Protein Deacetylase CobB Interplays with c-di-GMP

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Abstract

As a ubiquitous bacterial secondary messenger, c-di-GMP plays key regulatory roles in processes such as bacterial motility and transcription regulation. CobB is the Sir2 family protein deacetylase that controls energy metabolism, chemotaxis and DNA supercoiling in many bacteria. Using an *E.coli* proteome microarray, we found that c-di-GMP strongly binds to CobB. Protein deacetylation assays showed that c-di-GMP inhibits CobB activity and thereby modulates the biogenesis of acetyl-CoA. Through mutagenesis studies, residues R8, R17 and E21 of CobB were shown to be required for c-di-GMP binding. Next, we found that CobB is an effective deacetylase of YdeH, a major diguanylate cyclase (DGC) of *E.coli* that is endogenously acetylated. Mass spectrometry analysis identified YdeH K4 as the major site of acetylation, and it could be deacetylated by CobB. Interestingly, deacetylation of YdeH enhances its stability and cyclase activity in c-di-GMP production. Thus, our work establishes a novel negative feedback loop linking c-di-GMP biogenesis and CobB-mediated protein deacetylation.

Key words

c-di-GMP; CobB; diguanylate cyclase; protein acetylation; negative feedback loop

67 Introduction

68 Cyclic diguanosine monophosphate (c-di-GMP) was first identified in
 69 *Gluconacetobacter xylinus*, where it was found to regulate cellulose
 70 synthesis¹. Subsequently, c-di-GMP was shown to be involved in a wide
 71 range of bacterial biological processes such as bacterial motility, biofilm
 72 formation, virulence and transcription regulation²⁻⁴. However, these
 73 processes likely represent only a portion of the full scope of its diverse
 74 functions in the cell, owing to the typical challenges of unambiguously
 75 ascribing functionality directly to the activity of a specific small molecule
 76 second messenger⁵. A first step toward this understanding has often
 77 emerged from an identification of the proteins to which it strongly
 78 interacts, as exemplified in studies of c-di-GMP binding to YcgR⁶, CckA⁷,
 79 BldD⁸ and CheY-like (Che) proteins⁹. Thus, we speculated that a better
 80 understanding of the full repertoire of c-di-GMP functions in bacteria
 81 would emerge from a comprehensive knowledge of the complete range
 82 of proteins to which it binds.

83 c-di-GMP is synthesized by diguanylate cyclases (DGCs)¹⁰ and
 84 degraded by specific phosphodiesterases (PDEs)¹¹⁻¹³. In *E.coli*, the
 85 dominant DGCs are YdeH (also known as DgcZ)¹⁴ and DosC¹⁵, and the
 86 major PDEs are YhjH¹⁶ and DosP¹⁵. Each of these proteins has been
 87 shown to be modulated by “first” messengers such as light, oxygen and
 88 temperature². Yet, owing to the involvement of c-di-GMP in the

89 aforementioned fundamental biological processes that are all
90 well-regulated by many basic metabolic mechanisms^{17, 18}, there is also
91 the possibility that c-di-GMP biosynthesis is also regulated via
92 metabolism-related intracellular signals.

93 The Sir2 family protein CobB is a NAD⁺-dependent deacetylase that is
94 highly conserved in prokaryotes^{19, 20}. In *E.coli*, CobB is the sole Sir2
95 homolog, although there are two forms of CobB, namely, CobB and
96 CobB_s, the former of which has an additional 37 aa N-terminal tail²¹.
97 CobB exhibits protein deacetylation activity and it regulates a variety of
98 physiological functions. For example, CobB deacetylates lysine-609 of
99 acetyl-coenzyme A synthetase (Acs) to activate its activity²², resulting in
100 an increased cellular concentration of acetyl-coenzyme A (acetyl-CoA),
101 which is a central component of energy metabolism. In addition, CobB
102 regulates *E.coli* chemotaxis by deacetylating the chemotaxis response
103 regulator protein (CheY)²³, as well as the activity of N-hydroxyarylamine
104 O-acetyltransferase (NhoA)²⁴ and topoisomerase I (TopA)²⁵. Yet, despite
105 the critical role that CobB plays in many biological processes, its inherent
106 regulation remains poorly understood²⁶⁻²⁸.

107 To globally identify c-di-GMP effectors and explore new functions of
108 c-di-GMP, we employed an *E.coli* proteome microarray²⁹ for
109 proteome-wide identification of c-di-GMP binding proteins. Surprisingly,
110 we found that c-di-GMP strongly binds to CobB. Subsequent biochemical

analysis confirmed that c-di-GMP binds to CobB and inhibits its deacetylation activity both *in vitro* and *in vivo*. Furthermore, we found that the major DGC in *E.coli*, YdeH, is endogenously acetylated and CobB promotes the stability and activity of YdeH through deacetylation of lysine-4. Altogether, we have established an evidence-based regulation loop underlying the cytoplasmic concentration of c-di-GMP that involves its direct binding to, and thereby inhibition of CobB.

Results

Protein deacetylase CobB is a novel c-di-GMP effector

To identify novel effectors of c-di-GMP, an *E.coli* proteomic microarray was probed with biotin-c-di-GMP. In this way, CobB (specifically, the version of CobB with the extra N-terminal tail) was identified as a strong binder of c-di-GMP (**Fig. 1a**). To validate this interaction, we developed a simple *in vitro* assay in where purified CobB was incubated with biotin-c-di-GMP, UV crosslinked, and then probed with fluorescent streptavidin.^{30, 31} We found that c-di-GMP indeed exhibits strong binding to CobB, although trace bindings were also observed for cGMP and c-di-AMP (**Fig. 1b**). Mixing c-di-GMP with biotin-c-di-GMP before the two were added to CobB resulted in a significant reduction in biotin-c-di-GMP binding to CobB (**Fig. 1b**). To quantify the binding strength of this interaction, we performed Isothermal Titration

133 Calorimetry (ITC) and found that the c-di-GMP/CobB affinity constant, K_d ,
 134 is 21.6 μM with a binding stoichiometry of 0.97. We also performed ITC
 135 assays with cGMP and c-di-AMP, and no binding was detected with
 136 these two molecules (**Fig. 1c and supplementary Fig. 1a-c**). To confirm
 137 these results, we also determined the affinity constant using Microscale
 138 Thermophoresis (MST), which yielded a K_d of $22.7 \pm 1.63 \mu\text{M}$ for the
 139 interaction between c-di-GMP and CobB (**Supplementary Fig. 2**). We
 140 note however that while the affinity constants determined from ITC and
 141 MST are consistent with each other, we observed some polymerization
 142 of CobB in the presence of c-di-GMP (**Supplementary Fig. 3**), which may
 143 somewhat affect the measurement of the affinity for c-di-GMP.
 144 Nonetheless, taken together, these results clearly show that c-di-GMP
 145 specifically binds to CobB.

146

c-di-GMP inhibits the deacetylase activity of CobB and down-regulates the biogenesis of acetyl-CoA

149 With the demonstration of binding between c-di-GMP and CobB, we
 150 speculated that c-di-GMP may affect the deacetylase activity of CobB. To
 151 test this, we examined the activity of CobB in the presence of c-di-GMP
 152 by monitoring the deacetylation of the well-known CobB substrates,
 153 Acs²², CheY²³, and NhoA²⁴. Before incubation with CobB, we found that
 154 purified Acs from a CobB-deficient *E.coli* ($\Delta cobB$) is highly acetylated (**Fig.**

155 **2a**). Incubation with CobB resulted in significant reduction in acetylation,
 156 consistent with previous work²² (**Fig. 2a**). However, in the presence of
 157 c-di-GMP, the deacetylation of Acs was significantly inhibited in a dose
 158 dependent manner (**Fig. 2a and supplementary Figure 4a**). By contrast,
 159 neither the presence of cGMP nor c-di-AMP affected the CobB
 160 deacetylation of Acs. Similar results were obtained for CheY
 161 (**Supplementary Figure 4b**) and NhoA (**Supplementary Figure 4c**).

162 To examine the inhibition of c-di-GMP on CobB in more detail, we
 163 measured the kinetics of CobB deacetylation of an acetylated peptide³²
 164 at different concentrations of c-di-GMP. We found that c-di-GMP
 165 significantly reduces the maximal catalytic rate of CobB, with no changes
 166 to the Km values, yielding a K_i of c-di-GMP for CobB of 32.27 μ M (**Fig. 2b**).
 167 Thus, c-di-GMP noncompetitively inhibits the deacetylation activity of
 168 CobB, suggesting that the binding region of c-di-GMP on CobB is not in
 169 the catalytic pocket.

170 To further confirm the inhibition of c-di-GMP on CobB activity, we
 171 sought to alter the endogenous levels of c-di-GMP and test the
 172 deacetylation activity of CobB *in vivo*. In *E.coli*, YdeH¹⁴ is the major DGC
 173 that produces c-di-GMP. Thus, we examined an *E.coli* strain with
 174 overexpressed YdeH (*ydeH*⁺) and a strain expressing a dysfunctional
 175 mutant YdeH (*ydeH*^{G206A,G207A})¹⁴ to produce strains with high and low
 176 levels of c-di-GMP, respectively. To monitor the activity of CobB, we

177 examined the acetylation level of endogenous Acs to which a
 178 chromosomal C-terminal FLAG-tag was attached. We also examined a
 179 series of CobB-depleted strains, namely $\Delta cobB$, $\Delta cobB::cobB$ and
 180 $\Delta cobB::cobB ydeH^+$, to further investigate this interaction. As expected,
 181 high levels of c-di-GMP were clearly observed for strains with YdeH⁺
 182 **(Fig.2c)**. In these strains, we found a significant reduction of
 183 deacetylation of Acs **(Fig.2d)**. We found that this deacetylation of Acs is
 184 abolished by the deletion of CobB ($\Delta cobB$), and that this could be
 185 recovered by putting back CobB ($\Delta cobB::cobB$). Thus, these results
 186 indicate that c-di-GMP increases the acetylation levels of Acs in a
 187 CobB-dependent manner.

