

1 **Single phage proteins sequester TIR- and cGAS-generated signaling molecules**

2 Dong Li^{1,7}, Yu Xiao^{2,7}, Weijia Xiong^{1,7}, Iana Fedorova^{3,7}, Yu Wang^{1,7}, Xi Liu^{1,7}, Erin Huiting³, Jie Ren⁴,
3 Zirui Gao¹, Xingyu Zhao¹, Xueli Cao¹, Yi Zhang¹, Joseph Bondy-Denomy^{3,5,6,*}, Yue Feng^{1,*}

4

5 ¹Beijing Advanced Innovation Center for Soft Matter Science and Engineering, State Key Laboratory
6 of Chemical Resource Engineering, College of Life Science and Technology, Beijing University of
7 Chemical Technology, Beijing 100029, China

8 ²Ministry of Education Key Laboratory of Protein Science, Beijing Advanced Innovation Center for
9 Structural Biology, Beijing Frontier Research Center for Biological Structure, School of Life Sciences,
10 Tsinghua University, Tsinghua-Peking Center for Life Sciences, Beijing 100084, China

11 ³Department of Microbiology and Immunology, University of California, San Francisco, San Francisco,
12 CA, 94158, USA

13 ⁴State Key Laboratory for Biology of Plant Diseases and Insect Pests, Ministry of Agriculture, Institute
14 of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100081, China

15 ⁵Quantitative Biosciences Institute, University of California, San Francisco, San Francisco, CA 94158,
16 USA

17 ⁶Innovative Genomics Institute, Berkeley, CA 94720, USA

18 ⁷Authors contributed equally

19

20 *Correspondence: fengyue@mail.buct.edu.cn (Y. F.), Joseph.Bondy-Denomy@ucsf.edu (J.B.-D.)

21

22

23 **Abstract**

24 Prokaryotic anti-phage immune systems use TIR (toll/interleukin-1 receptor) and cGAS (cyclic GMP-
25 AMP synthase) enzymes to produce 1"-3'1"-2' glycocyclic ADPR (gcADPR) and cyclid di-/tri-
26 nucleotides (CDNs and CTNs) signaling molecules that limit phage replication, respectively ¹⁻³.
27 However, how phages neutralize these common systems is largely unknown. Here, we show that
28 Thoeris anti-defense proteins Tad1 ⁴ and Tad2 ⁵ both have anti-CBASS activity by simultaneously
29 sequestering CBASS cyclic oligonucleotides. Strikingly, apart from binding Thoeris signals 1"-3' and
30 1"-2' gcADPR, Tad1 also binds numerous CBASS CDNs/CTNs with high affinity, inhibiting CBASS
31 systems using these molecules *in vivo* and *in vitro*. The hexameric Tad1 has six binding sites for CDNs
32 or gcADPR, which are independent from two high affinity binding sites for CTNs. Tad2 also sequesters
33 various CDNs in addition to gcADPR molecules, inhibiting CBASS systems using these CDNs.
34 However, the binding pockets for CDNs and gcADPR are different in Tad2, whereby a tetramer can
35 bind two CDNs and two gcADPR molecules simultaneously. Taken together, Tad1 and Tad2 are both
36 two-pronged inhibitors that, alongside anti-CBASS protein 2, establish a paradigm of phage proteins
37 that flexibly sequester a remarkable breadth of cyclic nucleotides involved in TIR- and cGAS-based
38 anti-phage immunity.

39

40 **Introduction**

41 Bacteria encode numerous immune systems that protect them from phage infection ⁶⁻¹¹. In turn, phages
42 also developed mechanisms to antagonize these immune systems and effectively replicate, such as
43 expressing proteins with anti-immune activities, out of which anti-CRISPR (Acr) proteins have been
44 studied extensively ¹²⁻¹⁵. Up until now, phage anti-immune proteins have been discovered for many
45 different systems, including CRISPR-Cas, restriction-modification, and BREX, which largely rely on
46 protein-protein interactions to block immune function ¹⁶. However, recently discovered inhibitors of
47 cyclic nucleotide-based anti-phage systems, like CBASS, Thoeris, Pycsar, and Type III CRISPR-Cas,
48 have revealed the ability of phage proteins to sequester or degrade cyclic nucleotides ^{4,5,17-20}.

49

50 The Thoeris anti-phage system encodes ThsB, a protein with a Toll/interleukin-1 receptor (TIR)
51 domain, which senses phage infection and produces the 1"-3' gcADPR signaling molecule that
52 subsequently activates the NADase effector ThsA ^{1,2,4}. CBASS (cyclic-oligonucleotide-based anti-
53 phage signaling system) encodes a cGAS/DncV-like nucleotidyltransferase (CD-NTase) that produces
54 cyclic dinucleotides (CDNs) or cyclic trinucleotides (CTNs) upon phage infection ³. A broad diversity
55 of CD-NTases has been identified in bacteria ²¹, which are able to produce at least 12 different cyclic
56 oligonucleotide species ²¹⁻²⁶. These cyclic oligonucleotides also bind to a cognate effector, which is
57 proposed to kill the cell and stop successful phage replication.

58

59 Thoeris anti-defense proteins Tad1 and Tad2 antagonize immunity by sequestering the signaling
60 molecule 1"-3' gcADPR as a sponge protein ^{4,5}. For CBASS, two phage proteins have been discovered
61 that antagonize its immunity. Anti-cbass protein 1 (Acb1) degrades the cyclic oligonucleotide signals
62 ¹⁹, and Acb2 is a sponge for CDNs ^{17,18} and CTNs at a distinct binding site ²⁷. Here, we report the
63 surprising observation that Tad1 and Tad2 both also possess anti-CBASS activity by sequestering a
64 breadth of CBASS signals. Strikingly, apart from 1"-3' and 1"-2' gcADPR, Tad1 also binds to CBASS
65 CDNs 2',3'-/3',2'-/3',3'-cGAMP/cUA/cAA/cGG and CTNs cA3/3'3'3'-cAAG (cAAG) with high
66 affinity. Tad2 sequesters CBASS CDNs 3',3'/3',2'/2',3'-cGAMP/cGG/cUG in addition to gcADPR

67 molecules. CBASS is generally more common than Thoeris²⁸, thus these findings greatly broaden our
68 appreciation of the utility of these proteins to the many phages that encode them. Some bacterial
69 species also encode both Thoeris and CBASS immune systems^{11,29}, which Tad1 and Tad2 could inhibit
70 simultaneously. Tad1 and Tad2 are therefore two-pronged inhibitors that block Thoeris and CBASS
71 activity due to the similar nature of their immune signaling molecules despite the independent
72 evolutionary origins of the enzymes that create them.

73

74 Results

75 Tad1 sequesters CBASS cyclic trinucleotides and dinucleotides

76 Due to the overall similarities between signaling molecules used by multiple defense systems, we
77 asked whether anti-Thoeris Tad1 and Tad2 sponges also sequester cyclic nucleotides used in Pycsar,
78 CBASS, or Type III CRISPR-Cas signaling systems³⁰⁻³². Four cyclic mononucleotides (cCMP, cUMP,
79 cGMP and cAMP), eight CDNs (3',3'-cGAMP, cGG, cUG, cUA, cUU, cAA, 2',3'- and 3',2'-cGAMP),
80 two CTNs (cA₃ and cAAG), as well as cA₄ and cA₆ were tested. Surprisingly, native gel assays showed
81 a shift of both CbTad1 (from *Clostridium botulinum* prophage) and CmTad1 (from *Clostridioides*
82 *mangenotii* prophage) upon adding cA₃ or cAAG, and a shift of CbTad1 upon adding 2',3'-cGAMP or
83 3',2'-cGAMP (Extended Data Figure 1). These binding events were further verified by isothermal
84 calorimetry (ITC) experiments (Figure 1a, Extended Data Figure 2), which showed that CbTad1 and
85 CmTad1 bind cA₃ with a *K_D* of ~14.0 and 9.8 nM, respectively, and they bind cAAG with a *K_D* of
86 ~12.5 and 20.1 nM, respectively (Figure 1a). CbTad1 bound 2',3'-cGAMP and 3',2'-cGAMP with a
87 *K_D* of ~31.1 and 24.5 nM, respectively (Figure 1a, Extended Data Figure 2), however, only bound
88 weakly (*K_D* > 0.4 μM) to 3',3'-cGAMP/cGG/cUA/cAA (Figure 1a, Extended Data Figure 2). High-
89 performance liquid chromatography (HPLC) revealed that incubating CbTad1 with cA₃ or 2',3'-
90 cGAMP depleted detectable molecules, but they were detected again in unmodified form after
91 proteolysis of CbTad1 (Figure 1b). The strong binding values reported here are >1 order of magnitude
92 stronger than the reported CmTad1 binding affinity for 1"-2' gcADPR of 241 nM⁴, which was the first
93 identified ligand for this protein. Bioinformatic analysis of the *Clostridium* genus revealed CBASS
94 CD-NTases that produce cA₃/cAAG (CdnD) and 3',2'-cGAMP (CdnG) (Extended Data Figure 3a)
95^{21,22}, highlighting the likely biological driver for the observed binding spectra of CbTad1 and CmTad1.
96 Taken together, these results demonstrate that Tad1 also binds to and sequesters both CTNs and CDNs
97 used in CBASS immunity in addition to gcADPR molecules.

98

99 Tad1 forms a hexamer that binds cyclic trinucleotides and gcADPR with different binding sites

100 To understand how Tad1 interacts with CTNs, we first determined crystal structures of apo CmTad1
101 (2.56 Å) and its complex with cA₃ (2.80 Å) and cAAG (3.27 Å), respectively (Extended Data Table
102 1). Surprisingly, in all the structures solved, CmTad1 is a hexamer (Figure 1c), rather than a dimer as
103 previously proposed for CbTad1⁴. To verify the oligomeric state of Tad1 in solution, we performed
104 static light scattering (SLS) analysis of CmTad1 and CbTad1, which also showed that both are
105 hexamers in solution (Figure 1d). Then we re-examined the structural data of CbTad1 (PDB codes:
106 7UAV and 7UAW), which showed that a hexamer similar as that of CmTad1 could be generated by
107 symmetry operations for both the apo CbTad1 (PDB code: 7UAV) and CbTad1 complexed with 1"-2'
108 gcADPR (PDB code: 7UAW) (Extended Data Figures 4a, b). The Tad1 hexamer can be viewed as a
109 trimer of dimers with Dihedral D3 symmetry (Figure 1c). Analysis of the interface between two dimers
110 showed that each protomer interacts with protomers from the other two dimers (Figure 1e). In CmTad1,

111 R88 and K96 of protomer A interact with T95, Q98 and E99 of protomer C through polar interactions.
112 In turn, Q98 and E99 of protomer A interact with R88 and K96 of protomer E. Meanwhile, M102,
113 W103 and K106 of protomer A also interact with the same residues from protomer F through
114 hydrophobic interactions (Figure 1e). Similar interactions can also be found in the CbTad1 hexamer
115 (Extended Data Figure 4c). Notably, most of these interface residues are conserved among Tad1
116 homologs (Extended Data Figure 4d). Mutation of the interface residues of both CbTad1 and CmTad1
117 showed a significant backwards shift of the protein peak in gel filtration assay, and SLS analysis
118 revealed that both mutant proteins are disrupted into dimers (Figure 1f, Extended Data Figure 4e).
119 Structural superimposition showed that while both the N-terminal anti-parallel β -sheet and C-terminal
120 two helices (α 1 and α 2) align well between CmTad1 and CbTad1, most of the loops linking the β -
121 strands and helices display different conformations in the two proteins (Extended Data Figure 4f).
122

123 Next, we investigated the binding pockets of the CTNs. One Tad1 hexamer binds two CTNs with two
124 distinct binding pockets that are far from the binding pockets for gcADPR molecules (Figure 1g). As
125 expected, cA₃ and cAAG bind at the same pocket in CmTad1 (Extended Data Figures 5a, 5b). In
126 contrast to gcADPR molecules that bind in the pocket located in the interface of the dimer, CTNs are
127 bound in the trimeric interface of the three Tad1 dimers (Figures 1g, Extended Data Figure 5c).
128 Interestingly, the binding mode of CTNs in Tad1 is reminiscent of that of Acb2, which is also a hexamer
129 and similarly binds two CTNs²⁷. As in Acb2, the CTN in Tad1 is also bound mainly through its three
130 phosphate groups, each of which is coordinated by R88 of one CmTad1 protomer and T95 of another
131 protomer through hydrogen bonds (Figure 1h). These two conserved residues are not only involved in
132 binding of CTNs, but also hexamer formation of Tad1 (Extended Data Figure 4d). Consistently,
133 mutation of either of the two corresponding residues in CbTad1, R90A and T97A, markedly reduced
134 cA₃ binding as revealed by native gel assay (Figure 1i). Moreover, C87, A91 and M92 from each
135 CmTad1 protomer also form hydrophobic interactions with the bases of cA₃. The binding mode of
136 CTN indicates that hexamer formation is needed for its binding. As expected, CbTad1 and CmTad1
137 mutations that abolished the hexamer also lost the ability to bind CTNs (Extended Data Figure 6),
138 supporting that hexamer formation is the prerequisite for cA₃ binding.
139

140 To confirm that the binding sites of the CTNs and gcADPR molecules in Tad1 are independent of each
141 other, we tested the binding of 1"-2' gcADPR with the R90A or T97A CbTad1 mutant proteins. A
142 native gel assay showed similar shifts of the two CbTad1 mutants as WT CbTad1 upon adding 1"-2'
143 gcADPR (Figure 1i), suggesting that binding to gcADPR is not affected by the two mutations. In turn,
144 we tested whether binding of CTNs is affected by disruption of the binding sites of gcADPR. We also
145 solved the structure of CbTad1 complexed with 1"-3' gcADPR at 2.16 Å resolution (Extended Data
146 Table 1), whose structure has been recently reported in a preprint, but not released by the Protein Data
147 Bank⁵. The structure showed that one CbTad1 hexamer binds six 1"-3' gcADPR molecules (Extended
148 Data Figure 7a) and the binding mode of 1"-3' gcADPR is similar to that of 1"-2' gcADPR (Extended
149 Data Figure 7b)⁴. Notably, mutation of the binding pocket R109A/R113A or F82A/N92A (Extended
150 Data Figure 7b) exhibited a severely reduced binding to 1"-2' gcADPR (Extended Data Figure 7c).
151 However, these mutations did not interfere with the binding of cA₃ (Extended Data Figure 7c). Taken
152 together, these data collectively show that one Tad1 hexamer binds two CTNs through two pockets
153 independent of those that bind gcADPR molecules.
154

155 Structural alignment between apo CmTad1 and its complexes with CTNs showed that the binding of
156 CTNs does not induce a conformational change of CmTad1, with a root mean square deviation (RMSD)
157 of 0.305 and 0.219 Å (C α atoms) for CmTad1-cA₃ and CmTad1-cAAG compared to the apo CmTad1,
158 respectively (Extended Data Figure 5d, e). This suggests that Tad1 might be able to interact with CTNs
159 and gcADPR molecules simultaneously. Therefore, we co-crystallized CbTad1 with both cA₃ and 1"-
160 3' gcADPR and then solved its crystal structure at a resolution of 2.31 Å (Extended Data Table 1). The
161 structure clearly showed that one CbTad1 hexamer binds to two cA₃ and six 1"-3' gcADPR molecules
162 simultaneously (Figure 1j). Structural alignment between CbTad1-cA₃-1"-3' gcADPR and apo CbTad1
163 also showed little conformational changes except in the binding pocket of 1"-3' gcADPR (Extended
164 Data Figure 5f).

