

1 Title:

2

3 Cell differentiation controls iron assimilation in a choanoflagellate

4

5 Authors:

6

7 Fredrick Leon¹, Jesus M. Espinoza-Esparza¹, Vicki Deng^{1,3}, Maxwell C. Coyle^{2,4}, Sarah

8 Espinoza², David S. Booth^{1*}

9

10 Affiliations:

11

12 ¹Chan Zuckerberg Biohub &

13 Department of Biochemistry and Biophysics

14 University of California, San Francisco School of Medicine

15 San Francisco, CA 94143

16

17 ²Howard Hughes Medical Institute &

18 Department of Molecular and Cell Biology

19 University of California, Berkeley

20 Berkeley, CA 94720

21

22 ³Current Address: Department of Molecular Biosciences

23 University of Texas, Austin

24 Austin, TX 78712

25

26 ⁴Current Address: Department of Molecular and Cellular Biology

27 Harvard University

28 Cambridge, MA 02138

29

30 *Corresponding author: David.Booth@ucsf.edu

1 Abstract:

2

3 Marine microeukaryotes have evolved diverse cellular features that link their life histories to
4 surrounding environments. How those dynamic life histories intersect with the ecological
5 functions of microeukaryotes remains a frontier to understand their roles in essential
6 biogeochemical cycles^{1,2}. Choanoflagellates, phagotrophs that cycle nutrients through filter
7 feeding, provide models to explore this intersection, for many choanoflagellate species
8 transition between life history stages by differentiating into distinct cell types³⁻⁶. Here we report
9 that cell differentiation in the marine choanoflagellate *Salpingoeca rosetta* endows one of its cell
10 types with the ability to utilize insoluble ferric colloids for improved growth through the
11 expression of a cytochrome b561 iron reductase (*cytb561a*). This gene is an ortholog of the
12 mammalian duodenal cytochrome b561 (*DCYTB*) that reduces ferric cations prior to their uptake
13 in gut epithelia⁷ and is part of an iron utilization toolkit that choanoflagellates and their closest
14 living relatives, the animals, inherited from a last common eukaryotic ancestor. In a database of
15 oceanic metagenomes^{8,9}, the abundance of *cytb561a* transcripts from choanoflagellates
16 positively correlates with upwellings, which are a major source of ferric colloids in marine
17 environments¹⁰. As this predominant form of iron^{11,12} is largely inaccessible to cell-walled
18 microbes^{13,14}, choanoflagellates and other phagotrophic eukaryotes may serve critical ecological
19 roles by first acquiring ferric colloids through phagocytosis and then cycling this essential
20 nutrient through iron utilization pathways¹³⁻¹⁵. These findings provide insight into the ecological
21 roles choanoflagellates perform and inform reconstructions of early animal evolution where
22 functionally distinct cell types became an integrated whole at the origin of animal
23 multicellularity¹⁶⁻²².

1 Text:

2

3 Thecates display a different transcriptome profile than other cell types.

4 Choanoflagellates transition between different stages of their life history by differentiating
5 into distinct cell types³⁻⁵. In the emerging model choanoflagellate *S. rosetta*, diverse
6 environmental cues promote the transitions between different types of cells⁶. Under nutrient
7 replete conditions, sessile thecate cells differentiate into motile slow swimmers that can
8 proliferate as single cells or chains of cells connected through intercellular bridges^{6,23,24}. In
9 response to specific bacterial²⁵⁻²⁷ and algal²⁸ cues, slow swimmers develop into rosettes, which
10 are multicellular colonies that form through serial cell division²⁹. As rosettes or slow swimmers
11 starve, they become fast swimmers, and under prolonged nutrient limitation, fast swimmers
12 differentiate into thecates that can proliferate under similarly harsh conditions⁶. The
13 recapitulation of these cell type transitions in the laboratory make *S. rosetta* a promising model
14 to investigate how a marine microeukaryote integrates environmental signals to produce distinct
15 cell types. However, the environmental functions that *S. rosetta* cell types could perform remain
16 to be uncovered, as choanoflagellate cell types have mostly been defined through their
17 morphological features^{3,6,29,30} rather than molecular functions.

18 We refined previous *S. rosetta* transcriptomes³¹ to uncover functional differences
19 between cell types reflected in their gene expression profiles. To improve those transcriptomes,
20 we took advantage of methods to stably culture thecate, slow swimmer, rosette, and fast
21 swimmer cell types in monoxenic strains of *S. rosetta* that feed on the bacterium *Echinicola*
22 *pacifica*^{23,24,32}. We also developed a method to preferentially lyse *S. rosetta* while discarding their
23 feeder bacteria to harvest mRNA from *S. rosetta* with a single round of poly-A selection (Fig.
24 S1) rather than previous methods that relied on multiple rounds of selection to deplete
25 contaminating RNAs from bacteria^{1,10}. A principal component analysis of these improved
26 transcriptomes emphasized the difference between the thecate cell type compared to slow
27 swimmers, rosettes, and fast swimmers (Fig. 1B) with the first principal component accounting
28 for 86% of the variance and clearly separating thecates from all other cell types. While these
29 results are consistent with previous gene expression profiles that found thecates separated from
30 other types of cells^{31,34}, the replicate measures in our data do not show a clear difference
31 between slow swimmers, rosettes, and fast swimmers, suggesting that these three cell types
32 are phenotypically plastic states.

33 The contrast between thecates and other cell types prompted us to investigate what
34 unique functions thecates may perform. By comparing thecates to slow swimmers, we identified

a set of genes that reproducibly ($q < 0.01$, note that q is a P -value adjusted for multiple comparisons) displayed a greater than two-fold change in gene expression in thecate. We analyzed this set of genes for common gene ontologies (GO)^{35–39} that indicated functional categories enriched in thecate, keeping in mind that GO analysis may underestimate functional differences between cell types due to incomplete genome annotations in this emerging model system⁴⁰. Genes upregulated in thecate were clearly enriched for functional modules (Fig. S2) implicated in cell signaling (e.g. nucleotide binding, protein kinase, protein phosphatase, calcium binding, metal ion transporter, guanylate cyclase and G-protein coupled receptor), gene regulation (e.g. transcription factor and ribosome component), and metabolism (e.g. nucleotide binding, oxidoreductase, glycosyl hydrolase, metal ion transporter, carbohydrate binding, sulfuric ester hydrolase, and ascorbic acid binding). In contrast, swimmers expressed modules for cell division, RNA processing, and oxidative metabolism (Fig. S3). This comparative analysis highlighted a gene (PTSG_09715) with 479-fold higher expression in thecate compared to slow swimmers ($q < 10^{-40}$) that was annotated as an oxidoreductase (GO:0016491) composed of a single cytochrome b561 iron reductase domain (Pfam 03188). Given the role that oxidoreductases perform in diverse eukaryotes to regulate iron acquisition^{41–44}, the strong induction of PTSG_09715 (henceforth called *cytb561a*) led us to hypothesize that thecate may have a different capacity to acquire iron from the environment compared to other cell types.

***cytb561a* expression improves thecate cell growth with ferric colloids.**

Of the 2,820 genes that reproducibly displayed a two-fold or greater change in mRNA expression in thecate compared to slow swimmers, the change in *cytb561a* expression is in the ninety-eighth percentile (Fig. 2A). This large change in *cytb561a* expression may have been coupled to the thecate cell type program, may have been due to differences in strains to generate thecate and slow swimmer transcriptomes, or may have been an independent response to external nutrients that differed between the media for culturing slow swimmers and thecate for the transcriptomes (see methods). To distinguish among these possibilities, we measured *cytb561a* mRNA expression in slow swimmers and thecate from the same strain of *S. rosetta* after standardizing a culturing regime (Fig. 2B and Table S1). In this protocol, slow swimmers and thecate were grown in the same media formulation and then depleted of iron. Afterwards, we reintroduced iron either as soluble ferric EDTA (Fe^{3+} •EDTA) or as insoluble ferric colloids (Fe^{3+} (oxyhydr)oxides)⁴⁰. Both forms of iron are ecologically important, for models based on environmental sampling predict that 40% of the world's surface ocean waters rely on colloidal sources of iron and 18% on soluble iron chelates¹¹. With either source of iron, the expression of

1 *cytb561a* mRNA increased in thecates compared to slow swimmers (Fig. 2C and S4 A-C):
 2 89-fold with ferric EDTA and 66.2-fold with ferric colloids (two-way ANOVA, $P < 0.005$). This
 3 result supports the conclusion that thecates are a stable cell type that expresses *cytb561a* as
 4 part of its differentiation regulon.

5 To determine the functional consequence of increased *cytb561a* expression in thecates,
 6 we compared the growth of thecates and slow swimmers in the presence of 100 μM ferric EDTA
 7 or ferric colloids (Fig. 2D). Thecates grew to a two-fold higher cell density in the presence of
 8 ferric colloids (two-way ANOVA, $P < 10^{-4}$), yet slow swimmers showed no change (two-way
 9 ANOVA, $P > 0.1$). This effect was observed at lower concentrations of iron (50 μM), but higher
 10 concentrations of iron ($\geq 200 \mu\text{M}$), favored greater cell proliferation in ferric EDTA (Fig. S5A).
 11 Thecates also grew better than slow swimmers with other ferric chelates, such as ferric EGTA
 12 and ferric pyoverdine (Fig. S5B). Importantly, the differences in *S. rosetta* growth were unlikely
 13 due to indirect effects from the prey bacteria, for *E. pacifica* grew similarly in all iron conditions,
 14 likely due to the small concentration of iron ($3.43 \pm 1.01 \mu\text{M}$) that dissolved from the ferric
 15 colloids (Fig. S5 C-E).

16 The increased proliferation of thecates in the presence of ferric colloids did depend on
 17 the expression of *cytb561a*. With an improved genome editing pipeline (see methods and Fig.
 18 S6), we altered *cytb561a* expression by introducing a premature termination sequence⁴⁶ at
 19 position 151 of *cytb561a* to incorporate missense mutations and a polyadenylation signal (Fig.
 20 S7), which diminished mRNA expression in thecates by 3.6-fold compared to wild-type (Fig.
 21 S4D, one-way ANOVA, $P = 0.004$). This mutant allele, *cytb561a*^{PTS}, eliminated the difference in
 22 cell proliferation between slow swimmers and thecates (Fig. 2D, two-way ANOVA $P > 0.1$). A
 23 comparison of *cytb561a*^{PTS} and wild-type growth dynamics (Fig. S8) showed that the
 24 enhancement of thecate growth with ferric colloids was primarily due to a two-fold larger
 25 carrying capacity and a shorter doubling time (17.9 h vs 13.2 h, two-way ANOVA, $P = 0.035$). To
 26 derive iron from ferric colloids for enhanced cell growth, thecates can directly ingest iron
 27 particles, as seen in time-lapse images of thecates feeding on ferric colloids embedded with a
 28 fluorescent polysaccharide (Figs. 2E & S9). Taken together, these results suggest that the
 29 increased expression of *cytb561a* in thecates confers a cell-type-specific function to assimilate
 30 iron from insoluble ferric colloids for faster growth to higher cell densities.

31

32 **Eukaryotes have broadly conserved a core iron acquisition toolkit.**

33 Although *cytb561a* expression was necessary for the increased proliferation of thecates
 34 when grown with ferric colloids, we were uncertain how this iron reductase factored into the

1 pathway(s) for iron acquisition in *S. rosetta*, as iron transport pathways have not previously
2 been examined in choanoflagellates. Therefore, we compiled an inventory of iron import and
3 export proteins that have been characterized in diverse model eukaryotes (e.g. humans⁴⁴, *S.*
4 *cerevisiae*⁴⁷, *P. tricornutum*⁴¹, *C. reinhardtii*⁴³, Fig. S10) to survey the distribution of those
5 proteins across Holozoa (the taxonomic group that includes Animalia, Choanoflagellata,
6 Filasterea, and Teretosporea) and a curated set of eukaryotes⁴⁸. We found that nearly all of the
7 proteins for iron import, export, reduction, and oxidation are widely distributed among
8 eukaryotes, suggesting a deep ancestry of iron acquisition pathways from the last common
9 eukaryotic ancestor (Fig. 3A and S11).

10 Our searches also revealed notable evolutionary patterns that correspond to the unique
11 biology of certain eukaryotic lineages. For example, we found significant losses of iron toolkit
12 genes in some lineages. Apicomplexa, a taxon that includes the intracellular parasites
13 *Cryptosporidium parvum*, *Toxoplasma gondii*, and *Plasmodium falciparum* has a reduced set of
14 iron acquisition genes, consistent with the origin of *de novo* iron acquisition pathways for
15 siphoning iron from their hosts⁴⁹. Moreover, we observed that some iron utilization genes are
16 more taxonomically restricted than others: Genes first characterized in ascomycete yeasts were
17 either primarily found in Fungi and Archaeplastida (e.g. iron transporter⁵⁰) or only present in a
18 few eukaryotes (e.g. iron permease⁵¹). Similarly, transferrin and phytoferritin were primarily
19 found in Holozoa and Archaeplastida, respectively, as previously shown⁵². Notably, the presence
20 of transferrin in Animalia and Teretosporea indicated that transferrin was present in the last
21 common ancestor of Holozoa, yet the transferrin receptor that facilitates the cellular
22 internalization of iron-bound transferrin appears to have originated in Deuterostomes (Fig. S12),
23 highlighting the origin of a mechanism to share iron between cells in the multicellular bodies of
24 animals. Finally, we also observed that the presence of genes encoding vacuolar iron
25 transporters and ferritin, which are both involved in iron storage, was slightly anticorrelated ($r =$
26 -0.380 , $P < 10^{-8}$). Overall, this catalog of iron utilization proteins indicates that eukaryotes have
27 variably retained, amplified, or lost genes from an ancestral toolkit for the acquisition, storage,
28 and export of iron.

29 Choanoflagellates and animals appeared to share a core pathway for acquiring iron from
30 their external environment. Iron acquisition in animals has most extensively been studied in
31 vertebrate gut epithelia⁵³. The apical surface of gut epithelial cells absorbs dietary iron by
32 reducing ferric cations to ferrous cations through the activity of duodenal cytochrome B561
33 (DCYTB) before the divalent metal ion transporter (DMT1) brings ferrous cations inside of cells.
34 On the basal surface of gut epithelia, ferroportin (FPN) secretes ferrous cations into the

1 bloodstream, where they are oxidized to ferric cations near the membrane by hephaestin
2 (HEPH). In our catalog of iron utilization genes, all choanoflagellates possessed homologs of
3 *DCYTB*, *DMT1*, and *FPN*, but *HEPH* (Fig. 3A, row 8–ferroxidase type1) was only present in *S.*
4 *rosetta* and 9 other species (of 22 surveyed⁵⁴) – perhaps due to the lack of complete genomes
5 for many of those species. Differences between choanoflagellates and animals were evident in
6 iron storage mechanisms, as choanoflagellates mostly possessed vacuolar iron transport
7 proteins, which have been studied in *S. cerevisiae*^{55,56}; whereas, animals broadly conserved
8 ferritin. As we searched for homologs of iron acquisition genes in *S. rosetta*, we found single
9 copies of *dmt1*, *fpn*, and *heph* and identified two vacuolar iron transporters (*vit1* and *vit2*). Three
10 cytochrome b561 genes were present, yet among all the conserved iron utilization genes, only
11 *cytb561a* exhibited strong differential expression between cell types (Fig. 3B), emphasizing its
12 cell-type-specific role in thecaes.

