Supplementary information, Data S1

Supporting Text

Cells, media, antibodies, nucleotides, primers and general methods

HEK293T cells were cultured in DMEM supplemented with 10 % FBS (Gibco) and antibiotics. THP-1 cells were cultured in RPMI medium supplemented with 10 % FBS, L-glutamine, sodium pyruvate. All *V. cholerae* strains (Table S1A) used in this study were derived from E1 Tor C6706 unless otherwise noted [1], and were propagated in LB media supplemented with ampicillin (Amp, 100 μg/ml), choloramphinical (Cm, 3 μg/ml), tetracycline (Tc, 2 μg/ml), X-gal (50 μg/ml), IPTG (20 μg/ml) or polymixin B (PolB, 100- units/ ml) when necessary. Anti-Flag (Sigma, F3165), anti-HA (Sigma, H9658), anti-GAPDH (Santa Cruz, sc-25778), anti-p-IRF3 (Epitomics, 2562-1) antibodies were purchased as indicated. Antiserum against VCA0681, VCA0210 or VCA0931 was generated by immunizing mice with the full length recombinant protein produced in *E. coli*, at Beijing Biotop Biotechnology, China. All cloned genes were sequence-verified and primers were listed in Table S1B. The procedures for SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting, and luciferase reporter assay have been described previously [2]. Polynucleotide kinase (PNK), RNA ligase 1 and alkaline phosphatase (CIP) used in this study were obtained from New England Biology and used according to manufacture protocol.

Plasmids

cDNA encoding VC0179, VCA0956, VCA0785 and PDE candidates were amplified from the genomic DNA of *V. cholerae* strain N16961, and cloned into p3×Flag-CMV-14 (Sigma) or p3× Flag-CMV-7.1 (Sigma) to generate Flag-tagged proteins. Deleted, truncated and point mutants were generated by the KOD -Plus- Mutagenesis Kit as the polymerase (TOYOBO Bio-Technology) and the construct coding the wild-type protein as the template. Homo Flag-cGAS expression vector was kindly provided by Dr. Zhijian J. Chen (University of Texas Southwestern Medical Center, Dallas, USA). Other expression plasmids were described previously [2]. Each construct was confirmed by sequencing.

Recombinant proteins

cDNA encoding amino acids 85-1500 of perfringolysin o (PFO) amplified from genome of *Bacillus perfringens*, which was a gift from Dr. Yuming Guo (China Agricultural University), was inserted into a pET21b vector in which the 6×His tag was fused to the C-terminus of PFO. BL21 harboring the PFO plasmid was induced with 0.5 mM IPTG at 18 ℃ for 15hr, and protein was purified with His60 Ni superflow resin (sigma) as reported [3].

DNA sequences encoding V-cGAPs and other PDE candidates were amplified from the genomic DNA of *V. cholerae* strain N16961 and inserted into pET-21b vector like PFO. BL21 harboring

these plasmids were induced with 0.5 mM IPTG at 18 °C overnight and purified as mentioned above. When it comes to be inclusion body, protein was dissolved in solubilization buffer [50 mM CAPS, pH11, 0.3 % sodium N-lauroylsarcosine, 1 mm DTT], and then incubated with Ni resin in buffer A [50 mM Tris-Cl, pH8.0, 300 mM NaCl, 20mM Imidazole, 5mm β-mercaptoethanol, 0.3% sodium N-lauroylsarcosine]. After wash in buffer A, bound protein was eluted with buffer B [20 mM Tris-Cl, pH7.4, 150 mM NaCl, 300 mM Imidazole, 0.3% sodium N-lauroylsarcosine]. At last, the protein was dialyzed against 20 mM Tris, pH8.5, overnight to refold and stored in aliquots as 3 mg/ml at -80 °C.