165

166 **Tad1 binds cyclic dinucleotides and gcADPR with the same binding pocket**

167 To understand how CbTad1 interacts with CDNs, we determined the crystal structures of CbTad1
168 complexed with 2',3'-cGAMP at 2.37 Å (Figure 2a and Extended Data Table 1). Surprisingly, 2',3'-
169 cGAMP binds in the same binding pocket as gcADPR molecules in CbTad1 (Figure 2b), and therefore
170 a CbTad1 hexamer binds six 2',3'-cGAMP molecules in total with two in each CbTad1 dimer (Figure
171 2a). Interestingly, comparison with the structure of CbTad1-1"-3' gcADPR complex showed that in the
172 binding pocket, the adenosine monophosphate moiety of 2',3'-cGAMP almost completely overlaps
173 with the corresponding part of 1"-3' gcADPR (Figure 2b). On this side, the C-terminal residues 116-
174 122 of CbTad1 also form an ordered lid to cover the 2',3'-cGAMP molecule as in the CbTad1-1"-3'
175 gcADPR structure. However, the loop linking β 4- α 1 does not move to seal the binding pocket as it
176 does in the CbTad1-1"-3' gcADPR structure, instead keeps a similar conformation as the apo CbTad1
177 (Figure 2b). This makes sense because the same movement of this loop will cause steric clash to 2',3'-
178 cGAMP, especially that after the same movement R57 will even overlap with the guanine base of 2',3'-
179 cGAMP (Extended Data Figure 8a). In the binding pocket of 2',3'-cGAMP, most of the residues that
180 are involved in gcADPR binding also bind to 2',3'-cGAMP, such as F82, N92, R109, and R113 (Figure
181 3c). Consistently, the R109A/R113A and F82A/N92A mutations of CbTad1 which disrupt 1"-3'
182 gcADPR binding (Extended Data Figure 7c) also severely reduced 2',3'-cGAMP binding (Figure 2d).
183 Moreover, on the side of the guanine base, Q8 forms a hydrogen bond with the oxygen atom of the
184 base. The guanine base is also sandwiched by L13 of one protomer and L119 of the other protomer
185 through hydrophobic interactions (Figure 2c).

186

187 To understand why CbTad1 only shows high affinity to 2',3'- and 3',2'-cGAMP among the CDNs we
188 tested, we performed docking studies of 3',3'-/3',2'-cGAMP into the binding pocket of 2',3'-cGAMP
189 in CbTad1 (Extended Data Figure 8b). Structurally, in 2',3'- or 3',2'-cGAMP, a 5'-GMP unit is
190 connected with a 5'-AMP unit via a 2'- 5' (or 3'-5') and a 3'-5' (or 2'-5') phosphodiester bond to form
191 a cyclic structure, which result in the same structure of the cyclic phosphate-ribose backbone
192 (Extended Data Figure 8b). However, in 3',3'-cGAMP, a 5'-GMP unit is connected with a 5'-AMP
193 unit with two 3'-5' phosphodiester bonds, leading to a different structure of cyclic backbone, which
194 might explain its low affinity to CbTad1. Moreover, cUA/cGG/cAA has the same cyclic backbone as
195 3',3'-cGAMP and also has low affinity to CbTad1. Interestingly, while CbTad1 binds to multiple CDNs,
196 CmTad1 only shows weak binding to 3',3'-cGAMP and cUA. Comparison of the 2',3'-cGAMP
197 binding pocket between CbTad1 and CmTad1 showed that most of the loops surrounding 2',3'-cGAMP
198 display different lengths and conformations between the two proteins (Extended Data Figure 8c), and

199 these loops of CbTad1 except the C-terminal loop do not move upon 2',3'-cGAMP binding. Moreover,
200 CbTad1 residues involved in binding to the 5'-GMP moiety of 2',3'-cGAMP are also not conserved in
201 CmTad1 (Extended Data Figure 4d). Similarly as gcADPR, 2',3'-cGAMP binding does not induce a
202 conformational change in the binding pocket of CTNs either (Extended Data Figure 8d). Moreover,
203 mutation of the CTN binding residues does not affect binding of 2',3'-cGAMP (Figure 2d) and vice
204 versa (Extended Data Figure 7c). Then we moved on to co-crystallize CbTad1 with both cA₃ and 2',3'-
205 cGAMP and then solved its crystal structure at a resolution of 1.54 Å (Extended Data Table 1), which
206 clearly showed that a CbTad1 hexamer binds to two cA₃ and six 2',3'-cGAMP molecules
207 simultaneously (Figure 2e). Structural alignment between CbTad1-cA₃-2',3'-cGAMP and apo CbTad1
208 also showed little conformational changes except in the binding pocket of 2',3'-cGAMP (Extended
209 Data Figure 8e).

210

211 **Cyclic dinucleotide binding spectra are different among Tad1 homologs**

212 Different binding spectrum of CDNs between CbTad1 and CmTad1 indicates that Tad1 homologs
213 might show different CDN binding spectra. Generation of a phylogenetic tree using PSI-BLAST to
214 identify Tad1 homologs revealed numerous distinct clades of Tad1 and showed that CbTad1 and
215 CmTad1 are represented on distant branches of the Tad1 phylogenetic tree (Extended Data Figure 9).
216 Importantly, most Tad1 orthologs retain both CDN/gcADPR and CTN binding sites, whereas only 12%
217 and 6% of proteins have substitutions in CDN/gcADPR or CTN binding sites, respectively (Extended
218 Data Figure 9). To test the binding activities of diverse homologs, we purified Tad1 from *Bacillus*
219 *cereus* phage SBSphiJ7 (named SBS Tad1) and *Colidextribacter sp. OB.20* (named ColiTad1) and test
220 their binding to the same array of cyclic oligonucleotides by native gel (Extended Data Figure 10)
221 combined with ITC assays (Extended Data Figure 11). Both SBS Tad1 and ColiTad1 also bind to
222 cA₃/cAAG and cADPR isomers, demonstrating a broadly conserved function of this family.
223 Interestingly, SBS Tad1 binds to 3',3'-cGAMP/cUA with high affinity (K_D values of 48.7 and 53.9 nM,
224 respectively) and 2',3'-/3',2'-cGAMP with low affinity (Figures 3a, Extended Data Figure 10, 11),
225 which is the opposite of CbTad1. Notably, these K_D values are also comparable to the SBS Tad1 binding
226 affinity for 1"-2' and 1"-3' gcADPR of 284 and 210 nM, respectively (Figures 3a, Extended Data
227 Figure 11). Moreover, ColiTad1 binds to 2',3'-cGAMP with high affinity and 3',3'-/3',2'-cGAMP
228 with low affinity. Analysis of the well sequenced *Bacillus cereus* group, which is the bacterial hosts
229 for SBS Tad1 revealed multiple commonly encoded CBASS CD-NTases (i.e. CdnB, CdnD, CdnE, and
230 CdnG) that produce the spectrum of cyclic oligonucleotides that SBS Tad1 binds to (Extended Data
231 Figure 3d). None of the known CD-NTases used in our search (see Methods) were identified in
232 *Colidextribacter* genomes. Taken together, these combined biochemical and bioinformatic results
233 indicate that Tad1 homologs maintain a conserved ability to bind to CBASS CTN and gcADPR signals,
234 with a variable spectrum of high affinity binding to CBASS CDNs (Figure 3b).

235

236 **Tad1 antagonizes Type II-A and Type III-C CBASS immunity**

237 Since SBS Tad1 displays high affinity binding to 3',3'-cGAMP, we tested whether SBS Tad1 can
238 inhibit Type II-A CBASS immunity that uses 3',3'-cGAMP signaling molecules to activate a
239 phospholipase (CapV) effector protein. To this end, we first used *in vitro* CapV activity assay we set
240 up in our previous study ¹⁸. While CapV activity could be activated by 3',3'-cGAMP, its activity was
241 abrogated when SBSTad1 was preincubated with 3',3'-cGAMP (Figure 3c). Following proteolysis of
242 SBS Tad1, the released molecule again activated the CapV activity. The SBS Tad1 mutants

243 F100A/N110A and R127A/R131A, designed based on its conserved CDN binding sites, both displayed
244 decreased 3',3'-cGAMP binding (Extended Data Figure 12) and reduced inhibition on CapV activity
245 (Figure 3c). Compared to SBS Tad1, other Tad1 homologs did not show significant inhibition of CapV
246 activity, likely due to their weak binding to 3',3'-cGAMP (Figure 3d). These results demonstrate that
247 SBS Tad1 antagonizes Type II-A CBASS immunity *in vitro* through sequestering 3',3'-cGAMP
248 signaling molecules.

249

250 To determine whether SBS Tad1 can inhibit this same 3',3'-cGAMP-based CBASS system *in vivo*, the
251 different Tad1 proteins were expressed in the *Pseudomonas aeruginosa* strain BWHPSA011 (Pa011)
252 with an active Type II-A CBASS system¹⁸. We performed phage infection assays with the CBASS-
253 targeted phage PaMx41 that lacks the anti-CBASS gene *acb2* (PaMx41Δ*acb2*). We observed that SBS
254 Tad1, but not CmTad1 or CbTad1, which do not bind tightly to 3',3'-cGAMP, inhibited Type II-A
255 CBASS activity to nearly the same extent as the Acb2 positive control (Figure 3e). To confirm that all
256 assayed proteins express well and retain anti-Thoeris activity *in vivo*, we identified a canonical Thoeris
257 system in the *P. aeruginosa* strain MRSN390231 (Pa231) and expressed it from the chromosome of a
258 strain that naturally lacks all known cyclic nucleotide signaling systems (PAO1). Expression of this
259 Thoeris system reduced the titer of phage F10 by 5 orders of magnitude. However, co-expression of
260 SBS Tad1, CmTad1, and CbTad1 inhibited Thoeris activity and rescued F10 phage titer, whereas Acb2
261 had no impact on Thoeris (Figure 3e). Acb2 also had no observed binding to the gcADPR molecules
262 *in vitro* (Extended Data Figure 13). These data collectively demonstrates that the Pa231 Thoeris system
263 uses a canonical signaling gcAPDR molecule and that Tad1 proteins antagonize canonical Thoeris,
264 with one Tad1 homolog inhibiting a 3',3'-cGAMP CBASS system *in vivo*, consistent with *in vitro*
265 binding patterns.

266

267 Since all Tad1 homologs tested display high affinity binding to CTNs, we tested whether Tad1 can
268 inhibit Type III-C CBASS immunity that uses cA₃ signaling molecules to activate a non-specific
269 endonuclease (NucC) effector protein^{33,34}. Using the NucC enzyme from *P. aeruginosa* strain ATCC
270 27853 (Pa278), we showed that addition of cA₃ activates the DNA cleavage activity, whereas adding
271 WT CbTad1 significantly decreased NucC activity (Figure 3f). Moreover, following proteolysis of
272 CbTad1, the released cA₃ molecule again activated the NucC activity (Figure 3f). The R90A and T97A
273 CbTad1 mutant proteins, which almost lost cA₃ binding, displayed no inhibition of NucC activity. The
274 same Pa278 Type III-C CBASS operon was chromosomally integrated into the PAO1 strain described
275 above. CBASS Pa278 CBASS reduces the titer of phage JBD67Δ*acb2* by 3 orders of magnitude
276 (Figure 3g). Co-expression of SBS Tad1, CbTad1, CmTad1, or an Acb2 control all fully inhibited cA₃-
277 based CBASS activity and rescued JBD67Δ*acb2* phage titer. Together, these data provide *in vivo* and
278 *in vitro* evidence that Tad1 is a flexible anti-CBASS sponge protein, binding to both CDNs and CTNs
279 involved in immunity, as well as an effective anti-Thoeris sponge protein.

280

281 **HgmTad2 sequesters multiple CBASS cyclic dinucleotides**

282 Tad2 is a recently discovered anti-Thoeris sponge identified from *Bacillus cereus* phage SPO1 that
283 works by sequestering gcADPR through a completely different structural fold from Tad1⁵. Using the
284 same array of cyclic nucleotides that we used to study Tad1, we first tested SPO1 Tad2. While binding
285 to gcADPR molecules was confirmed by native gel (Extended Data Figure 14a), no significant shift of
286 SPO1 Tad2 was observed upon adding any of the other cyclic nucleotides (Extended Data Figure 14a),

287 suggesting that SPO1 Tad2 might not bind any of these signaling molecules.
288

289 The Tad2 family of proteins is quite widespread in numerous MGEs (mobile genetic elements) and
290 contains a domain of unknown function DUF2829. This domain was previously found in an anti-
291 CRISPR (Acr), AcrIIA7, derived from human gut metagenomic libraries ³⁵ (short for HgmTad2
292 hereafter). We decided to test whether HgmTad2 also sequesters gcADPR molecules. Interestingly,
293 during purification, HgmTad2 eluted in three separate peaks in the process of ion exchange
294 chromatography (Extended Data Figure 15a), which displayed different migrations in native gel. We
295 collected the three components separately and tested whether they bind to gcADPR molecules. Native
296 gel assay showed a shift of the purified HgmTad2 protein in all the three states upon adding 1''-
297 2'gcADPR (Extended Data Figure 15b), suggesting gcADPR binding. Then, we moved on to solve the
298 structures of HgmTad2, HgmTad2-1''-2' gcADPR as well as HgmTad2-1''-3' gcADPR complexes.
299 These complexes were obtained by expression of HgmTad2 alone or during co-expression with TIR
300 protein from *Brachypodium distachyon*, or co-expression with ThsB from *Bacillus cereus* MSX-D12
301 ⁴, respectively (Extended Data Table 1). Purified HgmTad2 in the three different states were used
302 separately during crystallization. Surprisingly, during structure solution, we found that a clear density
303 with a shape similar to that of 3',3'-cyclic di-GMP (cGG) was visible in all the solved structures using
304 HgmTad2 of States 2 and 3 (Figure 4a), which simultaneously contained gcADPR molecules at a
305 distinct site when co-expressed with gcADPR-producing enzymes. These enzymes and the
306 significance of cGG as a CBASS signaling molecule will be discussed below. However, there was no
307 such density in the structures using HgmTad2 in State 1. This suggested that HgmTad2 in States 2 and
308 3 contains cGG or other similar molecule bound during its expression in *E. coli*. To verify that the
309 density corresponds to cGG, purified HgmTad2 in States 2 and 3 was denatured by heating and filtered
310 to obtain the nucleotide within the protein. The filtered nucleotide showed a similar retention time as
311 cGG, but markedly different from that of 3',3'-cGAMP (Figure 4b), further supporting that the
312 nucleotide within purified HgmTad2 in States 2 and 3 is cGG. However, the sample of HgmTad2 in
313 State 1 after the same procedure showed no peak here (Figure 4b). Together, these findings show that
314 HgmTad2 can bind cGG and gcADPR simultaneously, and a large fraction of the purified HgmTad2
315 contains bound cGG from expression in *E. coli*.
316

317 Binding of cGG by HgmTad2 was unexpected and this inspired us to consider that HgmTad2 might
318 also bind other CDNs. To exclude the influence of bound cGG within HgmTad2 in binding assays, we
319 only collected HgmTad2 in State 1 to test its binding spectrum using native gel assays, which showed
320 a significant shift of HgmTad2 upon adding 3',2'-/3',3'-cGAMP/cGG/cUG and a minor shift upon
321 adding 2',3'-cGAMP, but no shift upon adding CTNs or other nucleotides (Figure 4c). These binding
322 events were further verified by surface plasmon resonance (SPR) experiments, which showed that
323 HgmTad2 binds to 3',2'-/3',3'-/2',3'-cGAMP and cUG with a K_D of ~0.51, 0.83, 670 and 1.01 nM,
324 respectively (Figures 4d, Extended Data Figure 15c). Surprisingly, the binding K_D of cGG to HgmTad2
325 was calculated as high as 24.2 pM, possibly explaining why HgmTad2 stably bound to endogenous
326 cGG during its expression in *E. coli*. HPLC assays demonstrated that HgmTad2 depletes 3',3'-cGAMP,
327 but doesn't degrade it (Figure 4e), while SPO1 Tad2 does not deplete the molecule (Extended Data
328 Figure 11b, c). Taken together, these results demonstrate that HgmTad2 specifically sequesters multiple
329 CDNs used in CBASS immunity in addition to gcADPR molecules.
330

331 **Tad2 binds cyclic dinucleotides and gcADPR with different binding pockets**

332 Next, we will introduce the structures of HgmTad2 and its complexes with gcADPR and CDNs. We
333 solved in total six structures of HgmTad2, which are in apo form (1.70 Å), 1''-2' gcADPR-bound (2.10
334 Å), cGG-bound (1.38 Å), 3',3'-cGAMP-bound form (2.11 Å), 1''-2' gcADPR-cGG-bound (2.28 Å)
335 and 1''-3' gcADPR-cGG-bound (1.98 Å) forms, respectively. Since SPO1 Tad2 structure has not been
336 released by the Protein Data Bank ⁵, we also solved its structure (2.27 Å) to compare with HgmTad2.
337 HgmTad2 forms a tetramer similarly to SPO1 Tad2 (Figure 4f), and the tetrameric state of HgmTad2
338 and SPO1 Tad2 were also verified by SLS analysis (Extended Data Figure 16a). A Tad2 tetramer can
339 be viewed as a dimer of dimers. Two Tad2 protomers interlock with each other to form an "X"-shaped
340 dimer with a buried surface of ~1400 Å². And then, two such dimers further interlock with each other
341 along the axis of helix α1 to form a tetramer, in which each protomer of the dimeric unit interacts with
342 the two protomers within the other unit (Figure 4f). Each HgmTad2 protomer also contains an N-
343 terminal α helix (α1) followed by an antiparallel five-stranded β sheet (β1–2, β5–7) as SPO1 Tad2
344 (Figure 4g). However, in the loop region linking β2 and β5, there are two α helices (α2–α3) and two β
345 strands (β3–β4) in HgmTad2 (Figure 4g, Extended Data Figure 16b), compared to only one α helix in
346 the corresponding loop region of SPO1 Tad2 ⁵. The C-terminal α helix (α4) of HgmTad2 is located
347 between β6–β7.