188 It is known that CobB activates Acs through deacetylation of K609,
 189 and Acs is responsible for the synthesis of acetyl-CoA, which is essential
 190 for cell growth²². It is also known that both acetate and propionate can
 191 serve as a donor for acetyl-CoA for cell growth^{33, 34} **(Fig.2e)**. To further
 192 confirm the regulatory role of c-di-GMP on acetyl-CoA synthesis, all of
 193 the aforementioned strains **(Fig.2f)** were cultured using acetate or
 194 propionate as the sole carbon source. For the CobB deficient cells
 195 ($\Delta cobB$), we observed an obvious growth defect, consistent with a lack of
 196 activated Acs. Similarly, overexpression of YdeH significantly inhibited
 197 cell growth in the $ydeH^+$ and $\Delta cobB::cobB ydeH^+$ strains. These results are
 198 consistent with an inhibition of CobB by high levels of c-di-GMP **(Fig.2f,**

199 **Supplementary Table 1).** We also found that the *ydeH*^{G206A,G207A} strain
 200 exhibited a similar growth as that of the WT strain. The inhibition of
 201 growth in the strains with *ydeH*⁺ was similar at concentrations of acetate
 202 or propionate of 10 mM or 30 mM (**Supplementary Fig. 5,**
 203 **Supplementary Table 1).** Thus, in addition to confirming the c-di-GMP
 204 inhibition of CobB *in vivo*, these results strongly suggest that c-di-GMP is
 205 a physiologically relevant effector of the regulation of acetyl-CoA
 206 biogenesis through the inhibition of the deacetylation activity of CobB.

207

208 **c-di-GMP globally affects CobB-dependent deacetylation in vivo**

209 To determine whether c-di-GMP could affect CobB-dependent
 210 deacetylation in a global setting, we applied Stable Isotope Labeling with
 211 Amino acids in Cell culture (SILAC) coupled with MS to quantitatively
 212 compare the levels of protein acetylation in WT and *ydeH*⁺ cells.
 213 Previously, Weinert *et al.*³⁴ identified 366 CobB regulated acetylation
 214 sites when *E.coli* was cultured in M9 media supplemented with 0.2%
 215 glucose. In addition, Cerezo *et al.*³⁵ identified 283 acetylation sites in
 216 *E.coli* under carbon-limited conditions. Since the acetylome of *E.coli*
 217 varies significantly under different culture conditions, to facilitate
 218 comparisons with previous work, we adopted the procedure developed
 219 by Weinert *et al.*³⁴ with slight modifications (see Methods). A total of 802
 220 acetylation sites (**Data set S1**) were identified, of which 107 (**Data set S2**)

221 exhibited enhanced acetylation upon overexpression of YdeH (**Fig. 3a, b,**
 222 **supplementary Fig. 6**). Of the CobB regulated acetylation sites identified
 223 by Weinert *et al.*³⁴ (**Data set S3**), 43 were discovered in our study, 28 of
 224 which were among those that exhibited greater acetylation in *ydeH*⁺ (**Fig.**
 225 **3c, data Set S4**). Hence, CobB regulated sites are enriched in *ydeH*⁺ cells.
 226 Furthermore, we mapped the 107 c-di-GMP upregulated sites to 87
 227 proteins and found that 42 of these proteins overlap with the 271 CobB
 228 regulated proteins identified previously³⁴ (**Fig. 3d, data Set S5**). We also
 229 compared the functional categories of the c-di-GMP upregulated
 230 proteins with the CobB regulated proteins and found that these two sets
 231 of proteins are highly similar in several classifications (**Supplementary**
 232 **Fig. 7**). We note that slight differences in our procedure from that
 233 described by Weinert *et al.* (particularly the absence of fractionation
 234 before MS analysis) may explain the lower numbers of acetylation sites
 235 identified here compared to the earlier work (802 vs. 3,680).
 236 Nevertheless, our data strongly indicate that c-di-GMP regulated
 237 acetylation is closely related to CobB dependent deacetylation. Thus,
 238 these results suggest that c-di-GMP globally affects CobB-dependent
 239 protein deacetylation *in vivo*.

240

241 **Mutagenesis and binding studies of the CobB and c-di-GMP interaction**

242 There is an additional 37 aa N-terminal tail in CobB compared to that of

243 CobB_S²¹ (**Fig. 4a**). Streptavidin blotting assays showed that truncation of
 244 the N-terminal fragment of CobB disrupts c-di-GMP binding, suggesting
 245 that residues 1-37 of CobB are essential for c-di-GMP interaction (**Fig.**
 246 **4b**). It is known that c-di-GMP binds to many of its effectors by Arg, Leu,
 247 Asp and Glu residues³⁶. To determine the exact binding sites on CobB,
 248 we mutated all Arg, Leu, Asp and Glu residues to Ala within the CobB
 249 N-terminal domain. We found that only CobB mutants with R8A, R17A
 250 and E21A exhibited a weakened interaction with c-di-GMP (**Fig. 4c**). We
 251 next performed ITC titration with these mutants and found that the K_d
 252 values are 1.8 mM, 1.6 mM and 0.32 mM for CobB^{R8A}, CobB^{R17A} and
 253 CobB^{E21A}, respectively (**Fig. 4d**), which is 15 to 83-fold lower than that of
 254 the wild type CobB (**Fig. 1c and supplementary Fig. 1d-f**). Despite a loss
 255 of c-di-GMP binding, CobB^{R8A}, CobB^{R17A} and CobB^{E21A} displayed similar
 256 deacetylase activity as CobB. Importantly, addition of c-di-GMP did not
 257 inhibit the activity of these mutants (**Fig. 4e**). Collectively, these data
 258 suggest that R8, R17 and E21 are important for c-di-GMP binding but do
 259 not directly participate in the catalytic activity of CobB.

260 To further validate the specific binding of c-di-GMP to CobB, we
 261 constructed strains of these three CobB mutants, *i.e.*, $\Delta cobB::cobB^{R8A}$
 262 $ydeH^+$, $\Delta cobB::cobB^{R17A}$ $ydeH^+$ and $\Delta cobB::cobB^{E21A}$ $ydeH^+$ based on an
 263 *E.coli* strain that includes the chromosomal Flag-tagged Acs. These
 264 strains were cultured with acetate or propionate and the c-di-GMP

265 concentrations were measured (**Supplementary Fig. 8a**). To monitor the
266 *in vivo* activity of CobB, we examined the acetylation of endogenous Acs.
267 As expected, the acetylation levels of Acs were significantly lower in the
268 strains with these CobB mutants, compared with that in the $\Delta cobB::cobB$
269 *ydeH*⁺ strain (**Fig. 4f**). Additionally, these CobB mutant strains showed
270 similar growth rates as $\Delta cobB::cobB$, but higher growth rates than
271 $\Delta cobB::cobB$ *ydeH*⁺ (**Fig. 4g, supplementary Fig. 8b-d**). Hence, these
272 results provide *in vivo* evidence for the role of these residues in CobB in
273 the binding of c-di-GMP.

274

275 **The binding and inhibition of the deacetylation activity of CobB by** 276 **c-di-GMP is conserved among prokaryotes**

277 CobB is a Sir2 homolog that is highly conserved in prokaryotes. We thus
278 hypothesized that the binding and inhibition of c-di-GMP observed with
279 CobB from *E.coli* is the same for the CobB homologues in other
280 prokaryotes. To test this hypothesis, CobB protein sequences from a
281 series of highly diverse bacteria were aligned (**Supplementary Fig. 9**).
282 We found that the c-di-GMP binding region is reasonably well conserved
283 in these bacteria (**Fig. 5a**). Examining *S. typhimurium* CobB as an
284 exemplary member of this conserved set, we found that CobB^{*S. typhimurium*}
285 binds to c-di-GMP, but not to c-di-AMP and cGMP (**Fig. 5b**). Furthermore,
286 we determined the affinity of *S. typhimurium* CobB and c-di-GMP to be

287 15.5 μ M (**Supplementary Fig. 10**), and that the CobB^{*S. typhimurium*}
 288 deacetylation of Acs could be clearly inhibited by c-di-GMP, but not
 289 cGMP and c-di-AMP (**Fig. 5c**).

290

291 **CobB deacetylates YdeH on K4 to activate its DGC activity**

292 Protein acetylation is one of the most abundant post-translational
 293 modifications in bacteria (and eukaryotes), with hundreds of acetylated
 294 proteins already identified in *E.coli*^{37, 38}. We speculated that some of the
 295 proteins involved in c-di-GMP metabolism are endogenously acetylated
 296 and could be deacetylated by CobB. We selected 4 DGCs (YdeH, YaiC,
 297 YegE and YeaJ), 2 PEDs (YahA, DosP), and 3 GTPases (Era, YihK and YihI)
 298 from *E.coli* to test this possibility. We found that the 2 DGCs (YdeH, YegE)
 299 and 2 GTPases (Era, YihK) were acetylated (**Fig. 6a**). Further, treating
 300 these proteins with CobB, we found that YdeH (DGC) and Era (GTPase)
 301 could be effectively deacetylated by CobB (**Fig. 6a**). To determine the
 302 residue(s) of YdeH targeted for deacetylation by CobB, both acetylated
 303 YdeH and CobB-treated deacetylated YdeH were subjected to
 304 mass-spectrometry analysis. In this way, YdeH K4, K170, K277 were
 305 determined as the sites that could be deacetylated by CobB (**Fig. 6b**,
 306 **supplementary Fig. 11**). To validate these sites, we mutated all 8 Lys
 307 residues of YdeH to Ala individually, and found that only the YdeH K4
 308 mutant exhibited a significantly decreased acetylation level (**Fig. 6c**).

309 These results indicate that K4 is the dominant acetylated site on YdeH. In
310 addition, two other acetylation sites were also identified on Era (K171)
311 **(Supplementary Fig. 12a)** and YegE (K936) **(Supplementary Fig. 12b)**.

312 To verify the functional role of YdeH K4 acetylation, we mutated the
313 Lys to Arg (YdeH^{K4R}), Gln (YdeH^{K4Q}) or Ala (YdeH^{K4A}). Western blotting
314 showed that these mutations significantly reduced the level of
315 acetylation of YdeH compared to that of WT YdeH **(Fig. 6d)**. Since YdeH
316 is the major DGC in *E.coli*, we then set to test whether CobB
317 deacetylation could affect the DGC activity of YdeH using UPLC-IM MS
318 **(Fig. 6e)**. Upon CobB treatment, WT YdeH showed a 2-fold increased
319 production of c-di-GMP, while the activities of YdeH^{K4R}, YdeH^{K4Q} and
320 YdeH^{K4A} were 69.5%, 29.5%, and 27.1% of that of the WT, respectively
321 **(Fig. 6f)**. Unexpectedly, the mutant YdeH^{K4R} that structurally resembles
322 the deacetylation form of YdeH also exhibited a lower DGC activity.
323 Evidently, the “K” to “R” mutation at residue 4 fails to fully mimic the
324 deacetylation status of this site. Nonetheless, these results suggest that
325 K4 is critical for the DGC activity of YdeH.

