Nitric oxide signaling controls collective contractions in a colonial choanoflagellate

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Although signaling by the gaseous molecule nitric oxide (NO) regulates key physiological processes in animals, including contractility[1-3], immunity[4,5], development[6-9] and locomotion[10,11], the early evolution of animal NO signaling remains unclear. To reconstruct the role of NO in the animal stem lineage, we set out to study NO signaling in choanoflagellates, the closest living relatives of animals[12]. In animals, NO produced by the nitric oxide synthase (NOS) canonically signals through cGMP by activating soluble guanylate cyclases (sGCs)[13,14]. We surveyed the distribution of the NO signaling pathway components across the diversity of choanoflagellates and found three species that express NOS, sGCs, and downstream genes previously shown to be involved in the NO/cGMP pathway. One of these, *Choanoeca flexa*, forms multicellular sheets that undergo collective contractions controlled by cGMP[15]. We found that treatment with NO induces sustained contractions in *C. flexa* by signaling through an sGC/cGMP pathway. Biochemical assays show that NO directly binds *C. flexa* sGC1 and stimulates its cyclase activity. The NO/cGMP pathway acts independently from other inducers of *C. flexa* contraction, including mechanical stimuli and heat, but sGC activity is required for contractions induced by light-to-dark transitions. The output of NO signaling in *C. flexa* – contractions resulting in a switch from feeding to swimming – resembles the effect of NO in sponges[1-3] and cnidarians[11,16,17], where it interrupts feeding and activates contractility. These data provide insights into the biology of the first animals and the evolution of NO signaling.

RESULTS

C. flexa encodes both NOS and sGC

C. flexa is a colonial choanoflagellate that forms concave sheets capable of global inversion of their curvature through collective contractility[15]. *C. flexa* inversion mediates a trade-off between feeding and swimming: relaxed colonies (with their flagella pointing inside) are slow swimmers and efficient feeders, while contracted colonies (with their flagella pointing outside) are inefficient feeders but fast swimmers[15] (Fig. 1A). Inversion has been shown to be induced by light-to-dark transitions through inactivation of a rhodopsin-phosphodiesterase (Rho-PDE) and accumulation of cGMP[15] (Fig. 1B). The involvement of cGMP in collective contraction in *C. flexa* and the connection between NO/cGMP signaling and tissue contraction in early-branching animals[18] led us to investigate whether NO signaling might exist and regulate sheet inversion in *C. flexa* (Fig. 1B).

By examining the genomes of two choanoflagellates (*M. brevicollis*[19] and *S. rosetta*[20]) and the transcriptomes of *C. flexa*[15] and 19 other choanoflagellates[21], we found that five choanoflagellates encode NOS homologs and 10 encode sGC homologs (Fig. 2A). Of these, three species – *S. infusionum, C. perplexa,* and *C. flexa* – express both NOS and sGCs, suggesting that some aspect of the physiology of these organisms might be regulated by NO/cGMP signaling.

Like metazoan NOS genes, choanoflagellate NOS genes encode the canonical oxygenase and reductase domains (Fig. 2B). Phylogenetic analysis revealed that one choanoflagellate NOS gene, that from *S. infusionum,* is closely related to those from metazoans and fungi (Fig. S1A). In contrast, all other choanoflagellate NOSs (including those from *C. flexa*) cluster with cyanobacterial NOSs, suggesting they might have been acquired through horizontal gene transfer from a cyanobacterial ancestor early in the evolution of choanoflagellates (Fig. S1A). These choanoflagellate NOS genes, like those of cyanobacteria, differ from metazoan NOS in that they encode an upstream globin domain with unknown function and lack the calmodulin-binding domain that mediates regulation of metazoan NOSs by Ca^{2+}, suggesting that calcium signaling does not regulate NO synthesis in *C. flexa*. The *C. flexa* transcriptome also encodes complete biosynthetic pathways for the NOS cofactors BH_4, FMN, FAD and NADPH (Fig. S2A) as well as downstream genes in the NO/cGMP signaling pathway: cGMP-dependent kinase (PKG), cGMP-gated ion channels (CNG) and cGMP-dependent phosphodiesterase (PDEG) (Fig. S2B), providing additional evidence that *C. flexa* employs NO signaling as part of its physiology.

Nearly all sGC genes from choanoflagellates, including *C. flexa,* encode the canonical domains observed in animal sGCs: the heme NO/O_2-binding domain (HNOB or H-NOX), the HNOB associated domain (HNOBA) and the C-terminal catalytic domain (guanylate cyclase)[22] (Fig. 2C). Phylogenetic analysis revealed that most choanoflagellate sGCs (including those of *C. flexa*) evolved from a single ancestral sGC found in the last common ancestor of choanoflagellates and metazoans which diversified separately into multiple paralogs in these two lineages (Fig. S1B). In contrast, the predicted sGC from one choanoflagellate species, *S. helianthica*, more closely resembles the sGCs of chlorophyte algae (which are the only protist group previously known to encode sGC proteins with a metazoan-like domain architecture[23]; Fig. S1B).

