Tiny organisms could reveal how animals evolved

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When life first evolved on Earth, it was microscopic and single-celled. Since then, multicellularity appears to have independently evolved several times among eukaryotes. In animals, multicellularity is thought to have first evolved about 600 million years ago—a transition that remains shrouded in mystery. "One of the challenges in studying the origin of animal multicellularity is that it is not well preserved in the fossil record," says Nicole King of University of California, Berkeley. To answer questions about early animal evolution, King turned instead to an unlikely source—microscopic water-dwelling organisms called choanoflagellates.

Choanoflagellates are tiny, transparent eukaryotes commonly found in all bodies of water. They are the closest living relatives to animals, meaning that both choanoflagellates and all multicellular animals—from sponges to humans—evolved independently from a single common ancestor. By studying these tiny organisms, researchers hope to understand the genomes and cell biology of this last common ancestor, suggesting the genes and pathways required for the evolution of the first multicellular animals. When King first started studying choanoflagellates as a postdoc in the early 2000s, little was known about them. "There just weren't a lot of people studying these enigmatic animal relatives," says King.

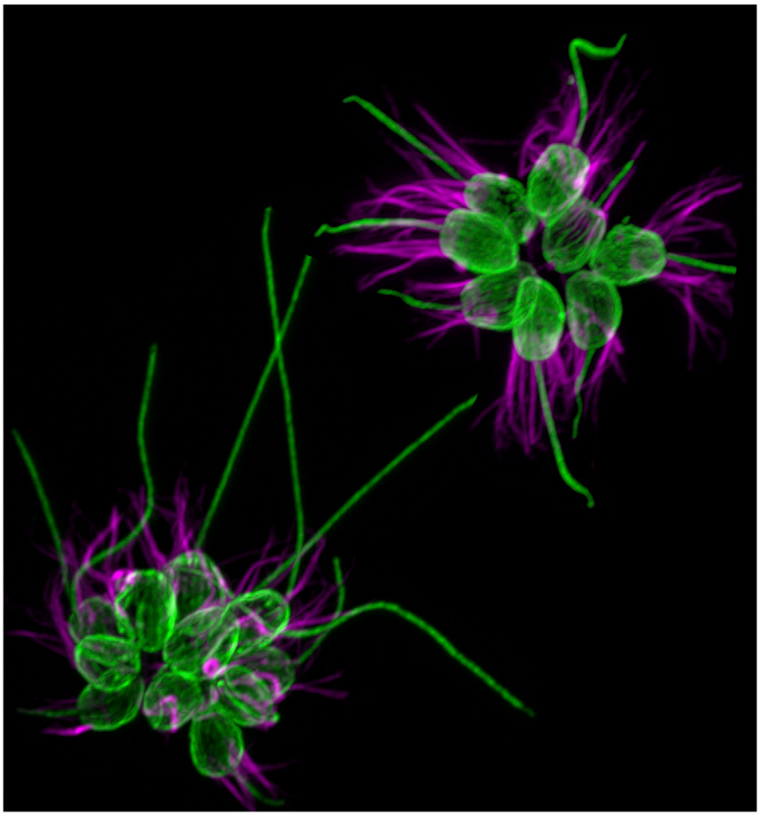
Crucial Pathways

"Choanoflagellates were about as far from a model system as it gets when [King] started," says Rick Grosberg of University of California, Davis, who has studied the fundamental features of the evolution of multicellularity. That is no longer the case, says Grosberg. "The choanoflagellate system really speaks directly to the question of the evolution of the multicellular toolbox in animals," he says.

Around the time King started studying choanoflagellates, new sequencing techniques enabled genomic studies of nonmodel organisms. Grosberg says King had the vision to see that these advances would allow scientists to take apart genomes and transcriptomes relatively quickly and cheaply. "It's completely changed, or should completely change, the way people think about the evolution of development," he adds.

King found that choanoflagellates expressed many genes and pathways that had been thought to be animal-specific. A key hypothesis at the time held that the evolution of multicellularity in animals hinged on genes involved in cell–cell signaling and cell adhesion, which play crucial roles in multicellular organisms. "What I found was that these types of genes predate animal origins," King says (1, 2). "That raised the interesting question of how these genes might have been functioning before the evolution of animal multicellularity."

It's not just in their genes that choanoflagellates resemble animals. Some of these organisms have the ability to switch from a single-celled form to multicellular rosette-shaped colonies that resemble early-stage animal embryos. King reasoned that studying rosette formation might provide insights into how multicellularity evolved. "We're trying to understand rosette development at a molecular and cellular level," she says.



Clues to the roots of animal evolution may lie in choanoflagellate rosettes, shown here with tubulin protein labeled in green (staining the cell body and flagellum) and actin labeled in magenta. Image courtesy of Arielle Woznica (University of California, Berkeley, CA).

