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Class III adenylyl cyclases generate the ubiquitous second messenger cAMP from ATP often in response to environmental or cellular cues. During evolution, soluble adenylyl cyclase catalytic domains have been repeatedly juxtaposed with signal-input domains to place cAMP synthesis under the control of a wide variety of these environmental and endogenous signals. Adenylyl cyclases with light-sensing domains have proliferated in photosynthetic species depending on light as an energy source, yet are also widespread in nonphotosynthetic species. Among such naturally occurring light sensors, several flavin-based photoactivated adenylyl cyclases (PACs) have been adopted as optogenetic tools to manipulate cellular processes with blue light. In this report, we report the discovery of a cyanobacteriochrome-based photoswitchable adenylyl cyclase (cPAC) from the cyanobacterium *Microcoleus* sp. *PCC 7113.* Unlike flavin-dependent PACs, which must thermally decay to be deactivated, cPAC exhibits a bistable photocycle whose adenylyl cyclase could be reversibly activated and inactivated by blue and green light, respectively. Through domain exchange experiments, we also document the ability to extend the wavelength-sensing specificity of cPAC into the near IR. In summary, our work has uncovered a cyanobacteriochrome-based adenylyl cyclase that holds great potential for the design of bistable photoswitchable adenylyl cyclases to fine-tune cAMP-regulated processes in cells, tissues, and whole organisms with light across the visible spectrum and into the near IR.

Class III adenylyl cyclases generate the ubiquitous second messenger cAMP from ATP often in response to chemical or environmental signals. In prokaryotes, cAMP frequently partners with the catabolite activator protein to regulate energy metabolism (1, 2), and also regulates motility and virulence (3). In eukaryotes, cAMP levels control crucial processes such as chromosome segregation, gene expression, and cellular metabolism (4). This regulatory plasticity arises from juxtaposition of adenylyl cyclase catalytic domains with a wide variety of "input" domains that sense different chemicals, nutrients, light, physical signals, and pH. Despite the signal input diversity, the protein-fold of homodimeric Class III adenylyl cyclase catalytic domains (termed cyclase homology domains (CHD))[3] and a catalytic mechanism that requires participation of two subunits across a dimer interface have been preserved from prokaryotes to eukaryotes for billions of years (5).

Cyanobacterial phototrophs possess a diverse complement of adenylyl cyclases that have been shown to regulate respiration (6), motility (7, 8), and heterocyst formation (9). Although blue light (B)-sensing adenylyl cyclases that utilize flavin are present in some cyanobacteria (10, 11), cyanobacterial adenylyl cyclases typically lack photoreceptor domains and those known to be under light control are indirectly regulated by other photoreceptors (12, 13). The cyanobacterial species *Microcoleus* sp. *PCC 7113* contains one of the largest complements of adenylyl cyclase genes with sensor regions recognized as a phospho-accepting response regulator, a heme containing NO-sensor, a ligand-binding periplasmic sensor, and one cGMP-specific phosphodiesterases/cyanobacterial adenylyl cyclases/formate hydrogen lyase transcription activator FhlA (GAF) domain sensor. GAF domains often mediate ligand-dependent protein–protein interactions; one of these adenylyl cyclase genes, *i.e.* MIC7113_2205 contains a GAF domain similar to those found

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This article contains Table S1 and Figs. S1–S9.

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[3] The abbreviations used are: CHD, cyclase homology domain; AC, adenylate cyclase; B, blue light; Bc, continuous blue light; BLUF, sensors of blue-light using FAD; BV, biliverdin IXα; CBCR, cyanobacteriochrome; DXCF, Asp-Xaa-Cys-Phe; G, green light; Gc, continuous green light; Rc, continuous red light; PAC, photoactivated adenylate cyclase; cPAC, cyanobacteriochrome photoswitchable adenylate cyclase; PCB, phycocyanobilin; PEB, phycoerythrobilin; RP-HPLC, reverse phase-high performance liquid chromatography; SEC, size exclusion chromatography; IPTG, isopropyl 1-thio-β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; β-gal, β-galactosidase; REC, response regulator; PAS, Per/Arnt/Sim.

in cyanobacteriochrome (CBCR) photoreceptors, distant relatives of red/far-red sensing plant phytochromes (14).