Importantly, not all animal sGCs are regulated by nitric oxide: in *Drosophila melanogaster* and *Caenorhabditis elegans*, so-called "atypical sGCs" preferentially bind soluble O_2 instead of NO and are thought to be involved in the regulation of feeding by oxygen concentration[24-26].

Discrimination between NO and O_2 is mediated by the presence of a hydrogen-bonding network, where a distal pocket tyrosine residue is critical for stabilizing the heme-O_2 complex[27]. We generated preferential binding predictions based on this motif[27,28] and found that both NO and O_2-preferential binding sGCs are widely distributed among choanoflagellates with no obvious pattern (Fig. 2A; Fig. S2D). Interestingly, predicted NO-selective sGCs are present in choanoflagellate species in which NOS was not detected, suggesting that these species might detect NO from an exogenous source (i.e. environmental bacteria or other protists), might possess an alternative NO-producing mechanism, or might encode an NOS that was not detected in the transcriptome. In *C. flexa*, one out of four sGC transcripts was predicted to be selective for NO and was named Cf-sGC1 (Fig. S2D). The other two choanoflagellate species found to possess both a NOS and sGCs, *C. perplexa* and *S. infusionum,* were also predicted to encode at least one NO-sensitive sGC (Fig. 2A).

NO/cGMP signaling controls colony contraction in *C. flexa.*

To test whether NO signaling regulates collective contractions in *C. flexa*, we treated *C. flexa* cultures with several NO donors[29], compounds capable of releasing NO in solution. We found that treatment of *C. flexa* with the NO donor proliNONOate led to an increase in intracellular NO as detected by the NO-sensitive fluorescent probe DAF-FM, demonstrating that it could be an effective reagent for studying NO signaling in *C. flexa in vivo* (Fig. 3A, S3A-C). Treatment of *C. flexa* with proliNONOate (Fig. 3B,C), DEANONOate (Fig. S3D) or NOC-12 (Fig. S3D) induced colony contraction within one to two minutes. Moreover, the inversion response of *C. flexa* to proliNONOate was concentration-dependent (Fig. 3D), reaching a plateau of nearly 100% inversion at a concentration of 0.1 μM. As a negative control, *C. flexa* did not invert in response to proline, the molecular backbone of proliNONOate and the end product of NO release (Fig. 3D). Taken together, these results indicate that NO is sufficient to induce colony contraction.

We next investigated whether NO triggers cGMP synthesis in *C. flexa*. We found that a pan-inhibitor of sGCs, ODQ[30], had no detectable effect on the percentage of contracted colonies 5 minutes after treatment with NO relative to cultures not treated with ODQ. However, a difference between the two conditions became evident 10 minutes after NO treatment, with nearly all ODQ-treated colonies relaxing into the uncontracted form, while all ODQ-untreated colonies remained

contracted at least twice as long (Fig. 3E). The observation of rapid colony relaxation in ODQ-
125 treated cultures was concentration-dependent (Fig. 3F).

These results demonstrate that the maintenance of NO-induced colony contraction requires sGC activity. If NO does indeed signal through the sGC/cGMP pathway, we predicted that treatment of colonies with an NO donor should increase intracellular cGMP concentration. We found that NO-treated cells contained consistently higher intracellular cGMP levels than untreated
130 cells (~2-fold increase on average, p=0.0237; Fig. 3G). Importantly, prior work has demonstrated that treatment of *C. flexa* with a cell-permeant form of cGMP (8-Br-cGMP) is sufficient to induce inversion[15]. These results further support the hypothesis that NO activates sGCs and stimulates the production of cGMP, which maintains *C. flexa* colony contraction.

135 **_C. flexa_ sGC1 is an NO-selective and catalytically active component of the _C. flexa_ NO/cGMP signaling pathway**

Of the four sGCs expressed by *C. flexa*, only one (Cf-sGC1) was predicted to selective for NO (Fig. 1A). To directly test the existence of a canonical NO/sGC/cGMP pathway in *C. flexa*, we characterized ligand binding and activity of Cf-sGC1 that had been heterologously expressed
140 in and purified from *E. coli* (Fig. 3H). The ligand specificity of sGCs can be determined by comparing the "Soret peak" maxima of the ultraviolet/visible (UV/vis) absorption after incubation with different gases, including NO, O_2 or CO. The Fe(II), unliganded form of Cf-sGC1 exhibited a Soret peak at 429 nm and a single broad plateau in the α/β region (500-600 nm), consistent with that of an NO-selective sGC[28] (Fig. 3I; table S1). In the presence of NO, the Soret peak shifted to
145 399 nm with two peaks in the α/β region, as characteristic for a 5-coordinate, high-spin NO-heme complex[28] (Fig. 3I; Table S1). On the other hand, when exposed to atmospheric oxygen in the absence of NO, the UV-vis absorption spectrum of Cf-sGC1 did not change (Fig. 3I; Fig. S4), consistent with our prediction that Cf-sGC1 is an NO-selective sGC and does not bind O_2. Moreover, like previously characterized animal sGCs, Cf-sGC1 also binds CO to form a 6-
150 coordinate, low spin CO-heme complex[31], evidenced by a Soret band maximum of 424 nm with two peaks in the α/β region (Fig. 3I; Table S1). Thus, the UV-vis spectroscopy results indicate that Cf-sGC1 binds diatomic gas ligands in a manner that resembles other well-characterized NO-selective sGCs.