13 Because the apico-basal localization of iron acquisition genes facilitates the
14 unidirectional import of iron in animal epithelia⁵³, we tested if iron transport in *S. rosetta* shared a
15 similar architecture (Fig. 3C). To do so, we used genome editing at endogenous loci to introduce
16 an epitope tag (ALFA-tag⁵⁷) in all the iron utilization genes (Fig. S7). Only *Cytb561a*, *Fpn*, and
17 *Heph* were visible in western blots of thecaes (Fig. S13A), so we focused on those proteins to
18 visualize by immunofluorescence. In thecaes, *Cytb561a* localized intracellularly toward the
19 apical half of the cell but not in any pattern resembling organelles⁵³. *Cytb561a* was undetectable
20 in slow swimmers (Fig. S13B), in contrast to thecaes that stably expressed *Cytb561a*
21 independent of iron availability (Fig. S14), both of which are consistent with mRNA expression
22 levels (Fig. 2C). In both slow swimmers and thecaes, *Fpn* and *Heph* localized near the apical
23 membrane inside of the collar (Fig. S15). The similar co-localization of *Fpn* and *Heph* would be
24 consistent with their coordinated function as an efflux pump and oxidase as described in
25 animals⁵³. Importantly, the basal localization of these proteins appears to be a feature of animal
26 epithelia that is not shared with choanoflagellates and may have evolved alongside the
27 mechanisms to establish and maintain cell polarity⁵⁹.

28 Based on the functions of homologous genes and the localization of *Cytb561a*, *Fpn*, and
29 *Heph*, we propose a model for iron acquisition in *S. rosetta* thecaes that may extend to other
30 choanoflagellates (Fig. 3D). After internalizing ferric colloids by phagocytosis, *Cytb561a* reduces
31 ferric cations in endosomes where we surmise that *Dmt1* transports ferrous cations into cells –
32 although *Dmt1* was undetectable by immunohistochemistry (Fig. S14). As slow swimmers do
33 not express *Cytb561a*, *S. rosetta* may acquire iron directly from bacterial prey digested in food
34 vacuoles or rely on *Cytb561b* or *Cytb561c* to reduce ferric cations. In both slow swimmers and

1 thecates, we hypothesize Fpn exports ferrous cations at the apical surface where Heph can
2 oxidize it.

3

4 **Cytb561 paralogs display distinct biochemical properties and global distributions.**

5 The variation in the number of cytochrome b561 paralogs across eukaryotes led us to
6 examine the evolution and function of the three cytochrome b561 paralogs that we identified in
7 *S. rosetta*. While the expression of *cytb561a* in thecates was clearly necessary for improved
8 growth in the presence of colloidal iron, slow swimmers still grew in those conditions as did
9 *cytb561a^{PTS}* thecates (Fig. 2D). These observations and the steady mRNA expression of
10 *cytb561b* and *cytb561c* across cell types (Fig. 3C) would be consistent with these paralogs
11 sustaining a baseline ability to acquire iron, even if we could not detect their protein products by
12 immunohistochemistry (Fig. S13). The potential for functional redundancy among these
13 paralogs also led us to consider if choanoflagellates and other eukaryotes display
14 lineage-specific expansions of this gene family that may have resulted in differing capacities to
15 acquire iron.

16 To better compare the cytochrome b561 paralogs in *S. rosetta*, we first built a
17 phylogenetic tree⁶⁰ from an alignment⁶¹ of Cytb561 proteins from Amorphea (Fig. 4A), a
18 eukaryotic supergroup that includes Holozoa and Amoeboae^{48,62}. The unrooted tree of
19 cytochrome b561 proteins robustly supported (ultrafast bootstrap⁶³ > 88) three distinct groups.
20 Groups B and C contained protein sequences from each major clade in Amorphea and its sister
21 group CRuMs (Collodictyonida, Rigiifilida and Mantamonadida). Strikingly, sequences from
22 Holozoa dominated Group A, as a single protein from Amoebozoa was the only sequence from
23 outside of Holozoa that fell into this group. Notably, Group A not only contained mammalian
24 DCYTB proteins but also Cytb561a from *S. rosetta*, supporting the annotation of this gene as an
25 DCYTB ortholog. The Cytb561b and Cytb561c proteins from *S. rosetta* fell into Groups B and C,
26 respectively.

27 Because each of the *S. rosetta* paralogs was part of a different group of cytochrome
28 b561 proteins, we examined⁶⁴ multiple sequence alignments (Figs. S16,17,19) and their
29 predicted structures⁶⁵ (Fig. S18) to evaluate if the deep evolutionary divergence was reflected in
30 their biochemical characteristics (Fig. 4B), using the crystal structure of human DCYTB as a
31 reference⁶⁶. DCYTB forms a homodimeric complex with each monomer folding into six
32 transmembrane helices that sandwich two heme groups in the interior of the protein. These
33 heme groups mediate the transfer of an electron from an ascorbate molecule on the cytosolic
34 side of the protein to ferric cations on the luminal side. Thus, in our comparisons of multiple

1 sequence alignments, we focused on the residues in DCYTB that bind ascorbate, mediate
2 homodimerization, and attract iron. First, all homologs appear to have conserved positively
3 charged residues that form the pocket to bind negatively charged ascorbate⁶⁶ (Fig. S16).
4 Second, only proteins from Group A conserve residues that mediate dimerization (Fig. S17),
5 which was also reflected in predicted dimeric structures of paralogs from *S. rosetta* (Fig. S18).
6 Third, among the Cytochrome b561 groups, we noticed significant differences on the luminal
7 surface that forms the binding site for ferric cations (Figs. 4B, arrow and S19). Homologs within
8 Group A, which contains Cytb561a and DCYTB, possess a mix of positive and negative
9 charges, indicating Group A paralogs can bind iron and coordinated ligands⁶⁶. Group C
10 homologs have largely anionic surface charges at the putative iron binding site, which could
11 plausibly bind iron as well. However, the positively-charged binding pockets conserved across
12 Group B homologs may repel rather than bind iron cations. Notably, the differences in charge
13 distribution among Cytochrome b561 groups appear to be general features of those groups (Fig.
14 S19C), yet electrostatic properties can diverge within those lineages (Fig. S19D). With each
15 paralog from *S. rosetta* possessing distinct biochemical properties near the iron binding site, we
16 hypothesize that the improvement of thecate growth with ferric colloids may not only be due to
17 the mass action from more Cytb561 proteins but also through the distinct biochemical activity of
18 Cytb561a.

19 If the biochemical activity and cell-type-specific expression of Cytb561 paralogs enable
20 choanoflagellates to acquire iron from different environmental sources, then we expect that the
21 expression of each paralog may be enriched in different ecological settings. To test this
22 hypothesis, we searched for each paralog in an open repository (Ocean Gene Atlas^{8,9}) of
23 metagenomes and metatranscriptomes from marine environments. When we visualized the
24 distribution of each paralog, we found that they varied in both location and transcript abundance
25 but were similarly present in metagenomic reads (Fig. 4C) The most abundant and
26 cosmopolitan paralog was *cytb561c*. Although present at most of the sites, the transcript
27 abundance of *cytb561b* was ~10-fold lower than *cytb561c*. The abundance of *cytb561a* had a
28 similarly low abundance as *cytb561b*, yet its distinct presence near coastlines and the equator
29 resembled the locations of marine upwellings⁶⁷ (Fig. S20A).

30 Importantly, upwellings are a major source of remineralized iron colloids rising from
31 ocean depths^{68,69}, and upwelling velocities may serve as a better proxy for the presence of
32 colloidal iron than predictions of soluble iron concentrations¹⁰. Therefore, we correlated the
33 abundance of the genomic and transcript reads for each paralog to average vertical velocities at
34 a depth of 100 meters for each sample location (Fig. 4C, S20B). Of all the comparisons, the

1 only robust correlation was between *cytb561a* transcript abundance and vertical velocity ($r =$
2 0.64, $P < 10^{-4}$), as the transcript abundances of the other paralogs exhibited only weak positive
3 correlations that were probably random ($P > 0.3$). The positive correlation between *cytb561a*
4 transcripts and upwelling velocities indicate ecological contexts where choanoflagellates may be
5 acquiring insoluble iron colloids for their growth. We cannot, however, determine if the
6 choanoflagellates in those regions are thecate expressing *cytb561a* as in *S. rosetta*, or
7 choanoflagellates with a different regulatory mechanism that evolved uncoupled *cytb561a*
8 expression from thecate development. Nonetheless, these observations connect *cytb561a*
9 expression to an environmental feature and places choanoflagellate biology in a global
10 ecological framework.

11

12 Iron is an essential micronutrient affecting the growth and productivity of
13 phytoplankton⁷⁰. Field measurements and ocean scale models have found that iron particulates
14 are the major source of iron for 40% of the world's surface ocean waters¹¹. In addition to
15 photolytic reduction^{14,71} and bacterial siderophores^{72,73}, the ingestion of iron particles by
16 phagotrophic protists is a major mechanism to solubilize iron and can alleviate iron limitation for
17 cell-walled organisms that rely on obtaining iron in soluble forms^{13–15}. As efficient
18 phagotrophs^{74,75,4,76}, choanoflagellates ingest iron particulates (Figs. 2E and S9), yet iron
19 assimilation from ferric colloids depends on the expression of a specific *Cytb561* paralog in only
20 one cell type of *S. rosetta*. This dependency emphasizes the importance of integratively
21 factoring protein evolution⁷⁷ and gene expression during life history transitions^{1,2} in models for
22 determining⁷⁸ the ecological roles of microeukaryotes in the acquisition and distribution of
23 limiting nutrients throughout their surrounding environment. Moreover, the synthesis of
24 cytological, genomic, and ecological data may help to interpret how the rise in oxygen that
25 resulted in the depletion of soluble ferrous cations from the oceans influenced eukaryotic
26 evolution^{77,79,80}, including the emergence of animals⁸¹.

27 The cell-type-specific expression of *cytb561a* not only illustrates how cellular
28 differentiation impacts the environmental function of a choanoflagellate but also informs how
29 cellular differentiation evolved as a defining feature of animal multicellularity^{16,19,17,82,20,21}.
30 Choanoflagellate cell types have been primarily defined by their morphology and motility^{4,6}. In
31 addition to those cell biological characteristics, we show here that thecate have a distinct ability
32 to utilize ferric colloids. The coupling of this function to cell differentiation stands in contrast to
33 the regulation of iron utilization in other microeukaryotes that transiently regulate iron utilization
34 genes in response to external iron concentrations^{56,83,84}. The functional difference between slow

1 swimmers and thecates more closely resembles the stable differentiation of cell types during
2 animal development^{85–87}. Taken together, these observations support models for the origin of
3 animal cell differentiation in which the last common holozoan ancestor differentiated into unique
4 cell types at each life history stage^{88,16,17,20,22}. Evolution along the animal stem lineage coupled
5 cell differentiation to multicellular development, resulting in multicellular bodies composed of
6 functionally distinct cells⁸⁹. Importantly, mechanisms to distribute resources from cells that
7 acquire nutrients from the external environment (e.g. epithelial cells⁹⁰) to cells that reside
8 internally helped to transform the functions of various cell types into a common organismal
9 physiology¹⁸. As the evolution of transferrin and its receptor illuminates (Figs. 3A and S12), the
10 multicellular physiology of animals has likely been cobbled together through the co-option of
11 mechanisms for nutrient acquisition and cellular trafficking.

1 Materials and Methods:

2

3 **Culturing *S. rosetta***

4 To grow cell types of *S. rosetta* for the generation of transcriptomes, slow swimmer and rosettes
 5 were grown in 5% Sea Water Complete (SWC) Media^{23,91} (Table S1), seeded at 10⁴ cells/ml and
 6 grown for 48 hours at 22°C. Rosettes were induced with outer membrane vesicles (OMVs)
 7 harvested from the bacterium *Algoriphagus machipongonensis*²⁶. Fast swimmers were prepared
 8 the same way as slow swimmers, but were grown for 72 hours at 22°C and transitioned to 30°C
 9 for 2 hours and 45 minutes. Thecates were grown from the strain HD1, a strain which is
 10 constitutively in the thecate cell type, in 10% Cereal Grass Media 3 (CGM3)^{23,91} (Table S1),
 11 seeded at 10⁴ cells/ml and grown for 48 hours at 22°C.

12

13 For iron acquisition experiments and standard culturing of *S. rosetta*, all cell types were grown in
 14 25% Red Algae (RA) Media⁹² (Table S1) with the feeder bacteria *Echinicola pacifica*. Cultures
 15 were grown at 18°C for maintaining cultures and shifted to 27°C one day prior to conducting iron
 16 acquisition experiments.

17

18 **RNA extraction**

19 **Protocol Link:** [dx.doi.org/10.17504/protocols.io.261ge4bojv47/v1](https://doi.org/10.17504/protocols.io.261ge4bojv47/v1)

20 In a previous paper⁹¹, we developed a lysis buffer to extract RNA from *S. rosetta* that has
 21 subsequently been used in other work for RNA^{30,93,94} and protein⁹⁵ extraction. Here we show that
 22 this buffer was developed to preferentially lyse *S. rosetta* in cultures that have an abundance of
 23 the prey bacterium *E. pacifica*. To preferentially lyse *S. rosetta*, we reasoned that sterol-based
 24 detergents, such as digitonin, would more effectively disrupt membranes with sterols, like those
 25 of *S. rosetta* and other eukaryotes⁹⁶ rather than bacterial membranes that largely lack the
 26 sterols⁹⁷. Additionally, the lysis buffer contains RNase inhibitors (RNaseIN and heparin) and
 27 translation inhibitors to decrease the degradation of mRNA. We evaluated the efficacy of this
 28 buffer by tracking ribosomal RNA from *S. rosetta* and *E. pacifica* in lysed samples that were
 29 centrifuged to separate the supernatant and pellet, in which we found that *S. rosetta* ribosomal
 30 RNAs were enriched in the supernatant and bacterial ribosomal RNAs were enriched in the
 31 pellet (Fig. S1). The optimization of this procedure resulted in the following method to obtain
 32 RNA samples from each cell type of *S. rosetta*:

33

1 After counting the cell concentration in a culture of *S. rosetta* feeding on *E. pacifica*, a volume
 2 yielding 10^7 cells was centrifuged at 2600xg for 5 min at 4°C. The pellet from this sample was
 3 resuspended in 100 µl of preferential lysis buffer (20 mM Tris-HCl, pH 8.0; 150 mM KCl; 5 mM
 4 MgCl₂; 250 mM Sucrose; 1 mM DTT; 10 mM Digitonin; 1 mg/ml Sodium Heparin; 1 mM Pefabloc
 5 SC; 100 µg/ml Cycloheximide; 1 tablet/ 5 ml buffer EDTA-free Protease Inhibitor Tablet
 6 [Sigma-Aldrich Cat. No. 11836170001] ; 0.5 U/µl Turbo DNase [Thermo Fisher Scientific, Cat.
 7 No. AM2239]; 1 U/µl SUPERaseIN [Thermo Fisher Scientific, Cat. No. AM2696]) and incubated
 8 on ice for 10 min. Cells were triturated through a 30G needle ten times. Afterwards, the
 9 insoluble debris was pelleted by centrifugation at 6000xg for 10 min at 4°C. The supernatant
 10 was removed and adjusted to a volume of 100 µl with RNase-free water. Total RNA was purified
 11 on a silica membrane with the RNA clean-up protocol from the RNAeasy kit (Qiagen, Cat. No.
 12 74104).