CBCRs possess heme-derived, linear-tetrapyrrole (bilin)-binding GAF domains that can perceive the complete range of the visible light spectrum, extending into the near UV and the near IR (14–16). All CBCRs utilize phycocyanobilin (PCB) that is spectrally tuned by key residues within the GAF domain scaffold (17–26). MIC7113_2205 belongs to the D*X*CF subfamily of CBCRs, characterized by two conserved cysteine residues, each of which can form covalent linkages to the PCB precursor to alter the π-conjugated chromophoric system to absorb blue light (18, 27). Although CBCRs are typically associated with histidine kinase, methyl chemotaxis, and cyclic di-GMP regulatory GGDEF or EAL output domains, MIC7113_2205 is the first adenylyl cyclase (Fig. S1) to be identified with a putative light-sensing CBCR GAF domain. MIC7113_2205 also contains an N-terminal response regulator (REC) and Per/Arnt/Sim (PAS) domains, both of which could represent additional input domains for other chemical or physical signaling systems. Atypical for widespread adenylyl cyclases with unrelated GAF domains, this novel adenylyl cyclase-associated CBCR gene appears unique to this *Microcoleus* species. The observation that the related cyanobacterium *Microcoleus chthonoplastes* PCC 7420 possesses a flavin-based, B-regulated adenylyl cyclase (10) suggests that light-regulation of cAMP production may be particularly critical for cyanobacteria in this genus.

The ability to manipulate adenylyl cyclase activity with light enables precise spatial and temporal control of cAMP levels within cells, organs, and tissues. Indeed, genetically-encoded photoactivated adenylyl cyclases (PAC) with Blue-Light sensors Using FAD (BLUF) domains, such as EuPAC, bPAC, BgPACs, LiPAC, NaPACs, and OaPAC (11, 28–38), or with flavin-binding LOV domains, such as mPAC (10), have been successfully deployed in mammalian cells. The utility of bilin-based PACs for similar applications was first documented by Gomelsky and co-workers (39), who fused a biliverdin IXα (BV)-dependent bacteriophytochrome to an adenylyl cyclase catalytic domain that could be reversibly controlled with red and far-red light (Fig. 1). As optogenetic tools, bistable phytochrome-based PACs offer the advantage that two wavelengths of light can be used to reversibly regulate cAMP production in cells in real time, rather than relying on thermal reversion pathways to inactivate the photoactivated state, *e.g.* for flavin-based PACs. Because of the small size of CBCR input domains (~200 amino acids), and extended spectral range, CBCR-based adenylyl cyclases hold particular promise as optogenetic reagents.

The present work was undertaken to test the hypothesis that MIC7113_2205 functions as a light-regulated adenylyl cyclase and to characterize the molecular basis of its light regulation. Our studies show that MIC7113_2205 is a bistable, blue/green (B/G)-regulated adenylyl cyclase that we hereafter refer to as cPAC for cyanobacteriochrome-based Photoswitchable Adenylyl Cyclase. We also test the interchangeability of CBCR GAF domains to alter the wavelength specificity of cPAC for its photoswitchable cAMP regulation *in vivo*, providing a roadmap for spectral engineering of a versatile new family of CBCR-based optogenetic actuators.

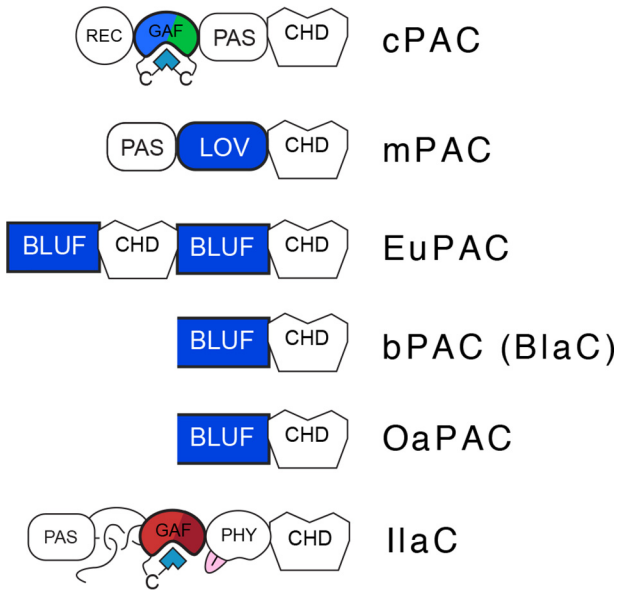

Figure 1. cPAC is a cyanobacterial photoactivated adenylyl cyclase. Shown are domain architectures of cPAC compared with those of other characterized PACs.