In animals, NO-selective sGCs display three levels of activity: 1) in the absence of NO, the
155 protein has a low basal guanylate cyclase activity; 2) when one NO molecule is bound at the heme moiety, the protein is partially activated (to several-fold the basal activity); and 3) in the presence of excess NO, the protein reaches maximal activation[32]. To characterize the enzymatic activity of Cf-sGC1, we measured cGMP production by purified Cf-sGC1 under unliganded, equimolar NO, and excess NO conditions using an endpoint activity assay. We found that Cf-sGC1 has an activity
160 profile similar to that of animal sGCs, namely a 2-fold increase in activity in the equimolar NO condition, and ~6-fold increase in the excess NO condition (Fig. 3J). Overall, Cf-sGC1 exhibits similar ligand binding properties and NO-stimulated activity profile to animal NO-specific sGCs[31]. These results further support the existence of NO/cGMP signaling in *C. flexa* and is consistent with it being mediated (at least in part) by Cf-sGC1.

165

NO/cGMP signaling acts independently from most other inducers of colony contraction.

In animals, NO signaling can be induced by a broad range of stimuli, which include chemical signals (for example, acetylcholine in mammalian blood vessels[33]), mechanical cues (for example, shear stress in blood vessels[34]), or heat shocks[35-38]. Interestingly, collar contractions in
170 choanoflagellates can often be induced by mechanical stimuli, such as flow and touch[39-42]. We thus set out to test whether NO signaling in *C. flexa* responds to or intersects with environmental inducers of inversion.

We observed that *C. flexa* colonies invert in a matter of seconds in response to agitation of culture flasks (which presumably combines the effect of flow and shocks with other colonies or
175 the walls of the flask) and to heat shocks (Fig. 4A,B). To test whether mechanically or heat-induced contraction requires NO/cGMP signaling, we incubated the colony cultures with the pan-sGC inhibitor ODQ and exposed them to either of the two different stressors. We found that inhibition of sGCs did not abolish mechanically induced or heat shock-induced contraction (Fig. 4A,B). Taken together, these findings suggest that the mechanosensitive, and thermosensitive pathways
180 that induce inversion in *C. flexa* are independent of sGCs, and hint at complex behavioral regulation in this choanoflagellate.

Previous work has shown that *C. flexa* colonies invert in response to light-to-dark transitions which they detect through a rhodopsin-cGMP pathway[15]. In the presence of light, a rhodopsin-phosphodiesterase hydrolyzes cGMP into GMP, thus preventing cGMP signaling. In

185 darkness, the rhodopsin-phosphodiesterase is inactivated, which allows cGMP to accumulate and trigger colony inversion (Fig. 1B). Interestingly, this pathway requires the presence of cGMP, which is presumably synthesized by either particulate (i.e. membrane-bound) or soluble (i.e. cytosolic) guanylate cyclases[43].

We next set out to answer whether sGCs are necessary for synthesizing the cGMP required
190 for phototransduction. To address this, we treated light-sensitive colonies with ODQ and found that this entirely abolished darkness-induced inversion (Fig. 4C). These results suggest that sGCs (either NO-dependent or O_2-dependent) are responsible for synthesizing baseline levels of cGMP that are then used during phototransduction. Even though ODQ-treated light-sensitive colonies did not invert in response to darkness, we confirmed that they could still respond to NO by undergoing
195 brief contractions (which were sustained for a much shorter time than in controls; Fig. 4D), consistently with earlier results (Fig. 3E).

DISCUSSION

Here we report the presence of NOS, sGCs and downstream components of NO/cGMP
200 signaling in three choanoflagellate species, at least two of which (*C. flexa* and *C. perplexa*) are capable of collective contractions[15,44]. To our knowledge, this is the first observation of both NOS and sGCs in a non-animal. We found that NO causes sustained colony contraction in *C. flexa* and an increase in cGMP concentration in live cells, while inhibition of sGCs (and thereby cGMP concentrations) accelerated colony relaxation. Moreover, *in vitro* experiments confirmed that NO
205 directly binds Cf-sGC1, which it activates with a two-step profile in response to NO concentration, as in animal sGCs[32].