13

14 **RNA library preparation and sequencing**

15 In preparation for sequencing, the quality of total RNA from *S. rosetta* samples was assessed by
 16 Agilent Bioanalyzer 2100 (Fig. S1C) with the Agilent RNA 6000 Nano Kit (Agilent, Cat. No.
 17 5067-1511). Then total RNA (500 ng per sample) was purified by one round of polyA mRNA
 18 selection with oligo-dT magnetic beads (NEB, Cat. No. S1550S), converted to cDNA using
 19 KAPA mRNA HyperPrep kit (KAPA biosystems, Cat. No. KK8580), and indexed-adapter ligated
 20 with KAPA single-indexed adapter kit (KAPA biosystems, Cat. No. KK8701). Final library quality
 21 was determined by Bioanalyzer 2100 using the Agilent High Sensitivity DNA Kit (Agilent, Cat.
 22 No. 5067-4626) and pooled together after normalizing samples based on their quantity from
 23 qPCR. Sequencing was carried out by the QB3-Berkeley Genomics core (QB3 Genomics, UC
 24 Berkeley, Berkeley, CA, RRID:SCR_022170), on the Illumina HiSeq 4000. All samples were
 25 pooled and run on a single lane with 12.4 to 61.3 million reads per sample. Reads were
 26 demultiplexed, and checked for quality by fastqc (Babraham Bioinformatics). Metadata for
 27 sequencing can be found in Table S2.

28

29 **RNA-seq quantification and gene enrichment analysis**

30 RNA-seq reads from each sample were mapped to a reference *S. rosetta* transcriptome
 31 ([https://ftp.ensemblgenomes.ebi.ac.uk/pub/protists/release-58/fasta/protists_choanoflagellida1_](https://ftp.ensemblgenomes.ebi.ac.uk/pub/protists/release-58/fasta/protists_choanoflagellida1_collection/salpingoeca_rosetta_gca_000188695/cdna/)
 32 [collection/salpingoeca_rosetta_gca_000188695/cdna/](https://ftp.ensemblgenomes.ebi.ac.uk/pub/protists/release-58/fasta/protists_choanoflagellida1_collection/salpingoeca_rosetta_gca_000188695/cdna/)) using kallisto⁹⁸. Transcript abundance
 33 and differential expression were quantified with sleuth⁹⁹. Gene enrichment for thecates was
 34 quantified by selecting genes with $q < 0.01$ and $\log_2(\text{fold-change} + 0.5) > 1$. The resulting genes

1 were assigned GO terms using DAVID Bioinformatics^{37,38} where *S. rosetta* gene IDs could be
2 identified by “ENSEMBLE_GENE_ID”. For ease of interpretation, assigned GO terms were
3 collapsed down using REVIGO¹⁰⁰ and further assigned to functional categories in *S. rosetta*
4 (TaxID: 946362) by GO Slim³⁹ (Table S3).

5

6 ***S. rosetta* growth with different sources of iron**

7 **Media Preparation.** To test the ability of *S. rosetta* cell types to differentially utilize iron sources,
8 we developed a method to culture *S. rosetta* under iron limitation. We created an iron depleted
9 media formulation named 4% Peptone Glycerol (PG) Media (Table S1). We added iron to this
10 media from two different sources: Ferric EDTA was prepared by dissolving FeCl₃ and EDTA to a
11 final concentration of 1.46 mM in artificial seawater (ASW [Ricca Chemical Company, Cat. No.
12 R8363000-20F]) and sterile filtering through a 0.22 µm polyethersulfone (PES) filter. Ferric
13 colloids were prepared by adding a small volume of 70% (v/v) ethanol to FeCl₃(s) to sterilize the
14 solid; after evaporating the liquid in a biological safety cabinet, 1.46 mM FeCl₃ was prepared in
15 ASW. The solution was heated to 50°C for 10 minutes to precipitate ferric (oxyhydr)oxides, or
16 ferric colloids⁴⁵.

17 **Fluorescent ferric colloid preparation.** Ferric colloids were prepared as above, except during
18 the 50°C incubation, a fluorescent dextran (10 kD anionic dextran conjugated to
19 tetramethylrhodamin [Thermo Fisher, Cat. No. D1868]) was added to a final concentration of 20
20 µg/ml. This dextran co-precipitated with the ferric colloids, thereby embedding fluorophores
21 within the colloids. The precipitated ferric colloids were pelleted by centrifugation at 4600 x g for
22 10 min at room temperature. The pellet was resuspended in ASW supplemented with 2% (v/v)
23 dextranase (Sigma aldrich, Cat. No. D0443) to digest any excess dextran during a 10 min
24 incubation at 60°C. After pelleting the ferric colloids again, they were resuspended to ~1.46 mM
25 in ASW. Finally, the resuspended colloids were ultrasonicated for two, 1 min cycles (pulsed for 1
26 sec on, 1 sec off) to create smaller particles for easier feeding and imaging, as larger particles
27 broke down slowly and often obscured cells during imaging.

28 **Determining iron concentration in media.** To determine the background concentration of
29 soluble iron in our newly formulated media and the amount of labile iron liberated from the ferric
30 colloids, we used a modified protocol¹³ to measured soluble iron concentrations in 4% PG and
31 4% PG with 100 µM ferric colloids. To measure these iron concentrations, 50 ml of 4% PG
32 media was incubated at 27°C for 6 hours with and without 100 µM ferric colloids. The media
33 was then centrifuged at 4600xg for 15 minutes at room temperature. The top 45 ml of
34 supernatant was taken for the following steps. To capture and concentrate the soluble iron in the

1 supernatant, 125 μ M 8-Hydroxyquinoline (oxine) was added to chelate labile iron, and 5 ml of
 2 1M MES, pH 6 was added to aid in oxine's solubility in the media and its binding to iron, which is
 3 facilitated by lower pH. These solutions were incubated for 24 hours protected from light, and
 4 then filtered through a Sep-Pak C18 column (Waters, Cat. No. WAT036800) to bind oxine-iron
 5 complexes. The column was then washed with 5 ml water and eluted with 1.4 ml methanol. To
 6 measure iron concentrations in the eluate, 50 μ l of eluate was measured with an colorimetric
 7 iron detection kit (Sigma-Aldrich, Cat. No. MAK025-1KT) following the provided protocol. Iron
 8 concentration was determined by measuring absorbance at 595 nm on a plate reader.

9 **Iron-limited *E. pacifica* pellets** were prepared by growing *E. pacifica* in 4% PG for 72 hours at
 10 225 rpm and 30°C, then pelleted, aliquoted, and frozen into 10 mg pellets, and stored at -80°C.
 11 *E. pacifica* pellets were resuspended in 1ml of media to achieve a 10mg/ml liquid bacteria stock
 12 to add to cultures.

13 **Culturing with different iron sources.** To set up the iron utilization assay, cultures grown in
 14 25% RA were passaged at a 1:60 dilution into 25% RA and grown for 72 hours at 18°C. This
 15 culture was then passaged at a 1:60 dilution into 25% RA and grown for 24 hours at 27°C.
 16 Finally, this culture was passaged at a 1:30 dilution into 4% PG (which had no iron added) and
 17 grown at 27°C for 24 hours. Cells from this final passage were twice washed by first centrifuging
 18 the culture at 2600xg for 4 minutes and then resuspending the pellet in 30 ml sterile ASW. After
 19 the final wash, the pellet was resuspended in 100 μ l ASW and counted. These washed cells
 20 were used to seed cultures at 10^4 cells/ml in 4% PG with the additions of 100 μ M iron (ferric
 21 EDTA or ferric colloids) and 50 μ g/ml of iron-limited *E. pacifica* pellets (see above). The resulting
 22 culture was grown at 27°C for 48 hours and harvested for each experiment.

23

24 **qPCR**

25 **Protocol Links:** [dx.doi.org/10.17504/protocols.io.ewov1nnzogr2/v1](https://doi.org/10.17504/protocols.io.ewov1nnzogr2/v1)

26 [dx.doi.org/10.17504/protocols.io.n2bvjnjongk5/v1](https://doi.org/10.17504/protocols.io.n2bvjnjongk5/v1)

27 **Primer design.** Primers were designed against *cytb561a*, the experimental gene of interest and
 28 *cofilin*, a highly expressed eukaryotic specific housekeeping gene with a consistent expression
 29 between *S. rosetta* cell types, as shown by the RNAseq analysis. Primers for quantitative
 30 polymerase chain reaction (qPCR) were designed by selecting 19-20 bp oligos spanning
 31 exon-exon junctions from cDNA sequences, (downloaded from:
 32 https://protists.ensembl.org/Salpingoeca_rosetta_gca_000188695/Info/Index). Primer
 33 sequences as follows: *cytb561a* forward primer 5'-CATGGAAGAAGCGCATGGTG-3', reverse
 34 primer 5'-GGGTCCGCAAGATCATTGAG-3', *cofilin* forward primer

1 5'-CAAGCTCCCCACGGACAAG-3', reverse primer 5'-GGGTCCATGCGAAGAAGAC-3'. In the
2 case of *cytb561a*, primers were selected downstream of the premature termination sequence
3 edited in by CRISPR/Cas9.

4 **Primer validation.** Target amplicons were amplified by polymerase chain reaction (PCR) from
5 the cDNA of slow swimmer and thecate cultures (Fig. S4A) with the Luna Universal qPCR
6 Master Mix (NEB, Cat, No. M3003). The amplicons were run on a 1% (w/v) agarose gel in
7 Tris-Borate-EDTA (TBE) buffer to verify that the primer sets amplified only one amplicon. The
8 amplification efficiency of primer sets was characterized across a serial dilution of ssDNA
9 standards for each target (Fig. S4B-C). ssDNA standards were generated from PCR products
10 amplified with a forward primer that was 5' phosphorylated to promote Lambda exonuclease
11 digestion of that strand (NEB, Cat. No. M0262S) and a reverse primer with phosphorothioate
12 bonds between the first four 5' nucleotides to block digestion. Those PCR products were then
13 digested with Lambda exonuclease following NEB's protocol. ssDNA concentrations were
14 determined by Qubit ssDNA Assay Kit (Thermo Fisher, Cat. No. Q10212) by creating a standard
15 curve with the provided standards and then reading fluorescence (Ex = 500 nm / Em=540 nm)
16 on a plate reader (Molecular Devices, SpectraMax iD5). The ssDNA standards were serially
17 diluted from 10⁵ to 10⁰ copies/μl in a solution with 10 ng/μl of *S. rosetta* RNA (Total RNA after
18 removing mRNAs) to account for any matrix effects.

19 **cDNA preparation.** Samples from slow swimmer or thecate cultures were pelleted and lysed
20 using the preferential lysis protocol, and total RNA was purified by RNeasy MinElute Cleanup
21 Kit (Qiagen, Cat. No. 74204). cDNA was prepared by SuperScript IV Reverse Transcriptase
22 (Thermo Fisher, Cat. No. 18090010) following the provided protocol using oligo d(T)₂₀ for
23 synthesis. However, the incubation temperature for cDNA synthesis was increased to 60°C to
24 account for the high GC content of the *S. rosetta* genome.

25 **qPCR.** 3 μl of cDNA samples or ssDNA standards were added to a 20μl reaction with Luna
26 Universal qPCR Master Mix (NEB, Cat, No. M3003) according to the provided protocol. The
27 samples were amplified in a single run on the QuantStudio 3 Real-Time PCR System (Thermo
28 Fisher, Cat. No. A28567).

29

30 **Genome editing**

31 **Protocol Link:** [dx.doi.org/10.17504/protocols.io.j8nlk86o5l5r/v1](https://doi.org/10.17504/protocols.io.j8nlk86o5l5r/v1)

32 **Culturing cells for transfection.** Mutant strains were generated with a modified protocol from a
33 previously published method⁴⁶. Cultures of *S. rosetta* slow swimmers were maintained in 15%
34 RA/2% PYG (called 15/2, Table S1) at 22°C, and 48 hours prior to transfection, cultures were

1 seeded at 10^4 cells/ml in 80 ml of 15/2 Media in 300 cm² vented tissue culture flasks (VWR, Cat.
2 No. 10062-884) and grown at 22°C.

3 **Cas9 RNP preparation.** On the day of transfection, Cas9 bound to a guide RNA (Cas9 RNP)
4 was prepared by combining 2 µl of 100 µM guide RNA (gRNA) with 2 µl of 20 µM EnGen
5 SpyCas9 NLS (NEB, Cat. No. M0646T), and incubated at room temperature for 2-4 hours.
6 (Note: Our gRNA was ordered as synthetic RNA oligonucleotides from Integrated DNA
7 Technologies that came as an Alt-R CRISPR-Cas9 crRNA and an Alt-R CRISPR-Cas9
8 tracrRNA. These two oligonucleotides were annealed together to form gRNA). Guide sequences
9 can be found in table S4.