348

349 Both SPO1 Tad2 and HgmTad2 tetramer bind two gcADPR molecules with two identical binding
350 pockets, which are located in the middle region of the tetramer at the interface of two protomers from
351 different dimeric units (Figure 5a). The gcADPR ligands are surrounded by loop L12 (between β1–
352 β2), helix α4 and the loop linking β6 and α4 of one protomer (a), and loop L12, L56, α4 and β6 of the
353 other (b) (Extended Data Figure 17a). Interestingly, the region between β1–β2 has two different
354 conformations in the apo HgmTad2 structure: Two protomers that will together bind one gcADPR both
355 form an extra helix (residues 19–24) away from each other in this region. The other two protomers both
356 form a loop much nearer to each other (Extended Data Figure 17b). Interestingly, in the gcADPR-
357 bound structure, all the four protomers form a loop in this region similar to that in the apo form
358 (Extended Data Figure 17b), suggesting that this region of HgmTad2 is flexible and binding of
359 gcADPR ligands will induce and stabilize it as a loop covering the ligand. Specifically, for 1''-2'
360 gcADPR, its adenine base is coordinated by hydrogen bonds from T92_a and water-mediated hydrogen
361 bonds from T82_b, N22_a and mainchain oxygen and nitrogen atoms of L88_b. Moreover, the adenine base
362 is also stabilized by hydrophobic interactions from M76_a, A78_a and V84_a (Figure 5b). The diphosphate
363 backbone is bound by N22 and G23 from both protomers. The free hydroxyls in the ribose–ribose
364 linkage are coordinated by hydrogen bonds from W87_a, L88_a, D93_a and D93_b (Figure 5b). Supporting
365 this, mutations W21A/N22A and S90A/T92A/D93A of HgmTad2 markedly reduced both its binding
366 to 1''-2' gcADPR (Extended Data Figure 17c) and its inhibition effect on 1''-2' gcADPR-activated
367 NADase activity of ThsA (Figure 5c).

368

369 HgmTad2 additionally binds to CDNs 3',3'-cGAMP/cGG/cUG and 3',2'-cGAMP in a distinct region
370 of the protein (Figure 5d). An HgmTad2 tetramer binds two CDNs with two identical binding pockets,
371 which are located at the top and bottom ends of the tetramer at the interface of two protomers within
372 one dimeric unit (Figure 5d). As expected, cGG and 3',3'-cGAMP bind at the same binding pocket
373 (Extended Data Figure 17d, e). Each pocket is a symmetrical one, surrounded by the loop between α2
374 and α3, β2, β3 and the loop between them, as well as the C-terminal residue of β6 from both protomers

375 (Extended Data Figure 17f). Notably, almost all of these are structural elements in the insertion
376 (residues 32-72) between β 2 and β 5 of HgmTad2 (Extended Data Figure 16b), which is nearly twice
377 the size of that in SPO1 Tad2 (residues 36-59). Binding of cGG causes some conformational changes
378 to the structural elements surrounding the molecule in the binding pocket (Extended Data Figure 17g).
379 In the HgmTad2-cGG complex, ligand binding is mediated by extensive hydrophobic and polar
380 interactions. The guanine base is stabilized by hydrophobic interactions from L36 and F70. Moreover,
381 it is coordinated by hydrogen bonds from R31 and N85 from one protomer, and mainchain carbonyls
382 of P32 from the other protomer (Figure 5e), as well as water-mediated interactions from D34 and main
383 chain carbonyls of T71 from the other protomer (Figure 5e). However, for the adenine base of 3',3'-
384 cGAMP, only one water mediated interaction can be formed by HgmTad2 apart from hydrophobic
385 interactions from L36 and F70 (Extended Data Figure 17h), which may explain the high binding
386 affinity of cGG and the inability of binding to cAA by HgmTad2. For the phosphate-ribose backbone,
387 the phosphate group is coordinated by polar interactions from S47 and mainchain nitrogen atom of
388 K46. To verify these residues, we mutated interacting residues of HgmTad2 (S47A, F70A,
389 R31A/N85A) and tested their binding to both cGG and 3',3'-cGAMP. Consistently, native gel showed
390 no shift of these mutants upon adding either cGG or 3',3'-cGAMP (Figure 5f, Extended Data Figure
391 17i).
392

393 To further confirm that the binding sites of the cyclic dinucleotides and gcADPR in HgmTad2 are
394 independent of each other, we tested the binding of 1"-2' gcADPR with S47A, F70A and R31A/N85A
395 mutant proteins, as well as the binding of cGG/3',3'-cGAMP with W21A/N22A and
396 S90A/T92A/D93A mutant proteins. The results showed that mutation of either binding site does not
397 decrease the binding of the other ligand (Figure 5g). This is also consistent with the fact that we
398 obtained the co-structures of HgmTad2-1"-2'-gcADPR-cGG (Figure 5h) and HgmTad2-1"-3'-
399 gcADPR-cGG (Figure 5i). Taken together, an HgmTad2 tetramer can bind to two cyclic dinucleotides
400 and two gcADPR molecules simultaneously.
401

402 **Tad2 binds cyclic dinucleotides with its insertion domain**

403 As mentioned above, HgmTad2 binds CDNs with its insertion between β 2 and β 5, which region is
404 much shorter in SPO1 Tad2. Structural superimposition shows that while HgmTad2 and SPO1 Tad2
405 are similar in the gcADPR-binding domain, they are highly different in the CDN binding domain
406 (Figure 6a). The insertion domain of HgmTad2 stretches out through an anti-parallel β sheet (β 3-4) to
407 create a cavity for binding of cyclic dinucleotides. However, the insertion in SPO1 Tad2 is much
408 smaller and displays a highly different conformation (Figure 6a). Since the binding to cGG results
409 from the insertion between β 2 and β 5 of HgmTad2 (Extended Data Figure 16b), we performed a
410 sequence-based analysis to search for Tad2 homologs with long insertions like HgmTad2 that may
411 enable binding to CDNs (Figure 6b, Extended Data Figure 18). Interestingly, two of such Tad2
412 homologs from *Sphingobacterium thalpophilum* (SptTad2) and *Salegentibacter* sp.BDJ18 (SaTad2)
413 with similarly long insertions also bind to CDNs with different affinities (Figures 6c-e, Extended Data
414 Figure 19). Notably, SptTad2 also binds cGG with a high affinity of 0.23 nM (Figure 6c), and purified
415 SptTad2 also contains cGG bound during expression. To investigate whether SptTad2 uses a similar
416 binding mode to bind CDNs, we solved the structure of SptTad2 bound with cGG (Figure 6f).
417 Structural superimposition showed that the cGG binding pocket is highly similar between HgmTad2
418 and SptTad2 (Figure 6g), thereby demonstrating that Tad2 homologs can bind CDNs with their large

419 insertion domain (35-41 residues) between β 2 and β 5. Interestingly, phylogenetic analysis showed that
420 the organization of this domain is highly variable in distant Tad2 homologs, which might reflect their
421 different CDN binding activity. The gcADPR binding site, however, is highly conserved with only 1%
422 of Tad2 proteins predicted to be non- functional in gcADPR binding (only short Tad2 versions of 120-
423 170 amino acids length were used for analysis) (Extended Data Figure 18).

424

425 **Tad2 antagonizes Type I-D and Type II-A CBASS *in vitro***

426 Diguanylate cyclases (DGCs) produce cGG in many Gram-negative bacteria that are distinct enzymes
427 from the CD-NTases that make CBASS nucleotides. cGG signaling in many bacteria controls motility
428 and biofilm formation ³⁶. Interestingly, HgmTad2 is encoded by *Bacteroides* phages. In *Bacteroides*,
429 these EAL-containing DGCs are absent, but instead cGG signaling is mediated by a CBASS CD-NTase
430 (CdnE) that signals to a TIR- or TM-STING fusion effector protein ³⁷. Upon further investigation of
431 *Bacteroides* and *Sphingobacterium* genomes, which are the bacterial hosts of HgmTad2- and SptTad2-
432 encoding phage, respectively, we found that the bacteria both encode CBASS CD-NTases that are
433 known to produce cGG (CdnE and CdnB) (Extended Data Figure 3b, c) ^{21,37}, providing a biologically
434 necessary role for these Tad2 proteins to strongly bind and sequester cGG signaling molecules. As
435 such, we tested whether HgmTad2 or SptTad2 can inhibit Type I-D CBASS immunity that uses cGG
436 signaling molecules with a previously reported TIR-STING activity assay ³⁷. While activity of TIR-
437 STING from *Sphingobacterium faecium* DSM 11690 could be activated by cGG, its activity was
438 abrogated when HgmTad2 or SptTad2 was preincubated with cGG (Figure 6h). The R31A/N85A,
439 S47A, and F70A HgmTad2 mutant proteins, which exhibited decreased cGG binding, displayed
440 reduced inhibition of TIR-STING activity (Figure 6g). Moreover, following proteolysis of HgmTad2,
441 the released cGG again partially activated the TIR-STING activity (Figure 6i). These results
442 demonstrate that Tad2 antagonizes Type I-D CBASS immunity *in vitro* through sequestering cGG
443 molecules.

444

445 Since HgmTad2/SptTad2/SaTad2 also display high affinity binding to 3',3'-cGAMP, we tested whether
446 they inhibit Type II-A CBASS immunity using the previous mentioned CapV activity assay. While
447 CapV activity is activated by 3',3'-cGAMP, it was abrogated when the cGG-free form of HgmTad2 or
448 SptTad2 or SaTad2 was preincubated with 3',3'-cGAMP (Extended Data Figure 20a). The
449 R31A/N85A, S47A and F70A HgmTad2 mutant proteins, which exhibited decreased 3',3'-cGAMP
450 binding, also displayed reduced inhibition of CapV activity (Extended Data Figure 20b). Moreover,
451 following proteolysis of HgmTad2, the released 3',3'-cGAMP molecule again activated the CapV
452 activity (Extended Data Figure 20b). These results demonstrate that Tad2 antagonizes Type II-A
453 CBASS immunity *in vitro* through sequestering the 3',3'-cGAMP molecule. Despite the Tad2 proteins
454 inhibiting Thoeris activity *in vivo* (Extended Data Figure 20c), we did not observe inhibition of the
455 Pa011 Type II-A CBASS activity *in vivo*, which is likely because the 3',3'-cGAMP binding site is
456 saturated with the highly abundant and common cGG nucleotide in *P. aeruginosa* (Extended Data
457 Figure 20d).

458

459 **Tad2 does not antagonize SpyCas9 activity**

460 We have demonstrated that HgmTad2 could simultaneously inhibit CBASS and Thoeris immunity as
461 a sponge protein with two different binding pockets. However, this protein has been previously
462 identified as an anti-CRISPR (Acr) protein, AcrIIA7 ³⁵, whose inhibitory mechanism is unknown.

463 HgmTad2 was previously shown to not interact with SpyCas9, but somehow inhibit its activity. It
464 seemed surprising to us that this protein might have three inhibitory activities. Therefore, to query this
465 activity and investigate the anti-CRISPR mechanism of HgmTad2, we first repeated the *in vitro*
466 SpyCas9 cleavage assay in Uribe et al. 2019. Despite many trials and optimization of the reaction
467 system, we still did not see Acr activity of HgmTad2 or the other Tad2 homologs in this study where
468 AcrIIA11 successfully inhibits SpyCas9-mediated DNA cleavage (Extended Data Figure 20e).
469 Consistent with this, chromosomal integration of SpyCas9 into the *P. aeruginosa* strain PAO1
470 demonstrated that the JBD30 phage is targeted³⁸, but HgmTad2 did not exhibit Acr activity (Extended
471 Data Figure 20f). Taken together, our data suggests that HgmTad2 inhibits CBASS and Thoeris, but
472 not the CRISPR-Cas9 system.

473

474 Discussion

475 Thoeris and CBASS are two anti-phage systems that use different signaling molecules to mediate
476 immunity. Two anti-immune proteins, Tad1 and Tad2, have been identified for Thoeris system and
477 two anti-immune proteins, Acb1 and Acb2, for CBASS. Here, we demonstrated that anti-Thoeris
478 proteins Tad1 and Tad2 also inhibit CBASS systems, which are generally more common, by
479 sequestering a broad array of CDNs and CTNs. Therefore, Tad1 and Tad2 are the first phage-encoded
480 sponge proteins that sequester multiple signaling molecules that are involved in two different anti-
481 phage immune systems. Notably, Tad1 and Tad2 sequester cyclic oligonucleotides with completely
482 distinct mechanisms. Tad1 is a hexamer that is assembled as a trimer of dimers. One Tad1 hexamer
483 sequesters two CTNs using two separate pockets formed only in the case of the hexameric assembly,
484 in which each pocket is composed of three interlocking protomers. In addition to CTNs, Tad1 also
485 sequesters CDNs using the same binding pocket as gcADPR molecules. By contrast, Tad2 is a tetramer
486 that binds two CDNs and two gcADPR molecules simultaneously. The binding pocket of CDNs in
487 Tad2 is far from that of gcADPR and is also different from those of other known CDN binding proteins.
488 Among the CDNs tested, HgmTad2 binds strongly to 3',3'-cGAMP/cGG/cUG and 3',2'-cGAMP, and
489 weakly to 2',3'-cGAMP. Notably, both Tad1 and Tad2 sequester 3',2'-cGAMP, a signaling molecule
490 that is not cleaved by Acb1, but has been recently implicated in both CBASS and cGAS-like signaling
491 systems in eukaryotes^{22,39,40}.

492

493 Surprisingly, HgmTad2 displayed a pM-range binding affinity to cGG, which is much higher than any
494 reported binding affinities to CDNs, and also much higher than affinities of HgmTad2 to other CDNs
495 (Figure 4d). This explains why purified HgmTad2 contains cGG that is bound during its expression in
496 *E. coli*. Moreover, cGG is a molecule that is not cleaved by Acb1 nor sequestered by Acb2. Therefore,
497 to our knowledge, HgmTad2 is the first phage anti-immune protein to act as a cGG sponge, which
498 might provide a useful reagent for studying cGG signaling not related to phage defense. In bacteria,
499 cGG is the most widespread CDN that functions as a signaling molecule, regulating multiple aspects
500 of bacterial growth and behavior, including motility, virulence, biofilm formation, and cell cycle
501 progression³⁶. In *Bacteroides*, however, there are at least two known CD-NTases that produce cGG as
502 a signaling molecule in CBASS immunity^{21,37}. Bioinformatic analyses demonstrate that cGG-based
503 CBASS immunity is found in bacteria that HgmTad2 and SptTad2-encoding phages may infect.

504

505 While the binding mode of CTNs is similar between Tad1 and Acb2, the assembly mechanism of the
506 hexamer and residues involved in binding are different between these proteins²⁷. Furthermore, our

507 recent study on Acb2 demonstrates that it functions as a sponge that binds to both CDNs and CTNs
508 used by a single bacterial anti-phage system. However, in the present study, we report that both Tad1
509 and Tad2 are sponge proteins that bind to a broad array of cyclic oligonucleotides from two
510 independent anti-phage systems. Therefore, since bacterial species may contain both CBASS and
511 Thoeris systems^{11,29}, Tad1 and Tad2 represent a unique class of proteins that are advantageous over
512 the pan-immune arsenal of their host. Altogether, our findings demonstrate the remarkable potency of
513 two anti-immune sponge proteins. Together with Acb2, these new data on Tad1 and Tad2 establish a
514 paradigm of anti-immune sponge proteins with >1 binding site. We predict that a broad distribution of
515 anti-immune sponges with multiple binding sites for signaling molecules may exist for anti-viral
516 immune signaling systems across all domains of life.