326 To test whether CobB can regulate the c-di-GMP levels *in vivo*, we
327 constructed several *ydeH*⁺ and Δ *cobB ydeH*⁺ strains, including those with
328 CobB mutants or with YdeH mutants, and then determined the YdeH
329 acetylation and c-di-GMP levels. We found that the acetylation level of
330 YdeH in *ydeH*⁺ is lower than that of Δ *cobB ydeH*⁺ and the c-di-GMP level

of *ydeH*⁺ is significantly higher than that of $\Delta cobB$ *ydeH*⁺ (**Fig. 6g**). These results confirm that CobB can regulate the DGC activity of YdeH through deacetylation. The strains with the YdeH mutants exhibited the lowest DGC activity, confirming that K4 is critical to the DGC activity of YdeH (**Fig. 6g**). Interestingly, the strains with the CobB mutants exhibit lower acetylation levels than WT CobB (**Fig. 6g**). These results indicate that c-di-GMP does not affect the activity of the CobB R8A, R17A and E21A mutants *in vivo*. Still, the CobB mutants exhibited higher c-di-GMP levels, further confirming that CobB promotes the DGC activity of YdeH through deacetylation.

341

342 **CobB enhances the solubility/stability of YdeH through deacetylation**

343 We found that the acetylation level of endogenous YdeH is significantly
344 decreased in WT cells compared to that of CobB-deficient cells (**Fig. 7a**).
345 In order to better characterize the endogenous stoichiometry of YdeH K4
346 acetylation, we used the absolute quantification (AQUA) method^{39, 40} to
347 quantify the level of the K4 acetylated peptide. As shown in **Fig. 7b**, the
348 YdeH K4 acetylation stoichiometry was 1.3 ± 0.2 % for the CobB deficient
349 cells, while it is undetectable for the WT cells.

350 Since it is known that acetylation can lead to protein degradation *in*
351 *vivo*⁴¹, we speculated that acetylation of YdeH may affect its stability.

352 Indeed, we found that the level of endogenous soluble YdeH decreased

353 40% in CobB deficient cells as compared to that of the WT cells (and also
 354 the CobB recovered cells) in exponential growth (**Fig. 7c**), and more
 355 significantly, a decrease of 70% soluble YdeH was observed for stationary
 356 growth (**Supplementary Fig. 16c**). Furthermore, for stationary growth, a
 357 significant amount of precipitated YdeH was observed for CobB deficient
 358 cells, but not for WT and CobB recovered cells (**Supplementary Fig. 16c**).
 359 We then examined the protein stability of YdeH mutants *in vitro* and
 360 found that YdeH^{WT} and YdeH^{K4Q} are less stable than YdeH^{K4A} and YdeH^{K4R}
 361 (**Supplementary figure 13**). In addition, we also noticed that the level of
 362 soluble YdeH following the addition of CobB is 2-fold of that in the
 363 absence of CobB (**Fig. 7e**).

364 Additionally, our data show that the c-di-GMP increase by 36% in WT
 365 cells than CobB deficient cells *in vivo* under exponential growth when
 366 YdeH was overexpressed (**Fig. 6g**). To understand the underlying
 367 mechanism, we then determined the level of YdeH when it was
 368 overexpressed in WT cells and CobB deficient cells. The results showed
 369 that the soluble YdeH level decreased ~45% in CobB deficient cells as
 370 compared to that of the WT cells (**Fig. 7f**), which is similar to that of the
 371 endogenous YdeH under exponential growth (**Fig. 7c**). We also measured
 372 the YdeH level in the sediment and observed significant amount of
 373 precipitated YdeH in CobB deficient cells as compared to that of WT and
 374 CobB recovered cells (**Fig. 7f**).

Thus, it appears as though YdeH K4 acetylation lead to protein aggregation/precipitation by reducing its solubility/stability (**Fig. 8a**). In WT cells, the acetylation of YdeH is removed by CobB and retained in soluble state, with little or undetectable protein sediment (**Supplementary Fig. 16c**). In CobB deficient cells, more acetylated YdeH will precipitate with both acetylated and/or deacetylated YdeH because of the lack of CobB deacetylation. Additionally, this model also explains why YdeH K4 may be frequently acetylated but cannot accumulate to high level *in vivo*. It is known that acetylation on specific sites promote protein aggregation^{42, 43}. For example, K280 acetylation causes the aggregation of tau. And tau promotes neuronal survival, thus the acetylation induced tau aggregation is pathologically significant. Interestingly, tau K280 belongs to a double lysine motif, *i.e.*, ²⁷⁵VQIINKK²⁸¹, and YdeH K4 belongs to a similar double lysine motif, *i.e.*, ¹MIKK⁴. Thus, it is possible that the underlying mechanism of lysine acetylation induced protein aggregation of YdeH may be similar to that of tau. However, because of the possible complexity, the detailed mechanism is yet to be discovered.

393

394 DISCUSSION

c-di-GMP is a key secondary messenger in prokaryotes. CobB is the first, and major, protein deacetylase identified in prokaryotes. Herein, we discovered that c-di-GMP specifically binds to CobB and inhibits its

deacetylase activity, and down-regulates the cellular concentration of acetyl-CoA through modulation of the acetylation levels of Acs. Interestingly, we also found the major *E.coli* DGC, YdeH, is endogenously acetylated, and CobB enhances the solubility/stability of YdeH, and activates the DGC activity of YdeH, through deacetylation. Thus, we established a negative feedback regulatory loop between c-di-GMP biogenesis and CobB dependent protein deacetylation.

The binding region and kinetics of CobB and c-di-GMP

The most well-known motif of c-di-GMP binding is the EXLXR motif⁴⁴. Interestingly, the c-di-GMP binding site on CobB contains RXLXE, which is precisely EXLXR in reverse. As expected in the EXLXR motif, both R17 and E21 are critical for c-di-GMP binding, while the L19 residue does not directly bind to c-di-GMP in the RXLXE motif. This motif is located in the N-terminal tail of this protein that we found is responsible for its dimerization (**Supplementary Figure 3**). Since many of the c-di-GMP binding proteins are dimers or tetramers^{4, 31, 45}, a plausible explanation for this effect of c-di-GMP described here is that c-di-GMP interferes with the dimerization of CobB.

A concern with our model though is that the K_d and K_i of c-di-GMP for CobB measured *in vitro* are larger than the sub- to micro-molar concentrations of c-di-GMP generally observed *in vivo*⁴⁶. While this may

be owing to presently unidentified molecular factors that reduce this affinity *in vivo*, we believe that this discrepancy could be explained by the spatially and temporally uneven distribution of c-di-GMP in the bacteria. Indeed, direct measurement of the concentration of c-di-GMP in bacteria using a FRET biosensor has revealed a wide range of concentrations within individual cells⁴⁷, including some resolution-limited locations ($\sim 200 \times 200 \times 1000 \text{ nm}^3$) that exhibit a local concentration significantly greater than $1 \text{ }\mu\text{M}$. We note that this concentration corresponds to only 25 molecules within this volume. Further, it is well known that the bacterial cytoplasm is extremely crowded⁴⁸, and recent work has shown that, as a result, effectively, there are caging effects on the free diffusion of particles⁴⁹. A single molecule of c-di-GMP within a “cage” of only $20 \times 20 \times 100 \text{ nm}^3$ is at a concentration of $40 \text{ }\mu\text{M}$. Thus, we speculate that it is indeed physically possible that the local concentration of c-di-GMP could exceed the measured affinity constants for CobB in the WT bacteria. In fact, owing to the mechanism described here, the negative regulatory loop involving CobB and YdeH may be principally responsible for keeping the local (and thus global) cytosolic concentration to the observed maximal levels.

439

c-di-GMP regulates physiological functions through inhibiting of CobB deacetylation

442 As the most important substrate of CobB, Acs is responsible for the
 443 synthesis of acetyl-CoA, which controls cell energy metabolism and
 444 global protein acetylation, and affects cell growth and proliferation⁵⁰⁻⁵².
 445 Our results indicate that c-di-GMP can lower the concentration of
 446 acetyl-CoA *in vivo* through inhibiting CobB and increasing the acetylation
 447 level of Acs. Previous studies have identified several possible
 448 overlapping effects of c-di-GMP and acetyl-CoA. For example, c-di-GMP
 449 affects the expression of acetate kinase (AckA) through the binding of
 450 the transcription factor that regulates AckA transcription in *B.*
 451 *burgdorferi*⁵³. In addition, acyl-CoA dehydrogenase, a key enzyme in
 452 acetyl-CoA metabolism pathways, is a genuine c-di-GMP effector in *B.*
 453 *bacteriovorus*⁵⁴. Here, we strengthened the link between c-di-GMP and
 454 acetyl-CoA by showing, for the first time, that c-di-GMP directly
 455 modulates the biogenesis of acetyl-CoA.

456 c-di-GMP has emerged as a key regulator in the decision between
 457 motile and sedentary forms of bacteria². Elevated c-di-GMP levels inhibit
 458 bacterial motility via effects on the flagella-associated protein⁶. Most
 459 recently, Nesper *et al.* found that c-di-GMP directly binds to the
 460 CheY-like regulators and tunes the bacterial flagellar motor⁹. According
 461 to our data, c-di-GMP can regulate the acetylation level of CheY via
 462 inhibition of the deacetylase activity of CobB. Thus, it is highly possible
 463 that c-di-GMP regulates bacterial motility through CobB-mediated

464 regulation of CheY activity.

465 CobB is a member of the sirtuin protein family, which are highly
466 conserved across prokaryotes to eukaryotes. Thus, it is possible that
467 c-di-GMP may also bind human sirtuins and play critical functional roles
468 in a variety of biological processes, such as host-pathogen interactions.