Results

cPAC is a bistable blue/green sensor

To analyze the photochemical properties of cPAC, we expressed a synthetic full-length cPAC gene in an *Escherichia coli* strain engineered to produce PCB from endogenous heme, and the recombinant holoprotein was purified as described previously (19, 40). In the dark-adapted P_b state, the PCB chromophore of cPAC adopted a 15*Z* configuration with an absorbance maximum at 410 nm in the B spectral region. Upon exposure to saturating B light, the chromophore photoconverted to its 15*E* configuration with an absorbance maximum at 520 nm in the G spectral region (Fig. 2*A*). P_g could be fully photoconverted to the B-absorbing P_b form with saturating G light. The B/G spectral properties of cPAC were consistent with those of other two-cysteine CBCRs in the D*X*CF subfamily, and its specific absorption ratio value of 0.18 indicated near saturated binding of PCB to the apoprotein (18, 19, 41). Dark reversion of cPAC was found to be slow on the timescale of biochemical assays, with ≤5% change over 90 min (Fig. S2A). Moreover, the difference spectrum for dark reversion did not match that for photoconversion (Fig. S2*B*). This difference indicates that other processes are occurring on the same timescale, such as changes in chromophore content (19) or oxidation of the D*X*CF Cys (42), making it likely that regeneration of the blue-absorbing 15*Z* dark state is overestimated by this approach. Taken together with the linearity of cPAC in AC assays lasting 60 min (Fig. 2*B*), it is clear that dark reversion is not a confounding factor in our experiments.

cPAC is a photoswitchable adenylyl cyclase

The C-terminal region of cPAC (MIC7113_2205) is annotated in GenBank™ as an "adenylyl or guanylyl cyclase domain." Further sequence analysis supports designation of cPAC as an adenylyl cyclase as both key adenosine-specific binding residues in the active site as well as all key metal and

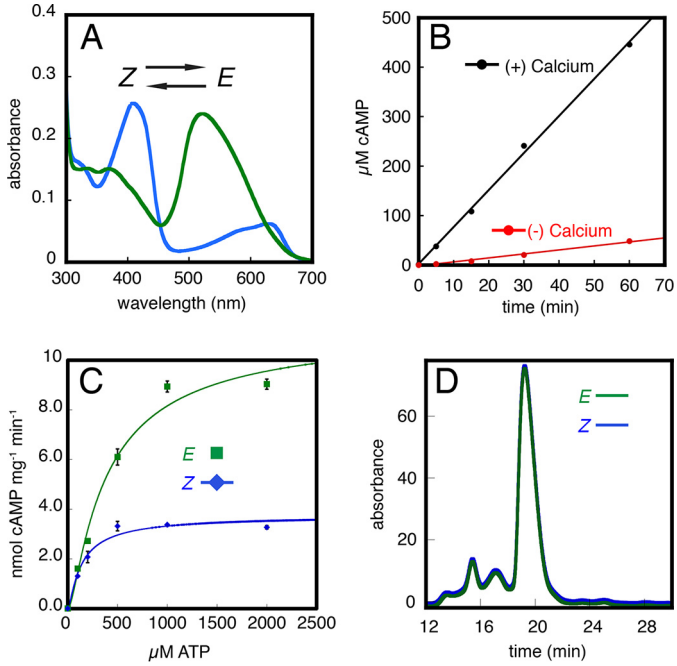

Figure 2. cPAC is blue/green photoswitchable adenylyl cyclase. *A*, spectra of cPAC displays the distinct B-absorbing (*15Z*) photostate and the G-absorbing (*15E*) photostate. *B*, time course assay of production of cAMP by the G-absorbing *15E* state of cPAC with or without calcium present in the media. *C*, initial rate kinetics of cPAC in the 15*E* and 15*Z* photostates. Data are fit to a second order fit derived from the Michaelis-Menten equation: $V_o = (V_{max}[S]^2)/(k^2 + 2k[S] + [S]^2)$. *D*, HPLC-SEC trace of full-length cPAC in the 15*E* and 15*Z* states. The major peak elutes at 19.6 min.

