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The cyclic dinucleotide 2'3'-cGAMP induces a broad anti-bacterial and anti-viral response in the sea anemone *Nematostella vectensis* — [Source link](#)

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30 **Abstract**

31 In mammals, cyclic dinucleotides (CDNs) bind and activate STING to initiate an anti-
32 viral type I interferon response. CDNs and STING originated in bacteria and are present
33 in most animals. By contrast, interferons are believed to have emerged in vertebrates;
34 thus, the function of CDN signaling in invertebrates is unclear. Here, we use a CDN,
35 2'3'-cGAMP, to activate immune responses in a model cnidarian invertebrate, the starlet
36 sea anemone *Nematostella vectensis*. Using RNA-Seq, we found that 2'3'-cGAMP
37 induces robust transcription of both anti-viral and anti-bacterial genes in *N. vectensis*.
38 Many of the anti-viral genes induced by 2'3'-cGAMP are homologs of vertebrate
39 interferon stimulated genes, implying that the interferon response predates the evolution
40 of interferons. Knockdown experiments identified a role for NF- κ B in specifically
41 inducing anti-bacterial genes downstream of 2'3'-cGAMP. Some of these putative anti-
42 bacterial genes were also found to be induced during *Pseudomonas aeruginosa*
43 infection. We characterized the protein product of one of the putative anti-bacterial
44 genes, the *N. vectensis* homolog of Dae4, and found that it has conserved anti-bacterial
45 activity. This work suggests that a broad anti-bacterial and anti-viral transcriptional
46 response is an evolutionarily ancestral output of 2'3'-cGAMP signaling in animals.
47

48 **Significance statement**

49 Cyclic dinucleotides are signaling molecules that originated in bacteria and were
50 subsequently acquired and co-opted by animals for immune signaling. The major cyclic
51 dinucleotide signaling pathway in mammals results in the production of anti-viral
52 molecules called interferons. Invertebrates such as sea anemones lack interferons, and
53 thus it was unclear whether cyclic dinucleotide signaling would play a role in immunity in
54 these animals. Here we report that in the anemone *Nematostella vectensis*, cyclic
55 dinucleotides activate both anti-viral and anti-bacterial immune responses, and do so
56 through a conserved pathway. These results provide insights into the evolutionary
57 origins of innate immunity, and suggest a broader ancestral role for cyclic dinucleotide
58 signaling that evolved toward more specialized anti-viral functions in mammals.

59 Introduction

60 The innate immune system is an evolutionarily ancient system that detects
61 pathogens and initiates their elimination. In mammals, the cGAS-STING pathway is
62 critical for sensing and responding to intracellular DNA, which is particularly important
63 for innate responses to DNA viruses (1, 2). The sensor protein in this pathway, cyclic-
64 GMP-AMP synthase (cGAS), is an enzyme that binds directly to cytosolic DNA and
65 produces 2'3'-cGAMP, a cyclic dinucleotide (CDN) second messenger that binds and
66 activates STING (3-8). Active STING uses its C-terminal tail (CTT) to recruit TBK1,
67 which then phosphorylates and activates the transcription factor IRF3 to induce the
68 expression of type I interferons (IFNs) (9-12). Type I IFNs are secreted cytokines that
69 signal via JAK-STAT signaling to induce transcription of hundreds of anti-viral genes
70 known as interferon-stimulated genes (ISGs) (13, 14). STING also activates NF- κ B,
71 MAP kinase (15), STAT6 (16), and autophagy-like pathways (17-20), as well as
72 senescence (21) and cell death (22-26), although the mechanism of activation of these
73 pathways, and their importance during infection, are less well understood.

74 Type I IFNs are thought to be a relatively recent evolutionary innovation, with
75 identifiable interferon genes found only in vertebrates (27). In contrast, STING and
76 cGAS are conserved in the genomes of most animals and some unicellular
77 choanoflagellates. Remarkably, CDN to STING signaling seems to have originated in
78 bacteria, where it may be important in bacteriophage defense (28, 29). Studies on the
79 function of STING in animals that lack type I IFN have been mostly limited to insects,
80 where STING seems to be protective during viral (30-33), bacterial (34), and
81 microsporidial (35) infection. In insects, STING may promote defense through activation
82 of autophagy (32, 35) and/or induction of NF- κ B-dependent defense genes (30, 31, 34).
83 Most ISGs are lacking from insect genomes and are not induced by CDN-STING
84 signaling (31). In addition, the biochemical mechanisms of STING activation and
85 signaling in insects remain poorly understood.

86 Biochemically, perhaps the best-characterized invertebrate STING is that of the
87 starlet sea anemone, *Nematostella vectensis*, a member of one of the oldest animal
88 phyla (Cnidaria). *N. vectensis* encodes a surprisingly complex genome that harbors
89 many gene families found in vertebrates but absent in other invertebrates such as
90 *Drosophila* (36). *N. vectensis* STING (nvSTING) and human STING adopt remarkably
91 similar conformations when bound to 2'3'-cGAMP, and nvSTING binds to this ligand
92 with high affinity ($K_d < 1$ nM) (37). The *N. vectensis* genome also encodes a cGAS
93 enzyme that produces 2'3'-cGAMP in mammalian cell culture (17). In vertebrates,
94 STING requires its extended CTT to initiate transcriptional responses (38, 39); however,
95 nvSTING lacks an extended CTT and thus its signaling mechanism and potential for
96 inducing transcriptional responses is unclear. Based on experiments with nvSTING in
97 mammalian cell lines, CTT-independent induction of autophagy has been proposed as
98 the 'ancestral' function of STING (17), but the endogenous function of STING in *N.*
99 *vectensis* has never been described.

100 Despite the genomic identification of many predicted innate immune genes (40, 41),
101 few have been functionally characterized in *N. vectensis*. The sole *N. vectensis* Toll-like
102 receptor (TLR) is reported to bind flagellin and activate NF- κ B in human cell lines, and is
103 expressed in cnidocytes, the stinging cells that define cnidarians (42). *N. vectensis* NF-
104 κ B (nvNF- κ B) binds to conserved κ B sites, is inhibited by *N. vectensis* I κ B (43), and
105 seems to be required for the development of cnidocytes (44). However, no activators of
106 endogenous nvNF- κ B have yet been identified. Recent work probing the putative anti-
107 viral immune response in *N. vectensis* found that double-stranded RNA (dsRNA)
108 injection into *N. vectensis* embryos leads to transcriptional induction of genes involved

109 in the RNAi pathway as well as genes with homology to ISGs (45). This response is
110 partially dependent on a RIG-I-like receptor, indicating deep conservation of anti-viral
111 immunity (45). However, no anti-viral or anti-bacterial effectors from *N. vectensis* have
112 been functionally tested.

113 Here, we characterize the response of *N. vectensis* to 2'3'-cGAMP stimulation.
114 Similar to the response of vertebrates to 2'3'-cGAMP, we find robust transcriptional
115 induction of putative anti-viral genes with homology to vertebrate ISGs. In addition, we
116 observed induction of numerous anti-bacterial genes that are not induced during the
117 vertebrate response to 2'3'-cGAMP. Although we were unable to show that the
118 response to 2'3'-cGAMP is nvSTING dependent, we did find a selective requirement for
119 nvNF- κ B in the induction of some of the anti-bacterial genes. Many of these genes are
120 also induced during *Pseudomonas aeruginosa* infection, suggesting a functional role in
121 anti-bacterial immunity. We selected and characterized the anti-bacterial activity of one
122 2'3'-cGAMP-induced *Nematostella* gene product, domesticated amidase effector
123 (nvDae4), a peptidoglycan cleaving enzyme that we found can kill Gram-positive
124 bacteria. This work demonstrates an evolutionarily ancient role for 2'3'-cGAMP in the
125 transcriptional induction of both anti-viral and anti-bacterial immunity.

126

127 **Results**

128 *Transcriptional response to 2'3'-cGAMP in Nematostella vectensis*

129 To assess the *in vivo* role of 2'3'-cGAMP signaling in *Nematostella vectensis*, we
130 treated 2-week-old polyps with 2'3'-cGAMP for 24 hours and performed RNA-Seq (Fig.
131 1A, Fig. S1). Thousands of genes were induced by 2'3'-cGAMP, many of which are
132 homologs of genes known to function in mammalian immunity. Despite the lack of
133 associated gene ontology (GO) terms for many of the differentially regulated genes,
134 unbiased GO term analysis revealed significant enrichment of immune-related terms
135 (Fig. 1B). We also treated animals with 3'3'-linked cyclic dinucleotides, which are
136 thought to be produced exclusively by bacteria, and which also bind to nvSTING *in vitro*,
137 albeit at lower affinity (37). Both 3'3'-cGAMP and cyclic-di-AMP treatment also induced
138 a smaller number of genes, although all of these genes were induced more strongly by
139 2'3'-cGAMP (Fig. S1). Interestingly, cyclic-di-GMP treatment led to almost no
140 transcriptional induction, despite having relatively high affinity for nvSTING *in vitro*. This
141 discrepancy may be due to differences in cell permeability among different CDNs, as
142 the ligands were added extracellularly. In order to be able to perform subsequent
143 genetic experiments, we confirmed that the immune gene induction downstream of by
144 2'3'-cGAMP also occurred in embryos (which are amenable to microinjection of
145 shRNAs). Quantitative reverse transcription PCR (qRT-PCR) on 48-hour-embryos
146 treated for 4 hours with a lower dose of 2'3'-cGAMP revealed that many immune genes
147 were also induced at this early developmental stage after a much shorter treatment (Fig.
148 1C).

149 Several interesting classes of genes were found to be upregulated in response to
150 2'3'-cGAMP. For example, several genes involved in the RNAi pathway were induced,
151 including homologs of Argonaute (AGO2), Dicer, and RNA-dependent RNA polymerase
152 (Rdrp1). In addition, many genes that are considered ISGs in mammals were also
153 induced in *N. vectensis*, including Viperin, RNase L, 2'-5'-oligoadenylate synthase
154 (OAS), interferon regulatory factors (IRFs), guanylate-binding proteins (GBPs), and the
155 putative pattern recognition receptors RIG-I-like receptor a (RLRa) and RLRb. These
156 results suggest a conserved role for 2'3'-cGAMP signaling in anti-viral immunity and ISG
157 induction, despite an apparent lack of conservation of type I interferons in *N. vectensis*.
158 Interestingly, we also found that many putative anti-bacterial genes were upregulated in

159 response to 2'3'-cGAMP, including homologs of LPS-binding protein (LBP), lysozyme,
160 perforin-2, Dae4, and mucins. These results indicate that 2'3'-cGAMP stimulation leads
161 to a broad immune response in *N. vectensis*.

162 To determine whether 2'3'-cGAMP signaled via nvSTING to induce these genes,
163 we injected shRNAs targeting nvSTING into 1-cell embryos and treated with 2'3'-
164 cGAMP 48 hours later. We extracted RNA and performed RNA-Seq on these samples,
165 and surprisingly, while nvSTING transcripts were reduced by ~50%, there was no
166 significant impact on 2'3'-cGAMP-induced gene expression (Fig. S2A, S2B). These
167 negative results were recapitulated in numerous independent qRT-PCR and Nanostring
168 experiments using 9 different shRNAs (3 shown in Fig. S2C). There are several
169 possible explanations for the failure to observe a requirement for nvSTING in 2'3'-
170 cGAMP signaling : (1) a 2-fold reduction in STING transcript levels may not result in a
171 reduction in STING protein levels if the protein is very stable; (2) even if STING protein
172 levels are reduced 2-fold, the reduction may not affect STING signaling due to threshold
173 effects; or (3) nvSTING may not be required for signaling downstream of 2'3'-cGAMP
174 due the presence of a redundant 2'3'-cGAMP sensor in *N. vectensis*. We generated an
175 anti-nvSTING antibody to validate knockdown efficiency at the protein level, but this
176 reagent did not appear to specifically detect nvSTING in anemone lysates. We also
177 tested whether an nvSTING translation-blocking morpholino could inhibit induction of
178 genes in response to 2'3'-cGAMP, but this also had no effect (Fig. S2D). Lastly, we
179 made multiple attempts to generate nvSTING mutant animals using CRISPR, using
180 multiple different guide RNAs, but the inefficiency of CRISPR in this organism and
181 issues with mosaicism prevented the generation of nvSTING null animals. We
182 previously solved the crystal structure of nvSTING bound to 2'3'-cGAMP and showed
183 that binding occurs with high affinity ($K_d < 1\text{nM}$) and in a similar mode as compared to
184 vertebrate STING (37). In addition, we found that when expressed in mammalian cells,
185 nvSTING forms puncta only in the presence of 2'3'-cGAMP, indicating some functional
186 change induced by this ligand (Fig. S2E). Thus, we hypothesize that 2'3'-cGAMP
187 signals via nvSTING, but technical issues and possible redundancy with additional
188 sensors prevent formal experimental evidence for this hypothesis.

