Cyclic di-nucleotides are important secondary signaling molecules in bacteria that regulate a wide range of processes. In this study, we found that *Caenorhabditis elegans* can detect and are attracted to multiple signal molecules produced by *Vibrio cholerae*, specifically the 3′,5′-cyclic diguanylate (c-di-GMP), even though this bacterium kills the host at a high rate. C-di-GMP is sensed through *C. elegans* olfactory AWC neurons, which then evokes a series of signal transduction pathways that lead to reduced activity of two key stress response transcription factors, SKN-1 and HSF-1, and weakened innate immunity. Taken together, our study elucidates the role of c-di-GMP in interkingdom communication. For *C. elegans*, bacterial c-di-GMP may serve as a cue that they can use to detect food. On the other hand, preexposure to low concentrations of c-di-GMP may impair their immune response, which could facilitate bacterial invasion and survival.
Bacteria are able to communicate with one another through a process known as quorum sensing (QS). This process mainly relies on the production and secretion of specific autoinducers (AIs), and allows the cells to make group decisions based on cell-density\(^1\). While the AIs are extracellular signaling molecules, bacteria also use a range of nucleotide-based intracellular signaling molecules known as second messengers to regulate physiological responses to cope with a changing environment. Molecules such as cyclic adenosine 3',5'-monophosphate (cAMP) and guanosine pentaphosphate or tetraphosphate ((p)pGpp) have been well studied for almost 50 years\(^2,3\). In the past two decades, the field of cyclic dinucleotides (CDNs) is expanding and has attracted more attention in different areas of research. 3',5'-cyclic diguanylate (c-di-GMP) was the first identified CDN and has been extensively studied since its discovery. Initially characterized as an activator of cellulose synthase in Acetobacter xylinum\(^4\), c-di-GMP is now known as a ubiquitous bacterial second messenger that regulates cell wall homeostasis, potassium ion channels, DNA integrity, as well as biofilm formation and virulence\(^7\). Adenosine-guanosine-3',5'-cyclic monophosphate (cGAMP) is the newest addition to the CDN list that has only been identified in very few bacteria. In *Vibrio cholerae* (\(*V.\) *cholerae*), cGAMP plays a role in efficient intestinal colonization\(^8\), and in *Geobacter*, it controls exoelectrogenesis\(^9\).

As more research has been conducted, it was discovered that the role of these signaling molecules is not limited to communication among bacterial cells. Bacteria and their eukaryotic hosts can also communicate with each other via these signaling molecules. This so-called interkingdom communication has recently become an expanding field of research with broad implications. Bacteria can sense and respond to mammalian hormones. For example, the classic stress hormones adrenaline and noradrenaline can induce bacterial growth and virulence expression. Conversely, bacterial AIs (specifically the acyl-homoserine lactones), can enter the mammalian cells and modulate host immune response and promote apoptosis\(^10\). Bacterial CDNs also have immunomodulation functions. It was reported that *Listeria monocytogenes* secretes c-di-AMP through a multidrug efflux pump and activates host type I interferon\(^11\). Whether c-di-GMP or cGAMP is secreted by bacteria is not known at this time. However, these signals can be detected by the endoplasmic-reticulum-resident protein STING (stimulator of interferon genes) in humans and mediate the type I interferon immune response\(^12,13\).

In the aforementioned cases, bacterial signals need to enter the host cells in order to elicit a specific response. Recent studies of bacteria-*C. elegans* interactions illustrated that bacterial AIs could function as chemical cues or odors that affect worms' behavior. Beale et al. first reported that *C. elegans* senses *Pseudomonas aeruginosa* acyl-homoserine lactones and are attracted by the signal. They also showed that *C. elegans* could acquire relatively long-lasting memory to avoid the AIs if they were pre-exposed to them for a short period\(^14\). *V. cholerae*, the etiologic agent of cholera, possesses multiple QS signaling molecules. CAC-1 and AI-2 are the two well-studied autoinducers. The CAI-1 molecule was found to chemoattract *C. elegans*, and it is sensed through the amphid AWCON neuron\(^15\). In this study, we show that in addition to CAI-1, c-di-GMP is another chemotractant that can be sensed by *C. elegans* by both AWCON and AWCOFF neurons. Sensing c-di-GMP elicits a series of signal transduction pathways in the host, which leads to a reduced innate immune response and a shortened lifespan by affecting the activity of two key stress response transcription factors, SKN-1 and HSF-1.

### Results

#### C. elegans are attracted to *V. cholerae* by signals other than the QS autoinducers.

In a previous study, we reported that water-soluble cranberry extract protects *C. elegans* from killing by different pathogenic bacteria, including *V. cholerae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Enterococcus faecalis*, and *Staphylococcus aureus*\(^16\). During these killing assays, an interesting phenomenon caught our attention. Worms fed on *V. cholerae* seemed to be allured by this type of bacterium and remained in the bacterial lawn until death. In contrast, worms fed on the other four pathogens showed a scattered behavioral pattern on the test plates. It is known that *C. elegans* can sense and avoid bacterial pathogens, but in our study, the worms were lured to a pathogen that significantly decreases their lifespan. A simple choice index (CI) assay was performed with the N2 wild type *C. elegans* over the course of 2 h to test whether the worms prefer the pathogenic *V. cholerae* wild type (wt) strain G6706 rather than *E. coli* OP50, the common food source for *C. elegans* used in the lab. As shown in Fig. 1a, the worms were readily attracted to *V. cholerae* (wt) compared to *E. coli* OP50.

