The cyclic dinucleotide 2′3′-cGAMP induces a broad antibacterial and antiviral response in the sea anemone *Nematostella vectensis*

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In mammals, cyclic dinucleotides (CDNs) bind and activate STING to initiate an antiviral type I interferon response. CDNs and STING originated in bacteria and are present in most animals. By contrast, interferons are believed to have emerged in vertebrates; thus, the function of CDN signaling in invertebrates is unclear. Here, we use a CDN, 2′3′- cyclic guanosine monophosphate-adenosine monophosphate (2′3′-cGAMP), to activate immune responses in a model cnidarian invertebrate, the starlet sea anemone *Nematostella vectensis*. Using RNA sequencing, we found that 2′3′-cGAMP induces robust transcription of both antiviral and antibacterial genes in *N. vectensis*. Many of the antiviral genes induced by 2′3′-cGAMP are homologs of vertebrate interferon-stimulated genes, implying that the interferon response predates the evolution of interferons. Knockdown experiments identified a role for NF-κB in specifically inducing antibacterial genes downstream of 2′3′-cGAMP. Some of these putative antibacterial genes were also found to be induced during *Pseudomonas aeruginosa* infection. We characterized the protein product of one of the putative antibacterial genes, the *N. vectensis* homolog of Dae4, and found that it has conserved antibacterial activity. This work suggests that a broad antibacterial and antiviral transcriptional response is an evolutionarily ancestral output of 2′3′-cGAMP signaling in animals.

**Significance**

Cyclic dinucleotides are signaling molecules that originated in bacteria and were subsequently acquired and co-opted by animals for immune signaling. The major cyclic dinucleotide signaling pathway in mammals results in the production of antiviral molecules called interferons. Invertebrates such as sea anemones lack interferons, and thus it was unclear whether cyclic dinucleotide signaling would play a role in immunity in these animals. Here, we report that in the anemone *Nematostella vectensis*, cyclic dinucleotides activate both antiviral and antibacterial immune responses and do so through a conserved pathway. These results provide insights into the evolutionary origins of innate immunity and suggest a broader ancestral role for cyclic dinucleotide signaling that evolved toward more specialized antiviral functions in mammals.
encodes a cGAS enzyme that produces 2′,3′-cGAMP in mammalian cell culture (17). In vertebrates, STING requires its extended CTT to initiate transcriptional responses (38, 39); however, nvSTING lacks an extended CTT, and thus its signaling mechanism and potential for inducing transcriptional responses are unclear. Based on experiments with nvSTING in mammalian cell lines, CTT-independent induction of autophagy has been proposed as the “ancestral” function of STING (17), but the endogenous function of STING in N. vectensis has never been described.

Despite the genomic identification of many predicted innate immune genes (40, 41), few have been functionally characterized in N. vectensis. The sole N. vectensis Toll-like receptor (TLR) is reported to bind flagellin and activate NF-κB in human cell lines and is expressed in cnidocytes, the stinging cells that define Cnidarians (42). N. vectensis NF-κB (nvNF-κB) binds to conserved κB sites, is inhibited by N. vectensis IκB (43), and seems to be required for the development of cnidocytes (44). However, no activators of endogenous nvNF-κB have yet been identified. Recent work probing the putative antiviral immune response in N. vectensis found that double-stranded RNA (dsRNA) injection into N. vectensis embryos leads to transcriptional induction of genes involved in the RNA interference (RNAi) pathway as well as genes with homology to ISGs (45). This response is partially dependent on a RIG-I-like receptor, indicating deep conservation of antiviral immunity (45). However, no antiviral or antibacterial effectors from N. vectensis have been functionally tested.

To assess the response of N. vectensis to 2′,3′-cGAMP stimulation. Similar to the response of vertebrates to 2′,3′-cGAMP, we find robust transcriptional induction of putative antiviral genes with homology to vertebrate ISGs. In addition, we observed induction of numerous antibacterial genes that are not induced during the vertebrate response to 2′,3′-cGAMP. Although we were unable to show that the response to 2′,3′-cGAMP is nvSTING dependent, we did find a selective requirement for nvNF-κB in the induction of some of the antibacterial genes. Many of these genes are also induced during Pseudomonas aeruginosa infection, suggesting a functional role in antibacterial immunity. We selected and characterized the antibacterial activity of one 2′,3′-cGAMP–induced Nematozostella gene product, domesticated amide dictate effector 4 (nvDa4e), a peptidoglycan (PG)-cleaving enzyme that we found can kill gram-positive bacteria. This work demonstrates an evolutionarily ancient role for 2′,3′-cGAMP in the transcriptional induction of both antiviral and antibacterial immunity.