The observation that colonies treated with the sGC inhibitor initially contracted in response to NO at levels matching untreated colonies, only to relax much more quickly was unexpected. We hypothesize that NO-induced contractions are mediated through at least two different
210 pathways: a slow pathway (described above) that maintains contraction and requires sGC/cGMP and an (unidentified) fast pathway independent of sGC/cGMP. Moreover, treatment of light-sensitive colonies with the sGC inhibitor abolished darkness-induced contractions (which are known to be mediated by cGMP[15]) but did not prevent NO-induced contractions, further supporting the existence of a second pathway. In other organisms, cGMP-independent NO
215 signaling can involve S-nitrosation, the modification of proteins through the formation of an S-

NO covalent bond[45,46], although the direct targets and functions of S-nitrosation in animals are incompletely understood compared to NO/cGMP signaling. It is possible that the same mechanism explains the cGMP-independent pathway underlying NO-induced colony contraction in *C. flexa*.

The control of multicellular behavior by NO/cCMP signaling in *C. flexa* is reminiscent of its function in animals, most notably in sponges[47]. In the demosponges *Tethya wilhelma*[1], *Ephydatia muelleri*[2], and *Spongilla lacustris*[3], NO induces global contractions and stops flagellar beating in choanocyte chambers, which interrupts feeding, allows expulsion of clumps of waste, and flushes the aquiferous canal system (a behavior sometimes called "sneezing")[48]. Recently, single-cell RNA sequencing in *Spongilla lacustris* revealed that pinacocytes (epithelial cells that cover and shape the sponge body) co-express NOS and sGC[3,49], the actomyosin contractility module and the transcription factor *Serum response factor* (Srf), a master regulator of contractility[3,50,51].

Control of motor and feeding behavior by NO/cGMP is also observed in cnidarians and some bilaterians. In the jellyfish *Aglantha digitale*, NO/cGMP signaling in neurons induces a switch from slow swimming (associated with feeding) to fast swimming (associated with escape) and inhibits tentacular ciliary beating[11]. In the sea pansy (a type of colonial cnidarian) *Renilla koellikeri*, NO/cGMP increases the amplitude of peristaltic contractions associated with the movement of body fluids through the gastrovascular cavity[17]. Finally, in the nudibranch *Clione limacine* and the snail *Lymnaea stagnalis,* NO activates both feeding and locomotory neural circuits[52-55]. Thus, as in *C. flexa*, the ancient functions of NO/cGMP signaling in animals may include the regulation of feeding and contraction[16,18,53,56-58]. Interestingly, NO signaling controls metamorphosis in sponges[7,59], gastropods[60], annelids[61], echinoderms[62,63] and ascidians[62,64,65], thus regulating a switch from swimming to feeding during irreversible developmental programs.

In the future, identifying the function of Cf-NOS will require gene knock-out, which is not yet possible in *C. flexa.* Moreover, studies on *Trichoplax* (in which NO/cGMP signaling has been predicted to exist based on genomic data[66]), additional animal phyla, and other choanoflagellates will help flesh out reconstitutions of the early evolution of animal NO signaling.

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Author contributions

JRR: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original draft, Writing - Review & editing, Visualization. YW: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original draft, Writing - Review & editing, Visualization. BGH: Conceptualization, Methodology. MM: Conceptualization, Methodology, Writing - Review & editing, Supervision, Project administration, Funding acquisition. NK: Conceptualization, Methodology, Writing - Review & editing, Supervision, Project administration, Funding acquisition. TB: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original draft, Writing - Review & editing, Supervision, Project administration, Funding acquisition.

Declaration of interests

The authors declare no competing interests.