189
190 *The N. vectensis NF- κ B homolog plays a role in the 2'3'-cGAMP response*

191 We next tested the role of conserved transcription factors that are known to
192 function downstream of STING mammals in the *N. vectensis* response to 2'3'-cGAMP.
193 Interestingly, many of these transcription factors are themselves transcriptionally
194 induced by 2'3'-cGAMP in *N. vectensis* (Fig. 1A). In mammals, the transcription factors
195 IRF3 and IRF7 induce type I IFN downstream of STING activation. While the specific
196 function of these IRFs in interferon induction are thought to have arisen in vertebrates,
197 other IRF family members, with conserved DNA binding residues, are present in *N.*
198 *vectensis* (Fig. S3). We microinjected 1-cell embryos with short hairpin RNAs (shRNAs)
199 targeting each of the 5 nvIRFs or a GFP control, treated with 2'3'-cGAMP, and
200 assessed gene expression by qRT-PCR and/or Nanostring. Knockdown of IRF
201 transcripts by 40-60% did not measurably impact gene induction by 2'3'-cGAMP (Fig.
202 S4). We similarly tested the role of the single *N. vectensis* STAT gene, as mammalian
203 STATs both induce anti-viral genes downstream of Type I IFN signaling, and may even
204 be directly activated by STING (16). Similar to the nvIRFs, we did not observe a
205 significant loss of gene induction by 2'3'-cGAMP in nvSTAT knockdown embryos by
206 both RNA-Seq and Nanostring (Fig. S4). There are several explanations for these
207 findings: (1) sufficient IRF or STAT protein may remain in knockdown animals to
208 transduce the signal, either due to low efficiency of the knockdowns, or to protein

209 stability; (2) the IRFs may act redundantly with each other, and therefore no effect will
210 be seen in single knockdown experiments; or (3) nvIRFs and nvSTAT may not play a
211 role in the response to 2'3'-cGAMP.

212 NF- κ B is also known to act downstream of mammalian STING, and appears to
213 be functionally conserved in *N. vectensis* (43). We found that NF- κ B signaling
214 components are transcriptionally induced by 2'3'-cGAMP (Fig. 1A). To test the role of
215 nvNF- κ B in the 2'3'-cGAMP response, we microinjected embryos with shRNAs targeting
216 nvNF- κ B, treated with 2'3'-cGAMP, and performed RNA-Seq (Fig. 2A). 241 genes were
217 transcribed at significantly lower levels in the nvNF- κ B knockdown embryos, and of
218 these, 98 were genes induced by 2'3'-cGAMP. Of these genes, 40 are uncharacterized,
219 and no GO terms were significantly enriched (data not shown). Of the induced genes
220 that were annotated in NCBI, we noticed many were homologs of anti-bacterial proteins,
221 including homologs of perforin-2/Mpeg-1, LPS-binding protein (LBP), linear gramicidin
222 synthase, and mucins. We confirmed that 2'3'-cGAMP-mediated induction of these
223 putative anti-bacterial genes was NF- κ B-dependent by performing qRT-PCR and
224 Nanostring (Fig. 2B; Fig. S5A). Of note, the induction of nvLysozyme was not nvNF- κ B
225 dependent (both by RNA-Seq and qRT-PCR; Fig. S5B), indicating either the existence
226 of another pathway for anti-bacterial gene induction, or that our knockdown experiment
227 was not able to affect expression of all nvNF- κ B dependent genes. In addition, all of the
228 putative anti-viral genes we examined appeared to be induced independent of nvNF- κ B
229 (Fig. S5).

230 We performed BLAST searches of unannotated 2'3'-cGAMP-induced, nvNF- κ B-
231 dependent genes and identified several other genes with predicted anti-bacterial
232 activity, including two homologs of bacterial *tae4* genes, and a putative guanylate
233 binding protein (GBP) (*N. vectensis* LOC5515806, hereafter nvGBP-806). The *Tae4*
234 homologs had been previously identified and will be referred to as nvDae4 proteins
235 (discussed further below; (46)). To confirm the identity of nvGBP-806 as a true GBP
236 homolog, we performed phylogenetic analysis. We identified four conserved *N.*
237 *vectensis* proteins harboring an N-terminal GBP GTPase domain with conserved GBP-
238 specific motifs, including nvGBP-806 (Fig. S6). All of the nvGBP homologs cluster with
239 vertebrate IFN-inducible GBPs and are themselves induced by 2'3'-cGAMP. Finally, we
240 identified several unannotated nvNF- κ B dependent, 2'3'-cGAMP-induced genes that
241 appeared to be cnidarian-specific with no identifiable homologs in other animal phyla
242 (Table S1).

243 To test directly whether nvNF- κ B is activated in *N. vectensis* upon 2'3'-cGAMP
244 treatment, we treated polyps with cGAMP and performed immunostaining for nvNF- κ B
245 (Fig. 2C). Inactive NF- κ B is localized to the cytosol, and we observed sparse, cytosolic
246 staining of ectodermal cells in untreated animals, as has been previously reported (43).
247 In contrast, in 2'3'-cGAMP treated animals, we found many more nvNF- κ B-positive
248 cells, and in almost all of these, nvNF- κ B was found in the nucleus. We performed
249 automated quantification of nuclear nvNF- κ B staining, and found that ~3-20% of nuclei
250 captured in our images were positive for nvNF- κ B (Fig. 2D). In sum, 2'3'-cGAMP leads
251 to nvNF- κ B nuclear localization, and nvNF- κ B appears to be required for expression of
252 many putative anti-bacterial, but not anti-viral, genes. Our results demonstrate the first
253 NF- κ B agonist in *N. vectensis*, and indicate a conserved immune function for NF- κ B in
254 this organism.

255
256 *Gene induction during Pseudomonas aeruginosa challenge*

257 In order to test whether the putative anti-bacterial, NF- κ B-dependent genes are
258 induced during bacterial infection, we infected *N. vectensis* with *Pseudomonas*

259 *aeruginosa*, a pathogenic Gram-negative bacterium. *P. aeruginosa* can infect a range of
260 hosts, including plants, mammals, and hydra (47, 48), though infections of *N. vectensis*
261 have not previously been reported. Infection of *N. vectensis* polyps with the *P.*
262 *aeruginosa* strain PA14 led to polyp death in a dose and temperature dependent
263 manner (Fig. 3A). 48 hours after infection, we isolated RNA from infected polyps and
264 assayed gene expression. Interestingly, nvSTING expression was induced during PA14
265 infection (Fig. 3B), and many of the putative anti-bacterial genes we identified as 2'3'-
266 cGAMP-induced were also induced during infection (Fig. 3C), although this expression
267 was not sufficient to protect from death. In addition, some putative anti-viral genes were
268 also induced in some animals (Fig. S5B), perhaps reflecting a broader immune
269 response in *Nematostella*. Importantly, the PA14 genome is not known to encode any
270 proteins that produce CDNs other than c-di-GMP. Since c-di-GMP was not sufficient to
271 robustly activate gene expression in *N. vectensis*, we believe that it is likely that the
272 response to PA14 is independent of bacterial CDNs, although we cannot rule out an
273 effect from PA14-produced c-di-GMP. In addition, we have no reason to believe that
274 PA14 in activating these genes via nv-cGAS, as we do not know the activator of this
275 enzyme. Nevertheless, taken together, these results indicate that the putative anti-
276 bacterial genes we identified as induced by 2'3'-cGAMP are also induced after bacterial
277 challenge.

278
279 *nvDae4 is a peptidoglycan-cleaving enzyme with anti-bacterial activity*

280 We decided to investigate directly whether any of the genes induced by both 2'3'-
281 cGAMP and bacterial infection are in fact anti-bacterial. Type VI secretion amidase
282 effector (Tae) proteins are bacterial enzymes that are injected into neighboring cells to
283 cleave peptidoglycan, an essential component of bacterial cell walls, leading to rapid
284 cell death (49). While the *tae* genes originated in bacteria, they have been horizontally
285 acquired multiple times in evolution by eukaryotes, and at least one of these so-called
286 “domesticated amidase effectors” (Daes) also has bactericidal activity (46, 50). The *N.*
287 *vectensis* genome has two *tae4* homologs, both of which were upregulated by 2'3'-
288 cGAMP in an nvNF- κ B-dependent manner. However, only one of the *N. vectensis* Dae
289 proteins is predicted to encode a conserved catalytic cysteine (46) required for
290 peptidoglycan hydrolysis. Therefore, we focused our efforts on this homolog, which we
291 call nvDae4 (GI: 5507694). We first tested whether nvDae4 has conserved bactericidal
292 properties by expressing nvDae4 in *E. coli* either with or without a periplasm-targeting
293 signal sequence and measuring bacterial growth (assessed by OD₆₀₀) over time (Fig.
294 4A). *E. coli* are Gram-negative bacteria and thus have peptidoglycan
295 compartmentalized within the periplasmic space. Consistent with the predicted
296 peptidoglycan-cleaving function of nvDae4, only periplasmic wild-type (WT) but not
297 catalytic mutant (C63A) nvDae4 expression led to bacterial lysis. In order to test directly
298 whether nvDae4 cleaves peptidoglycan, we produced recombinant protein in insect
299 cells. Since nvDae4 encodes a secretion signal, recombinant nvDae4 was secreted by
300 the insect cells and purified from the cell supernatant. Purified nvDae4 protein was
301 incubated with purified peptidoglycan from either *E. coli* or *Staphylococcus epidermis*.
302 Analysis by high performance liquid chromatography (HPLC) showed that nvDae4
303 cleaves both Gram-negative (Fig. 4B) and Gram-positive (Fig. S7) derived
304 peptidoglycan. Finally, we wondered whether nvDae4 could directly kill Gram-positive
305 bacteria, as these bacteria contain a peptidoglycan cell wall that is not protected by an
306 outer membrane and is therefore accessible to extracellular factors. We treated *B.*
307 *subtilis* with recombinant nvDae4 and found that bacteria treated with WT but not C63A
308 nvDae4 protein were killed (Fig. 4C) in a dose-dependent manner (Fig. 4D). Overall

309 these results show that the 2'3'-cGAMP-induced protein nvDae4 is a peptidoglycan-
310 cleaving enzyme with the capacity to kill bacteria.

311

312 Discussion

313 In this study, we identified hundreds of *N. vectensis* genes that are induced by
314 the STING ligand 2'3'-cGAMP. Despite over 600 million years of divergence and the
315 absence of interferons, *N. vectensis* responds to 2'3'-cGAMP similarly to mammals by
316 inducing a variety of anti-viral genes. Similarly, Lewandowska et al. (45) recently
317 reported that *N. vectensis* responds to the synthetic double-stranded RNA poly(I:C), a
318 viral mimic and pathogen-associated molecular pattern (PAMP). In *N. vectensis*,
319 poly(I:C) induced both RNAi pathway components and genes traditionally thought of as
320 vertebrate ISGs. Our combined findings indicate that the pathways linking PAMP
321 detection to ISG expression existed prior to the vertebrate innovation of type I IFNs.
322 Interestingly, some invertebrate species have protein-based anti-viral signaling
323 pathways that perform similar functions to type I IFNs in vertebrates. For example,
324 mosquito cells secrete the peptide Vago upon viral infection, which signals through the
325 JAK-STAT pathway to activate anti-viral immunity (51). Additionally, the oyster
326 *Crassostrea gigas* is thought to have an IFN-like system, but no secreted proteins have
327 yet been identified in this organism (52). *N. vectensis* may also encode an undiscovered
328 IFN-like protein; at a minimum, *N. vectensis* encodes several IRF-like genes (Fig. S3).
329 One attractive hypothesis is that these IRFs are important for the anti-viral response of
330 *N. vectensis*; however, we were unable to see any impact of single knockdown
331 experiments on the induction of genes by 2'3'-cGAMP, though this may be explained by
332 redundancy or technical limitations of our knockdown approach. Nevertheless, an
333 important conclusion of our work is that induction of a broad transcriptional program is
334 an ancestral function of 2'3'-cGAMP signaling, similar to what has been seen in
335 *Drosophila* (31) and choanoflagellates (53). This ancestral transcriptional response
336 complements an additional autophagy response to 2'3'-cGAMP that was previously
337 reported to be induced by nvSTING in mammalian cells (17), and has now also been
338 shown to be induced by 2'3'-cGAMP and STING in choanoflagellates (53).