Results

Transcriptional Response to 2′,3′-cGAMP in N. vectensis. To assess the in vivo role of 2′,3′-cGAMP signaling in N. vectensis, we treated 2-wk-old polyps with 2′,3′-cGAMP for 24 h and performed RNA sequencing (RNA-Seq) (Fig. L4 and SI Appendix, Fig. S1). Thousands of genes were induced by 2′,3′-cGAMP, many of which are homologs of genes known to function in mammalian immunity. Despite the lack of associated gene ontology (GO) terms for many of the differentially regulated genes, unbiased GO term analysis revealed significant enrichment of immune-related terms (Fig. 1B). We also treated animals with 3′-linked CDNs, which are thought to be produced exclusively by bacteria and which also bind to nvSTING in vitro, albeit at lower affinity (37). Both 3′-cGAMP and cyclic-dGAMP treatment also induced a smaller number of genes, although all of these genes were induced more strongly by 3′-cGAMP (SI Appendix, Fig. S1). Interestingly, cyclic diguanylate monophosphate (c-di-GMP) treatment led to almost no transcriptional induction despite having relatively high affinity for nvSTING in vitro. This discrepancy may be due to differences in cell permeability among different CDNs, as the ligands were added extracellularly. In order to be able to perform subsequent genetic experiments, we confirmed that the immune gene induction downstream of 2′,3′-cGAMP also occurred in embryos (which are amenable to microinjection of short hairpin RNAs [shRNAs]). qRT-PCR on 48-h embryos treated for 4 h with a lower dose of 2′,3′-cGAMP revealed that many immune genes were also induced at this early developmental stage after a much shorter treatment (Fig. 1C).

Several interesting classes of genes were found to be up-regulated in response to 2′,3′-cGAMP. For example, several genes involved in the RNAi pathway were induced, including homologs of Argonaute (AGO2), Dicer, and RNA-dependent RNA polymerase (Rdrp1). In addition, many genes that are considered ISGs in mammals were also induced in N. vectensis, including Viperin, RNase L, 2′,5′-oligoadenylate synthase (OAS), interferon regulatory factors (IRFs), guanylate-binding proteins (GBP), and the putative pattern recognition receptors RIG-I-like receptor a (RLRa) and RLRb. These results suggest a conserved role for 2′,3′-cGAMP signaling in antiviral immunity and ISG induction despite an apparent lack of conservation of type I IFNs in N. vectensis. Interestingly, we also found that many putative antibacterial genes were up-regulated in response to 2′,3′-cGAMP, including homologs of LPS-binding protein (LBP), lysozyme, perforin-2, Dae4, and mucins. These results indicate that 2′,3′-cGAMP stimulation leads to a broad immune response in N. vectensis.

To determine whether 2′,3′-cGAMP signaled via nvSTING to induce these genes, we injected shRNAs targeting nvSTING into one-cell embryos and treated with 2′,3′-cGAMP 48 h later. We extracted RNA and performed RNA-Seq on these samples, and surprisingly, while nvSTING transcripts were reduced by ∼50%, there was no significant impact on 2′,3′-cGAMP–induced gene expression (SI Appendix, Fig. S2 A and B). These negative results were recapitulated in numerous independent qRT-PCR and Nanostring experiments using nine different shRNAs (three shown in SI Appendix, Fig. S2C). There are several possible explanations for the failure to observe a requirement for nvSTING in 2′,3′-cGAMP signaling: 1) a twofold reduction in nvSTING transcript levels may not result in a reduction in STING protein levels if the protein is very stable; 2) even if nvSTING protein levels are reduced twofold, the reduction may not affect nvSTING signaling due to threshold effects; or 3) nvSTING may not be required for signaling downstream of 2′,3′-cGAMP due the presence of a redundant 2′,3′-cGAMP sensor in N. vectensis. We generated an anti-nvSTING antibody to validate knockdown efficiency at the protein level, but this reagent did not appear to specifically detect nvSTING in anemone lysates. We also tested whether an nvSTING translation-blocking morpholino could inhibit induction of genes in response to 2′,3′-cGAMP, but this also had no effect (SI Appendix, Fig. S2D).

