diffusivity, and work has shown that Physarum can reorganize its structure to optimize Taylor dispersion (118, 119). Additionally, behavioral coordination arises in a self-organizing manner as the cell lacks any sort of centralized organizational structure (109–111, 117, 119, 120). Quantitative analysis of Physarum dynamics in conjunction with mathematical modeling of flows have shown how advected cues, likely intracellular Ca²⁺ (121), in conjunction with changes in network structure and tube diameter affected by those signals are sufficient to account for the observed foraging behaviors (109, 117, 119, 122). In this way, the cell is a kind of active reaction diffusion system (123, 124).
In Physarum, this biophysical approach has provided mechanistic insight into the coordination of complex behaviors. It has also allowed for the testing of competing hypotheses as to the underlying molecular mechanisms of signal propagation (i.e. elastic waves vs. electrical impulses vs. advected molecular signals) (119). Insights from this work may inform our understanding of systems beyond Physarum including hyphal networks by which fungi grow and feed, which share many physical similarities. More generally, insight may also inform our understanding of how cells and other “simple” systems are able to coordinate and control complex behavior and of how natural systems compute (110, 111, 119, 125).

1.2.4 Conclusion

Although the model systems and ideas and their application to understanding the mechanistic basis of cellular function highlighted in the previous sections are by no means new, recent technological developments have greatly facilitated or enabled the kinds of quantitative measurements needed to test theory and have also made large scale simulations and numerical calculations possible. There has also been a marked increase in interdisciplinary work necessary to proceed with the combination of theory and experiments underlying this approach to understanding function at the bio-physical interface. As such, we expect progress and discoveries to continue to accelerate.

1.3 Evolution at the intersection of biology and physics

While considerable work on the interplay between physical constraints and cellular behavior has identified mechanisms of robust biological function, considerably less work has focused on the role of this interplay in evolution. There is certainly a substantial precedent for such studies, although most work has focused on animals and plants at the organism or tissue scale without explicit treatment of cells. Work has also tended to emphasize phenotypic limitations and optimization of function.

For example, work on the evolution of ammonite shell coiling explored why certain shell morphologies existing in a theoretical morphospace (126) were never observed in nature (127). The missing shell morphologies turned out to correspond to a transition from shells with overlapping whorls, which corresponds to a significant increase in drag and therefore decrease in swimming efficiency (127). Interestingly, this work predicted the existence of a hydrodynamic efficiency optimum that was not densely populated in the original analysis of shell morphologies from the fossil record (126, 127). Analysis of coiling morphologies in additional species revealed that a large number of morphologies did indeed cluster around the optimum (128). This work demonstrates that consideration of the role of physical constraints in the context of evolutionary processes can generate fruitful predictions.

Another prominent example comes from the investigation of the evolution of plant morphologies. A powerful simulation framework using a rule-based model of plant growth grounded in physical and physiological constraints was developed to capture land plant evolution (129–132). The model takes into account physical constraints such as those imposed by mechanical stress on branches and photosynthetic efficiency and implements morphogenesis based in simple rules for branch growth. Simulations generated diverse plant morphologies, and impressively, captured much of the evolutionary trajectory of land plant evolution (131, 133). Furthermore, the work generated specific predictions about how the intensity and diversity of
selective pressures (the roughness of the fitness landscape) along with developmental constraints affect the evolution of morphological diversity and complexity (131). Hence, this work also provided insight into how physical constraints affect adaptive trajectories through a morphospace (131, 133).

1.3.2 Explicit consideration of cellular behavior

While the previous two examples do not explicitly treat the behavior of cells underlying morphogenesis, evolution at the intersection of cellular behavior and physical constraints with a more explicit treatment of cellular behavior has also been considered. The theory of Turing patterns with explicit treatment of cellular behavior, such as migration and haptotaxis, and mechanics (67, 134–137), has recently begun to be tested and built upon in the context of vertebrate limb (138–142) and tooth evolution and development (74, 143). In the case of limb development, a variety of different molecular mechanisms have been proposed to underlie digit formation and subsequent changes in patterning during evolution. However, all are grounded in self-organization via Turing pattern formation, which ultimately may serve as a unifying framework (138). While details in terms of biological and physical mechanisms certainly remain to be filled in, the genetic and molecular changes underlying limb evolution are inextricable from the resultant cellular behavior and physical constraints of the Turing mechanism.

In the case of the evolution of tooth morphogenesis, building on a substantial body of work on genetic and molecular mechanisms, a framework for modeling tooth morphogenesis that incorporates the proliferation of layers of mesenchymal and epithelial cells along with genetic and molecular mechanisms regulating growth was developed (73, 74, 144). In the model, all of these processes occur in the context of physical constraints including the diffusion of signals and geometrical constraints imposed by tissue geometry (73, 74, 144). The model was able to accurately capture patterns in the evolution of tooth cusp size and position by tuning parameters corresponding to underlying changes in a relatively small number of parameters corresponding to aspects of genetic regulation and cellular responses (73, 74, 143, 144). These results show how even in the case of a very complex biological process, the underlying parameters controlling morphogenesis and the evolution of different morphologies may not be so numerous (143).

Although they do not deal with protists, these examples highlight that sharpening focus on essential molecular or genetic components is one of the key advantages of appealing to physical mechanisms. Investigation of physical mechanisms also naturally fits into an evolutionary context because biophysical models can generate predictions about the relationships between changes in cellular properties or behaviors and resultant phenotypes.

1.3.2 Optimality and performance

Approaches combining perspectives from biology and physics to understand protist evolution at the cellular level are much less developed compared to the processes described so far in this section. This work has also tended to focus primarily on adaptation and optimal performance. Work on the evolution of volvocine algae in the context of physical constraints has perhaps received the most attention (81). Experiments and theory together have suggested important physical constraints on colony size scaling. First, experiments showed that external fluid flow is important for *Volvox* colony metabolism. Specifically, germ cells of deflagellated colonies were shown to not grow well, but growth could be rescued by increasing external flow by stirring
Experiments in conjunction with theory then demonstrated that *Volvox* colonies were able to maintain nutrient flux through phenotypic plasticity (increasing cell spacing and flagellar length) even in the face of reduced nutrient growth media (146). The unicellular relative *Chlamydomonas* in contrast did not show compensatory phenotypic plasticity in the same conditions (146). Theory suggested that for large spherical colonies, transport of nutrients by flagellar driven flow should play an important role (81, 147). This is because nutrient uptake will scale proportionally with radius while metabolic needs of the colony will scale as the square of the radius (81, 147). Therefore, there will come a point at which the metabolic needs outpace the increase in diffusive transport of nutrients with increasing colony size. Increasing swimming speed, however can circumvent this constraint by increasing nutrient transport (81, 147). Together, these results and arguments suggest that, independently of other selective pressures and constraints, large colonies should optimize flow around colonies through swimming in order to optimize growth rates. Interestingly, the predicted point of decreasing diffusive uptake with colony size sits near the middle of the size *Volvox* colonies (81, 147).

Outside of *Volvox*, work has also considered optimality of protist morphology for swimming and feeding. Examples include combined theoretical and experimental investigation of the choanoflagellate *Salpingoeca rosetta* (148, 149) and *Chlamydomonas* (150). Studies of *Chlamydomonas* have found that flagellar lengths on average sit near the optimal length for swimming speed and that the modes of swimming employed by *Chlamydomonas* are themselves close to optimal for the size and shape of the organism (150–152). Work on choanoflagellates has argued that colony morphology in terms of relative cell positions and orientations may be optimized for cooperative feeding (148). However, this result is contentious, with theoretical work arguing that different environmental tradeoffs exist for different life history stages (149). Sophisticated computational fluid mechanics modeling was also used to test hypotheses about the function of lorica, silica cages produced by some choanoflagellates (153). Simulations, calibrated by data, showed that the lorica does not enhance feeding by increasing drag and preventing feeding current recirculation as previously hypothesized (153, 154). Instead, the authors suggest that by stabilizing cellular motion, the lorica may increase prey capture efficiency (153), although this hypothesis awaits experimental testing.

It is worth noting that due to the ubiquity of tradeoffs in biological function in conjunction with the fact that evolution is a satisficing process (biological systems need only work well enough to persist in any given situation), it is not clear that biological systems will often function anywhere close to optimally in terms of physical constraints. However, physical limits on performance certainly present universal bounds on cell behavior and a possible class of null hypotheses for essential cellular functions.

### 1.3.3 Conserved and convergent mechanisms: “rules” in evolution

In addition to explaining limitations on evolutionary outcomes, the interplay between active processes and physical constraints can also bring deeper or more general understanding of mechanism, both in terms of conserved and convergent processes. In particular, the generality of the physical context of many evolutionary processes can lead to the emergence of “rules” in evolutionary dynamics. Experimental evolution constitutes a powerful method by which to understand these rules.

In the multicellular context of biofilm formation, the wrinkly spreader phenotype in *Pseudomonas fluorescens* provides a fascinating illustrative example of phenotypic convergence
in the face of physical constraints. Under conditions of oxygen deprivation, the adaptive mutant “wrinkly spreader” phenotype reproducibly evolves and is characterized by a wrinkled surface that increases surface area and hence increases oxygen absorption area at the liquid-air or agarose-air interface where the biofilm grows (155–157). Although the multicellular phenotype is identical and the regulated overproduction of cellulose (a component of the extracellular matrix) is a shared phenotype at the cellular level (156, 158), the underlying evolutionary trajectories and pathways regulating morphogenesis of the phenotype are not conserved (156). Convergence or parallel evolution is widespread in biology, but this example in particular powerfully illustrates the potential for flexibility at the level of genes and gene regulation (albeit in this case constrained to three pathways\(^3\) (156)) with significant constraints at the physical and cellular, phenotypic level.

In the context of the evolution of multicellularity, recent work has argued that constraints imposed by cell packing may be unavoidable in the evolution of simple multicellularity (159). In the experimental evolution of snowflake yeast under selection for larger colony size, cells reproducibly evolve to become skinnier (changed their aspect ratio) in order to reduce accumulated stress that would otherwise fragment colonies at larger colony sizes (159). The authors argue that accumulated stress may in fact be a generic feature of developmental, multicellular systems (159). In this case, accumulated residual stress acts as both a constraint (by limiting overall colony size) and a mechanism of multicellular reproduction by causing fragmentation of colonies. Another study of experimental evolution of snowflake yeast in conjunction with mathematical modeling argued that accounting for cell packing geometry was key to understanding the counterintuitive evolution of increasing rates of apoptosis (160). In both studies, geometrical constraints proved to play a key role in explaining evolutionary processes.

These examples, though not all dealing with protists, illustrate how understanding of physical constraints can lead to the emergence of “rules” in evolution. Furthermore, in several cases, physical constraints played a key role in clarifying the functional and adaptive significance of cellular modification.

1.3.3 Conclusion

Together, the examples considered here illustrate the power in combining perspectives from physics and biology to understand principles of function and evolution in biological systems. Additionally, these examples serve to illustrate the importance of close collaboration between theory and experiment. As we have seen, physical constraints can act not only as limitations but can also facilitate robust mechanisms and can also elucidate convergence, shared mechanisms, and stasis in evolution. Notably, compared to work in animal or bacterial systems, we see a relative paucity of work focusing specifically on evolutionary cell biology in protists from a biophysical perspective. Filling this gap will be essential for developing our understanding of evolutionary cell biology and for elucidating general principles of cell biology.

1.4 Outlook

1.4.1 General approaches to combining cellular biophysics and evolutionary cell biology

\(^3\) It should be noted that these studies have also revealed genetic constraints play an important role in the evolution of pathways regulating wrinkly spreader morphogenesis, and the existence of genetic constraints presents another evolutionary dimension to consider. But such considerations ultimately lie beyond the bounds of this work.
Understanding the evolution of cellular behavior and its functional consequences is a key challenge in evolutionary cell biology. The examples presented here demonstrate both the power and potential for investigating biological function in terms of the regulated interplay between cellular behavior and physical constraints to drive mechanistic insight. How might such a perspective be productively applied moving forward?

Of course, understanding at the genomic, genetic, and molecular level is necessary to mechanistically characterize evolutionary processes, as evolution is in an important sense fundamentally about changes in relative gene frequencies. Comparative genomics approaches grounded in mechanistic cell biology have also shown exciting promise in advancing our understanding of evolutionary cell biology. For example, these approaches have been used to examine the evolution of amoeboid motility and predict its existence in organisms where it had previously never been observed (16, 35). We have presented a complementary perspective here of how insight can also be driven by investigation of cellular function at the interface of cell biology and physics, in particular by focusing on the interplay between cellular behavior and physical constraints. Furthermore, advancing our understanding of how eukaryotic phenotypes arise from genomes will require a much more comprehensive understanding of this interplay. We have argued here how this complementary perspective can drive mechanistic insight into particular biological processes along with insight into what might be more general as well.

Combining perspectives from physics with experimental evolution represents another opportunity for deriving new insights in evolutionary cell biology. Investigations of bacterial evolution have tended to dominate work in experimental evolution for obvious reasons including rapid growth rates and high cell densities in culture, relative ease of sequencing and genome assembly, extensive understanding of genetic pathways, and relative ease of molecular manipulation. However, eukaryotic systems including protists stand to provide insight as well. Yeast and algae have already provided insight into the evolution of multicellularity and demonstrate the potential of such systems. However, the broad spectrum of cell biology represented by microbial eukaryotes along with the development of new model systems points to the potential for a great expansion of research in this direction. Experimental evolution using protists would greatly benefit from high quality genomes and defined culture conditions in addition to high throughput methods for quantifying diverse phenotypes.

In general, protists present a rich, comparatively unexplored region of cell biology ripe for investigation by quantitative methods. We are only beginning to understand the principles of cell biology beyond the most intensively studied systems. In addition to seeking out new biological insight, the historical cell biology literature represents a rich resource for interesting observations and questions that can now be effectively addressed with modern methods. Advances in microscopy and computation along with molecular techniques stand to drive deep mechanistic insight into fundamental cellular and evolutionary processes across the stunning diversity of eukaryotic cells (Fig. 1.1, 2).

1.4.2 Physical constraints, cellular behavior, and regulation of form in choanoflagellates

The choanoflagellates, the closest living relatives of animals (161, 162), constitute a simple system in which to study the evolution of multicellularity. Owing to their unique phylogenetic position, insights into the biology of choanoflagellates, including their multicellular lifestyles, have begun to shed light on the origin of animals (18, 22, 163–166). Choanoflagellates are as diverse as animals (22), and many species form multicellular colonies of various shapes and sizes
(154). Understanding the regulation of form in diverse multicellular choanoflagellates stands to elucidate how the earliest animals may have taken shape through developmental processes. Furthermore, the relative simplicity of choanoflagellate multicellularity makes it amenable to quantitative approaches that are necessary for analyses grounded in physics. An understanding of physical constraints relevant to the regulation of choanoflagellate form stands to lend insight beyond the origin of animals to principles underlying multicellular morphogenesis, which has independently evolved many times (18), more generally.

The subsequent chapters herein investigate the regulation of form in multicellular choanoflagellates using theory and experiments from field-based natural history to quantitative microscopy and chemical perturbations. This work provides new insight into the origin and evolution of morphogenetic processes in terms of the interplay between active cellular behaviors and physical constraints. In particular, I show how the regulated basal secretion of extracellular matrix drives a robust, stereotyped morphogenetic progression in the model choanoflagellate *Salpingoeca rosetta* and further demonstrate that this mechanism is sufficient to generate a diverse range of multicellular morphologies, many of which are found among diverse species of choanoflagellates. With collaborators, I also show how light-regulated apical contractility mediates a rapid and reversible shape change in the previously undescribed choanoflagellate *Choanoeca flexa*. This work demonstrates how collective apical contractility may have convergently evolved in both choanoflagellates and animals to drive multicellular shape change and provides strong evidence that apical contractility was present in the last common ancestor between choanoflagellates and animals. Taken together, these studies show how simple cellular behaviors in conjunction with physical constraints provide robust yet tunable mechanisms underlying the evolution of the regulation of multicellular form.
1.5 Figures

Figure 1.1 Protists display a great diversity of cellular form and function. Various protists from historical literature (drawings) and from environmental samples illustrate this diversity across various phylogenetic distances. (A) *Isthmia salina* diatom collected from a rock pool in Half Moon Bay, CA. (B) *Anthophysa vegetans*, a chrysophyte often found in rivers (167). (C) *Fabrea salina* ciliate collected from a splash pool in Curaçao. (D) *Ornithocercus magnificus*
dinoflagellate noted for its particularly complex morphology (167). (E) *Rhipidodendron splendidum*, a spongomonad found in freshwater (167). (F) *Euplotes sp.* ciliate labeled using the vital dye SiR-tubulin and imaged by confocal microscopy. (G) *Oxyrrhis marina*, a globally distributed dinoflagellate (168). (H) Unidentified amoeba collected in a tide pool near Half Moon Bay, CA. (I) *Euplotes harpa* ciliate (168). (J) *Trichomonas intestinalis*, a parabasalid intestinal parasite (167). (K) Termite gut endosymbionts including *Trichonympha sp.* (large central cell) collected from the hindgut of termites from the forests near UC Berkeley. (L) Unidentified ciliate collected from a high salinity splash pool in Curaçao. (M) Test of an unidentified foraminiferan collected from a saltwater aquarium filter. (N) Unidentified radiolarian collected from near shore waters in Curaçao. (O) *Rhizosphaera leptomita* radiolarian (169) (P) Unidentified foraminiferan crawling with extended pseudopods collected from a tide pool near Tomales Bay in California. (Q) Rosette colonies of an undescribed *Salpingoea sp.* choanoflagellate collected from Mono Lake in California.
Figure 1.2 Protists constitute the majority of phylogenetic diversity among Eukaryotes. This graphic represents our current understanding of eukaryotic diversity and evolutionary relationships synthesized from morphological, phylogenetic, and phylogenomic analyses. The colors denote the eukaryotic “supergroups”. All supergroups contain many protist lineages. Images display a few representative organisms from each supergroup. The placement of lineages outside of the supergroups is contentious. Clockwise from right to left, the images are as follows: archaeplastids- rhodophyte, chlorophyte, streptophyte; amoebozoa- tubulinid, arcellinid, myctezoan; opisthokonts- fungus, microsporidian, choanoflagellate, cnidarian, bilaterian; excavates- parabasalian, oxymonad, euglenid; rhizaria- acantharian, foraminiferan, chlorarachniophyte; alveolates- ciliate, dinoflagellate; stramenopiles- labyrinthulid, synurophyte, diatom, phaeophyte, actinophryid; unassigned- cryptomonad, katablepharid, haptophyte. Reprinted with permission from AAAS (170).
Figure 1.3 Biophysical approaches to understanding form and behavior in protist systems have led to mechanistic insight. Studies in these systems are particularly well-developed and have driven fundamental insights into the regulation of form and function in cellular systems. (A-C) Embryo inversion in the green alga *Volvox* serves as a model for morphogenesis based on the bending of cellular sheets. (A) *Volvox* forms large, spherical colonies composed of biflagellate cells connected by cytoplasmic bridges and embedded in an extracellular matrix. Colonies have differentiated germ and somatic cells, and Embryos form and develop inside the colonies. Embryos are depicted here as circles within the colony. (B) Developing embryos turn themselves inside out during development in a process called inversion. Inversion is driven by coordinated cell shape changes from spindle to flask to columnar cells. Cytoplasmic bridges (br) are also displaced and
bridge tension ultimately leads to the bending of the sheet of cells. (C) Overview of shape change during embryo inversion. Different species of Volvox undergo different types of inversion processes. The one depicted here is the most extensively studied and known as “B” type. Gray shading represents the surface of the embryo with flagella pointing outward. Illustrations based in part on figures from (171). (D) Dictyostelium morphogenesis under starvation conditions is controlled by reaction-diffusion pattern formation stemming from cAMP activity. Arrows indicate the progression of the starvation response from aggregation of amoeboid cells (i) leading to mound formation (ii) to slug (iii) and/or stalk formation (iv). In each stage, waves of cAMP activity, indicated in gray, orchestrate cellular behaviors including directed migration, ECM secretion, adhesion, and differentiation in order to robustly and reproducibly form the multicellular structure. Based in part on figures and information from (54, 172, 173). (E) The green alga Chlamydomonas serves as a model for cellular motility and navigation in the context of fluid mechanics. The top image depicts the wave forms of flagellar beating the biflagellate Chlamydomonas cell. Bottom images indicate synchronous and asynchronous flagellar dynamics, which play a role in phototaxis. Synchronous flagellar activity leads to helical trajectories while asynchronous activities causes abrupt changes in swimming direction. Tuning the sequences of roughly linear “runs” and direction changes, “tumbles”, can give rise to robust searching and directional swimming along gradients too shallow to be sensed across the cell body. Flagellar waveforms based in part on (174). (F) The slime mold Physarum polycephalum searching for food and suitable local environments in its natural habitat. The large cell, up to centimeters in size, is organized as a series of contractile tubes with dynamic structure through which cytoplasm is pumped. Due to its ability to select optimal paths connecting resources in complex environments, Physarum has long served as a model for cellular decision-making. Image by Frankenstoen obtained on Wikimedia Commons under a created commons license.
Chapter 2: Biophysical principles of choanoflagellate self-organization

Abstract

Inspired by the patterns of multicellularity in choanoflagellates, the closest living relatives of animals, I quantify the biophysical processes underlying the morphogenesis of rosette colonies in the choanoflagellate *Salpingoeca rosetta*. I find that rosettes reproducibly transition from an early stage of 2D growth to a later stage of 3D growth, despite the underlying stochasticity of the cell lineages. We postulate that the extracellular matrix (ECM) exerts a physical constraint on the packing of proliferating cells, thereby sculpting rosette morphogenesis. Perturbative experiments coupled with biophysical simulations demonstrate the fundamental importance of a basally-secreted ECM for rosette morphogenesis. In addition, this yields a morphospace for the shapes of these multicellular colonies, consistent with observations of a range of choanoflagellates. Overall, this biophysical perspective on rosette development complements previous genetic perspectives and thus helps illuminate the interplay between cell biology and physics in regulating morphogenesis.
2.1 Introduction

Nearly all animals start life as a single cell (the zygote) that, through cell division, cell differentiation, and morphogenesis, gives rise to a complex multicellular adult form (175, 176). These processes in animals require regulated interplay between active cellular processes and physical constraints (177–183). A particularly interesting system in which to study this interplay is the choanoflagellates, the closest relatives of animals (19, 161, 162). Choanoflagellates are aquatic microbial eukaryotes whose cells bear a diagnostic “collar complex” composed of an apical flagellum surrounded by an actin-filled collar of microvilli (18, 154) (Fig. 2.1). The life histories of many choanoflagellates involve transient differentiation into diverse cell types and morphologies (184, 185). For example, in the model choanoflagellate *Salpingoeca rosetta*, solitary cells develop into multicellular colonies through serial rounds of cell division (186), akin to the process by which animal embryos develop from a zygote (Fig. 2.1A). Therefore, choanoflagellate colony morphogenesis presents a simple, phylogenetically-relevant system for investigating multicellular morphogenesis from both a biological and a physical perspective (18).