transition state residues are present (31, 43) (Fig. S1). To resolve whether cPAC is an adenylyl or guanylyl cyclase, the activity of the 15*Z*-P_b and 15*E*-P_g states were compared using Mg-ATP or Mg-GTP as substrate. cPAC was able to convert Mg-ATP to cAMP (Fig. 2*B*); however, no cGMP was detected by reverse phase-HPLC (RP-HPLC) when Mg-GTP was used as substrate. The 15*E*-P_g state also produced more cAMP than the 15*Z*-P_b state indicating that cPAC is light regulated. In view of the cyanobacterial adenylyl cyclase literature, we next tested whether cPAC activity was affected by addition of calcium or bicarbonate. These studies showed that 5 mM calcium greatly increased the activity of cPAC in both 15*Z*-P_b and 15*E*-P_g states (Fig. S3*A*). For the more active 15*E*-P_g state, the presence of calcium resulted in a >10-fold greater cAMP accumulation compared to reaction mixtures lacking added calcium (Fig. 2*B*). By contrast, cPAC activity was stimulated only slightly (~2.5-fold) by the addition of 50 mM bicarbonate, nor did bicarbonate further stimulate cPAC activity more than calcium alone (Fig. S3*B*). This indicates that Ca^{2+} could stimulate cPAC activity *in vivo*, but probably it is not regulated by bicarbonate.

Initial rate measurements for both *E* and *Z* states of cPAC were next performed as a function of Mg-ATP concentration in the optimized buffer medium (Fig. 2*C*). A modified Michaelis-Menten analysis using a second-order reaction model fit the data slightly better than the standard analysis (44) (Fig. S4*A*, Table S1). The specific activity of cPAC in the 15*Z*-P_b dark state was estimated to be 3.8 nmol of cAMP min^{-1} mg^{-1}, whereas that of the 15*E*-P_g active state was estimated to be 11.4 nmol of cAMP min^{-1} mg^{-1}. These specific activities are comparable with those of flavin-based adenylyl cyclases in the activated state (Table 1). As has been seen previously for other soluble adenylyl cyclases (45), cPAC turnover was substrate inhibited at high Mg-ATP concentrations (Fig. S4*B*).

Table 1
Activity comparison of cPAC and flavin binding PACs
cPAC specific activity was measured as described under "Experiment procedures." Enzyme activity data points were analyzed in triplicate, and S.D. was calculated.

Adenylyl cyclase	Specific activity	
	Activated state	Dark state
	nmol cAMP min^{-1} mg^{-1}	
cPAC	11.4 ± 0.5	3.8 ± 0.2
bPac	10 ± 2	0.03 ± 0.005
OaPac	9 ± 1	0.5[a]
mPac	30 ± 20	1.0 ± 0.6
EuPac	3.5	0.3[a]
BlaC[b]	57	0.5[a]

[a] Data not reported: estimated value from enzymatic data.
[b] BlaC and bPAC are the same protein, but were analyzed and documented separately (30, 31).

The REC domain is necessary for cPAC activity

To analyze how the domain architecture of cPAC affects its activity, several truncations were constructed. The first two of these, cPAC(ΔR2) and cPAC(ΔR3), lacked the N-terminal REC domain, while retaining two or three predicted helices at the N terminus of the GAF domain, respectively. Two of these helices are typically found necessary for proper GAF domain folding. Two other constructs, cPAC(ΔRG) lacked both the REC and GAF domains, whereas the cPAC(ΔRGP) construct contained only the cyclase domain (Fig. S5*A*). Truncated cPAC(ΔR2) and cPAC(ΔR3) proteins were both folded and photoactive, exhibiting P_b and P_g spectra nearly indistinguishable from those of the full-length cPAC (Fig. 2*A* and Fig. S5). By contrast, adenylyl cyclase activities of these and all truncated cPAC constructs were less than 5% of WT, and adenylyl cyclase activities of cPAC(ΔRG) and cPAC(ΔRGP) were barely detectable (Fig. 3*A*). It is also interesting that the two REC-less constructs cPAC(ΔR2) and cPAC(ΔR3) did retain some light–regulated adenylyl cyclase activity although the significance of the differences is unclear due to their low AC activities. Taken together, these data indicated that the REC domain is critical for cPAC activity in either state.

The regulatory activity of REC domains of two-component response regulator proteins is typically modulated by phosphorylation. REC domain phosphorylation could affect the monomer–dimer equilibrium of canonical response regulator proteins to influence, *e.g.* their DNA-binding affinity, and/or could affect the conformation/dynamics/activity of other associated output domains (46). To test the first possibility, full-length cPAC and all four truncated constructs were analyzed by HPLC-size exclusion chromatography (HPLC-SEC). As seen in Fig. 2*D*, full-length cPAC migrates as a dimer in both 15*Z*-P_b and 15*E*-P_g states (Table 2). By contrast, all truncated cPAC constructs appear monomeric under these conditions (Table 2, Fig. S6); these results suggest that the REC domain is critical for homodimer formation, which in turn is necessary for cPAC catalytic activity.