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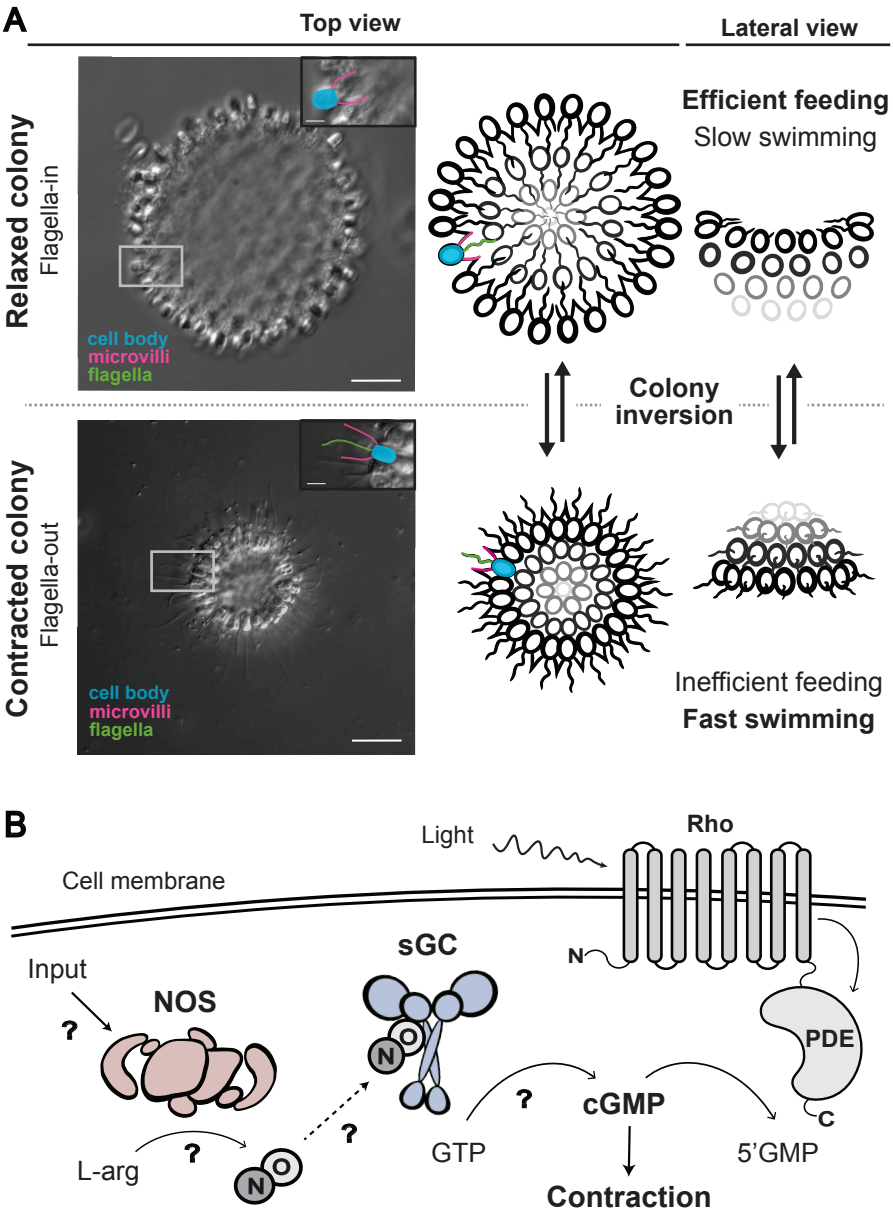
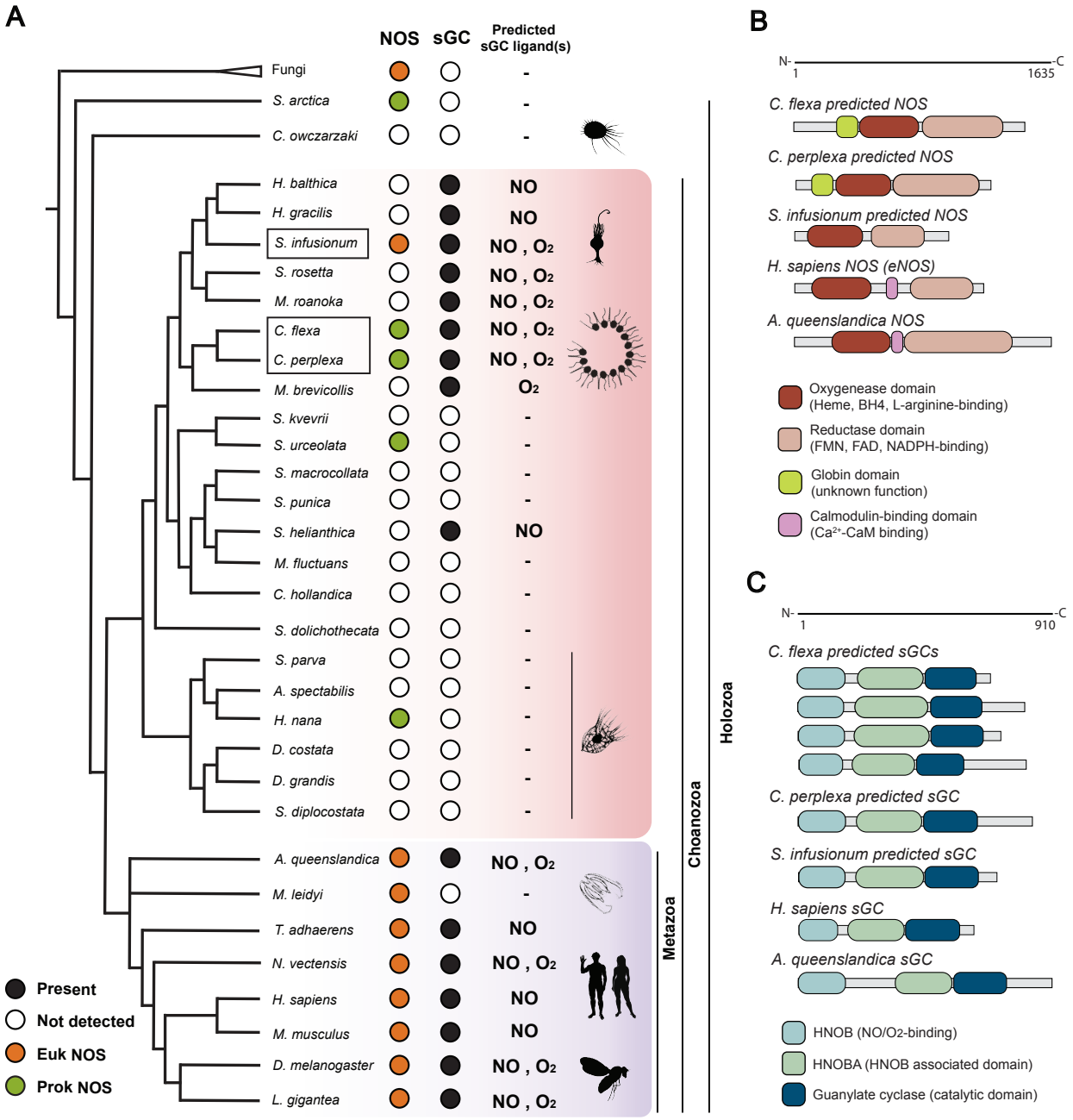