*S. rosetta* forms planktonic rosette-shaped colonies (“rosettes”), in which the cells are tightly packed into a rough sphere that resembles a morula-stage animal embryo (186). Because the cell division furrow forms along the apical-basal axis, thereby dissecting the collar, all of the cells in rosettes are oriented with their flagella and collars facing out into the environment and their basal poles facing into the rosette interior (Fig. 2.1B). Interestingly, all three genes known to be required for rosette development are regulators of the extracellular matrix (ECM): a C-type lectin called *rosetteless* (187) and two predicted glycosyltransferases called *jumble* and *couscous* (188). Nonetheless, little is known about either the mechanistic role of the ECM or the extent to which rosette morphogenesis is shaped by physical constraints.

A critical barrier to understanding the biological and physical mechanisms underlying rosette morphogenesis has been the absence of a detailed characterization of the morphogenetic process. For example, it is not known whether rosettes form through the development of invariant cell lineages akin to those seen in *C. elegans* (189) or through stochastic cell divisions, as occurs, for example, in sponges and mice (190, 191). Moreover, it is not known whether there are identifiable developmental stages in rosette development. To quantify the principles of rosette morphogenesis, we used a combination of quantitative descriptions of rosette development, experimental perturbations, and biophysical simulations that together reveal the importance of the regulated secretion of basal ECM in physically constraining proliferating cells and thereby sculpting choanoflagellate multicellularity.
2.2 Results

2.2.1 Rosette morphogenesis displays a stereotyped transition from 2D to 3D growth

To constrain our search for mechanistic principles, we first quantified the range of sizes and spectrum of morphologies of S. rosetta rosettes by measuring the population-wide distribution of rosette size in terms of cell number. S. rosetta cultured solely in the presence of the rosette-inducing bacterium A. machipongonensis (192), lead to a population with a stationary cell number distribution. While some rosettes contained as many as 25 cells, the most common rosette size was 8 cells/rosette, with 51% of rosettes containing between 6-8 cells (Fig. 2.2A). While rosettes grow through cell division, their ultimate size is determined by either colony fission (as previously reported; 16) or cell extrusion (Fig S1). In each case, the rosettes contained 8 or more cells, suggesting that these rosette size decreasing phenomena are more common in larger rosettes.

We next quantified defining features of the 3D morphology of rosettes containing between four (following (193), we defined four cells as the smallest cell number clearly identifiable as a rosette) to twelve cells (representing 90% of rosettes at steady state, Methods; Fig. 2.2B, C). This analysis revealed that rosettes increased in volume and diameter as cell number increased (Fig. 2.2D). Although the average cell volume reduced between the four-cell and five-cell stages of rosette development, average cell volume did not change substantially with increasing cell number after the five-cell stage (Fig. 2.S2), suggesting that cells in rosettes grow between cell divisions. This contrasts with cleavage in the earliest stages of animal embryogenesis, in which cell volume steadily decreases as cell divisions proceed with no cell or overall tissue growth (176).

Our analyses revealed that rosette morphogenesis displays two distinct, but previously undescribed phases: (1) a 2D phase of growth from four to seven cells, during which the overall shape of rosettes changed substantially with increasing cell number and (2) a 3D phase from eight to twelve cells, during which rosettes expanded nearly isotropically (Fig. 2.2C-E). Interestingly, the most common rosette size (8 cells) corresponded to the transition between the two phases of growth.

Transitions from 2D to 3D growth can be driven by the constrained growth of cell layers leading to increasing mechanical stresses (48, 194–197). We hypothesized that the physical packing of cells in rosettes might constrain cell growth and proliferation and help explain the growth transition during rosette morphogenesis. Indeed, cell packing initially increased (as indicated by an increase in the number of nearest neighbor cells, Fig. 2F, and suggested by the reduced average sphericity of cells, Fig. 2.S2). Following the growth transition at the 8-cell stage, cell packing continued to increase with increasing cells/rosette, although the rate of increase slowed as a function of the number of cells/rosette (Fig. 2.2F). Therefore, the transition to isotropic 3D growth in eight-cell rosettes may occur in response to the accumulation of stress caused by the increase in cell packing in growing rosettes.

2.2.3 Rosette developmental dynamics are stochastic

The influence of cell packing on rosette morphogenesis did not preclude the possibility that the rosette developmental program might also involve specific patterns of cell division that result in well-defined cell lineages. We therefore documented cell lineages in live, developing rosettes (Fig. 2.3). Consistent with the single previous published observation of live rosette development (186), the cells maintained polarity throughout development, with their division planes oriented
along the apical-basal axis. Relative to the cell division times in linear chains (Fig. 2.1A), which form when rosette inducing bacteria are absent, we observed a slight but statistically significant increase in division rate in rosettes (p=0.03 by Wilcoxon rank sum test Fig. 2.S3). In addition, we found that both the order and timing of cell divisions differed among different rosettes (Fig. 2.3B, C), ruling out the possibility that cell lineages are invariant. This process of apparently unpatterned cell divisions resembles the dynamics of early embryogenesis in diverse animals, including sponges and mice (190, 191).

Although division patterns were variable between rosettes, ruling out the possibility of invariant cell lineages, in no rosette did cells from the first, second, or third cell division give rise to more than 60% of cells (Fig. 2.3D). Moreover, cell division remained balanced throughout rosette morphogenesis, with no cell lineage coming to dominate. Importantly, the cell lineages of chains showed the same kind of stochasticity and variability as rosettes (Fig. 2.3D). These observations suggest that rosette morphogenesis does not require the strongest forms of cell cycle control or coordination (i.e. the synchronous divisions or deterministic division timing or order observed in the development of some animals such as C. elegans, Xenopus, Drosophila, and zebrafish (198–201) and in the green alga Volvox (39, 202, 203)).

2.2.4 ECM constrains proliferating cells in rosettes

To reconcile the stereotyped 3D growth transition (Fig. 2.2) with the stochastic developmental dynamics of rosette formation (Fig. 2.3), we set out to test the “ECM constraint hypothesis (Fig. 2.4A, B).” This hypothesis was motivated by the idea that physical constraints imposed by the geometry and mechanics of cell packing play a key role in morphogenesis and that the source of the physical constraint in growing rosettes is the ECM, which is known to be required for rosette morphogenesis and connects all cells in a rosette, filling the rosette center (165, 185, 187, 188). The phenomenon of physically constrained morphogenesis suggests that the amount of ECM secreted during rosette development is an important factor in sculpting rosette morphogenesis (Fig. 2.4A, B). We visualized and quantified the volume of the ECM by staining with fluorescein-conjugated Jacalin, a galactose-binding lectin (188, 204). Importantly, Jacalin does not stain chains, so its target is likely specific to rosette ECM (188). We found that the relative amount of space occupied by basal ECM (ECM volume/total cell volume, denoted by \( f \)) in developing rosettes was constant and maintained at roughly 6% (Fig. 2.4C). Therefore, we infer that cells in rosettes produce ECM at a constant rate relative to the growth of cells, either through synthesis and secretion alone or through a balance of regulated synthesis, secretion, and degradation.

A key prediction of the ECM constraint hypothesis (Fig. 2.4A, B) is that compressive stress on cells, balanced by stress in the ECM, should increase with cell number. Alternatively, cell-cell connections mediated by lateral cell-cell adhesion or cytoplasmic bridges formed during incomplete cytokinesis (165, 185) (Fig. 2.1B) might be primarily responsible for the structural integrity of rosettes. If cell-cell connections dominate over ECM in holding together rosettes, we would expect cells to be under tension such that measured stresses would be in the opposite direction to those predicted by the ECM constraint hypothesis.

To probe the balance of forces in developing rosettes, we performed laser ablation experiments, which provided a readout of the relative magnitude and direction of stresses within rosettes (205–208). Upon ablation of a single cell in a rosette, we found that the remaining cells immediately became more rounded and moved closer together, reducing the size of the gap left by
the ablated cell (Fig. 2.4E. This result demonstrated that residual elastic stress (as measured by initial recoil velocity after ablation, (205)) is maintained in rosettes, with cells under compressive stress balanced by an additional component of residual stress. If rosettes were primarily held together by strong cell-cell adhesion or constrained by cytoplasmic bridges (Fig. 2.1B), the expected recoil would have been in the opposite direction, causing a larger gap to open in rosettes, due to cells increasing contact area with remaining neighbors in the former case and tension in bridges in the latter. Moreover, as the number of cells in rosettes increased, the measured residual stress increased (Fig. 2.4F), consistent with the ECM constraint hypothesis (Fig. 2.4A, B). These results ruled out strong cell-cell adhesion or constraint by cytoplasmic bridges as the dominant physical mechanisms underlying rosette integrity and morphogenesis.

Additionally, residual stress (as measured by initial recoil velocity (181, 205, 209)) displayed a sharp increase, by nearly a factor of two, at the 8-cell stage (Fig. 2.4F), coinciding with the 3D growth transition (Fig. 2.2). In conjunction with the observed increase in cell packing (Fig. 2.2F), this result suggested that the packing of cells is mechanically constrained in developing rosettes such that cells are increasingly compressed against one another with increasing cell number. We reasoned that the shared ECM secreted from the basal end of cells, adhesion to which is likely essential for rosette formation (187, 188), might be the source of this constraint. While we have ruled out bridges as a dominant component of the structural integrity of rosettes, they could play a role in stabilizing cell orientation to hinder out of plane growth during the 2D phase of rosette morphogenesis.

2.2.5 Material properties of ECM affect morphogenesis

We next sought to test the ECM constraint hypothesis through perturbative experiments. While the hypothesis entails that changing geometrical properties such as cell shape and relative amount of ECM should have a substantial effect on rosette morphogenesis, these properties could not be experimentally tuned. However, we could perturb the mechanical properties of the ECM. To do so, we treated developing rosettes with strontium chloride (SrCl$_2$). Strontium is a divalent cation that can stiffen hydrogels, including animal ECM, by increasing crosslinking density (210–214). Importantly, we found that SrCl$_2$ has no detectable effect on cell growth at up to twice the highest concentration used during this set of experiments (Fig. S4). Under our ECM constraint hypothesis, we predicted that increased ECM stiffness would alter morphogenesis by further constraining cell packing, thus holding cells in a more compact arrangement along with a relative increase in residual stress. Consistent with our hypothesis, we found that rosettes became more compact with increasing SrCl$_2$ concentration (Fig. 2.5A, B), and the 3D transition shifted to lower cell numbers, occurring at the five-cell stage for the highest SrCl$_2$ concentration (Fig. 2.5C). Additionally, the transition to isotropic growth at the 8-cell stage was abolished (Fig. 2.5B). Together, these analyses reveal that morphogenesis is altered.

Using laser ablation experiments, we found that relative residual stress as determined by maximum initial recoil velocity (as in Fig. 2.4E, F) was significantly increased for SrCl$_2$-treated 4-7 cell rosettes relative to untreated rosettes (Fig. 2.5D). The increase in residual stress in conjunction with the 2D to 3D growth transition at lower cell numbers, supported the hypothesis that ECM-constrained proliferation is a key driver of the 3D transition in rosette morphogenesis. Interestingly, for the 8-cell-stage and higher, we did not find a significant increase in residual stress in SrCl$_2$-treated rosettes compared to untreated rosettes. This is consistent with cells exerting maximum growth pressure on their neighbors at the 8-cell stage and above. Taken together, these
results confirm important model predictions by demonstrating that material properties of the ECM can affect morphogenesis, which highlights the central role of the ECM in sculpting rosette morphology.

2.2.6 Amount of ECM, cell shape, and ECM stiffness as control parameters for morphogenesis

To formalize and test our hypothesis of morphogenesis shaped by ECM constraint (Fig. 4A, B), we next developed a cell-based computational model to simulate rosette development. Because development involves few cells (ruling out continuum modeling) in a low Reynolds number environment where inertial forces play a negligible role (80, 215) we developed particle-based simulations akin to Brownian dynamics, but neglected the role of thermal fluctuations given the large size of the cells and aggregates (51). In the model, the ECM and cells were represented by a system of interacting spherical particles (Fig. 6A). This particle representation also allowed us to capture the discrete and stochastic nature of cell division and the stochastic nature ECM secretion as well the polarity of cell division and ECM secretion. Each cell in the model was composed of three linked spheres to capture cell shape and for computational tractability, with a small sphere representing the basal pole of the cell, a larger sphere representing the cell body, and the largest representing the collar exclusion region. Cells interacted stERICally with one another. The ECM was modelled as a system of small spheres with attractive interactions in order to capture the complex shapes the ECM can take on (Fig 2.4C) as well as its deformability. ECM particles similarly shared attractive interactions with the basal poles of cells. Cells in the model were allowed to divide stochastically, with the division plane orientation around the apico-basal axis determined by the previous division (consistent with observations of rosette development from Fig. 2.3 and (186)), and ECM particles were secreted stochastically at a constant rate from the basal pole of non-dividing cells (see Methods for a more detailed description of the model and simulations).

In this simplified model of rosette morphogenesis, three main parameters characterized the system: cell aspect ratio (length along apical/basal axis vs. equatorial diameter), \( \alpha \), amount of ECM relative to total cell volume, \( \phi \), and relative stiffness of the ECM (in terms of the strength of ECM-ECM adhesion bonds relative to the force exerted by growing and dividing cells), \( \sigma \). Simulations with parameter values constrained by cell and ECM morphology data collected as part of this study showed that this simple model was sufficient to recapitulate rosette morphogenesis, including the expected 3D transition at the 8-cell stage (Fig. 2.S5). Furthermore, simulations showed that rosette morphogenesis was robust to a range of scaled ECM stiffness values (Fig. 2.6B-E, S5) and to the stochasticity of cell divisions (Fig. 2.S5). We did find that simulations fail to recapitulate all aspects of rosette morphogenesis, most saliently, the growth scaling (Fig. 2D) and the absolute magnitudes of flatness and sphericity (Fig. 2.2E, S5). We expect, however, that a more detailed treatment of the mechanics of cells and ECM may capture these aspects of rosette morphogenesis more accurately, but such a detailed model is beyond the scope of the present study.

Exploration of the effects of different parameter values revealed that the model captures a range of different colonial morphologies (Fig. 2.6B, F). This space of forms and associated model parameters constitutes a theoretical morphospace (126) of ECM-based colonial choanoflagellate morphologies given this simplified model of morphogenesis. Interestingly, some of the simulated forms resembled colonies, such as tree-like structures (found in Codosiga cymosa (216) and an uncharacterized Salpingoeca sp., Fig. 2.6Gt, t1) or cups (found in Codosiga umbellata (217) and another uncharacterized Salpingoeca sp., Fig. 2.6Gc, c5), that have been previously reported in...
other choanoflagellate species (Fig. 2.6F, G). We found that colony morphogenesis is particularly sensitive to $\alpha$ and $\phi$, changes in each of which can lead to dramatic changes in predicted multicellular forms. For example, holding the other two parameters fixed, increase in $\phi$ alone would be predicted to drive a change from rosettes to disks or cups and from cones to trees (Fig. 2.6C, D). Colony morphogenesis was also affected by changes in $\sigma$, but the effects tended to be subtler, such as changes in cell packing over a relatively wide range of values (Fig. 2.S5). In contrast with changes in $\phi$, increase in $\sigma$ alone was either not predicted to lead to any transitions in predicted colony morphology type or, at most, lead to single transitions such as from rosettes to disks (Fig. 2.6D, E). These results demonstrate that basal secretion of a shared ECM constitutes a robust yet flexible mechanism for regulating multicellular morphogenesis. Furthermore, these results made specific predictions about different colony morphologies corresponding to specific cell morphologies and relative ECM volumes and stiffnesses.
2.3 Discussion

Our quantitative analyses, experimental perturbations, and simulations allowed us to understand the process by which single cells of *S. rosetta* gives rise to multicellular rosettes. We found that the earliest stages of rosette morphogenesis proceed through 2D anisotropic growth, which is stereotypically followed by a transition to 3D isotropic growth. In particular, we found that the basal ECM secreted by cells during rosette development physically constrains proliferating cells, and thereby drives a stereotyped morphogenetic progression in the absence of strict cell lineage specification and division timing. Simulations showed that this simple mechanism, the regulated basal secretion of ECM, is sufficient to not only recapitulate rosette morphogenesis but yield a morphospace that can not only explain the multicellular morphology of *S. rosetta* but also that of other species of colonial choanoflagellates. These results emphasize the importance of the choanoflagellate ECM for morphogenesis and should encourage future studies of its composition, physical properties, and regulation.

The importance of the basal ECM revealed in this study may generalize to other choanoflagellate species and colonial morphologies. Our simulations predict that differences in ECM levels (resulting from differing rates of biosynthesis, secretion, or degradation), cell shape, and in ECM stiffness relative to cells are sufficient to explain the existence of radically different colony morphologies across diverse choanoflagellates. Measurements and comparisons of ECM levels (\(\phi\)), cell shape (\(\alpha\)), and ECM stiffness (\(\sigma\)), in diverse colonial choanoflagellates will be crucial to validate the model, and deviations from the predictions of the model could point to additional regulatory mechanisms.

From a broader perspective, rosette morphogenesis shows interesting parallels to mechanisms underlying morphogenesis in diverse other taxa. In terms of physical mechanisms, the constrained proliferation of cells that occurs during rosette development generates crowding stresses like those that regulate morphogenesis by animal epithelia (218, 219), snowflake yeast (159), and bacterial biofilms (195). In epithelia, compaction of cells due to crowding has been proposed as a general signal for cellular processes underlying tissue homeostasis such as apoptosis and extrusion (220–223). Further, accumulation of stress due to crowding of cells produces a jamming-like behavior that has been proposed as a generic constraint on the development of multicellular systems with fixed cell geometry (159). Due to the generality of physical constraints on cell packing, it is plausible that such phenomena acted both as constraints and regulatory mechanisms in the development and morphogenesis of early animals and their ancestors.

Cellular mechanisms of rosette morphogenesis are also shared with other multicellular systems. Our results demonstrate that the regulation of basal ECM sculpts the multicellular morphology of rosettes. Thus, our biophysical studies have converged on results from genetic screens in *S. rosetta* that implicated animal ECM gene homologs in the regulation of rosette development, including a C-type lectin (187) and predicted glycosyltransferases (188). The basal ECM of rosettes is reminiscent of the basal lamina, a basally secreted layer of ECM that underpins animal epithelia and regulates tissue morphogenesis by constraining cell proliferation (197) including in *Drosophila* wing and egg chamber development (103, 218, 224), branching growth during lung and salivary gland development (225, 226), notochord expansion (227), lumen elongation (228), and in tumor growth in mammary epithelia (211). The ECM also sculpts morphogenesis in *Volvox*, in which defects in ECM composition disrupt morphogenesis (40, 229), and in bacterial biofilms, in which the ECM can constrain cells and thereby drive 3D
morphogenesis (195, 230). Remarkably, some bacteria form multicellular rosettes in a process that is mediated by basal ECM secretion (231, 232).