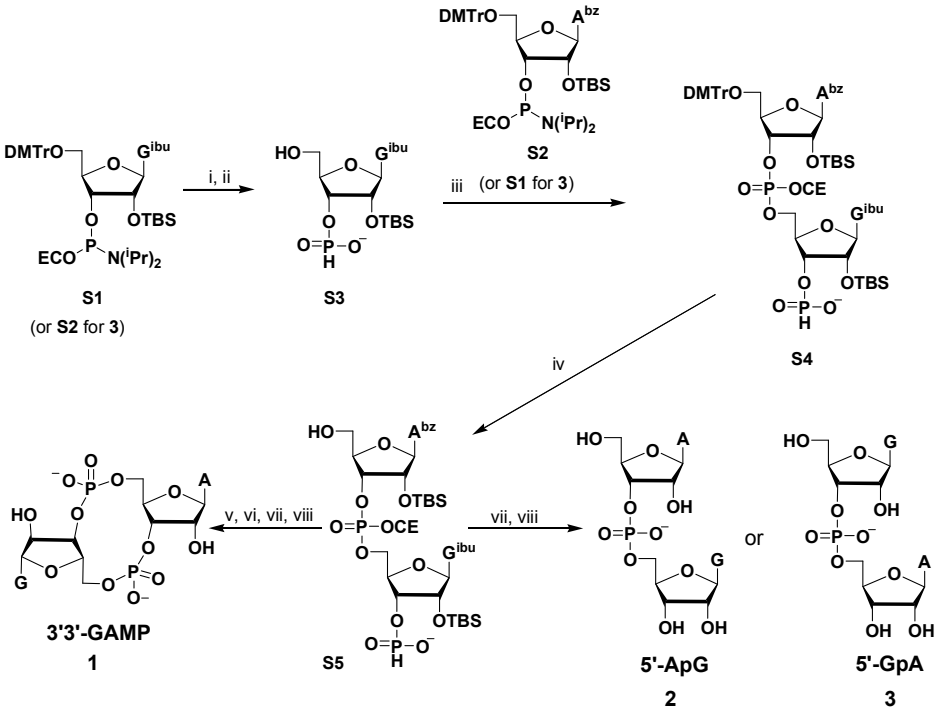
***In vitro* cGAMP activity assay**

Purified V-cGAPs were incubated with different cGAMPs in the reaction mixture [20 mM HEPES, pH7.0, 1 mg/ml bovine serum albumin, 1 mM DTT, and 5 mM $MgCl_2$] at 30 °C for the indicated time. After heated at 95 °C for 5 min, the mixture was centrifuged at 12,000 rpm for 5 min. 2 μl supernatant was mixed with 10^6 PFO-permeabilized THP1 cells to detect the phosphorylation of IRF3 as reported [3]. To isolate 3'3'-cGAMP from 293T cells expressing DncV, the supernatant (S100) of dounced cells was prepared according to the reported protocol（Wu et al., 2013), and concentrated by drying at 65 °C before PFO assay.

To isolate endogenous 3'3'-cGAMP from *V. cholerae* colonies on chemotaxis plate, equal wet weight of these strains was re-suspended in culture medium respectively. After ultrasonication, the mixture were heated at 95°C for 5 min and centrifuged at 14,000 rpm for 5 min before PFO assay.

Chemical synthesis of cGAMPs, 5'-ApG, and 5'-GpA

All moisture-sensitive reactions were performed under an argon atmosphere using oven-dried glassware. Reagents and solvents were purchased and used without further purification unless otherwise stated. **S1** and **S2** and all other amidites were purchased from Wuhu Huaren Scientific Co. and used as received. Anhydrous DMF, acetonitrile and pyridine (from Aldrich) were dried over 4A molecular sieves. Silica gel column chromatography were performed on Qingdao 300-400 gel, TLC-analysis was carried out on pre-coated Silica Gel 60 F254 (Merck), with detection by UV light.



(i) pyridinium trifluoroacetate; *tert*-$BuNH_2$; (ii) dichloroacetic acid; (iii) **S2**; 2-Butanone peroxide; (iv) dichloroacetic acid; (v) pyridinium trifluoroacetate; DMOCP; I_2, H_2O; (vi) *tert*-$BuNH_2$; (vii) TEA-3HF; (viii) CH_3NH_2-EtOH.

3'3'-cGAMP **1** was chemically synthesized by following the similar procedure reported by Jones et al [4]. The other three cGAMP isomers (3'2'-cGAMP; 2'2'-cGAMP; 2'3'-cGAMP) and c-di-GMP and c-di-AMP were synthesized by the same way as 3'3'-cGAMP.