469

470 **CobB regulates the YdeH DGC activity *in vitro***

471 The overexpressed YdeH was used for DGC activity assay *in vitro*. We
472 determined the K4 acetylation stoichiometry of the overexpressed YdeH
473 following the same AQUA procedure that we used for the endogenous
474 YdeH. The results showed that the overexpressed YdeH K4 acetylation
475 stoichiometry is $28.7 \pm 4.9\%$ (**Supplementary Fig. 14**). Assuming that all
476 the K4 acetylation could be deacetylated by CobB, we could thus expect
477 a ~1.4-fold activity increase upon CobB deacetylation. But it is still could
478 not fully explain the 2-fold activity increase upon CobB deacetylation (**Fig.**
479 **6f**). To this end, we sought to determine, with a simple model, whether
480 the loss of stable YdeH could completely account for the 2-fold difference
481 in c-di-GMP. In particular, based on the stability data (**Fig. 7e**), we
482 considered the case in which the difference in c-di-GMP is completely
483 owing to the decrease in soluble protein. Assuming that the substrate is
484 not limiting throughout the assay and that steady state is achieved after
485 a very short time, the lower amount of c-di-GMP produced would simply

486 be a result of the lower amount of soluble protein at any time point. We
 487 found that the decrease in soluble protein is well-described by a single
 488 exponential (**Supplementary Fig. 15**)

$$C \sim e^{-kt}$$

489 where C is the concentration of YdeH and k is the rate of the protein loss,
 490 determined to be 0.49 hr^{-1} in the fit. Thus, the fold-increase, F , expected
 491 for the fully stable (CobB treated) YdeH relative to the more unstable
 492 version can be calculated (for the 2 hrs assay) by

$$F = \frac{\int_0^2 dt}{\int_0^2 e^{-kt} dt} = \frac{2}{\left(\frac{1}{k}\right)(1 - e^{-2k})} = 1.57$$

493 with the aforementioned value of k . Thus, simple loss of the active
 494 protein would result in a 1.57-fold difference in c-di-GMP. Since we
 495 observed a 2-fold difference, we conclude that the loss of soluble protein
 496 can account for ~78% ($1.57/2$) of the difference measured with the
 497 acetylated and deacetylated YdeH. Hence, a difference in the inherent
 498 activity between acetylated-YdeH and deacetylated-YdeH is needed to
 499 fully account for observed difference in c-di-GMP produced.

500 We noticed that the K4 acetylation stoichiometry of overexpressed
 501 YdeH is much high that of endogenous YdeH. The endogenous
 502 acetylation stoichiometry of YdeH K4 was measured using the
 503 exponential growth of *E. coli*. While as a common practice, when
 504 overexpress a protein in *E. coli*, the strain is usually induced at

505 exponential growth, and then cultured for another ~2 hours for fast
506 protein producing. At the time of protein purification, the strain is
507 already at stationary phase. Thus, it is possible that the big difference of
508 acetylation stoichiometry between the overexpressed YdeH and the
509 endogenous one is at least partially due to the difference of the growth
510 phases.

511 To test this possibility, we measured the YdeH K4 acetylation
512 stoichiometry using *E. coli* of stationary growth. The results showed that
513 the YdeH K4 acetylation stoichiometry is $1.1 \pm 0.3\%$ in WT cells and $4.2 \pm$
514 0.5% in CobB deficient cells (**Supplementary Fig. 16a-b**). For CobB
515 deficient cells, the YdeH K4 acetylation stoichiometry is 3-fold
516 ($4.2\%/1.3\%$) in stationary growth than that of exponential growth. At
517 least, this data could partially explain the stoichiometry gap between the
518 endogenous and overexpressed YdeH.

519

520 **CobB regulates the YdeH DGC activity *in vivo***

521 It is known that the expression of YdeH is tightly regulated by the RNA
522 binding protein CsrA and the *csrA* mutant can increase the
523 transcription of YdeH in 15-fold and another DGC YcdT in 45-fold⁵⁵. Thus,
524 to artificially generate a condition of YdeH high expression for functional
525 analysis, the *csrA* mutant has often been applied⁵⁶. However, the *csrA*
526 mutation can also activate the expression of another DGC, *i.e.*, YcdT,

527 which could cause high c-di-GMP background when studying YdeH. So,
 528 we chose to overexpress YdeH in *E. coli* to study the regulation role of
 529 CobB to YdeH inside the cell. Our data show that the c-di-GMP increase
 530 by 36% in WT cells than CobB deficient cells *in vivo* under exponential
 531 growth when YdeH was overexpressed (**Fig. 6g**), which is consistent with
 532 the observed 45% decrease of soluble YdeH level in CobB deficient cells
 533 than that of WT cells (**Fig. 7f**). Hence, the 36% lower c-di-GMP in CobB
 534 deficient cells is mainly due to the instability/ precipitation of YdeH.

535 Though it is clear now that the major functional consequence of K4
 536 acetylation is the decreasing of YdeH stability, the direct activity change
 537 upon acetylation and deacetylation by CobB may still play important role.
 538 Our results showed that YdeH^{K4A}, which has comparable stability with
 539 WT YdeH, also exhibited a loss ~50 % of its DGC activity (**Fig. 6f**). It is
 540 possible that the YdeH K4 acetylation may significant affect the structure
 541 of YdeH. To understand the mechanism, we proposed a working model
 542 that illustrates how CobB could regulate the structure of YdeH, based on
 543 an analysis of the atomic structure of YdeH⁵⁶. It has been reported that
 544 dimerization and proper conformational rearrangement of the active
 545 center are required for optimal YdeH DGC activity (**Supplementary Fig.**
 546 **17a**). Reside K4 is located within a “hinge” region of YdeH and may
 547 potentially regulate the conformational rearrangement of the active
 548 center upon dimer formation (**Supplementary Fig. 17b-d**). In the

549 acetylated form of K4, the two GGDEF catalytic domains of YdeH are
 550 misaligned that may cause the aggregation and then lead to precipitate.
 551 **(Supplementary Fig. 17b)** Besides, this misaligned region could prevent
 552 productive ligation of the two GTP molecules, each captured by a GGDEF
 553 domain. According to our enzymatic studies, it is very likely that
 554 deacetylation of YdeH K4ac triggers the proper realignment of the
 555 GGDEF dimer so that the active center is rearranged to allow the
 556 formation of intermolecular phosphoester bonds between the two GTP
 557 substrates **(Supplementary Fig. 17c)**. Interestingly, the proposed model
 558 is close to the Zn-regulation mechanism in YdeH, *i.e.*, inhibits YdeH's DGC
 559 activity by hindering its dimerization⁵⁶. Thus, at least in part, CobB may
 560 regulate the active/inactive switch of DGC activity of YdeH through
 561 deacetylation of YdeH on K4 **(Supplementary Fig. 17d)**.

562

563 **The interplay between CobB and c-di-GMP**

564 Our data show that c-di-GMP can significantly reduce of the decetylation
 565 activity of CobB through direct binding, and that the deacetylation of
 566 overexpressed YdeH by CobB leads to an increase of c-di-GMP by 36% *in*
 567 *vivo*. In addition, c-di-GMP is synthesized from GTP by DGC. CobB
 568 regulates the levels of acetyl-CoA through deacetylating Acs and then
 569 modulates the generation of GTP in the tricarboxylic acid (TCA) cycle.
 570 Thus, to a certain extent, the biogenesis of c-di-GMP could be activated

571 by CobB through its effects on the TCA cycle. We also found that a
572 GTPase Era and the DGC YegE are also endogenously acetylated. These
573 results further demonstrate that the biogenesis of c-di-GMP is regulated
574 by (de)acetylation.

575 The interplay between c-di-GMP and CobB may play important roles
576 in bacteria. It is known that c-di-GMP participates in motility, biofilm
577 formation and virulence, all of which are regulated by energy
578 metabolism^{57, 58}. We found that c-di-GMP modulates the bacterial
579 energy metabolism through inhibiting the deacetylation activity of CobB,
580 thus a connection between important bacterial processes and energy
581 metabolism could be established. In addition, CobB is prominently
582 implicated in the regulation of metabolism⁵⁹ and c-di-GMP is involved in
583 GTP metabolism and purine metabolism, suggesting a possible two-way
584 regulation between c-di-GMP and CobB at the metabolic level.

585 Taken together, we established a novel feedback regulation loop
586 between c-di-GMP and the deacetylation activity of CobB (**Fig. 8b**). The
587 findings of both directions (c-di-GMP inhibits CobB and CobB promotes
588 c-di-GMP biogenesis) are novel. From this loop, we can envision a tightly
589 regulated balance between the levels of c-di-GMP and the protein
590 deacetylase activity of CobB. We strongly believe that our findings will
591 facilitate future functional studies of both c-di-GMP and CobB-based
592 regulation of protein acetylation.

593

594 **Methods**

595 **Bacterial Strains, Plasmids, and CobB Mutant Construction**

596 In this study, we used *E.coli* BW25113 as the wild type strain and the
597 plasmid pSUMO10 for the overexpression of CobB, pET32a for the
598 rescue experiment, and pCA24N for the overexpression of YdeH, Acs,
599 CheY, NhoA, Era, YeaJ, YaiC, YahA, DosP, YihI, YegE and YihK. We
600 performed the CobB mutations using the QuikChange® Site-Directed
601 Mutagenesis Kit (Agilent Technologies, Santa Clara, USA).

602

603 ***E.coli* Proteome Microarray Screening and Data Processing**

604 The *E.coli* proteome microarrays were prepared as described
605 previously²⁹. The proteome microarrays were first blocked with blocking
606 buffer (3% BSA in 0.1% Tween 20; TBST) for 1 h at room temperature.
607 Bio-c-di-GMP was then diluted to 1 μ M incubated on the microarray at
608 room temperature for 1 h, and the same concentration of biotin was
609 included as a negative control. The microarrays were next washed with
610 TBST three times for 5 min each and then were incubated with
611 Cy3-Streptavidin at a 1:1000 dilution (Sigma-Aldrich, Darmstadt,
612 Germany) for 1 h at room temperature, followed by three washes with
613 TBST for 5 min each. The microarrays were spun dry at 250 $\times$ g for 3 min
614 and were scanned with a GenePix pro 6.1 microarray scanner to visualize

615 and quantify the results. The raw data have been published in the
616 Protein Microarray Database (www.proteinarray.cn) with the accession
617 number, PMDE271. The auto-analysis tool in Protein Microarray
618 Database (<http://www.proteinarray.cn/index.php/analysis-toolkits>) was
619 used to process the microarray data.

620

621 **Preparation of c-di-GMP**

622 It is known that c-di-GMP (BIOLOG Life Science Institute, Bremen,
623 German) may form dimer or polymer, affects the binding with its
624 effectors. Thus, we first determined the c-di-GMP monomer population.
625 The OD₂₇₆ and OD₂₈₉ of c-di-GMP were measured using the cuvette
626 mode of Nanodrop 2000c (Thermo Fisher Scientific, MA, USA) with a
627 volume of 100 µL. To calculate the ratio of monomer of c-di-GMP (P_{mono})
628 at various concentrations, we applied the equation ($p_{mono} = 1.15$
629 $(A_{276}/A_{289}) - 1.64$) developed by Gentner *et al.*⁶⁰ To eliminate the
630 possible effect of c-di-GMP polymer, we heated the c-di-GMP solution in
631 a water bath at 60°C for 1 h to depolymerize ~80% c-di-GMP to
632 monomer before testing.