Altogether, the principles that we can glean from the simplicity of choanoflagellate morphogenesis holds the promise of revealing general principles by which biological and physical mechanisms shape morphogenesis more broadly.
2.4 Methods

2.4.1 Choanoflagellate strains and culture

Two strains of S. rosetta were used for the experiments in this study: one grown solely in the presence of the non-rosette inducing bacterium Echinicola pacifica (233), a strain called SrEpac (234) and the other grown solely in the presence of the rosette inducing bacterium Algoriphagus machipongonensis (235), a strain called PX1 (164, 192).

SrEpac was grown in 5% Sea Water Complete (SWC) media at 22°C. Sea Water Complete media consisted of 250 mg/L peptone, 150 mg/L yeast extract, 150µL/L glycerol in artificial sea water and was diluted to 5% by volume in artificial sea water to make 5% Sea Water Complete media. Artificial sea water (ASW) consisted of 32.9 g Tropic Marin sea salts (Wartenberg, Germany) dissolved in 1L distilled water for a final salinity of 32-27 parts per thousand. SrEpac was passaged either 1:10 into 9mL fresh 5% SWC once a day or 1:20 every other day into 9mL fresh 5% SWC to stimulate rapid proliferation and maintain log-phase growth. Cells were grown in 25cm² cell culture flask (Corning).

PX1 was grown in 25% Cereal Grass media (CGM3) at 22°C. Cereal Grass media consisted of Cereal Grass (Basic Science Supplies) added to ASW at 5g/L, steeped for 3.5 hours and then filtered. This media was then diluted to 5% by volume in ASW in order to make 25% CGM3. PX1 was passaged 1:5 into 9mL of fresh 25% CGM3 every two to three days to stimulate rapid proliferation and maintain log-phase growth. Cells were grown in 25cm² cell culture flask (Corning).

2.4.2 Rosette induction

Rosette development from single cells was stimulated by the addition of outer membrane vesicles (OMVs) isolated from Algoriphagus bacteria (192) to SrEpac cultures. To isolate OMVs, Algoriphagus was first grown in 200mL of SWC at 30°C for 48 hrs. on a shaker. Bacterial cells were then pelleted, and the cell free supernatant was sterile filtered, then spun at 36,000 x g for three hours at 4°C (Type 45 Ti rotor, Beckman Coulter) to pellet OMVs. Finally, OMVs were resuspended in 1.5 mL ASW. To induce rosette development, OMVs were added to SrEpac at a concentration of 1:2000 by volume. This concentration led to >90% of cells in rosettes by 48 hrs. post-induction.

2.4.3 Electron microscopy

Algoriphagus OMV induced SrEPac cultures (48 hours post-induction) were concentrated by centrifugation (1200xg for 5 min). Colonies were resuspended in 5% BSA in ASW, high pressure frozen using a Leica EM PACT2, and fixed by freeze substitution in 0.01% OsO4 + 0.2% uranyl acetate in acetone (236). Samples were resin embedded in Epon Araldite (Embed-812) (237), cut into 80 nm sections, and then imaged using an FEI Tecnai 12 transmission electron microscope.

2.4.4 Rosette cell number quantification
Rosettes from rapidly growing PX1 cultures (*S. rosetta* co-cultured with *Algoriphagus*) were concentrated to 5x by centrifugation (1500xg for 10 min) and resuspended by vigorous pipetting in fresh 25% CGM3 media. Rosettes were then gently adhered to a poly-D-lysine coated coverslip (FluoroDish, World Precision Instruments, Inc), which had been washed three times using 25% CGM3. Rosettes were observed using a Leica DMIL microscope with a 20x objective (Leica, N Plan, 0.35 NA), and cells were manually counted. In general, rosettes were not overlapping, and cells were deemed to belong to a rosette when oriented radially outward about a central focus.

### 2.4.5 Quantitative morphology analysis pipeline

For morphological analysis of rosettes, SrEpac cultures were first induced to form rosettes as described above. After 24 hours, developing rosettes were pelleted by centrifugation (1500xg for 10 min) and resuspended in fresh ASW by vigorous pipetting in order to minimize bacteria and to break apart any chains that might be mistaken for rosettes. Rosettes were then deposited onto a poly-D-lysine coated coverslip (FluoroDish, World Precision Instruments, Inc), which had been washed three times using ASW. Rosettes were stained by overloading with LysoTracker Red DND-99 (ThermoFisher Scientific) at 1:200 dilution, which reliably stains the entire cell body. Next, z-stack images of stained rosettes were acquired on a Zeiss 880 laser scanning confocal microscope using a 40x water immersion objective (Zeiss, C-Apochromat, 1.2 NA) and illumination with a 561 nm laser (Zeiss). Importantly, pure water, and not water immersion oil, was used to minimize coverslip deflection during imaging.

After image acquisition, z-stacks were registered using the Stackreg plugin in FIJI (238, 239). Aligned z-stacks were deconvolved using the Parallel Iterative Deconvolution v1.12 plugin in FIJI (238, 239). For deconvolution, the Wiener Filter Preconditioned Landweber method (WPL) with stock settings and a theoretical pointspread function for the imaging system generated using the Diffraction PSF 3D plugin in FIJI (238, 239) were used. Aligned, deconvolved z-stacks were then segmented using Imaris v3.8 (Bitplane, Belfast). First, the images were median filtered with a 3x3x1 kernel and smoothed using a Gaussian filter with a sigma of 0.24 microns. Intensity thresholds for local intensity segmentation and thresholds for size and shape filters to exclude extraneous objects such as bacteria within the analysis region were then chosen based on segmentation of a few rosettes from each sample and then kept the same for all rosettes in the sample. Individual cells in rosettes were segmented using the Split Touching Objects option. Segmentation of each rosette was manually inspected, and any improperly segmented cells were manually split and fused as necessary. Statistics of segmented rosettes, including number of cells, cell positions, orientations, sizes, and shapes were exported to MATLAB release 2016a (Mathworks, Natick) for additional morphological analysis.

Rosette volume was measured by determining the convex hull of cell positions. Maximum rosette width was measured by the maximum distance between cells in rosettes. To further evaluate rosette morphology, principle axes of rosettes were determined by principle components analysis of cell positions. Flatness ($F$) and Sphericity ($S$) of rosettes were computed from these principle components where $F = 1 - C/B$, and $S = \sqrt[3]{BC/A^2}$ where $A$, $B$, and $C$ are the principle axes in descending order of magnitude. The packing of cells was then quantified by the average number of neighboring cells over all cells in rosettes as determined by a Voronoi tessellation (240) of cell positions. Finally, rosettes were then binned by cell number for the final analysis of morphological progression.
2.4.6 Cell lineage analysis

Rapidly growing SrEpac cultures were induced to form rosettes, and 9 hours post induction, induced cells were concentrated to 5x by centrifugation and resuspension in 5% SWC (1200xg for 5 min, initial volume 15mL resuspended in 3mL) and then deposited in a 200 µL droplet on a poly-D-lysine coated coverslip (FluoroDish, World Precision Instruments, Inc). Cells were imaged in phase contrast or DIC on either a Zeiss Axio Observer Z1 with a 20x (Zeiss, Plan-Apochromat, 0.8 NA) objective or a Leica DMI6000B with a 20x (Leica, Plan-Apochromat, 0.7 NA) objective at 1 frame/minute for 16 hours. Cell positions were tracked using the Manual Tracking plugin in FIJI (238, 239), and division events were tracked and recorded manually. For analysis of chain cell lineages, the cells were not induced to form rosettes, but otherwise all previous steps were followed.

2.4.7 ECM measurements

Rosettes were prepared as in the “Quantitative morphology analysis pipeline” section (QMAP). Additionally, to label the ECM, fluorescein labeled Jacalin (Vector Labs, FL-1151) at a 1:400 dilution was added to the concentrated rosettes. Imaging also followed the QMAP with additional sequential illumination with a 488 nm laser to excite the fluorescein. Z-stack images were processed and analyzed following QMAP with the exception of post-processing in MATLAB, as cell and ECM volumes were exported directly from Imaris.

2.4.8 Laser ablation

For laser ablation, an upright Olympus BX51WI microscope (Olympus Corporation) equipped with Swept Field Confocal Technology (Bruker) and a Ti:Sapphire 2-photon Chameleon Ultra II laser (Coherent) were used. The 2-photon laser was set to 770 nm and ablation was performed using three 20 ms pulses. A 60x water dipping objective (Olympus, LUMPlanFL N, 1.0 NA) was used for imaging. Images were captured using an EM-CCD camera (Photometrics). The following emission filter was used: Quad FF-01-446/523/600/677-25 (Semrock). PrairieView Software (v. 5.3 U3, Bruker) was used to acquire images.

Rosettes were gently adhered to a coverslip using poly-D-lysine and stained with lysotracker (as described above). Individual cells in rosettes were ablated, and the subsequent recoil, which is proportional to the elastic stress (181, 205, 209), was recorded at a frame rate of 1/0.48 s. Images were registered using the StackReg plugin in FIJI (238, 239) to correct for small movements of the rosette colony due to flagellar motion during acquisition of images. Recoil velocities were measured in the frames following ablation by particle image velocimetry (PIV) using PIVlab software in MATLAB (241). Settings for PIV included four direct Fourier transform correlation passes with window sizes of 64, 32, 16 and 8 pixels and corresponding step sizes of 32, 16, 8, and 4 pixels. To reject noise and erroneous velocities, filters of 7 standard deviations about the mean and local median filters with a threshold of 5 and epsilon of 0.1 were applied. Finally, any remaining velocity measurements not corresponding to displacements of cells in rosettes were manually rejected. Recoil velocities were measured in the subsequent 3 frames following ablation by radial scans about the circumference of the rosette, and the maximum measured velocity was selected.
2.4.9 Strontium treatment

For strontium treatment of rosettes, SrEpac cultures were centrifuged at 1500g for 10 to pellet all cells and resuspended in 5% SWC media containing added SrCl₂ to a final concentration of either 0, 2.5, or 5 mM. These cells were then induced to form rosettes as described above. Morphological analysis and laser ablation were also conducted as described above.

For cell growth assays (Fig. 2.4), SrEpac cultures were prepared as described in the preceding paragraph but were not induced to form rosettes. Cells were then plated into 12-well plates (Falcon) at an initial density of 20000 cells/mL. To determine cell density, cells were counted using a hemocytometer (Hausser Scientific) viewed in phase contrast on a Leica DMIL microscope with a 20x (Leica, N Plan, 0.35 NA) objective. Cells were counted at 4, 24, and 28 hours. Growth rates were then determined by exponential fits to the log-phase of growth obtained using the Curve Fitting application in MATLAB.

2.4.10 Simulations

Cells and ECM were modelled as spherical particles (Fig. 2.6) with interactions that allowed us to tune the various morphological and material properties we wished to investigate. The particle representation allowed us to capture both the relevant geometric aspects of colony formation including polarized cell divisions and ECM secretion as well as the discrete and stochastic nature of these processes.

2.4.10.1 Cells

Each cell was composed of three linked particles with diameters \(d_1\), \(d_2\), and \(d_3\) representing the basal pole, cell body, and collar and in ascending order of magnitude, to capture cell geometry. Cell particles interacted sterically with one another via the hard-sphere Weeks-Chandler-Andersen (WCA) potential (242):

\[
V_{WCA}(R) = 4\varepsilon_{WCA} \left( \frac{d_i}{R} \right)^{12} - \left( \frac{d_i}{R} \right)^{6} + \Delta V(R) \text{ for } R < R_{cut},
\]

\[
V_{WCA}(R) = 0 \text{ for } R > R_{cut}
\]

where \(R\) is the interparticle distance; \(d_i\) with \(i = 1, 2, 3\) is the cell particle diameter; \(\varepsilon_{WCA}\) sets the force of repulsion upon overlap; \(\Delta V(R) = V_{LJ}(R_{cut})\) where \(V_{LJ}\) is the Lennard-Jones potential (242):

\[
V_{LJ}(R) = 4\varepsilon \left[ \left( \frac{d_i}{R} \right)^{12} - \left( \frac{d_i}{R} \right)^{6} \right]
\]

with \(\varepsilon = \varepsilon_{WCA}\); and \(R_{cut} = \frac{1}{2} d_i\) is the cutoff distance for the potential set to the diameter of the cell particle. For a given cell, cohesion of particles was maintained using a finitely extensible nonlinear elastic (FENE) potential:

\[
V_{FENE}(R) = \frac{1}{2} k_{FENE} R_0^2 \left( 1 - \left( \frac{R - \Delta}{R_0} \right)^2 \right) + V_{WCA}(R)
\]

where \(k_{FENE}\) sets the strength of the potential (set high enough to maintain cohesion); \(R_0 = d_1\); \(R\) is the interparticle distance; \(\Delta = \frac{d_i + d_j}{2} - 1\) where \(d_k\) is the diameter of the \(k^{th}\) particle; and
\[ V_{WCA}(R) = 4\varepsilon_{WCA} \left( \left(\frac{d_1}{R-\Delta}\right)^{12} - \left(\frac{d_1}{R-\Delta}\right)^6 \right) \text{ for } R - \Delta < \frac{1}{2} \Delta, \] and
\[ V_{WCA}(R) = 0 \text{ for } R - \Delta \geq \frac{1}{2} \Delta. \]

Additionally, a harmonic potential acting between the basal and apical cell particle was used to keep cells straight and elongated:
\[ V_h(R) = \frac{1}{2} k (R - R_E)^2 \]
where the spring constant \( k = k_h \) sets the strength of the potential, and rest length \( R_E = 2(d_1/2 + d_2 + d_3/2) \) was chosen to be large enough to ensure cell elongation. For simplicity, the mass of all cell particles was the same, and the friction coefficient was otherwise determined by viscosity, \( \eta \) and the particle diameter, \( d : \gamma = 6\pi\eta d \).

### 2.4.10.2 ECM

ECM was composed of small particles with diameter \( d_{ECM} \ll d_1 \). To maintain ECM cohesion (while preventing divergence in energy) and volume, allow for ECM deformations and shape transformation, and for computational tractability, ECM-ECM particle interactions were also modeled by a modified Lennard-Jones potential (243), which reduces interparticle repulsion and better describes a condensed state, such as ECM:
\[ V_{MLJ}(R) = 4\varepsilon \left( \left(\frac{d_{ECM}}{R}\right)^{12} - \left(\frac{d_{ECM}}{R}\right)^6 \right) \text{ for } R < R_{cut}, \]
\[ V_{MLJ}(R) = 0 \text{ for } R \geq R_{cut} \]
where \( R \) is the interparticle distance, \( \varepsilon = \varepsilon_{ECM} \) sets the strength of ECM adhesion, and \( R_{cut} = d_3 \) is the cutoff distance for the potential.

### 2.4.10.3 Cell-ECM interactions

To capture adhesive interactions while preventing particle overlap and for computational tractability, cell-ECM adhesion was modeled with a modified Lennard-Jones potential between basal cell particles and ECM particles:
\[ V_{SLJ}(R) = 4\varepsilon_{ECM} \left( \left(\frac{d_{ECM}}{R-\Delta}\right)^{12} - \left(\frac{d_{ECM}}{R-\Delta}\right)^6 \right) \text{ for } R < R_{cut} + \Delta, \]
\[ V_{SLJ}(R) = 0 \text{ for } R \geq R_{cut} + \Delta. \]

### 2.4.10.4 Cell division

Cells were allowed to divide stochastically, with probability \( p_{Div}(t) = \frac{1}{1-e^{-\mu(t-t_{Div})}} \) where \( t_{Div} \) is the cell cycle time, and \( \mu = \frac{t_{Div}}{10} \) sets the variability in division timing. The division plane orientation around the apico-basal axis was set by the previous division. During division, cell particles are replicated and shifted slightly (starting with a separation \( 0.25d_i \) for particle \( i \)) in the direction perpendicular to the division plane by an offset \( D_{Div} \). Dividing cell particles then push one another apart under the influence of a harmonic potential with increasing rest length: \( V_h(R) = \frac{1}{2} k (R - R_p)^2 \) where the spring constant \( k = k_{Div} \) sets the strength of the potential, and hence, how much force the cells can exert during growth and division; and rest length \( R_p \) increases with each
timestep in the simulation by an amount $\delta R = \frac{1}{g_{\text{DIV}}^{\frac{d_i}{2}}-1}$ where $g_{\text{DIV}}$ is the number of timesteps over which division occurs for particle $i$. Cell division is complete once $R_p \geq d_i$ for all particles. With this implementation of cell division, we approximated both cell growth (while particles are still overlapping) and division.

2.4.10.5 ECM secretion

ECM particles were secreted stochastically from non-dividing cells at a constant rate with a probability $1 - p_{\text{Div}}(t)$ from the basal pole of non-dividing cells. Secretion always occurred in the direction of the apico-basal axis of cells. Similarly to division, ECM particles were extended from the basal pole according to a harmonic potential with $k = k_{\text{ECM}}$ setting the force with which ECM particles were secreted; the rest length $R_p$ increased with every time step by $\delta R = \frac{1}{g_{\text{SEC}}^{\frac{d_{\text{ECM}}}{2}}-1}$ where $g_{\text{SEC}}$ is the number of timesteps over which secretion occurs until $R_p = \frac{d_{\text{ECM}}}{2}$.

2.4.10.6 Running simulations

Simulations were carried out using Fortran and followed Brownian dynamics (244):

$$\dot{X} = \frac{\gamma k_B T D(t) - \nabla U(X)}{\gamma},$$

where $X$ and $\dot{X}$ are position and velocity; $U(X)$ is the sum of all interaction potentials acting on a given element of the system (particle), so $-\nabla U(X)$ with $\nabla$ as the gradient operator, is the force resulting from the total interaction potential on a given element of the system (particle); $\gamma$ is the friction coefficient, $k_B$ is Boltzmann’s constant, $T$ is temperature, and $D(t)$ is a delta correlated, stationary Gaussian process with 0 mean. For simplicity, the mass of all particles was the same, and the friction coefficient was otherwise determined by viscosity, $\eta$ and the particle diameter, $d$: $\gamma = 6\pi\eta d$. A Verlet integration algorithm was used to update the positions of the spheres at each timestep in the simulation (245).

2.4.10.7 Simulation analysis

In the model, three main parameters corresponding to physical aspects of choanoflagellate cells and rosettes describe the system: 1) cell aspect ratio, defined to be the length to width ratio of the three particle system: $\frac{d_1 + d_2 + d_3}{d_3}$; 2) scaled ECM stiffness relative to the force exerted during cell growth and division: $\frac{k_{\text{ECM}}}{k_{\text{DIV}}^{d_{1}}}$; and 3) The ECM volume relative to cell volume secreted by a cell between divisions: $\frac{n d_{\text{ECM}}^{d_{2}}}{2(d_{1}^{2}+d_{2}^{2}+d_{3}^{2})}$ where $n = \frac{\tau_{\text{DIV}}}{\tau_{\text{Sec}}}$ with $\tau_{\text{Sec}} = \frac{R_p}{\delta R}$ is the average number of ECM particles secreted between divisions.