Previously, Werner KM et al. reported that *V. cholerae* QS CAI-1 is a chemoattractant sensed by *C. elegans* amphid sensory...
neuron AWCON. To test whether other signal molecules are involved in chemotraction, we utilized three mutant strains: *cqsA* (no CAI-1 production), *luxS* (no AI-2 production), and a *cqsA*/luxS* double deletion. Each mutant was tested against *E. coli* OP50 using the standard CI assay. *C. elegans* preference was observed towards all three mutant strains (Fig 1a), conveying that signals other than the two autoinducers play a role. Interestingly, the choice index of any of the mutant strains over *E. coli* was comparable to that of the wild type over *E. coli*. This observation implies that rather than CAI-1, other signal molecules play a predominant role in attracting *C. elegans*. Next, we mixed the supernatant of the overnight culture of wild type *V. cholerae* and the *cqsA*/luxS* double mutant with *E. coli* OP50, respectively, and tested worms’ preference over *E. coli* OP50. As expected, worms were attracted to *E. coli* OP50 mixed with the supernatant from either the wild type or the double mutant strain (Fig 1b). This result confirms the existence of other chemotraction signals present in the *V. cholerae* cell-free supernatant.

C-di-GMP is the major signaling molecule that attracts *C. elegans*. QS autoinducers and the cyclic dinucleotides are essential signaling molecules that regulate numerous physiological functions in bacteria. Unlike the QS autoinducers, which are secreted into the environment, the cyclic dinucleotides are generally known as intracellular second messengers. We questioned whether c-di-GMP and cGAMP, the two cyclic dinucleotides characterized in *V. cholerae*, could function as extracellular signals that attract *C. elegans*. To test this, we purchased pure solutions of c-di-GMP and cGAMP. CI assays were conducted as usual, except that *E. coli* OP50 was mixed with two concentrations (0.1 and 1 nM) of each cyclic dinucleotide to observe preference. These concentrations were tested because it was theorized that low levels of these molecules would be present outside of the cells. Figure 2a shows that at 1 nM, both c-di-GMP and cGAMP were able to trigger an attractive behavior in *C. elegans*. As a control, c-di-AMP (1 nM) was also tested in the same assay. In contrast to c-di-GMP and cGAMP, c-di-AMP caused an apparent avoidance in *C. elegans*. We also tested similar signaling molecules, GMP and cGMP, and showed that neither cGMP nor GMP could cause any chemotraction or repulsion at concentrations similar to which elicited a response from c-di-GMP and cGAMP (Fig 2a).

We next harvested the supernatant and the cell pellet from the wild type *V. cholerae* overnight culture (~ 10⁹ CFU/ml) by centrifugation, and measured the concentration of c-di-GMP and cGAMP from the two portions using liquid chromatography–mass spectrophotometry (LC-MS) assay. Pure c-di-GMP and cGAMP solutions were used as standards for this assay. As shown in Fig 2b, c-di-GMP was detected in both the cell lysate and the supernatant. The concentration was calculated as 177.3 ± 49.4 nM in the cell lysate and 19.3 ± 4.6 nM in the supernatant. No cGAMP was detected in the cell lysate or the supernatant. These results suggest that c-di-GMP may be the additional signal that attracts *C. elegans* towards *V. cholerae*.

To further confirm the role of c-di-GMP in chemotraction, we sought to manipulate the concentration of c-di-GMP in *V. cholerae*. C-di-GMP is synthesized by the diguanylate cyclases containing a GGDEF domain and degraded by the phosphodiesterases containing either an EAL or HD-GYP domain. pAT1568, a plasmid that overexpresses the phosphodiesterase (EAL domain) of the vieA gene from the *E. coli*-inducible pBAD promoter, was introduced into the *cqsA*/luxS* strain by electroporation. The resulting transformant was grown overnight in the presence of 0.2% *E. coli* arabinose to induce phosphodiesterase expression and was used in CI assays against *E. coli* OP50. As a control, the empty vector pBAD33 was also introduced into the *cqsA*/luxS* strain and cultivated in a similar fashion. Figure 2c shows the CI result. With pAT1568 (i.e., decreased c-di-GMP level), *C. elegans* was no longer attracted to the *cqsA*/luxS* strain (choice index = 0), while with the empty vector pBAD33, a strong preference was still observed. This observation revealed the critical role of c-di-GMP production and presence in the chemotactic behavior of *C. elegans*. Combining the results from Fig. 2a, b, we consider that c-di-GMP is the major signaling molecule that attracts *C. elegans*.

*C. elegans* senses c-di-GMP through the AWC neurons and the cGMP-gated TAX-2/TAX-4 channel. The chemosensory system in *C. elegans* allows the organism to detect food, develop, avoid danger, mate, etc. There are 11 pairs of amphid chemosensory neurons in *C. elegans*. Of these, the ASE gustatory neurons are known to sense salts and water-soluble attractants, and the AWA and AWC olfactory neurons are required to sense attractants with volatile odors. Different chemical signals are sensed by different G protein-coupled chemoreceptors in the chemosensory neurons, and then passed to two major signal transduction sensory channels, the cGMP-gated TAX-2/TAX-4 channel and the lipid-sensing OSM-9/OCR-2 TRPV channel. It was reported that *C. elegans* senses *V. cholerae* CAI-1 through the AWC³ strain and uses the TAX-2/TAX-4 channel. We hypothesized that the same neuronal sensory pathway could be involved in sensing c-di-GMP. To test this, different *C. elegans* mutant strains were assessed to determine if they were able to similarly respond to c-di-GMP as the wild type strain. The tax-2/tax-4 mutant was no longer attracted to c-di-GMP, while the osm-9/ocr-2 mutant was still attracted, signifying that the cGMP-gated TAX-2/TAX-4 channel is required (Fig. 3). Mutation to ceh-36 causes defects in developing functional AWC and ASEL neurons, and mutation to che-1 causes an inability to develop functional ASE and ASER neurons. Mutations to nys-5 and nys-1 cause an inability to develop the AWC³ and AWC⁰ neurons, respectively. Figure 3 shows that loss of functional AWC or ASE neuron (che-36) kept the worms from the attraction, and worms without the functional ASE and ASER neuron (che-1) were still attracted, indicating the AWC neuron is involved. Further assay with the nys-5 and nys-1 mutants revealed that the AWC³ and AWC⁰ neurons are both necessary because no preference was observed when studying these mutants. Based on these results, it can be concluded that *C. elegans* senses c-di-GMP through the AWC³ and AWC⁰ neurons and the cGMP-gated TAX-2/TAX-4 channel.