We previously solved the crystal structure of nvSTING bound to 2′,3′-cGAMP and showed that binding occurs with high affinity (K<sub>d</sub> < 1nM) and in a similar mode as compared to vertebrate STING (37). In addition, we found that when expressed in mammalian cells, nvSTING forms puncta only in the presence of 2′,3′-cGAMP indicating some functional change induced by this ligand (SI Appendix, Fig. S2E). Thus, we hypothesize that 2′,3′-cGAMP signals via nvSTING, but technical issues and possible redundancy with additional sensors prevent formal experimental evidence for this hypothesis.

The N. vectensis NF-κB Homolog Plays a Role in the 2′,3′-cGAMP Response. We next tested the role of conserved transcription factors that are known to function downstream of STING in
mammals in the *N. vectensis* response to 2′,3′-cGAMP. Interestingly, many of these transcription factors are themselves transcriptionally induced by 2′,3′-cGAMP in *N. vectensis* (Fig. 1A). In mammals, the transcription factors IRF3 and IRF7 induce type I IFN downstream of STING activation. While the specific functions of these IRFs in interferon induction are thought to have arisen in vertebrates, other IRF family members, with conserved DNA-binding residues, are present in *N. vectensis* and may even be directly activated by STING (16). Similar to the IRFs, we did not observe a significant loss of gene induction by 2′,3′-cGAMP in nvSTAT knockdown embryos by both RNA-Seq and Nanostring (SI Appendix, Fig. S4 C and D). There are several explanations for these findings: 1) sufficient IRF or STAT protein may remain in knockdown animals to transduce the signal, either due to low efficiency of the knockdowns or to protein stability; 2) the IRFs may act redundantly with each other, and therefore no effect will be seen in single knockdowns or to protein stability; 2) the IRFs may act redundantly with each other, and therefore no effect will be seen in single knockdown experiments; or 3) nvIRFs and nvSTAT may not play a role in the response to 2′,3′-cGAMP.

NF-xB is also known to act downstream of mammalian STING and appears to be functionally conserved in *N. vectensis* (43). We found that NF-xB signaling components are transcriptionally induced by 2′,3′-cGAMP (Fig. 1A). To test the role of the 2′,3′-cGAMP response, we microinjected one-cell embryos with shRNAs targeting the mammalian NF-xB, treated with 2′,3′-cGAMP, and performed RNA-Seq (Fig. 24). A total of 241 genes were transcribed at significantly lower levels in the mammalian NF-xB knockdown embryos, and of these, 98 were genes induced by 2′,3′-cGAMP. Of these genes, 40 are uncharacterized, and no GO terms were significantly enriched. Of the induced genes
Fig. 2. The induction of many antibacterial genes by 2′3′-cGAMP is νNF-κB dependent. (A) Heatmap showing all genes that are significantly (adjusted p value [padj] < 0.05; log₂ fold change [FC] < −1) down-regulated in 2′3′-cGAMP–treated embryos microinjected with νNF-κB shRNA versus GFP shRNA. Genes with predicted antibacterial function are labeled. (B) qRT-PCR of antibacterial genes in νNF-κB shRNA– or control GFP shRNA–treated samples after induction by 2′3′-cGAMP. Fold change was calculated relative to untreated, GFP shRNA injected as 2^ΔΔCt and each point represents one biological replicate. Unpaired t test performed on ΔΔCt before log transformation. *P ≤ 0.05; **P ≤ 0.01. (C) Whole mount immunofluorescence of polyps stained with anti-νNF-κB antiserum. Right two panels are enlargements of the boxed regions indicated in the left two panels. (D) Quantification of cells with nuclear localization of νNF-κB after treatment with cGAMP (representative images shown in C). Each point represents a single polyp, in which at least 1,500 cells were analyzed. Statistical analysis was performed by unpaired t test; *P = 0.0481.
that were annotated in the National Center for Biotechnology Information (NCBI), we noticed many were homologs of antibacterial proteins, including homologs of perforin-2/Mpeg-1, LBP linear granicidin synthase, and mucins. We confirmed that 2’3’-cGAMP-mediated induction of these putative antibacterial genes was nvNF-xB dependent by performing qRT-PCR (Fig. 2B). Notably, the induction of nvLysozyme was not nvNF-xB dependent (both by RNA-Seq and qRT-PCR; SI Appendix, Fig. S5B), indicating either the existence of another pathway for antibacterial gene induction or that our knockdown experiment was not able to affect expression of all nvNF-xB–dependent genes. In addition, all of the putative antiviral genes we examined appeared to be induced independently of nvNF-xB (SI Appendix, Fig. S5A).