633

634 **Streptavidin blotting assay**

635 In this assay, CobB (0.5 mg/mL, 16.13 µM) and 10 µM bio-c-di-GMP were
636 incubated in the deacetylation buffer (50 mM Tris-HCl, 4 mM MgCl₂, 50

637 mM NaCl, 50 mM KCl, 1 mM NAD⁺, pH 8.0) at 37°C for 1 h and the same
 638 amount of biotin, cGMP, c-di-AMP were included as negative controls.
 639 The samples were then UV-cross linked on ice with 10 min for 1.2
 640 mJ/cm² energy to further link the c-di-GMP to CobB. These samples were
 641 then analyzed by Western blotting. After incubation with IRDye 800-CW
 642 Conjugated Streptavidin (LI-COR Biosciences, Nebraska, USA) for 2 h, the
 643 membranes were washed with TBST three times and visualized with an
 644 Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).
 645 And the protein level was determined by Ponceau S staining is the
 646 (Sangon Biotech, Shanghai, China).

647

648 **ITC assay**

649 In the ITC assay, c-di-GMP and the wild-type CobB and mutant CobB
 650 proteins were prepared in the titration buffer (20 mM Tris, 250 mM NaCl,
 651 2 mM DTT, and pH 7.5). Protein concentrations were measured based on
 652 the UV 280nm absorption. The ITC titrations were performed using a
 653 MicroCal iTC200 system (GE Healthcare, Pittsburgh, USA) at 25°C. Each
 654 titration consisted of 17 successive injections (the first at 0.4 μL and the
 655 remaining 16 at 2.4 μL). The stock c-di-GMP, cGMP and c-di-AMP at 1.5
 656 mM were titrated into wild-type or mutant CobB (0.1 mM) in the sample
 657 cells of 200 μL volume individually. c-di-GMP, cGMP and c-di-AMP of 1.5
 658 mM were titrated into 200 μL titration buffer as controls for data

659 processing. The resultant titration curves were processed using the
660 Origin 7.0 software program (OriginLab) according to the “one set of
661 sites” fitting model.

662

663 **CobB deacetylase activity assay**

664 CobB (50 µg/mL, 1.6 µM) and c-di-GMP (at either 0.25 or 0.5 mM) were
665 incubated in 20 µL deacetylation buffer (50 mM Tris-HCl, 4 mM MgCl₂,
666 50 mM NaCl, 50 mM KCl, 1 mM NAD⁺, pH 8.0) at 37°C for 0.5 h. For
667 negative controls, we used 0.5 mM cGMP and c-di-AMP. The CobB
668 substrates (4.16 µM Acs, 1.41 µM CheY, 0.62 µM NhoA) were then
669 added and incubated at 37°C for 1.5 h. These proteins were analyzed by
670 both silver staining and Western blotting. Membranes were incubated
671 with a pan anti-acetyl antibody (Cell Signaling Technology, MA, USA,
672 with a 1:1000 dilution) for at 4°C for 12 h and then incubated with an
673 IRDye 800 secondary antibody at room temperature for 1 h. The
674 membranes were visualized with an Odyssey Infrared Imaging System.

675

676 **Measuring the catalytic kinetics of CobB**

677 CobB (9.3 µg/mL, 0.3 µM) was incubated with 0, 10, 20 and 80 µM
678 c-di-GMP at 37°C for 20 min in 80 µL deacetylation buffer, and then 20
679 µL gradient concentrations acylated peptide
680 (LEQIAELAGVSK^{ac}TNLLYYFPSK)³² (1, 2, 4, 8, 16, 32, 50, 75, 100, 150 and

200 μ M) were added and co-incubated at 37°C for 30 min. 100 mM HCl and 160 mM acetic acid were added to stop the reactions and spun for 10 min at 18,000 x g to separate the enzyme from the reactions. These samples were analyzed by HPLC. Briefly, samples were injected onto a C-18 column (Alltima™ C18 4.6 x 250 mm) and analyzed by reversed-phase HPLC (Shimadzu, Japan). Solution A (0.065% trifluoroacetic acid in 100% water (v/v)) and solution B (0.05% trifluoroacetic acid in 100% acetonitrile (v/v)) were used in a gradient program (0.01 min with 5% Solution B, 25 min with 65% Solution B, 25.01 min with 95% Solution B, 31 min with 95% Solution B, 31.01 min with 5% Solution B, 40 min with 5% Solution B and stop in 40.01 min) with a flow rate of 1 mL/min. Peptides were detected at 220 nm wavelength. This assay was performed three preparations and V_{max} , K_i values were calculated by curve-fitting the plot using GraphPad Prism 6.

Construction of an *E.coli* strain (BW25113) harboring chromosomal 3xFLAG-tagged Acs and 3xFLAG-tagged YdeH

E.coli strain (BW25113) harboring chromosomal 3xFLAG-tagged Acs and 3xFLAG-tagged YdeH were constructed using the Red recombination system⁶¹, as described previously³².

Isolation and Quantification of c-di-GMP in *E.coli*

703 The c-di-GMP isolation methods were described previously⁶². Briefly, the
 704 total amount of *E.coli* cells with a 50 OD were harvested and
 705 re-suspended in 2 mL ddH₂O. To extract intracellular c-di-GMP, 8 mL
 706 extract mixture of 50% methanol and 50% acetonitrile were added. We
 707 also added 1 μM cGMP as the reference. The mixture was incubated in
 708 boiling water for 10 min and then centrifuged at 10,000 rpm for 10 min.
 709 The supernatant was transferred to a new tube for freeze-drying. The
 710 freeze-dried pellet was re-suspended in 100 μL water containing 50%
 711 methanol. Samples were analyzed by UPLC-IM-MS (Ultra
 712 high-performance liquid chromatography coupled ion mobility mass
 713 spectrometry). UPLC-IM-MS was performed using a Waters UPLC I-class
 714 system equipped with a binary solvent delivery manager and a sample
 715 manager, coupled with a Waters VION IMS Q-TOF Mass Spectrometer
 716 equipped with an electrospray interface (Waters Corporation, Milford,
 717 USA) at the Instrumental Analysis Center of Shanghai Jiao Tong
 718 University. UPLC was performed on a ZIC-HILIC column (100 mm × 2.1
 719 mm i.d., 3.5 μm; Merck). The column was eluted with gradient solvent
 720 from A: B (5: 95) to A: B (40: 60) at a flow rate of 0.40 mL/min, where A is
 721 50 mM ammonium formate and B is acetonitrile. We employed the
 722 following MS experimental parameters: a negative polarity with 2.0 kV
 723 capillary voltage, 20 V sampling cone and 6 eV collision energy. c-di-GMP
 724 was detected in M-1 ion of m/z 689.086 with a fragment ion of m/z

725 344.040, and cGMP as an input in M-1 ion of m/z 344.039.

726

727 **The SILAC MS assay of quantitative acetylation proteomics**

728 *E.coli* BW25113 ($\Delta lysA$) and BW25113 with YdeH overexpression ($\Delta lysA$
729 $ydeH^+$) were subjected for SILAC MS assay. The two strains were
730 activated in LB medium, and then were cultured in 2% glucose M9
731 minimal media supplemented with heavy isotopes of lysine
732 ($^{13}C_6^{14}N_2$ -lysine) or light isotopes of lysine ($^{12}C_6^{14}N_2$ -lysine) (Silantes,
733 Munich, Germany) for $\Delta lysA$ and $\Delta lysA ydeH^+$, respectively. Strains were
734 induced by 0.1 mM IPTG during exponential growth (OD 600nm = ~0.4)
735 and the cells were harvested after inducing for 4 h (OD 600nm = ~1.0).
736 These cells (~30 OD) were added with 1 mL lysis buffer (8 M Urea, 100
737 mM NH_4HCO_3 , 2 mM sodium butyrate, 5 mM nicotinamide, 1x protease
738 inhibitor (Roche, Basel, Switzerland), pH 8.0) and lysed for 2 min at 4°C
739 by an Ultrasonic Cell Disruptor (Cheng-cheng Weiye Science and
740 Technology, Beijing, China). Protein concentration was determined by
741 BCA kit (Pierce, MA, USA). The labeling efficiency of *E.coli* cultured in
742 “heavy” medium was checked before sequential proteomic experiments.

743 Light-labeled and heavy-labeled lysate were equally mixed. Cysteine
744 bonds were reduced by 5 mM dithiothreitol (DTT) at 56°C for 30 min and
745 followed by alkylation reaction with 15 mM iodoacetamide at room
746 temperature in darkness for 30 min. The alkylation reaction was

747 quenched by 30 mM cysteine. The protein solution was diluted to less
748 than 2 M Urea by addition of 100 mM NH_4HCO_3 (pH 8.0) and then
749 digested with sequencing grade trypsin at a trypsin-to-protein ratio of 1:
750 50 (w/w) at 37°C for 16 h. For complete digestion, additional trypsin was
751 added at trypsin-to-protein ratio of 1: 100 (w/w) at 37°C for another four
752 hours. The tryptic peptides were desalted through SepPak C18 cartridges
753 (Waters, MA, USA) and vacuum dried.

754 To enrich the lysine acetylated peptides, 2 mg desalted peptides
755 were dissolved in NETN buffer (600 mM NaCl, 1 mM EDTA, 50 mM
756 Tris-HCl, 0.5% NP-40, pH 8.0) and incubated with 10 μL drained
757 pre-washed anti-acetyl beads (Immunechem, Burnaby, Canada) at 4°C
758 overnight with gentle shaking. The beads were gently washed for four
759 times with NETN buffer and twice with deionized water. The bound
760 peptides were eluted with 0.1% TFA and vacuum dried. The eluted
761 peptides were desalted with C18 ZipTips (Millipore, MA, USA) according
762 to the manufacturer's instructions.

763 Enriched acetylated peptides were analyzed by nano flow LC-MS/MS
764 using an EASY-nLC 1000 system connected to Orbitrap Fusion mass
765 spectrometer (Thermo Fisher Scientific, MA, USA). Peptides were
766 dissolved in solvent A (0.1% FA in 2% ACN) and separated using a
767 homemade reverse-phase C18 analytical column (75 μm ID $\times$ 18 cm
768 length, 3 μm particle size) with a 90-min gradient from 5% to 80%

769 solvent B (0.1% FA in 90% ACN) at a constant flow rate of 300 nL/min.
 770 Intact peptides with m/z 300-1400 were detected at a resolution of
 771 120,000 at m/z 200. Ions with intensity above 5,000 were isolated and
 772 sequentially fragmentized by Higher Collision Dissociation (HCD) with
 773 normalized collision energy of 32% in top speed mode. The automatic
 774 gain control (AGC) targets were set at 5.0e5 for full scan and 7.0e3 for
 775 MS/MS scan, respectively. The dynamic exclusion duration was set as
 776 30s.