5'-ApG **2** and 5'-GpA **3** were prepared through compound **S5**, the same intermediate product in making 3'5'-cGAMP **1,** by using slightly modified method described as below. The solution of **S1** (0.58 g, 0.6 mmol, 1 equiv) in CH_3CN (5 mL) was added water (0.022 ml, 1.2 mmol, 2 equiv) and pyridinium trifluoroacetate (0.140 g, 0.72 mmol, 1.2 equiv) with stirring under Ar. Shortly after the addition, tert-$BuNH_2$ (3 mL) was added and the mixture was stirred for 15 min at room temperature. Then the reaction mixture was concentrated under vacuum, dissolved in 7 mL of CH_2Cl_2. To this solution, H_2O (0.108 ml, 6 mmol, 10 equiv) and 6 % dichloroacetic acid in CH_2Cl_2 (7 mL, 6 mmol) was added sequentially. The mixture was stirred for 15 min, following by addition of pyridine (0.87 ml, 10.8 mmol, 2 equiv rel to DCA) to quench the reaction. The mixture was concentrated under vacuum, co-evaporated twice in CH_3CN (2x 3mL) to give G H-phosphonate **S3** in hemi-solid form.

S3 was then re-dissolved in 2mL of anhydrous CH_3CN. To this solution was added a solution of A amidite, **S2** (0.756 g, 0.78 mmol, 1.3 equiv) in 3 ml of anhydrous CH_3CN under protecting Ar-atmosphere. After 30 min stirring at rt, 2-Butanone peroxide (1.2 ml, 1.8 mmol, 3 equiv) was added and the mixture was stirred for further 40 min, then 0.189 g Na_2SO_3 in 0.4 ml H_2O was

added. The mixture was stirred for 5 min, and concentrated under vacuum (at 30 ^{o}C bath temp.) to give intermediate compound **S4**.

S4 was dissolved in 10 ml of CH_2Cl_2, followed by addition of H_2O (0.108 ml, 6 mmol, 10 equiv) and the 10 ml of 6 % DCA (6 mmol) in CH_2Cl_2. After 15 min, the reaction was quenched by addition of 6 ml of pyridine. The mixture was concentrated to a small volume, purified by column chromatography on silica gel by using 0 to 25 % CH_3OH-CH_2Cl_2 as gradient elution, to give product crude **S5**.

Compound 3'3'-cGAMP (**1**) can be synthesized from **S5** based on the reported procedure [4]. For making compound **2** & **3**, **S5** was treated directly with 18 ml of CH_3NH_2 in anhydrous EtOH (33 % by weight, 127 mmol, 212 equiv rel to the amino protecting groups). After 4 h of stirring at room temperature, the mixture was concentrated to a solid form and co-evaporated thrice with 3 ml of pyridine and 1 ml of Et_3N by rotary vacuum evaporator. To the obtained oil was added 1 ml of pyridine, and the flask was placed in an oil bath at 55 ^{o}C. Et_3N (4.5 ml, 30 mmol) and $Et_3N{\cdot}3HF$ (2 ml, 36 mmol F^-, 30 eq rel to each TBS) were added simultaneously. The mixture was stirred at 55 ^{o}C for 12 hr, the flask was removed from the oil bath and cooled to room temperature. To the mixture, acetone (70 ml) was slowly added with stirring. After 10 min the precipitate solid was collected by filtration, washed 5× with 3 ml portions of acetone, and air dried under vacuum. This crude product **2** or **3** was dissolved in water and purified by C18 reverse-phase HPLC system using preparative sepax Amethyst C18-H (21.2×150 mm) at 5 mL/min flow rate, with a gradient of B (CH_3CN) in A (50 mM TEAA pH 7.2) (0-20 % of B over 15 min, 20-30 % of B over 10 min, 30-50% of B over 10 min). Evaporation of collected fractions after preparative column separation gave compound **2** and **3** as the triethylamine salt. Yield 147 mg, 40% for **2**. MS (ESI) $[M-H]^-$: 611.1 for both **2** and **3**.

HPLC and MS

Reverse phase high-performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-20A HPLC system. Samples were purified or fractionated by analytical Inertsil ODS-3 C18 column (250×4.6 mm, 5 μm) at 1 mL/min flow rate, with a gradient of B (CH3CN) in A (50 mM TEAA pH 7.2) (0-20 % of B over 15 min, 20-30 % of B over 10 min). Mass Spectra (ESI) was obtained by using Bruker APEX IV instrument.

Construction of mutants and DNA manipulations in *V. cholerae*.