We performed basic local alignment search tool (BLAST) searches of unannotated 2’3’-cGAMP–induced, nvNF-xB–dependent genes and identified several other genes with predicted antibacterial activity, including two homologs of bacterial tae4 genes and a putative GBP (N. vectensis LOC5515806, hereafter nvGBP-806). The Tae4 homologs had been previously identified and will be referred to as nvDae4 proteins [discussed further innvDae4 Is a PG-Cleaving Enzyme with Antibacterial Activity (46)]. To confirm the identity of nvGBP-806 as a true GBP homolog, we performed phylogenetic analysis. We identified four conserved N. vectensis proteins harboring an N-terminal GBP GTPase domain with microhomology (46). To confirm the identity of nvGBP-806 as a true GBP homolog, we performed phylogenetic analysis. We identified four conserved N. vectensis proteins harboring an N-terminal GBP GTPase domain with conserved GBP-specific motifs, including nvGBP-806 (SI Appendix, Fig. S6). All of the nvGBP homologs cluster with vertebrate IFN-inducible GBPs and are themselves induced by 2’3’-cGAMP. Finally, we identified several unannotated nvNF-xB–dependent, 2’3’-cGAMP–induced genes that appeared to benidarian specific with no identifiable homologs in other animal phyla (SI Appendix, Table S1).

To test directly whether nvNF-xB is activated in N. vectensis upon 2’3’-cGAMP treatment, we treated polyps with cGAMP and performed immunostaining for nvNF-xB (Fig. 2C). Inactive NF-xB is localized to the cytosol, and we observed sparse, cytosolic staining of ectodermal cells in untreated animals, as has been previously reported (43). In contrast, in 2’3’-cGAMP–treated animals, we found many more nvNF-xB–positive cells, and in almost all of these, nvNF-xB was found in the nucleus. We performed automated quantification of nuclear nvNF-xB staining and found that ~3 to 20% of nuclei captured in our images were positive for nvNF-xB (Fig. 2D). In sum, 2’3’-cGAMP leads to nvNF-xB nuclear localization, and nvNF-xB appears to be required for expression of many putative antibacterial, but not antiviral, genes. Our results demonstrate that 2’3’-cGAMP is a potent NF-xB agonist in N. vectensis and indicate a conserved immune function for NF-xB in this organism.

**Gene Induction During P. aeruginosa Challenge.** In order to test whether the putative antibacterial, NF-xB–dependent genes are induced during bacterial infection, we infected N. vectensis with P. aeruginosa, a pathogenic gram-negative bacterium. P. aeruginosa can infect a range of hosts, including plants, mammals, and hydra (47, 48), though infections of N. vectensis have not previously been reported. Infection of N. vectensis polyps with the P. aeruginosa strain PA14 led to polyp death in a dose- and temperature-dependent manner (Fig. 3A). A total of 48 h after infection, we isolated RNA from infected polyps and assayed gene expression. Interestingly, nvSTING expression was induced during PA14 infection (Fig. 3B), and many of the putative antibacterial genes we identified as 2’3’-cGAMP–induced were also induced during infection (Fig. 3C), although this expression was not sufficient to protect them from death. In addition, some putative antiviral genes were also induced in some animals (SI Appendix, Fig. S5C), perhaps reflecting a broader immune response in Nematostella. Importantly, the PA14 genome is not known to encode any proteins that produce CDNs other than c-di-GMP. Since c-di-GMP was not sufficient to robustly activate gene expression in N. vectensis, we believe that it is likely that the response to PA14 is independent of bacterial CDNs, although we cannot rule out an effect from PA14-produced c-di-GMP. In addition, we have no reason to believe that PA14 is activating these genes via nv-cGAS, as we do not know the activator of this enzyme. Nevertheless, taken together, these results indicate that the putative antibacterial