339 We found that in addition to an anti-viral response, *N. vectensis* responds to 2'3'-
340 cGAMP by inducing a variety of anti-bacterial genes, including lysozyme, Dae4,
341 perforin-2-like, LPB, and GBPs. With the exception of GBPs, which have dual anti-viral
342 and anti-bacterial activity, these anti-bacterial genes are not induced by 2'3'-cGAMP in
343 vertebrates; thus, the anti-bacterial response appears to be a unique feature of 2'3'-
344 cGAMP signaling in *N. vectensis*, and it will be interesting to see whether this proves
345 true in other invertebrates, or in additional cell types or contexts in vertebrates. Indeed,
346 a recent study found that during oral *L. monocytogenes* infection of mice, a STING-
347 dependent and IFN-independent response helps clear bacteria. Several of the anti-
348 bacterial genes are also induced by poly(I:C) in *Nematostella* (45), and we found that at
349 least one putative anti-viral gene was also induced during *P. aeruginosa* infection,
350 perhaps indicating a broader anti-pathogen response to PAMPs in *N. vectensis*.
351 Interestingly, we found that nvNF- κ B was specifically required for the induction of many
352 of the anti-bacterial genes. This suggests that nvNF- κ B activation downstream of 2'3'-
353 cGAMP signaling may have been present in the most recent common ancestor of
354 cnidarian and mammals, and confirms a role for nvNF- κ B in *N. vectensis* immunity.
355 Consistent with this speculation, *Drosophila* STING also appears to activate NF- κ B (30,
356 31, 34).

357 To further establish that 2'3'-cGAMP induces proteins with anti-bacterial activity, we
358 functionally characterized one 2'3'-cGAMP-induced, nvNF- κ B-dependent protein,

359 nvDae4. We found that nvDae4 is a peptidoglycan-cleaving enzyme with direct
360 bactericidal activity against Gram-positive bacteria. Many of the 2'3'-cGAMP-induced
361 NF- κ B dependent genes are not recognizable homologs of proteins of known function;
362 thus, they represent good candidates for the discovery of novel anti-bacterial genes in
363 *N. vectensis*.

364 Using shRNAs to knockdown nvSTING failed to confirm an essential role for
365 nvSTING in the response to 2'3'-cGAMP. However, our previous biochemical and
366 structural studies showed nvSTING binds 2'3'-cGAMP with high affinity ($K_d < 1$ nM) and
367 in a very similar manner as vertebrate STING (37). STING is essential for the response
368 to 2'3'-cGAMP in diverse organisms, including vertebrates, choanoflagellates (53), and
369 insects (31). In addition, nvSTING is highly induced by 2'3'-cGAMP. So despite our
370 negative results, we favor the idea that nvSTING is at least partially responsible for the
371 response of *N. vectensis* to 2'3'-cGAMP. It is possible that *N. vectensis* encodes a
372 redundant 2'3'-cGAMP sensor, but such a sensor would have had to evolve specifically
373 in Cnidarians, or be lost independently from choanoflagellates, insects and vertebrates.
374 It is likely that technical limitations of performing shRNA knockdowns in *N. vectensis*
375 accounts for our inability to observe a role for nvSTING in the response to 2'3'-cGAMP,
376 though we cannot exclude the possibility that *N. vectensis* utilizes a distinct 2'3'-
377 cGAMP-sensing pathway.

378 If indeed 2'3'-cGAMP is signaling via nvSTING, this presents several mechanistic
379 questions. First, in mammals, all known transcriptional responses downstream of
380 STING, including those requiring NF- κ B activation, require the CTT (38, 39), leading to
381 the question of how invertebrate STING proteins, which lack a discrete CTT, can
382 activate this pathway. Also, nvNF- κ B knockdown did not impact the vast majority of 2'3'-
383 cGAMP-induced genes, which may imply the existence of other signaling pathways
384 downstream of nvSTING. How these unidentified pathways become activated is another
385 interesting question and one that could also shed light on mammalian STING signaling.
386 Finally, mammalian STING can also be activated by direct binding to bacterial 3'3'-
387 linked CDNs (54), and nvSTING also binds to these ligands, albeit with lower affinity
388 (37). We found that treatment of *N. vectensis* with these ligands also led to induction of
389 many of the same genes, likely through the same pathway. This perhaps indicates a
390 role for the nvSTING pathway in bacterial sensing, though our preliminary attempts to
391 observe an impact of 2'3'-cGAMP-induced gene expression on bacterial colonization of
392 *N. vectensis* were unsuccessful. Further development of a bacterial infection model for
393 *N. vectensis* will be required to study the anti-bacterial response of this organism *in*
394 *vivo*.

395 A crucial remaining question is what activates nvcGAS to produce 2'3'-cGAMP.
396 Double-stranded DNA did not seem to activate this protein *in vitro* (37), but this could be
397 due to the absence of cofactors. This protein is also constitutively active when
398 transfected into mammalian cells, but this could be due to overexpression.
399 Unlike human cGAS, nvcGAS does not have any clear DNA-binding domains, although
400 this does not necessarily exclude DNA as a possible ligand. The *Vibrio* cGAS-like
401 enzyme DncV is regulated by folate-like molecules (55), so there is a diverse range of
402 possible nvcGAS activators. Understanding the role of CDN sensing pathways in
403 diverse organisms can shed light on the mechanisms of evolution of viral and bacterial
404 sensing, and on unique ways divergent organisms have evolved to respond to
405 pathogens.

406

407 **Methods**

408

409 ***Nematostella vectensis* culture and spawning**

410 *N. vectensis* adults were a gift from Mark Q. Martindale (University of Florida)
411 and were cultured and spawned as previously described (56). Briefly, animals were kept
412 in 1/3x seawater (12ppt salinity) in the dark at 17°C and fed freshly hatched *Artemia*
413 (Carolina Biological Supply Company) weekly. Spawning was induced every two weeks
414 by placing animals at 23°C under bright light for 8 hours, followed by 2 hours in the
415 dark, and then finally moved to the light where they were monitored for spawning. Egg
416 masses were de-jellied in 4% L-Cysteine (pH 7-7.4) in 1/3x sea water for 10-15 minutes
417 and washed 3 times with 1/3x sea water. Water containing sperm was added to the
418 washed eggs and these were either used immediately for microinjection or allowed to
419 develop at room temperature.

420

421 **CDN treatment**

422 For the RNA sequencing experiment on polyps (Fig. 1 and Fig. S1), ~4 week old
423 polyps were treated in duplicate in a bath of 500µM c-di-AMP, c-di-GMP, 2'3'-cGAMP,
424 or 3'3'-cGAMP (all InvivoGen) in 1/3x sea water for 24 hours. For remaining cGAMP
425 treatment experiments, 50-100 48-hour old embryos were treated with 100µM 2'3'-
426 cGAMP (InvivoGen) in 1/3x sea water for 4 hours.

427

428 **RNA sequencing**

429 For the initial CDN treatment experiment using polyps, total RNA was extracted
430 using Qiagen RNeasy Mini kits according to the manufacturer's protocol. Libraries were
431 prepared by the Functional Genomics Laboratory at UC Berkeley using WaferGen
432 PrepX library prep kits with oligo dT beads for mRNA enrichment according to the
433 manufacturer's protocol, and 50 nt single-end sequencing was carried out on the
434 HiSeq4000 (Illumina) by the Vincent J.Coates Genomics Sequencing Laboratory. For all
435 other RNA sequencing experiments on 48 hour embryos, RNA was extracted using
436 Trizol (Thermo Fisher Scientific) according to the manufacturer's protocol. Libraries
437 were prepared by the Functional Genomics Laboratory at UC Berkeley as follows: oligo
438 dT beads from the KAPA mRNA Capture Kit (KK8581) were used for mRNA
439 enrichment; fragmentation, adapter ligation and cDNA synthesis were performed using
440 the KAPA RNA HyperPrep kit (KK8540). Libraries were pooled evenly by molarity and
441 sequenced by the Vincent J.Coates Genomics Sequencing Laboratory on a
442 NovaSeq6000 150PE S4 flowcell (Illumina), generating 25M read pairs per sample.
443 Read quality was assessed using FastQC. Reads were mapped to the *N. vectensis*
444 transcriptome (NCBI: GCF_000209225.1) using kallisto and differential expression was
445 analyzed in R with DESeq2. Differential expression was deemed significant with a log₂
446 fold change greater than 1 and an adjusted p-value less than 0.05. GO term analysis
447 was performed using goseq with GO annotations from
448 [https://figshare.com/articles/dataset/Nematostella_vectensis_transcriptome_and_gene_](https://figshare.com/articles/dataset/Nematostella_vectensis_transcriptome_and_gene_models_v2_0/807696)
449 [models_v2_0/807696](https://figshare.com/articles/dataset/Nematostella_vectensis_transcriptome_and_gene_models_v2_0/807696). The EnhancedVolcano package
450 (<https://github.com/kevinblighe/EnhancedVolcano>) was used to generate volcano plots.
451 Heatmaps are based on regularized log-transformed normalized counts and Z-scores
452 are scaled by row. All RNA-Seq results can be found in Supplementary Dataset 1. Raw
453 sequencing reads and normalized gene counts can be found at the NCBI GEO under
454 accession GSE175984.

455

456 **Quantitative Real-Time PCR (qRT-PCR)**

457 Embryos and polyps were lysed in TRIzol (Invitrogen) and RNA was extracted
458 according to the manufacturer's protocol. 500ng of RNA was treated with RQ1 RNase-

459 free DNase (Promega) for and reverse transcribed with Superscript III (Invitrogen).
460 Quantitative PCR was performed using SYBR Green (Thermo Fisher Scientific) with 0.8
461 μM of forward and reverse primers on a QuantStudio 5 Real-Time PCR System
462 (Applied Biosystems) with the following cycling conditions: 50°C 2 min; 95°C 10 min;
463 [95°C 15 sec, 60°C 1 min] x 40; 95°C 15 sec; 60°C 1 min; melt curve: step 0.075 °C/s to
464 95°C. Fold changes in expression levels were normalized to actin and calculated using
465 the $2^{-\Delta\Delta\text{Ct}}$ method. Student's t-tests were performed on ΔCt values. All primer sequences
466 used in this study can be found in Supplementary Dataset 2.

467

468 **shRNA microinjection**

469 Short hairpin RNAs for microinjection were prepared by *in vitro* transcription as
470 previously described (57). Briefly, unique 19 nucleotide targeting motifs were identified
471 and used to create oligonucleotides with the following sequence: T7 promoter—19nt
472 motif—linker—antisense 19nt motif—TT. RNA secondary structure was visualized using
473 mfold (<http://www.unafold.org/mfold/applications/rna-folding-form.php>) to ensure a
474 single RNA conformation. Both sense and anti-sense oligonucleotides were synthesized
475 and mixed to a final concentration of 25 μM , heated to 98°C for 5 minutes and cooled to
476 24°C before use as template for *in vitro* transcription using the Ampliscribe T7-Flash
477 Transcription Kit (Lucigen). Reactions were allowed to proceed overnight, followed by a
478 15 minute treatment with DNase and subsequent purification with Direct-zol™ RNA
479 MiniPrep Plus (Zymo Research). All shRNAs used in this study can be found in
480 Supplementary Dataset 2.

481 Microinjections of one-cell embryos were carried out as previously described
482 (58). shRNAs were diluted to a concentration of 500-900 ng/ μl (ideal concentrations
483 were determined experimentally) in RNase-free water with fluorescent dextran for
484 visualization. Injected embryos were monitored for gross normal development at room
485 temperature and used for experiments 48 hours later unless otherwise indicated.
486 Knockdowns for each gene were performed using at least two different shRNAs and
487 phenotypes were confirmed in at least 3 independent experiments.