DncV (VC0179) and V-cGAPs deletion mutants were constructed by allelic exchange using strain C6706 as wild-type precursor. All primers were designed based on the DNA sequence of the *V. cholerae* N16961 genome downloaded from the TIGR database (http://cmr.tigr.org). The flanking sequences of the target genes were amplified from genomic DNA of strain N16961. The

chromosomal fragments containing a deletion of the target genes were obtained with bridge PCR using the mixture of the up and down flanking sequences products as templates and cloned into suicide vector pWM91 as BamHI/XhoI fragments. Amplicons and restriction fragments were purified from agarose gels by using the Qiagen QIAquick gel extraction kit. The resulting suicide plasmid was constructed in *E. coli* SM10λpir and mobilized into receptor strain C6706. Exconjugants were selected on LB agar containing PolB (100 unit/ml) and Amp (150 μg/ml) and streaked on LB agar containing 15% (w/v) sucrose. Sucrose-resistant colonies were tested for Amp sensitivity and deletion mutants were confirmed by DNA sequencing.

For ectopic expression, DncV (VC0179) was amplified from C6706 chromosomal DNA and cloned into plasmid pBAD24 and pBAD33 after digestion with EcoRI/XbaI and KpnI/PstI, respectively. V-cGAPs were amplified from C6706 chromosomal DNA and cloned into plasmid pMalc2x after digestion with NdeI/XbaI. The resulting plasmids were constructed in E. coli Top10 and electroporated into receptor *V. cholerae* strains. Primers are listed in Table S1B.

Quantitative PCR

Total RNA from *V. cholerae* was extracted using the Trizol Plus RNA purification system (TaKaRa) followed by the DNase treatment (Ambion) to remove residual chromosomal DNA contamination. The purity and integrity of RNA samples was verified by UV spectrophotometry and agarose gel electrophoresis. cDNA was synthesized by reverse transcription with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA equivalent to 20ng total RNA was analyzed by quantitative real-time RT-PCR (qRT-PCR) with SYBR Green (TaKaRa). Relative expression values (R) were calculated using the equation $R = 2^{-(\Delta Ct\ target\ -\Delta Ct\ reference)}$ where C_t is the fractional threshold cycle. The *recA* mRNA was used as reference. A control mixture lacking reverse transcriptase was performed for each reaction to exclude chromosomal DNA contamination.

Examination of chemotactic behavior of *V. cholerae*

Chemotactic behavior of *V. cholerae* was examined in AKI [5] or LB medium containing 0.25 % agar. Taking V-cGAP1 for example, briefly, overnight culture of *V. cholerae* Δ*dncV*, Δ*V-cGAP1*, Δ*dncV* and Δ*V-cGAP1* expressing DncV from arabinose-inducible plasmid, Δ*V-cGAP1* expressing DncV from arabinose-inducible plasmid and V-cGAP1 (wild type or mutants) from IPTG-inducible plasmid simultaneously with and without induction were stabbed in motility or chemotaxis plates as described above and incubated at 30ºC for 24 to 48 hr.

Biofilm formation

Biofilm formation was measured by the crystal violet staining method and results normalized for growth and expressed as the A570/OD600 ratio. Briefly, overnight culture from fresh colonies was

diluted 1: 50 in fresh medium and transferred to glass tubes. The tubes were incubated for 24 h at 30°C for biofilm development.

Infant mouse colonization assays

Intestinal colonization competition assay in 4- to 5-day-old CD-1 suckling mice (Beijing HFK Bioscience) was performed basically as described previously[6]. Briefly, strains were grown on LB-agar plates with Sm overnight at 30°C. Wild-type and mutant strains were mixed together in PBS. 100 μl of this competition mixture (10^5 bacteria) was inoculated into a 4- to 5-day-old CD1 mouse pup. One strain carried an active *lacZ* allele. 100 μl of serial dilutions of the competition mixture were dropped in LB + Sm100 + X-gal and enumerated to determine the input ratio of wild-type and mutant strain. After incubation at 30 °C for 18 hr the mouse pups were sacrificed and small intestines were removed and homogenized in 4 ml of PBS. 100 μl of serial dilutions were dropped in LB + Sm100 + Xgal and enumerated to determine the output ratio of wild-type and mutant strain. The *in vivo* competitive indices were defined as the output ratio of mutant/wild-type strain divided by the input ratio of mutant/wild-type strain. Statistical significance was determined by comparing the resulting ratio to the ratio of WT versus WT *lacZ*−.

Statistical analysis.

We performed statistical analysis by using an unpaired Student's t-test for all studies unless otherwise indicated. We considered $P<0.05$ to be statistically significant.

Supporting references

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