![Fig. 3.](https://doi.org/10.1073/pnas.2109022118)
genes we identified as induced by 2′,3′-cGAMP are also induced after bacterial challenge.

nvDae4 is a PG-Cleaving Enzyme with Antibacterial Activity. We decided to investigate directly whether any of the genes induced by both 2′,3′-cGAMP and bacterial infection are, in fact, antibacterial. Type VI secretion amidasie effector (Tae) proteins are bacterial enzymes that are injected into neighboring cells to cleave PG, an essential component of bacterial cell walls, leading to rapid cell death (49). While the tae genes originated in bacteria, they have been horizontally acquired multiple times in evolution by eukaryotes, and at least one of these so-called “domesticated amidasie effectors” (Daes) also has bactericidal activity (46, 50). The N. vectensis genome has two tae4 homologs, both of which were up-regulated by 2′,3′-cGAMP in an nvNF-xB-dependent manner. However, only one of these homologs is predicted to encode a conserved catalytic cysteine (46) required for PG hydrolysis. Therefore, we focused our efforts on this homolog, which we call nvDae4 (GI: 5507694).

We first tested whether nvDae4 has conserved bactericidal properties by expressing nvDae4 in *E. coli* with buffer alone, nvDae4 WT, or catalytic mutant C63A enzyme. (C) Partial HPLC chromatograms of *E. coli* PG sacculi after overnight incubation with buffer only (no enzyme) or 1 μM nvDae4 WT or C63A enzyme. (C) *B. subtilis* CFU after 2 h incubation with buffer alone, nvDae4 WT, or catalytic mutant C63A (25 μM). Error bars ± SEM; n = 3. Unpaired t test performed on log-transformed values; **P ≤ 0.01. (D) Dose-dependent killing of *B. subtilis* by WT nvDae4 enzyme (same assay as in C). Error bars ± SD; n = 2 per concentration.

Overall, these results show that the 2′,3′-cGAMP-induced protein nvDae4 is a PG-cleaving enzyme with the capacity to kill bacteria.

**Discussion**

In this study, we identified hundreds of *N. vectensis* genes that are induced by the STING ligand 2′,3′-cGAMP. Despite over 600 million years of divergence and the absence of interferons, *N. vectensis* responds to 2′,3′-cGAMP similarly to mammals by inducing a variety of antiviral genes. Similarly, Lewandowska et al. (45) recently reported that *N. vectensis* responds to the synthetic dsRNA A polyinosinic-polyribidylic acid [poly(I:C)], a viral mimic and pathogen-associated molecular pattern (PAMP). In *N. vectensis*, poly(I:C) induced both RNAi pathway components and genes traditionally thought of as vertebrate ISGs. Our combined findings indicate that the pathways linking PAMP detection to ISG expression existed prior to the vertebrate innovation of type I IFNs. Interestingly, some invertebrate species have protein-based antiviral signaling pathways that perform similar functions to type I IFNs in vertebrates. For example, mosquito cells secrete the peptide Vago upon viral infection, which signals through the JAK-STAT pathway to
activate antiviral immunity (51). Additionally, the oyster *Crassostrea gigas* is thought to have an IFN-like system, but no secreted proteins have yet been identified in this organism (52). *N. vectensis* may also encode an undiscovered IFN-like protein; at a minimum, *N. vectensis* encodes several IRF-like genes (SI Appendix, Fig. S5). One attractive hypothesis is that these IRFs are important for the antiviral response of *N. vectensis*; however, we were unable to see any impact of single knockdown experiments on the induction of genes by 23′-cGAMP, though this may be explained by redundancy or technical limitations of our knockdown approach. Nevertheless, an important conclusion of our work is that induction of a broad transcriptional program is an ancestral function of 23′-cGAMP signaling, similar to what has been seen in *Drosophila* (31) and choanoflagellates (53). This ancestral transcriptional response complements an additional autophagy response to 23′-cGAMP that was previously reported to be induced by nvSTING in mammalian cells (17) and has now also been shown to be induced by 23′-cGAMP and STING in choanoflagellates (53).