Natural units for the system are the length $d_1$, time $\tau_{\text{DIV}}$, and energy $k_B T$. For all simulations, the following values in system units were held fixed: $d_1 = 1$, $k_{\text{FENE}} = 500 \frac{k_B T}{d_1^2}$, $\eta = 50 \frac{k_B T \tau_{\text{DIV}}}{d_1^2}$, $k_n = 200 \frac{k_B T}{d_1^2}$, $g_{\text{DIV}} = 5000 \frac{d_1^3}{\tau_{\text{DIV}}}$, and $g_{\text{SEC}} = 5000 \frac{d_3^3}{\tau_{\text{DIV}}}$. All other parameters not
already fixed in the previous paragraphs were varied to explore the morphospace (Fig. 2.6B), with axes corresponding to the three main parameters detailed in the previous section. The timestep for simulations was $0.001\tau_{Div}$. Simulation snapshots for Fig. 2.6 were rendered for the visual inspection of morphologies using Python. Rosettes were defined as structures with cells completely surrounding a central region of ECM; disks were defined as structures that maintained a closed ring of cells pointing radially outward along the colony circumference with an open central region of ECM; cups and cones were defined as structures with cells clustered together, oriented in roughly the same direction, opposed to an open ECM emanating away from the basal pole of all cells; and trees were defined as structures with cells oriented in a similar fashion to those in cones but with the ECM displaying a dichotomous branching structure. Quantitative analyses of simulation results (Fig. 2.S5) were carried out in MATLAB.
Figure 2.1. The choanoflagellate Salpingoea rosetta develops from a single cell into multicellular colonies through serial rounds of cell divisions (186). (A) All choanoflagellate cells bear a diagnostic “collar complex” composed of an apical flagellum (f) surrounded by an actin-filled collar of microvilli (c) (18, 154). S. rosetta produces two different colonial forms depending on environmental conditions: compact, mechanically robust, roughly spherical rosette colonies (Rosette) that form in the presence of specific bacterially produced Rosette Inducing Factors (RIFs; (185, 186, 192, 193)), and fragile, linear chain colonies (Chain) that form during rapid cell growth in the absence of RIFs (185). Both types of colonies form developmentally by serial cell divisions. Single cell image adapted from (185), and rosette and chain images adapted from (188). (B) A thin section through the equator of a rosette, imaged by transmission electron microscopy, reveals the subcellular architecture of a rosette. Cells in rosettes are packed close to one another around a central focus with the collar complex of each cell facing outward into the environment (c=collar and f=flagellum). Most cells are connected to one another by thin cytoplasmic bridges (br, only two of which are visible in this section) (165), which are also present in chains (185). The center of rosettes is devoid of cells but is filled with a secreted extracellular matrix (e, faintly visible here as granular material), into which cells extend filopodia (fi) (185, 187). All scale bars = 3 µm.
Figure 2.2. Quantitative analysis of rosette morphology reveals that rosettes undergo a reproducible 2D-3D growth transition. (A) In cultures grown under conditions of constant rosette induction, *S. rosetta* existed as unicells, doublets, triplets, and rosettes containing between 4 – 25 cells (an “individual” refers to any unicell or group of cells in each of these categories), with the most common rosette size being 8 cells/individual. Shown is the mean percentage of total cells in a population (y-axis) found in single cells, cell doublets, cell triplets, and rosettes of increasing size, plotted by number of cells/rosette (x-axis). Error bars indicate standard deviations from measurements obtained on three different days. N = 511. (B) Our image analysis pipeline allowed us to quantify and compare rosette morphology and is illustrated here for two
representative rosettes. From left to right, for each rosette, (i) cell positions were extracted from segmented images and then used to determine aspects of rosette morphology including (ii) rosette size, including volume (measured by generating a convex hull), (iii) shape, including flatness and sphericity (the former quantified by $1 - C/B$ and the latter by $\sqrt{BC/A^2}$ where $A$, $B$, and $C$ are the principle axes in descending order by magnitude of a principle components analysis-based ellipsoid fit of cell positions), (iv) and cell packing (neighbor number determined by Voronoi tessellation(240)). (C) Representative rosettes are shown in three roughly orthogonal views for size classes ranging from four to 12 cells/rosette, with the numbers above each image column indicating the number of cells/rosette. Following previous work (193), we defined four cells as the smallest number of cells clearly identifiable as a rosette. (D) Rosettes transition from an early phase of major shape change (dark grey; scaling exponent ~8) to a later phase of approximately isotropic growth (light grey; scaling exponent ~3), as shown by a log-log plot of rosette volume (y-axis) vs. maximum rosette width (x-axis). (E) Rosettes transition from a relatively flat morphology during the 4-6 cell stage (dark grey; mean flatness $\approx 0.5 - 0.7$ and mean sphericity $\approx 0.4 - 0.6$, with flatness = 1.0 perfectly flat and sphericity = 1.0 perfectly spherical) to a more spheroidal morphology during the 8-12 cell stage (light grey; mean flatness $\approx 0.2 - 0.3$ and mean sphericity $\approx 0.7-0.8$). (F) Packing increases with number of cells at a decreasing rate. Points on plots D-F represent mean values; error bars indicate standard error of the mean. N = 100 rosettes, with at least 8 rosettes from each cell-number class, pooled from three different samples. All scale bars = 3 $\mu$m.
Figure 2.3. Stochasticity of developmental dynamics revealed by lineage analysis. (A) Lineage analysis for a representative rosette, imaged live by time lapse microscopy. Individual daughter cells were marked to record their relationship to their parent lineage (e.g. 1.1 and 1.2 are daughters of cell 1). Scale bar is 3 µm. (B) Representative cell lineages during rosette development. (C) 3D visualizations showing rosette and chain formation over time. (D) Schematic representation of rosette and chain structures with numbers indicating their counts.
illustrating differences in both the order and timing of cell divisions, with the first two on the left having the same division order but with large differences in division times, and the lineage on the right showing differences in both division order and timing. Branch lengths scale with time and are set to zero based on the first division. (C) Lineages a-c from (B) displayed as space-time plots illustrate cell division variability between rosettes in both space and time. Plots also demonstrate that cells remain in place after divisions, with no large rearrangements, moving apart only slightly as they grow. Colors from dark to light gray indicate the order of cell divisions. (D) Cell lineages that form during rosette and chain development are balanced, although the specific times of cell divisions in different lineages and different rosettes or chains can differ. In rosettes and chains, imbalanced lineage structures (i.e. with significantly different numbers of cells) were not observed at any stage. Shown here are results for four and five-cell rosettes and chains. The dashed line with an “x” indicates that this division pattern was never observed. Furthermore, this uneven branching pattern was never observed in sub-lineages of any developing chain or rosette. Data were pooled from three different rosette induction experiments (for rosettes) and three different experiments with uninduced cells (for chains).
Figure 2.4. Extracellular matrix constrains proliferating cells during rosette morphogenesis. (A-B) Cartoons depicting ECM constraint hypothesis. (A) As cells grow, compressive forces exerted on neighboring cells (top set of arrows) may be balanced by stress in the basally-secreted ECM (blue), which resists deformation (bottom set of arrows). (B) If ECM is limiting, rosettes may undergo a jamming-like transition and accumulate residual stress as cells continue to grow and divide. (C) Representative images of 4 different rosettes of various sizes (of 6, 7, 8, and 10 cells/rosette ordered from left to right, top to bottom) with ECM tagged using Jacalin. (D) Ratio of total ECM volume to total cell volume as a function of cells/individual is maintained at a constant level over the course of rosette development. Points represent mean values, and error bars are bootstrap 95% confidence intervals. Data were collected from 93 rosettes pooled from two experiments, with at least 5 rosettes from each size class. Image processing and
analysis proceeded similarly to that described in Fig. 2.1. (E) Laser ablation revealed increasing stress as a function of cells/rosette. Single cells in rosettes were ablated, and recoil velocities were measured by particle image velocimetry (PIV). Arrows in the images indicate the direction and magnitude of the velocity of the recoil as determined by PIV. Rosettes were found to always close when recoil was observed (in all cases beyond the 5-cell stage). (F) Recoil velocities (rescaled by the length scale of the average cell diameter of 5 µm and the time scale of the average division time of 6 hrs) increased with increasing cells/rosette, indicating increasing stress as a function of cells/rosette (181, 205, 209). These results are consistent with the ECM constraint hypothesis and inconsistent with cytoplasmic bridges or cell-cell adhesion as the dominant factors stabilizing rosette structure. Points indicate mean values; error bars indicate standard error of the mean. Data were collected from 47 rosettes pooled from 3 different rosette inductions, with at least 4 rosettes from each size class. Scale bars = 5 µm.
Figure 2.5. Material properties of the ECM affect morphogenesis, consistent with predictions of the ECM constraint hypothesis. Treatment of rosettes with SrCl$_2$, which stiffens hydrogels by increasing crosslinking density (210–214), alters rosette morphogenesis. (A) Representative images of untreated and SrCl$_2$-treated rosettes illustrate the change in rosette morphology. Cells were packed more tightly in SrCl$_2$ treated rosettes, leading to differences in rosette size and shape. Scale bar = 4 µm. (B) The scaling relationship between maximum rosette width and volume revealed that SrCl$_2$ abolished the transition to approximately isotropic growth observed in untreated rosettes. As in Fig. 2.2D, this is a log-log plot of rosette volume vs. maximum rosette width, with each point representing average values for a rosette cell-number class from 4-12 cells/rosette. Error bars are standard error of the mean. This analysis also revealed that rosettes became increasingly compact with increasing SrCl$_2$ concentration. (C) Quantification of rosette flatness (as in Fig. 2.1), showed that SrCl$_2$ shifts the 3D growth transition to lower cell numbers. In the case of the highest SrCl$_2$ concentration, the transition occurred by the 5-cell stage. For both (B) and (C), results were from a total of 100 rosettes pooled from 3 experiments, with at least 8 rosettes for each size class for both SrCl$_2$ concentrations. (D) Relative residual stress, as measured by maximum initial recoil velocity (rescaled, as in 4E, F, by the length scale of the average cell diameter of 5 µm and the time scale of the average division time of 6 hrs) after laser ablation of
single cells (as in figure 4E, F), increased in rosettes of 4-7 cells under 5mM SrCl₂ treatment compared to untreated rosettes. These data demonstrate that increased residual stress is correlated with altered cell packing and hence, altered rosette morphology. Points represent means and error bars represent standard error of the mean from 41 total measurements pooled from two experiments, with at least 4 rosettes from each size class.
**Figure 2.6.** A simple model shows that amount of ECM, cell shape (aspect ratio), and ECM stiffness tune multicellular morphogenesis. The model incorporates simple cellular and physical interactions, including ECM secretion and cell division, cell-cell steric interactions, and ECM adhesion. Three main parameters describe the system: cell aspect ratio, $\alpha$, scaled ECM stiffness, $\sigma$, and relative ECM volume, $\phi$. (A) An image of a choanoflagellate (adapted from (185)) next to a simulation snapshot to illustrate how cell geometry is modeled by three linked spheres (black) and ECM is modeled by small spheres (grey) secreted at the basal pole of cells. In the model, cells interact stERICALLY with one another, and ECM spheres have adhesive interactions with one another and with basal cell particles. Scale bar = 5 $\mu$m. (B) The morphospace of ECM-based colonial morphologies generated by simulations can be broken into four regions: rosettes, disks, cones/cups, and trees as denoted by colors as indicated in the legend. The lower-case letters indicate approximately the point in the morphospace occupied by the corresponding simulated colony in panel F. (C-E) Orthogonal planes through the displayed morphospace, with the parameter of fixed value noted above each plot, illustrate how changing two parameters while keeping the third fixed affects morphology. Colors indicate morphological classification as in panel B. (C) Scaled ECM stiffness is constant ($\sigma=0.6$). (D) Cell aspect ratio is constant ($\alpha=0.8$) (E) Relative ECM volume is constant ($\sigma=0.6$). (F) Representative simulated colonies for each of the regions are displayed in two orthogonal views ($r_1$=rosette with $\alpha=0.7$, $\sigma=0.8$, and $\phi=0.04$; $d_1$=disk with $\alpha=0.75\sigma=0.85$, and $\phi=0.075$; $d_2$=disk with $\alpha=0.8$, $\sigma=0.15$, and $\phi=0.02$; $c_1$=cone with $\alpha=0.55$, $\sigma=0.5$, and relative $\phi=0.08$; $c_2$=cup with $\alpha=0.6$, $\sigma=0.2$, and $\phi=0.09$; $c_3$=cup with $\alpha=0.9$, $\sigma=0.12$, and $\phi=0.13$; and $t_1$=tree with $\alpha=0.65$, $\sigma=0.9$, and $\phi=0.12$). Note that $d_2$ and $c_3$ represent extreme ends of the morphospace to better illustrate, along with the other representative simulation snapshots, how changing the model parameters affects the simulated morphologies. (G) Simulated colonial morphologies are reminiscent of morphologies of colonial choanoflagellates found in nature. ($r_2$) *Codonosiga botrytis* (167). ($r_3$) *Salpingoeca rosetta* (22). ($d_3$) *Proterospongia haeckelii* (246) (Ertl after Lackey). ($d_4$) *Salpingoeca amphoridium* (161). ($c_4$) *Codosiga umbellata* (217). ($c_5$) Uncharacterized environmental isolate collected from Mono Lake by Daniel Richter, *Salpingoeca* sp. ($t_2$) *Codosiga cymosa* (216) (Calkins after Kent). ($t_3$) Uncharacterized environmental isolate from a tide pool in Curaçao, *Salpingoeca* sp. Scale bars = 10 $\mu$m.
Figure S1. Rosettes can decrease in cell number by extrusion of single cells. Snapshots from a time series collected by DIC imaging illustrating the process by which cells are extruded from a developing rosette and swim away as solitary cells. Black arrowhead indicates location of extrusion event and white arrowheads indicate extruded cells. Scale bar = 5 µm.
Figure 2.S2. Cell size and shape changes during colony development. (A) Average size of cells in rosettes decreases significantly from the 4 to 5-cell stage and then experience a modest, progressive decrease in cell size after the 8-cell stage. Error bars are standard error of the mean. (B) Sphericity of cells as measured by $\frac{\frac{1}{3}(6V)^{2}}{A}$ (247), where V is cell volume and A is cell area, decreases in rosettes while remaining constant in chains. This is consistent with cells in rosettes either actively changing shape during development, becoming deformed by compressing due to cell packing, or a combination of the two. Data is pooled from 100 rosettes and 110 chains, with at least 8 colonies from each size class. Error bars are standard error of the mean.
Figure 2.S3. Division times in developing rosettes, induced cells, are slightly but significantly different from those of uninduced cells (p=0.03 by Wilcoxon rank sum method). While the range of division times is the same, induced cells show a small increase in division rate (mean division time of 5.5 hrs for induced cells vs. 5.9 hrs for uninduced cells). Data is pooled from 20 different colonies for both the induced and uninduced conditions.
Figure 2. S4. SrCl$_2$ does not affect cell growth rates at up to twice the concentration used in the experiments in this study (Fig. 2.5). The plot displays growth curves on a log scale for various concentrations of SrCl$_2$ and the bottom table displays corresponding growth rates derived from exponential fits of the log-phase of each growth curve.
Figure 2.S5. Simulations of colony development with parameters constrained by measurements of rosette and ECM morphology recapitulate rosette morphogenesis including the 3D transition at the 8-cell stage. Simulated rosette morphogenesis is robust to the stochasticity of cell divisions and to a range of different values of scaled ECM stiffness, $\sigma$, and rosettes are increasingly 3D at lower cell numbers with increasing $\sigma$, consistent with results from experiments (Fig. 2.5C). The mean of 10 different simulation runs for each $\sigma$ value is plotted here, and error bars are the standard error of the mean.
Chapter 3: Light-regulated collective contractility in a multicellular choanoflagellate

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Abstract
Collective cell contractions that generate global tissue deformations are a signature feature of animal movement and morphogenesis. Nonetheless, the ancestry of collective contractility in animals remains mysterious. While surveying the Caribbean island of Curaçao for choanoflagellates, the closest living relatives of animals, with collaborators, I isolated a previously undescribed species (here named Choanoeca flexa sp. nov.), that forms multicellular cup-shaped colonies. The colonies rapidly invert their curvature in response to changing light levels, which they detect through a rhodopsin-cGMP pathway. Inversion requires actomyosin-mediated apical contractility and allows alternation between feeding and swimming behavior. C. flexa thus rapidly converts sensory inputs directly into multicellular contractions. In this respect, it may inform reconstructions of hypothesized animal ancestors that existed before the evolution of specialized sensory and contractile cells.
3.1 Introduction

The evolution of animals from single-celled ancestors involved several major evolutionary innovations, including multicellularity, spatial cell differentiation, and morphogenesis (18, 248). Efforts to reconstruct the origin of animal multicellularity have benefited from the study of choanoflagellates, the closest living relatives of animals (19, 154, 162). Choanoflagellates are microbial eukaryotes that feed on bacteria and live in aquatic environments around the world; many species differentiate over their life history into diverse cell types, including unicellular and multicellular forms (154, 165, 184, 185). Comparative genomics and transcriptomics have revealed that many gene families once thought to be unique to animals (e.g. cadherins, C-type lectins, and receptor tyrosine kinases) are also present in choanoflagellates (21, 22, 162, 187, 249). Moreover, laboratory studies of the model choanoflagellate Salpingoeca rosetta (185) have revealed diverse responses to environmental cues, such as pH-taxis (250), aerotaxis (107), and bacterial regulation of life history transitions (multicellular development (251) and mating (36)). However, S. rosetta is only one of approximately 380 known species (252) and choanoflagellates are at least as genetically diverse as animals (22). Choanoflagellate diversity thus represents a largely untapped opportunity to investigate environmental regulation of cell behavior, the principles that broadly underpin multicellularity, and the evolution of animal cell biology.

3.2 Photic cues induce multicellular sheet inversion in a colonial choanoflagellate

During a survey of choanoflagellate diversity on the Caribbean island of Curaçao in April 2018 (Fig. 3.1A, B), we collected large, cup-shaped colonies of protozoa (~100 µm diameter) from shallow pools in the splash zone above the tide line of a rocky coastal area (Fig. 3.1B). Each colony was composed of a monolayer (“sheet”) of up to hundreds of flagellated cells (Fig. 3.1C, Movie 3.S1). Upon closer inspection, we observed that the cells bore the characteristic collar complex of choanoflagellates (18, 154), in which a “collar” of microvilli surrounds a single apical flagellum (Fig. 3.1D). However, unlike in most choanoflagellate colonies (154), the apical flagella pointed into the interior of the colony (Fig. 3.1E), resembling the orientation of collar cells in the choanocyte chambers of sponges (253). While observing colonies, we were surprised to see them invert their curvature rapidly (within ~30 s from initiation to completion) while maintaining their cell topology, such that the flagella now pointed outward along the radius of curvature of the colony (Fig. 3.1F, G, Movie S2). The colonies tended to remain in the inverted form (“flagella-out”) for several minutes before reverting to their initial, relaxed conformation (“flagella-in”) in a similarly rapid process (Fig. 3.1H, Movie S3).

To start laboratory cultures of this choanoflagellate, we manually isolated several representative colonies away from the other microbial eukaryotes present in the original splash pool sample (e.g., presumptive Oxyrrhis sp. dinoflagellates; Fig. 3.1C, Movie S1) and transferred them into nutrient-supplemented artificial seawater along with co-isolated bacteria (which choanoflagellates need as a food source). Cells from the isolated colonies proliferated and served as the foundation of all downstream laboratory cultures. Phylogenetic analyses of 18S rDNA sequences indicated that it is the sister-species of the previously described species Choanoeca perplexa (also known as Proterospongia choanojuncta; Fig. 1I, Fig. 3.S1), which has a dynamic life history that includes single cells and colonies (184, 254). In reference to the striking sheet bending behavior of the new species, we named it Choanoeca flexa. Interestingly, a similar inversion behavior was briefly mentioned in a 1983 study of C. perplexa (184). The cell line from
that study subsequently stopped forming colonies in the laboratory and could never be revived nor re-isolated (154), preventing mechanistic study of the process. Our re-observation of this process in the newly discovered sister-species of *C. perplexa* confirms and extends that early report. This behavior, representing a global change in multicellular form, is reminiscent of concerted movement and morphogenesis in animals (e.g., muscle contraction or gastrulation). Because of the potential evolutionary implications of rapid shape change in *C. flexa*, we set out to investigate (1) how colony inversion is regulated, (2) the mechanisms underlying colony inversion, and (3) the ecological consequences of colony inversion.

Unexpectedly, several lines of evidence indicated that *C. flexa* colony inversion is regulated by light. While imaging live *C. flexa* sheets for long periods of time (>1hr) under constant illumination, we noted that colony inversions became less frequent. In contrast, after the microscope illumination was turned off, the colonies would invert almost immediately (Movie S7). To test whether light-to-dark transitions consistently induce *C. flexa* inversion, we established a quantitative assay based on the observation that the projected area of a *C. flexa* sheet decreases by as much as 50% during inversion (Fig. 3.2A-C, Movie S4). Using this assay, we confirmed that a rapid decrease in illumination reliably induced inversion of *C. flexa* colonies from flagella-in to flagella-out within thirty seconds (Movie 3.S5; Fig. 2D). Thus, *C. flexa* colony inversion can be triggered by light-to-dark transitions. To our knowledge, this represents the first observation of light-responsive behavior in a choanoflagellate.

### 3.3 A rhodopsin-cGMP pathway regulates colony inversion in response to light-to-dark transitions

We next sought to understand how *C. flexa* colonies detect and respond to a photic stimulus. Although choanoflagellates are unpigmented and transparent, at least four choanoflagellates (22) encode a choanoflagellate-specific rhodopsin-phosphodiesterase fusion protein (Fig. 3.S2), RhoPDE, that has been investigated for its potential as an optogenetic tool (255–258). RhoPDE proteins consist of an N-terminal type I (bacterial) rhodopsin (a class of photosensitive transmembrane proteins broadly involved in light detection (259)) fused to a C-terminal phosphodiesterase (PDE) that catalyzes light-dependent cyclic nucleotide hydrolysis (Fig. 3.3A). Based on *in vitro* studies (255–258), RhoPDE from *S. rosetta* appears capable of converting a photic stimulus into a biochemical signal within seconds, similar to the time scale of the *C. flexa* response to light-to-dark transitions.