10 **Transfection.** During the Cas9 RNP incubation, cells were harvested for transfection by
11 centrifuging the cultured cells at 2400xg for 3 minutes at 4°C. The supernatant was discarded,
12 and cells were resuspended in 50 ml of Cell Wash Buffer (420 mM NaCl; 50 mM MgCl₂; 30 mM
13 Na₂SO₄; 10 mM KCl; titrated to pH 8.0 with ~2.4 mM NaHCO₃). The cells were centrifuged again
14 at 2400xg and 4°C for 3 minutes. The supernatant was removed, and the pellet was
15 resuspended in 100 µl of Cell Wash Buffer. Cells were counted and diluted to 5×10^7 cells/ml,
16 split into 100 µl aliquots, and centrifuged for 2 min at 800xg and room temperature. The
17 supernatant was removed and replaced with 100 µl priming buffer (40 mM HEPES-KOH, pH
18 7.5; 34 mM Lithium Citrate; 15% (w/v) PEG 8000; 50 mM Cysteine; 1.5 µM Papain). Cells were
19 primed for 45 minutes at room temperature. Afterwards, the priming was quenched with 10 µl of
20 50 mg/ml bovine serum albumin. The primed cells were centrifuged at 1200xg for 4 min at room
21 temperature. After removing the supernatant, the cells were resuspended in 25 µl ice cold SF
22 buffer (Lonza Cat. No. V4SC-2096). Nucleofection reactions were prepared by combining 16 µl
23 ice cold SF buffer, 4 µl Cas9 RNP, 2 µl of repair oligo, and 2 µl of the resuspended and primed
24 cells. This reaction mix was loaded into a 96-well nucleofection plate (Lonza Cat. No.
25 AAF-1003S, AAF-1003B), and pulsed with CU 154 (Fig. S4). 100 µl of ice cold recovery buffer
26 (10 mM HEPES-KOH, pH 7.5; 900 mM Sorbitol; and 8% (w/v) PEG 8000) was added
27 immediately to each pulsed well and incubated for 5 minutes at room temperature. Afterwards
28 the entire content from each nucleofection well was transferred into 2 ml of 15/2 Media with the
29 addition of 50 µg/ml *E. pacifica* pellet.

30

31 **Strain isolation and Screening**

32 **Protocol Link:** [dx.doi.org/10.17504/protocols.io.14egn6n2pl5d/v1](https://doi.org/10.17504/protocols.io.14egn6n2pl5d/v1)

33 We adapted the Cas12a DETECTR genotyping assay¹⁰¹ to screen for cells with desired
34 mutations from Cas9 genome editing. Following overnight recovery post-nucleofection, cells

1 were counted and diluted to 45 cells/ml in 10% RA (Table S1) with 50 µg/ml *E. pacifica* pellet
2 and aliquoted into 96-well plates. Plates were then grown at 27°C for 72 hours to propagate
3 cells. Afterwards 12 µl from each well was added into 36 µl DNAzol Direct (20 mM potassium
4 hydroxide; 60% (w/v) PEG 200, pH 13.3-13.7– Note: It is important to test a range of pH values
5 to establish the optimal pH for your own use)⁴⁶ that was pre-aliquoted into 96-well PCR plates,
6 which we called the lysed sample. The lysed samples were then incubated at 80°C for 10
7 minutes. The target DNA was amplified (primer sequences in Table S4) in PCR reactions
8 prepared with GoTaq Clear Master Mix (Promega, Cat. No. M7123) using 2 µl of the lysed
9 samples per 25 µl PCR reaction. Cycling conditions followed Promega guidelines and melt
10 temperatures for oligos were calculated using
11 (<https://www.promega.com/resources/tools/biomath/tm-calculator/>). During the PCR, a Cas12a
12 mastermix was assembled in two parts: First, Cas12a RNP: (13 µl water; 2 µl r2.1 buffer [NEB,
13 Cat. No. B6002S]; 2.5 µl 100 µM gRNA [ordered as a synthesized oligo]; 2 µl 100 µM
14 LbaCas12a [NEB, Cat. No. M0653T]) was incubated for 5 minutes at room temperature.
15 Second, the Cas12a RNP was combined with the rest of the components (for one 96-well plate:
16 486 µl water; 60 µl r2.1 buffer; 18 µl Cas12a RNP; 36 µl 5 µM ssDNA probe [IDT, Cat. No.
17 11-04-02-04]) and then incubated for 5 minutes at room temperature. 5 µl of the mastermix was
18 added to each 25 µl PCR and incubated at 37°C for 1 hour. The fluorescent signal was then
19 measured in the VIC channel on a QuantStudio 3 Real-Time PCR System. Wells with high
20 signal (≥10-fold above background) were recovered in 25% RA overnight at 27°C, counted,
21 diluted to 3 cells/ml in 10% RA with 50 µg/ml *E. pacifica* pellet, and plated in a 96-well plate with
22 100 µl/well, which corresponds to 0.3 cells/well. Plates were grown at 27°C for 72-96 hours.
23 Finally, these clonal isolates were screened by digesting amplicons with a restriction enzyme
24 (RE). 12 µl per isolate was added to 36 µl DNAzol, incubated at 80°C to make a lysed sample. 2
25 µl of the lysed samples were added to 25 µl PCR reactions with GoTaq Green mastermix
26 (Promega, Cat. No. M7123). After amplification, 1 µl of RE was added to each 25 µl PCR
27 reaction, and incubated following manufacturers' protocols. RE digestions were run on 1%
28 agarose gels with wild-type or undigested controls. For positive RE digestion hits, PCR products
29 were Sanger sequenced to confirm the sequence of the edited site (Table S5).

30

31

32

33 **Growth curves**

1 **S. rosetta**. We monitored cell density at times points over 48 hours to assess the population
2 dynamics of wild type and *cytb561^{PTS}* thecates in iron utilization assay conditions. Cells were
3 plated in 24-well plates with 100 μ M ferric EDTA or ferric colloids sources in 4% PG or in
4 nutrient replete media (25% RA) as a control. At each time point, the entire contents of one well
5 was harvested by scraping the bottom and then transferring the liquid to a microfuge tube. Cells
6 were fixed with 5 μ l of 37.5% paraformaldehyde (PFA) and then counted on a Reichert
7 Bright-Line Metallized Hemacytometer (Hausser Scientific, Cat. No. 1483). Growth curves were
8 fit to a logistic growth equation that explicitly models the lag time with a heaviside step
9 function²⁸.

10 **E. pacifica** growth curves were set up in 4% PG media inoculated with 50 μ g/ml iron-limited *E.*
11 *pacifica* pellet and a ferric EDTA titration in clear bottom 96-well plates, and grown for 48 hours
12 at 27°C. Growth was assessed by monitoring the optical density at 600 nm on a plate reader
13 with orbital shaking before every reading. Ferric colloids were not included in the growth curves
14 as iron particulates would shift optical density readings. To assess *E. pacifica* growth with ferric
15 colloids, 4% PG media was inoculated with 50 μ g/ml iron-limited *E. pacifica* pellet and 100 μ M
16 ferric EDTA or ferric colloids in 6-well plates and grown for 48 hours at 27°C. Wells were
17 scraped and 1 ml aliquots were centrifuged at 500xg for 2 minutes to settle iron particulates.
18 The top 500 μ l of supernatant was gathered for measuring the optical density at 600 nm.

19

20 **Immunofluorescent Staining**

21 **Protocol Link:** [dx.doi.org/10.17504/protocols.io.5jyl85k38l2w/v1](https://doi.org/10.17504/protocols.io.5jyl85k38l2w/v1)

22 Cultures of wild-type and ALFA-tagged strains were maintained in 25% RA at 18°C for thecates
23 and 22°C for slow swimmers, as higher temperatures helped to maintain slow swimmers. 24
24 hours prior to imaging, cultures were seeded at 10⁴ cells/ml in 6 ml of 25% RA in 25cm² vented
25 tissue culture flasks (VWR Cat. No. 10062-874) and grown at 27°C. On the day of imaging, prior
26 to preparing cells, 50 μ l of 10 mg/ml Poly D-lysine hydrobromide (MP Biomedicals, Cat. No.
27 102694) was added to each well of a glass bottom 96-well plate (Thermo Scientific, Cat. No.
28 165305) and incubated for $\geq$ 15 min at room temperature. To prepare cells, the supernatant of
29 thecate cultures was decanted, and replaced with 6 ml filtered ASW, then scraped with a cell
30 scraper, and centrifuged at 500xg for 5 minutes at room temperature. For swimmer cultures, the
31 cells were centrifuged 500xg for 5 minutes at room temperature. The supernatant removed
32 leaving 0.5 ml remaining, and resuspended in 5 ml filtered ASW and centrifuged 500xg for 5 min
33 at room temperature. Both thecate and swimmer cultures follow the same protocol henceforth.
34 The supernatant was removed, leaving 0.5 ml, and the tubes gently swirled to resuspend cells.

1 The poly D-lysine coated wells were washed 3 times with 50 μ l ASW, and then 50-100 μ l of cells
2 were added to each well using wide-bore pipette tips. Cells settled on the well surface for 15
3 minutes. All subsequent steps use gel loading tips. After settlement, the supernatant was
4 removed leaving 25 μ l behind to avoid drying and damaging delicate cell structures. 70 μ l of Fix
5 buffer (10 mM MES-KOH, pH 6.1; 138 mM KCl; 3 mM $MgCl_2$; 2 mM EGTA; 3% (v/v) PFA; 15%
6 (w/v) sucrose) was slowly added to the top of each well and incubated for 5 min at room
7 temperature. 70 μ l of supernatant was removed from the opposite side from which solution was
8 added. This will be referred to as a “wash”. Next, wells were washed with Fix/tween buffer (Fix
9 buffer with the addition of 0.07% (v/v) Tween-20) and incubated for 5 min at room temperature.
10 Wells were then washed with a quench buffer (10 mM MES-KOH, pH 6.1; 138 mM KCl; 3 mM
11 $MgCl_2$; 2 mM EGTA; 15% (w/v) sucrose; 0.3M glycine) and washed immediately with
12 Permeabilization-MeOH buffer (8% (v/v) MeOH; 1% (v/v) Tween-20; in LICOR Intercept
13 Blocking Buffer [LI-COR Biosciences Cat. No. 927-70001]) and incubated for 15 minutes. (Note:
14 LICOR Intercept Blocking buffer increased detection of AFLA epitopes during testing.) After,
15 wells were washed with Permeabilization buffer (1% (v/v) Tween-20; LICOR Intercept Blocking
16 Buffer) and then washed twice in antibody mix (1:200 FluoTag-X2 anti-ALFA Alexa647
17 [Nanotag, Cat No. N1502-AF647-L]; 1:200 phalloidin Alexa546 [Invitrogen, Cat. No. A22283];
18 1:500 alpha Tubulin Monoclonal Antibody, DM1A [Invitrogen, Cat. No. 62204]; 1:500 alpaca
19 anti-mouse IgG1, recombinant VHH, Alexa488 [Chromotek, Cat. No. sms1AF488-1]) diluted in
20 the Permeabilization buffer and incubated for 1 hour protected from light. Afterwards, wells were
21 washed twice with PEM (100 mM PIPES-KOH, pH 6.9; 1 mM EGTA; 1 mM $MgSO_4$) and
22 immediately imaged.

23

24 **Microscopy**

25 **Time-lapse of ferric colloid ingestion.** 100 μ l of 1.46 μ M fluorescent ferric colloids were added
26 to 2 ml of wild-type thecate cultures, previously grown in glass bottomed dishes (World
27 Precision Instruments, Cat. No. FD35-100) for 24 hours at 22°C. Samples were imaged by
28 widefield microscopy. The microscope was a Nikon Eclipse Ti2-E inverted microscope outfitted
29 with a D-LED light source, Chroma 89401 Quad Filter Cube, and 60x CFI Plan Apo VC NA1.2
30 water immersion objective. Time-lapse images were acquired every 4 sec over 2 hours with 50
31 msec exposure time for brightfield and fluorescent (TRITC channel) images. In three
32 independent experiments, a total of 4 feeding events were found in a field of 200 cells, although
33 this was not an exhaustive search for ferric colloid intake frequency. One cell that was oriented
34 on its side to best visualize feeding was selected for further processing. Images were processed

1 in FIJI by cropping individual cells in 100x100 pixel bounds, and image contrast was manually
2 adjusted. Ferric colloid particles were automatically tracked, first by setting a threshold to
3 produce a binarized image (image > adjust > manual threshold) and then by finding particles
4 (analyze > analyze particles > size = 10-300 pixels, circularity = 0.2-1.0).
5 **Immunofluorescence Imaging.** Samples were imaged by widefield microscopy. The
6 microscope was a Nikon Eclipse Ti2-E inverted microscope outfitted with a D-LED light source,
7 Chroma 89401 Quad Filter Cube, and 60x CFI Plan Apo VC NA1.2 water immersion objective.
8 Single focal plane and Z-stacks of samples were imaged with two-fold binning (120 μm /pixel) on
9 a Nikon Digital Sight 50 M Camera with 100-400 msec exposure times for each channel and
10 variable illumination intensities to extend the dynamic range of signal without photobleaching
11 samples. Single focal-plane images were processed within the Nikon imaging software. Images
12 were background subtracted (120 pixel, rolling ball radius), and deconvolved using the
13 Landweber deconvolution algorithm with 12-16 iterations (autostopping engaged), and
14 automatic background subtraction. Deconvolved images were cropped in 150x150 pixel bounds
15 in FIJI and merged images were combined using maximum intensity composite. Supplementary
16 images were processed in FIJI by cropping individual cells in 150x200 pixel bounds, projecting
17 the maximal intensity, subtracting the background (75 pixel, sliding paraboloid radius),
18 enhancing the contrast (saturation 0.02% of the pixels), and applying a minimum filter of 0.5
19 pixels. Merged images were combined using maximum intensity composite.

20

21 **Western blots**

22 **Protocol Link:** [dx.doi.org/10.17504/protocols.io.3byl4987zgo5/v1](https://doi.org/10.17504/protocols.io.3byl4987zgo5/v1)

23 Cultures were grown in 25% RA or 4% PG (without supplemented iron) for 24 hours at 27°C to
24 test whether external iron conditions (replete or deplete) resulted in any post-translational
25 regulation of protein expression. Cultures were scraped and then lysed according to the
26 preferential lysis protocol, with the following modifications: TurboDNase and SUPERasein were
27 replaced with 15 μl of Pierce universal nuclease (Thermo Scientific Cat. No. 88702) per 1 ml
28 buffer, and heparin and cycloheximide were removed. After clearing the lysate, Tween-20 was
29 added to a final concentration of 1% (v/v), and the sample was incubated for 10 minutes at room
30 temperature to aid in liberating membrane-bound proteins. Protein concentration was
31 determined by Bradford assay (Thermo Fisher Scientific Cat. No. 23236) and samples were
32 normalized to the lowest concentration. To prepare for SDS-PAGE, approximately 20 μg or 40
33 μg of total protein for thecates or slow swimmers, respectively, was denatured with 4x Laemmli
34 SDS loading buffer and incubated for 10 minutes at room temperature (Note: 80°C caused

1 membrane proteins to crash out, so room temperature incubation was essential). Samples were
2 loaded into a Novex wedgewell 10% Tris-Glycine Mini Gel (Thermo Fisher Scientific Cat. No.
3 XP00105BOX) along with a 1:10 diluted PageRuler Plus Prestained Protein Ladder (Thermo
4 Fisher Scientific Cat. No. 26619). Gels were run at 225 V for 30 minutes, and afterwards the gel
5 was transferred to a 0.45 µM nitrocellulose membrane (Bio-rad Cat. No. 1620115) at 50 V for 30
6 minutes, then 100 V for 60 minutes in transfer buffer (25 mM tris base; 192mM glycine; 10%
7 methanol) at 4°C. Afterwards the membrane was stained with total protein stain (LI-COR
8 Biosciences Cat. No. 926-11010) and then imaged in the 700 nm channel with a 30 second
9 exposure on a LI-COR Odyssey Fc. Afterwards, the membrane was blocked in LICOR Intercept
10 Blocking Buffer (LI-COR Biosciences Cat. No. 927-70001) for 1 hour at 37°C and then overnight
11 at 4°C. The following day the membrane was immunostained with FluoTag-X2 anti-ALFA
12 LI-COR IRDye 800CW (NanoTag Biotechnologies Cat. No. N1502-Li800-L) diluted 1:10,000 in
13 LI-COR PBS block plus 0.2% Tween-20 for 2 hours at room temperature. (Note: Both the
14 LI-COR PBS block and anti-ALFA IRDye 800CW were essential for visualization, other blocks
15 and fluorophores lead to significantly lower signal.) The membrane was washed 3x in PBST
16 (PBS with 0.2% Tween-20) and then imaged in the 800 nm channel with a 10 minute exposure.
17 This western protocol was adapted from a previous study⁹⁵.