We found that in addition to an antiviral response, *N. vectensis* responds to 23′-cGAMP by inducing a variety of antibacterial genes, including lysozyme, Dae4, perforin-2-like, LBP, and GBP5. With the exception of GBP5, which have dual antibacterial and antiviral activity, these antibacterial genes are not induced by 23′-cGAMP in vertebrates. Thus, the antibacterial response appears to be a unique feature of 23′-cGAMP signaling in *N. vectensis*, and it will be interesting to see whether this proves true in other invertebrates or in additional cell types or contexts in vertebrates. Indeed, a recent study found that during oral *Listeria monocytogenes* infection of mice, a STING-dependent and IFN-independent response helps clear bacteria. Several of the antibacterial genes are also induced by poly(I:C) in *Nematostella* (45), and we found that at least one putative antiviral gene was also induced during *P. aeruginosa* infection, perhaps indicating a broader antipathogen response to PAMPs in *N. vectensis*. Interestingly, we found that nvNF-κB was specifically required for the induction of many of the antibacterial genes. This suggests that nvNF-κB activation downstream of 23′-cGAMP signaling may have been present in the most recent common ancestor of cnidarians and mammals and could have played a role in the antiviral response of *N. vectensis*. Consistent with this speculation, *Drosophila* STING also appears to activate NF-κB (30, 31, 34).

To further establish that 23′-cGAMP induces proteins with antibacterial activity, we functionally characterized one 23′-cGAMP–induced, nvNF-κB–dependent protein, nvDae4. We found that nvDae4 is a PG-cleaving enzyme with direct bactericidal activity against gram-positive bacteria. Many of the 23′-cGAMP–induced nvNF-κB–dependent genes are not recognizable homologs of proteins of known function; thus, they represent good candidates for the discovery of novel antibacterial genes in *N. vectensis*.

Using shRNAs to knockdown nvSTING failed to confirm an essential role for nvSTING in the response to 23′-cGAMP. However, our previous biochemical and structural studies showed nvSTING binds 23′-cGAMP with high affinity (Kd < 1nM) and in a very similar manner as vertebrate STING (37). STING is essential for the response to 23′-cGAMP in diverse organisms including vertebrates, choanoflagellates (53), and insects (31). In addition, nvSTING is highly induced by 23′-cGAMP. So, despite our negative results, we favor the idea that nvSTING is at least partially responsible for the response of *N. vectensis* to 23′-cGAMP. It is possible that *N. vectensis* encodes a redundant 23′-cGAMP sensor, but such a sensor would have had to have evolved specifically in cnidarians or been lost independently from choanoflagellates, insects, and vertebrates. It is likely that technical limitations of performing shRNA knockdowns in *N. vectensis* accounts for our inability to observe a role for nvSTING in the response to 23′-cGAMP, though we cannot exclude the possibility that *N. vectensis* utilizes a distinct 23′-cGAMP-sensing pathway.

If, indeed, 23′-cGAMP is signaling via nvSTING, this presents several mechanistic questions. First, in mammals, all known transcriptional responses downstream of STING, including those requiring NF-κB activation, require the CTT (38, 39), leading to the question of how invertebrate STING proteins, which lack a discrete CTT, can activate this pathway. Also, nvNF-κB knockdown did not impact the vast majority of 23′-cGAMP–induced genes, which may imply the existence of other signaling pathways downstream of nvSTING. How these unidentified pathways become activated is another interesting question and one that could also shed light on mammalian STING signaling. Finally, mammalian STING can also be activated by direct binding to bacterial 3′3′-linked CDNs (54), and nvSTING also binds to these ligands, albeit with lower affinity (37). We found that treatment of *N. vectensis* with these ligands also led to induction of many of the same genes, likely through the same pathway. This perhaps indicates a role for the nvSTING pathway in bacterial sensing, though our preliminary attempts to observe an impact of 23′-cGAMP–induced gene expression on bacterial colonization of *N. vectensis* were unsuccessful. Further development of a bacterial infection model for *N. vectensis* will be required to study the antibacterial response of this organism in vivo.