To test for the presence of RhoPDE or other candidate photosensitive proteins in *C. flexa*, we sequenced and assembled the *C. flexa* transcriptome (Figshare DOI 10.6084/m9.figshare.8216291). 56 million reads were assembled into 50,463 predicted transcripts encoding 20,477 predicted unique proteins, of which four appeared to be RhoPDE homologs (Fig. 3.S2; GenBank accession numbers MN013138, MN013139, MN013140 and MN013141). No other rhodopsins were detected in the *C. flexa* transcriptome. The only other candidate photoreceptor protein domain found was a member of the cryptochrome family of photosensitive transcription factors (260), which act on the timescale of transcriptional regulation (at least several minutes (261, 262)) and therefore appear unlikely to mediate the light-to-dark transition response. Thus, we focused our attention on the RhoPDEs.

The hypothesized role of RhoPDE as the regulator of the light-to-dark transition response offered two testable predictions. First, depletion of the rhodopsin chromophore, retinal (263),
should prevent the response by abolishing rhodopsin activity. Second, artificially increasing the cellular concentration of cGMP or cAMP (which are degraded by the enzymatic activity of PDEs) should mimic the effect of darkness and therefore be sufficient to trigger sheet inversion.

Retinal is a carotenoid chromophore whose isomerization underlies rhodopsin photodetection (263–265). Plants and some bacteria can synthesize carotenoids, but transcriptome analysis revealed that C. flexa lacks a key enzyme in the retinal biosynthesis pathway (Fig. 3.3S3); therefore, like animals, C. flexa must receive retinal or its biochemical precursor, beta-carotene, from its food. Because C. flexa sheets are grown with diverse co-isolated environmental bacteria, it is possible that they take up retinal or beta-carotene from their bacterial prey. To test whether bacterially produced carotenoids are required for light-regulated colony inversion, we established a monoxenic culture containing only C. flexa and a co-isolated bacterial species, Pseudomonas oceani, that lacks genes in the retinal biosynthesis pathway (Fig. 3.3S3) (266). This culture, referred to as “ChoPs” (for Choanoeca + Pseudomonas), was expected to be devoid of carotenoids, thereby abolishing rhodopsin activity. As predicted, C. flexa sheets in ChoPs cultures did not invert in response to darkness (Fig. 3.3B). Inoculating ChoPs cultures with a mixture of additional co-isolated environmental bacteria restored the light-to-dark response, demonstrating that a bacterial factor is necessary for this behavior. Moreover, addition of exogenous retinal to ChoPs cultures was sufficient to restore the wild-type light-to-dark response in C. flexa (Fig. 3.3C). The requirement of retinal for the inversion response and the fact that the only rhodopsin-containing genes in the C. flexa genome encode RhoPDEs suggest that one (or more) RhoPDEs are required for the light-to-dark-induced inversion.

We next investigated whether cyclic nucleotide signaling plays a role in C. flexa phototransduction. Treatment of C. flexa sheets with two inhibitors of phosphodiesterase activity, caffeine (267) and IBMX (268), caused colonies to invert even in the absence of a photic stimulus (Fig. 3.3D). Moreover, incubating C. flexa sheets with a cell-permeant analog of cGMP induced colony inversion in a dose-responsive manner, while a cell-permeant analog of cAMP had no effect (Fig. 3.3E), suggesting that cGMP acts as a second messenger in phototransduction and is the endogenous trigger of colony inversion.

Together, these results provide evidence that the C. flexa response to light-to-dark transitions relies on a rhodopsin as a photoreceptor and on cGMP as a second messenger. The simplest interpretation of these findings is that a RhoPDE protein controls C. flexa phototransduction. However, direct validation of this hypothesis will require targeted disruption of the RhoPDE homologs encoded by C. flexa (Fig. 3.3S2), which is not currently possible.

### 3.4 Sheet inversion mediates a trade-off between feeding and swimming

What are the functional and ecological roles of sheet inversion in C. flexa? Flagella-in sheets showed little to no motility, either slowly drifting or settling to the bottom of culture flasks (Movie S6). In contrast, inverted (flagella-out) sheets swam rapidly (Fig. 3.4A, B, S5, Movie S7). Thus, one important consequence of sheet inversion is increased motility, which could allow rapid escape from environmental hazards (including predators).

In choanoflagellates, flagellar beating in unattached, single cells typically results in motility, while flagellar beating in cells attached to surfaces or other cells (e.g. in colonies) has been hypothesized to enhance feeding currents that draw bacterial prey to the outside of the collars for phagocytosis (148, 154). Hence, the enhanced motility of flagella-out colonies might come at a cost – reduced feeding efficiency. To test for the existence of a tradeoff between swimming and
feeding, we used bacteria-sized fluorescent beads (269) to quantify particles ingested by cells of flagella-in and flagella-out sheets (Fig. 3.4C-F). While cells in flagella-in sheets fed efficiently (>75% cells/sheet internalizing beads, Fig. 3.4G), cells in flagella-out sheets did not (~10% cells/sheet internalizing beads on average, Fig. 3.4G). Moreover, the flow generated by the sheets during inversion was visualized by tracking fluorescent beads in suspension in sea water. In relaxed sheets, the flow converged toward the center of the colony (carrying bacterial prey toward the cells), while in inverted sheets, the flow was directed away from the colony – allowing swimming, but not feeding (Fig. 3.6).

Because sheets swim slowly when relaxed (flagella-in) and rapidly when inverted (flagella-out), we suspected that darkness-induced inversion might allow sheets to accumulate in bright areas, effectively undergoing phototaxis. To test for phototaxis, we illuminated chambers containing C. flexa sheets with directional light and found that sheets tended to accumulate in the brightest areas near the illumination source compared to a control in which no illumination was provided (Fig. 3.4H, Fig. 3.5S7). Further, we found that neither colonies from ChoPs cultures nor single cells from dissociated sheets were capable of phototaxis, suggesting that rhodopsin activity, multicellularity, and sheet inversion are all required for phototaxis (Fig. 3.4H). These results demonstrate that sheet inversion mediates a tradeoff between feeding (flagella-in) and swimming (flagella-out) that is plausibly ecologically relevant (Fig. 3.4I).

3.5 Sheet inversion requires apical actomyosin contractility

How do cells in sheets interact with their neighbors and what cellular mechanisms allow sheet inversion? By employing DIC microscopy on live colonies, as well as confocal microscopy and electron microscopy on fixed colonies, we found that cells in C. flexa sheets are linked by direct contacts between collar microvilli (Fig. 3.5A-C, S8). Importantly, we found no evidence for the intercellular bridges, shared ECM, or filopodial contacts that mediate multicellularity in other choanoflagellate species (184, 185, 187, 188).

Interestingly, we also observed that collar morphology tended to differ between relaxed and inverted sheets. In relaxed sheets (flagella-in; Fig. 3.5C), the microvilli on each cell assembled into a barrel-shaped collar whose diameter varied little from base to tip. In inverted sheets (flagella-out; Fig. 3.5C), the microvilli formed a flared, cone-shaped collar whose diameter increased from base to tip. This contrast in collar shape suggests a potential mechanism for sheet inversion: active “opening out” of the collar, by increasing the surface area of the apical side of the sheets relative to their basal side, might force a change in sheet curvature. Consistent with this, we found that, as in intact sheets, dissociated C. flexa cells treated with caffeine opened their collar into a conical shape, while untreated controls maintained a barrel-shaped collar (Fig. 3.5D-G). Additionally, caffeine treatment caused the microvilli to straighten and the base of the collar to slide toward the equator of the cell (Fig. 3.5S9). These data indicate that the changes in collar geometry observed during inversion are actively generated by individual cells, and do not require interactions among neighboring cells.

If C. flexa cells modulate the shape of their collars, what is the underlying cellular mechanism? In animal epithelial tissues, sheet bending during morphogenesis is frequently due to a process called apical constriction, in which contraction of an apical actomyosin network reduces the surface area of the apical side of the cell (51, 270). Apical constriction is mediated by molecular motors belonging to the myosin II family, which is of ancient eukaryotic origin (271) and is
represented in all previously sequenced choanoflagellate genomes (272) and transcriptomes (22). The C. flexa transcriptome encodes homologs of the myosin II regulatory light chain (GenBank accession number MK787241) and heavy chain (GenBank accession number MK787240) (Fig. 3.S4) whose protein sequences are respectively 78% and 63% similar to their human counterparts.

We thus investigated the actomyosin cytoskeleton of C. flexa. Like in choanoflagellates, the apical side of animal epithelial cells is defined by the presence of a cilium/flagellum and/or microvilli, and the apicobasal axis of both types of cells is broadly accepted to be homologous (273). Confocal imaging of sheets labelled with fluorescent phalloidin revealed the presence of a pronounced F-actin ring at the apical pole of each cell, from which the microvillar collar extends (Fig. 3.5H). Directly connected to this ring, and perpendicular to it, we detected a small number of longitudinal actin fibers (usually two or three) pointing toward the basal pole. Diameter measurements showed that the actin ring was consistently smaller in inverted, flagella-out sheets compared with relaxed, flagella-in sheets (Fig. 3.5I-L). The same was true for dissociated, caffeine-treated cells compared with the corresponding negative controls (Fig. 3.5I, L), consistent with the ring actively constricting during sheet inversion. During inversion and in response to caffeine, some (but not all) cells transiently acquired a “bottle cell” morphology with a narrow apex and a bulbous base (Fig. 3.S9), reminiscent of animal cells undergoing pronounced apical constriction (274, 275). Interestingly, caffeine treatment also induced shortening of the longitudinal actin fibers (Fig. 3.S9G-H), suggesting that fiber contraction pulls the collar toward the basal pole.

Using five different commercial myosin II antibodies, including two raised against the activated phosphorylated form (Fig. 3.S10), we found that C. flexa cells contain myosin that overlaps in regions with the apical actin ring (Fig. 3.5M-P, Fig. 3.S10), longitudinal fibers, and base of the microvilli (Fig. 3.5Q-S), consistent with the idea that the apical actin network is contractile. Finally, blebbistatin (which inhibits the ATPase activity of myosin II (276)) entirely abolished ring constriction in caffeine-treated dissociated cells (Fig. 3.S11) and prevented sheet inversion (Fig. 3.5T), as did latrunculin B (which inhibits dynamic actin polymerization (277)) and ML-7 (which prevents activation of myosin by phosphorylation of the Myosin Regulatory Light Chain (278)) (Fig. 3.5T). None of these drugs affected flagellar beating (which was used as a visual control of cell survival, see Material and Methods), consistent with them specifically targeting actomyosin. Together, these results suggest that sheet inversion requires apical constriction of an actomyosin network at the base of the collar (Fig. 3.5U).

3.6 The ancestry of apical constriction

The discovery of sheet bending driven by apical constriction in a multicellular choanoflagellate has several potentially important evolutionary implications. Epithelial sheet bending is a fundamental mechanism underlying animal embryonic development (51, 279, 280) and multicellular contractility also plays a fundamental role in the behavior of adult animals by allowing fine-tuned body deformations (281). As both embryonic and adult tissue contractility are found in nearly all animal lineages, including sponges (282, 283), ctenophores (284, 285), placozoans (286), cnidarians (274, 287) and bilaterians (51, 270, 288), both were likely present in the last common animal ancestor. By contrast, collective contractility and apical constriction were hitherto unknown in close relatives of animals, making their origin mysterious.

The existence of actomyosin-mediated apical constriction in C. flexa raises the possibility that this cellular module might have been present in the last common ancestor of choanoflagellates
and animals (which together comprise the choanozoans (18)). Collar contractions have been reported in the unicellular stages of three other choanoflagellate species: *Codosiga pulcherrima* (289), *Monosiga gracilis* (290), and the sessile form of *C. perplexa* (184), the sister-species of *C. flexa*. As part of this study, we observed collar contractions in *C. flexa* sheets (Fig. 3.5G), dissociated cells from sheets (Fig. 3.5D, E), and in naturally solitary “thecate” cells (Movie S12). We expanded this taxonomic sampling by investigating four other choanoflagellate species, *Monosiga brevicollis*, *S. rosetta*, *Salpingoeca urceolata* and *Diaphanoeca grandis*, which together cover the three main branches of the choanoflagellate phylogenetic tree (252). All four species displayed spontaneous changes in collar geometry occurring at the scale of a few seconds. *S. urceolata* (Movie S8) and *M. brevicollis* (Movie S9) showed spontaneous and reversible opening/closing of the collar (similar to *C. flexa*), while *S. rosetta* (Movie S8) and *D. grandis* (Movie S11) displayed subtler shape changes (reorientation of individual microvilli and modulation of collar curvature, respectively) (Figure 3.6A, S12). In all four species, immunostaining revealed the presence of an apical actomyosin ring at the base of the collar. This suggests that the apical actomyosin ring is a conserved feature of choanoflagellate biology (Figure 3.6B-C) and that unicellular apical constriction was present in the last common ancestor of choanoflagellates and animals.

What might be the function of apical constriction in single cells? In some sessile choanoflagellates – including in the thecate form of *C. perplexa* – collar contraction happens in response to physical contact with an external object, and allows retraction of the cell inside an extracellular structure (called a theca) (254), suggesting it represents a defensive withdrawal reflex from predators or other threats. In free-swimming cells, collar contraction might fine-tune the hydrodynamics of swimming and/or feeding: for example, a closed collar might reduce drag and facilitate locomotion, while a spread collar could slow down swimming and increase collar area, thereby facilitating prey capture. Validation of these functional hypotheses will require direct testing.

These observations suggest that apical actomyosin mediated cell constriction evolved on the choanozoan stem lineage (Figure 3.6B). Could this cellular module be even more ancient? Polarized actomyosin contractions have been implicated in multicellular morphogenesis in the fruiting body of the slime mold *Dictyostelium* (291) and may be homologous to those observed in choanoflagellates and animals. However, the absence of comparable processes in the intermediate branches between *Dictyostelium* and choanozoans raises the possibility that polarized cell contractions in *Dictyostelium* and apical constriction in choanozoans evolved independently (292). Finally, the ichthyosporean *Sphaeroforma arctica*, a close relative of choanozoans, forms large multinucleated spores that partition into distinct cells in an actomyosin-dependent process (293), providing an independent example of actomyosin-dependent multicellular development.

In contrast to single-cell apical constriction, the multicellular sheet bending observed in *C. flexa* and *C. perplexa* (184) has not been reported in other choanoflagellates. This suggests that apical constriction was present in solitary cells in the last choanozoan common ancestor, and was independently converted into multicellular sheet bending through the evolution of intercellular junctions in animals (163) and the evolution of microvillar adhesions in *C. flexa*. Interestingly, multicellular inversion has been proposed to have been part of the developmental repertoire of ancient animals (18, 294), based on the existence of whole-embryo inversion (from flagella-in to flagella-out) during calcareous sponge development (295). It is also intriguing that a similar inversion (but much slower – about an hour-long) takes place during the development of the alga *Volvox* (43). Given the large evolutionary distance between choanoflagellates and volvocalean
green algae, along with the absence of inversion in intervening branches, inversion likely evolved independently in both groups (18).

In animals, the control of multicellular contractions invariably relies either on the cooperation of multiple cell types (as in adult organisms (287, 288, 296)) or on complex programmed signaling cascades (as in embryos (51, 279, 280)). By contrast, *C. flexa* directly converts sensory stimuli into collective contractions, without observable spatial cell differentiation, and evokes some hypotheses of early animal evolution that envisioned the first contractile tissues as homogeneous myoepithelia of multifunctional sensory- contractile cells (297).

The fact that contractility in *C. flexa* can be controlled by light represents another intriguing parallel to animal biology. Indeed, rhodopsin-cGMP pathways similar to that in *C. flexa* also underlie phototransduction in some animal cells (e.g. bilaterian ciliary photoreceptors (264, 265) and cnidarian photoreceptors (298, 299)), as well as in fungal zoospores (300). In contrast with choanoflagellates, however, phototransduction in animal photoreceptors relies on a type II (eukaryotic) rhodopsin that activates a separate phosphodiesterase through a G-protein intermediary (264, 265) (Fig. 3.S13). Meanwhile, fungal zoospores use a distinct rhodopsin fusion protein (a type I rhodopsin fused to a guanylyl cyclase) to increase cellular cGMP in response to light (300) (Fig. 3.S13). If a RhoPDE fusion protein controls *C. flexa* phototransduction, this would represent a third unique solution to the problem of transducing information from a change in illumination into a change in cyclic nucleotide signaling.

Much remains to be discovered concerning the ecological function, mechanical underpinnings, and molecular mechanisms of phototransduction and apical constriction in *C. flexa*. A deeper understanding will require the development of molecular genetic tools, which have only recently been established in *S. rosetta* (188, 301) and *D. grandis* (302). Nonetheless, *C. flexa* demonstrates how the exploration of choanoflagellate diversity can reveal unexpected biological phenomena and provides an experimentally tractable model for studying multicellular sensory- contractile coupling.
3.7 Materials and Methods

3.7.1 Species description

Order Craspedida Cavalier-Smith 1997 (303).
Genus Choanoeca Ellis 1930 (304)
*Choanoeca flexa* sp. nov.

**Etymology**: from the Latin ‘flexa’, nominative feminine singular of ‘flexus’ which means ‘bending’ or ‘transition, change’.

**Type locality**: splash pool of the Curaçao rocky coast (12.22831° N, -69.01353° W), from which water was collected with 25 cm² canted U-shaped culture flasks (T25; ThermoFischer Scientific 1012628) in April 2018.

**Description**: cell body is about 4-5 μm long. Microvillous collar is about 10 μm long. Microvilli of resting cells appear markedly curved. Cells form two-dimensional curved sheets linked by direct contact between their collars. Resting sheets are cup-shaped hemispheres with flagella pointing on the inner side of the curvature. Cells in their thecate form (Movie S12) are characterized by a stalkless theca, a bulbous cell base, a narrow and pointed apex, and a widely spread collar without a flagellum, as in the thecate form of *Choanoeca perplexa* (254).

**Holotype**: Figure 1E.

3.7.2 Initial isolation and culture of *Choanoeca flexa*

Samples containing *Choanoeca flexa* were first isolated by the three co-first authors from multiple splash pools on the northern rocky coast of Curaçao (12°14’12.1″ N, 69°01’34.8″ W). Individual colonies were manually isolated by pipetting 0.1 μL with a P2 micropipette under a Leica DMIL transmitted light microscope. Isolation was confirmed by pipetting the resulting droplet onto a microscopy slide and visually confirming the presence of single colonies, which were then transferred into 24-well plates containing 1 mL culture medium. Each well was seeded with 3 or 4 individual colonies. Wells were visually inspected 24 to 48 hours after transfer, and those containing colonies were transferred into a T25 culture flask containing 10 mL culture medium. Sheets were cultured in 1% to 10% Cereal Grass Medium (hereafter CGM3) in artificial seawater (hereafter ASW; as in (305)). Cultures were maintained at 22°C under a light-dark cycle (12:12 hours) in a VWR Scientific model 2005 low temperature incubator equipped with a lamp (Venoya Full Spectrum 150W Plant Growth LED) controlled by a programmable timer (Leviton VPT24-1PZ Vizia). For long-term storage, *C. flexa* cultures were frozen and kept in a liquid nitrogen dewar following (306). Cells could be successfully revived by thawing from frozen stocks (306).

3.7.3 Establishment and propagation of the ChoPs (*Choanoeca-Pseudomonas*) monoxenic strain

We define the ChoPs strain (Fig. 3.3B) as a monoxenic culture of *C. flexa* with the co-isolated bacterium *Pseudomonas oceani* (*P. oceani*). ChoPs was established by elimination of all environmental bacteria from the original polyxenic isolate by antibiotic treatment, followed by re-seeding with *P. oceani*. More specifically, (1) a polyxenic isolate was treated with a
combination of 6 antibiotics to prevent growth of environmental bacteria: carbenicillin (100 μg/mL), erythromycin (160 μg/mL), kanamycin (100 μg/mL), lincomycin (200 μg/mL), rifampicin (20 μg/mL), and streptomycin (200 μg/mL).

To confirm that the antibiotic combination prevented proliferation of all co-isolated bacteria, we inoculated 100 μL of the original polyxenic culture, with or without antibiotics, into 5 mL 1% CMG3 with 20 μg/mL cycloheximide (to prevent eukaryotic growth). Cells were grown in a bacterial incubator at 22°C under 300 rpm agitation and cell growth was monitored by measuring 600 nm optical density.

Figure 3.M1. Growth of environmental bacteria co-isolated with C. flexa, monitored by optical density measurements, in the presence or in the absence of antibiotics.