18

19 **Protein database searches and tree construction**

20 We compiled a list of iron acquisition proteins (Table S6) from these model organisms: *Homo*
21 *sapiens*⁴⁴, *Saccharomyces cerevisiae*⁴⁷, *Chlamydomonas reinhardtii*⁴³, *Arabidopsis thaliana*¹⁰²,
22 and *Phaeodactylum tricornutum*⁴¹. For each protein, we retrieved the corresponding Pfam
23 hidden markov model (HMM) from Interpro (<https://www.ebi.ac.uk/interpro/>)¹⁰³. Using HMMER
24 v3.4 (hmmer.org¹⁰⁴), we searched proteomes from EukProt v3¹⁰⁵, focusing on proteomes from
25 “The Comparative Set” (TCS), which consisted of 196 species of phylogenetic relevance with
26 high BUSCO completion. We supplemented the TCS with the transcriptomes of 18
27 choanoflagellate species⁵⁴ and other Holozoa and Fungi. To count the number of unique
28 proteins that conformed to the HMM profile from ‘hmmsearch’, we aligned hits with ‘hmmalign’
29 and retained only one sequence from hits with >99% identity (Note: this was important for
30 removing multiple isoforms and reference sequences from highly annotated genomes). We
31 additionally filtered hits based on length with ‘esl-alimanip’ to retain protein sequences that only
32 possessed the HMM profile, which eliminated multidomain proteins that only had the HMM
33 profile as a one of its domains (Table S3 for length filter thresholds). (Note: The number of hits
34 prior to filtering can be found in Table S3) For HMM profiles that yielded a high number of

1 protein hits that were not well-filtered (e.g. Transferrin Receptor), we aligned sequences with
 2 MAFFT-DASH⁶¹, removed sites that were uninformative or contained 90% gaps with ClipKIT¹⁰⁶
 3 (kpic-gappy 0.9), and generated phylogenetic trees with IQ-TREE¹⁰⁷ using ModelFinder¹⁰⁸ to
 4 choose the appropriate substitution model for each set of protein alignments. This procedure
 5 was also used to determine the phylogenetic relationships of Cytb561 paralogs (Fig. 4A), and
 6 the chosen substitution model was LG+F+R7. Trees were visualized with iTOL¹⁰⁹. Multiple
 7 sequence alignments were visually inspected and curated for supplemental figures using
 8 Jalview v2¹¹⁰. Cytb561 structure models were predicted with AlphaFold v3⁶⁵ and visualized using
 9 UCSF ChimeraX⁶⁴.

10

11 Gene distribution maps and correlations with upwellings.

12 **cytb561 paralog search.** The abundance and location of *S. rosetta* cytb561 paralogs was
 13 searched for using the Ocean Gene Atlas^{8,9}. Protein sequences of each paralog from *S. rosetta*
 14 were searched for in the MATOUv1+G (for metagenomes) and MATOUv1+T (for
 15 metatranscriptomes) databases, using tblastn with the following search parameters: expected
 16 threshold E-values < 1⁻¹⁰, and abundance as percent of total reads. The Ocean Gene Atlas
 17 automatically assigns protein sequences to phyla and classifies them on expected organism
 18 size, such that the results could be filtered first by size (0.8-5 µm) and then by phyla to identify
 19 only choanoflagellate specific hits. Therefore to identify choanoflagellate specific hits and the
 20 location they were gathered from, three tables were gathered from each search: the distribution
 21 of E-values (E-values per hit and their assigned phyla), abundance matrix for the 0.8-5 µm
 22 fraction (the abundance as a fraction of total reads and the sample site ID for each hit), and
 23 environmental parameters for the 0.8-5 µm fraction (all measured and inferred environmental
 24 measurements for each hits sample location). From these three tables, choanoflagellate
 25 assigned hits, abundances, and sample locations were compiled.

26 **Upwelling velocities.** To identify average vertical velocities at the sample sites from the Tara
 27 Oceans Expedition¹¹¹, from which the Ocean Gene Atlas acquires its data, upward seawater
 28 velocities were gathered from the Copernicus Marine Services' Global Ocean Physics Analysis
 29 and Forecast model's (<https://doi.org/10.48670/moi-00016>) dataset
 30 (cmems_mod_glo_phy-wcur_anfc_0.083deg_P1M-m). The coordinates for each sample site
 31 were compiled¹¹¹ and vertical velocities were gathered from the dates 10/31/2020 - 5/20/2024.
 32 Then the search was done using a modified script offered by the Copernicus Marine Services
 33 (<https://help.marine.copernicus.eu/en/articles/7970637-how-to-download-data-for-multiple-point>

1 [s-from-a-csv](#)). The vertical velocity at each sample site was then averaged across the 3.5 year
2 range to account for temporal variation (Fig. S8B).

3 **Correlations and maps.** Choanoflagellate paralog abundances were tabulated with their
4 sample site ID, sample coordinates, and average upwelling velocity (from above). First, the
5 abundances and average vertical velocities were correlated by the Pearson function in Microsoft
6 Excel. To calculate the associated P value for each Pearson correlation, a T statistic was
7 calculated using the formula: $t = (r \cdot [n-2]^{1/2}) \cdot (1-r^2)^{-1/2}$, where r is the calculated Pearson
8 correlation, n is the sample number, t is the T statistic. This statistic was then input into the t
9 distribution function (TDIST) in Excel, along with the degrees of freedom ($n-2$), to calculate the P
10 values. Next, the abundances and average vertical velocities were plotted in a scatter plot to
11 manually inspect the data for outliers. If a datum was suspected of being an outlier, a Dixon's Q
12 test was conducted for that datum with the formula: $Q = |gap|/range$, where gap = the difference
13 between the suspected outlier and the next nearest datum, and range = the range of the data
14 set. The value is considered an outlier and removed if $Q > Q_{table}$, where Q_{table} is a reference
15 value determined by the sample size and confidence level. Of all tested outliers, only one datum
16 was removed, belonging to *cytb561a* transcript abundance at TARA site 52 (-16.96° , 53.99°),
17 with a $Q = 0.8718$ and where $Q_{table} = 0.3323$ for 99% confidence level for a sample size of 33.
18 Without this outlier removed the statistics for *cytb561a*'s correlation would be $r = 0.09$ and $P =$
19 0.58 . Maps were plotted with the compiled table of paralog abundances and coordinates, using
20 matplotlib with modified scripts generated by ChatGPT (<https://chat.openai.com>).

1 Data Availability:

2 RNA sequencing data is available in the public repository NCBI Gene Expression Omnibus
3 (GEO) database under requisition ID: GSE267344

4

5 Author Contributions:

6 FL and DSB conceived of the project, designed experiments, conducted research, performed
7 data analysis, and wrote the manuscript. JMEE and VD generated strains and developed the
8 genotyping pipeline. MCC and SE performed an early analysis of RNA-seq data.

9

10 Acknowledgements:

11 We thank Nicole King for supporting an early phase in this project and providing feedback. Alain
12 Garcia de las Bayonas, Flora Rutaganira, Ben Larson, members of the Booth lab, and the
13 students at the Marine Biological Laboratories (MBL) 2023 Physiology Course provided
14 essential experimental advice. We also appreciate helpful discussions from Elizabeth
15 Blackburn, Jeremy Reiter, Wallace Marshall, Sandy Johnson, Carol Gross, and Dyche Mullins.
16 FL was supported by a National Science Foundation Graduate Research Fellowship, a UCSF
17 Discovery Fellowship, and the UCSF Tetrad Graduate Program Training Grant T32GM139786.
18 This work was supported by awards to DSB from the Chan Zuckerberg Biohub, the UCSF
19 Sandler Program for Breakthrough Biomedical Research, a David and Lucile Packard
20 Foundation Fellowship in Science and Engineering, and a Maximizing Investigators' Research
21 Award for Early Stage Investigators from the National Institute of General Medical Sciences
22 R35GM147404. Sequencing was additionally supported by an instrumentation grant from the
23 National Institutes of Health S10OD018174 to the QB3 Vincent J. Coates Genomics
24 Sequencing Laboratory at University of California, Berkeley.

1 Figures:

2

3 **Figure 1:** Thecates from the choanoflagellate *S. rosetta* are a distinct cell type.

4 **(A)** *S. rosetta* differentiates into morphologically distinct cell types. This schematic shows four of
5 the cell types from *S. rosetta* that were stably cultured to produce transcriptome profiles. All of
6 these cell types display the common choanoflagellate cell architecture in which an apical
7 flagellum is encircled by a collar of actin-filled microvilli (indicated on the slow swimmer) that
8 enables choanoflagellates to phagocytose bacteria and particulate matter. When nutrients are
9 abundant, slow swimmers (green) respond to bacterial and algal cues to develop into
10 multicellular rosettes (blue) that form by serial cell division. Cultures of slow swimmers also form
11 chains of cells through serial cell divisions, but those chains are easily disrupted by mechanical
12 force. Under starvation, slow swimmers and rosettes become fast swimmers (grey), which have
13 a reduced cell body and collar with a longer flagellum. Under sustained nutrient deprivation, fast
14 swimmers differentiate into thecates (red), a type of cell that adheres to substrates by
15 constructing an extracellular apparatus called a theca. Thecates still proliferate in low nutrient
16 conditions by dividing into swimmers that build their own theca. By taking the supernatant of
17 thecate cultures in high nutrient conditions, thecates can then differentiate into slow swimmers.
18 **(B)** The transcriptome profile of thecates stands apart from all other cell types. A principal
19 component analysis of triplicate RNA-seq profiles from each cell type (panel A) shows that the
20 86% of the variance between samples is attributed to the thecate transcriptome profile. All other
21 cell types cluster closely together.

22

23 **Figure 2:** Thecates utilize ferric colloids through the expression of an iron reductase, *cytb561a*.

24 **(A)** Thecates highly express an iron reductase. The $\log_2$ transformed ratio of transcript
25 abundances (counts) from thecates and slow swimmers (x-axis) plotted against q -values
26 (y-axis) highlights transcriptome changes in each cell type.. Among the genes with transcript
27 abundances that reliably ($q < 0.01$) changed more than two-fold in thecates (red) compared to
28 swimmers (green), PTSG_09715 is in the 98th percentile. This gene, which is annotated as an
29 iron reductase, encodes a single domain: cytochrome b561 (Pfam 03188).

30 **(B)** A method to swap iron sources that support choanoflagellate growth. In preparation for iron
31 limitation assays, *S. rosetta* was passaged in low-nutrient media and depleted of iron. Cells
32 were then washed multiple times and inoculated with iron-limited feeder bacteria. At the same
33 time, ferric EDTA (Fe^{3+} •EDTA) or ferric colloids (Fe^{3+}) were provided. Cultures grew for 48 hours
34 at 27°C before assaying *cytb561a* expression (Fig. 2C and S2) and growth (Figs. 2D and S4)

1 **(C)** *cytb561a* expression is part of the thecate regulon, and not determined by external iron
2 conditions. The expression of *cytb561a* was monitored by RT-qPCR in cultures of slow
3 swimmers (green) or thecates (red) that were grown with either ferric EDTA or ferric colloids.
4 The expression of *cytb561a* was normalized to *cofilin* (PTSG_01554), a eukaryotic gene that
5 displays high, consistent expression across all *S. rosetta* cell types. Independent triplicates were
6 performed and *P*-values were calculated from a two-way ANOVA.

7 **(D)** Thecates require *cytb561a* for increased proliferation with ferric colloids. To account for
8 differences in growth between slow swimmers (green) and thecates (red), the cell density of
9 cultures grown with ferric colloids was normalized to cultures grown with ferric EDTA. With this
10 metric, a ratio greater than one indicates that the cell type displays increased growth with ferric
11 colloids; whereas, a ratio less than one indicates the converse. A premature termination
12 sequence introduced at position 151 in *cytb561a* with CRISPR/Cas9 genome editing produced
13 a mutant allele (*cytb561a*^{PTS}) with a ten-fold reduction in *cytb561a* mRNA levels and stop
14 codons that would truncate the protein translated from this transcript (Fig. S2 and Table 2).
15 Unlike the wild-type strain (circles), thecate cells with *cytb561a*^{PTS} displayed no improved
16 growth with ferric colloids (triangles).

17 **(E)** Thecates ingest ferric colloids through phagocytosis. Time courses of wild-type thecates
18 incubated with fluorescently labeled ferric colloids (see methods on ferric colloid labeling).
19 Fluorescent ferric colloid particles were tracked (magenta outline) and observed being ingested
20 and internalized through phagocytosis. The zero time point indicates initial contact with the ferric
21 colloid particle.

22

23 **Figure 3:** Iron acquisition pathways in choanoflagellates and animals evolved from a toolkit
24 widely conserved in eukaryotes.

25 **(A)** Distribution of iron acquisition proteins across diverse eukaryotes. Iron acquisition proteins
26 characterized in model eukaryotes were compiled into a list to search a curated database of
27 proteomes predicted from the genomes and/or transcriptomes of diverse eukaryotes. The iron
28 acquisition proteins are sorted into these categories from the top to bottom rows: transporters,
29 reductases, oxidases, iron storage, and sharing/binding. Each column indicates a single
30 species, and major eukaryotic lineages are denoted by color. The number of unique protein hits
31 identified in each species is shown in greyscale.