488

489 **Immunohistochemistry, imaging, and quantification**

490 Polyps treated for 4 hours with 100 μM 2'3'-cGAMP were stained for nvNF- κB as
491 previously described (59). Briefly, polyps were fixed in 4% paraformaldehyde in 1/3x
492 sea water overnight at 4°C with rocking, and subsequently washed 3 times with wash
493 buffer (1x PBS, 0.2% Triton X-100). Antigen retrieval was performed by placing
494 anemones in 95°C 5% urea for 5 minutes and allowing them to cool to room
495 temperature before washing 3 times in wash buffer. Samples were blocked overnight at
496 4°C in blocking buffer (1x PBS, 5% normal goat serum, 1% bovine serum albumin,
497 0.2% Triton X-100). Samples were stained with anti-nvNF- κB (1:100; gift of Thomas
498 Gilmore, Boston University) in blocking buffer for 90 minutes at room temperature and
499 washed 4 times in wash buffer. Samples were then incubated in FITC-anti-Rabbit IgG
500 (1:160; F9887, Sigma-Aldrich) in blocking buffer for 90 minutes at 37°C. Finally,
501 samples were washed in wash buffer, stained with 1 $\mu\text{g}/\text{mL}$ of DAPI for 10 minutes,
502 washed again, and mounted in Vectashield HardSet Mounting medium and imaged on a
503 Zeiss LSM 710 AxioObserver. Imaris 9.2 (Bitplane) was used to create 3D surfaces
504 based on DAPI expression, and surface statistics were exported and analyzed in
505 FlowJo (BD) to quantify nuclear nvNF- κB expression as previously described (60).

506

507 **Bacterial infection**

508 Single colonies of *Pseudomonas aeruginosa* strain PA14-GFP were cultured
509 overnight in LB with 50µg/ml of carbenicillin, centrifuged for 5 minutes at 3000 x *g*,
510 resuspended to an OD₆₀₀ of 0.1, 0.01, or 0.001 in 1/3x sea water and used to infect
511 polyps, which were kept at room temperature or 28°C as indicated. Inputs were plated
512 to calculate CFU/ml. Polyp survival was monitored daily. For expression analysis,
513 polyps were homogenized in Trizol and RNA extraction and qPCR were performed as
514 indicated above.

515

516 **Protein purification**

517 nvDae4 lacking its signal peptide was cloned from cDNA. These fragments were
518 then cloned into the pAcGP67-A baculovirus transfer vector for secreted, His-tagged
519 protein expression. The plasmid for expressing mutant nvDae4 (C63A) was made from
520 the pAcGP67-A-nvDae4 plasmid using Q5 site-directed mutagenesis (NEB) according
521 to the manufactures protocol. Plasmids were transfected into Sf9 insect cells (2x10⁶
522 cells/ml in 2ml) using Cellfectin II Reagent (Gibco) along with BestBac 2.0 v-cath/chiA
523 Deleted Linearized Baculovirus DNA (Expression Systems) for 6 hours, after which
524 media was replaced and cells were left for 1 week at 25°C. Supernatants were
525 harvested and 50 µL were used to infect 7x10⁶ Sf9 cells in 10ml of media for 1 week at
526 25°C. Supernatants containing secondary virus were harvested, tested, and used to
527 infect High Five cells (2 L at 1.5x10⁶ cells/ml) for 72 hours at 25°C with shaking.
528 Supernatants containing protein were harvested by centrifuging for 15 min at 600 x *g* at
529 4°C and subsequently passing through a 0.45 µm filter to remove all cells. Supernatants
530 were buffered to 1x HBS (20mM HEPES pH 7.2, 150mM NaCl), mixed with 2 mL of Ni-
531 NTA agarose were per liter, and rotated at 4°C for 2 hours. Ni-NTA resins with bound
532 protein were collected on a column by gravity-flow and washed with 30x column volume
533 of wash buffer (20mM HEPES, 1M NaCl, 30mM imidazole, 10% glycerol). Protein was
534 eluted in 1 mL fractions using 1xHBS supplemented with 200mM imidazole. Buffer was
535 exchanged to 1xHBS+ 2mM DTT using Econo-Pac10DG Desalting Prepacked Gravity
536 Flow Columns (Bio-rad) according to the manufacturers protocol, and proteins were
537 concentrated using 10kDa concentrators (Millipore).

538

539 **Bacterial killing assays**

540 For expression in *E. coli*, nvDae4 WT and C63A lacking the endogenous signal
541 sequence were cloned into the pET28a vector for inducible cytosolic expression, or the
542 pET22b vector for inducible periplasmic expression. *E. coli* (BL21 DE3 strain) were
543 freshly transformed with the vectors and grown overnight in LB with 50 µg/mL
544 carbenicillin shaking at 37°C. Overnight cultures were backdiluted in LB to the same
545 OD₆₀₀ and grown to log phase before induction with 0.25mM IPTG. Plates were kept
546 shaking at 37°C and OD₆₀₀ was read every 5 minutes for 3 hours.

547 *Bacillus subtilis*-GFP (derivative of strain 168; BGSC accession #1A1139) was
548 grown to log-phase in LB with 100 µg/mL spectinomycin, centrifuged, resuspended in
549 0.5xHBS, and incubated alone or with nvDae4 WT or C63A at indicated concentrations
550 for 2-3 hours at 37°C. Serial dilutions were plated on LB agar with 100 µg/mL
551 spectinomycin to determine CFU.

552

553 **Peptidoglycan cleavage assays**

554 Peptidoglycan (PG) was purified and analyzed as previously described (50).
555 Briefly, *Escherichia coli* BW11325 (from Carol Gross, UCSF) and *Staphylococcus*
556 *epidermis* BCM060 (from Tiffany Scharschmidt, UCSF) were grown to an OD₆₀₀ of 0.6,
557 harvested by centrifugation, and boiled in SDS (4 % final concentration) for 4 hours with

558 stirring. After washing in purified water to remove SDS, the peptidoglycan was treated
559 with Pronase E for 2 h at 60°C (0.1 mg/ml final concentration in 10 mM Tris-HCl pH7.2
560 and 0.06 % NaCl; pre-activated for 2 h at 60 °C). Pronase E was heat inactivated at
561 100°C for 10 min and washed with sterile filtered water (5 x 20 min at 21k x g). PG from
562 Gram-positive bacteria (Se) was also treated with 48% HF at 37°C for 48 h to remove
563 teichoic acids, followed by washes with sterile filtered water. nvDae4 enzyme (WT and
564 C63A) was added (1-10 µM in 10 mM Tris-HCl pH 7.2 and 0.06% NaCl) and incubated
565 O/N at 37°C. Enzymes were heat inactivated at 100°C for 10 min. Mutanolysin (Sigma
566 M9901, final concentration 20 µg/ml) was added to the purified peptidoglycan and
567 incubated overnight at 37 °C. The peptidoglycan fragments were reduced, acidified,
568 analyzed via HPLC (0.5 ml/min flow rate, 55°C with Hypersil ODS C18 HPLC column,
569 Thermo Scientific, catalog number: 30103-254630).

570

571 **Supplemental methods**

572

573 **Phylogenetic analysis**

574 Protein sequences containing domains of interest were downloaded from NCBI,
575 with the exception of nvGBP6 and nvGBP7, for which RNA-seq data showed additional
576 nucleotide usage relative to the reference sequence (all sequences can be found in
577 Supplementary Dataset 2). These were aligned using on phylogeny.fr using MUSCLE
578 and manipulated in Geneious. For the GBP and IRF alignments, only the domain of
579 interest was used for phylogenetic analysis. Maximum likelihood phylogenetic trees
580 were generated with PhyML using 100 bootstrap replicates. Alignments shown were
581 made in Geneious using Clustal Omega.

582

583 **Nanostring gene expression analysis**

584 A custom codeset targeting 36 genes of interest and 4 housekeeping genes for
585 normalization (Supplementary Dataset 2) was designed by NanoString Technologies
586 (Seattle, WA) for use in nCounter XT CodeSet Gene Expression Assays run on an
587 nCounter SPRINT Profiler (NanoString Technologies). RNA was isolated as it was for
588 qRT-PCR experiments, and hybridized to probes according to the manufacturer's
589 protocol using 50ng/µL of total RNA. Quality control, data normalization, and
590 visualization was performed in nSolver 4.0 analysis software (NanoString Technologies)
591 according to the manufacturer's protocol.

592

593 **Mammalian cell immunofluorescence and confocal microscopy**

594 Glass coverslips were seeded with 293T cells and grown to ~50% confluency.
595 Cells were transfected for 24-48 hours with a total of 1.25 µg of DNA and 3 µL
596 Lipofectamine 2000. Each well contained the following: pcDNA4-STING (10 ng),
597 pEGFP-LC3 (5 ng) and either empty vector or pcDNA4 with the indicated cyclic-
598 dinucleotide synthase (1,235 ng). Cells were washed once in PBS, fixed for 15 minutes
599 in 4% paraformaldehyde, washed once in PBS, and permeabilized for 5 minutes in
600 0.5% saponin in PBS. Cells were then washed once in PBS, and treated with 0.1%
601 sodium borohydride/0.1% saponin/PBS for 5 – 10 minutes in order to consume any
602 remaining paraformaldehyde. Cells were then washed 3 times in PBS, and blocked with
603 blocking buffer (1% BSA/0.1% saponin/PBS) for 45 minutes. Cells were then incubated
604 HA antibody (1:200 dilution, Sigma 11867423001 rat IgG from Roche) in blocking buffer
605 for one hour and washed 3 times in PBS. Each well was then incubated with 0.1%
606 saponin/PBS and secondary antibody (1:500 dilution, Jackson ImmunoResearch, Cy3
607 affinipure donkey anti-rat IgG, 712-165-153) for 45 minutes. Finally, cells were washed

608 3 times in PBS, mounted using VectaShield with DAPI, and dried overnight. Images
609 were acquired using a Zeiss LSM 780 NLO AxioExaminer.

610

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783

784 Figure Legends

785

786 **Figure 1: 2'3'-cGAMP induces many putative immune genes in *Nematostella*** 787 ***vectensis***

- 788 A) Volcano plot showing differential gene expression (DE) in *N. vectensis* polyps
789 untreated vs. treated with 2'3'-cGAMP for 24 hours. A positive fold-change
790 indicates higher expression in polyps treated with 2'3'-cGAMP. Genes of interest
791 with homologs known to be involved in immunity in other organisms are labeled.
792 B) Breakdown of DE genes into categories based on known GO terms. Gene set
793 enrichment analysis shows a clear enrichment of GO terms associated with
794 immunity.
795 C) qRT-PCR measuring genes of interest in 48-hour-old *N. vectensis* embryos
796 untreated or treated with 2'3'-cGAMP for 4 hours. Fold changes were calculated
797 relative to untreated as $2^{-\Delta\Delta Ct}$ and each point represents one biological replicate.
798 Unpaired t test performed on $\Delta\Delta Ct$ before log transformation. * $p \leq 0.05$;
799 ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

800

801 **Figure 2: The induction of many anti-bacterial genes by 2'3'-cGAMP is nvNF- κ B** 802 **dependent**

- 803 A) Heatmap showing all genes that are significantly ($p_{adj} < 0.05$, $\log_2 FC < -1$)
804 downregulated in 2'3'-cGAMP -treated embryos microinjected with NF- κ B shRNA
805 vs. GFP shRNA. Genes with predicted anti-bacterial function are labeled
806 B) qRT-PCR of anti-bacterial genes in nvNF- κ B shRNA or control GFP shRNA
807 treated samples after induction by 2'3'-cGAMP. Fold change was calculated
808 relative to untreated, GFP shRNA injected as $2^{-\Delta\Delta Ct}$ and each point represents
809 one biological replicate. Unpaired t test performed on $\Delta\Delta Ct$ before log
810 transformation. * $p \leq 0.05$; ** $p \leq 0.01$.
811 C) Whole mount immunofluorescence of polyps stained with anti-nvNF- κ B
812 antiserum. Right two panels are enlargements of the boxed regions indicated in
813 the left two panels.
814 D) Quantification of cells with nuclear localization of nvNF- κ B after treatment with
815 cGAMP (representative images shown in C). Each point represents a single
816 polyp, in which at least 1500 cells were analyzed. Statistical analysis was
817 performed by unpaired t test; * $p = 0.0481$.