517

518 **Acknowledgments**

519 We thank the staff at beamlines BL02U1 and BL19U1 of the Shanghai Synchrotron Radiation Facility
520 for their assistance with data collection. We thank the Tsinghua University Branch of China National
521 Center for Protein Sciences Beijing and Dr. Shilong Fan for providing facility support for X-ray
522 diffraction of the crystal samples. We thank Drs. Yuanyuan Chen, Zhenwei Yang, Bingxue Zhou at the
523 Institute of Biophysics, Chinese Academy of Sciences for technical help with ITC and SPR
524 experiments. Y. F. is supported by National key research and development program of China
525 (2022YFC3401500 and 2022YFC2104800), the National Natural Science Foundation of China
526 (32371329 and 32171274), Beijing Nova Program (20220484160) and the Fundamental Research
527 Funds for the Central Universities (QNTD2023-01). E.H. is supported by the National Science
528 Foundation Graduate Research Fellowship Program [Grant No. 2038436]. Any opinions, findings, and
529 conclusions or recommendations expressed in this material are those of the authors and do not
530 necessarily reflect the views of the National Science Foundation. J.B.-D. is supported by the National
531 Institutes of Health [R21AI168811, R01GM127489], the Vallee Foundation, and the Searle
532 Scholarship.

533

534 **Author contributions**

535 Y.F. and J.B.-D. conceived and supervised the project and designed experiments. D.L., W.X., Y.W.,
536 X.L., Z.G., X.Z. and X.C. purified the proteins, grew and optimized the crystals, collected the
537 diffraction data and performed *in vitro* activity analysis and binding assays. Y.X. solved the crystal
538 structures with the help of Y.F. and Y.Z.. I.F. performed *in vivo* phage experiments, strain engineering,
539 and Tad1/Tad2 protein bioinformatics. J. R. performed HPLC assays. E.H. executed cyclase
540 bioinformatics. Y.F. wrote the original manuscript. J.B.-D., Y.F., I.F., and E.H. revised the manuscript.

541

542 **Declaration of interests**

543 J.B.-D. is a scientific advisory board member of SNIPR Biome and Excision Biotherapeutics, a
544 consultant to LeapFrog Bio and BiomX, and a scientific advisory board member and co-founder of
545 Acrigen Biosciences. The Bondy-Denomy lab received research support from Felix Biotechnology.

546

547 **Data Availability**

548 The accession numbers for the coordinate and structure factors reported in this paper are PDB: 8KBB
549 (apo-CmTad1), 8KBC (CmTad1-cA₃), 8KBD (CmTad1-cAAG), 8KBE (CbTad1-1",3'-gcADPR) ,
550 8KBF (CbTad1-1",3'-gcADPR-cA₃), 8KBG (CbTad1-2',3'-cGAMP), 8KBH (CbTad1-2',3'-cGAMP-

551 cA₃), 8KBI (apo-HgmTad2), 8KBJ (HgmTad2-1",2'-gcADPR) 8KBK (HgmTad2-1",2'-gcADPR-
552 cGG), 8KBL (HgmTad2-1"-3'-gcADPR-cGG), 8KBM (HgmTad2-cGG), 8WJC (HgmTad2-3',3'-
553 cGAMP), 8WJD (SptTad2-cGG) and 8WJE (apo-SPO1 Tad2). This paper does not report original code.
554 Any additional information required to reanalyze the data reported in this paper is available from the
555 corresponding authors upon request.
556

557 Materials and Methods

558 Bacterial strains and phages

559 The *P. aeruginosa* strains (BWHP011, ATCC 27853, MRSN390231, PAO1) and *E. coli* strains
560 (DH5a) were grown in Lysogeny broth (LB) medium at 37°C both with aeration at 225 r.p.m. Bacteria
561 plating was performed on LB broth supplemented with gentamicin for maintaining pHERD30T
562 plasmid (50 µg ml⁻¹ for *P. aeruginosa* and 20 µg ml⁻¹ for *E. coli*), as well as with 10 mM MgSO₄ for
563 phage spot assays. Gene expression in *P. aeruginosa* was induced by the addition of 0.2% L-arabinose
564 or 0.3 mM isopropyl-β-D-thiogalactopyranoside IPTG unless stated otherwise. The *E. coli* BL21 (DE3)
565 strain was used for recombinant protein overexpression and grown in Lysogeny broth (LB) medium.
566 The cells were grown at 37°C until OD_{600nm} reached 0.8 and then induced at 18°C for 12 h.
567

568 Protein expression and purification

569 The *Clostridium botulinum* Tad1, *Clostridioides manganotii* Tad1, *SBSphiJ7* Tad1, *Colidexitribacter*
570 Tad1, *Sphingobacterium thalpophilum* Tad2, *Salegentibacter* sp. BDJ18 Tad2, SPO1 Tad2, *P.*
571 *aeruginosa* BWHP011 CapV, *P. aeruginosa* ATCC 27853 NucC, *Bacillus cereus* MSX-D12 ThsA,
572 *Sphingobacterium faecium* DSM 11690 STING and *S. pyogenes* Cas9 genes were synthesized by
573 GenScript and codon-optimized for expression in *E. coli*. The full-length CmTad1, SBS Tad1,
574 ColiTad1, SptTad2, SaTad2, EcTad2, ThsA, CapV, NucC, SfSTING and SpyCas9 gene was amplified
575 by PCR and cloned into a modified pET28a vector in which the expressed protein contains a His6 tag
576 or His6-SUMO tag. The full-length CbTad1 gene was amplified by PCR and cloned into a modified
577 pRSFDuet vector in which the expressed CbTad1 protein contains a His6 tag. The Tad1 or Tad2
578 mutants were generated by two-step PCR and were subcloned, overexpressed and purified in the same
579 way as wild-type protein. All the proteins were expressed in *E. coli* strain BL21 (DE3) and induced by
580 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the cell density reached an OD_{600nm} of 0.8.
581 After growth at 18°C for 12 h, the cells were harvested, resuspended in lysis buffer (50 mM Tris-HCl
582 pH 8.0, 300 mM NaCl, 10 mM imidazole and 1 mM PMSF) and lysed by sonication. The cell lysate
583 was centrifuged at 20,000 g for 50 min at 4°C to remove cell debris. The supernatant was applied onto
584 a self-packaged Ni-affinity column (2 mL Ni-NTA, Genscript) and contaminant proteins were
585 removed with wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 30 mM imidazole). Then the protein
586 was eluted with elute buffer (50 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole). The eluant of
587 protein was concentrated and further purified using a Superdex-200 increase 10/300 GL (GE
588 Healthcare) column equilibrated with a buffer containing 10 mM Tris-HCl pH 8.0, 200 mM NaCl and
589 5 mM DTT. The purified proteins were analyzed by SDS-PAGE. The fractions containing the target
590 protein were pooled and concentrated. Specifically, SBS Tad1 was purified in the same approach as
591 above, but the buffer pH was 8.8, with 500 mM NaCl and 10% glycerol throughout the whole
592 purification process.
593

594 The cells expressing CapV were resuspended with lysis buffer containing 50 mM phosphate buffer pH
595 7.4, 300 mM NaCl, 10% glycerol (v/v). The CapV proteins bound to Ni-NTA beads were washed with
596 a buffer containing 50 mM phosphate buffer pH 7.4, 300 mM NaCl, 10% glycerol (v/v), 30 mM
597 imidazole and then eluted with the 50 mM phosphate buffer pH 7.4, 300 mM NaCl, 10% glycerol (v/v),
598 300 mM imidazole. The eluant of CapV was concentrated and further purified using a Superdex-200
599 increase 10/300 GL (GE Healthcare) column equilibrated with a reaction buffer containing 50 mM
600 phosphate buffer pH 7.4, 300 mM NaCl, 10% glycerol (v/v). The purified protein was analyzed as
601 described above. The fusion protein of NucC with His6-SUMO tag was digested with Ulp1 on the Ni-
602 NTA column at 18°C for 2 h after removing contaminant proteins with wash buffer. Then the NucC
603 protein was eluted with wash buffer. The eluant of NucC was concentrated and further purified as His-
604 tagged proteins as described above.
605

606 The full-length HgmTad2 and AcrIIA11 gene was synthesized by GenScript and amplified by PCR
607 and cloned into pGEX6p-1 to produce a GST-tagged fusion protein with a PreScission Protease
608 cleavage site between GST and the target protein. The HgmTad2 mutants were subcloned,
609 overexpressed and purified in the same way as wild-type protein. The proteins were expressed and
610 induced similarly as above. After growth at 16°C for 12 h, the cells were harvested, re-suspended in
611 lysis buffer (1×PBS, 2 mM DTT and 1 mM PMSF) and lysed by sonication. The cell lysate was
612 centrifuged at 18,000 g for 50 min at 4°C to remove cell debris. The supernatant was applied onto a
613 self-packaged GST-affinity column (2 mL glutathione Sepharose 4B; GE Healthcare) and contaminant
614 proteins were removed with wash buffer (1×PBS, 2 mM DTT). The fusion protein was then digested
615 with PreScission protease at 16°C for 2 hours. The protein with an additional five-amino-acid tag
616 (GPLGS) at the N-terminus was eluted with buffer containing 25 mM HEPES pH 7.5, 200 mM NaCl,
617 and 2 mM DTT. The eluant was concentrated and further purified using a Superdex-200 (GE
618 Healthcare) column equilibrated with a buffer containing 10 mM Tris-HCl pH 8.0, 200 mM NaCl, and
619 5 mM DTT. And then, the HgmTad2 protein was desalted into QA buffer containing 25 mM Tris pH
620 8.0, 10 mM NaCl and 2 mM DTT by a desalting column (GE Healthcare), and was further purified by
621 ion exchange chromatography with Resource Q column (GE Healthcare). The protein bound to the
622 column was eluted with a gradient concentration of 10-100 mM NaCl, and then protein purity and
623 states were verified with native PAGE and SDS-PAGE, respectively, together with the protein sample
624 flowed through the column. Selenomethionine (Se-Met)-labelled HgmTad2 was expressed in *E. coli*
625 B834 (DE3) cells grown in M9 minimal medium supplemented with 60 mg/L SeMet (Acros) and
626 specific amino acids: Ile, Leu and Val at 50 mg/L; Lys, Phe and Thr at 100 mg/L. The SeMet protein
627 was purified as described above. The four Acb2 homologs were cloned and purified as described
628 previously²⁷.
629

630 Crystallization

631 All the protein samples in this study were diluted in buffer containing 10 mM Tris-HCl pH 8.0, 200 mM
632 NaCl and 5 mM DTT before crystallization. Each protein was crystallized at 18°C using the following
633 conditions:

634

635 (1) apo CmTad1/HgmTad2:

636 The concentration of both proteins was 30 mg/mL. The crystals of CmTad1 were grown for 3-4 days
637 using reservoir solution containing 2.0 M ammonium sulfate, 0.1 M sodium HEPES pH 7.5 and 1.4%

638 v/v PEG 400. The crystals of HgmTad2 were grown for 2-3 days using reservoir solution containing
639 1.0 M lithium chloride, 0.1 M citrate pH 4.0, 20% w/v PEG 6000. Before being harvested, the crystals
640 were cryoprotected in the reservoir solution containing 20% glycerol before flash-freezing in liquid
641 nitrogen.

642

643 (2) CmTad1 complexed with cA₃/cAAG, CbTad1 complexed with cA₃/2',3'-cGAMP+cA₃:
644 Prior to crystallization, cA₃ or cAAG was mixed with protein at a molar ratio of 0.8:1, and 2',3'-cGAMP
645 was mixed with protein at a molar ratio of 1.2:1. The crystals of CmTad1-cA₃ and CmTad1-cAAG
646 grew to full size in about 4-5 days, their reservoir solution contains 1.6 M ammonium sulfate, 10% v/v
647 1,4-Dioxane. The crystallization condition of CbTad1-cA₃ was 0.1 M MIB (sodium malonate dibasic
648 monohydrate, imidazole, boric acid) pH 6.0, 55% v/v MPD, and the crystallization condition of the
649 CbTad1-cA₃ was 0.1 M PCTP (sodium propionate, sodium cacodylate trihydrate, Bis-Tris propane)
650 pH 8.0, 60% MPD.

651

652 (3) CbTad1 complexed with 1"-3' gcADPR/1"-3' gcADPR+cA₃:
653 CbTad1 co-expressed with ThsB' was purified, and then was mixed with cA₃ at a molar ratio of 1: 0.8.
654 The crystals of purified CbTad1 or its mix with cA₃ was grown in reservoir solution containing 3.2 M
655 ammonium sulfate and 0.1 M citrate pH 5.0 for 4-5 days. They were stored in antifreeze containing
656 20% glycerol and quick frozen with liquid nitrogen.

657

658 (4) HgmTad2 complexed with cGG/1"-2' gcADPR/1"-2' gcADPR+cGG/3',3'-cGAMP:
659 These four structures were crystallized in the same condition containing 1.0 M lithium chloride, 0.1 M
660 Citrate pH 4.0, 20% w/v PEG 6000. For HgmTad2 complexed with cGG, purified HgmTad2 in the
661 cGG-bound state was used. For HgmTad2 complexed with 1"-2' gcADPR or 1"-2' gcADPR+cGG,
662 HgmTad2 co-expressed with BdTIR was purified, and no-cGG or cGG-bound form was used,
663 respectively. For HgmTad2 complexed with 3',3'-cGAMP, HgmTad2 in the no-cGG state was used and
664 mixed with 3',3'-cGAMP at a molar ratio of 1:1.2.

665

666 (5) HgmTad2 complexed with 1"-3' gcADPR+cGG:
667 HgmTad2 co-expressed with ThsB' was purified and the cGG-bound form was used in crystallization.
668 The crystallization condition was 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6,
669 25% w/v PEG 4000.

670

671 (6) apo SPO1 Tad2:
672 After purifying SPO1 Tad2, the protein was diluted to 24 mg/mL, and then grown under the conditions
673 that 0.5 M ammonium sulfate, 1.0 M sodium citrate tribasic dihydrate pH 5.6, 1.0 M lithium sulfate
674 monohydrate conditions for about 1 week.

675

676 (7) SptTad2-cGG:
677 The SptTad2 protein purified from *E. coli* Bl-21 naturally carries c-di-GMP. The protein was diluted
678 to 24 mg/mL, and then grown under the condition containing 0.3 M magnesium nitrate hexahydrate, 0.1
679 M Tris pH 8.0, 23% w/v PEG2000 for 4-5 days, and then transferred into antifreeze and then flash-
680 freezing in liquid nitrogen.