C-di-GMP suppresses the innate immunity of *C. elegans*. Since it has been shown that c-di-GMP can cause initial attraction of *C. elegans* over the course of a couple of hours, it was next of interest to investigate if detection of this signal molecule plays a role in the health of the host. Lifespan assays were performed in which NGM-FUDR media was supplemented with 1 nM of c-di-GMP to test the lifespan of *C. elegans* N2, when exposed to c-di-GMP throughout their entire life. The results in Fig. 4a and Table 1 show that the presence of c-di-GMP significantly affects the lifespan of *C. elegans* N2, when exposed to c-di-GMP throughout their entire life. The mean number of days lived of N2 worms at 25 °C when exposed to c-di-GMP was 11.30 ± 0.48, whereas nonexposure was 13.66 ± 0.39, an 17.0% decrease (*p < 0.001*). The lifespan shortening effect is specific to the c-di-GMP molecule, as neither cGAMP nor c-di-AMP at 1 nM affected the normal lifespan of N2 worms (Fig. 4a and Table 1). We also found that the presence of live bacteria is requisite, as c-di-GMP only caused a slight decrease in the
Fig. 2 C-di-GMP is the major signal in *V. cholerae* supernatant that chemoattracts *C. elegans*. **a** Preference towards *E. coli* OP50 supplemented with 1 nM c-di-GMP and cGAMP. Avoidance behavior was observed with 1 nM c-di-AMP. No preference was observed with either cGMP or GMP. Results are the average of three independent experiments, and error bars are standard error of the mean representing 95% confidence intervals. Individual values are shown as black dots. Unpaired Student T-test was used to calculate the p values (0.1 nM vs. 1 nM). *p < 0.05. **b** LC-MS assay of c-di-GMP and cGAMP from the cell lysate and supernatant of wild type *V. cholerae* overnight culture. c-di-GMP and cGAMP were monitored by electrospray ionization mass spectrometry in positive mode with multiple reaction monitoring (MRM) at the transitions of m/z 691.10 → 152.10, 691.10 → 539.90, 691.10 → 248.05, and m/z 675.10 → 136.10, 675.10 → 524.00, 675.10 → 330.05, respectively. **c** Decreasing intracellular c-di-GMP (cqsA−/luxS− with pAT1568) abolishes *C. elegans* preference towards *V. cholerae*. Results are the average of three independent experiments, and error bars are standard error of the mean representing 95% confidence intervals. Individual values are shown as black dots. p values were calculated by using the unpaired Student T-test (pAT1568 vs. pBAD33). *p < 0.05.*
lifespan when the N2 worms were fed on heat-killed *E. coli* OP50 (Fig. 4b and Table 1).

Younger worms have robust grinders that can break down bacteria effortlessly, thus live bacteria are often absent from the lumen. There is a decline in grinder function as the worms age, which allows bacteria to easily escape to the lumen of the gut and proliferate\textsuperscript{20,21}. Effective curb of intestinal bacterial accumulation is an indicator of strong gut immunity, and also an important causative factor of lifespan determination\textsuperscript{20,21}. We therefore compared the gut colonization of *E. coli* OP50 from day-6 (equivalent to middle age) N2 worms in the presence and absence of c-di-GMP. A substantial increase of bacterial number was observed with the addition of 1 nM c-di-GMP (Fig. 4c). Incubating *E. coli* OP50 with the same concentration of c-di-GMP in vitro neither increases the growth rate nor enhances bacterial surface attachment (Supplementary Figs. 1 and 2). Moreover, when *E. coli* OP50 was cultured with 1 nM c-di-GMP overnight and then fed to N2 worms (no c-di-GMP added to the assay plates), the resulting lifespan remains the same as the control (Supplementary Fig. 3). These observations indicate that the increased gut colonization is largely attributed to a weakened gut immunity in the host.

We speculated that the expression of *C. elegans* innate immune response genes might be inhibited by c-di-GMP based on previous results. To examine this, we selected a few innate immune genes (C23G10.1, clec-46, clec-71, col-41, dct-5, fmo-2, pqr-5, and dod-22) that are reported to be upregulated during bacterial infections\textsuperscript{22,23} and analyzed their expression by qRT-PCR. Figure 5 shows that when synchronized L4-stage N2 worms were exposed to 1 nM c-di-GMP for only 10 min, expression of these genes was generally reduced 2-fold to 5-fold except for *dod-22*, which decreased by 1.6-fold. This was consistent with our hypothesis that the presence of this signaling molecule is modulating innate immunity.

C-di-GMP acts through SKN-1 and HSF-1 to impact immunity and lifespan. The FOXO family protein DAF-16, the SKN-1 protein, and the heat shock protein HSF-1 are the three major stress response transcription regulators in *C. elegans*. DAF-16, the downstream transcription factor of the insulin/insulin-like growth factor-1 signaling (IIS) pathway, plays a key role in modulating longevity and immunity\textsuperscript{24-26}. SKN-1 is the downstream effector of the major immune-signaling p38 mitogen-activated protein kinase (MAPK) pathway and controls numerous genes involved in stress response and lifespan regulation\textsuperscript{27}.