777 MS/MS data files were processed with MaxQuant software (version
 778 1.5.3.8) against *Escherichia coli* (strain K12) database from Uniprot
 779 (proteome ID: UP000000625, 4309 sequences, last modified on May
 780 13th, 2017) with a reversed decoy database. SILAC was selected as
 781 “doublets” and “Heavy labels” panel was selected as heavy lysine (Lys6).
 782 Trypsin/P was chosen as the digestion enzyme and two maximum
 783 missing cleavages was allowed. Carbamidomethyl (C) was specified as
 784 the fixed modification and variable modifications were oxidation (M),
 785 acetylation (Protein N-term) and acetylation (K). False discovery rates
 786 (FDR) at protein, peptide and modification level were all set as 1%. For
 787 quantitative analysis, the normalized H/L ratio of each acetylated
 788 peptide exported by MaxQuant software was corrected at the protein
 789 level to eliminate the protein abundance difference. The SILAC mass
 790 spectrometry proteomics raw data have been deposited to the

791 ProteomeXchange⁶³ Consortium via the PRIDE⁶⁴ partner repository with
792 the dataset identifier PXD007616 (Username: reviewer16485@ebi.ac.uk,
793 Password: dD4exhSO).

794

795 **Identification of deacetylation sites by Q Exactive plus MS**

796 Five proteins of the DGC pathway, YdeH, Era, YegE, YeaJ and YihK were
797 constructed to Pca24N and purified from *E.coli* BL21. After SDS-PAGE
798 separation and tryptic digestion, these proteins were mixed and
799 analyzed by mass spectrometry. Briefly, nanoLC–MS/MS-experiments
800 were performed on an EASY-nLC system (Thermo Scientific, Odense,
801 Denmark) connected to a Q Exactive Plus (Thermo Scientific, Bremen,
802 Germany) through a nanoelectrospray ion source. Samples (1 µL) were
803 loaded by an autosampler onto a 2-cm packed pre-column (75 µm ID x
804 360 µm OD) in 0.1% HCOOH/water (buffer A) at a flow rate of 1 µL/min
805 for 5 min. Analytical separation was performed over a 15-cm packed
806 column (75 µm ID x 360 µm OD) at 300 nL/min with a 60 mins gradient
807 of increasing CH₃CN (buffer B, 0.1% HCOOH/CH₃CN). Both pre-column (5
808 µm diameter, 200 Å pore size) and analytical column (3 µm diameter,
809 100 Å pore size) were packed with C18-reversed phase silica
810 (DIKMA-inspire TM, CA, USA) using a pressure bomb. Following sample
811 loading, buffer B was increased rapidly from 3% to 6% over 5min and
812 then shallowly to 22% over 36 min, and then to 35% over 9 min followed

813 by a quick increase to 95% over 3min, and hold at 95% for 7 min. The
 814 total acquisition duration lasted for 60 min. The Q Exactive Plus mass
 815 spectrometer was operated in the data dependent mode to
 816 automatically switch between full scan MS and MS/MS acquisition.
 817 Survey full scan MS spectra (m/z 350–1800) were acquired in the
 818 Orbitrap with 70 000 resolution (m/z 200) after accumulation of ions
 819 to a 3×10^6 target value based on predictive AGC from the previous full
 820 scan. Dynamic exclusion was set to 60 s. The 15 most intense multiply
 821 charged ions ($z \geq 2$) were sequentially isolated and fragmented in the
 822 octupole collision cell by higher-energy collisional dissociation (HCD)
 823 with affixed injection time of 55 ms and 17500 resolutions for the fast
 824 scanning method. Typical mass spectrometric conditions were as follows:
 825 spray voltage, 1.7 kV; heated capillary temperature, 320°C; normalized
 826 HCD collision energy 27%. The MS/MS ion selection threshold was set to
 827 9×10^3 counts. A 1.6 Da isolation width for the samples was chosen. The
 828 mass spectrometry raw data have been submitted to PRIDE^{63, 64} with
 829 project accession PXD007651 (User: reviewer60424@ebi.ac.uk,
 830 password: 6rxEMkjm). And the raw data file was named
 831 “20161202_ZHN_AC.raw” with the search file
 832 “20161202_ZHN_AC-01.ms”.
 833

834 YdeH was chosen for in-depth quantitative MS analysis using Q
 Exactive plus mass spectrometer. YdeH was overexpressed in *E. coli* and

835 affinity purified. YdeH was treated with CobB for deacetylation and
 836 untreated WT YdeH as the control. After SDS-PAGE separation and
 837 trypsin digestion, the samples were analyzed by Q Exactive plus mass
 838 spectrometer under the same experiment condition. The MS raw data
 839 was processed using Protein Discovery software (ThermoFisher) to
 840 identify the lysine acetylation sites. The acetylation sites, which were
 841 identified in untreated WT YdeH sample but not in CobB treated sample,
 842 were considered to be deacetylated by CobB. The mass spectrometry
 843 raw data have been submitted to PRIDE with project accession
 844 PXD008113 (Username: reviewer00527@ebi.ac.uk, password:
 845 jKBngFLH).

846

847 **Determination of the deacetylation activity of CobB *in vivo***

848 The strains, $\Delta cobB$, WT, $ydeH^+$, $ydeH^{G206A,G207A}$, $\Delta cobB::cobB$, Δ
 849 $cobB::cobB ydeH^+$ were used for to assay the deacetylation activity of
 850 CobB. These strains were grown in Vogel-Bonner medium (0.81 mM
 851 $MgSO_4 \cdot 7H_2O$, 43.8 mM K_2HPO_4 , 10 mM $C_6H_8O_7 \cdot H_2O$, 16.7 mM
 852 $NaNH_4HPO_4 \cdot 4H_2O$) with 10 mM acetate at 25°C for 12 h and induced by
 853 0.2 mM IPTG at 25°C for 12 h. 20 OD cells were harvested and
 854 repeatedly freeze-thawed three times. The cells were then treated with
 855 4mL lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole
 856 (Sigma-Aldrich), 1mg/mL lysozyme (Sangon Biotech, Shanghai, China), 1x

857 CellLytic B (Sigma-Aldrich), 50 units/mL of Benzonase (Sigma-Aldrich) and
 858 1 mM PMSF (Sigma-Aldrich), pH 8.0) at 4°C for 20 min with vigorous
 859 shaking. After lysis and centrifugation at 10,000 rpm for 5 min, 20 µL
 860 anti-FLAG antibody (Sigma-Aldrich) and 50 µL protein G conjugated
 861 agarose beads (Roche) were added at 4°C for 2 h with gentle agitation to
 862 enrich the 3xFLAG-tagged Acs. Protein G conjugated agarose was
 863 harvested and washed three times by buffer A (50 mM NaH₂PO₄, 300
 864 mM NaCl, 10 mM imidazole, pH 8.0). The 3xFLAG-tagged Acs was then
 865 eluted by heating at 95°C for 10 min. The amount of Acs protein and
 866 deacetylation level of these samples were analyzed by western blotting.
 867 Membranes were incubated with the pan anti-acetyl antibody (Cell
 868 Signaling Technology with a 1:1000 dilution) for at 4°C for 16 h and an
 869 anti-FLAG antibody (Sigma-Aldrich with a 1:2000 dilution) at 4°C for 16 h.
 870 The IRDye 800 antibody was used as a secondary antibody, and
 871 membranes were incubated for 1 h at room temperature. The
 872 membranes were washed three times in TBST between each antibody
 873 incubation step. Final visualization was performed using an Odyssey
 874 Infrared Imaging System.

875

876 **Determination of the strain growth curve in Vogel-Bonner medium**

877 The strains described above were grown in Vogel-Bonner medium with
 878 10 or 30 mM acetate or propionate at 25°C for 12 h and then induced by

879 0.2 mM IPTG at 25°C for 20 h. During the entire 32 h growth period, the
880 cell concentrations were measured at OD₆₀₀ using Nanodrop 2000s at 8,
881 12, 16, 24 and 32 h. The growth curves were then drawn using GraphPad
882 Prism 6.

883

884 **Homology analysis, phylogenetic tree construction, and sequence** 885 **alignment**

886 The 25 bacteria that we selected for homology analysis were *Escherichia*
887 *coli*, *Salmonella typhimurium*, *Yersinia pestis*, *Shigella dysenteriae*,
888 *Citrobacter amalonaticus*, *Erwinia tracheiphila*, *Vibrio parahaemolyticus*,
889 *Klebsiella oxytoca*, *Cedecea neteri*, *Achromobacter sp.ATCC35328*,
890 *Siccibacter colletis*, *Lelliottia amnigena*, *Buttiauxella brennerae*, *Kluyvera*
891 *ascorbata*, *Mangrovibacter sp.MFB070*, *Cronobacter dublinensis*, *Hafnia*
892 *alvei*, *Chania multitudinisentens*, *Serratia symbiotica*, *Yokenella*
893 *regensburgi*, *Trabulsiella odontotermis*, *Leclercia adecarboxylata*,
894 *Pantoea dispersa*, *Shimwellia blattae*, *Kosakonia radicincitans*. The CobB
895 sequences were examined at the NCBI website and the phylogenetic
896 tree was constructed by EMBL-EBI Clustalw2 online tool. Sequence
897 alignment was performed using DNAMAN 2.0.

898

899 **Determination of the YdeH DGC activity**

900 The purified WT YdeH 5 uM (0.14mg/ml) protein was incubated with

901 CobB 1.6 μ M (0.05mg/ml) for deacetylating at 37°C for 30 min. For DGC
 902 activity determination, the WT YdeH, deacetylated YdeH and YdeH
 903 mutants were incubated with 1 mM GTP, 5 mM MgCl₂ in 100 μ l at 30°C
 904 for 2 h. The reaction was terminated with heating at 95°C for 10 min
 905 and spun for 10 min at 18,000 g to separate the enzyme from the
 906 reactions. Before analyzed, we add 2 μ M cGMP to these reactions as the
 907 control. The c-di-GMP concentrations were determined by UPLC-IM-MS
 908 with the same parameters as mentioned earlier.