Reyes-Rivera et al. Figure 1

Figure 1. The inversion behavior of *C. flexa*, its control by light and hypothesized control by NO

(A) Micrograph (left) and graphical depiction (right) of *C. flexa* inversion behavior. Cells are linked by their collars and form a cup-shaped monolayer or sheet; scale bar: 15 µM. Insets: Pseudo-colors highlight the characteristic morphological features of choanoflagellates and the orientation of the flagella (flagella from relaxed colony not shown, out of focus); scale bar: 5 µM. Relaxed colonies (top half) have their flagella pointing towards the inside of the colony and are efficient feeders and slow swimmers. Contracted colonies (bottom half) have their flagella pointing towards the outside of the colony and are inefficient feeders but fast swimmers.

(B) *C. flexa* colony inversion is controlled by light-to-dark transitions, mediated by a rhodopsin-phosphodiesterase fusion protein (Rho-PDE) upstream cGMP signaling. In the presence of light, Rho-PDE is active and constantly converting cGMP into 5'GMP. We hypothesized that NO/cGMP signaling might also be able to induce inversion. Mechanisms tested in this paper are highlighted with question marks: a primary input activates the nitric oxide synthase (NOS), which converts L-arginine into NO and L-citrulline. NO diffuses away and activates soluble guanylate cyclase (sGC) which convert GTP into cGMP, causing colony contraction.



Reyes-Rivera et al. Figure 2

Figure 2. NO synthase (NOS) and soluble guanylate cyclases (sGC) predicted to bind either NO or O_2 are broadly distributed across choanozoans, and all three are present in *C. flexa*.

530 **(A)** Phylogenetic distribution of NOS and sGC across opisthokonts. *C. flexa*, its sister species *C. perplexa*, and *S. infusionum* encode both NOS and NO-sensitive sGCs, as do animals.

(B) Choanoflagellate NOSs have the metazoan canonical oxygenase and reductase domain but lack the calcium-calmodulin binding domain. *C. flexa* and other choanoflagellate NOS encode an upstream globin domain with unknown function, also observed in cyanobacterial NOS.

535 **(C)** *C. flexa* sGCs have the same domain architecture as animal sGCs.