HSF-1 is another versatile transcription factor that regulates a multitude of genes involved not only in stress response but also in development, metabolism, as well as lifespan and immunity modulation\textsuperscript{28-30}. To test whether c-di-GMP acts through these regulators, lifespan assays in the presence and absence of c-di-GMP were conducted in mutant *daf-16* and *hsf-1* strains. In the case of *skn-1*, RNA interference (RNAi) was utilized to knock down the gene expression, and the empty vector pl4440 was used in parallel to serve as the control. We reasoned that if c-di-GMP acts through a particular transcriptional regulator to shorten the lifespan, mutating or knocking down expression of the gene will acts through a particular transcriptional regulator to shorten the lifespan, mutating or knocking down expression of the gene will

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**Fig. 3** *C. elegans* senses c-di-GMP through the AWC neurons and uses the Tax-2/Tax-4 channel. Choice index (CI) results are the average of three independent experiments, and error bars are standard error of the mean representing 95% confidence intervals. Individual values are shown as black dots. *p* values were calculated by using the unpaired Student *T*-test (each mutant vs. N2). *p* < 0.05.

**Fig. 4** C-di-GMP affects *C. elegans* lifespan and gut colonization. 
(a) Lifespan of N2 worms fed with live *E. coli* OP50 in the presence of c-di-GMP, c-di-AMP, and cGAMP. 
(b) Lifespan of N2 worms fed with heat-killed *E. coli* OP50 in the presence of c-di-GMP. The lifespan assays were repeated in at least three independent trials with similar results. The data shown in the figure are representatives from one of the trials. Quantitative data and statistical analyses for these trials are included in Table 1. 
(c) Average CFU/N2 worm (aged to Day 6) when fed with *E. coli* OP50 at 25°C in the presence or absence of 1 nM c-di-GMP. Results are the average of three independent experiments, and error bars are standard error of the mean representing 95% confidence intervals. Individual values are shown as black dots. *p* values were calculated by using the unpaired Student *T*-test (N2 vs. N2 + c-di-GMP). *p* < 0.05.
by c-di-GMP in the hsf-1 mutant and the skn-1 RNAi knockdown strains, meaning that SKN-1 and HSF-1 are required for c-di-GMP to exert its effect.

Next, two representative target genes of DAF-16 (C10G11.5 and F52H3.5), two of SKN-1 (gcs-1 and gst-4), and two of HSF-1 (hsp-16.2 and hsp-70) were measured for their expression levels in the presence and absence of c-di-GMP. Figure 6d shows that c-di-GMP triggered downregulation of the SKN-1 and HSF-1 target genes but not the DAF-16 targets. These results suggest that sensing c-di-GMP by the chemoreceptors in the sensory neuron elicited signal transduction, that eventually led to reduced activity of SKN-1 and HSF-1.

p38 MAPK(PMK-1) and RTK-Ras-ERK MAPK (MPK-1) are required in the c-di-GMP-elicited signal transduction pathway. MAP kinases are central components of a series of signal transduction pathways that control many vital cellular processes. MAP kinases in mammals are grouped into three families: p38/SAPKs (stress-activated protein kinases), JNKs (Jun amino-terminal kinases), and ERKs (extracellular-signal-regulated kinases)31. The three corresponding MAPKs in C. elegans are PMK-1, JNK-1, and MPK-1. Activity and cellular localization of SKN-1 and HSF-1 are modified by phosphorylation. Phosphorylated SKN-1 and HSF-1 are translocated into the nucleolus where they activate their target genes. Previous studies reported that either PMK-132 or MPK-133 possess the ability to directly phosphorylate SKN-1 at the same site. The kinases that phosphorylate HSF-1 remains elusive. Using the pmk-1 and jnk-1 mutant strains as well as the mpk-1 RNAi, we examined whether these MAPKs are involved in the signal transduction pathway elicited by c-di-GMP. Lifespans of these mutant worms were compared in the presence and absence of 1 nM c-di-GMP and shown in Fig. 7a–c and Table 1. In the pmk-1 mutant and the mpk-1 RNAi worms, adding c-di-GMP in the medium no longer reduced the lifespan as compared to that in the N2 or N2/pL4440 worms. While in the jnk-1 mutant, a 10% decrease in lifespan was observed. Our data suggest that the two MAP kinases, PMK-1 from the p38 MAPK pathway and MPK-1 from the RTK-Ras-ERK pathway, are required in the c-di-GMP-elicited signal transduction.

Discussion

Using the V. cholerae-C. elegans interaction model, we discovered that c-di-GMP acts as an interkingdom communication signaling molecule to attract C. elegans and suppress the host’s innate immunity. Sensing c-di-GMP is through the olfactory AWC\textsuperscript{ON} and AWC\textsuperscript{OFF} neurons and the cGMP-gated TAX-2/TAX-4 channel. This is different from the case of sensing V. cholerae CAI-1, which only requires the AWC\textsuperscript{ON} neuron35. Unlike the gustatory neurons, olfactory neurons are able to detect long-range signals, usually at nanomolar concentrations. In consensus with this, the concentration of c-di-GMP from the overnight V. cholerae culture was measured to be around 20 nM.

Though the two species may not intersect in nature, we think our findings could have universal implications. Being a soil-dwelling nematode, C. elegans feeds on bacteria and has co-evolved with bacteria over the years, and thus has developed a particularly intimate relationship with bacteria. We believe that the production of c-di-GMP by bacteria, or its presence outside of the bacterial cell, acts as a cue attracting C. elegans towards a food source. That initial response and attraction from the worms by c-di-GMP could signal a favorable environment. While this attraction may decrease immune response initially, degradation and weakening of this signal over time could lead to a return to baseline immunity under noninfectious conditions in vivo. However, when this signal is produced by a pathogen, such as V. cholerae, this initial attraction could be all it takes for C. elegans to begin feeding and thus become infected. Pathogens such as V. cholerae and P. aeruginosa possess a powerful signaling network that utilizes c-di-GMP for its success. C-di-GMP has been considered to be prevalent in Gram-negative bacteria, but its existence and function in Gram-positive bacteria have been gradually revealed in recent years, especially in those commonly found in the soil, such as Bacillus spp.34-37, Streptomyces coelicolor\textsuperscript{38}, Clostridium difficile\textsuperscript{39}, and Mycobacterium smegmatis\textsuperscript{40}. With the attraction of C. elegans towards low concentrations of c-di-GMP, this can allow it to find numerous bacteria in the environment, Gram-positive or Gram-negative, pathogen or not.