To isolate P. oceani, 70 μL of the original polyxenic strain were spread on a 4% agar plate supplemented with CGM3 culture medium. A single colony was picked, grown in 5 mL 100% CGM3 for 3 days, and identified by 16S sequencing as P. oceani. We then passaged the antibiotic-treated C. flexa polyxenic line 5 times at 1:10 dilution in the 6-antibiotic combination, re-seeding every time with 1:20 volume of P. oceani culture (to progressively dilute out all other bacterial species, and feed choanoflagellates with a constant supply of pre-grown bacteria). After 5 passages, antibiotic treatment was replaced by treatment with 20 μg/mL rifampicin only (to which P. oceani is resistant) and active re-seeding with novel P. oceani was stopped. Monoxenicity of the resulting ChoPs line was validated by PCR amplification of the 16S locus followed by sequencing of 50 individual clones, as well as by iTag sequencing (Table S1 and section “Characterization of bacterial communities by iTag sequencing”). ChoPs was cultured in the constant presence of rifampicin to prevent contamination.

3.7.4 C. flexa DNA extraction, rDNA locus amplification and phylogenetic analysis

C. flexa was grown to 10^6 cells/mL in 200 mL culture medium in 6-layer culture flasks, and pelleted by centrifugation for 15 minutes at 2,000 rpm at 4°C. DNA was extracted using the DNEasy Blood & Tissue Kit (Qiagen 69504). The 18S locus was amplified by PCR with Q5 DNA Polymerase (New Englands Biolab M0491L) and the following degenerate primers:
<table>
<thead>
<tr>
<th>PCR Type</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>First PCR</td>
<td>CTCAARGAYTAAGCCATGCA</td>
<td>CCGCCCCAGYCAAACTCCC</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>GAAACTGCGAATGGCT</td>
<td>ACCTACGAAACCTTGTTACG</td>
</tr>
</tbody>
</table>

**Table M1.** Primer sequences for 18S cloning.

The following PCR programs were used: (1) for the first round of PCR: 1. 98 °C, 2 minutes; 2. 98 °C, 10 seconds; 3. 48 °C, 30 seconds; 4. 55 °C, 30 seconds; 5. 72 °C, 3 minutes; 6. repeat steps 2-5, 30x total; 7. 72 °C, 11 minutes 11 seconds; (2) for the nested PCR: 1. 98 °C, 2 minutes; 2. 98 °C, 10 seconds; 3. 48 °C, 30 seconds; 4. 52 °C, 30 seconds; 5. 72 °C, 1 minute; 6. repeat steps 2-5, 35x total; 7. 72 °C, 7 minutes 21 seconds.

rDNA sequences from *C. flexa* and other opisthokonts were aligned with ClustalX 2.0 (307), trimmed with gBlocks ([http://phylogeny.lirmm.fr/phylo.cgi/one_task.cgi?task_type=gblocks](http://phylogeny.lirmm.fr/phylo.cgi/one_task.cgi?task_type=gblocks)) under minimally stringent parameters, and a Maximum Likelihood phylogenetic tree was produced with SeaView v. 4.7 (308) using PhyML with the GTR model, empirical nucleotide equilibrium frequencies, optimized proportion of invariable sites, optimal rate variation across sites, and best of NNI & SPR tree search method. Trees were visualized and edited with FigTree v. 1.4.4 ([http://tree.bio.ed.ac.uk](http://tree.bio.ed.ac.uk)) and further edited with Adobe Illustrator CC 2018.

**3.7.5 C. flexa RNA extraction, transcriptome sequencing and assembly**

*C. flexa* was grown to 10⁶ cells/mL in 200 mL culture medium in 6-layer culture flasks and pelleted by centrifugation for 15 minutes at 2,000 rpm at 4°C. Cells were lysed by selective lysis as in (301) and RNA was extracted using a RNEasy kit (Qiagen 7404). 150 paired-end RNAseq libraries were prepared after poly-A selection by the QB3 Functional Genomics Laboratory at UC Berkeley with poly-A selection and sequenced on a HiSeq 4000 sequencer ([Illumina, San Diego, California, United States](http://www.illumina.com)) at the Vincent J. Coates Genomics Sequencing Laboratory at the California Institute for Quantitative Biosciences (Berkeley, California, United States). Read quality was assessed with FastQC ([https://www.bioinformatics.babraham.ac.uk/projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) and transcriptome assembly was performed as in (22) with Trinity v. 2.5.1, with the ‘-trimmomatic’ option and ‘-min_contig_length’ set to 150. Predicted protein sequences were generated with Transdecoder (309) with a predicted minimum protein sequence length of 50 aminoacids and redundant protein sequences were eliminated using CD-HIT (310). The reads generated were uploaded onto the NCBI website with the BioSample accession number SAMN11533889, BioProject ID PRJNA540068, and SRA accession number PRJNA540068. Transcriptome and non-redundant predicted proteome are available on Figshare at [DOI: 10.6084/m9.figshare.8216291](https://doi.org/10.6084/m9.figshare.8216291). Phylogenetic trees for proteins of interest were generated following the same procedure as for rDNA (see above ‘C. flexa genome extraction, rDNA locus amplification and phylogenetic analysis’) with aminoacid sequences instead of nucleotide sequences.

**3.7.6 Characterization of bacterial communities by iTag sequencing**

A *C. flexa* culture was pelleted at 4500xg and bacterial DNA was extracted using a DNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer’s Gram-Positive Bacteria Protocol. Library construction and sequencing were then performed by the UC Berkeley Functional Genomics Laboratory. Amplicon library construction was carried out in two distinct PCR steps:
briefly, PCR1 used modified gene-specific primers to create amplification products of the V5-V6 region of the bacterial 16S gene flanked by stub sequences (to provide priming templates for PCR2); PCR2 primers added dual-matched index sequences, sequencing primer binding sites, and Illumina p5 and p7 adapter sequences to the 5' and 3' ends of each amplicon.

PCR1 was carried out in 25 μl reactions including 1 μl genomic DNA, 0.5 μl of each 10 μM primer, 10 μl 2.5x 5PRIME HotMasterMix (QuantaBio, Beverly, MA), 1μl BSA (New England Biolabs, Ipswitch, MA) (to a final concentration of 10μg/μL) and 12 μl nuclease free water. We amplified the 16S V4 hypervariable region using the primer set 515f from Parada et. al (311) (GTGYCAGCMGCGCGGTAA) and 806r from Apprill et al. (312) (GGACTACNVGGGTWTCTAAT). All PCR reactions were set up on ice and using Hot Start polymerase master mix to minimize non-specific amplification and primer dimerization. PCR conditions were: denaturation at 94°C for 3 mins; 30 amplification cycles of 45 sec at 94°C, 1 min at 50°C and 90 sec at 72°C; followed by a 10 min final extension at 72°C. PCR products were visualized for successful amplification and correct sizing using gel electrophoresis.

PCR2 was carried out in 25 μl reactions including 5 μl genomic DNA, 2 μl of combined, μM forward and reverse indexing primers, 10 μl 2.5x 5PRIME HotMasterMix (QuantaBio, Beverly, MA), and 8 μl nuclease free water. PCR2 primers added dual--matched 8bp unique barcodes to each end of the amplicons, such that each forward and reverse primer pair carried the same index sequence. PCR conditions were: denaturation at 94°C for 3 mins; 8 amplification cycles of 45 sec at 94°C, 1 min at 52°C and 90 sec at 72°C; followed by a 10 min final extension at 72°C. PCR products were visualized and quantified using an Advanced Analytical Fragment Analyzer (Agilent, Santa Clara, CA), and individual samples were pooled equimolarly based on Fragment Analyzer concentrations.

The final pool of all individually indexed amplicon libraries was cleaned with Agencourt AMPure XP magnetic beads using a 0.8X bead ratio. The cleaned pool was quantified with qPCR using the KAPA Illumina Library Quant Kit and Universal qPCR Mix (KAPA Biosystems, Wilmington, MA). Amplicon libraries were sequenced on an Illumina MiSeq 300PE v3 run spiked with 10% PhiX in order to achieve sufficient sample heterogeneity.

3.7.7 Light microscopy

Sheets were imaged in FluoroDishes (World Precision Instruments FD35-100) by differential interference contrast (DIC) microscopy using a 40x (water immersion, C-Apochromat, 1.1 NA), 63x (oil immersion, Plan-Apochromat, 1.4 NA), or 100x (oil immersion, Plan-Apochromat, 1.4 NA) Zeiss objective mounted on a Zeiss Observer Z.1 with a Hamamatsu Orca Flash 4.0 V2 CMOS camera (C11440-22CU).

3.7.8 Transmission electron microscopy (TEM)

Sheets were concentrated by centrifugation (200xg for 5 min) and then resuspended in 5% BSA in artificial seawater. The resuspended sheets were then high pressure frozen using a Leica EM PACT2 and fixed by freeze substitution in 0.01% OsO₄ + 0.2% uranyl acetate in acetone. Samples were resin embedded in Epon Araldite (Embed-812) (313), cut into 80 nm sections, and then imaged using an FEI Tecnai 12 transmission electron microscope.

3.7.9 Scanning electron microscopy (SEM)
*C. flexa* sheets were concentrated by pelleting 6 mL of culture for 15 minutes at 200 g and gently resuspending the pellet in 600 μL final volume. Sheets were then pipetted onto silicon wafers coated with poly-D-lysine (Sigma Aldrich P6407-5MG), fixed for 2 hours in 2% glutaraldehyde in 0.1M Sodium cacodylate buffer pH 7.2, rinsed 3 times (for 15 minutes each) in 0.1M sodium cacodylate buffer pH 7.2, post-fixed for 2 hours in 1% osmium tetroxide in 0.1M sodium cacodylate buffer pH 7.2, and rinsed 3 times (for 5 minutes each) in 0.1M sodium cacodylate buffer, pH 7.2. Samples were dehydrated in the following steps: 35% ETOH (5 min), 50% ETOH (5 min), 70% ETOH (5 min), 80% ETOH (10 min), 95% ETOH (10 min), 100% ETOH (10 min) and 100% ETOH (10 min). Samples were critical point dried for 60 minutes on a Tousimis AutoSamdri 815 Critical Point Dryer, mounted on stubs using conductive carbon tape, and sputter coated before imaging on a Hitachi S-5000 Scanning electron microscope.

### 3.7.10 Sheet fixation and FM143-FX/phalloidin/immunofluorescence stainings

FluoroDishes (World Precision Instruments FD35-100) were pre-treated with a handheld Corona surface treater (Electro-Technic Products BD-20AC), coated with poly-D-lysine (Sigma Aldrich P6407-5MG) diluted to the provider’s specifications, and rinsed twice in ASW. Sheet colonies were then transferred into the treated dishes and left to adhere to the bottom surface for 30 minutes. Fixation was performed by adding 1:3 volume ice-cold 16% paraformaldehyde (PFA; reference) to the FluoroDish (to a final concentration of 4%) for 2 hours at room temperature.

#### 3.7.10.1 FM143-FX staining

Sheets were fixed with 5 μg/mL FM143-FX together with PFA in the fixation solution. This procedure was chosen because FM143-FX was observed to quickly trigger dissociation of live sheets, but to be compatible with the preservation of fixed samples. The fixation solution was then washed out three times carefully with ASW and the samples were imaged directly in FluoroDishes.

#### 3.7.10.2 Phalloidin staining

Cells were stained with 0.66 units/mL Alexa 488-phalloidin (ThermoFischer Scientific A12379) or rhodamine-phalloidin (ThermoFischer Scientific R415) in 0.3% Triton X/ASW overnight and under agitation at 4°C. The fixation solution was then washed out three times carefully with ASW and the samples were imaged directly in FluoroDishes.

#### 3.7.10.3 Immunofluorescence

Samples were blocked for 30 minutes at room temperature with blocking solution (1% bovine serum albumin in PEM buffer (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM MgSO4)/0.3% Triton-X), and then stained with antibodies diluted in blocking buffer (see below). For antibodies against the heavy chain of myosin (or full-length myosin), we selected antibodies that were reported to bind both smooth and striated myosin isoforms, based on the following reasoning: as the divergence between both myosin paralogs predates the choanoflagellate/animal divergence, we expected any antibody that broadly targets both smooth
and striated myosin of animals to be likely to bind to the choanoflagellate myosin heavy chain as well. Additionally, we investigated antibodies targeting the myosin regulatory light chain (either in all its forms or in its phosphorylated form only), motivated by its high level of sequence conservation (>80% identity between *C. flexa* and *H. sapiens*) and by conservation of the Ser19 phosphorylation residue. The following antibodies were tested:

<table>
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<th>Provider</th>
<th>Antibody</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmental Hybridoma Bank</td>
<td>CMII23</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>Sigma Aldrich</td>
<td>M7648</td>
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<td>Abgent</td>
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<tr>
<td>Bioss Inc.</td>
<td>bs-3295R</td>
<td>pSer19-Myosin Regulatory Light Chain</td>
</tr>
<tr>
<td>Sigma Aldrich</td>
<td>F1804-50UG</td>
<td>FLAG (negative control)</td>
</tr>
</tbody>
</table>

Primary antibodies were diluted in blocking solution to the manufacturer’s specifications. Primary antibody incubation was performed overnight at 4°C under agitation. Samples were then washed three times in PEM buffer, incubated with secondary antibodies diluted 1:300 in blocking solution (Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 488) for 2 hours at room temperature, washed three times with PEM, and imaged directly in FluoroDishes.

All samples were imaged using a Zeiss LSM 880 Axio Examiner with Airyscan and a 63x, 1.4 NA C Apo oil immersion objective (Zeiss) and excitation provided by a 488 nm laser (Zeiss).

### 3.7.11 Light-induced sheet inversion assays

Light-controlled inversion assays were performed in 24-well plates (Fischer Scientific 09-761-146) containing 2 mL sheet culture per well and imaged with a Leica DMIL LED transmitted light microscope coupled to a Leica DFC 350FX camera. Images were acquired with the MicroManager software at a frame rate of 1 image/second. The “light on” condition corresponded to maximal illumination by the microscope lamp and imaging with 1 ms exposure time. The histogram of detected light intensities was monitored with MicroManager and was found, under these conditions, to be a narrow bell curve centered around 50% light intensity. The “light off” condition was established by raising the exposure time to 400 ms and manually decreasing light intensity until the histogram of detected light intensities closely matched the one of the “light on” condition (sharp peak at 50%) – thus indicating that light intensity had been reduced down to close to 1:400 of its initial value. Each sheet was imaged for 120 seconds (55 to 60 seconds with the light off and 60 to 65 seconds after the light was switched off). The light reduction procedure itself lasted about 1 second (resulting in one white slice (see Movie S5) which was excluded from the area quantification pipeline – see below). This protocol was found to reliably induce inversion of polyxenic sheets during the sheets’ subjective day.

### 3.7.12 Sheet area quantification and image analysis

Sheet projected area was quantified in ImageJ 1.46r. Stacks produced by live imaging of sheets undergoing light-induced inversion were cropped around the sheet of interest, split into 2 parts (before and after switching off the light, to correct for minimal differences in exposure), and
processed with the “Make Binary” command followed by 2 to 5 iterations of the “Close” command and “Dilate” command in succession. Iterations were stopped when the resulting shape contained no gaps and were performed an identical number of times for both parts of each movie. Areas were quantified by measuring the mean gray value on each slice with the measureStack plugin (http://www.optinav.info/MeasureStack.htm). Resulting measurements were stored in .xls files and combined into a single table using a Python or R script (3.14). Area=f(time) curves were plotted using the ggplot2 package in R studio (https://ggplot2.tidyverse.org/). Measurements were smoothened using a rolling average over a 5-second time window. For each individual sheet, areas were normalized by either (1) for the “light on” time window, the time average of areas measured over the entire window or (2) the initial area (for the “light off” time window).

### 3.7.13 Drug treatments and all-trans-retinal rescue assays

<table>
<thead>
<tr>
<th>Name (target)</th>
<th>Stock concentration</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX (PDE)</td>
<td>1 M in DMSO</td>
<td>1 mM</td>
</tr>
<tr>
<td>Caffeine (PDE)</td>
<td>77 mM in H₂O</td>
<td>5 mM</td>
</tr>
<tr>
<td>Y-27632 (ROCK kinase)</td>
<td>14 mg/mL in H₂O</td>
<td>43.7 μM</td>
</tr>
<tr>
<td>ML-7 (MRLC kinase)</td>
<td>10 mg/mL in DMSO</td>
<td>22 μM</td>
</tr>
<tr>
<td>All trans-retinal</td>
<td>88 mM in ethanol</td>
<td>88 μM</td>
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<tr>
<td>Latrunculin B (F-actin)</td>
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<td>Blebbistatin (myosin II)</td>
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<tr>
<td>8-Br-cGMP</td>
<td>50 mg/mL in H₂O</td>
<td>1E-5M to 1E-3M</td>
</tr>
</tbody>
</table>

Sheets were pre-treated with actomyosin and ion channel inhibitors for 30 minutes before behavioral assays. Y-27632 did not observably affect inversion. EGTA was observed to instantly prevent sheet inversion after addition and, on long time scales (<30 minutes), led to sheet dissociation. All behavioral assays with EGTA were performed <1 minute after addition in the culture medium. Finally, caffeine, IBMX, and 8-Br-cGMP were observed to induce inversion in a few seconds after addition (if the culture medium was actively mixed by gentle swirling). For quantification of the number of inverted sheets, sheets were fixed by addition of 1:3 volume ice-cold 16% PFA, and manually counted under a Leica DMIL LED transmitted light microscope.

### 3.7.14 Bacterial rescue protocol

#### 3.7.14.1 Isolation of bacteria from monoxenic and polyxenic cultures

To isolate bacteria from the monoxenic ChoPs culture (*P. oceani*) or polyxenic culture (“env. bacteria”), 20 μl of either culture was inoculated into 100% CGM3 with 20 μg/ml cycloheximide (to inhibit eukaryotic growth). Cultures were grown shaking at 22 degrees for 48 hours. Bacteria were then pelleted at 20,000xg, washed with ASW, and resuspended to 1 O.D.600/ml in ASW.

#### 3.7.14.2 Pre-treatment of recipient culture
To reduce bacterial load, the recipient ChoPs culture was treated for 24 hours with an antibiotic cocktail (20 μg/ml rifampicin, 200 μg/ml streptomycin, 100 μg/ml kanamycin, 160 μg/ml erythromycin, 100 μg/ml carbenicillin, and 200 μg/ml lincomycin). After 24 hours, choanoflagellates were pelleted at 1000xg, washed once in ASW, then resuspended in 10%CGM3.

3.7.14.3 Bacterial rescue

Pre-treated Chops cultures were seeded into 10% CGM3 at 4E4 cells/ml; isolated bacteria (either *P. oceani* or “env. bacteria”) were diluted into the culture at 1:1000 and grown for 48 hours. Cultures that received *P. oceani* were subsequently treated with 20ng/μl rifampicin for one week to ensure absence of contaminants. Photic response was then quantified as described below (see “Light-induced sheet inversions assays”).

3.7.15 Retinal rescue protocol

Monoxenic ChoPs culture was grown in 10% CGM3 + 20 ng/μl rifampicin + 0 nM, 125 nM or 500 nM all-trans-retinal. After one week treatment, photic response was quantified as described above (see “Light-induced sheet inversion assays”).

3.7.16 Sheet dissociation

A dense (1E6 cells/mL) sheet culture was reconcentrated 10x by centrifugation (2,000 rpm for 15 min at 4°C) and dissociated by vortexing for 30 seconds followed by filtration through a Tisch Scientific syringe-top 5 μm filter (SPEC18191). Dissociated cells were immediately transferred into an Ibidi 8-well plate imaging chamber (80826) coated with poly-D-lysine and washed twice with ASW (200 μL of cell suspension per chamber).

3.7.17 Quantification of actin ring diameter

Imaging of phalloidin labeled samples (both single cells and intact sheets) for actin ring quantification was performed as detailed in the “Sheet fixation and FM143-FX/phalloidin/immunofluorescence stainings” section. Actin ring quantification for both intact and dissociated sheets was performed using Imaris v3.8 (Bitplane, Belfast). Images were first segmented with a global threshold chosen manually for each condition on one image and then held constant for all subsequent images. Diameters were measured using the “Measure Points” function, with three diameters manually chosen and the longest measured distance chosen as the ring diameter. Only completely intact rings with associated intact collars were chosen for analysis.

3.7.18 Sheet tracking

Sheets were imaged on a Zeiss Axio Zoom.V16 (generously lent by Zeiss to the 2018 Physiology course at the Marine Biology Laboratory in Woods Hole). Individual sheets were tracked on Fiji v. 2.0 (315) using the Manual Tracking option of the Tracking plugin.

3.7.19 Movement quantification
Movies of sheets swimming were produced on a Leica DMIL LED transmitted light microscope coupled to a Leica DFC 350FX camera, and movement was quantified by measuring inter-frame Pearson correlation using RStudio (316). Sheet inversion was induced by a rapid (ca. 1 sec transition time) reduction in light intensity (see “Light-induced sheet inversion” section).