909

910 **Determination of the YdeH K4 acetylation stoichiometry using AQUA** 911 **quantification**

912 We used the absolute quantification (AQUA) method^{39, 40} to quantify the
 913 peptide levels. We synthesized the AQUA peptides for deacetylated
 914 (KTTEIDAIL(¹³C₆, ¹⁵N)LNLNK and TTEIDAIL(¹³C₆, ¹⁵N)LNLNK) and acetylated
 915 peptides (K^{ac}TTEIDAIL(¹³C₆, ¹⁵N)LNLNK) of YdeH K4. To acquire the
 916 endogenous YdeH protein, we inserted a chromosomal C-terminal
 917 Flag-tag to YdeH and immuno-precipitated by an anti-FLAG antibody.
 918 The strains, Δ *cobB* and WT, were used for to assay the YdeH K4
 919 acetylation stoichiometry. These strains were grown in LB medium
 920 overnight and transfer to 1 L VB-E medium with 1:500 dilution and
 921 grown to OD₆₀₀ = ~0.3 at 25°C for 16 h as the exponential growth cells
 922 and to OD₆₀₀ = ~0.6 at 25°C for 42 h as the stationary phase cells. These

923 cells were harvested and lyzed by high pressure with 50 mL lysis buffer
 924 (50 mM NaH₂PO₄, 300 mM NaCl, and 1 mM PMSF (Sigma-Aldrich), pH
 925 8.0). After lysis and centrifugation at 10,000 rpm for 5 min, 100 µL
 926 anti-FLAG antibody (Sigma-Aldrich) was added and incubated at 4°C for
 927 20 h with gentle agitation to enrich the 3xFLAG-tagged YdeH. 300 µL
 928 protein G conjugated agarose beads (Roche) were added and incubated
 929 at 4°C for 4 h with gentle agitation to enrich the anti-Flag antibody.
 930 Protein G conjugated agarose was harvested and washed for three times
 931 by buffer A (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). The 3xFLAG-tagged
 932 YdeH was eluted by 2 mL 0.2 mg/mL Flag peptide at 4°C for 4 h. The free
 933 Flag peptide was separated through dialysis.

934 To acquire the overexpressed YdeH, we constructed the YdeH to
 935 pCA24N in $\Delta cobB$ strain ($\Delta cobB ydeH^+$). This strain was grown in LB
 936 medium overnight and transfer to 100 mL LB medium with 1:100
 937 dilution and grown to OD₆₀₀ = ~0.6 at 37°C for 2 h, 0.1 mM IPTG was
 938 added to induce at 37°C for 2 h. The cell was harvested and lyzed with
 939 35 mL lysis buffer. After lysis and centrifugation at 10,000 rpm for 5 min,
 940 1 mL Ni-IDA beads (Senhuimicrosphere, Suzhou, China) was added and
 941 incubated at 4°C for 1 h with gentle agitation to enrich the His-tagged
 942 YdeH. The His-tagged YdeH was eluted by 250 mM imidazole at 4°C for
 943 20 min. The free imidazole was separated through dialysis.

944 For YdeH acetylation stoichiometry, affinity purified YdeH proteins

945 were digested by trypsin overnight after cysteine reduction and
 946 alkylation reaction. Then tryptic peptides were desalted. The synthetic
 947 AQUA standard peptides were spiked into the digested YdeH samples
 948 with the close MS intensity to the native peptides. Peptide mixture
 949 containing AQUA peptides were analyzed by Q-Exactive mass
 950 spectrometer with three independent measurements. Full MS scan
 951 mode was used and the scan range was set as 700 to 900 m/z,
 952 containing all three targeted peptides. Extracted ion chromatography
 953 (XIC) peak areas of native peptide and corresponding heavy labeled
 954 peptide were used for stoichiometry calculation. The mass spectrometry
 955 proteomics raw data have been deposited to the ProteomeXchange⁶³
 956 Consortium via the PRIDE⁶⁴ partner repository with the dataset identifier
 957 PXD007616 (Username: reviewer16485@ebi.ac.uk, Password:
 958 dD4exhSO).

959

960 **Determination of stability of YdeH *in vitro***

961 After overexpressed in CobB deficient cells, YdeH was purified and
 962 diluted to 0.14 mg/mL, followed with or without CobB treatment. These
 963 samples were incubated at 37°C for deacetylation for 0.5 h. Then 1 mM
 964 GTP was added, the samples were incubated at 30°C for another 1 h
 965 and 2h. These samples were centrifuged at 12,000 g for 10 min to
 966 separate the soluble protein from the precipitation.

For YdeH mutants, these were diluted to 0.14 mg/mL incubated at 37°C for 0.5 h. Then 1 mM GTP was added, the samples were incubated at 30°C for another 1 h and 2h. These samples were centrifuged at 12,000 g for 10 min to separate the soluble protein from the precipitation.

Statistical analysis

Graphs were plotted using GraphPad Prism 6 and the statistical analyses were performed using Excel. Pairwise comparisons were performed using two-tailed Student's *t*-test, and statistical significance was set at *P* < 0.05. Error bars represent the mean ± standard errors of mean (S.E.M.).

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1131

1132

1133 **Acknowledgements**

1134 We thank Prof. Yufeng Yao of Shanghai Jiao Tong University for providing

1135 *S. typhimurium* CobB overexpression plasmid. We thank Prof. Yan Wei

1136 and Liyun Ji of Shanghai Jiao Tong University for performing the Q

1137 Exactive plus assay. We thank Ademi Zhakyp of Nazarbayev University

1138 for proof-reading the manuscript. This study was supported in part by

1139 The National Key Research and Development Program of China Grant

1140 2016YFA0500600, National Natural Science Foundation of China Grants

1141 31670831, 31370813, to S.C.T., 31370750, 31670722 to D.M.C., and

1142 31670066 to M.J.T., and National Basic Research Program of China (973

1143 Program) (No. 2014CBA02004) to M.J.T..

1144

1145 **Author Contributions Statement**

1146 S.C.T. conceived the idea. L.J.B. provided key reagents. Z.W.X. and C.X.L.

1147 performed interaction assay and functional analysis. Z.W.X. and C.X.L.

1148 performed enzyme activity assay. Z.W.X., H.N.Z., X.R.Z. and H.T.L.

1149 performed structure analysis and protein mutation. H.N.Z. and X.R.Z.

1150 performed the ITC and MST assay. Z.W.X., H.N.Z., X.R.Z., H.W.J., S.J.G and

1151 F.L.W. prepared the figures with the help of S.H.W. L.L.Q. and M.J.T.

1152 performed the SILAC-MS. F.L. performed the UPLC-IM-MS analysis. J.L.H.

1153 performed Nano LC maXis impact UHR-TOF MS analysis. Z.W.X., H.N.Z.,
1154 X.R.Z., D.M.C. and S.C.T. wrote the manuscript.

1155

1156 **Additional Information**

1157 **Competing financial interests:** The authors declare that we have no
1158 conflict of interest.

1159

1160 **Figure legends**

1161 **Figure 1. Protein deacetylase CobB is a novel c-di-GMP effector. (a)**

1162 The *E.coli* proteome microarrays were probed with bio-c-di-GMP,
1163 followed by incubation with Cy3-conjugated streptavidin. A control
1164 experiment was carried out with biotin. Obvious binding difference of
1165 CobB on the microarrays incubated with bio-c-di-GMP vs. biotin was
1166 observed. There are two spots per protein, SNR (+) is the average signal
1167 to noise ratio of the two duplicate spots. **(b)** Streptavidin blotting assay.

1168 Affinity purified CobB was incubated with 10 μ M bio-c-di-GMP. 10 μ M
1169 biotin, biotin-cGMP and biotin-c-di-AMP were included as negative
1170 controls. In the 3rd lane, CobB was incubated with 10 μ M bio-c-di-GMP
1171 and 20 μ M c-di-GMP. The bindings of the biotinylated ligands were
1172 visualized by streptavidin. Silver staining showed equal amounts of CobB
1173 were included for each reaction. **(c)** ITC analysis of the binding between
1174 c-di-GMP and CobB. 1.5 mM c-di-GMP (purple line) was titrated into

1175 CobB, in parallel assays, equal amount of cGMP (green line) and
1176 c-di-AMP (red line) were included as the negative controls. The K_d of
1177 c-di-GMP and CobB binding was determined as 21.6 μ M.

1178

1179 **Figure 2. c-di-GMP inhibits the deacetylase activity of CobB and**
1180 **down-regulates the biogenesis of acetyl-CoA. (a)** CobB activity assay
1181 was performed using Acs as substrate. The loss of the acetylation of Acs
1182 indicates CobB's deacetylase activity, as shown by the pan anti-acetyl
1183 antibody. (three preparations; $**P < 0.01$, two-tailed Student's t-test).
1184 The protein levels of Acs and CobB were determined by silver staining. **(b)**
1185 The kinetics of CobB enzyme-catalyzed reactions. The CobB catalytic
1186 kinetics were performed with the addition of 0, 10, 20, 80 μ M c-di-GMP
1187 and an acetylated peptidewas used as the substrate. The acetylated and
1188 deacetylated peptides were quantified by HPLC with three replicates and
1189 these curves were fitting by michaelis-menten equation using GraphPad
1190 Prism 6. **(c)** The endogenous c-di-GMP levels of the 6 *E.coli* strains were
1191 determined by UPLC-IM-MS (three preparations; $*P < 0.05$, two-tailed
1192 Student's t-test). **(d)** CobB's deacetylation activity was monitored using
1193 endogenous Acs as substrate for the 6 strains. 3xFLAG-tagged Acs was
1194 enriched by an anti-FLAG antibody and the acetylation level was
1195 determined by the pan anti-acetyl antibody. The expression of DGC and
1196 CobB were determined using an anti-His antibody. The bar graph showed

1197 the quantitation of the acetylation level of Acs. (three preparations; $*P <$
 1198 0.05 and $**P < 0.01$, two-tailed Student's t-test) **(e)** c-di-GMP modulates
 1199 the synthesis of acetyl-CoA through inhibiting CobB to deacetylate Acs. **(f)**
 1200 The growth curves of the 6 strains were determined. The *E.coli* strains
 1201 were cultured in Vogel-Bonner medium. The growth was measured at 8,
 1202 12, 16, 20, 24 and 32 h with three replicates.