Our observation seems contradictory to the current knowledge that CDNs trigger the innate immune system by activating interferon genes12. However, we do not think it is a contradiction. C. elegans lacks the c-di-GMP receptor STING41 and the robust interferon system, therefore c-di-GMP will not trigger the canonical STING pathway in the C. elegans model. Only within the last decade has significant research been conducted investigating the binding of bacterial CDNs to the human STING receptor. Initially, this was implicated in the innate immune response to pathogens, but more recently, it has been shown to detect tumor-derived DNA and direct antitumor immunity through the versatile type I IFN42. The human STING receptor has a higher binding affinity with cGAMP and c-di-GMP than it does to c-diAMP43. Its affinity is even higher when binding endogenous mammalian cGAMP, which possesses a 2′-5′ phosphodiester linkage rather than the microbial 3′-5′ linkage13. Through these studies, it was found that STING has the affinity to bind to the bacterial c-di-GMP at a concentration of about 4.4 μM, which initiates the downstream signal transduction. Efforts have been made in attempts to modify STING for higher affinity, and while this proved successful, the concentration of c-di-GMP needed for binding was still high, 0.461 ± 0.053 μM43.

In our study, we found that c-di-GMP, as low as 1 nM, is being sensed by C. elegans, and this decreases its immune response. We also found that this behavioral response occurred at similar concentrations towards microbial cGAMP. Although not uncovered in this work, there is a possibility that C. elegans may possess a receptor with a higher affinity for CDNs than the mammalian STING. If this were the case, finding this receptor and studying its structure will provide valuable information to the STING research field. The ability to bind CDNs at nanomolar concentrations would remarkably improve STING’s sensitivity to foreign and cancer cell-derived DNA. This is an exciting new area of research, and it has the possibility to be far-reaching, especially in the fight against cancer and improving immunotherapies.
We showed that c-di-GMP acts through two key stress response transcription factors in *C. elegans*, SKN-1 and HSF-1. SKN-1 is the downstream effector of the major immune-signaling p38 MAPK pathway and controls numerous genes involved in stress response and lifespan regulation. HSF-1 is a multifaceted transcription factor involved in stress response, development, metabolism, as well as lifespan and immunity modulation. The possible MAP kinases (PMK-1, JNK-1, and MPK-1) that regulate SKN-1 and HSF-1 activity and nucleus localization were investigated. PMK-1 from the p38 MAPK pathway and MPK-1 from the RTK-Ras-ERK pathway are required in the c-di-GMP-elicited signal transduction. It is known that both PMK-1 and MPK-1 can phosphorylate SKN-1 to modulate its function, while the kinase in *C. elegans* to phosphorylate HSF-1 is still not clear. Our data propose the possibility that PMK-1 or MPK-1 might be the upstream kinase for HSF-1. Of course, further studies are needed to address this interesting question.

Although we believe that we have successfully defined this new role for bacterial second messengers and their effect on eukaryotes, more work still needs to be done. Communications between bacteria and their hosts likely have played an indispensable role during their coevolution over millions of years. Bacteria have evolved to release signaling molecules to improve their survival and spread by interfering with the host's behavior and immune response. In conjunction, the hosts have developed receptors for these signals to trigger immune responses to defend themselves. This has contributed to the symbiotic relationship that exists between bacteria and their hosts. Manipulation of such signaling can provide valuable insight to researchers looking to develop novel methods to treat infection and combat persistence. The better we understand this "language", the better we can start to control the conversation rather than reacting.

### Methods

**Plasmids, bacterial strains, and growth conditions.** The plasmid pBAD33 (pACYC184 araC *P araBAD* Cam^-^) was obtained from Coli Genetic Stock center, plasmid pBAD38 (pBAD33::NTF3::lacA-His^6^) was provided by Dr. Andrew Camilli (Tufts University). The *V. cholerae* strains used in this study were all derived from the wild-type C6706 strain (O1 serotype El Tor isolated from Peru) were cultured in Lysogeny broth (LB) with 100 μg/mL of streptomycin. Chloramphenicol (5 μg/mL) was used for the selection of pBAD33 and pAT1568 in *E. coli OP50*. The *V. cholerae* strains were grown in Brain Heart Infusion broth (BHI) at 30 °C and were supplemented with 25 μg/mL of kanamycin unless otherwise noted. The *E. coli* strains were grown in LB medium supplemented with 50 μg/mL of ampicillin. The *C. elegans* wild-type N2 strain and various mutant strains at 25 °C in the absence and presence of 1 nM of c-di-GMP.