To do so, the researchers needed a robust model system. Unfortunately, laboratory-grown choanoflagellates didn't consistently form multicellular rosettes. "There were really basic questions that we couldn't address because we couldn't get them to be multicellular in the lab," King says. That's when the researchers had a stroke of luck.

Complex Interactions

As a student in King's laboratory prepared a culture of the choanoflagellate *Salpingoeca rosetta* for sequencing, he treated it with a mix of antibiotics to reduce the diversity of prey bacteria. To his surprise, he noticed that rosette formation increased. The researchers hypothesized that the antibiotics killed off some dominant bacteria, allowing other bacteria, that induced rosette-formation,

www.pnas.org/cgi/doi/10.1073/pnas.1615928113

Rosanna Alegado and her students collect choanoflagellate samples from a Hawaiian fishpond, later extracting DNA to assess the microbial communities. Image courtesy of Rosie Alegado (University of Hawaii at Manoa, Honolulu).

to thrive. The scientists went on to identify bacteria called *Algoriphagus machipongonensis* that induced *S. rosetta* rosette colony formation. "It's an amazing example of how environmental bacteria are regulating a choanoflagellate life history transition," says King. "But it was also an important technological breakthrough."

To look for the bacterial and molecular cues that induce rosette colony formation, King began collaborating with Harvard Medical School's John Clardy. "It's a simple problem to describe, but tough to deal with," says Clardy. The researchers had to separate the bacterial cultures into different fractions, assess each fraction's rosette-inducing activity, and purify the active molecule from a selected fraction through several rounds of chromatography. They eventually identified a bacterial lipid molecule responsible for rosette formation, which they named rosette-inducing factor 1 (RIF-1) (3). "It's a very obscure kind of lipid," notes Clardy. RIF-1 turned out to be an unusual relative of sphingolipids, which are important signaling molecules in animals, plants, and fungi. RIF-1 represented just 0.015% of all of the *A. machipongonensis* sphingolipids.

The researchers found that RIF-1's stereochemistry—the 3D arrangement of its atoms—was crucial to rosette formation. Only one such "stereoisomer" of RIF-1 was capable of inducing rosette formation, despite all of them having the same chemical equation (4). "It tells us that we're looking at a very specific signaling system," says Clardy.

RIF-1 induced rosette colony formation in *S. rosetta* cultures, but it didn't recapitulate the full rosette-forming activity of *A. machipongonensis*, which meant there were likely other interactions between the bacteria and *S. rosetta* yet to be identified.

To try to get a more comprehensive picture of *A. machipongonensis*' chemical cues, King and Clardy took different fractions of lipids from the bacteria, mixed them together, and then examined the effects (5). They identified another rosette-inducing factor, which they named RIF-2. RIF-2 induced higher levels of rosette formation than RIF-1, but at greater concentrations.

The researchers found other chemical cues being produced by the bacteria. Although these didn't affect rosette development by themselves, they either enhanced or inhibited the rosette-inducing activity of RIF-1 or RIF-2 (5, 6). These studies "dig deeply into the molecular mechanisms regulating unicellular to multicellular transitions," says Grosberg.

"It's clear that there is a lot of complex lipid signaling going on that we don't understand," says Clardy. "The story continues to grow and get more complex and also much more interesting."

Natural Studies

In nature, choanoflagellates swim around in a rich soup of bacterial cues. *A. machipongonensis* alone produces at least three different classes of lipids that exert different effects on rosette development. "Think about the natural environment, where there's not just one species but thousands of different species of bacterial molecules," says King.

However, that natural environment remains obscure. Like all microscopic eukaryotes, says King, their size makes them difficult to observe directly in nature. Rosanna Alegado, a former postdoc in King's laboratory and first author of the RIF-1 study, has been conducting some preliminary metagenomics studies looking at choanoflagellates and associated bacterial species in their natural environment, in estuaries near University of Hawaii, where she's now based. Alegado has, so far, identified several local species, some of which form rosette colonies. She has sampled 14 sites over the past 18 months, attempting to discern what role choanoflagellates play in the food web and what the ecological role of rosette formation might be. It's still early days, but she hopes to have some answers within the next couple of years.

There's also the question of whether these complex bacterial cues may have contributed to animal evolution. "There's no doubt bacteria were an important part of the environment for the first animals, and the important question is how they influenced early animal biology," says King. "Right now it's a mystery."

King and her colleagues are currently trying to identify the choanoflagellate receptors for bacterial signaling molecules, and hope to piece together their downstream signaling pathway. "If that pathway is conserved in animals," says King, "there's a stronger argument to be made about how bacteria might have influenced the evolution of animal multicellularity."

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