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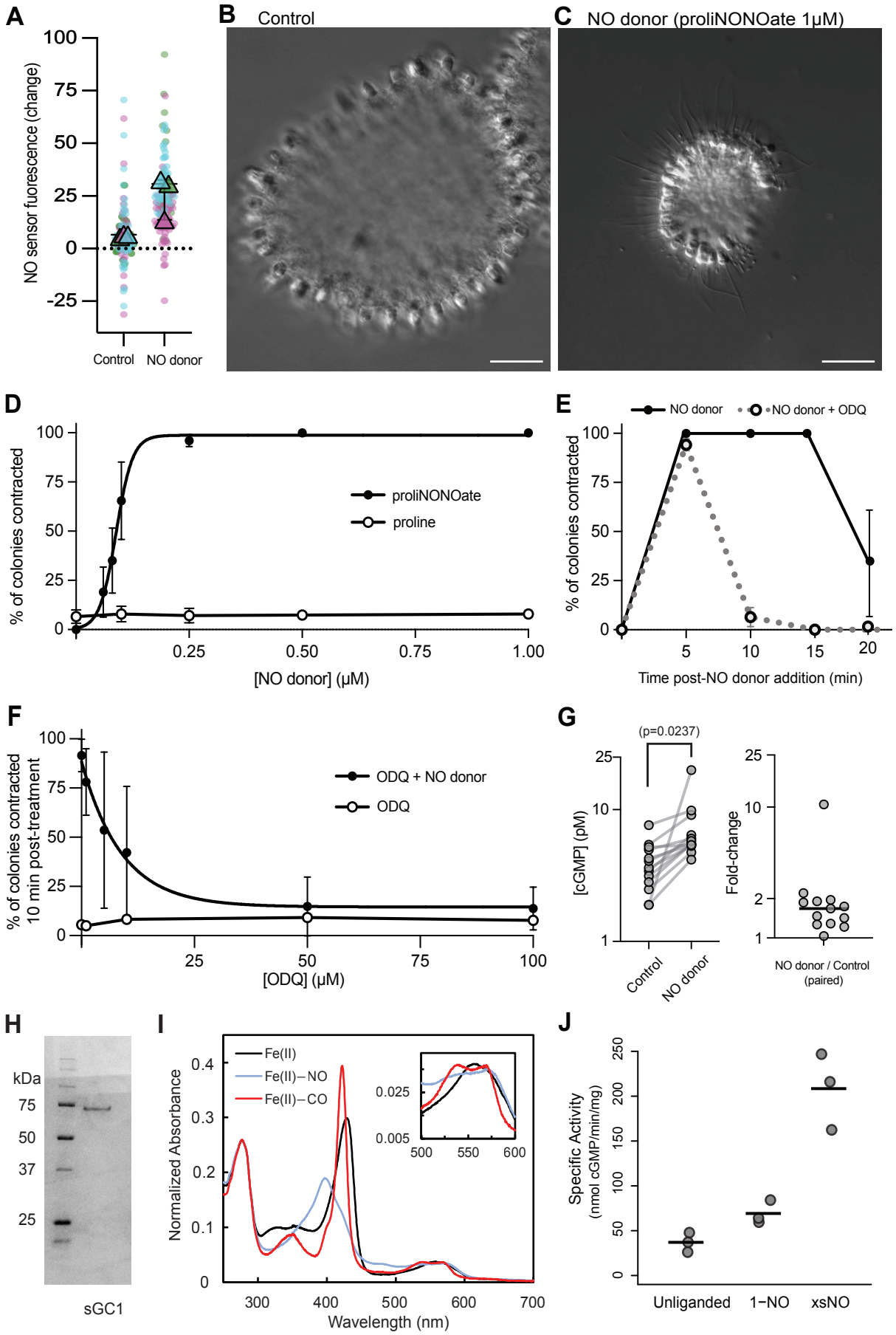

Reyes-Rivera et al. Figure 3

560 **Figure 3. NO induced sustained contraction in *C. flexa* through sGC activation and cGMP synthesis.**

(A) NO release by proliNONOate was visualized by loading cells with the NO-sensitive fluorescent probe DAF-FM and measuring the change in intracellular fluorescence intensity over 30 minutes. Cells treated with NO donor showed a higher increase in fluorescence. Graph is a

565 SuperPlot[67] of N=3 biological replicates per condition, with sample sizes n=27, 33, 47 for the three control groups and n=26, 57, 42 for the three treated groups. p=0.0331 by an unpaired t-test.

(B-C) NO induces colony contraction. While colonies treated with a negative control compound (proline) remained relaxed, colonies treated with 0.25 μM of the NO donor proliNONOate contracted within 1-2 minutes.

570 **(D)** The NO donor proliNONOate induces contractions within ~1 minute in a dose-dependent manner. A closely related molecule incapable of releasing NO (proline) had no effect over the same concentration range.

(E) Inhibition of sGCs with 50 μM ODQ did not abolish NO-induced contraction at early time points but greatly reduced its duration. ODQ-treated colonies were contracted 5 minutes post-

575 treatment with NO donor but relaxed soon after, while untreated colonies remain contracted for at least 5 more minutes.

(F) Inhibition of sustained contraction by ODQ is dose-dependent. Colonies were incubated with different concentrations of ODQ for 1 hour before treatment with 0.25 μM of the NO donor proliNONOate. We quantified the percentage of contracted colonies 10 minutes after NO-

580 treatment. In **(D-F)** each point represents the mean value of N=3 biological replicates with at least 30 colonies scored per biological replicate. Error bars are standard deviations.

(G) Treatment of cells with a NO donor increased intracellular cGMP concentration almost 2-fold as quantified by ELISA. N=13 pairs of control/treated samples, p=0.024 by a paired T-test.