| Strain | Mean ± SE (Day) | Median (Day) | # of worms | p-value | % change |
|--------|-----------------|--------------|------------|---------|----------|
| N2²    | 12.90 ± 0.45    | 14.00        | 59         |         |          |
| N2 + c-di-GMP² | 11.20 ± 0.48    | 11.00        | 60         | <0.001  | −17.0    |
| N2 + c-di-AMP² | 13.53 ± 0.30    | 13.00        | 62         | 0.583   | −1.2     |
| N2 + cGAMP² | 13.71 ± 0.26    | 14.00        | 65         | 0.577   | 0.1      |
| N2 (heat-killed OP50)³ | 14.02 ± 0.37    | 14.00        | 57         |         |          |
| N2 + c-di-GMP (heat-killed OP50)³ | 13.14 ± 0.38    | 13.00        | 58         | 0.063   | −6.3     |
| N2⁴    | 13.70 ± 0.45    | 14.00        | 59         |         |          |
| N2 + c-di-GMP⁴ | 11.20 ± 0.48    | 11.00        | 60         | <0.001  | −18.2    |
| daf-16(mgD50)⁵ | 8.64 ± 0.22     | 8.00         | 61         |         |          |
| daf-16(mgD50) + c-di-GMP⁵ | 7.20 ± 0.23     | 7.00         | 61         | <0.001  | −14.9    |
| EV⁶    | 12.94 ± 0.36    | 13.00        | 62         | <0.001  | −15.5    |
| EV + c-di-GMP⁶ | 10.93 ± 0.36    | 11.00        | 60         | <0.001  | −15.5    |
| skn-1 RNAi⁶ | 11.20 ± 0.29    | 11.00        | 61         |         |          |
| skn-1 RNAi + c-di-GMP⁶ | 11.75 ± 0.32    | 12.00        | 60         | 0.080   | 4.9      |
| N2³    | 12.84 ± 0.41    | 13.00        | 62         | <0.001  | −21.1    |
| N2 + c-di-GMP⁷ | 10.13 ± 0.41    | 11.00        | 62         | <0.001  | −21.1    |
| hsf-T(sy44)r³ | 6.31 ± 0.20     | 7.00         | 61         | 0.495   | −3.3     |
| hsf-T(sy44)r + c-di-GMP⁷ | 6.10 ± 0.21     | 6.00         | 61         |         |          |
| N2³    | 13.40 ± 0.41    | 14.00        | 60         | 0.950   | −1.1     |
| N2 + c-di-GMP² | 10.37 ± 0.44    | 11.00        | 60         | <0.001  | −22.6    |
| pmk-k(ks2)³ | 12.08 ± 0.52    | 13.00        | 60         | <0.001  | −22.6    |
| pmk-k(ks2) + c-di-GMP² | 11.95 ± 0.54    | 13.00        | 61         | 0.950   | −1.1     |
| N2³    | 14.03 ± 0.37    | 14.00        | 59         |         |          |
| N2 + c-di-GMP³ | 11.53 ± 0.41    | 12.00        | 59         | <0.001  | −17.8    |
| jnk-1(gk7)³ | 12.43 ± 0.29    | 12.00        | 60         |         |          |
| jnk-1(gk7) + c-di-GMP³ | 11.17 ± 0.28    | 11.00        | 59         | 0.003   | −10.1    |
| EV³    | 12.88 ± 0.40    | 13.00        | 60         |         |          |
| EV + c-di-GMP³ | 11.15 ± 0.36    | 11.00        | 60         | <0.001  | −13.4    |
| mpk-1 RNAi³ | 11.40 ± 0.30    | 11.00        | 60         |         |          |
| mpk-1 RNAi + c-di-GMP³ | 11.63 ± 0.31    | 12.00        | 59         | 0.518   | 2.0      |

All the worms were fed on live E. coli OP50 unless otherwise noted. The lifespan experiments were repeated at least three times with similar results, and the data for representative experiments are shown. The lifespan data were analyzed using the log-rank test and p-values for each individual experiment are shown. The p-value was calculated by comparing c-di-GMP-treated to the untreated worms.

¹Results presented in Fig. 4a.
²Results presented in Fig. 4b. The worms were fed with heat-killed E. coli OP50.
³Results presented in Fig. 4a.
⁴Results presented in Fig. 6a.
⁵Results presented in Fig. 6b.
⁶Results presented in Fig. 6c.
⁷Results presented in Fig. 7a.
⁸Results presented in Fig. 7b.
⁹Results presented in Fig. 7c.

We showed that c-di-GMP acts through two key stress response transcription factors in *C. elegans*, SKN-1 and HSF-1. SKN-1 is the downstream effector of the major immune-signaling p38 MAPK pathway and controls numerous genes involved in stress response and lifespan regulation. HSF-1 is a multifaceted transcription factor involved in stress response, development, metabolism, as well as lifespan and immunity modulation. The possible MAP kinases (PMK-1, JNK-1, and MPK-1) that regulate SKN-1 and HSF-1 activity and nucleus localization were investigated. PMK-1 from the p38 MAPK pathway and MPK-1 from the RTK-Ras-ERK pathway are required in the c-di-GMP-elicited signal transduction. It is known that both PMK-1 and MPK-1 can phosphorylate SKN-1 to modulate its function, while the kinase in *C. elegans* to phosphorylate HSF-1 is still not clear. Our data propose the possibility that PMK-1 or MPK-1 might be the upstream kinase for HSF-1. Of course, further studies are needed to address this interesting question.

Although we believe that we have successfully defined this new role for bacterial second messengers and their effect on eukaryotes, more work still needs to be done. Communications between bacteria and their hosts likely have played an indispensable role during their coevolution over millions of years. Bacteria have evolved to release signaling molecules to improve their survival and spread by interfering with the host’s behavior and immune response. In conjunction, the hosts have developed receptors for these signals to trigger immune responses to defend themselves. This has contributed to the symbiotic relationship that exists between bacteria and their hosts. Manipulation of such signaling can provide valuable insight to researchers looking to develop novel methods to treat infection and combat persistence. The better we understand this "language", the better we can start to control the conversation rather than reacting.
**Fig. 6** C-di-GMP acts through SKN-1 and HSF-1, but not DAF-16. Lifespan of **a** *daf-16(mgD50)*, **b** *skn-1* RNAi, and **c** *hsf-1(* sy441) worms when supplemented with 1 nM c-di-GMP. Each lifespan experiment was repeated in at least three independent trials with similar results. Quantitative data and statistical analyses for all trials are included in Table 1. **EV** stands for "empty vector pL4440". **d** qRT-PCR analysis of the representative target genes of DAF-16 (*C10G11.5* and *F52H3.5*), SKN-1 (*gcs-1* and *gst-4*), and HSF-1 (*hsp-16.2* and *hsp-70*), in response to 1 nM c-di-GMP (normalized to *act-1*). Results are the average of three independent experiments, and error bars are standard error of the mean representing 95% confidence intervals. Individual values are shown as black dots. *p* values were calculated by using the unpaired Student *T*-test (each gene vs. *act-1*). *p* < 0.05.