3.7.20 Microbead feeding assays, flow visualization and quantification

0.2 μm green fluorescent microbeads were used for both feeding assays and flow visualization (FluoSpheres™ Carboxylate-Modified Microspheres, 0.2 μm, red fluorescent (580/605), 2% solids; ThermoFischer Scientific F8810) at a 1:100 dilution. For flow visualization, live sheets were observed in FluoroDishes on a Zeiss Z.1 Observer (see “Light microscopy” section). For feeding assays, 1 mL sheet culture were pipetted into wells in a 24-well plate with microbeads at 1:100 dilution, and put on a Reliable Inc. rocking shaker (see “Mechanically induced sheet inversion assays”) under level 10 agitation for 1 hour. Sheets were then fixed by addition of 1:3 volume 16% PFA at 4°C (to a final concentration of 4% PFA) and carefully transferred into poly-D-lysine-coated FluoroDishes for observation, using a P1000 micropipette with a truncated pipet tip (to limit shear forces). Sheets were imaged on a Zeiss Z.1 Observer in both DIC (to observe cells) and green fluorescence (to reveal beads), and cells having phagocytosed beads were manually counted for each sheet.

3.7.21 Phototaxis assays and analysis

Phototaxis assays were performed in Ibidi 8-well plate imaging chambers (80826). Wells were completely filled with dense C. flexa cultures, and a coverslip (FisherScientific, 12-545-D) was used to cover the well in order to both prevent evaporation and flatten the background illumination intensity profile over the full extent of the well. Samples were then allowed to settle in ambient overhead light conditions for 30min. Next, a phase contrast reference image of the entire well was acquired using a Zeiss Z.1 Observer and Hamamatsu Orca Flash 4.0 V2 CMOS camera (C11440-22CU) by tiling with a 10x, 0.45 NA Plan-Apochromat (Zeiss) objective. The room was then darkened by turning off or blocking all sources of light. Except for the negative control condition, samples were then immediately exposed to directional illumination supplied by a white LED (Elegoo) powered by an Elegoo Uno R3 Arduino supplying 5V through across a 10 kΩ (Elegoo) resistor and focused onto one edge of the well containing the sample using a 15x magnification lens (TV-15 Triview, Carson Optical). This illumination scheme created a conical gradient of decreasing light intensity along the well in the direction of illumination due to increasing distance from the focal point at the edge of the well. Negative controls received no directional illumination. After one hour, a final image of the entire well was acquired as before. For single cell phototaxis experiments, prior to the settling step, sheets were dissociated by vortexing in a 1.5 mL Eppendorf tube using a Vortex Genie 2 (Scientific industries) for one minute.

All image processing was performed using Fiji (315). First, images were background subtracted using Gaussian blurred (σ = 100 pixles) duplicate images. Images were then cropped to remove the edges of well, and a five-pixel median filter was applied. Images were then imported into MATLAB release 2016a (Mathworks, Natick) where subsequent analysis was performed. For each pair of reference and final images (see “Phototaxis assays” section), a phototaxis index (PI) was calculated, with PI defined as:
where $I_{pr}$ is the integrated intensity of the first 1/3 of the reference image proximal to the phototaxis light source, $I_{dr}$ is the integrated intensity of the 1/3 of the reference image most distal to the phototaxis light source, $I_{pf}$ is the integrated intensity of the first 1/3 of the final image proximal to the phototaxis light source, and $I_{df}$ is the integrated intensity of the 1/3 of the final image most distal to the phototaxis light source. Because choanoflagellate cells are bright under phase contrast, PI is a readout of the relative change in abundance of choanoflagellate cells in the region proximal versus distal to the light source over the course of the phototaxis assay.

3.7.22 Live imaging of Diaphanoeca grandis, Salpingoeca rosetta, Salpingoeca urcoelata and Monosiga brevicollis

*D. grandis, S. rosetta, S. urceolata* and *M. brevicollis* cultures were obtained by thawing frozen stocks stored in liquid nitrogen in the King lab (following (306)) and maintained in 1% CGM3/ASW medium. Live cells were mounted in FluoroDishes treated with a handheld Corona surface treater, coated with poly-D-lysine, and rinsed three times with ASW. Imaging was performed in DIC optics at 100x magnification on a Zeiss Observer Z.1 platform using a Hamamatsu Orca Flash 4.0 V2 CMOS camera (C11440-22CU).
Figure 3.1. *Choanoeca flexa* sp. nov., a choanoflagellate discovered in splash pools on the island of Curaçao, forms colonies that rapidly and reversibly invert their curvature.
(A-C) *Choanoeca flexa* was discovered in splash pools on the northern shore of Curaçao. (A) Map of the Caribbean Sea with arrowhead highlighting the island of Curaçao. Inset, magnified map of Curaçao with arrowhead indicating the sampling site (12°14’12.1” N, 69°01’34.8” W). (B) Photograph of the sampling site. Water samples were collected from splash pools (such as that indicated by the red arrow) within reach of ocean spray. (C) Light microscopy of freshly collected splash pool samples revealed a diverse microbial eukaryotic community, including dinoflagellates (*Oxyrrhis* sp.; arrowhead) and cup-shaped colonies of a previously unknown choanoflagellate (arrows), each comprising a monolayer of uniflagellate cells, that appeared to spontaneously invert their curvature (see Movie S1). The species name *flexa* was given in reference to this striking behavior. Shown is a still frame from Movie S1, recorded in Curaçao soon after sample collection. (D) *C. flexa* was recognizable as a choanoflagellate based on its cell morphology, visualized here by DIC microscopy. Each cell has a single apical flagellum surrounded by a collar of microvilli. (E and F) *C. flexa* colonies can adopt two distinct orientations, flagella-in and flagella-out, as visualized here through three-dimensional reconstruction of fixed colonies that were stained with a membrane marker (FM1-43FX). In the flagella-in orientation (E), the flagella project into the interior of the approximately hemispherical colony. In the flagella-out orientation (F), the flagella point outward from the hemisphere (same scale as E). (G and H) *C. flexa* colonies rapidly and reversibly invert their curvature while maintaining contacts among neighboring cells. (G) A flagella-in colony inverts to the flagella-out orientation and seals into a nearly closed sphere over the course of six seconds. Shown are still frames from Movie S2. (H) A flagella-out colony reverts to the flagella-in orientation through a similar process, as shown in still frames from Movie S3. Some frames have been rotated to facilitate tracking individual cells between images. (I) Summary schematic depicting the inversion process (in which sheets transit from flagella-in to flagella-out) and of the converse relaxation process (from flagella-out to flagella-in). (J) Phylogenetic analysis of 18S rDNA sequence confirmed that *Choanoeca flexa* (red arrow) is nested within the choanoflagellates and revealed that it is sister to the species *Choanoeca perplexa* (254). Fig. 3.S1 shows the phylogeny with branch lengths to scale.
Figure 3.2. Light-to-dark transition induces *C. flexa* colony inversion. (A-C) Colony inversion can be tracked across a population at low magnification because it correlates with a decrease in the projected area of each colony. Time-lapse microscopy (Movie S4) shows a single *C. flexa* colony as it spontaneously inverted from the flagella-in (A) to flagella-out (B) orientation. Insets correspond to the boxed region of the colony, with pseudocoloring to highlight the orientation of the cell and its apical flagellum. Importantly, the orientation of the flagellum relative to the curvature of the colony inverts without the cell breaking its contacts with neighboring cells. (C) Projected surface area of the colony shown in panels (A) and (B) plotted as function of time. This inversion corresponded to a 50% decrease in area over ~30 seconds. Normalized sheet area is defined as the area of a sheet divided by its initial value (at t = 0). (D) Colonies sense and respond
to changes in light intensity, reliably undergoing inversion in response to light-to-dark transitions. Colony inversion can be quantified as a decrease in projected area. Area for $n = 5$ colonies, normalized by the initial value for each colony (at $t=0$), is plotted as a function of time before and after light reduction (vertical dotted line). See Movie S5 for a representative example. The line represents the mean projected area (rolling average over 5-second windows) and the ribbon represents standard deviation.
Figure 3.3. *C. flexa* cells transduce light stimuli through a rhodopsin-cGMP pathway using bacterial carotenoids. (A) RhoPDE (blue), a choanoflagellate-specific enzyme rhodopsin and a candidate regulator of sheet inversion in response to light-to-dark transitions. *C. flexa* encodes four putative homologs of RhoPDE (Fig. 3.3), each comprising a type I (bacterial) rhodopsin (“RhoI”) fused to a cyclic nucleotide phosphodiesterase (“PDE”). Photodetection by rhodopsin requires the cofactor retinal (yellow hexagon, “R”), a covalently bound chromophore that undergoes isomerization in response to light (263). When light levels are high (left panel), the
rhodopsin domain activates the PDE domain, which hydrolyzes cGMP to 5’GMP (255–258), keeping cellular cGMP levels low. When light is reduced (right panel), the PDE domain is inactive, allowing cellular cGMP levels to rise. We show below (E) that increased cellular cGMP leads to inversion. (B) A bacterially produced factor is required for light-regulated sheet inversion. C. flexa sheets were grown in the presence of different combinations of bacteria and their photic response was quantified as in Fig. 3.2D. C. flexa sheets grown in a “Polyxenic” culture containing diverse co-isolated environmental bacteria (Table 3.S1) inverted to flagella-out in response to decreased illumination, as measured by a decrease in colony projected area. By contrast, sheets grown in a monoxenic culture that contains only C. flexa and the bacterium P. oceani (“ChoPs” culture), did not respond to changes in illumination. When the ChoPs culture was inoculated with environmental bacteria from the polyxenic culture (“ChoPs + env. bacteria”), the photic response was restored, showing that a bacterial factor is necessary for light-regulated inversion. As a negative control, ChoPs inoculated with only P. oceani bacteria (“ChoPs + P. oceani”) did not show restoration of the inversion response. Shown are data from n = 4 ChoPs colonies, n = 3 Polyxenic colonies, n = 3 colonies of ChoPs + env. bacteria, and n = 3 colonies of ChoPs + P. oceani. (C) The rhodopsin chromophore retinal (or its carotenoid precursors) is the bacterial molecule required for the photic response. The ChoPs culture, which is insensitive to changes in illumination, was treated with varying concentrations of retinal, and the photic response was quantified as in Fig. 3.2D. Treatment with 125 nM or 500 nM retinal was sufficient to restore light-regulated inversion. Thus, the light-insensitive phenotype of the ChoPs strain is due to the absence of a bacterial carotenoid, which indicates that rhodopsin activity is required for inversion in response to light-to-dark transitions. Shown are data from n = 4 ChoPs colonies, n = 4 Polyxenic colonies, n = 5 colonies of ChoPs + 125 nM retinal, and n = 5 colonies of ChoPs + 500 nM retinal. (D) Phosphodiesterase activity suppresses sheet inversion in C. flexa. Treatment with the phosphodiesterase inhibitors caffeine (10 mM) or IBMX (1 mM) caused C. flexa colonies to invert to the flagella-out orientation in the absence of a photic stimulus (n = 3 independent trials; N = 52, 55, and 38 for controls; N = 23, 31, and 40 for caffeine; N = 42, 37, and 27 for IBMX). (E) cGMP acts as a second messenger in the C. flexa phototransduction pathway. Treating the light-unresponsive ChoPs culture with increasing concentrations of a cell-permeant cGMP analog (8-Br-cGMP) caused sheets to invert into the flagella-out orientation in a dose-dependent manner in the absence of a photic stimulus. By contrast, treating with 8-Br-cAMP did not have an effect. Thus, increased cellular cGMP concentration is sufficient to cause sheet inversion.
**Figure 3.4. Sheet inversion results in a trade-off between swimming and feeding.** (A and B) Flagella-out sheets swim faster than flagella-in sheets. (A) Following light-to-dark induced inversion, flagella-out sheets swam faster than they did in their relaxed, flagella-in form. Swimming speed increased quickly after darkness-induced inversion (Movie S7), as quantified by an increase in the measured amount of movement. Movement was defined as 1-correlation (where “correlation” refers to the Pearson correlation between two consecutive frames of a given movie; see Material and Methods). Movement was normalized between 0 and 1 for each of $n = 9$ time-lapse movies. Error bars represent standard deviation. (B) Sheets swim faster after caffeine-induced inversion. Here, caffeine treatment (5 mM) was used to chemically induce inversion in all sheets under constant light, presumably by inhibiting PDE activity. Caffeine treatment thus enabled sustained experiments with inverted sheets by preventing relaxation. Movement was quantified as in (A). $n = 9$ time-lapse movies for the control condition (populations of relaxed sheets imaged under constant ambient light) and $n = 10$ movies of sheet populations in which inversion has been induced by 5 mM caffeine. (C-G) Flagella-in sheets feed more efficiently than flagella-out sheets. Choanoflagellates feed by phagocytosis of bacterial prey captured from the water column. Internalization of 0.2 μm fluorescent beads was used to quantify phagocytic activity of cells in sheets. (C-F) Detection of beads phagocytosed by flagella-in sheets (untreated) and flagella-out sheets (treated with 5 mM caffeine) that were fixed after incubation for 1 hour with fluorescent microbeads. Cells were visualized by DIC transmitted light (C and E) and beads by green fluorescence (C-F). Arrowheads: fluorescent beads (inside the cells in C, stuck to a flagellum in E). (G) Proportion of cells having phagocytosed beads in $n = 17$ sheets with flagella in compared to $n = 21$ sheets with flagella out. $p$-value is by the chi-square test. (H) Sheet phototaxis requires retinal and multicellularity. Polyxenic sheets (capable of perceiving light) migrate toward a lateral light source over 1 hour ($n = 12$ experiments). By contrast, no directional accumulation was observed in polyxenic sheets without directional light ($n = 12$ experiments). Interestingly, dissociated single cells ($n = 9$ experiments) were not capable of phototaxis. Likewise, retinal-deprived monoxenic cultures (ChoPs) that do not invert in response to light-to-dark transitions are also incapable of phototaxis ($n = 10$ experiments). $p$-values are by an ANOVA with Dunnett’s correction. (I) Model of how sheet inversion mediates a swimming-feeding tradeoff. In flagella-in sheets, flagellar beating generates a feeding flow that carries bacteria toward the basal side of the cells (Fig. 3.S7), allowing phagocytosis. In flagella-out sheets, flagellar beating allows swimming, and the basal side of the cells faces the inner side of the colony, preventing it from coming in contact with bacterial prey.
Figure 3.5. Sheet inversion requires apical actomyosin cell contraction. (A and B) Cells within a sheet are linked by direct contacts between their collars. (A) Transmitted light DIC microscopy image, showing direct contact between the microvillous collars of neighboring cells. (m): microvilli, (f): flagellum. Dotted line indicates approximate plane of transverse section in panel B. (B) Transmission electron microscopy of a transverse section through the microvillar collars of neighboring cells reveals close contacts microvilli. (C) Collar morphology differs between flagella-in sheets (top) and flagella-out sheets (bottom). Cells in flagella-in sheets have collars that are cylindrical (with the microvilli nearly parallel) while the microvilli of flagella-out sheets are flared, producing conical collars. Collars were stained with fluorescent phalloidin in fixed samples and imaged by confocal microscopy. Both panels are maximum projections of Z-stacks. (D-G) Caffeine treatment of dissociated cells caused the collar to flare out, resembling the collars found in flagella-out sheets. (D and E) Two representative individual cells imaged by DIC microscopy, either without (D) or with (E) caffeine treatment. Arrowheads: microvilli. (F) The differences in collar morphology where quantified in terms of the collar angle (defined by the tip of two bilateral microvilli and the base of the flagellum). (G) Collar angles in caffeine-treated cells are significantly larger than those in control cells. Collar angles measured in \( n = 16 \) untreated dissociated cells and \( n = 28 \) caffeine-treated cells. Data are presented as violin boxplots, showing the median value (black horizontal lines inside white boxes), interquartile range (white boxes), and total range (thin lines). Surrounding the boxplots are kernel density traces plotted symmetrically (violin plots). \( p \)-value by Mann-Whitney’s U test.

(H) An actin ring connected to a small number of longitudinal fibers is present at the base of each collar, visualized here in a sheet stained with fluorescent phalloidin and imaged by confocal microscopy (maximum projection of a Z-stack). Inset: higher magnification shows actin rings and a longitudinal fiber extending below collar microvilli.

(I-L) The actin ring constricts during inversion in intact sheets and in response to caffeine in isolated cells. (I and J) Actin ring observed by phalloidin staining in an untreated cell (I) and in a cell treated with 5 mM caffeine (J). (K) Ring diameter is consistently larger in flagella-in sheets \( (n = 8 \text{ sheets, } N = 124 \text{ cells}) \) than in flagella-out sheets \( (n = 7 \text{ sheets, } N = 110 \text{ cells}) \). \( p = 1.52*10^{-6} \) by Mann-Whitney’s U test. (L) Ring diameter is consistently smaller in 10 mM caffeine-treated dissociated cells \( (n = 74) \) and 5 mM caffeine-treated dissociated cells \( (n = 82) \) than in untreated dissociated cells \( (n = 89) \). \( p \)-values by Dunnett’s test for comparing several treatments with a control. (M to S) Antibodies against myosin II localize to the apical actin ring, to the longitudinal fibers, and to the base of microvilli in immunostained sheets. (M) Immunostained sheet, (N to P) close-up views showing the apical ring, (Q to S) close-up view showing the fibers and base of microvilli. Green: Sigma M7648 rabbit anti-myosin II antibody, magenta: rhodamine-phalloidin. (Also see Fig. 3.S10.) (T) Treatment with inhibitors of actin polymerization (latrunculin B, \( n = 6 \) colonies), myosin contractility (blebbistatin, \( n = 6 \) colonies), or myosin activation by phosphorylation (ML-7, \( n = 9 \) colonies) prevented sheet inversion in response to light-to-dark transitions. \( n = 13 \) colonies for the DMSO-treated (negative control) condition. Photic response was quantified as in Fig. 3.2D. (U) Summary schematic of the proposed inversion mechanism. Contraction of the apical actomyosin network (comprising ring, fibers, and base of the microvilli) correlates with, and is required for, collar flaring and sheet inversion.
**Figure 3.6. Apical constriction is conserved in choanoflagellates** (A) Spontaneous collar contractions observed in *Salpingoeca urceolata* and *Monosiga brevicollis* by time-lapse DIC microscopy. Dark and gray traces represent cell outline before and after contraction (3 to 7 seconds later), respectively. *S. urceolata* shows global and reversible collar closure correlated with retraction of the cell within its theca (Movie S8). *M. brevicollis* shows transient and reversible opening of its collar (Movie S9). (th): theca. (B) An apical actomyosin ring is detected at the base of the collar in four different choanoflagellate species: *Diaphanoeca grandis, S. urceolata, M. brevicollis* and *Salpingoeca rosetta*. F-actin stained by rhodamine-phalloidin. Myosin II stained...
with the Sigma M7648 antibody. Composite shows overlay of F-actin and myosin II staining patterns. Note that myosin II was generally not detected in the microvilli, except in *S. urceolata*. (m): microvilli (C) Apical constriction of individual cells was present in the last common ancestor of choanoflagellates and animals, and independently gave rise to multicellular apical constriction in *C. flexa* and in animals (see Figs. 6A and S13 for supporting data.). *C. perplexa*, the sister-species of *C. flexa*, can undergo transient inversions of colony curvature that were briefly reported in an earlier study (184). Based on our study of *C. flexa*, we hypothesize that these inversions reflect conservation of collective apical constriction with that observed *C. perplexa*. Unfortunately, the currently available *C. perplexa* strains (from frozen stocks stored in the ATCC and in our lab) no longer form colonies in culture.
3.9 Supplementary Materials

Other Supplementary Materials for this chapter include the following:
Movies S1 to S12