818

819 **Figure 3: *Pseudomonas aeruginosa* infection induces putative anti-bacterial** 820 **genes**

- 821 A) Survival curves of *N. vectensis* polyps infected with *P. aeruginosa* at indicated
822 dose and temperature.
823 B+C) qRT-PCR of nvSTING (B) or putative anti-bacterial genes (C) assayed at 48
824 hours post *Pa* infection (2×10^7 CFU/ml). Each point represents one biological
825 replicate; unpaired t test performed on $\Delta\Delta Ct$ before log transformation. ** $p \leq 0.01$;
826 *** $p \leq 0.001$; **** $p \leq 0.0001$.

827

828 **Figure 4: A 2'3'-cGAMP induced, nvNF- κ B-dependent protein has anti-bacterial** 829 **activity**

- 830 A) Growth of *E. coli* expressing either periplasmic (Peri-) or cytosolic (Cyto-) nvDae4
831 (WT or C63A) induced with 250 μ M IPTG. Error bars +/- SD; n=3. Unpaired t test;
832 **p= 0.0063.
833 B) Partial HPLC chromatograms of *E. coli* peptidoglycan sacculi after overnight
834 incubation with buffer only (no enzyme), or 1 μ M nvDae4 WT or C63A enzyme.
835 C) *Bacillus subtilis* CFU after 2 hour incubation with buffer alone, nvDae4 WT or
836 catalytic mutant C63A (25 μ M). Error bars +/- SEM; n=3. Unpaired t test
837 performed on log-transformed values; **p \leq 0.01.
838 D) Dose dependent killing of *B. subtilis* by WT nvDae4 enzyme (same assay as in
839 C). Error bars +/- SD; n=2 per concentration.

840

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- 842 A) Heatmap showing differentially expressed genes in response to c-di-AMP, c-di-
843 GMP, and 3'3'-cGAMP. Almost all of these are also significantly induced by 2'3'-
844 cGAMP.
845 B-D) Volcano plots of differential gene expression in *N. vectensis* polyps untreated
846 vs. treated with cyclic-di-AMP (B), cyclic-di-GMP (C) and 3'3'-cGAMP (D) for 24
847 hours.

848

849 **Figure S2: nvSTING knockdown does not impact the induction of genes by 2'3'- 850 cGAMP**

- 851 A) Volcano plot showing differential gene expression in 48 hour embryos treated
852 with 2'3'-cGAMP that were injected with GFP shRNA or nvSTING shRNA.
853 Positive fold-change indicates higher expression in GFP shRNA injected
854 embryos.
855 B) Clustered heatmap showing the expression of the top 1000 varied genes by
856 RNA-Seq between embryos injected with either GFP or nvSTING shRNA and
857 either untreated or treated with 2'3'-cGAMP.
858 C) Fold change of nvSTING, nvOAS, and nvLBP assayed by Nanostring from
859 experiments using 3 different shRNAs to knock down nvSTING expression.
860 D) qRT-qPCR measuring genes of interest in 48-hour-old embryos injected with a
861 control (ctrl) or nvSTING translation-inhibiting morpholino (MO) and treated with
862 2'3'-cGAMP. Fold changes were calculated as $2^{-\Delta\Delta C_t}$ and each point represents
863 one biological replicate. Unpaired t test performed on $\Delta\Delta C_t$ before log
864 transformation; no significant differences.
865 E) Immunofluorescence images of 293T cells transfected with plasmids encoding
866 nvSTING-HA, LC3-GFP, and either empty vector, human cGAS or *V. cholera*
867 DncV. Human cGAS is activated by the transfected DNA to produce 2'3'-cGAMP,
868 and DncV, which produces 3'3'-cGAMP, is constitutively active in 293T cells.

869

870 **Figure S3: Phylogenetic study of *N. vectensis* IRFs**

- 871 A) Phylogenetic tree of all human and *N. vectensis* IRF proteins. 3 nvIRFs cluster
872 with members of the human IRF1 supergroup, while the other 2 nvIRFs cluster
873 with the IRF4 supergroup.
874 B) Full protein alignment of sequences in A). The DNA-binding domain is highly
875 conserved between all *N. vectensis* and human IRFs. Among *N. vectensis*
876 paralogs, only nvIRF5 contains an IAD1 domain.

877 C) Alignment of all nvIRF DNA-binding domains with conserved tryptophan pentad
878 outlined in red

879

880 **Figure S4: Knockdowns of nvIRFs or nvSTAT have no effect on 2'3'-cGAMP-**
881 **induced gene expression**

882 A) Fold changes in gene expression as determined by Nanostring in embryos
883 microinjected with shRNAs targeting EGFP, nvIRF1, or nvIRF5 either untreated
884 or treated with 2'3'-cGAMP.

885 B) Fold changes in gene expression as determined by qRT-PCR in samples
886 microinjected with shRNAs targeting EGFP, nvIRF2, nvIRF-67, or nvIRF4 either
887 untreated or treated with 2'3'-cGAMP. Note that IRF2 is not induced by cGAMP
888 and was mostly undetected in all samples; therefore it is possible that the
889 knockdowns were unsuccessful.

890 C) Volcano plot showing differential gene expression as determined by RNA-Seq in
891 48 hour embryos treated with 2'3'-cGAMP that were injected with GFP shRNA or
892 nvSTAT shRNA. Positive fold-change indicates higher expression in GFP shRNA
893 injected embryos. The GFP shRNA samples here are the same as those shown
894 in Figure 2A.

895 D) Fold changes in gene expression as determined by Nanostring in embryos
896 microinjected with shRNAs targeting EGFP or nvSTAT either untreated or treated
897 with 2'3'-cGAMP.

898

899 **Figure S5: Anti-viral gene induction is not dependent on nvNF- κ B**

900 A) Fold changes in gene expression as determined by Nanostring in embryos
901 microinjected with shRNAs targeting EGFP or nvNF- κ B, either untreated or
902 treated with 2'3'-cGAMP. The GFP shRNA samples here are the same as those
903 shown in Figure S4. GBP-806 expression included to show anti-bacterial gene
904 induction is lower in these samples.

905 B) Fold changes in nvLysozyme expression as determined by qRT-PCR in embryos
906 microinjected with shRNAs targeting EGFP or nvNF- κ B, either untreated or
907 treated with 2'3'-cGAMP.

908 A+B) No significant differences in gene expression are observed between any
909 cGAMP treated samples.

910 C) qRT-PCR of putative anti-viral genes assayed at 48 hours post *Pa* infection
911 (2×10^7 CFU/ml). Each point represents one biological replicate; unpaired t test
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913

914 **Figure S6: Phylogenetic study of *N. vectensis* GBPs**

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916 with the full protein sequences. Branches with mammalian interferon-induced
917 GTPases and cGAMP-induced *N. vectensis* GBPs are colored red; these tend to
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919 B) Alignment of the GTPase domains of all *N. vectensis* GBPs and select
920 mammalian GTPase. Conserved GBP and atlastin specific residues are
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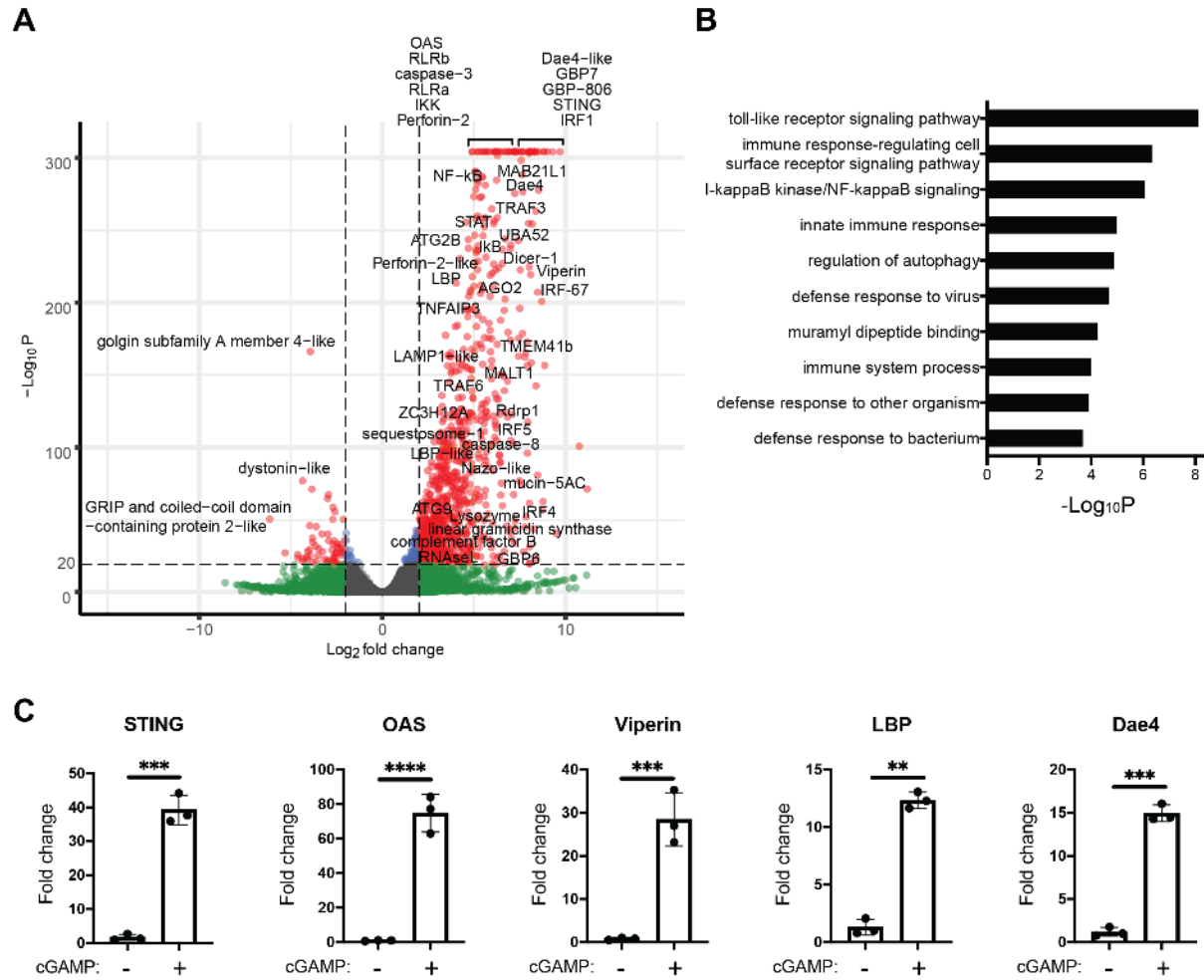


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- A) Volcano plot showing differential gene expression (DE) in *N. vectensis* polyps untreated vs. treated with 2'3'-cGAMP for 24 hours. A positive fold-change indicates higher expression in polyps treated with 2'3'-cGAMP. Genes of interest with homologs known to be involved in immunity in other organisms are labeled.
- B) Breakdown of DE genes into categories based on known GO terms. Gene set enrichment analysis shows a clear enrichment of GO terms associated with immunity.
- C) qRT-PCR measuring genes of interest in 48-hour-old *N. vectensis* embryos untreated or treated with 2'3'-cGAMP for 4 hours. Fold changes were calculated relative to untreated as $2^{-\Delta\Delta C_t}$ and each point represents one biological replicate. Unpaired t test performed on $\Delta\Delta C_t$ before log transformation. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