681

682 **Data collection, structure determination and refinement**
683 All the data were collected at SSRF beamlines BL02U1 and BL19U1, integrated and scaled using the
684 HKL2000 package ⁴¹. The initial model of CbTad1 was used from PDB: 7UAV. The initial models of
685 CmTad1, SPO Tad2 and SptTad2 were obtained using AlphaFold2 ⁴². The structure of apo HgmTad2
686 was solved by SAD phasing, using Autosol in PHENIX ⁴³. The structures of protein complexed with
687 cyclic oligonucleotides were solved through molecular replacement and refined manually using COOT
688 ⁴⁴. All the structures were further refined with PHENIX ⁴³ using non-crystallographic symmetry and
689 stereochemistry information as restraints. The final structure was obtained through several rounds of
690 refinement. Final Ramachandran statistics: 96.75% favoured, 3.25% allowed and 0% outliers for apo-
691 CmTad1-Zn structure; 96.62% favoured, 3.38% allowed and 0% outliers for CmTad1-Zn-cA₃; 96.75%
692 favoured, 3.25% allowed and 0% outliers for CmTad1-Zn-cAAG structure; 97.59% favoured, 2.41%
693 allowed and 0% outliers for CbTad1-1",3'-gcADPR structure; 96.37% favoured, 3.63% allowed and
694 0% outliers for CbTad1-1",3'-gcADPR-cA₃ structure; 96.99% favoured, 3.01% allowed and 0%
695 outliers for CbTad1-2",3'-cGAMP structure; 97.82% favoured, 2.18% allowed and 0% outliers for
696 CbTad1-2",3'-cGAMP-cA₃ structure; 97.55% favoured, 2.45% allowed and 0% outliers for apo-
697 HgmTad2 structure; 96.32% favoured, 3.68% allowed and 0% outliers for HgmTad2-1",2'-gcADPR
698 structure; 95.34% favoured, 4.66% allowed and 0% outliers for HgmTad2-1",2'-gcADPR-cGG
699 structure; 97.06% favoured, 2.94% allowed and 0% outliers for HgmTad2-1"-3'-gcADPR-cGG
700 structure; 97.06% favoured, 2.94% allowed and 0% outliers for HgmTad2-cGG structure; 98.77%
701 favoured, 1.23% allowed and 0% outliers for HgmTad2-3",3'-cGAMP structure; 97.98% favoured, 2.02%
702 allowed and 0% outliers for SptTad2-cGG structure; 98.63% favoured, 1.37 allowed and 0% outliers
703 for apo-SPO1 Tad2 structure. Structural illustrations were generated using PyMOL (<https://pymol.org/>).
704 Data collection and structure refinement statistics are summarized in Extended Data Table 1.
705

706 **Isothermal titration calorimetry binding assay**
707 The dissociation constants of binding reactions of CmTad1/CbTad1 with cA₃/cAAG/3",2"-
708 cGAMP/2",3"-cGAMP/3",3"-cGAMP/cAA/cGG/cUG/cUA/cUU, SBS Tad1 with cA₃/cAAG/3",2"-
709 cGAMP/2",3"-cGAMP/3",3"-cGAMP/cUA/1"-2" gcADPR/1"-3" gcADPR, and ColiTad1 with
710 cA₃/cAAG/3",2"-cGAMP/2",3"-cGAMP/3",3"-cGAMP were determined by isothermal titration
711 calorimetry (ITC) using a MicroCal ITC200 calorimeter. All the protein and cyclic-oligonucleotides
712 were desalted into the working buffer containing 20 mM HEPES pH 7.5 and 200 mM NaCl. The
713 titration, for example, was carried out with 19 successive injections of 2 μ L cA₃/cAAG at 25 μ M
714 concentration, spaced 120 s apart, into the sample cell containing CbTad1 with a concentration of 5
715 μ M by 700 rpm at 25°C. Correspondingly, 3",2"-cGAMP/2",3"-cGAMP at 150 μ M concentration was
716 titrated into 50 μ M CbTad1. cA₃/cAAG at 150 μ M concentration was titrated into 30 μ M CmTad1,
717 and 3",2"-cGAMP/2",3"-cGAMP/3",3"-cGAMP/cAA/cGG/cUG/cUA/cUU at 300 μ M concentration
718 was titrated into 100 μ M CmTad1. For SBS Tad1, 3",2"-cGAMP/3",3"-cGAMP/2",3"-
719 cGAMP/cUA/1"-2" gcADPR/1"-3"gcADPR at 300 μ M concentration was titrated into 100 μ M SBS
720 Tad1, and 3",2"-cGAMP/3",3"-cGAMP/2",3"-cGAMP at 300 μ M concentration was titrated into 100
721 μ M ColiTad1. For both SBS Tad1 and ColiTad1, cA₃/cAAG at 150 μ M concentration was titrated into
722 30 μ M SBS Tad1 or ColiTad1. All of the above titration experiments were performed in the same
723 experimental procedure. The Origin software was used for baseline correction, integration, and curve
724 fitting to a single site binding model.
725

726 **ThsA NADase activity assay**

727 NADase assay was performed by using ThsA enzyme from *Bacillus cereus* MSX-D12, which was
728 expressed and purified as described previously, as a reporter for the presence of cyclic ADPR isomers.
729 NADase reaction was performed in a black, 96-well plate (Corning 96-well half area black non-treated
730 plate with a flat bottom) at 37 °C in a 95 µL reaction volume, and the final concentration of ThsA and
731 1''-3' gcADPR was 50 and 5 nM, respectively. Next, 5 µL of 2 mM ε-NAD solution was added to each
732 well immediately before measurement and mixed by pipetting rapidly. ε-NAD was used as a
733 fluorogenic substrate to report ThsA enzyme NADase activity by monitoring increase in fluorescence
734 (excitation 300 nm, emission 410 nm) using EnSpire Multimode Plate Reader (PerkinElmer) at 37 °C.
735 To examine the inhibitory effect of HgmTad2 or its mutants on ThsA, HgmTad2 or its mutants (40 nM
736 of each) was incubated with 5 nM 1''-3' gcADPR in incubation buffer (50 mM Tris pH 7.5 and 50 mM
737 NaCl) at room temperature for 5 min in advance. Then ThsA was added at a final concentration of 50
738 nM. After an incubation for 5 minutes, ε-NAD was added to start the reaction.
739

740 **Surface Plasmon Resonance assay**

741 The SPR analysis was performed using a Biacore 8K (GE Healthcare) at room temperature (25 °C).
742 Equal concentrations of HgmTad2/SPO1 Tad2/SptTad2/SaTad2 were immobilized on channels of the
743 carboxymethylatedextran-modified (CM5) sensor chip to about 280 Response Unit (RU). To collect data
744 for kinetic analysis, a series of concentrations (12.5 nM, 25 nM, 50nM, 100 nM, 200 nM) of 3',3'-
745 cGAMP/3',2'-cGAMP/2',3'-cGAMP/cGG/cUG/cA₃ diluted in binding buffer (20 mM HEPES pH 7.5,
746 200 mM NaCl and 0.05% (v/v) Tween-20) was injected over the chip at a flow rate of 30 µL/min. The
747 protein-ligand complex was allowed to associate for 60 s and dissociate for 600 s. Data were fit with a
748 model describing a bivalent analyte. Kinetic rate constants were extracted from this curve fit using
749 Biacore evaluation software (GE healthcare).
750

751 **High-performance liquid chromatography (HPLC)**

752 For analysis of ligand sequestering, 40 µM Tad1 or Tad2 protein was pre-incubated with 4 µM cA₃,
753 2',3'-cGAMP or 3',3'-cGAMP for 30 min at 18°C. And then, for Tad1 series, proteinase K was
754 subsequently added to the reaction system at a final concentration of 0.5 µM and the reaction was
755 performed at 58°C for 1 h. For Tad2, the sample was first heated at 100°C for 10 min, and then
756 proteinase K was subsequently added to the reaction system at a final concentration of 25 µM and the
757 reaction was performed at 58°C for 3 h. For analysis of intrinsically bound nucleotide in HgmTad2
758 during expression, 40 µM HgmTad2 in different states was treated as reported for Tad2 in the above.
759 4 µM 3',3'-cGAMP and cGG were used as standards.
760

761 Reaction samples were transferred to Amicon Ultra-15 Centrifugal Filter Unit 3 kDa and centrifuged
762 at 4°C, 4,000 g. The products obtained by filtration were further filtered with a 0.22 µm filter and
763 subsequently used for HPLC experiments. The HPLC analysis was performed on an Agilent 1200
764 system with a ZORBAX Bonus-RP column (4.6 × 150 mm). A mixture of 2% acetonitrile and 0.1%
765 trifluoroacetic acid solution in water (98%) were used as mobile phase with 0.8 mL/min. For cA₃, 5%
766 acetonitrile and 0.1% trifluoroacetic acid solution in water (95%) were used as mobile phase. The
767 compounds were detected at 254 nm.
768

769 **Fluorogenic biochemical assay for CapV activity**

770 The enzymatic reaction velocity was measured as previously described¹⁸. Briefly, the esterase activity
771 of the 6×His-tagged CapV was probed with the fluorogenic substrate resorufin butyrate. The CapV
772 protein was diluted in 50 mM sodium phosphate pH 7.4, 300 mM NaCl, 10% (v/v) glycerol to a final
773 concentration of 2 μM. To determine the enzymatic activity of CapV activated by 3',3'-cGAMP,
774 0.8 μM of 3',3'-cGAMP was added to DMSO solubilized resorufin butyrate (stock of 20 mM mixed
775 with 50 mM sodium phosphate pH 7.4, 300 mM NaCl, 10% v/v glycerol reaching a final concentration
776 of 100 μM). Subsequently, the purified 6×His-tagged CapV was added to the reaction solution
777 containing 3',3'-cGAMP to a final assay volume of 50 μL, and fluorescence was measured in a 96-
778 well plate (Corning 96-well half area black non-treated plate with a flat bottom). Plates were read once
779 every 30 s for 10 min at 37°C using a EnSpire Multimode Plate Reader (PerkinElmer) with excitation
780 and emission wavelengths of 550 and 591 nm, respectively.

781

782 To determine the function of inhibitory proteins, 8 μM protein was pre-incubated with 0.8 μM 3',3'-
783 cGAMP for 10 min at 18°C, and the subsequent detection method was as described above. To examine
784 whether the released molecule from HgmTad2 or SBS Tad1 is still able to activate CapV, 0.8 μM
785 3',3'-cGAMP was incubated with 8 μM HgmTad2 or SBS Tad1 for 10 min at 18°C. Proteinase K was
786 subsequently added to the reaction system at a final concentration of 25 μM and the reaction was
787 performed at 58°C for 3 h. Reaction products were transferred to Amicon Ultra-4 Centrifugal Filter
788 Unit 3 kDa and centrifuged at 4°C, 4,000 g. Filtered products were used for CapV activity assay as
789 described above.

790

791 ***SfTIR-STING* NAD⁺ cleavage activity analysis**

792 The enzymatic reaction velocity was measured as previously described⁴⁵. The enzymatic activity of
793 *SfTIR-STING* was activated by cGG. 500 μM ε-NAD and 50 nM cGG were mixed in a 96-well plate
794 format with reaction buffer (50 mM Tris pH 7.5, 50 mM NaCl). Subsequently, purified 6×His-tagged
795 *SfTIR-STING* was added to the reaction to a final assay volume of 50 μL and plates were read once
796 every 15 s for 10 min at 37°C using a EnSpire Multimode Plate Reader (PerkinElmer) with excitation
797 and emission wavelengths of 410 and 300 nm, respectively. Reaction rate was calculated from the
798 linear part of the initial reaction.

799

800 To determine the function of inhibitory proteins, 1 μM HgmTad2, SPO1 Tad2, SptTad2 and SaTad2
801 was pre-incubated with 50 nM cGG for 20 min at 18°C, and the subsequent detection method was as
802 described above. To determine the function of HgmTad2 mutants, 200 nM HgmTad2 and its mutants
803 was pre-incubated with 50 nM cGG for 20 min at 18°C. To examine whether the released molecule
804 from HgmTad2 is still able to activate *SfTIR-STING*, 50 nM cGG was incubated with 200 nM
805 HgmTad2 for 10 min at 18°C. Proteinase K was subsequently added to the reaction system at a final
806 concentration of 1 μM and the reaction was performed at 58°C for 1 h. Reaction products were
807 transferred to Amicon Ultra-4 Centrifugal Filter Unit 3 kDa and centrifuged at 4°C, 4,000 g. Filtered
808 products were used for *SfSTING* activity assay as described above.

809

810 ***In vitro* NucC activity assay**

811 The nuclease activity assay was measured as previously described²⁷. Plasmid pUC19 was used as
812 substrate. NucC (10 nM) and cA₃ molecules (5 nM) were mixed with 0.4 μg DNA in a buffer
813 containing 25 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 mM MgCl₂, and 2 mM DTT (20 μL reaction

814 volume), incubated at 37°C for 20 min, then separated on a 1% agarose gel. Gels were stained with
815 Goldview and imaged by UV illumination.

816
817 To determine the function of CbTad1, 200 nM CbTad1 or its mutants were pre-incubated with the
818 other components at 18°C for 30 min, and the subsequent reaction and detection method was as
819 described above. To examine whether the released molecule from CbTad1 is able to activate NucC, 5
820 nM cA₃ was incubated with 200 nM CbTad1 at 18°C for 20 min. Proteinase K was subsequently added
821 to the reaction system at a final concentration of 1 μM and the reaction was performed at 58°C for 1 h,
822 then the proteinase K-treated samples were heated with 100°C for 10 min to extinguish proteinase K
823 and the subsequent detection method was as described above.

824
825 ***In vitro* SpyCas9 DNA cleavage assay**
826 SpyCas9 sgRNA was generated by *in vitro* T7 transcription kit (Vazyme). 100 nM SpyCas9 and 150
827 nM sgRNA was incubated with 10 μM purified Tad2 or AcrIIA11 in cleavage buffer (20 mM HEPES-
828 KOH pH 7.5, 75 mM KCl, 10% glycerol, 1 mM DTT, and 10 mM MgCl₂) for 30 min at 37°C. Plasmid
829 pUC57 containing the target protospacer 25 sequence inserted using *KpnI/XbaI* was linearized by ScaI
830 digestion. Linearized plasmid was added to the Cas9/sgRNA complex at 10 nM final concentration.
831 The reactions were incubated at 37°C for 10 min and extinguish by 1 μM proteinase K for 15 min at
832 58°C, then separated on a 1% agarose gel. Gels were stained with Goldview and imaged by UV
833 illumination.

834
835 *SpyCas9_sgRNA_DNA template*
836 ATGTAATACGACTCACTATAGGAAATTAGGTGCGCTTGGCGTTTAGAGCTAGAAATAG
837 CAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
838

839 *Cleavage assay DNA sequence*
840 TCGGTGCGGGCCTCTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCG
841 ATTAAGTTGGGTAACGCCAGGGTTTCCCAGTCACGACGTTGAAAACGACGGCCAGTG
842 CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCAATCCCAGCCAAGCGCACCT
843 AATTCCGAATTCGTAATCATGGTCATAGCTGTTCTGTGAAATTGTTATCCGCTCA
844 CAATTCCACACACATACGAGCCGGAAGCATAAA
845

846 **Native-PAGE assay**
847 For ligand binding native-PAGE assay, proteins were pre-incubated with cyclic nucleotides for 20 min
848 at 18°C, where protein was 20 μM and the concentrations of cyclic nucleotides was 5, 10 or 20 μM,
849 respectively. Products of the reaction were analyzed using 5% native polyacrylamide gels and
850 visualized by Coomassie blue staining.

851
852 **Multi-angle light scattering (MALS)**
853 MALS experiments were performed in 10 mM Tris pH 8.0, 200 mM NaCl and 2 mM TCEP using a
854 Superdex-200 10/300 GL size-exclusion column from GE Healthcare. All protein concentrations were
855 diluted to 1.7 mg/mL. The chromatography system was connected to a Wyatt DAWN HELEOS Laser
856 photometer and a Wyatt Optilab T-rEX differential refractometer. Wyatt ASTRA 7.3.2 software was
857 used for data analysis.

858

859 **Episomal gene expression**

860 The shuttle vector pHERD30T that replicates in *P. aeruginosa* and *E. coli*⁴⁶ was used for episomal
861 expression of Acb2 and Tad proteins in *P. aeruginosa* strains. pHERD30T has an arabinose-inducible
862 promoter and a selectable gentamicin marker. Vector was digested with NcoI and HindIII restriction
863 enzymes. Inserts were amplified by PCR using bacterial overnight culture or synthesized by Twist
864 Bioscience and joined with the digested vector using Hi-Fi DNA Gibson Assembly (NEB) following
865 the manufacturer's protocol. The resulting plasmids were transformed into *E. coli* DH5a. All plasmid
866 constructs were verified by whole plasmid sequencing. *P. aeruginosa* cells were electroporated with
867 the pHERD30T constructs and selected on gentamicin.

868

869 **Chromosomal Thoeris integration**

870 For chromosomal insertion of the MRSN390231 Thoeris SIR2 (Pa231) operon at the Tn7 locus in *P.*
871 *aeruginosa* PAO1(PAO1 Tn7:Thoeris SIR2), the integrating vector pUC18-mini-Tn7T-LAC⁴⁷
872 carrying Thoeris operon and the transposase expressing helper plasmid pTNS3⁴⁸ were used. pUC18-
873 mini-Tn7T-LAC empty vector was used for the creation of the control strain (PAO1 Tn7:empty). The
874 vector was linearized using around-the-world PCR (in positions of KpnI and BamHI sites), treated
875 with DpnI, and then purified. The insert was amplified using MRSN390231 overnight culture as a
876 DNA template and joined with linearized pUC18-mini-Tn7T-LAC vector using Hi-Fi DNA Gibson
877 Assembly (NEB) following the manufacturer's protocol. The resulting plasmids were used to
878 transform *E. coli* DH5a. All plasmid constructs were verified by whole plasmid sequencing. *P.*
879 *aeruginosa* PAO1 cells were electroporated with pUC18-mini-Tn7T-LAC and pTNS3 and selected on
880 gentamicin-containing plates. Potential integrants were screened by colony PCR with primers PTn7R
881 and PgImS-down⁴⁸. Electrocompetent cell preparations, transformations, integrations, selections,
882 plasmid curing, and FLP-recombinase-mediated marker excision with pFLP were performed as
883 described previously⁴⁹.