**Figure 3.S1.** Full 18S rDNA phylogenetic tree of *C. flexa*, several other choanoflagellates, and representative outgroup opisthokonts (including animals and fungi). Maximum Likelihood phylogenetic tree of 18S rDNA sequences from *C. flexa* and other opisthokonts. Support values on nodes: approximate likelihood ratio test (aLRT) (317). Nodes with support values <0.5 were collapsed into polytomies (e.g. the branching order between the sponge *Amphimedon queenslandica*, the ctenophore *Pleurobrachia pileus* and other metazoans, which represents a currently uncertain point of higher-order metazoan phylogeny (318)).
**Figure 3.S2. C. flexa encodes four putative homologs of RhoPDE.** (A) Domain architectures of RhoPDE proteins across choanoflagellates. Shown are all choanoflagellate genes found to contain a phosphodiesterase domain and the 7 helices characteristic of bacterial (type I) rhodopsins. Phosphodiesterase domains were annotated using PFAM. Rhodopsin transmembrane helices of the S. rosetta protein were annotated following Lamarche et al. (257); those of the other homologs were determined by alignment with the S. rosetta protein. *The 8th transmembrane helix of A. spectabilis RhoPDE lacks the conserved lysine residue required to covalently bind retinal, and therefore likely lacks rhodopsin activity. (B and C) Comparison of conserved amino acids between S. rosetta RhoPDE, C. flexa RhoPDEs, and various type I rhodopsins and phosphodiesterases. Proteins and residues were selected as described by Yoshida et al. (256). Red and blue letters indicate acidic and basic amino acids, respectively. (B) Comparison of conserved amino acids between choanoflagellate RhoPDEs and selected type I rhodopsins. Notably, all of the C. flexa homologs contain the conserved lysine residue of the 8th transmembrane helix, which is required for covalent binding of the chromophore retinal. (C) Comparison of conserved amino acids between choanoflagellate RhoPDEs and selected cyclic nucleotide phosphodiesterases. The substrate specificities of the phosphodiesterases are indicated in the leftmost column. S. rosetta RhoPDE hydrolyzes cGMP ~10-fold more efficiently than cAMP based on *in vitro* studies (256).
Neither *C. flexa* nor the bacterium *Pseudomonas ocean* encodes the complete retinal biosynthesis pathway. The *C. flexa* transcriptome, as well as the genomes of the bacterial species found in the polyxenic culture (Table S1), were searched for genes encoding enzymes in the retinal biosynthesis pathway using BLASTP. For each step in the pathway, multiple bacterial, plant (“p”), fungal (“f”), and/or animal (“a”) genes were used as queries, and the highest returned score is shown. Notably, *C. flexa* does not encode a recognizable lycopene cyclase, the enzyme that synthesizes beta-carotene, the immediate precursor of retinal. Thus, *C. flexa* must receive retinal or beta-carotene from its food. *Pseudomonas ocean*, the bacterium in the monoxenic ChoPs culture, lacks any recognizable homologs of genes in this pathway, indicating that it likely cannot produce beta-carotene or retinal. The *Bordetella* sp. bacterium shown in Table S1 was not included in this analysis because the precise species could not be identified.
Smooth muscle/ non-muscle myosin heavy chain

Striated muscle myosin heavy chain

Spizellomyces punctatus MRLC (XP_016605380.1)

Amphimedon queenslandica MRLC (XP_011409933.1)

Ephydatia muelleri MRLC (ALB75315.1)

Suberites domuncula MRLC (CAH49894.1)

Oscarella lobularis MRLC (ALB75323.1)

Homo sapiens ML12B

Hydra vulgaris MRLC (XP_002156446.1)

Nematostella vectensis MRLC (XP_001636892.1)

Salpingoeca rosetta MRLC (XP_00499924.1)

Chooanoeca flexa MRLC (TRINITY_DN6526_c1.g1)

Monosiga brevicollis MRLC (XP_001743432).
**Figure 3.S4. Phylogenetic trees of predicted proteins encoded by the *Choanoeca flexa* transcriptome.** (A) Phylogenetic tree of microbial rhodopsin domains, based on sequences compiled by Avelar et al. (300) to which sequences from five choanoflagellates with microbial rhodopsins (*C. flexa* red arrows) and sequences from Richter et al. (22) – see Fig. 3.S2) were added. Choanoflagellate microbial rhodopsins are all fused to phosphodiesterase domain (see Fig. 3.S4) and are sister to a clade of fungal microbial rhodopsins fused to a guanylyl-cyclase, in agreement with the phylogenetic tree reconstructed by Avelar et al (300). (B) Phylogenetic tree of myosin heavy chains (MHC). The *C. flexa* transcriptome appears to encode a single predicted MHC homolog (red arrow) which, like in other studied choanoflagellates (272, 287), belongs to the smooth muscle/non-muscle family. (C) Phylogenetic tree of myosin regulatory light chains (MRLC). The *C. flexa* transcriptome appears to encode a single predicted MRLC homolog.
Figure 3.S5. Sheets with flagella out are more motile than sheets with flagella in. (A) Five sheets observed at low magnification in a constant level of transmitted light (frames from Movie S6). Sheets remained flagella-in and nearly immotile. Colored curves represent tracks of individual sheets. (B) Five different sheets observed in transmitted light following a rapid (~1 sec) reduction in light intensity (frames from Movie S7). Following inversion, the sheets became actively motile. Colored lines represent tracks of individual sheets.
Figure 3.6. The flow field generated by flagellar beating is reoriented during sheet inversion. (A) Feeding flow generated by a sheet with flagella in (visualized by observation of 0.2 μm fluorescent beads in the water and computed by Particle Image Velocimetry; see Material and Methods). The flow is predominantly directed toward the center of the sheet, consistent with it carrying bacterial prey toward the cell bodies. (B) Flow generated by the same sheet after inversion. Note that the flow is predominantly directed parallel to border of the sheet, making it unlikely to support efficient feeding.
Figure 3.S7. Sheet phototaxis requires retinal and multicellularity. Top row: sheets imaged at low magnification at t=0 by transmitted light. Bottom row: sheets imaged at t=1 hour by transmitted light. Polyxenic sheets (cultured with a diversity of co-isolated bacterial species) under directional illumination accumulated toward the side of the flask near the light source (on the right side of the image), while no directional accumulation was observed in ChoPs monoxenic sheets (without retinal and unable to perceive light), polyxenic sheets without directional illumination, and single cells.
Figure 3.8. Neighboring cells within sheets are linked by their collars. (A) Cross-section of a sheet imaged by transmitted electron microscopy. Note the close proximity between neighboring collars (arrows), and the absence of visible basal extracellular matrix, basal filopodia, or intercellular bridges. (B) Sheet imaged by scanning electron microscopy (SEM). Collars are spread out on the EM disk with cell bodies sticking up. Note the direct contact between neighboring collars (arrows) and the lack of any other structure connecting neighboring cells. (C) Contact between microvilli (arrow) belonging to two neighboring cells imaged by SEM. (D) Same individual as (C) imaged at higher magnification. Note the close apposition of the microvilli belonging to two neighboring cells (arrow), which appear closer to each other than microvilli are within the collar of the same cell.
**Figure 3.59. Caffeine treatment results in cell-autonomous collar deformations.**

(A) Schematic summarizing the values measured and calculated to quantify collar shape. 

(B) Caffeine treatment leads to a decrease in collar curvature in dissociated cells. \( n = 30 \) untreated cells, \( n = 35 \) cells treated with 5 mM caffeine, and \( n = 59 \) cells treated with 10 mM caffeine. \( p = 4.6 \times 10^{-5} \) (control vs. 5 mM caffeine) and \( p = 2.9 \times 10^{-6} \) (control vs. 10 mM caffeine) by Dunnett’s test for comparing several treatments with a control. 

(C) Caffeine treatment leads to sliding of the collar toward the basal pole in dissociated cells. \( n = 27 \) untreated cells, \( n = 35 \) cells treated with 5 mM caffeine, and \( n = 57 \) cells treated with 10 mM caffeine. \( p = 1.6 \times 10^{-8} \) (control vs. 5 mM caffeine) and \( p = 5.7 \times 10^{-5} \) (control vs. 10 mM caffeine) by Dunnett’s test. 

(D to F) Untreated (D) and caffeine-treated (E and F) dissociated cells. In untreated cells, the base of the collar (arrowhead) appears close to the apical side of the cell (arrow; where the flagellum emerges), while it is significantly displaced toward the base in caffeine-treated cells. Note the “bottle cell”-like morphology of the cell in (F) (with a narrow apex and a bulbous base), which is characteristic of a fraction of caffeine-treated cells (or cells in intact sheets during inversion) and is considered diagnostic of apical constriction in animal cells (275, 319, 320). 

(G and H) Longitudinal actin (yellow arrowheads) fibers shorten in response to caffeine treatment in dissociated cells. (G) Cells stained for F-actin by fluorescent phalloidin. Yellow arrowheads: longitudinal fibers. (H) Fiber length quantification. \( n = 28 \) control cells, \( n = 16 \) cells treated with 5 mM caffeine, and \( n = 20 \) cells treated with 10 mM caffeine. \( p = 5.2 \times 10^{-5} \) (control vs. 5 mM caffeine) and \( p = 3.9 \times 10^{-8} \) (control vs. 10 mM caffeine) by Dunnett’s test.
Fig. 3.S10. Five distinct anti-myosin II antibodies stain the apical actin ring in *Choanoeca flexa*. Sheets immunostained by the antibody of interest (Developmental Hybridoma Bank CMII23 against full-length myosin; Abgent AP19667a-ev against Myosin Regulatory Light
Chain; and Cell Signalling Technology 3671S and Bioss USA bs-3295r against phosphorylated pS19-Myosin Regulatory Light Chain) and co-stained with rhodamine-phalloidin (targeting F-actin). All antibodies stain a domain nested in (and partly overlapping with) the apical actin ring, while a negative control antibody (Sigma Aldrich F1804 anti-FLAG) gives no detectable pattern under the same imaging conditions and rendering parameters. In sheets stained with the negative control antibody, only unspecific staining in the cell body is apparent if one increases the brightness of the corresponding channel with ImageJ.
Figure 3.S11. Myosin II is required for apical ring constriction in response to caffeine. Ring diameter measured in dissociated cells, untreated, treated with 2 μM blebbistatin (which inhibits myosin II), treated with 5 mM caffeine, or treated with both 2 μM blebbistatin and 5 mM caffeine. Blebbistatin treatment prevents caffeine-induced ring constriction. $n = 164$ control cells, $n = 106$ blebbistatin-treated cells, $n = 77$ caffeine-treated cells, and $n = 99$ cells treated with both blebbistatin and caffeine, respectively. $p = 0.0111$ (caffeine vs. control), $p = 0.0034$ (caffeine vs. blebbistatin) and $p = 0.0097$ (caffeine vs. blebbistatin+caffeine) by Kruskal-Wallis test for multiple comparisons. Other comparisons show no significant difference: $p = 1.00$ (blebbistatin vs. control and blebbistatin vs. blebbistatin+caffeine) and $p = 0.76$ (blebbistatin vs. blebbistatin+caffeine).
Figure 3.S12. Spontaneous deformations of the collar in the choanoflagellates *Diaphanoeca grandis* and *Salpingoeca rosetta*. Time-lapse DIC imaging of live *S. rosetta* cells shows spontaneous reorientation of individual microvilli (Movie S10), notably following an increase in light intensity. In the loricate *D. grandis*, which is encased in a self-secreted silicon-based extracellular lodge, spontaneous and reversible changes in microvilli curvature are observed (Movie S11). Both types of collar deformation can be quantified as a change in collar angle (yellow doted lines).
Figure 3.S13. Phototransduction pathways in choanoflagellates, animals, and fungi.

(A) Many choanoflagellates (Fig. 3.S2) encode a fusion protein, RhoPDE, composed of a type I (bacterial) rhodopsin fused to a cyclic nucleotide phosphodiesterase (PDE). When illuminated, the rhodopsin activates the PDE domain, resulting in hydrolysis of cGMP (255, 256).

(B) A similar rhodopsin-cGMP pathway controls phototransduction in vertebrate photoreceptor cells. Light activates a type II (eukaryotic) rhodopsin in the disk membrane, which activates a cGMP-specific phosphodiesterase via a G-protein intermediary (Gt). The G-protein and the PDE are tethered to the membrane by lipid modifications (264, 294).

(C) Like choanoflagellates, some fungal zoospores use a type I rhodopsin fusion protein for phototransduction. However, this fungal protein, RhoGC, comprises a rhodopsin fused not to a PDE but to a guanylyl cyclase (GC), which catalyzes light-dependent synthesis of cGMP from GTP. Thus, illumination results in increased cellular cGMP levels (300).
<table>
<thead>
<tr>
<th>Phylum</th>
<th>Species</th>
<th>Reads</th>
<th>Representative 16S Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gammaproteobacteria</td>
<td><em>Pseudoalteromonas</em> sp. <em>(distincta, hodoensis, marina, nigrifaciens, or translucida)</em></td>
<td>147,406 (57.1%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
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<tr>
<td>Gammaproteobacteria</td>
<td><em>Alteromonas macleodii</em></td>
<td>62,765 (24.3%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
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<tr>
<td>Gammaproteobacteria</td>
<td><em>Pseudomonas oceani</em></td>
<td>21,464 (8.3%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
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<tr>
<td>Gammaproteobacteria</td>
<td><em>Pseudoalteromonas aliena</em></td>
<td>1,6271 (6.3%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td><em>Muricauda</em> sp. <em>(aquimarina or lutimaris)</em></td>
<td>5,419 (2.1%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
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<tr>
<td>Gammaproteobacteria</td>
<td><em>Pseudoalteromonas arabiensis</em></td>
<td>4,567 (1.8%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td><em>Bordetella</em> sp.</td>
<td>443 (0.171%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td><em>Rhodanobacter</em> sp. <em>(lindanilasticus, glycinis, or terrae)</em></td>
<td>10 (0.004%)</td>
<td>AGCGTTAATCTGGGCGTTAAAGGGCACTCACGGCGCTTT TGGTAAAGCTGGAGATGGTAAAGCCCCGGCTCAACCTGGGAACCTGC ATTCGTTAATCTGGGCGAAAGCTAGGATAGAGGGTGGTAGAATT TCCAGGGTACGGGTTGAAATCCGCATATCGGGAAGGAAATT CGTGCGAAGCCGACCCACCTGGGTCAACACTGACGCTCATGACGA AAGACTGCA</td>
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**Table 3.1. Bacterial composition of polyxenic sheet culture determined by 16S sequencing.** The bacterial species present in the polyxenic *C. flexa* culture were identified by iTag sequencing of 16S rDNA (321). Shown are all species for which at least 10 reads were recovered. Due to the short length of the 16S amplicon (rightmost column), some reads were compatible with multiple bacterial species (e.g., first row). The 16S sequence of the *Bordetella* bacterium did not match any species in the NCBI database.
Supplementary movies S1-S12 can be found online at https://www.biorxiv.org/content/10.1101/661009v1

**Movie S1.** Sheet inversion and relaxation observed in an environmental splash pool sample.

**Movie S2.** Sheet inversion (from flagella-in to flagella-out) observed in DIC transmitted light microscopy.

**Movie S3.** Sheet relaxation (from flagella-out to flagella-in) observed in DIC transmitted light microscopy.

**Movie S4.** Spontaneous sheet inversion, showing decrease in projected area. Time lapse movie of a sheet observed in DIC transmitted light microscopy.

**Movie S5.** Light-to-dark transitions reliably induce sheet inversion, which can be quantified as a decrease in projected area. Left: time lapse movie of a sheet observed in phase contrast microscopy. Right: projected area. The light-to-dark transition is visible as a white frame at t=52 seconds, and corresponds to a 75-fold decrease in the intensity of incident light. Camera exposure time was increased correspondingly to allow continued imaging.

**Movie S6.** Sheets in constant light display have low mobility. Sheet culture observed with a Zeiss AxioZoom transmitted light dissecting microscope.

**Movie S7.** Light-to-dark transitions induces sheet inversion followed by fast swimming. Sheet culture observed with a Zeiss AxioZoom transmitted light dissecting microscope.

**Movie S8.** Spontaneous collar contractions in *Salpingoeca urceolata*. Cells are attached to the substrate by a stalked, cup-shaped extracellular lodge called a theca. The most spectacular contraction event is visible shortly after t=610 seconds and corresponds to a rapid decrease in collar angle, concomitant with retraction of the cell body inside the theca. The cell then reverts to its resting shape in the following 20 seconds.

**Movie S9.** Spontaneous collar contractions in *Monosiga brevicollis*. Bacteria co-cultured with *M. brevicollis* are visible around the cell body.

**Movie S10.** Spontaneous reorientation of microvilli in *Salpingoeca rosetta*.

**Movie S11.** Spontaneous changes in collar curvature in *Diaphanoeca grandis*. The cell is encased within an extracellular lodge called a lorica, composed of self-secreted silicon strips.

**Movie S12.** Spontaneous collar contractions in the thecate form of *Choaneca flexa*. Note that the thecate form lacks a flagellum, as in *C. perplexa* (254), the sister-species of *C. flexa.*
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Appendix: Integrating cellular biophysics, cell biology, and natural history to understand the regulation and evolution of shape and movement in eukaryotes

“Nature loves to hide” -Heraclitus (B123)  

Taken together as a whole, this thesis represents the synthesis of approaches from cellular biophysics, cell biology, and natural history to address a fundamental question in evolutionary cell biology: How do cells regulate shape and movement in order to thrive in various environments? In particular, we have investigated how form is regulated in multicellular choanoflagellates, how this capacity evolves within the choanoflagellates, and what implications these findings may have with respect to the evolutionary origins of animals.

In Chapter 2, I showed how the basal extracellular matrix secreted by cells during development of S. rosetta rosettes physically constrains proliferating cells to drive a stereotyped 2D-3D growth transition that is robust to stochasticity in the dynamics of cellular proliferation. Based on these findings, I developed, along with collaborators, a simple, biophysical model of colony morphogenesis based on the regulated basal secretion of extracellular matrix in conjunction with physical interactions between cells. Beyond capturing the 2D-3D growth transition in rosette morphogenesis, tuning parameters corresponding to cell shape, relative rate of ECM secretion, and relative ECM stiffness was sufficient to capture a range of colonial morphologies. Many of these predicted morphologies are reminiscent of colonial choanoflagellates found in nature. These experimental and theoretical results suggest that the regulated basal secretion of extracellular matrix in conjunction with physical constraints on cell packing is sufficient for robust colony morphogenesis and that changes in ECM secretion, the material properties of ECM, and cell shape may underlie the evolution of different colony morphologies. Moving forward, it will be very interesting to test theoretical predictions of the model against quantitative measurements across diverse choanoflagellates with colonial morphologies captured by simulations. Additionally, we know little about the material properties of the ECM of choanoflagellates and diverse animals. Animal ECM is unique in its composition (18, 248). Understanding the role of the material properties of the ECM in choanoflagellate and animal morphogenesis and function broadly in conjunction with how those properties evolve stands to shed new light on animal origins.

In Chapter 3, I presented a description of the new colonial choanoflagellate C. flexa, serendipitously isolated during field work in Curaçao. With collaborators, I demonstrated that rapid and reversible cup-shaped colony inversions are triggered by changes in light transduced by a unique choanoflagellate protein (RhoPDE) that inversions mediate a feeding swimming tradeoff and can give rise to phototaxis, and that collective acto-myosin mediated apical contractility of cells mediates inversion. These findings inform reconstructions of hypothesized animal ancestors by demonstrating how multifunctional cells can transduce environmental signals to trigger coordinated multicellular shape and behavioral change. Furthermore, the cellular module, an apical acto-myosin contractile apparatus, may have evolved before the divergence of choanoflagellates and animals, and therefore may have existed in the cells of the earliest animal ancestors. Work elucidating the molecular details of how cellular shape changes are orchestrated will be essential for placing collective contractility in C. flexa in its full evolutionary context. I expect that clarifying the physical mechanisms by which inversion occurs will aid in determining the underlying cellular

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4 This fragment may be more accurately translated as “Nature is ever hidden” (324), but the most common translation presented here is a bit more evocative.
mechanisms. This work will necessitate quantitative approaches to analyze the dynamics of inversion at the cellular level using high resolution microscopy in conjunction with mathematical modeling of the mechanics of inversion.

Contrary to the colonies considered in Chapter 2, *C. flexa* colonies do not have a structural basal ECM. What are the relative tradeoffs and constraints on form and function imposed by these different modes of multicellularity? Animals are unique in having large, flexible bodies capable of rapid locomotion. The structural integrity of animal bodies and physical properties of tissues are conferred in large part by gel-like extracellular matrix (322). Indeed, the most abundant protein by weight in our own bodies, and the most abundant animal protein, is collagen, an integral, animal specific ECM component (323). Most movements in animal in locomotion and morphogenesis are driven by cellular shape change or contractility (322). It is possible that unique properties of animal ECM in conjunction with cells capable of rapid shape changes triggered by external stimuli were a key step in the evolutionary emergence of the kinds of large, flexible, mobile bodies unique to animals. Deeper understanding of the composition and material properties of the choanoflagellate ECM and its regulation, mechanistic understanding of the regulation of rapid cell shape changes in choanoflagellates, and broader understanding of the material properties of animal ECM across animal diversity will be essential for sharpening and testing this hypothesis.

Much choanoflagellate biology remains to be discovered. Detailed, mechanistic understanding in a few choanoflagellate species combined with broad contextualization across the diversity of choanoflagellates presents a way forward in clarifying animal origins. Such a research program will require an interdisciplinary approach and the integration of results from disciplines across biology and biophysics. This approach will be greatly facilitated by advances in microscopy, computation, genomics, transcriptomics, and gene editing. Regardless of the particular relevance to animal origins, choanoflagellates stand to serve as a simple system for investigating the cellular and biophysical principles of multicellularity.

Beyond choanoflagellates, the diversity of cellular form and function in protists presents an incredibly rich resource for new discoveries in cell biology. We have argued here that understanding how function emerges from the regulated interplay between cellular behavior and physical constraints in the context of eukaryotic diversity represents a promising direction for advancing evolutionary cell biology. My work has demonstrated the utility of such a research program combining elements of field work based natural history with cell biology and biophysics. Who knows what awaits discovery in the lab and in the field?