32 **(B)** Cell-type expression of iron acquisition genes identified *S. rosetta* cell types. Genes are
33 denoted by unique identifiers for *S. rosetta* genes and gene names that were given in this work
34 based on their homology with animals. Expression values are averaged triplicate values of

1 transcripts per million (TPM) from cell type transcriptomes (Fig. 1). Of all genes and paralogs,
2 *cytb561a* exhibits the most striking differential regulation.

3 **(C)** The localization of iron acquisition proteins in thecates. An ALFA epitope was engineered
4 into endogenous genetic loci at the carboxy terminus of the protein coding sequences. Although
5 this tag was engineered into all iron acquisition genes (except the vacuolar transporters), only
6 the proteins displayed in this panel were visible in western blots (Fig. S7). Immunofluorescent
7 staining of ALFA-tags, tubulin and phalloidin indicate the localization of tagged protein relative to
8 cytoskeletal markers of cellular architecture. Images of individual cells are representative of
9 more than three independent staining experiments in which six or more images were captured
10 of fields containing more than ten cells. Scale bar is 5 μ m.

11 **(D)** A putative iron acquisition pathway in *S. rosetta*. Iron is internalized by phagocytic vesicles
12 derived at the base of the collar. Inside, Cytb561a reduces iron for transport into the cytosol by
13 Dmt1. Iron is then assimilated by the cell. To maintain iron homeostasis, Fpn exports iron out of
14 the cell, and Heph aids in maintaining a favorable concentration gradient for export.

15

16 **Figure 4:** Cytb561 paralogs possess distinct biochemical properties and appear at different
17 oceanic locations.

18 **(A)** Phylogeny of iron reductases reveals Cytb561a is an ortholog of animal DCYTB. Each
19 paralog of *S. rosetta* falls into distinct clades of cytochrome b561. Circles on each branch are
20 proportional to bootstrap values greater than sixty. Scale bar indicates the average number of
21 substitutions per site. Branch colors correspond to clades as shown in the legend:
22 choanoflagellata (choano.), tereto. Teretosporea (tereto.), amoeba (amoeb.), rhizari (rhiz.),
23 rhodophyta (rhodo.), chromista (chr.), haptophyta (hapt.), metamonoda (metam.)

24 **(B)** Predicted structures of *S. rosetta* Cytb561 paralogs show differences in dimerization and
25 substrate-binding interfaces. Alphafold (v3) predictions show Cytb561a as the only paralog
26 forming homodimers. Predicted electrostatic surfaces Cytb561 paralogs show different charge
27 distributions around the substrate binding pocket (arrows). Models are angled 45° for viewing
28 the lumenal surface where iron binds.

29 **(C)** *cytb561a* transcript abundance correlates with ocean upwelling velocities. Maps (left panels)
30 show the genomic (grey) and transcript (blue) abundance of each paralog. Plots show genomic
31 (middle panels) and transcript (right panels) abundance on the y-axis and average upwelling
32 velocities on the x-axis. Note, the x-axis for *cytb561c* transcript abundance is scaled differently
33 than for *cytb561a* and *cytb561b*. *r* indicates Pearson correlation and *P*-values were calculated
34 from a t-test.

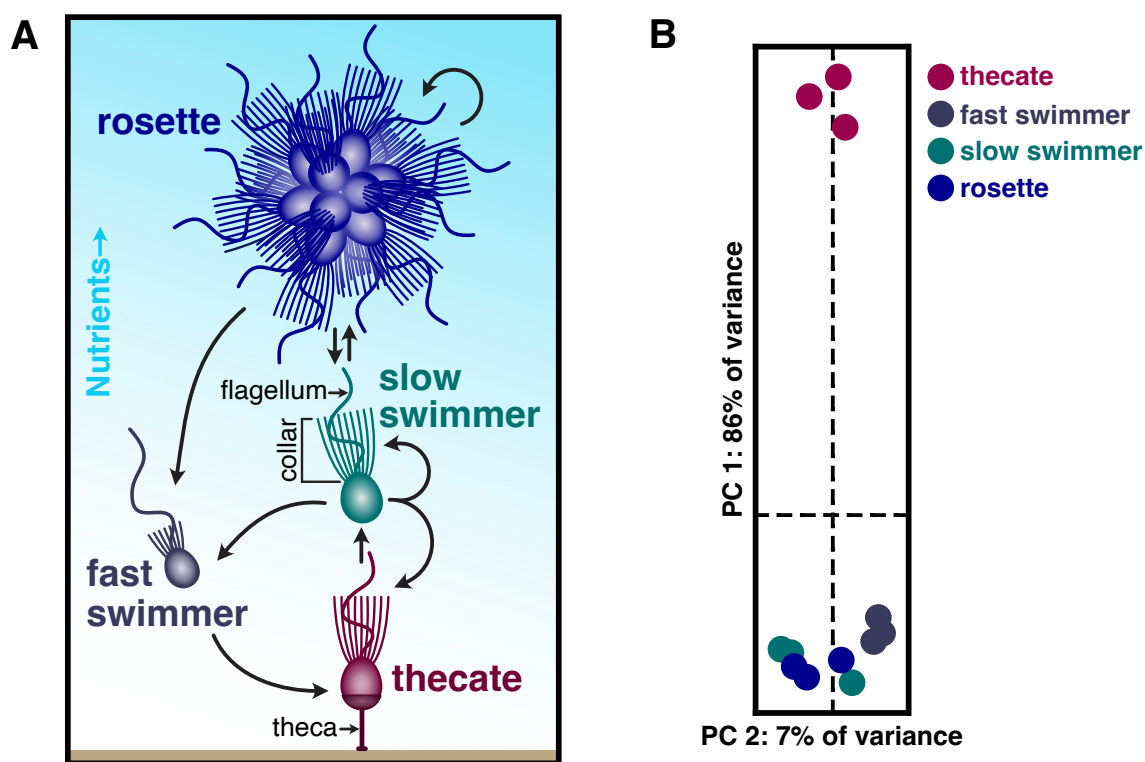


Figure 1: Thecates from the choanoflagellate *S. rosetta* are a distinct cell type.

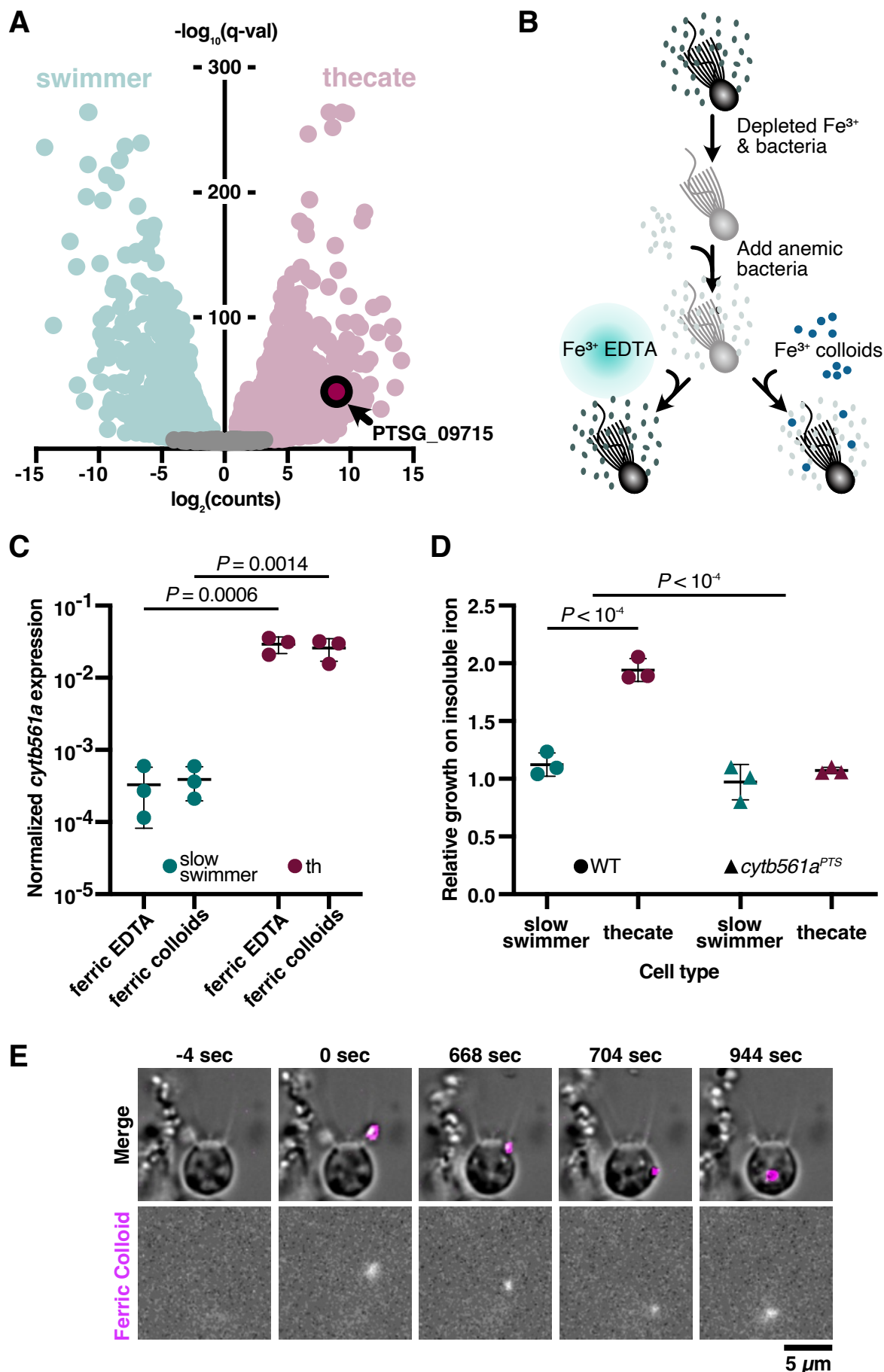
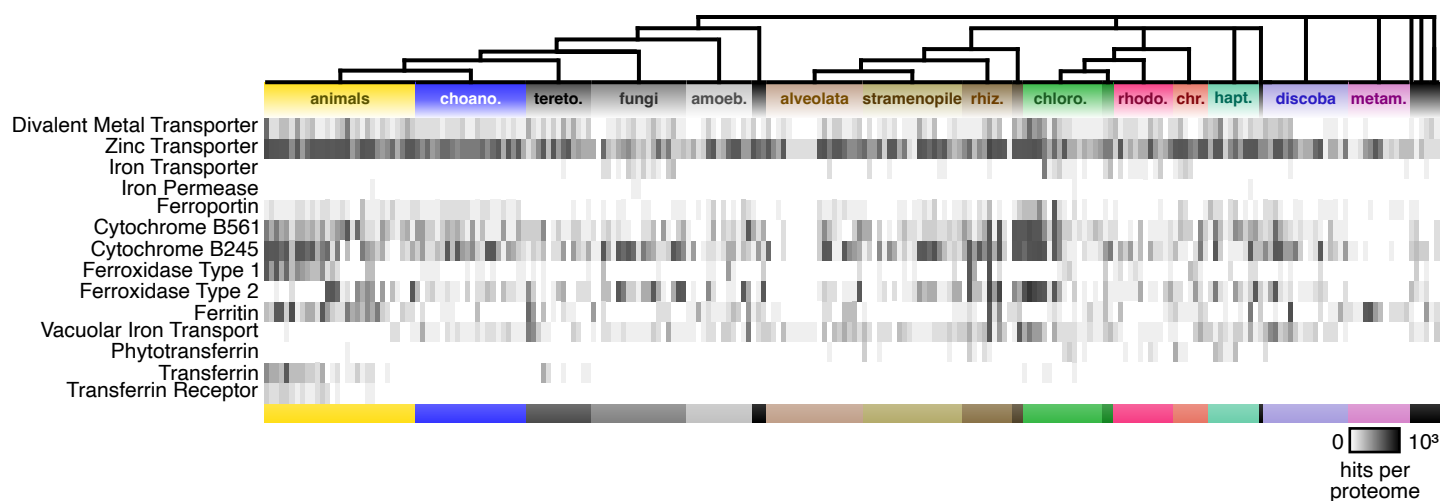
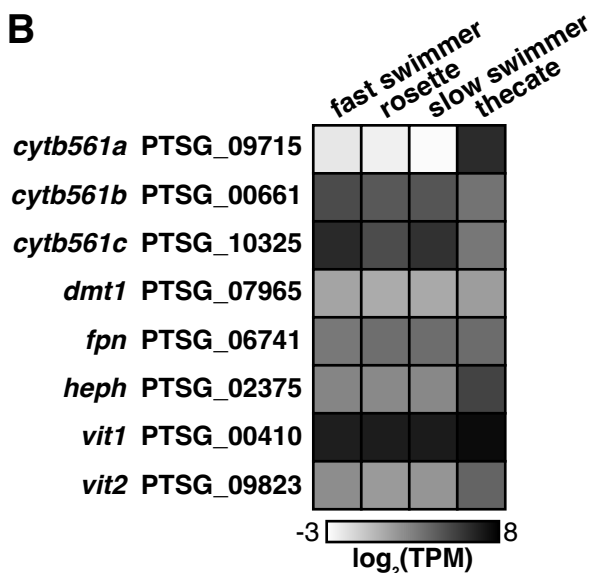


Figure 2: Thecates utilize ferric colloids through the expression of an iron reductase, *cytb561a*.

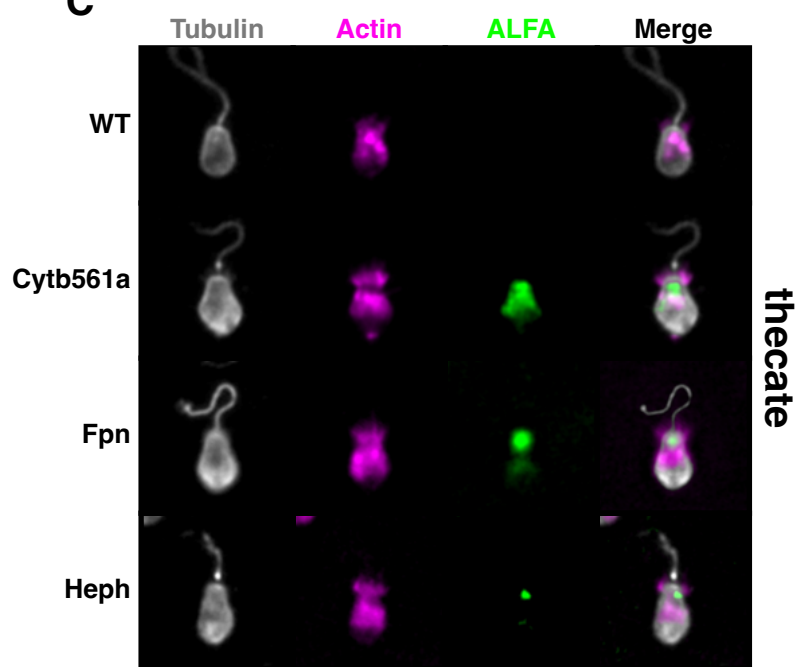
A



B



C



D

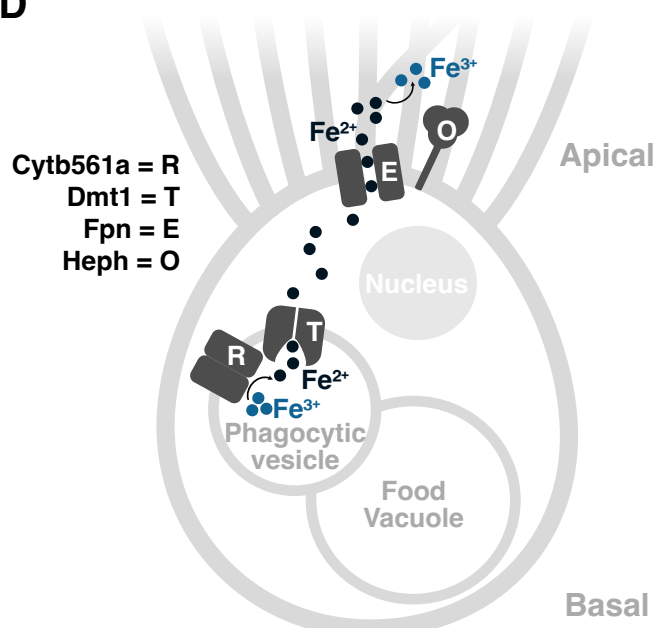


Figure 3: Choanoflagellates and animals share core iron acquisition genes.