We next examined whether the P_b to P_g photoconversion could affect the quaternary state of the protein. As shown in Fig.

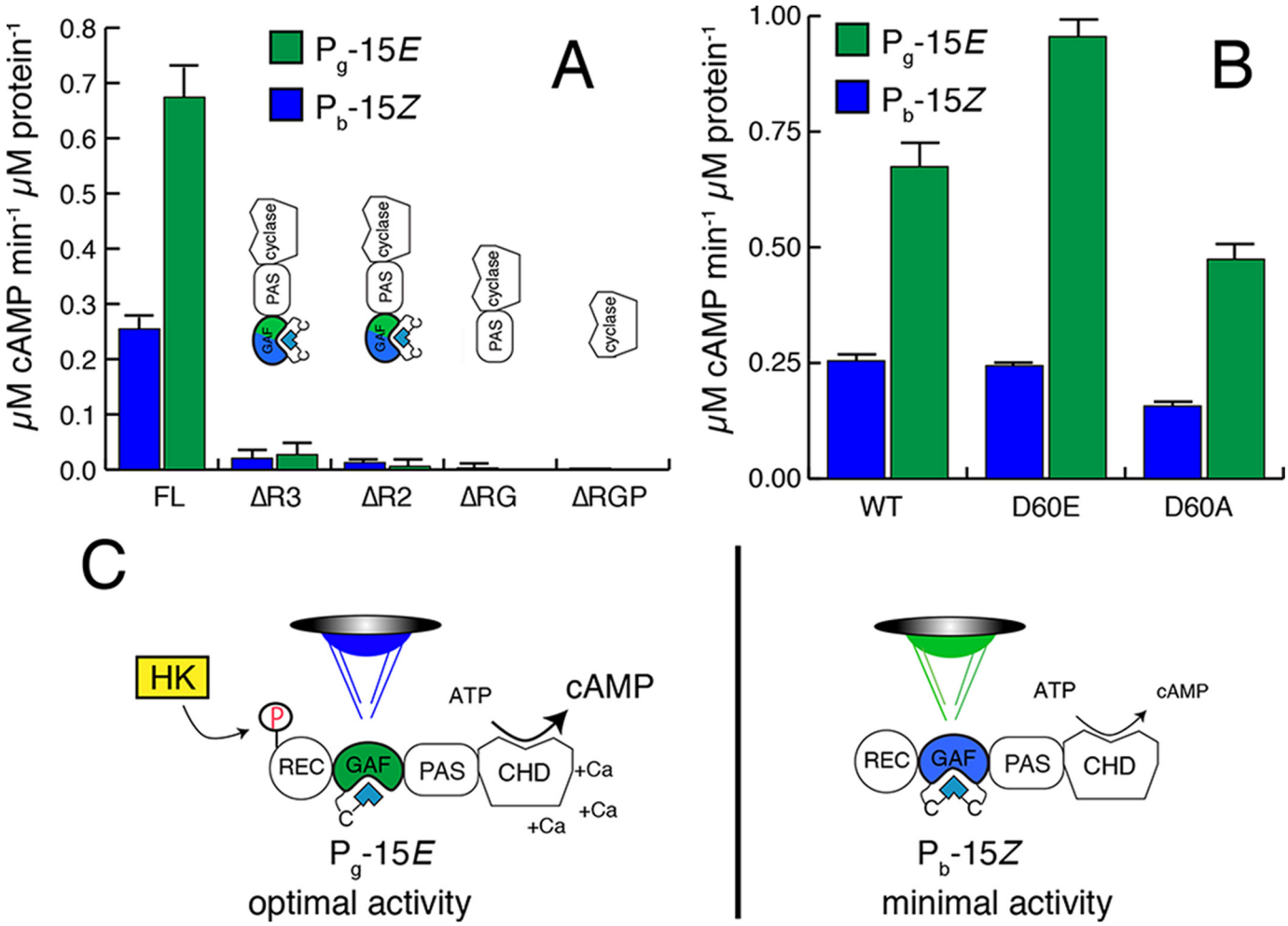

Figure 3. REC domain of cPAC is essential for optimal activity. *A,* bar graph of enzyme activity of full-length cPAC and subsequent domain truncations. Δ*R3* and Δ*R2* denote truncation of the REC domain leaving 3 or 2 helices of the N-terminal GAF domain, respectively, Δ*RG* denotes the construct of the PAS–cyclase domain and Δ*RGP* denotes only the cyclase domain (see text for details). *B,* bar graph of enzyme activity comparing WT cPAC to cPAC(D60E) and cPAC(D60A) mutations. *C,* schematic of optimal activity conditions for cPAC (B light triggering the 15*E*-P_g state, phosphorylation of the REC domain in the presence of 5 mM Ca^{2+}) as compared with conditions for minimal activity (G light triggering the 15*Z*-P_b state, with buffer lacking Ca^{2+}).