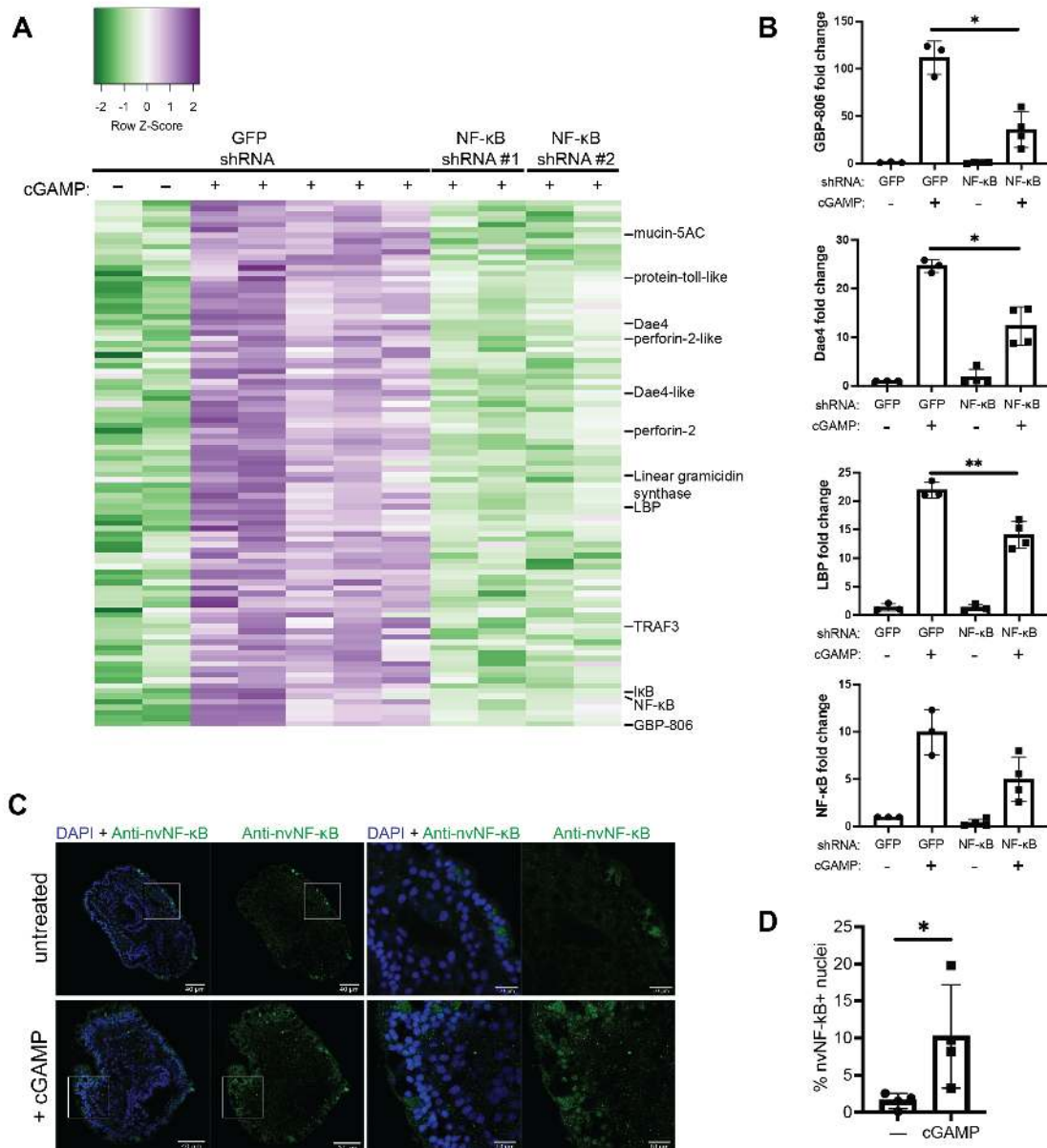


Figure 2: The induction of many anti-bacterial genes by 2'3'-cGAMP is nvNF- κ B dependent

- A) Heatmap showing all genes that are significantly ($p_{adj} < 0.05$, $\log_2FC < -1$) downregulated in 2'3'-cGAMP -treated embryos microinjected with NF- κ B shRNA vs. GFP shRNA. Genes with predicted anti-bacterial function are labeled
- B) qRT-PCR of anti-bacterial genes in nvNF- κ B shRNA or control GFP shRNA treated samples after induction by 2'3'-cGAMP. Fold change was calculated relative to untreated, GFP shRNA injected as $2^{-\Delta\Delta Ct}$ and each point represents one biological replicate. Unpaired t test performed on $\Delta\Delta Ct$ before log transformation. * $p \leq 0.05$; ** $p \leq 0.01$.

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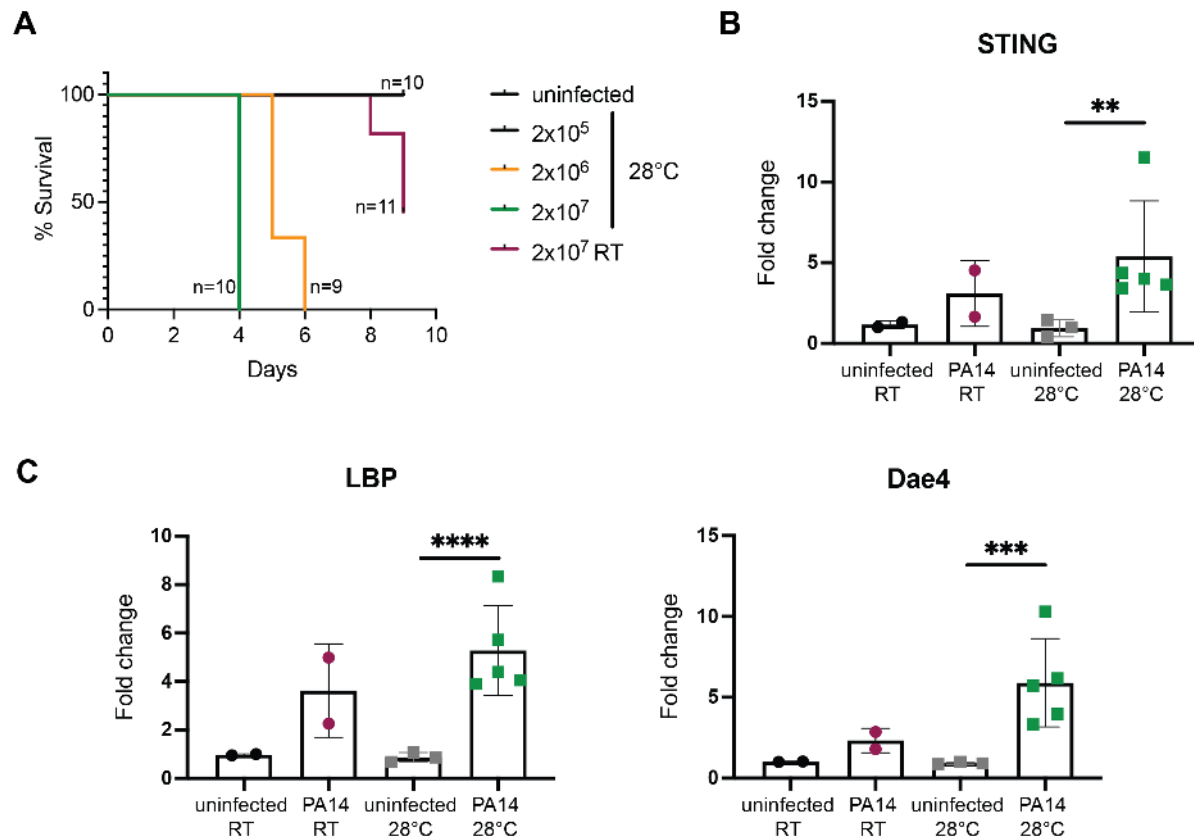


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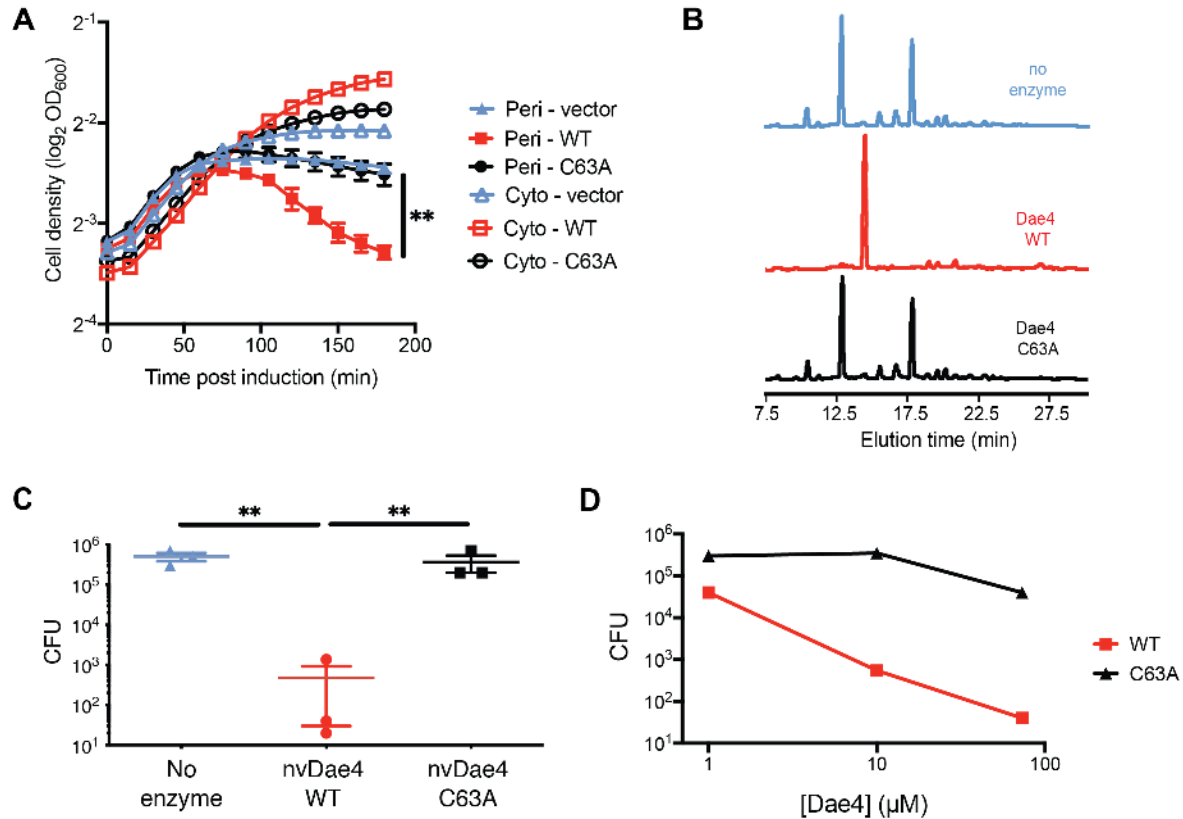


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- A) Growth of *E. coli* expressing either periplasmic (Peri-) or cytosolic (Cyto-) nvDae4 (WT or C63A) induced with 250 μM IPTG. Error bars +/- SD; n=3. Unpaired t test; **p = 0.0063.
- B) Partial HPLC chromatograms of *E. coli* peptidoglycan sacculi after overnight incubation with buffer only (no enzyme), or 1 μM nvDae4 WT or C63A enzyme.
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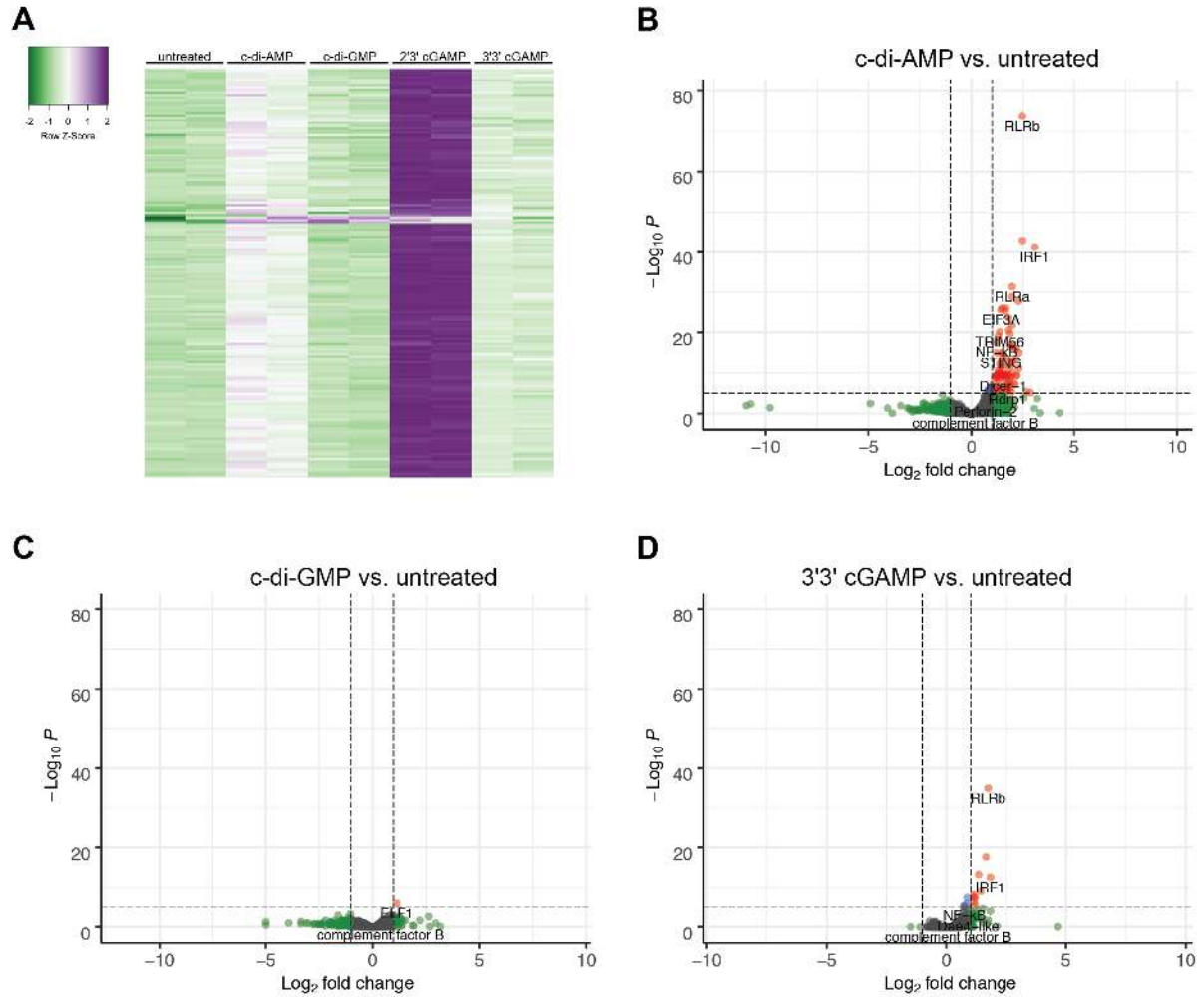