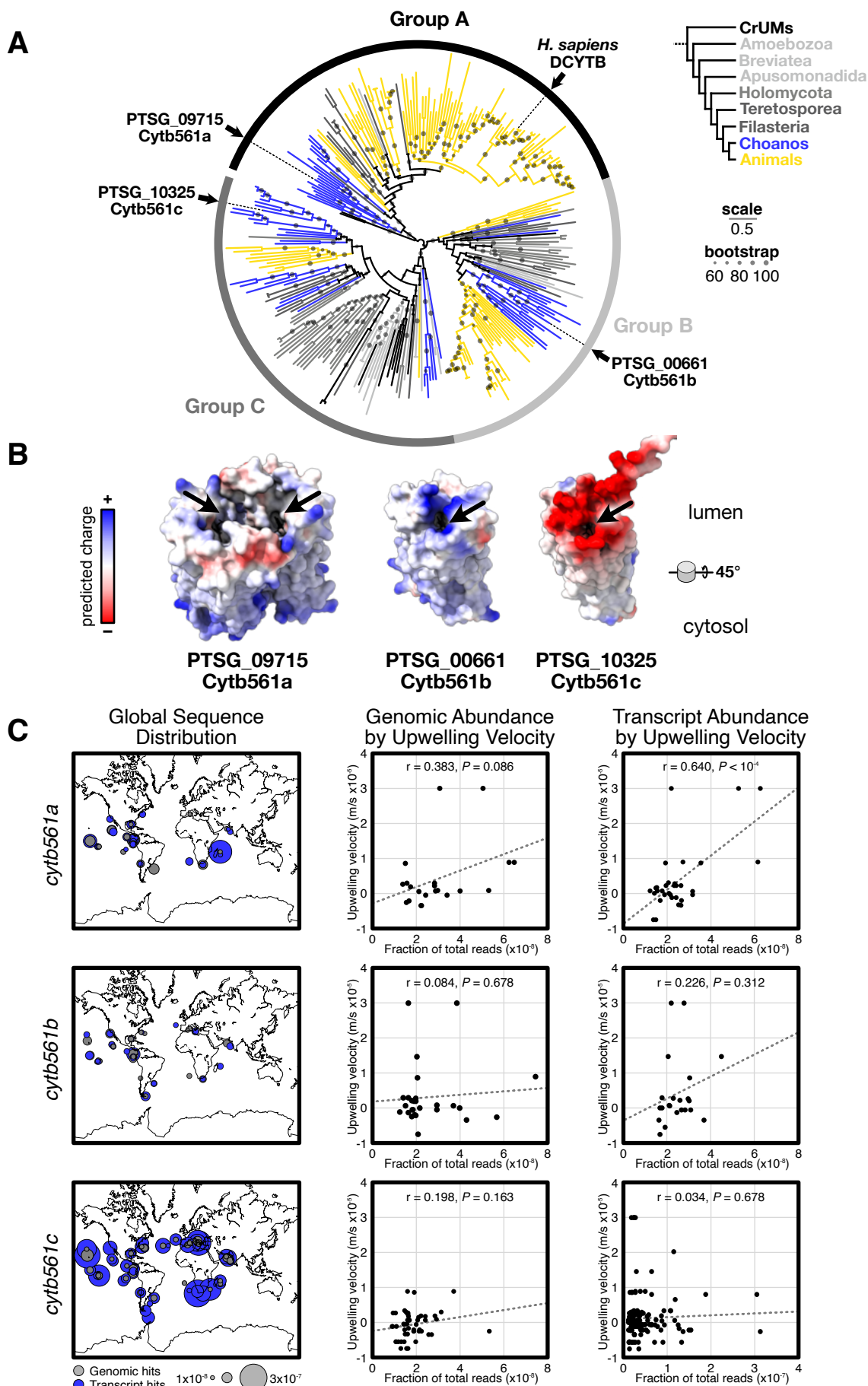


Figure 4: Cytb561 paralogs possess distinct biochemical properties and appear at different oceanic locations.

1 References:

- 2 1. Worden, A. Z. *et al.* Rethinking the marine carbon cycle: Factoring in the multifarious
3 lifestyles of microbes. *Science* **347**, (2015).
- 4 2. Keeling, P. J. Combining morphology, behaviour and genomics to understand the evolution
5 and ecology of microbial eukaryotes. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20190085
6 (2019).
- 7 3. Leadbeater, B. S. C. Observations on the life-history and ultrastructure of the marine
8 choanoflagellate *Choanoeca perplexa* Ellis. *J. Mar. Biol. Assoc. U. K.* **57**, 285–301 (1977).
- 9 4. Leadbeater, B. S. C. *The Choanoflagellates: Evolution, Biology, and Ecology*. (Cambridge
10 University Press, Cambridge, United Kingdom, 2015).
- 11 5. Reyes-Rivera, J. *et al.* Nitric oxide signaling controls collective contractions in a colonial
12 choanoflagellate. *Curr. Biol.* **32**, 2539-2547.e5 (2022).
- 13 6. Dayel, M. J. *et al.* Cell differentiation and morphogenesis in the colony-forming
14 choanoflagellate *Salpingoeca rosetta*. *Dev. Biol.* **357**, 73–82 (2011).
- 15 7. McKie, A. T. *et al.* An Iron-Regulated Ferric Reductase Associated with the Absorption of
16 Dietary Iron. *Science* **291**, 1755–1759 (2001).
- 17 8. Carradec, Q. *et al.* A global ocean atlas of eukaryotic genes. *Nat. Commun.* **9**, 373 (2018).
- 18 9. Vernet, C. *et al.* The Ocean Gene Atlas v2.0: online exploration of the biogeography and
19 phylogeny of plankton genes. *Nucleic Acids Res.* **50**, W516–W526 (2022).
- 20 10. Capone, D. G. & Hutchins, D. A. Microbial biogeochemistry of coastal upwelling regimes
21 in a changing ocean. *Nat. Geosci.* **6**, 711–717 (2013).
- 22 11. Tagliabue, A. *et al.* Authigenic mineral phases as a driver of the upper-ocean iron cycle.
23 *Nature* **620**, 104–109 (2023).
- 24 12. Sofen, L. E. *et al.* Authigenic Iron Is a Significant Component of Oceanic Labile
25 Particulate Iron Inventories. *Glob. Biogeochem. Cycles* **37**, e2023GB007837 (2023).
- 26 13. Barbeau, K., Moffett, J. W., Caron, D. A., Croot, P. L. & Erdner, D. L. Role of protozoan

- 1 grazing in relieving iron limitation of phytoplankton. *Nature* **380**, 61–64 (1996).
- 2 14. Barbeau, K. & Moffett, J. W. Laboratory and field studies of colloidal iron oxide
- 3 dissolution as mediated by phagotrophy and photolysis. *Limnol. Oceanogr.* **45**, 827–835
- 4 (2000).
- 5 15. Barbeau, K., Kujawinski, E. B. & Moffett, J. W. Remineralization and recycling of iron,
- 6 thorium and organic carbon by heterotrophic marine protists in culture. *Aquat. Microb. Ecol.*
- 7 **24**, 69–81 (2001).
- 8 16. Mikhailov, K. V. *et al.* The origin of Metazoa: a transition from temporal to spatial cell
- 9 differentiation. *BioEssays* **31**, 758–768 (2009).
- 10 17. Seb  -Pedr  s, A., Degnan, B. M. & Ruiz-Trillo, I. The origin of Metazoa: a unicellular
- 11 perspective. *Nat. Rev. Genet.* **18**, 498–512 (2017).
- 12 18. Brunet, T. & King, N. The Origin of Animal Multicellularity and Cell Differentiation. *Dev.*
- 13 *Cell* **43**, 124–140 (2017).
- 14 19. Arendt, D. *et al.* The origin and evolution of cell types. *Nat. Rev. Genet.* **17**, 744–757
- 15 (2016).
- 16 20. Najle, S. R. & Ruiz-Trillo, I. The Protistan Origins of Animal Cell Differentiation. in *Origin*
- 17 *and Evolution of Metazoan Cell Types* (CRC Press, 2021).
- 18 21. M  rquez-Zacar  as, P. *et al.* Evolution of Cellular Differentiation: From Hypotheses to
- 19 Models. *Trends Ecol. Evol.* **36**, 49–60 (2021).
- 20 22. Brunet, T. & King, N. The Single-Celled Ancestors of Animals: A History of Hypotheses.
- 21 in *The Evolution of Multicellularity* (CRC Press, 2022).
- 22 23. Levin, T. C. & King, N. Evidence for Sex and Recombination in the Choanoflagellate
- 23 *Salpingoeca rosetta*. *Curr. Biol.* **23**, 2176–2180 (2013).
- 24 24. Levin, T. C., Greaney, A. J., Wetzel, L. & King, N. The Rosetteless gene controls
- 25 development in the choanoflagellate *S. rosetta*. *eLife* **3**, (2014).
- 26 25. Alegado, R. A. *et al.* A bacterial sulfonolipid triggers multicellular development in the

- 1 closest living relatives of animals. *eLife* **1**, e00013 (2012).
- 2 26. Woznica, A. *et al.* Bacterial lipids activate, synergize, and inhibit a developmental switch
- 3 in choanoflagellates. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 7894–7899 (2016).
- 4 27. Peng, C.-C., Dormanns, N., Regestein, L. & Beemelmans, C. Isolation of
- 5 sulfonosphingolipids from the rosette-inducing bacterium *Zobellia uliginosa* and evaluation of
- 6 their rosette-inducing activity. *RSC Adv.* **13**, 27520–27524 (2023).
- 7 28. Perotti, O., Esparza, G. V. & Booth, D. S. A red algal polysaccharide influences the
- 8 multicellular development of the choanoflagellate *Salpingoeca rosetta*. 2024.05.14.594265
- 9 Preprint at <https://doi.org/10.1101/2024.05.14.594265> (2024).
- 10 29. Fairclough, S. R., Dayel, M. J. & King, N. Multicellular development in a
- 11 choanoflagellate. *Curr. Biol.* **20**, R875–R876 (2010).
- 12 30. Brunet, T. *et al.* A flagellate-to-amoeboid switch in the closest living relatives of animals.
- 13 *eLife* **10**, e61037 (2021).
- 14 31. Fairclough, S. R. *et al.* Premetazoan genome evolution and the regulation of cell
- 15 differentiation in the choanoflagellate *Salpingoeca rosetta*. *Genome Biol.* **14**, R15 (2013).
- 16 32. Nedashkovskaya, O. I. *et al.* *Echinicola pacifica* gen. nov., sp. nov., a novel
- 17 flexibacterium isolated from the sea urchin *Strongylocentrotus intermedius*. *Int. J. Syst. Evol.*
- 18 *Microbiol.* **56**, 953–958 (2006).
- 19 33. Richter, D. J., Fozouni, P., Eisen, M. B. & King, N. Gene family innovation, conservation
- 20 and loss on the animal stem lineage. *eLife* **7**, e34226 (2018).
- 21 34. Fumagalli, M. R., Zapperi, S. & Porta, C. A. L. Gene regulatory programs in the life
- 22 history of *Salpingoeca rosetta*. 2023.06.12.544615 Preprint at
- 23 <https://doi.org/10.1101/2023.06.12.544615> (2023).
- 24 35. Binns, D. *et al.* QuickGO: a web-based tool for Gene Ontology searching. *Bioinformatics*
- 25 **25**, 3045–3046 (2009).
- 26 36. Huntley, R. P. *et al.* The GOA database: Gene Ontology annotation updates for 2015.