**Fig. 7** PMK-1 and MPK-1 are required in the c-di-GMP-elicited signal transduction pathway. Lifespan of **a** *pmk-1(km25)*, **b** *jnk-1(gk7)*, and **c** *mpk-1* RNAi worms when supplemented with 1 nM c-di-GMP. Each lifespan experiment was repeated in at least three independent trials with similar results. Quantitative data and statistical analyses for all trials are included in Table 1. **EV** stands for "empty vector pL4440".
C. elegans strains and growth conditions. All C. elegans strains were obtained from the Caenorhabditis Genetics Center, University of Minnesota, USA. Strains used in this study were: N2 Bristol (wild type), AUS (n10954), FG100/PR678 (tax-2(kx10)/tax-2(kx678)), FG125 (ocr-2(ok47), tns-9(ky10), ocr-1(kx66)), FK311 (ceh-36(kx665)), PR674 (che-1(p674), PR674 (che-1(p674)), GR1370 (daf-16 [mgDf50]), PS5351 (hft-1(yk411), KU25 (pmk-1(kn25)), and VC8 (pjk-1(qk7)).

All strains were maintained at their permissive temperature on nematode growth medium (NGM) seeded with E. coli OP50 feeding strain. NGM for maintenance was prepared via standard protocol in 60 mm plates. E. coli OP50 was dropped on the center of the plates the night prior to transfer of worms. Bacterial strains were dropped onto the center of the plates 2 h prior to worm transfer. NGM-FUDR 35 mm plates were used for lifespan assays. Fluorodeoxyuridine (FUDR) is an inhibitor of DNA synthesis, and at a concentration of 50 μg/mL it prevents the reproduction and development of progeny but does not interfere with the lifespan and the physiology of adult C. elegans.5,6 Five times concentrated bacterial cultures were dropped in 100 μL aliquots onto the center of plates 2 h prior to worm transfer. NGM or NGM-FUDR plates with 1 nM C-di-GMP were created by adding correct concentrations of stock C-di-GMP (1 mM) to warm NGM mixtures after autoclaving.

Preparation of cyclic di-nucleotides. Cyclic diadenosine monophosphate (c-di-AMP), cyclic diadenosine monophosphate (c-di-GMP), and cyclic adenosine monophosphate–guanine monophosphate (cGAMP) were purchased from BioLog Life Science Institute (catalog numbers are C088, C057, and C117, respectively). Stock concentrations of these sodium salt compounds were made by dissolving in ddH2O. The stock samples were stored in −20°C freezer. Serial dilutions were made from frozen stocks to obtain desired concentration before experiments were conducted.

Liquid chromatography-mass spectrophotometry (LC-MS) assay. V. cholerae C6706 was grown shaking at 37°C for 22 h in 100 mL of LB broth. The culture was centrifuged for 10 min at maximum speed to separate the supernatant and the cell pellet. The supernatant and the cell lysate were tested for the presence of CDNs, corresponding to the extracellular and intracellular concentrations, respectively. The supernatant was treated with final concentration of 10 mM EDTA for 10 min and then passed through 0.45-µm filter. The filtrate was freeze-dried, resuspended in 500 μL of sterile ddH2O, and centrifuged in Amicon Ultra-0.5 mL centrifugal filter device to remove any DNA or protein molecules larger than 3 KDa. The sample was lyophilized and stored at room temperature before LC-MS assay.

C-di-GMP and cGAMP in supernatant and cell pellet were quantified by HPLC with online tandem mass spectrometry (MS). The CDNs were resolved on a C18 column (150 × 2 mm, Prodigy™ 5 μm ODS-2 150 Å) (Phenomenx) using a gradient generated between 0.2% formic acid in water (A) and 0.2% formic acid in methanol (B) at a flow rate of 0.25 mL/min starting from 0% B over 2 min, then linearly to 20% B over 4 min, then to 100% B over 0.5 min, followed by this solution for 3 min, then back to 0% B. CDNs were analyzed on a Shimadzu 8050 triple-quadrupole mass spectrometer interfaced to a Shimadzu UHPLC multiplexing system using electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM) and characteristic daughter ions m/z 675.10 → 136.10 for cGAMP and m/z 691.10 → 152.10 for c-di-GMP. Several other parent and characteristic daughter ions m/z 675.10 → 524.00, 675.10 → 330.05 and m/z 691.10 → 539.90, 691.10 → 248.05 were used as reference to confirm the defined cGAMP and c-di-GMP, respectively. The MS parameters were optimized by using their respective standards. Series concentrations of CDNs standards were monitored by the above UHPLC/MS analysis and regression equations to calculate the concentrations in supernatant and cell pellet.

Choice index (CI) assays. C. elegans were grown at 25°C on E. coli OP50 under well-fed and un-crowded conditions. CI assays were performed on standard LB agar plates. The plates were divided in half to reveal the center point in each plate. Bacterial strains that were used for this experiment were grown in a shaking incubator overnight in the proper growth medium and temperature at approximately 20 rpm. Overnight cultures were then seeded onto each end of the plate 6 cm apart, and a 2 cm radius is drawn around each lawn (Fig. 8). The lawns are allowed to dry for 2 h before experiments were conducted. After the lawns were able to dry, 1 μL of 10 mM sodium azide (NaN3) was dropped on the center of each lawn to paralyze C. elegans to make sure they did not change bacterial lawns once one was initially chosen. Between 50 and 150 well-fed N2 worms were then placed in the center of the assay plate to begin the choice experiment. Worms present within the 2 cm radius of each bacterial lawn were counted after 1 and 2 h. Each assay was performed independently and in at least triplicate. Choice index was calculated as follows:

\[ CI = \left( \frac{\text{# of worms on test strain}}{\text{# of worms on } E. \ coli \ OP50} \right) \]

*Positive values indicate preference towards the test strain, while negative values indicate preference towards E. coli OP50. If there were no preference, the equation would yield a value close to 0.

Average and standard error were calculated from three independent experiments.

Unpaired Student’s t-test (between two groups) and one-way balanced ANOVA (for more than two groups) were used for statistical analysis. A p-value < 0.05 was accepted as statistically significant.