1203

1204 **Figure 3. c-di-GMP globally affects CobB-dependent deacetylation.**

1205 c-di-GMP affects *E.coli* acetylation. Two strains, *i.e.*, WT and *ydeH*⁺ were
 1206 included for quantitative acetylation analysis using SILAC-MS. **(a)** The
 1207 mass spectra show light (*ydeH*⁺) and heavy (WT) signal for a
 1208 representative peptide. **(b)** Histogram shows the SILAC ratio distribution
 1209 of acetylation sites in *ydeH*⁺ cells compared to that of the WT cells.
 1210 The levels of 107 acetylated peptides were upregulated in *ydeH*⁺. **(c)** The
 1211 pie charts show the overlap of the c-di-GMP regulated acetylation sites
 1212 and the known CobB regulated acetylation sites reported by Weinert *et*
 1213 *al.* **(d)** The charts show the overlap of c-di-GMP regulated acetylation
 1214 proteins and the known CobB regulated acetylation proteins reported by
 1215 Weinert *et al.*

1216

1217 **Figure 4. Determination of the binding sites of c-di-GMP on CobB. (a)**

1218 CobB domain architecture shows the N-terminal domain (colored yellow).

1219 While for the truncation version of CobB, *i.e.*, CobB_S, there is no
 1220 N-terminal domain. **(b)** CobB_S lost the binding with c-di-GMP. Both CobB
 1221 and CobB_S were incubated with bio-c-di-GMP. After UV cross linking, the
 1222 reactions were subjected for streptavidin blotting. **(c)** Streptavidin
 1223 blotting assays for WT and CobB mutants. It is known that c-di-GMP
 1224 tends to bind residues Arg (R) and Glu (E). We individually mutated all
 1225 the R and E residues to pinpoint the binding sites. The c-di-GMP binding
 1226 assays were carried out with all these mutated CobB, the bindings were
 1227 visualized by streptavidin. The loadings were monitored by Ponceau S
 1228 staining. **(d)** ITC assay to measure the binding kinetics of c-di-GMP and
 1229 three CobB mutants. **(e)** *In vitro* deacetylation assay of the three CobB
 1230 mutants using Acs as substrate with three replicates. **(f)** The
 1231 deacetylation activity of CobB mutants were monitored using
 1232 endogenous Acs as substrate *in vivo*. (three preparations; two-tailed
 1233 Student's t-test, **P* < 0.05) **(g)** The growth curves of the CobB mutant
 1234 strains were determined. The growth was measured at 8, 12, 16, 20, 24
 1235 and 32 h with three replicates.

1236

1237 **Figure 5. c-di-GMP binds CobB and inhibits its activity is conserved in**
 1238 **prokaryotes. (a)** CLUSTALW alignment of the binding motif of c-di-GMP
 1239 and CobB. Residues involved in c-di-GMP binding R8, R17 and E21 were
 1240 boxed (Gray) and the depth of color indicated the degree of conservative

1241 of the residues. **(b)** c-di-GMP binding assay of *E.coli* and *S. typhimurium*
 1242 CobB. The *E.coli* and *S. typhimurium* CobB were incubated with
 1243 bio-c-di-GMP, same amount of biotin, cGMP and c-di-AMP were included
 1244 as controls. **(c)** c-di-GMP inhibits *S. typhimurium* CobB's deacetylase
 1245 activity *in vitro*. CobB^{*S. typhimurium*} activity assay was performed using *E.coli*
 1246 Acs as the substrate. The acetylation level of Acs was detected by the
 1247 pan anti-acetyl antibody. (three preparations; **P* < 0.05, two-tailed
 1248 Student's t-test).

1249

1250 **Figure 6. CobB up-regulates the level of c-di-GMP through**
 1251 **deacetylation of the major diguanylate cyclase YdeH in *E.coli*.** **(a)** The
 1252 acetylation levels of four c-di-GMP related enzymes of *E. coli* with or
 1253 without CobB treatment was monitored by the pan anti-acetyl antibody.
 1254 **(b)** Mass spectrometry (MS) analysis to determine the acetylation site/s
 1255 of YdeH that could be deacetylated by CobB. The affinity purified YdeH
 1256 with or without CobB treatment were subjected for MS analysis. K4 was
 1257 discovered as the deacetylation site. **(c)** Mutagenesis of YdeH confirmed
 1258 that K4 is the major acetylation site. All the 8 Lys residues of YdeH were
 1259 mutated to Ala individually. The acetylation was visualized using the
 1260 anti-acetyl antibody. **(d)** Mutagenesis of YdeH K4 confirms the
 1261 acetylation of K4. Three single YdeH mutants, K4R, K4Q and K4A were
 1262 constructed. **(e)** CobB modulates the synthesis of c-di-GMP through

1263 deacetylating YdeH. **(f)** The DGC activity of the three mutants along with
 1264 WT YdeH and CobB treated WT YdeH were analyzed using UPLC-IM-MS *in*
 1265 *vitro* with three replicates, the peak areas represent the amounts of
 1266 c-di-GMP in these samples. **(g)** YdeH's acetylation levels and c-di-GMP
 1267 concentrations were measured with CobB mutants and YdeH mutants.
 1268 The acetylation level of purified YdeH was detected by the pan
 1269 anti-acetyl antibody and the protein levels of YdeH and CobB were
 1270 determined by the anti-His antibody. (three preparations; * $P < 0.05$, ** P
 1271 < 0.01 , two-tailed Student's t-test).

1272
 1273 **Figure 7. YdeH is endogenous acetylated and this acetylation is**
 1274 **regulated by CobB. (a)** The endogenous YdeH acetylation level of 3 *E.coli*
 1275 strains. A 3xFLAG tag was chromosomally inserted at the 3'-end of Acs
 1276 coding sequence. 3xFLAG-tagged YdeH was enriched by an anti-FLAG
 1277 antibody and the acetylation levels were determined by the pan-Ack
 1278 antibody. **(b)** The mass spectra show AQUA quantification of the
 1279 endogenous acetylation of YdeH K4 using AQUA peptides. **(c)** The level of
 1280 the endogenous YdeH. The protein levels were determined by the
 1281 anti-FLAG antibody and GroEL was applied as the loading control. The
 1282 bar graph showed the quantitation of the protein levels of YdeH with
 1283 three replicates. **(d)** The relative mRNA repression of endogenous YdeH.
 1284 **(e)** The stability of YdeH with or without CobB treatment. These samples

1285 were separated at 0.5 h, 1.5h and 2.5 h and the protein levels were
1286 determined by the anti-His antibody. The bar graph showed the
1287 quantitation of the protein level of YdeH with three replicates. **(f)** The
1288 level of the overexpressed YdeH in the supernatant and sediment. The
1289 protein levels were determined by the anti-His antibody and GroEL was
1290 applied as the loading control. The bar graph showed the quantitation of
1291 the protein level of supernatant YdeH with three replicates.

1292

1293 **Figure 8. The regulating model of c-di-GMP and CobB interplay. (a)**
1294 YdeH K4 acetylation reduce YdeH's stability and cause precipitation. **(b)**
1295 The overview of c-di-GMP and CobB interplay. c-di-GMP inhibits the
1296 deacetylation activity of CobB and then regulates Ac-CoA synthesis. In
1297 another direction, CobB activates YdeH's DGC activity through
1298 deacetylation to prevent the precipitation of YdeH, and also activate
1299 YdeH. In addition, Ac-CoA modulates GTP, the precursor of c-di-GMP
1300 generation through TCA. Quantitatively, c-di-GMP abolishes 72% of CobB's
1301 decetylation activity through direct binding of CobB, and CobB promotes
1302 c-di-GMP level to 36% more through deacetylation of YdeH.

a.

Protein	Bio-c-di-GMP	Biotin	SNR(+)
CobB			2.16

b.

c.

Xu et al. Figure 1.

a.

b.

c.

d.

e.

f.

Xu et al. Figure 2.

a.

c.

b.

d.

Xu et al. Figure 3.

Xu *et al.* Figure 4.

a.

	R8	R17	E21
<i>Escherichia coli</i>	ML.SRRGHRLSRFRKNKRRRLRERLRQRIFFRDK		
<i>Salmonella typhimurium</i>	MQ.SRRFHLRSRFRKNKRLRERLRQRIFFRDR		
<i>Yersinia pestis</i>	...MRIRHRLCRFRKSKHVRHQRFSRIFHRDS		
<i>Shigella dysenteriae</i>	ML.SRRGHRLSRFRKNKRRRLRERLRQRIFFRDK		
<i>Citrobacter amalonaticus</i>	ML.SRRGHRLSRFRKNKRRRLRERLRQRIFFRDR		
<i>Erwinia tracheiphila</i>	MRNPRRRLRLARYKKNNRQVHQRFRQRIFFERDR		
<i>Vibrio parahaemolyticus</i>	ML.SRRGHRLSRFRKNKRRRLRERLRQRIFFRDR		
<i>Klebsiella oxytoca</i>	ML.SRRGHRLSRFRKNKRRRLRERLRQRIFFRDK		
<i>Cedecea neteri</i>	ML.SRRQHLRSRFRKNKRLRQRLRQRIFFRDR		
<i>Achromobacter sp. ATCC35328</i>	ML.SRRGHRLSRFRKNKRRRLRERLRQRIFFRDK		
<i>Siccibacter colletis</i>	MQ.SRRSHRLIRFKKNKRRRLRDLRQRIFFRDT		
<i>Lelliottia amnigena</i>	ML.SRRQGLSRFRKNKRRRLRERLRQRIFFRDR		
<i>Kluyvera ascorbata</i>	MQ.SRRLHRLSRFRNNKRLRQRLRQRIFFTD		
<i>Cronobacter dublinensis</i>	MQ.SRRLHRLGRFRKNKRRRLRERLRQRIFFRDR		
<i>Chania multitudinisentens</i>	...MHTRHRLCRFRKNKHVLRHQRFSRIFHRDT		
<i>Yokenella regensburgei</i>	MQ.SRRLHRLTRFRLNKRRRLRDLRQRIFFRDS		
<i>Pantoea dispersa</i>	MRQPRRRLRLARYKKNNRQVHQRFRQRIFFERDR		
<i>Shimwellia blattae</i>	MP.SRGLHRLCRFRKNKRRRLRARLRQRTFFRDE		
<i>Kosakonia radicincitans</i>	MQ.SRRLHRLSRFRNNKRRRLRERLRQRIFFRDR		

b.

CobB <i>S.typhimurium</i>	-	+	+	+	+	+
CobB <i>E.coli</i>	+	-	-	-	-	-
Bio-c-di-GMP (μM)	20	20	-	20	-	-
Bio-cGMP (μM)	-	-	-	-	20	-
Bio-c-di-AMP (μM)	-	-	-	-	-	20
Biotin (μM)	-	-	20	-	-	-
c-di-GMP (μM)	-	-	-	40	-	-

CobB		SA
		Silver Staining

Xu *et al.* Figure 6.

Xu *et al.* Figure 7.

a.

b.

Xu *et al.* Figure 8.