(H-J) Purification, ligand binding properties and specific activity of Cf-sGC1. **(H)** Coomassie-

585 stained SDS-PAGE gel of recombinant Cf-sGC1 expressed in *E. coli* and purified. Band in lane "sGC1" represents Cf-sGC1 with a monomeric molecular weight of 75.7 kDa. Left lane, molecular weight ladder. **(I)** UV-visible absorption spectra of *Cf* sGC1 under unliganded (black), NO-bound (blue), and CO-bound (red) conditions. Soret maxima: NO-bound: 429 nm; CO-bound: 423 nm; NO-bound: 399 nm. Inset, α/β bands show increased splitting upon ligand binding. **(J)** Specific

590 activity of Cf-sGC1 under unliganded, equimolar quantities of NO (1-NO) and excess NO (xsNO) conditions. Initial rates were measured from activity assays performed at 25°C, pH 7.5 with 1.5

mM Mg^{2+}-GTP substrate and 40 nM enzyme. Horizontal bars represent mean of three biological replicates.

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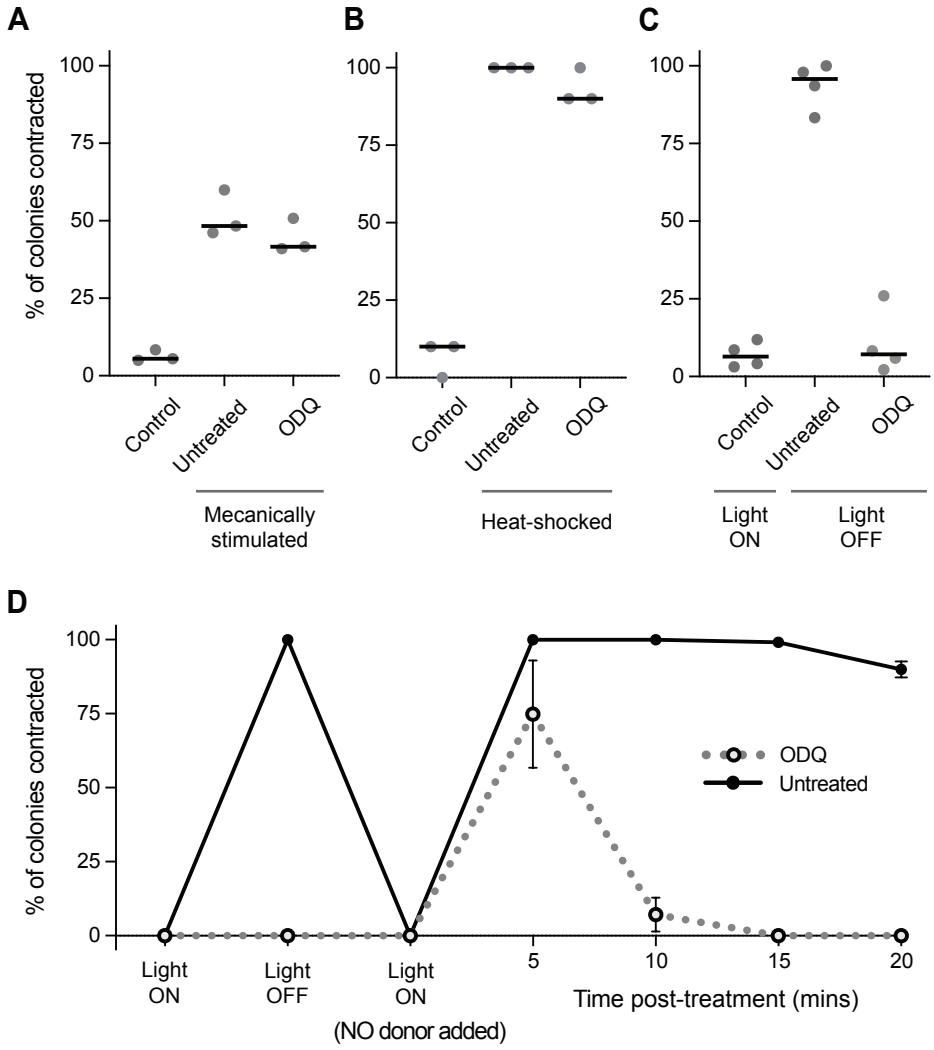

Reyes-Rivera et al. Figure 4

Figure 4. NO/cGMP acts independently of light-regulated, mechanically induced and heat shock-induced contractions in *C. flexa*.

(A-B) Mechanical stimuli and heat-shock induce *C. flexa* colony contraction. Inhibition of sGC (50 μM ODQ) did not have an effect on mechanically or heat shock-induced contractions.

600 **(C)** Inhibition of sGC (50 μM ODQ) abolished darkness-induced inversion ($p<0.0001$ by an unpaired t-test).

(D) Colonies treated with ODQ did not respond to on/off changes in light but briefly contracted when exposed to NO donor, consistent with earlier results (Figure 3D-F). 10 minutes after treatment, ODQ-treated colonies were relaxed, while untreated colonies remained contracted for a

605 longer period. In **(A-D)** each point is the average of N=3 **(A-B)** or N=4 **(C-D)** biological replicates with at least n=30 colonies scored per biological replicate. Error bars are standard deviations.