- 1 *Nucleic Acids Res.* **43**, D1057–D1063 (2015).
- 2 37. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of
- 3 large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).
- 4 38. Sherman, B. T. *et al.* DAVID: a web server for functional enrichment analysis and
- 5 functional annotation of gene lists (2021 update). *Nucleic Acids Res.* **50**, W216–W221
- 6 (2022).
- 7 39. Thomas, P. D. *et al.* PANTHER: Making genome-scale phylogenetics accessible to all.
- 8 *Protein Sci.* **31**, 8–22 (2022).
- 9 40. Gaudet, P. & Dessimoz, C. Gene Ontology: Pitfalls, Biases, and Remedies. in *The Gene*
- 10 *Ontology Handbook* (eds. Dessimoz, C. & Škunca, N.) 189–205 (Springer, New York, NY,
- 11 2017). doi:10.1007/978-1-4939-3743-1_14.
- 12 41. Sutak, R., Camadro, J.-M. & Lesuisse, E. Iron Uptake Mechanisms in Marine
- 13 Phytoplankton. *Front. Microbiol.* **11**, (2020).
- 14 42. Caza, M. & Kronstad, J. W. Shared and distinct mechanisms of iron acquisition by
- 15 bacterial and fungal pathogens of humans. *Front. Cell. Infect. Microbiol.* **3**, 80 (2013).
- 16 43. Allen, M. D., del Campo, J. A., Kropat, J. & Merchant, S. S. FEA1, FEA2, and FRE1,
- 17 Encoding Two Homologous Secreted Proteins and a Candidate Ferrioreductase, Are
- 18 Expressed Coordinately with FOX1 and FTR1 in Iron-Deficient *Chlamydomonas reinhardtii*.
- 19 *Eukaryot. Cell* **6**, 1841–1852 (2007).
- 20 44. Kaplan, J. Mechanisms of Cellular Iron Acquisition: Another Iron in the Fire. *Cell* **111**,
- 21 603–606 (2002).
- 22 45. Wells, M. L., Mayer, L. M. & Guillard, R. R. L. A chemical method for estimating the
- 23 availability of iron to phytoplankton in seawater. *Mar. Chem.* **33**, 23–40 (1991).
- 24 46. Booth, D. S. & King, N. Genome editing enables reverse genetics of multicellular
- 25 development in the choanoflagellate *Salpingoeca rosetta*. *eLife* **9**, e56193 (2020).
- 26 47. Ramos-Alonso, L., Romero, A. M., Martínez-Pastor, M. T. & Puig, S. Iron Regulatory

- 1 Mechanisms in *Saccharomyces cerevisiae*. *Front. Microbiol.* **11**, (2020).
- 2 48. Richter, D. J. *et al.* EukProt: A database of genome-scale predicted proteins across the
- 3 diversity of eukaryotes. *Peer Community J.* **2**, (2022).
- 4 49. Wunderlich, J. *et al.* Iron transport pathways in the human malaria parasite *Plasmodium*
- 5 *falciparum* revealed by RNA-sequencing. 2024.04.18.590068 Preprint at
- 6 <https://doi.org/10.1101/2024.04.18.590068> (2024).
- 7 50. Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D. & Dancis, A. A
- 8 Permease-Oxidase Complex Involved in High-Affinity Iron Uptake in Yeast. *Science* **271**,
- 9 1552–1557 (1996).
- 10 51. Dix, D. R., Bridgham, J. T., Broderius, M. A., Byersdorfer, C. A. & Eide, D. J. The FET4
- 11 gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae*. *J. Biol.*
- 12 *Chem.* **269**, 26092–26099 (1994).
- 13 52. McQuaid, J. B. *et al.* Carbonate-sensitive phytoferritin controls high-affinity iron
- 14 uptake in diatoms. *Nature* **555**, 534–537 (2018).
- 15 53. Pantopoulos, K., Porwal, S. K., Tartakoff, A. & Devireddy, L. Mechanisms of Mammalian
- 16 Iron Homeostasis. *Biochemistry* **51**, 5705–5724 (2012).
- 17 54. Richter, D. J., Fozouni, P., Eisen, M. B. & King, N. Gene family innovation, conservation
- 18 and loss on the animal stem lineage. *eLife* **7**, e34226 (2018).
- 19 55. Bode, H.-P., Dumschat, M., Garotti, S. & Fuhrmann, G. F. Iron Sequestration by the
- 20 Yeast Vacuole. *Eur. J. Biochem.* **228**, 337–342 (1995).
- 21 56. Philpott, C. C. & Protchenko, O. Response to Iron Deprivation in *Saccharomyces*
- 22 *cerevisiae*. *Eukaryot. Cell* **7**, 20–27 (2008).
- 23 57. Götzke, H. *et al.* The ALFA-tag is a highly versatile tool for nanobody-based bioscience
- 24 applications. *Nat. Commun.* **10**, 4403 (2019).
- 25 58. Laundon, D., Larson, B. T., McDonald, K., King, N. & Burkhardt, P. The architecture of
- 26 cell differentiation in choanoflagellates and sponge choanocytes. *PLOS Biol.* **17**, e3000226

- 1 (2019).
- 2 59. Brunet, T. & Booth, D. S. Chapter One - Cell polarity in the protist-to-animal transition. in
- 3 *Current Topics in Developmental Biology* (ed. Tepass, U.) vol. 154 1–36 (Academic Press,
- 4 2023).
- 5 60. Minh, B. Q. *et al.* IQ-TREE 2: New Models and Efficient Methods for Phylogenetic
- 6 Inference in the Genomic Era. *Mol. Biol. Evol.* **37**, 1530–1534 (2020).
- 7 61. Rozewicki, J., Li, S., Amada, K. M., Standley, D. M. & Katoh, K. MAFFT-DASH:
- 8 integrated protein sequence and structural alignment. *Nucleic Acids Res.* **47**, W5–W10
- 9 (2019).
- 10 62. Torruella, G., Galindo, L. J., Moreira, D. & López-García, P. Phylogenomics of neglected
- 11 flagellated protists supports a revised eukaryotic tree of life. 2024.05.15.594285 Preprint at
- 12 <https://doi.org/10.1101/2024.05.15.594285> (2024).
- 13 63. Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q. & Vinh, L. S. UFBoot2:
- 14 Improving the Ultrafast Bootstrap Approximation. *Mol. Biol. Evol.* **35**, 518–522 (2018).
- 15 64. Pettersen, E. F. *et al.* UCSF ChimeraX: Structure visualization for researchers,
- 16 educators, and developers. *Protein Sci. Publ. Protein Soc.* **30**, 70–82 (2021).
- 17 65. Abramson, J. *et al.* Accurate structure prediction of biomolecular interactions with
- 18 AlphaFold 3. *Nature* **630**, 493–500 (2024).
- 19 66. Ganasen, M. *et al.* Structural basis for promotion of duodenal iron absorption by enteric
- 20 ferric reductase with ascorbate. *Commun. Biol.* **1**, 1–12 (2018).
- 21 67. Marsh, R. & van Sebille, E. Chapter 3 - Surface drift, gyres, and the fate of plastic. in
- 22 *Ocean Currents* (eds. Marsh, R. & van Sebille, E.) 63–102 (Elsevier, 2021).
- 23 doi:10.1016/B978-0-12-816059-6.00012-7.
- 24 68. Fitzwater, S. E. *et al.* Iron, nutrient and phytoplankton biomass relationships in upwelled
- 25 waters of the California coastal system. *Cont. Shelf Res.* **23**, 1523–1544 (2003).
- 26 69. von der Heyden, B. P. & Roychoudhury, A. N. A review of colloidal iron partitioning and

- 1 distribution in the open ocean. *Mar. Chem.* **177**, 9–19 (2015).
- 2 70. Behrenfeld, M. J., Bale, A. J., Kolber, Z. S., Aiken, J. & Falkowski, P. G. Confirmation of
3 iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. *Nature* **383**,
4 508–511 (1996).
- 5 71. Wells, M. L., Mayer, L. M., Donard, O. F. X., de Souza Sierra, M. M. & Ackelson, S. G.
6 The photolysis of colloidal iron in the oceans. *Nature* **353**, 248–250 (1991).
- 7 72. Bundy, R. M., Jiang, M., Carter, M. & Barbeau, K. A. Iron-Binding Ligands in the
8 Southern California Current System: Mechanistic Studies. *Front. Mar. Sci.* **3**, (2016).
- 9 73. Manck, L. E. *et al.* Petrobactin, a siderophore produced by *Alteromonas*, mediates
10 community iron acquisition in the global ocean. *ISME J.* **16**, 358–369 (2022).
- 11 74. Tranvik, L. J., Sherr, E. B. & Sherr, B. F. Uptake and utilization of ‘colloidal DOM’ by
12 heterotrophic flagellates in seawater. *Mar. Ecol. Prog. Ser.* **92**, 301–309 (1993).
- 13 75. Gonzalez, J. M. & Suttle, C. A. Grazing by marine nanoflagellates on viruses and
14 virus-sized particles: ingestion and digestion. *Mar. Ecol. Prog. Ser.* **94**, 1–10 (1993).
- 15 76. Wilken, S. *et al.* Choanoflagellates alongside diverse uncultured predatory protists
16 consume the abundant open-ocean cyanobacterium *Prochlorococcus*. *Proc. Natl. Acad. Sci.*
17 *U. S. A.* **120**, e2302388120 (2023).
- 18 77. Dupont, C. L., Butcher, A., Valas, R. E., Bourne, P. E. & Caetano-Anollés, G. History of
19 biological metal utilization inferred through phylogenomic analysis of protein structures. *Proc.*
20 *Natl. Acad. Sci.* **107**, 10567–10572 (2010).
- 21 78. Kiørboe, T., Visser, A. & Andersen, K. H. A trait-based approach to ocean ecology. *ICES*
22 *J. Mar. Sci.* **75**, 1849–1863 (2018).
- 23 79. Knoll, A. H. & Nowak, M. A. The timetable of evolution. *Sci. Adv.* **3**, e1603076 (2017).
- 24 80. Wade, J., Byrne, D. J., Ballentine, C. J. & Drakesmith, H. Temporal variation of planetary
25 iron as a driver of evolution. *Proc. Natl. Acad. Sci.* **118**, e2109865118 (2021).
- 26 81. Sperling, E. A., Knoll, A. H. & Girguis, P. R. The Ecological Physiology of Earth’s Second

- 1 Oxygen Revolution. *Annu. Rev. Ecol. Evol. Syst.* **46**, 215–235 (2015).
- 2 82. Sebé-Pedrós, A. *et al.* Early metazoan cell type diversity and the evolution of
- 3 multicellular gene regulation. *Nat. Ecol. Evol.* **2**, 1176–1188 (2018).
- 4 83. Allen, A. E. *et al.* Whole-cell response of the pennate diatom *Phaeodactylum tricornutum*
- 5 to iron starvation. *Proc. Natl. Acad. Sci.* **105**, 10438–10443 (2008).
- 6 84. Glaesener, A. G., Merchant, S. S. & Blaby-Haas, C. E. Iron economy in *Chlamydomonas*
- 7 *reinhardtii*. *Front. Plant Sci.* **4**, (2013).
- 8 85. Arendt, D. The evolution of cell types in animals: emerging principles from molecular
- 9 studies. *Nat. Rev. Genet.* **9**, 868–882 (2008).
- 10 86. Tanay, A. & Sebé-Pedrós, A. Evolutionary cell type mapping with single-cell genomics.
- 11 *Trends Genet.* **37**, 919–932 (2021).
- 12 87. Zeng, H. What is a cell type and how to define it? *Cell* **185**, 2739–2755 (2022).
- 13 88. Brunet, T. & King, N. The Single-Celled Ancestors of Animals: A History of Hypotheses.
- 14 (2020) doi:10.20944/preprints202011.0302.v1.
- 15 89. Knoll, A. H. The Multiple Origins of Complex Multicellularity. *Annu. Rev. Earth Planet.*
- 16 *Sci.* **39**, 217–239 (2011).
- 17 90. Richter, D. J. & King, N. The Genomic and Cellular Foundations of Animal Origins. *Annu.*
- 18 *Rev. Genet.* **47**, 509–537 (2013).
- 19 91. Booth, D. S., Szmidt-Middleton, H. & King, N. Transfection of choanoflagellates
- 20 illuminates their cell biology and the ancestry of animal septins. *Mol. Biol. Cell* **29**, 3026–3038
- 21 (2018).
- 22 92. Perotti, O., Viramontes, G. & Booth, D. A red algal polysaccharide influences the
- 23 multicellular development of the choanoflagellate *S. rosetta*. (2024).
- 24 93. Woznica, A. *et al.* STING mediates immune responses in the closest living relatives of
- 25 animals. *eLife* **10**, e70436 (2021).
- 26 94. Coyle, M. C. *et al.* An RFX transcription factor regulates ciliogenesis in the closest living

- 1 relatives of animals. *Curr. Biol.* **33**, 3747–3758.e9 (2023).
- 2 95. Rutaganira, F. U. N., Scopton, A. P., Dar, A. C. & King, N. A small molecule screen
- 3 reveals the essentiality of p38 kinase for choanoflagellate cell proliferation.
- 4 2022.08.26.505350 Preprint at <https://doi.org/10.1101/2022.08.26.505350> (2022).
- 5 96. Harayama, T. & Riezman, H. Understanding the diversity of membrane lipid composition.
- 6 *Nat. Rev. Mol. Cell Biol.* **19**, 281–296 (2018).
- 7 97. Sohlenkamp, C. & Geiger, O. Bacterial membrane lipids: diversity in structures and
- 8 pathways. *FEMS Microbiol. Rev.* **40**, 133–159 (2016).
- 9 98. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq
- 10 quantification. *Nat. Biotechnol.* **34**, 525–527 (2016).
- 11 99. Pimentel, H., Bray, N. L., Puente, S., Melsted, P. & Pachter, L. Differential analysis of
- 12 RNA-seq incorporating quantification uncertainty. *Nat. Methods* **14**, 687–690 (2017).
- 13 100. Supek, F., Bošnjak, M., Škunca, N. & Šmuc, T. REVIGO Summarizes and Visualizes
- 14 Long Lists of Gene Ontology Terms. *PLOS ONE* **6**, e21800 (2011).
- 15 101. Chen, J. S. *et al.* CRISPR-Cas12a target binding unleashes indiscriminate
- 16 single-stranded DNase activity. *Science* **360**, 436–439 (2018).
- 17 102. Tabata, R. *et al.* Systemic Regulation of Iron Acquisition by Arabidopsis in Environments
- 18 with Heterogeneous Iron Distributions. *Plant Cell Physiol.* **63**, 842–854 (2022).
- 19 103. Paysan-Lafosse, T. *et al.* InterPro in 2022. *Nucleic Acids Res.* **51**, D418–D427 (2023).
- 20 104. Eddy, S. R. Accelerated Profile HMM Searches. *PLOS Comput. Biol.* **7**, e1002195
- 21 (2011).
- 22 105. Richter, D. J. *et al.* EukProt: A database of genome-scale predicted proteins across the
- 23 diversity of eukaryotes. 2020.06.30.180687 Preprint at
- 24 <https://doi.org/10.1101/2020.06.30.180687> (2022).
- 25 106. Steenwyk, J. L., Iii, T. J. B., Li, Y., Shen, X.-X. & Rokas, A. ClipKIT: A multiple sequence
- 26 alignment trimming software for accurate phylogenomic inference. *PLOS Biol.* **18**, e3001007

- 1 (2020).
- 2 107. Nguyen, L.-T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: A Fast and
- 3 Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol.*
- 4 *Evol.* **32**, 268–274 (2015).
- 5 108. Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A. & Jermini, L. S.
- 6 ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods* **14**,
- 7 587–589 (2017).
- 8 109. Letunic, I. & Bork, P. Interactive Tree of Life (iTOL) v6: recent updates to the
- 9 phylogenetic tree display and annotation tool. *Nucleic Acids Res.* gkae268 (2024)
- 10 doi:10.1093/nar/gkae268.
- 11 110. Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. & Barton, G. J. Jalview
- 12 Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**,
- 13 1189–1191 (2009).
- 14 111. Pesant, S. *et al.* Open science resources for the discovery and analysis of Tara Oceans
- 15 data. *Sci. Data* **2**, 150023 (2015).
- 16 112. Bloh, K. *et al.* Deconvolution of Complex DNA Repair (DECODR): Establishing a Novel
- 17 Deconvolution Algorithm for Comprehensive Analysis of CRISPR-Edited Sanger Sequencing
- 18 Data. *CRISPR J.* **4**, 120–131 (2021).