C. elegans lifespan assay. Lifespan assays were carried out at 25°C. Worms were synchronized by transferring 20 gravid worms to 60 mm NGM plates seeded with E. coli OP50 2 days prior to the start of assays. Worms were allowed to lay eggs for 4 h, and then parent worms were removed from plates, leaving only synchronized eggs. Worms were then incubated until L4 stage. Overnight bacterial cultures were concentrated by centrifugation and removal of 50% of supernatant. Cultures were then resuspended in remaining medium. When necessary, E. coli OP50 was heat killed to reduce larval stage (1 h at 80°C). Aliquots of 100 μL of E. coli OP50 were dropped on the center of NGM-FUDR 35 mm plates (with and without 1 nM c-di-GMP) and allowed to dry for 2 h. Worms in L4 stage were transferred to assay plates; 20 worms per plate. If the worms were transferred onto plates with heat-killed E. coli OP50, then prior to transferring to the assay plates they were allowed to roam on LB gentamicin (50 μg/mL) plates for 1 h to rid external live bacteria. Plates were subsequently checked daily. Dead worms were removed from the plates after being counted and recorded. The day of transfer was defined as day zero. Statistical analysis was carried out through SPSS software under the Kaplan–Meier lifespan analysis. The p-values were determined via log rank test, and a p < 0.05 was accepted as statistically significant.

Gut colonization of C. elegans. E. coli OP50 was grown overnight in LB broth with 100 μg/mL of streptomycin broth. Aliquots of 100 μL of E. coli OP50 was dropped on NGM with 1 nM c-di-GMP and control NGM plates. Five C. elegans N2 gravid worms were transferred onto each plate and incubated at 20°C for 4 h. Synchronized egg populations were allowed to grow to L4 stage (~40 h), and then transferred to NGM-FUDR and NGM-FUDR with 1 nM c-di-GMP plates with 100 μL of respective overnight cultures at 2× concentration. This was designated as Day 0, and tests were conducted on Day 6 aged worms on each bacterial species +/- c-di-GMP. Ten worms were randomly chosen from each plate and transferred to LB gentamicin (50 μg/mL) plates for one hour to rid N2 worms of external bacteria. Worms were then transferred to Eppendorf tubes containing 400 μg of St. C. car体会到 heads and 1 mL of M9. Tubes were vortexed for 1 min and then centrifuged at max rpm for 2 min. After, tubes were then quickly vortexed and serially diluted in M9 and plated on LB streptomycin (100 μg/mL) plates. Colony-forming
RNA interference (RNAi) constructs targeting mpk-1 and skn-1 were used for analysis, and a one-way ANOVA (for more than two groups) was used for statistical analysis. Each qPCR experiment was repeated three times using independent RNA preparations.

The data were pooled and analyzed using unpaired Student’s *t*-test (between each target gene and the control act-1), and a *p*-value < 0.05 was accepted as statistically significant. The primers used in this study are listed in Table 2.

**Table 2 Oligonucleotides used in quantitative real-time PCR.**

| Gene   | Sequence of the oligonucleotide                      |
|--------|------------------------------------------------------|
| act-1  | Forward 5′-CCAGAATGCTGATCGTATGCAGAAA-3′ Reverse 5′-TGGAGAGGACCGAGGATAGA-3′ |
| C10G11.5 | Forward 5′-AGTAGATCCACGAGCAAGAGGATG-3′ Reverse 5′-AAAGCTACGAGCAGATCTGCTG-3′ |
| C23G10.1 | Forward 5′-CCATCCACTTGGTTCGCTT-3′ Reverse 5′-TACCTGCTTTTCCTTTTCT-3′ |
| clec-46 | Forward 5′-CTTCTTGCTGTCTGACATT-3′ Reverse 5′-GGGTTTCCCAACAAACAC-3′ |
| clec-71 | Forward 5′-TTGCTGCTTGAAGCATTAACTC-3′ Reverse 5′-TCATTGGGAATCCGTTATCC-3′ |
| col-41 | Forward 5′-CACCAGAATCTCGAGGA-3′ Reverse 5′-GTGGGTTGTCCTGCTTCT-3′ |
| dct-5  | Forward 5′-GCTCGAATGGATTGAAATGA-3′ Reverse 5′-AAAGTTTGGGCCAGATCCGAC-3′ |
| dod-22 | Forward 5′-TTTGTTGCCCAATGTTCAAC-3′ Reverse 5′-AAAGAATCTGCGGCTTCAG-3′ |
| F52H3.5 | Forward 5′-GCATCCACGCCTATGACGTTAGTT-3′ Reverse 5′-GCTTCACTACCAGGTAACCC-3′ |
| fmo-2  | Forward 5′-TGCCTGTCATTAGGCTTCGTT-3′ Reverse 5′-GGAATTCCACGGAATGGCT-3′ |
| gcs-1  | Forward 5′-CATCTAGCCGCTCAAAAACAA-3′ Reverse 5′-CATGAGACTATCTGCTC-3′ |
| gtc-1  | Forward 5′-CAGTACATGTTCCTCCTC-3′ Reverse 5′-CGGCTCAGCGTCTGACATT-3′ |
| gtx-1  | Forward 5′-CTGCGGCAAAATGAGCTTG-3′ Reverse 5′-GAGTTTTGGGCACAGTCCAG-3′ |
| hsp-16.2 | Forward 5′-TTGCCATCAATCTCAACGTC-3′ Reverse 5′-CTTTTTTTGGGCTGGCCACCT-3′ |
| hsp-70  | Forward 5′-CCTTCCGAGAATGCGTACCCGACG-3′ Reverse 5′-TTATCAAATCTCTGTTGTTG-3′ |
| pgn-5  | Forward 5′-GTCGACGCACCAACAAACATCA-3′ Reverse 5′-CTTGGACGTTGATCGACATT-3′ |

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