884

885 **Phage growth**

886 Phages F10 and JBD67 Δ acb2 were grown on *P. aeruginosa* PAO1, which lacks CBASS and Thoeris
887 systems. Phage PaMx41 Δ acb2 was grown on *P. aeruginosa* BWHPSA011 (Pa011) Δ CBASS strain.
888 For phage propagation 100 μ l of *P. aeruginosa* overnight cultures were infected with 10 μ l of low titer
889 phage lysate ($>10^{4-7}$ pfu/ml) and then mixed with 3 ml of 0.35% top agar 10 mM MgSO₄ for plating
890 on the LB solid agar (20 ml LB agar with 10 mM MgSO₄). After incubating 37 °C overnight, 2.5 ml
891 SM phage buffer was added on the solid agar lawn and then incubated for 10 minutes at room
892 temperature. The whole cell lysate was collected, a 10% volume of chloroform was added, and the
893 tubes were left for 20 minutes at room temperature with gentle shaking, followed by centrifugation at
894 maximum speed for 3 min 4°C to remove cell debris. The supernatant phage lysate was stored at 4°C
895 for downstream assays.

896

897 **Plaque assays**

898 Plaque assays were conducted at 37 °C with solid LB agar plates supplemented with 10 mM MgSO₄,
899 50 μ g ml⁻¹ gentamicin, 0.2% L-arabinose, and 0.3 mM IPTG for PAO1 strains with CBASS or Thoeris
900 operon chromosomal integration, and the same conditions except without IPTG for the native CBASS
901 and Thoeris strains. 100 μ L of overnight bacterial culture was mixed with top agar (0.35% agar in LB)

902 and plated. Phage lysates were diluted 10-fold then 2.5 μ L spots were applied to the top agar after it
903 had been poured and solidified. The plates were incubated overnight at 37 °C.

904

905 Bioinformatic analysis of CD-NTases

906 CD-NTases were identified within the bacterial hosts relevant for each Tad protein by using a protein
907 BLAST (blastp) search. A previously curated list of CD-NTase sequences²¹ was queried against
908 *Clostridium* (taxid:1485), *Clostridioioides* (taxid:1870884), *Bacteroides* (taxid:816),
909 *Sphingobacterium* (taxid:28453), and *Bacillus cereus* group (taxid:86661). There is only one CD-
910 NTase record for *Salegentibacter* (taxid:143222) and zero for *Colidextribacter* (taxid:1980681), so a
911 list of CD-NTase across bacterial taxonomies was used from Whiteley et al. 2019. These lists of CD-
912 NTases were queried against the NCBI non-redundant protein database of each respective bacterial
913 genus as indicated above. A genus-level analysis was chosen due to the diversity of CD-NTase
914 sequences associated with the different clades, which are known or predicted to produce specific
915 cyclic oligonucleotides. Hits from the blastp search with >24.5% amino acid identity, >50% coverage,
916 and an E value of <0.0005 were identified as CD-NTases. Two or more CD-NTases per CD-NTase
917 clade per bacterial genus were queried using Defense Finder^{50,51}, which revealed that all CD-NTases
918 identified are a part of a CBASS system. 183 CD-NTase hits were identified in *Clostridium* and nine
919 hits in *Clostridioioides*, so their results were combined as 202 total hits in Extended Data Figure 3. A
920 total of 107 hits were identified in *Bacteroides*, 71 in *Sphingobacterium*, and 270 in *Bacillus cereus*
921 group. Six hits were found in *Salegentibacter* and zero hits for *Colidextribacter*.

922

923 Phylogenetic tree analysis

924 Tad1 and Tad2 homologs were identified using SBSTad1 (NCBI: P0DW57) and SPO1Tad2 (NCBI:
925 YP_002300464.1), respectively, as query proteins to seed a position-specific iterative blast (PSI-
926 BLAST) search of the NCBI non-redundant protein database. Three rounds of PSI-BLAST searches
927 were performed with a max target sequence of 5,000 and E value cut-off of 0.005 for inclusion in the
928 next search round, BLOSUM62 scoring matrix, gap costs settings existence 11 and extension 1, and
929 using conditional compositional score matrix adjustment. Hits from the third search round of PSI-
930 BLAST with >70% coverage, E value of < 0.0005 and length less than 190 amino acids (for Tad1) and
931 length 70-120 amino acids (for Tad2) were clustered using MMSeq2⁵² to remove protein redundancies
932 (minimum sequence identity=0.9 for Tad1 and 0.8 for Tad2, minimum alignment coverage=0.9), which
933 resulted in 410 and 667 representative Tad1 and correspondingly, Tad2 homolog sequences. MAFFT
934 (FFT-NS-I iterative refinement method)⁵³ was used to create protein alignment. Manual analysis of
935 the MAFFT protein alignment was performed to ensure the presence of at least one of the cyclic
936 oligonucleotide binding site regions and to remove non-relevant sequences. The final aligned 385 and
937 568 sequences (Tad1 and Tad2 correspondingly) were used to construct a phylogenetic tree using
938 FastTree⁵⁴ and then visualized and annotated in iTOL⁵⁵.

939

940

941 References

1. Manik, M.K. et al. Cyclic ADP ribose isomers: Production, chemical structures, and immune signaling. *Science* **377**, eadc8969 (2022).
2. Ofir, G. et al. Antiviral activity of bacterial TIR domains via immune signalling molecules. *Nature* **600**, 116-120 (2021).

945

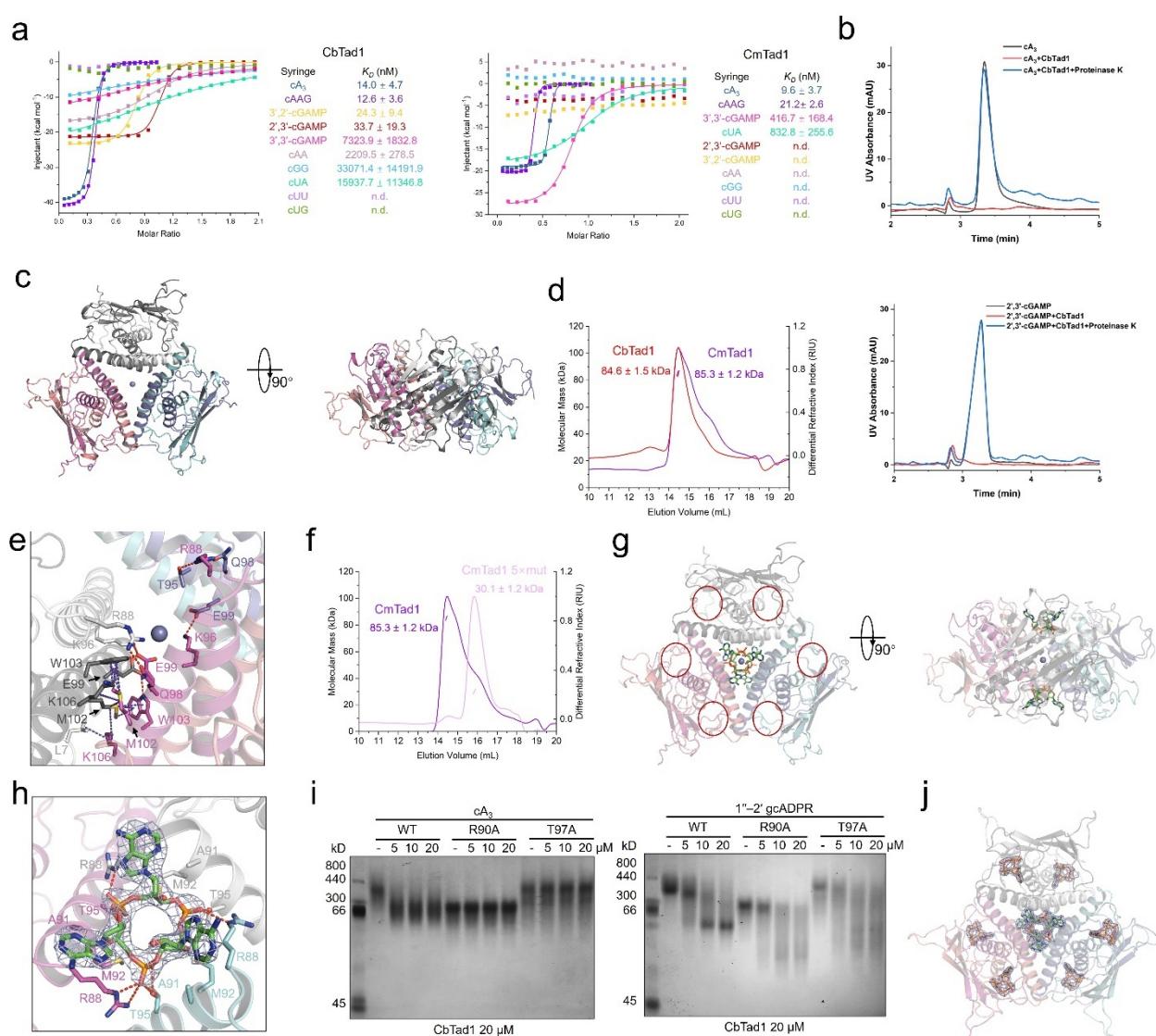
- 946 3. Cohen, D. et al. Cyclic GMP-AMP signalling protects bacteria against viral infection. *Nature*
947 **574**, 691-695 (2019).
- 948 4. Leavitt, A. et al. Viruses inhibit TIR gcADPR signaling to overcome bacterial defense. *Nature*
949 **611**, 326-331 (2022).
- 950 5. Yirmiya, E. et al. Phages overcome bacterial immunity via diverse anti-defense proteins.
951 *bioRxiv* <https://doi.org/10.1101/2023.05.01.538930> (2023).
- 952 6. Doron, S. et al. Systematic discovery of antiphage defense systems in the microbial pangenome.
953 *Science* **359** (2018).
- 954 7. Gao, L. et al. Diverse enzymatic activities mediate antiviral immunity in prokaryotes. *Science*
955 **369**, 1077-1084 (2020).
- 956 8. Millman, A. et al. An expanded arsenal of immune systems that protect bacteria from phages.
957 *Cell Host Microbe* **30**, 1556-1569.e5 (2022).
- 958 9. Rousset, F. et al. Phages and their satellites encode hotspots of antiviral systems. *Cell Host*
959 *Microbe* **30**, 740-753.e5 (2022).
- 960 10. Vassallo, C.N., Doering, C.R., Littlehale, M.L., Teodoro, G.I.C. & Laub, M.T. A functional
961 selection reveals previously undetected anti-phage defence systems in the *E. coli* pangenome.
962 *Nat Microbiol* **7**, 1568-1579 (2022).
- 963 11. Johnson, M.C. et al. Core defense hotspots within *Pseudomonas aeruginosa* are a consistent
964 and rich source of anti-phage defense systems. *Nucleic Acids Research* **51**, 4995-5005 (2023).
- 965 12. Stanley, S.Y. & Maxwell, K.L. Phage-Encoded Anti-CRISPR Defenses. *Annu Rev Genet* **52**,
966 445-464 (2018).
- 967 13. Li, Y. & Bondy-Denomy, J. Anti-CRISPRs go viral: The infection biology of CRISPR-Cas
968 inhibitors. *Cell Host Microbe* **29**, 704-714 (2021).
- 969 14. Jia, N. & Patel, D.J. Structure-based functional mechanisms and biotechnology applications of
970 anti-CRISPR proteins. *Nat Rev Mol Cell Biol* **22**, 563-579 (2021).
- 971 15. Davidson, A.R. et al. Anti-CRISPRs: Protein Inhibitors of CRISPR-Cas Systems. *Annu Rev*
972 *Biochem* **89**, 309-332 (2020).
- 973 16. Gao, Z. & Feng, Y. Bacteriophage strategies for overcoming host antiviral immunity. *Front*
974 *Microbiol* **14**, 1211793 (2023).
- 975 17. Jenson, J.M., Li, T., Du, F., Ea, C.K. & Chen, Z.J. Ubiquitin-like Conjugation by Bacterial
976 cGAS Enhances Anti-phage Defence. *Nature* **616**, 326-331 (2023).
- 977 18. Huiting, E. et al. Bacteriophages inhibit and evade cGAS-like immune function in bacteria.
978 *Cell* **186**, 864-876 e21 (2023).
- 979 19. Hobbs, S.J. et al. Phage anti-CBASS and anti-Pycsar nucleases subvert bacterial immunity.
980 *Nature* **605**, 522-526 (2022).
- 981 20. Athukoralage, J.S. et al. An anti-CRISPR viral ring nuclease subverts type III CRISPR
982 immunity. *Nature* **577**, 572-+ (2020).
- 983 21. Whiteley, A.T. et al. Bacterial cGAS-like enzymes synthesize diverse nucleotide signals.
984 *Nature* **567**, 194-199 (2019).
- 985 22. Fatma, S., Chakravarti, A., Zeng, X. & Huang, R.H. Molecular mechanisms of the CdnG-Cap5
986 antiphage defense system employing 3',2'-cGAMP as the second messenger. *Nat Commun* **12**,
987 6381 (2021).
- 988 23. Millman, A., Melamed, S., Amitai, G. & Sorek, R. Diversity and classification of cyclic-
989 oligonucleotide-based anti-phage signalling systems. *Nat Microbiol* **5**, 1608-1615 (2020).

- 990 24. Burroughs, A.M., Zhang, D., Schäffer, D.E., Iyer, L.M. & Aravind, L. Comparative genomic
991 analyses reveal a vast, novel network of nucleotide-centric systems in biological conflicts,
992 immunity and signaling. *Nucleic Acids Res* **43**, 10633-54 (2015).
- 993 25. Davies, B.W., Bogard, R.W., Young, T.S. & Mekalanos, J.J. Coordinated regulation of
994 accessory genetic elements produces cyclic di-nucleotides for *V. cholerae* virulence. *Cell* **149**,
995 358-70 (2012).
- 996 26. Duncan-Lowey, B., McNamara-Bordewick, N.K., Tal, N., Sorek, R. & Kranzusch, P.J.
997 Effector-mediated membrane disruption controls cell death in CBASS antiphage defense. *Mol
998 Cell* **81**, 5039-5051.e5 (2021).
- 999 27. Cao, X. et al. Phage anti-CBASS protein simultaneously sequesters cyclic trinucleotides and
1000 dinucleotides. *bioRxiv* [https://doi.org/10.1101/2023.06.01.543220\(2023\)](https://doi.org/10.1101/2023.06.01.543220).
- 1001 28. Tesson, F. et al. Systematic and quantitative view of the antiviral arsenal of prokaryotes. *Nature
1002 Communications* **13**, 2561 (2022).
- 1003 29. Wu, Y. et al. Defence systems provide synergistic anti-phage activity in *E. coli*. *bioRxiv* [https://doi.org/10.1101/2022.08.21.504612\(2022\)](https://doi.org/10.1101/2022.08.21.504612).
- 1004 30. van Beljouw, S.P.B., Sanders, J., Rodriguez-Molina, A. & Brouns, S.J.J. RNA-targeting
1005 CRISPR-Cas systems. *Nature Reviews Microbiology* (2022).
- 1006 31. Tal, N. et al. Cyclic CMP and cyclic UMP mediate bacterial immunity against phages. *Cell* **184**,
1007 5728-5739 e16 (2021).
- 1008 32. Molina, R., Sofos, N. & Montoya, G. Structural basis of CRISPR-Cas Type III prokaryotic
1009 defence systems. *Curr Opin Struct Biol* **65**, 119-129 (2020).
- 1010 33. Lau, R.K. et al. Structure and Mechanism of a Cyclic Trinucleotide-Activated Bacterial
1011 Endonuclease Mediating Bacteriophage Immunity. *Mol Cell* **77**, 723-733 e6 (2020).
- 1012 34. Ye, Q. et al. HORMA Domain Proteins and a Trip13-like ATPase Regulate Bacterial cGAS-
1013 like Enzymes to Mediate Bacteriophage Immunity. *Mol Cell* **77**, 709-722 e7 (2020).
- 1014 35. Uribe, R.V. et al. Discovery and Characterization of Cas9 Inhibitors Disseminated across Seven
1015 Bacterial Phyla. *Cell Host & Microbe* **25**, 233-+ (2019).
- 1016 36. Jenal, U., Reinders, A. & Lori, C. Cyclic di-GMP: second messenger extraordinaire. *Nature
1017 Reviews Microbiology* **15**, 271-284 (2017).
- 1018 37. Morehouse, B.R. et al. STING cyclic dinucleotide sensing originated in bacteria. *Nature* **586**,
1019 429-+ (2020).
- 1020 38. Mahendra, C. et al. Broad-spectrum anti-CRISPR proteins facilitate horizontal gene transfer.
1021 *Nature Microbiology* **5**, 620-629 (2020).
- 1022 39. Slavik, K.M. et al. cGAS-like receptors sense RNA and control 3'2'-cGAMP signalling in
1023 *Drosophila*. *Nature* **597**, 109-113 (2021).
- 1024 40. Cai, H. et al. A novel virus-induced cyclic dinucleotide, 2'3'-c-di-GMP, mediates STING-
1025 dependent antiviral immunity in *Drosophila*. *bioRxiv* (2023).
- 1026 41. Otwinowski, Z. & Minor, W. [20] Processing of X-ray diffraction data collected in oscillation
1027 mode - ScienceDirect. *Methods in Enzymology* **276**, 307-326 (1997).
- 1028 42. Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583-
1029 589 (2021).
- 1030 43. Adams, P.D., Grosse-Kunstleve, R.W., Hung, L.W., Ioerger, T.R. & Terwilliger, T.C. PHENIX:
1031 building new software for automated crystallographic structure determination. *Acta
1032 Crystallographica Section D Biological Crystallography* **58**, 1948-1954 (2002).
- 1033