A crucial remaining question is what activates nv-cGAS to produce 23′-cGAMP. Double-stranded DNA did not seem to activate this protein in vitro (37), but this could be due to the absence of cofactors. This protein is also constitutively active when transfected into mammalian cells, but this could be due to overexpression. Unlike human cGAS, nv-cGAS does not have any clear DNA-binding domains, although this does not necessarily exclude DNA as a possible ligand. The *Vibrio* cGAS-like enzyme DncV is regulated by folate-like molecules (55), so there is a diverse range of possible nv-cGAS activators. Understanding the role of CDN sensing pathways in diverse organisms can shed light on the mechanisms of evolution of viral and bacterial sensing and on unique ways divergent organisms have evolved to respond to pathogens.

**Methods**

*N. vectensis* Culture and Spawning. *N. vectensis* adults were a gift from Mark Q. Martindale (University of Florida, St. Augustine, FL) and were cultured and spawned as previously described (56). Briefly, animals were kept in 1/3× sea water (12 ppt salinity) in the dark at 17 °C and fed freshly hatched Artemia (Carolina Biological Supply Company) weekly. Spawning was induced every 2 wk by placing animals at 23 °C under bright light for 8 h, followed by 2 h in the dark, and then finally moved to the light, where they were monitored for spawning. Egg masses were de-jellied in 4% L-cysteine (pH 7 to 7.4) in 1/3× sea water for 10 to 15 min and washed three times with 1/3× sea water. Water containing sperm was added to the washed eggs, and these were either used immediately for microinjection or allowed to develop at room temperature.

**CDN Treatment.** For the RNA-Seq experiment on polyps (Fig. 1 and SI Appendix, Fig. S1), ~4-wk-old polyps were treated in duplicate in a bath of 500 μM cdi-AMP, cdi-GMP, 23′-cGAMP, or 3′3′-cGAMP (all InvivoGen) in 1/3× sea water for 24 h. For remaining cGAMP treatment experiments, 50 to 100 48-h-old embryos were treated with 100 μM 23′-cGAMP (InvivoGen) in 1/3× sea water for 4 h.

**RNA-Seq.** For the initial CDN treatment experiment using polyps, total RNA was extracted using Qiagen RNeasy Mini kits according to the manufacturer’s protocol. Libraries were prepared by the Functional Genomics Laboratory at the University of California (UC), Berkeley using WaverGen PrepX library prep kits with oligo DT beads for messenger RNA (mRNA) enrichment according to the manufacturer’s protocol, and 50-nt single-end sequencing was carried out on the HiSeq4000 (Illumina) by the Vincent J. Coates Genomics Sequencing Laboratory. For all other RNA-Seq experiments on 48-h embryos, RNA was extracted using TRIzol (Thermo Fisher Scientific) according to the
expression levels were normalized to actin and calculated using the 2−ΔΔCt method. Relative cycling conditions: 50°C (Thermo Fisher Scientific) was treated with RQ1 RNase-free DNase (Promega) and reverse transcribed

Embryos and polyps were lysed in TRIzol (Invitrogen), and RNA was extracted using the TRIzol Reagent (Invitrogen). RQ1 RNase-free DNase (Promega) and reverse transcription were performed using q2PCR. RT-PCR was performed using gosqRT with GO annotations from https://fipsarchive.com/articles/dataset/Nematostella_vestimentata_transcriptome_and_gene_models_v2_0807696. The EnhancedVolcano package (https://github.com/kevinbligh/EnhancedVolcano) was used to generate volcano plots. Heatmaps are based on regularized log-transformed normalized counts, and Z-scores are scaled by row. All RNA-Seq results can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2. Raw sequencing reads and normalized gene counts can be found in Dataset S1. Raw sequencing reads and normalized gene counts can be found in Dataset S2.
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