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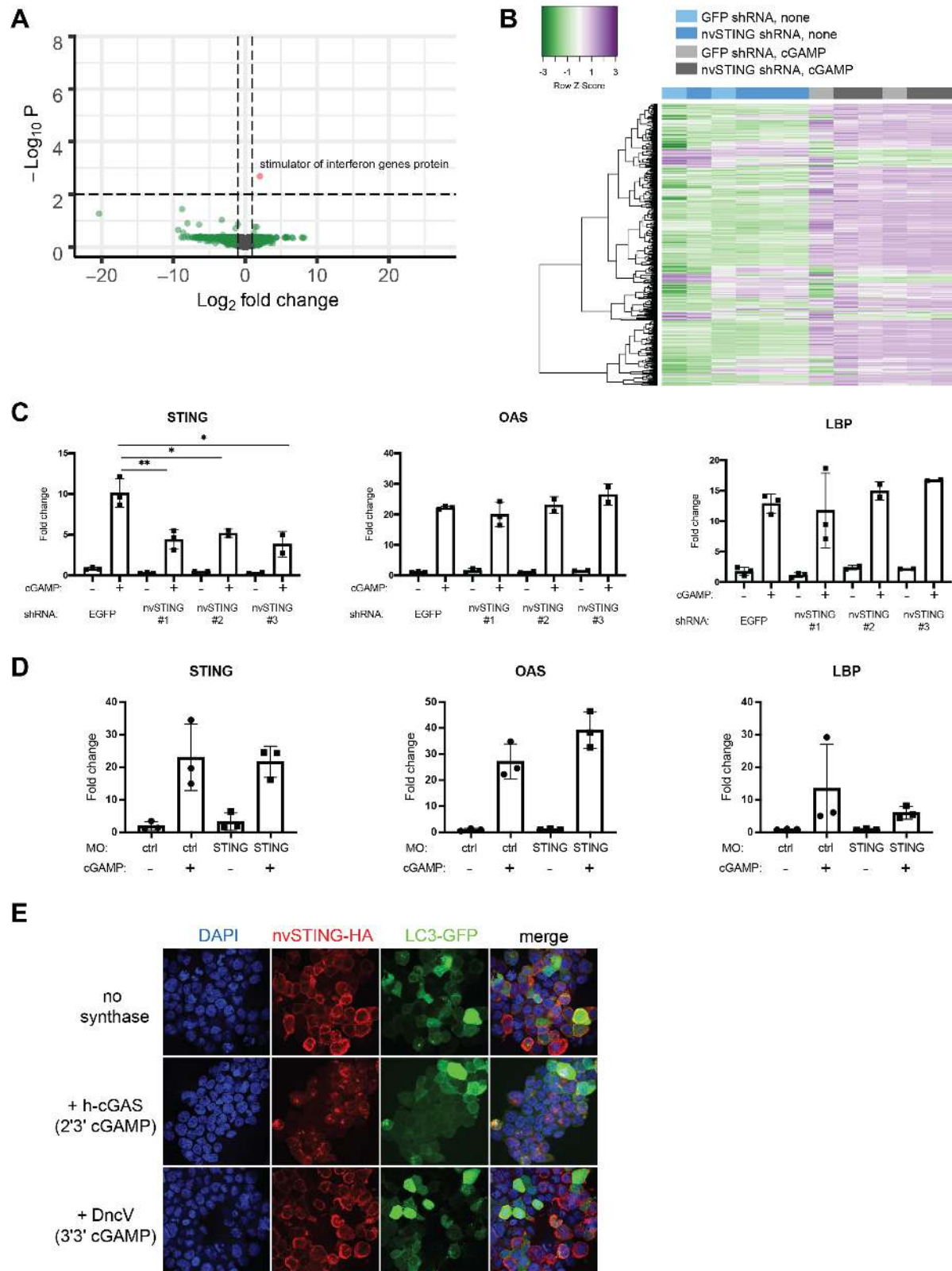


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- E) Immunofluorescence images of 293T cells transfected with plasmids encoding nvSTING-HA, LC3-GFP, and either empty vector, human cGAS or *V. cholera* DncV. Human cGAS is activated by the transfected DNA to produce 2'3'-cGAMP, and DncV, which produces 3'3'-cGAMP, is constitutively active in 293T cells.