- 1034 44. Emsley, P., Lohkamp, B., Scott, W. & Cowtan, K. Features and development of Coot. *Acta*
1035 *Crystallogr.* (2010).
- 1036 45. Morehouse, B.R. et al. STING cyclic dinucleotide sensing originated in bacteria. *Nature* **586**,
1037 429-433 (2020).
- 1038 46. Qiu, D., Damron, F.H., Mima, T., Schweizer, H.P. & Yu, H.D. PBAD-based shuttle vectors for
1039 functional analysis of toxic and highly regulated genes in Pseudomonas and Burkholderia spp.
1040 and other bacteria. *Appl Environ Microbiol* **74**, 7422-6 (2008).
- 1041 47. Choi, K.H. & Schweizer, H.P. mini-Tn7 insertion in bacteria with single attTn7 sites: example
1042 Pseudomonas aeruginosa. *Nat Protoc* **1**, 153-61 (2006).
- 1043 48. Choi, K.H. et al. Genetic tools for select-agent-compliant manipulation of Burkholderia
1044 pseudomallei. *Appl Environ Microbiol* **74**, 1064-75 (2008).
- 1045 49. Choi, K.-H. & Schweizer, H.P. mini-Tn7 insertion in bacteria with single attTn7 sites: example
1046 Pseudomonas aeruginosa. *Nature Protocols* **1**, 153-161 (2006).
- 1047 50. Tesson, F. et al. Systematic and quantitative view of the antiviral arsenal of prokaryotes.
1048 **13**(2022).
- 1049 51. Abby, S.S., Néron, B., Ménager, H., Touchon, M. & Rocha, E.P. MacSyFinder: a program to
1050 mine genomes for molecular systems with an application to CRISPR-Cas systems. *PLoS One*
1051 **9**, e110726 (2014).
- 1052 52. Steinegger, M. & Söding, J. MMseqs2 enables sensitive protein sequence searching for the
1053 analysis of massive data sets. *Nature Biotechnology* **35**, 1026-1028 (2017).
- 1054 53. Katoh, K., Rozewicki, J. & Yamada, K.D. MAFFT online service: multiple sequence alignment,
1055 interactive sequence choice and visualization. *Brief Bioinform* **20**, 1160-1166 (2019).
- 1056 54. Price, M.N., Dehal, P.S. & Arkin, A.P. FastTree 2--approximately maximum-likelihood trees
1057 for large alignments. *PLoS One* **5**, e9490 (2010).
- 1058 55. Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree
1059 display and annotation. *Nucleic Acids Res* **49**, W293-w296 (2021).
- 1060
- 1061
- 1062
- 1063
- 1064
- 1065
- 1066
- 1067
- 1068
- 1069
- 1070
- 1071
- 1072
- 1073
- 1074
- 1075
- 1076
- 1077

1078

Figures



1079

Figure 1. Tad1 is a hexamer to bind to two molecules of cyclic trinucleotides.

1080 **a**, ITC assays to test binding of cyclic oligonucleotides to CbTad1 and CmTad1. Representative
1081 binding curves and binding affinities are shown. The K_D values are mean ± s.d. (n=3). Raw data for
1082 these curves are shown in Extended Data Figure 2.

1083 **b**, The ability of CbTad1 to bind and release cA₃ and 2',3'-cGAMP when treated with proteinase K
1084 was analyzed by HPLC. cA₃ and 2',3'-cGAMP standard was used as controls. The remaining
1085 nucleotides after incubation with CbTad1 were tested.

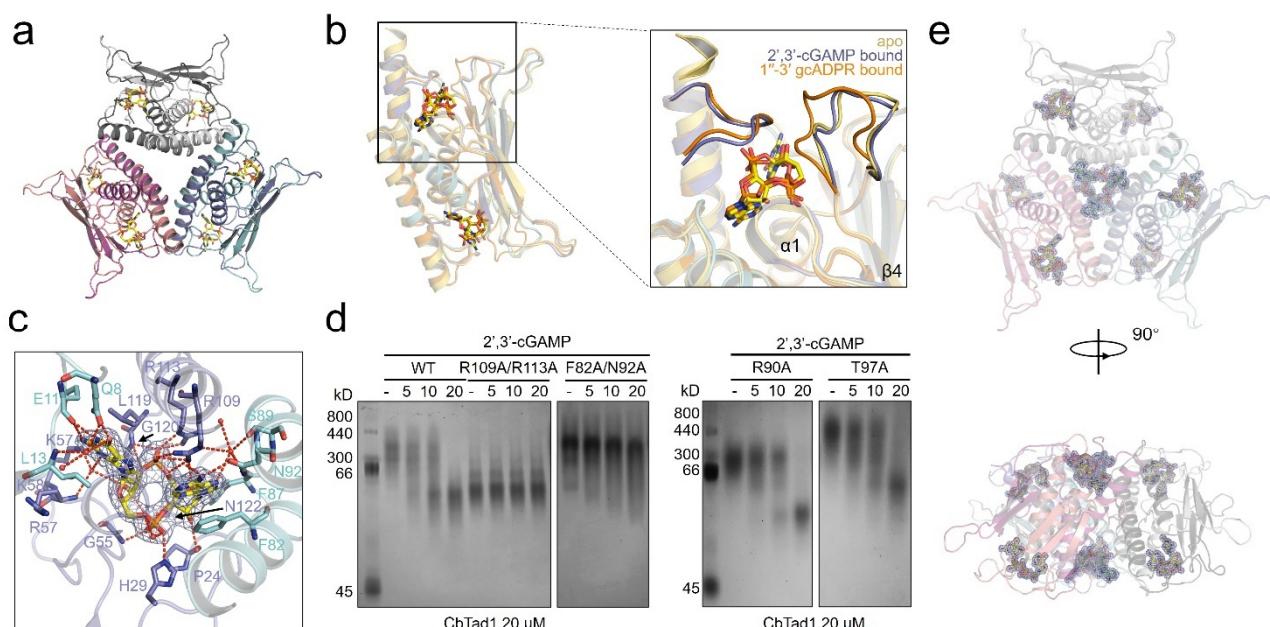
1086 **c**, Overall structure of CmTad1 hexamer. The Zn ion is shown as a sphere. Three views are shown.

1087 **d**, Static light scattering (SLS) studies of purified CbTad1 and CmTad1. Calculated molecular weight
1088 is shown above the peaks.

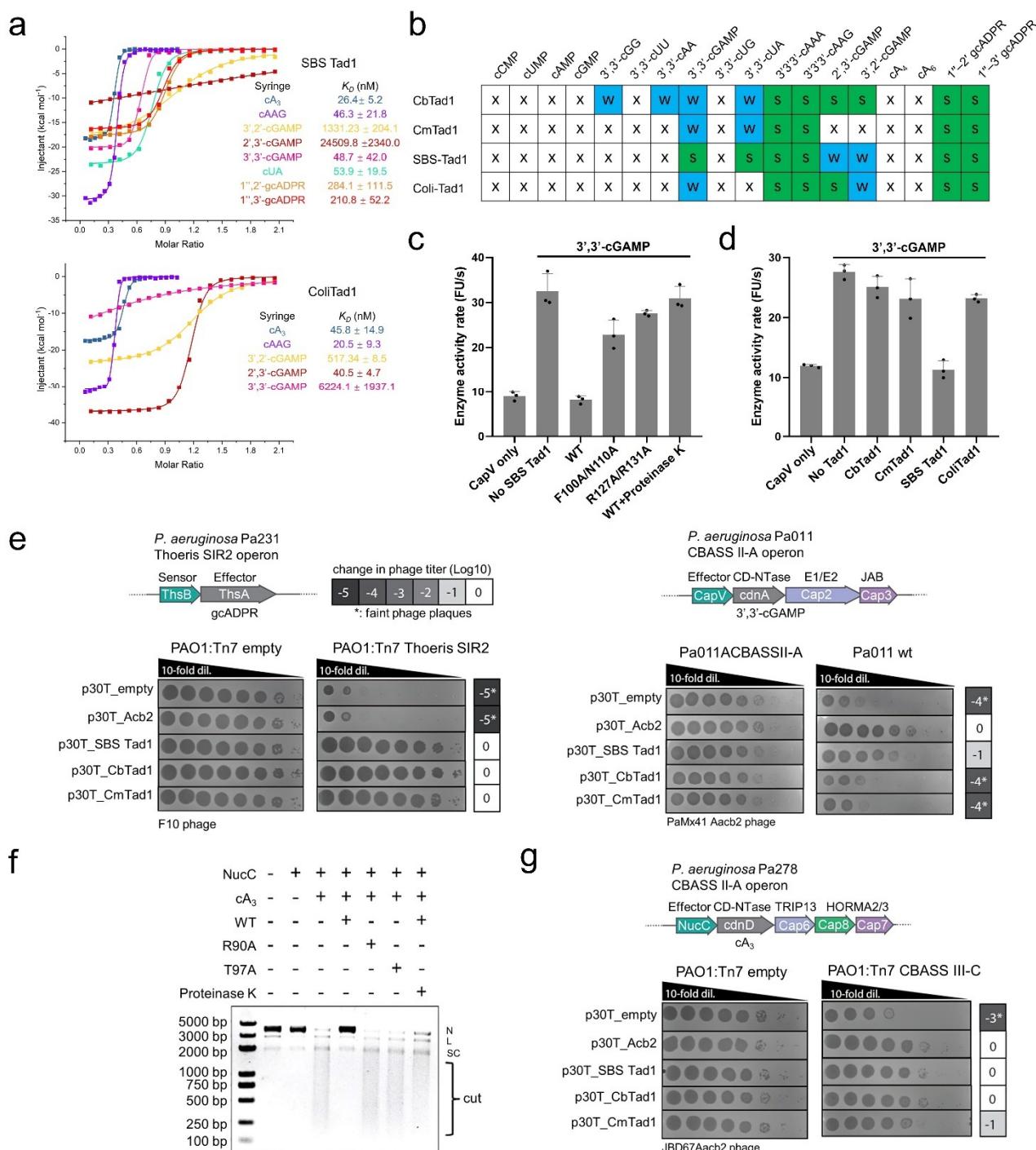
1089 **e**, Detailed binding in the hexamer interface of CmTad1. Residues involved in hexamer formation are
1090 shown as sticks. Red dashed lines represent polar interactions.

1091 **f**, SLS studies of purified CmTad1 and its Q98A/E99A/M102A/W103A/K106A mutant. Calculated
1092 molecular weight is shown above the peaks. 5× mut represents the above mutant with 5 residues
1093 mutated.

1095 **g**, Overall structure of CmTad1 hexamer bound to cA₃. Two views are shown.
1096 **h**, Detailed binding between CmTad1 and cA₃. Residues involved in cA₃ binding are shown as sticks.
1097 Red dashed lines represent polar interactions. 2Fo-Fc electron density of cA₃ within one binding pocket
1098 is shown and contoured at 1 σ .
1099 **i**, Native PAGE showed the binding of CbTad1 and its mutants to cA₃ and 1''-2' gcADPR.
1100 **j**, Overall structure of CmTad1 hexamer bound to cA₃ and 1''-3' gcADPR. cA₃ and 1''-3' gcADPR are
1101 shown as green and orange sticks, respectively. 2Fo-Fc electron density of cA₃ and 1''-3' gcADPR
1102 within CbTad1 hexamer contoured at 1 σ .
1103
1104



1105
1106 **Figure 2. Tad1 binds to 2',3'-/3',2'-cGAMP using the same binding pocket as gcADPR molecules.**
1107 **a**, Overall structure of CbTad1 hexamer bound to 2',3'-cGAMP, which is shown as yellow sticks.
1108 **b**, Structural superimposition of apo, 1''-3' gcADPR-bound and 2',3'-cGAMP-bound CbTad1 protein.
1109 1''-3' gcADPR and 2',3'-cGAMP are shown as orange and yellow sticks, respectively. The two loops
1110 that undergo conformational changes upon ligand binding are highlighted.
1111 **c**, Detailed binding between CbTad1 and 2',3'-cGAMP. Residues involved in 2',3'-cGAMP binding
1112 are shown as sticks. Red dashed lines represent polar interactions. 2Fo-Fc electron density of 2',3'-
1113 cGAMP within one binding pocket is shown and contoured at 1 σ .
1114 **d**, Native PAGE showed the binding of CbTad1 and its mutants to 2',3'-cGAMP.
1115 **e**, Overall structure of CmTad1 hexamer complexed with cA₃ and 2',3'-cGAMP. cA₃ and 2',3'-cGAMP
1116 are shown as green and yellow sticks, respectively. Two views are shown. 2Fo-Fc electron density of
1117 cA₃ and 2',3'-cGAMP within CbTad1 hexamer contoured at 1 σ .
1118
1119



1120

1121 **Figure 3. Tad1 antagonizes Type II-A and Type III-C CBASS immunity.**

1122 **a**, ITC assays to test binding of cyclic oligonucleotides to SBS Tad1 and ColiTad1. Representative
1123 binding curves and binding affinities are shown. The K_D values are mean \pm s.d. (n=3). Raw data for
1124 these curves are shown in Extended Data Figure 2.

1125 **b**, Summary of the binding results of Tad1 homologs. Words in black: verified only by native PAGE.
1126 X: no binding; W: binding K_D higher than 400 nM. S: shift in native gel or binding K_D lower than 400
1127 nM by ITC or SPR.

1128 **c**, CapV enzyme activity in the presence of 3',3'-cGAMP and resorufin butyrate, which is a

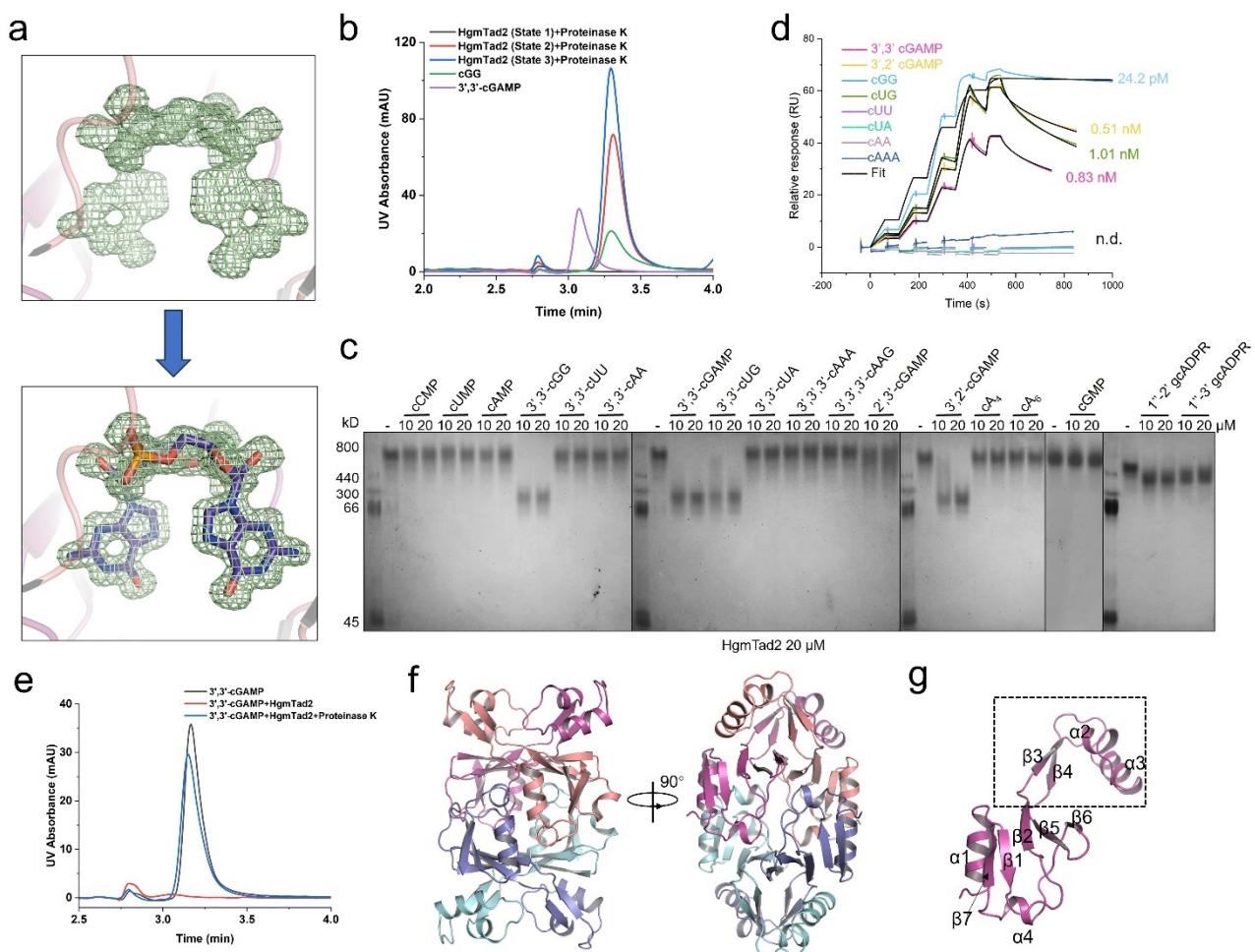
1129 phospholipase substrate that emits fluorescence when hydrolyzed. The enzyme activity rate was
1130 measured by the accumulation rate of fluorescence units (FUs) per second. To test the effects of Tad1
1131 homologs to sequester 3',3'-cGAMP, Tad1 or its mutants (8 μ M) was incubated with 3',3'-cGAMP
1132 (0.8 μ M) for 30 min. Filtered nucleotide products were used for the CapV activity assay. Data are mean
1133 \pm SD (n=3).