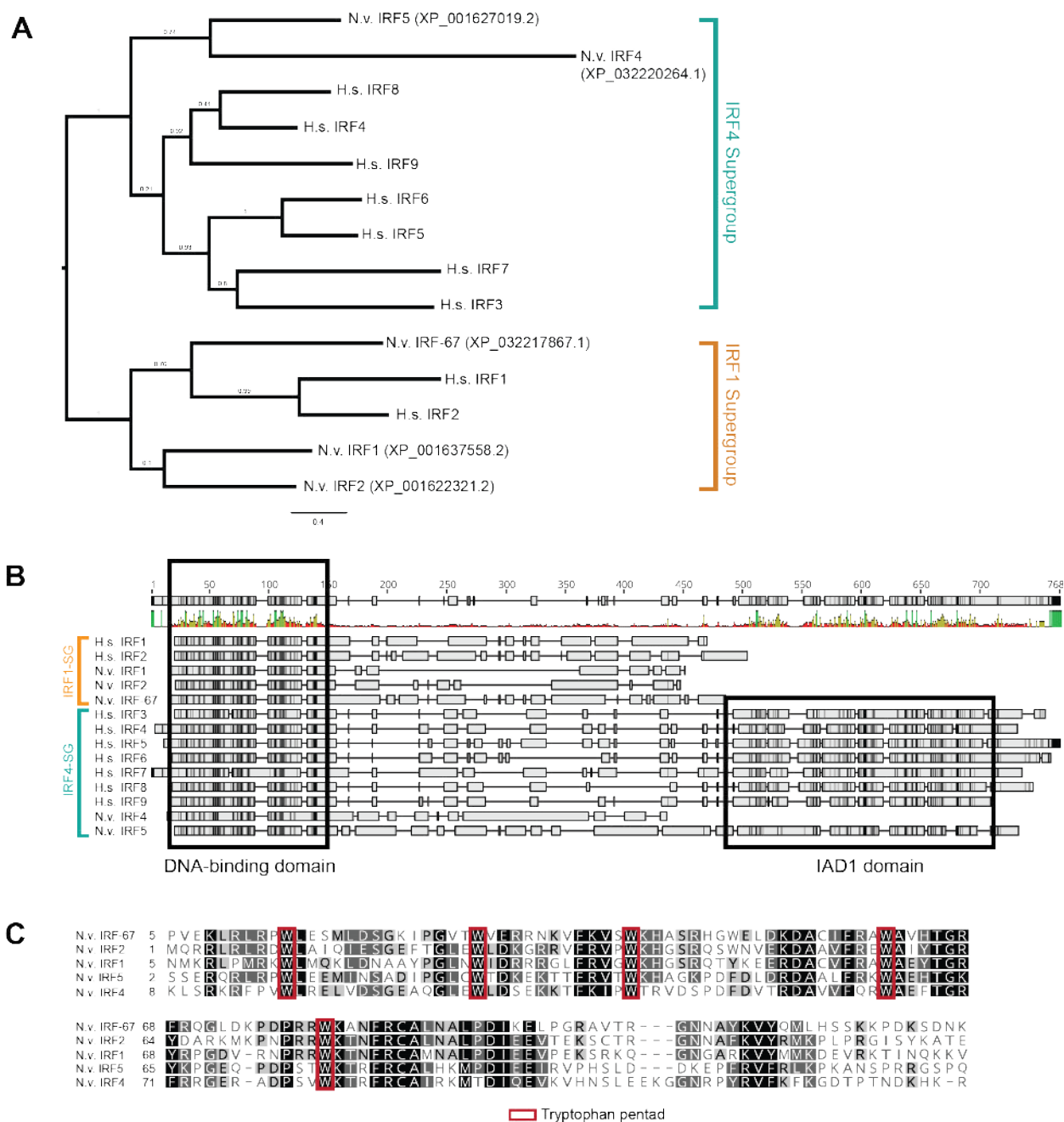


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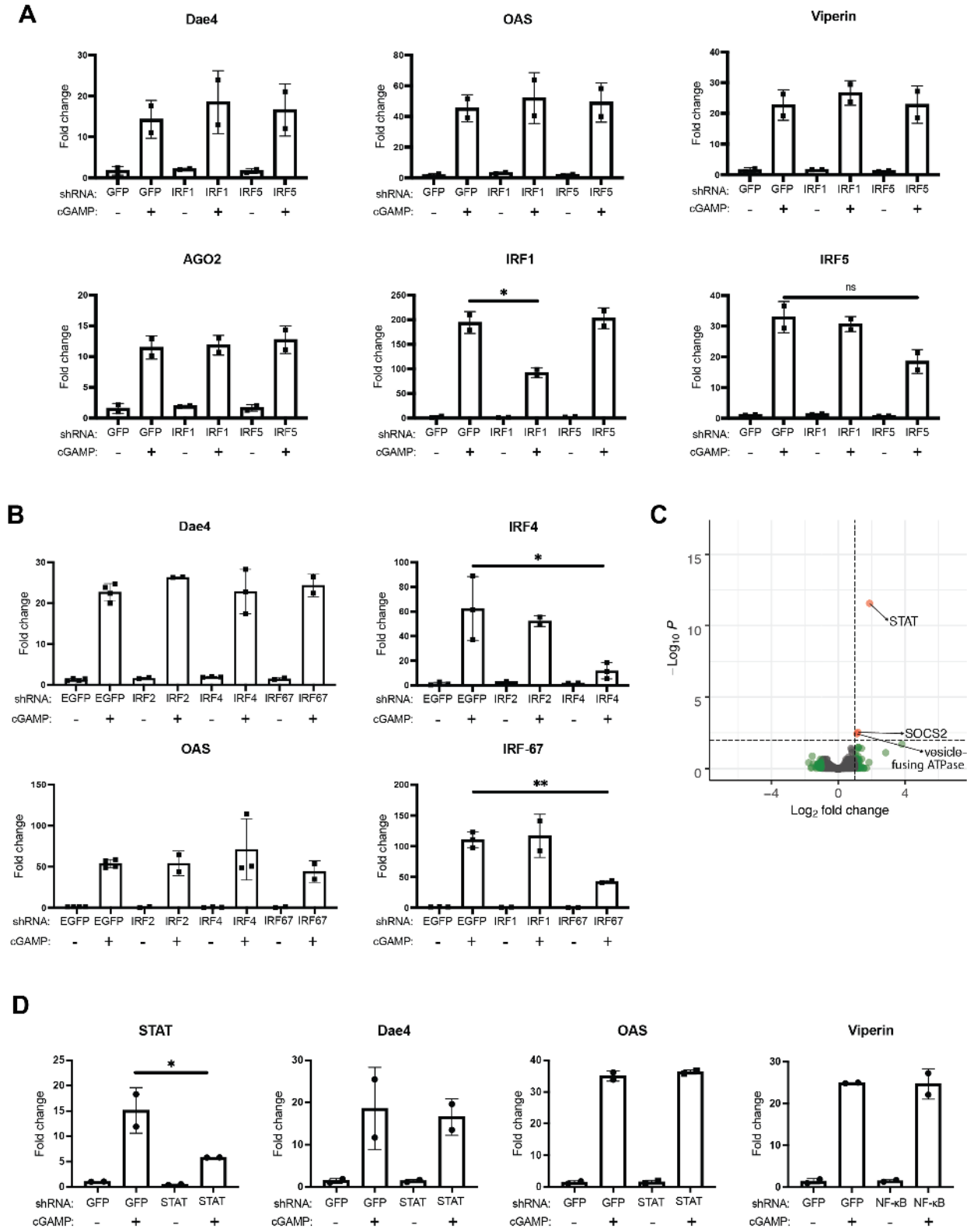
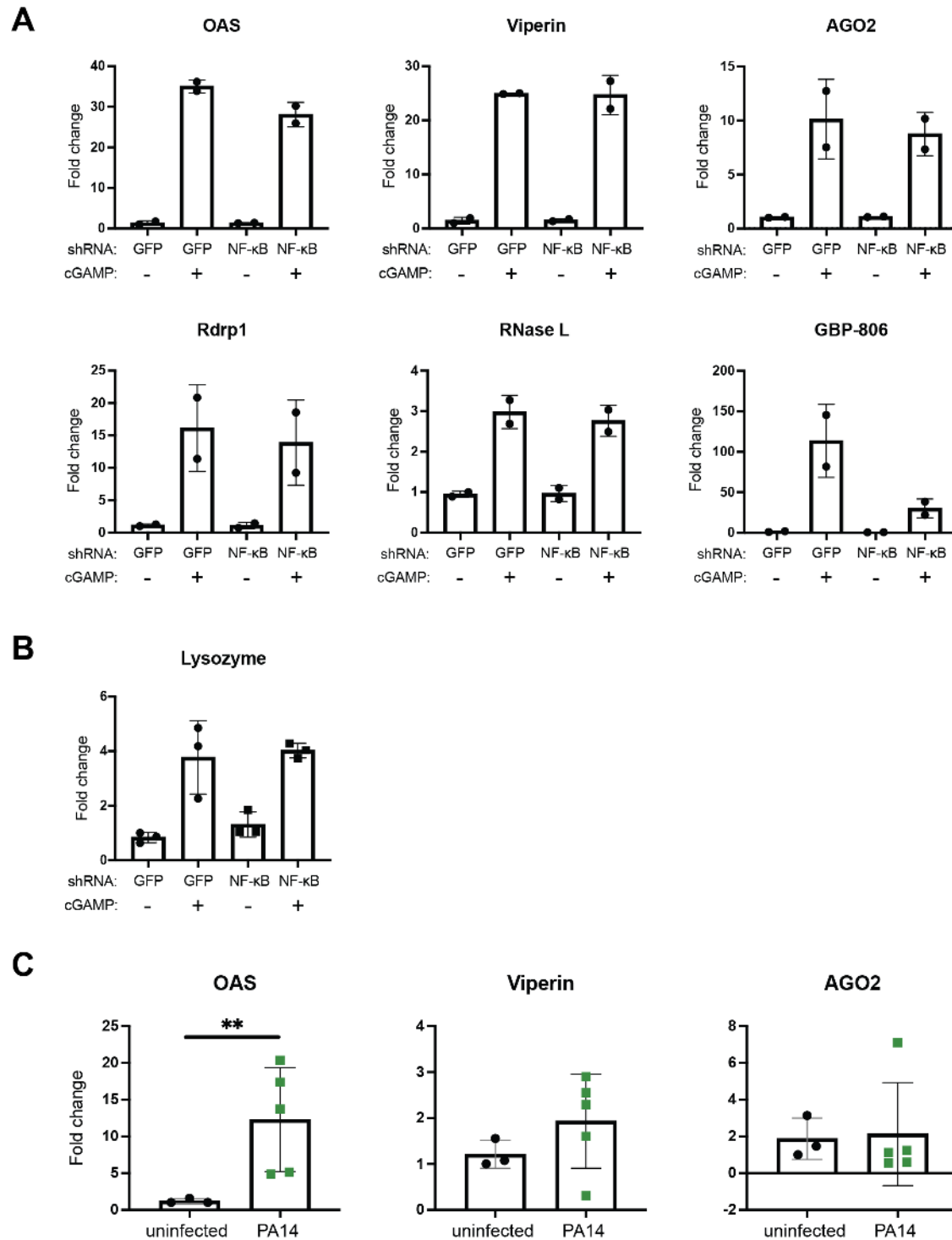


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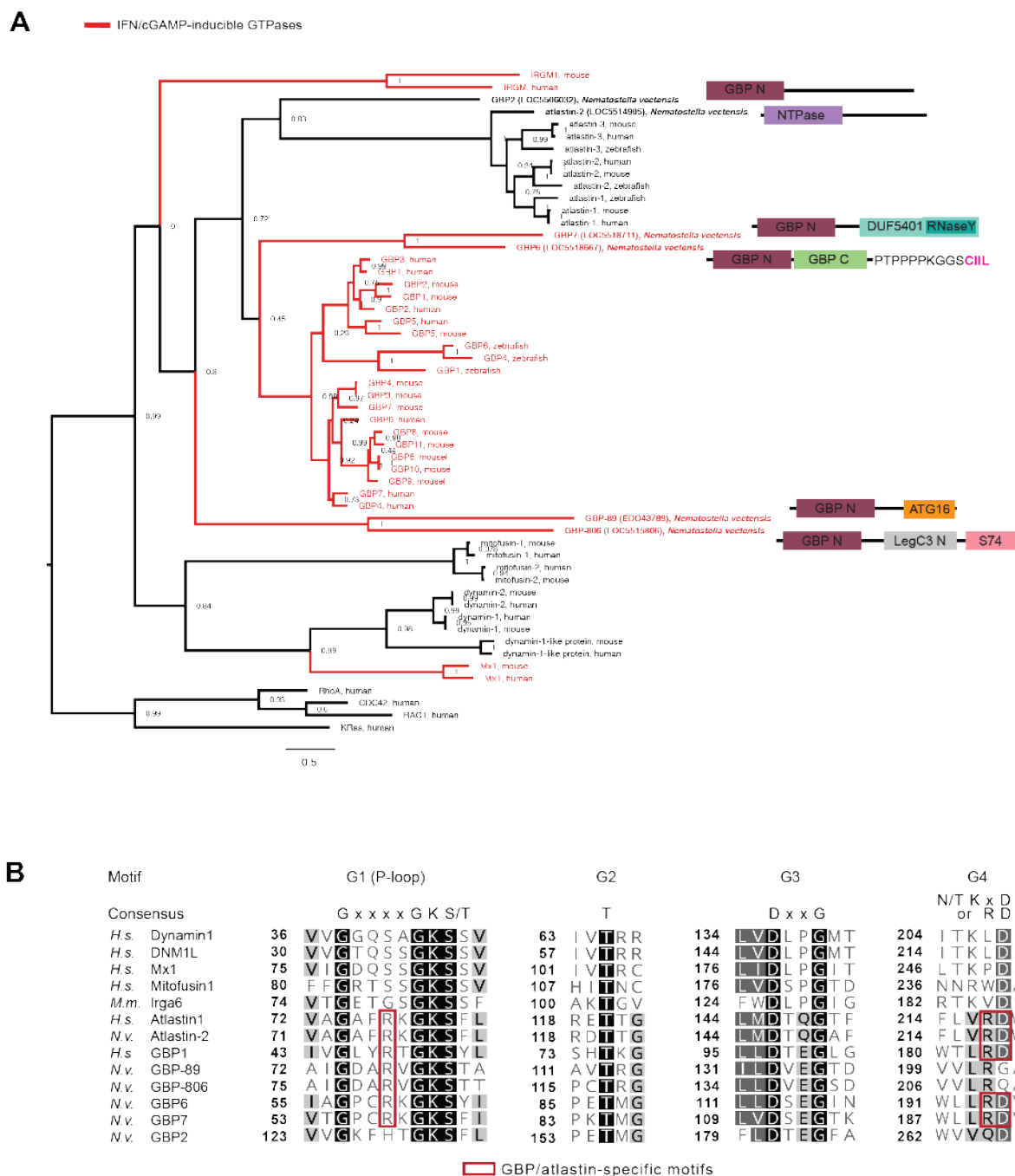


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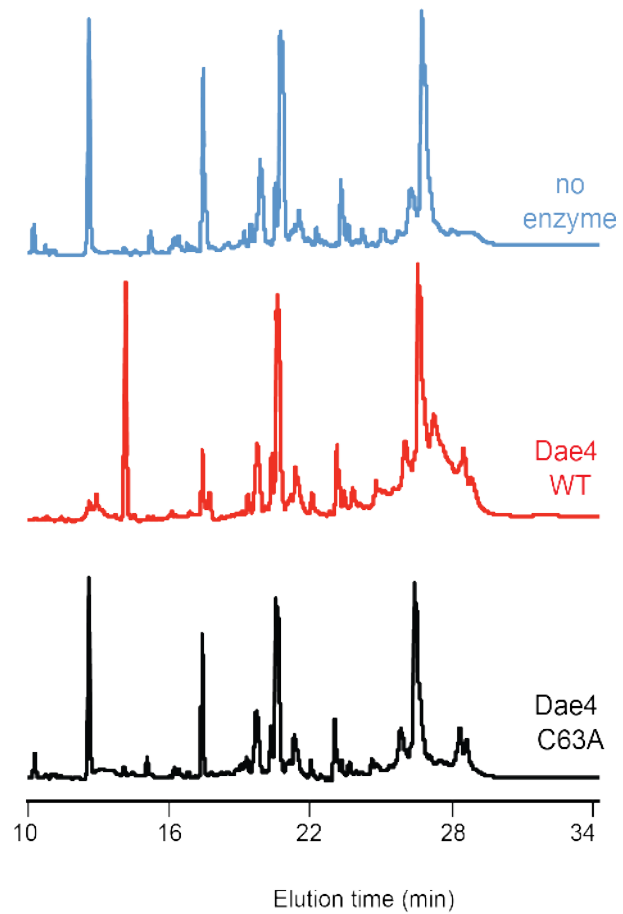


Figure S7: nvDae4 cleaves peptidoglycan from Gram-positive bacteria. Partial HPLC chromatograms of *Staphylococcus epidermidis* peptidoglycan sacculi products resulting from incubation with buffer only (no enzyme), or 1 μ M nvDae4 WT or C63A enzyme.

Table S1: Cnidarian-specific genes that are induced by 2'3'-cGAMP in an ν NF- κ B-dependent manner

NCBI Gene ID	Domains	Only in Cnidaria?	Only in Anthozoa?	Homolog found in immune cells in <i>Stylophora pistillata</i> (1)?
5501851	none	yes	yes	no
5504224	MDN1 super family	yes	yes	no
5516219	none	yes	yes	no
5518710	none	yes	yes	no
116603205	none	yes	yes	No homolog in <i>Stylophora pistillata</i>
116603727	none	yes	yes	No homolog in <i>Stylophora pistillata</i>
116604070	none	yes; except Bacillus spore coat proteins	yes; except Bacillus spore coat proteins	No homolog in <i>Stylophora pistillata</i>
116604505	none	yes	yes	No homolog in <i>Stylophora pistillata</i>
116612667	none	yes	yes	no
116613998	none	yes	yes	no
116616875	none	yes	yes; similar to 116620239	No homolog in <i>Stylophora pistillata</i>
116619128	none	yes	no	yes
116620239	none	yes	yes; similar to 116616875	No homolog in <i>Stylophora pistillata</i>

Dataset 1: DESeq2 results for all RNA-Seq experiments

Dataset 2: All primers, oligos, and probes used in this study

SI References

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