1134 **d**, CapV enzyme activity with Tad1 homologs. The experiment was performed as in c.

1135 **e**, Plaque assays to test the activity of Tad1 against Thoeris and Type II-A CBASS immunity *in vivo*.
1136 Organization of *P. aeruginosa* Pa231 Thoeris and *P. aeruginosa* Pa011 CBASS II-A operons shown.
1137 F10 phage was spotted in 10-fold serial dilutions on a lawn of *P. aeruginosa* cells expressing Thoeris
1138 operon genes (PAO1:Tn7 Thoeris SIR2), or without Thoeris (PAO1:Tn7 empty). PaMx41 Δ acb2 was
1139 spotted on a lawn of Pa011 cells with deletion of CBASS operon (Pa011 Δ CBASS II-A) or Pa011 wild
1140 type cells (Pa011 wt), electroporated with pHERD30T plasmids carrying Tad1 genes or empty vector.
1141 **f**, Effect of CbTad1 or its mutants on cA₃-activated NucC effector protein function. After treatment
1142 with proteinase K, the released cA₃ also showed the ability to activate the nuclease activity of NucC.
1143 The concentration of NucC, cA₃, CbTad1 and proteinase K is 10 nM, 5 nM, 200 nM and 1 μ M,
1144 respectively. N denotes nicked plasmid, SC denotes closed-circular supercoiled plasmid, and cut
1145 denotes fully digested DNA.

1146 **g**, Plaque assays to test the activity of Tad1 against Type III-C CBASS immunity *in vivo*. Organization
1147 of *P. aeruginosa* Pa278 Type III-C CBASS operon shown. JBD67 Δ acb2 phage was spotted in 10-fold
1148 serial dilutions on a lawn of *P. aeruginosa* cells expressing Pa278 CBASS operon genes (PAO1:Tn7
1149 CBASS III-C), or without the system (PAO1:Tn7 empty), electroporated with pHERD30T plasmids
1150 carrying Tad1 genes or empty vector.

1151



1152

1153 **Figure 4. Tad2 binds an array of cyclic dinucleotides.**

1154 **a**, The Fo-Fc density around the putative cGG in the structure of HgmTad2 of State 3 contoured at 2.5σ . The density itself and with cGG placed are shown in the upper and lower panels, respectively.

1155 **b**, The molecules in HgmTad2 of three states released when treated with proteinase K was analyzed

1156 by HPLC. 3',3'-cGAMP and cGG standard was used as controls.

1157 **c**, Native PAGE showed the binding of HgmTad2 of State 1 to cyclic oligonucleotides and gcADPR

1158 molecules.

1159 **d**, Overlay of sensorgrams from surface plasmon resonance (SPR) experiments, used to determine

1160 kinetics of HgmTad2 binding to CDNs. Data were fit with a model describing one-site binding for the

1161 ligands (black lines).

1162 **e**, The ability of HgmTad2 of State 1 to bind and release 3',3'-cGAMP when treated with proteinase

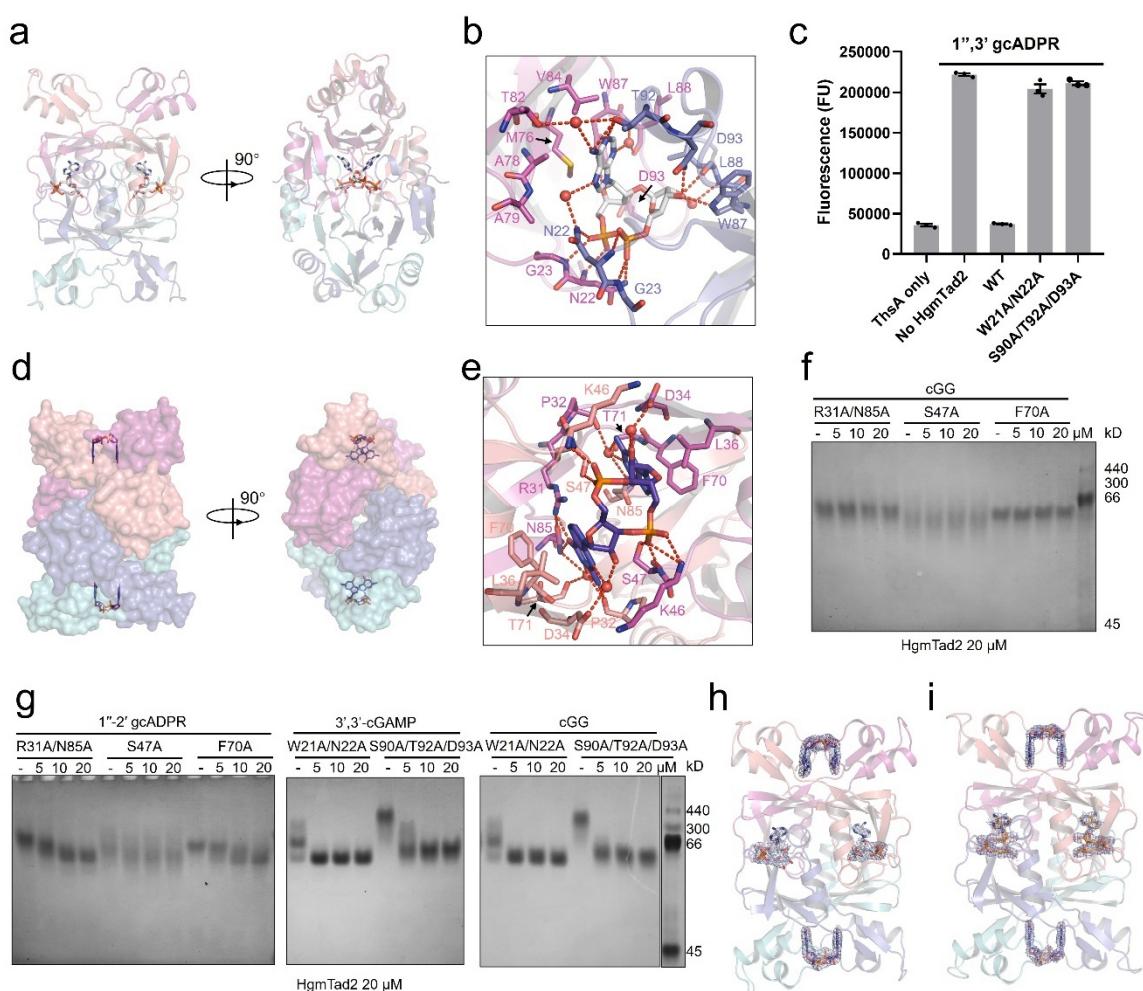
1163 K was analyzed by HPLC. 3',3'-cGAMP standard was used as a control. The remaining nucleotides

1164 after incubation with HgmTad2 was tested.

1165 **f**, Overall structure of HgmTad2 tetramer. Two views are shown.

1166 **g**, Structure of a protomer of HgmTad2. Secondary structures are labelled.

1167
1168



1169

1170 **Figure 5. Tad2 binds cyclic dinucleotides and gcADPR molecules simultaneously.**

1171 **a**, Overall structure of HgmTad2 tetramer bound to 1''-2' gcADPR, which is shown as gray sticks.

1172 **b**, Detailed binding between HgmTad2 and 1''-2' gcADPR. Residues involved in ligand binding are

1173 shown as sticks. Red dashed lines represent polar interactions.

1174 **c**, ThsA enzyme activity in the presence of 1''-3' gcADPR and ϵ -NAD. Wild-type (WT) and mutated

1175 HgmTad2 at 40 nM were incubated with 5 nM 1''-3' gcADPR. And then the reactions were filtered

1176 and their ability to activate ThsA NADase activity was measured. Bars represent the mean of three

1177 experiments, with individual data points shown. Data are mean \pm SD (n=3).

1178 **d**, Overall structure of HgmTad2 tetramer bound to cGG, which is shown as purple sticks. HgmTad2

1179 is shown as surface model.

1180 **e**, Detailed binding between HgmTad2 and cGG. Residues involved in ligand binding are shown as

1181 sticks. Red dashed lines represent polar interactions.

1182 **f**, Native PAGE showed the binding of HgmTad2 mutants to cGG.

1183 **g**, Native PAGE showed the binding of HgmTad2 mutants to 1''-2' gcADPR, 3',3'-cGAMP or cGG.

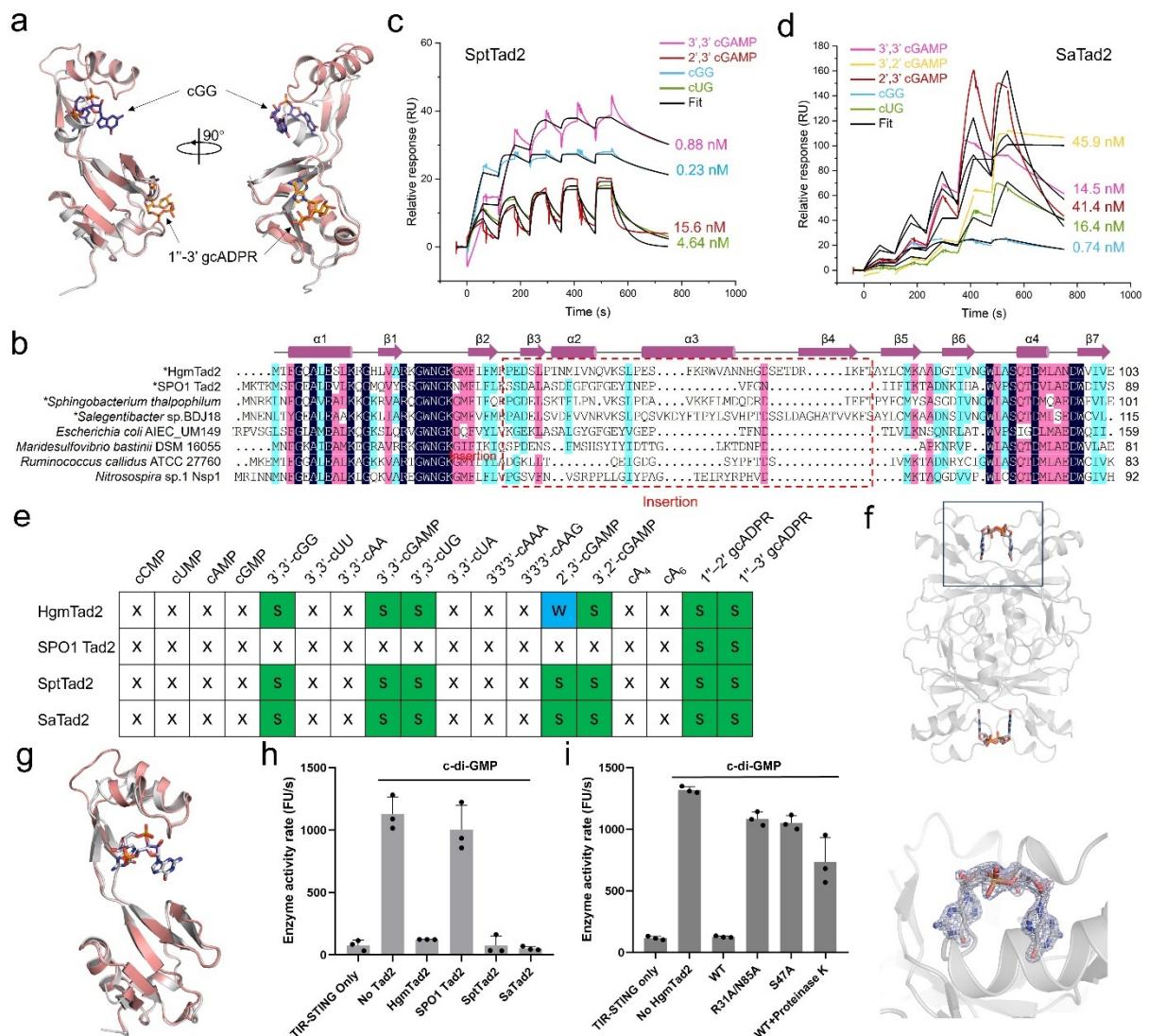
1184 **h-i**, Overall structure of HgmTad2 tetramer bound to cGG and 1''-2' gcADPR simultaneously (**h**), or

1185 cGG and 1''-3' gcADPR simultaneously (**i**), cGG, 1''-2' gcADPR and 1''-3' gcADPR are shown as

1186 purple, gray and orange sticks, respectively. 2Fo-Fc electron density of the ligands within HgmTad2

1187 tetramer is contoured at 1 σ .

1188



1189

Figure 6. Tad2 antagonizes Type I-D CBASS immunity that uses cGG.

1190 **a**, Structural superimposition between HgmTad2-cGG-1''-3'-gcADPR and SPO1 Tad2. HgmTad2 and
1191 small molecules are colored as in Fig. 6I. SPO1 Tad2 is colored gray.

1192 **b**, Sequence alignment among Tad2 homologs. Residues with 100 % identity, over 75 % identity and
1193 over 50 % identity are shaded in dark blue, pink and cyan, respectively. Secondary structural elements
1194 of HgmTad2 are shown above the alignment. The insertion region (residues 32-72) between β 2 and β 5
1195 of HgmTad2 or between β 2 and β 3 of SPO1 Tad2 (residues 36-59) is marked with a rectangle.
1196 Biochemically studied Tad2 homologs are marked with an asterisk before its species name.
1197

1198 **c-d**, SPR assay of SptTad2 (**c**) and SaTad2 (**d**).

1199 **e**, Summary of the binding results of Tad1 homologs. The figure is labelled as in Figure 3b.

1200 **f**, Overall structure of SptTad2 bound to cGG. A close view of the bound cGG with 2Fo-Fc electron
1201 density contoured at 1 σ is shown in the lower panel.

1202 **g**, Structural superimposition between HgmTad2-cGG and SptTad2-cGG. HgmTad2 and cGG are
1203 colored as in Fig. 6I. SptTad2 and its bound cGG are colored gray.

1204 **h, i**, TIR-STING NAD⁺ cleavage activity in the presence of cGG and nicotinamide 1,N⁶-ethenoadenine
1205 dinucleotide (ϵ NAD), which emits fluorescence when cleavage. The enzyme activity rate was

1206 measured by the accumulation rate of fluorescence units (FUs) per second. To test the effects of
1207 HgmTad2 or its homologs to bind cGG, HgmTad2 or its homologs (1 μ M) was incubated with cGG
1208 (50 nM) for 20 min. To test the effects of HgmTad2 or its mutants to bind and release cGG, HgmTad2
1209 or its mutants (200 nM) was incubated with cGG (50 nM) for 20 min and then proteinase K (28.3
1210 μ g/mL) was added to release the nucleotide from the HgmTad2 protein, Filtered nucleotide products
1211 were used for the TIR NADase activity assay. Data are mean \pm SD (n=3).
1212