Table 2

Predicted size and oligomeric state of cPAC and truncated variants

HPLC-SEC measurements were performed in duplicate (n = 2) and predicted molecular weight averaged as described under "Experimental procedures."

Construct	HPLC-SEC measurement	Predicted monomer molecular mass
cPAC (full-length)	143 ± 8 kDa	77 kDa
cPAC(ΔR3)	68 ± 5 kDa	60 kDa
cPAC(ΔR2)	66 ± 4 kDa	58 kDa
cPAC(ΔRG)	45 ± 4 kDa	41 kDa
cPAC(ΔRGP)	30 ± 2 kDa	27 kDa

2*D*, the HPLC-SEC elution profiles of both states of full-length cPAC were superimposable. Based on this result, we conclude that photoactivation of cPAC does not lead to a major change in overall protein conformation. This also implies that small light-induced structural changes are responsible for the regulation of activity of cPAC, a result similar to that reported for BLUF containing PACs (11, 37, 38). Our HPLC-SEC data also suggests that homodimerization is required for efficient catalytic turnover. However, dimerization is not required for the cPAC photocycle, because both cPAC(ΔR2) and cPAC(ΔR3) constructs retained the same spectral properties as the full-length protein (Fig. S5C). As all truncated proteins are monomeric, the enzyme activity data suggest that REC domain dimerization restricts conformational sampling of CHD domains that disfavor formation of the catalytically competent dimer or, alternatively, that REC-dependent dimerization is necessary to increase the local CDH concentration to support dimerization and catalytic turnover (5).

cPAC activity is modulated by REC domain phosphorylation

Because the REC domain is required for cPAC function, we next performed experiments to test the role of REC domain phosphorylation on its catalytic activity. The transmitter kinase responsible for its phosphorylation is unknown, so we constructed site-directed variants of the conserved predicted phospho-accepting Asp-60 residue of cPAC (as categorized for a REC domain consensus sequence, CDD 238088) to mimic its phosphorylated and nonphosphorylated states (47). To do so, we introduced phosphomimetic D60E "gain-of-function" and D60A "loss-of-function" substitutions; such functional substitutions have been described in the response regulator receiver family previously (48–50). Both cPAC variants exhibited photochemical properties identical with those of the WT (Fig. S5*C*), demonstrating that these substitutions did not perturb

the structure or light-sensing function of the CBCR GAF domain of cPAC. Surprisingly, the 15*E*-P_g state of D60E exhibited 150% AC activity of the WT; by comparison, the 15*E*-P_g state of D60A was 30% less active than the WT. However, the activities of the 15*Z*-P_b states of WT and D60E mutant were nearly identical (Fig. 3*B*), suggesting that phosphorylation selectively enhances the activity of the photoproduct state. This conclusion must be tempered by the possibility that the 15*Z*-P_b state of WT cPAC from *E. coli* may already be partially phosphorylated when isolated. Taken together, our data suggest that phosphorylation of the REC domain likely plays a regulatory role on cPAC activity *in vivo*. The interplay between REC phosphorylation and light activation of the GAF domain remains an interesting subject for future study. Taken together, our enzyme data indicate that the maximum signal output from cPAC is achieved following B light treatment, when the REC domain is phosphorylated and calcium levels are elevated (Fig. 3*C*).

In vivo light–regulated manipulation of cAMP levels by cPAC

To test the efficacy of cPAC as an optogenetic tool *in vivo*, we co-expressed cPAC with a PCB biosynthetic plasmid in *cya*$^-$ *E. coli* strain VJS711 that lacks endogenous adenylyl cyclase activity (51). This strain allows us to measure the cAMP-dependent activation of the endogenous β-gal (*lacZ*) gene via the plasmid-derived cPAC without interference of endogenous CYA-dependent cAMP production. When spread on LB plates containing X-Gal/IPTG, only strains that produce sufficient levels of cAMP will appear blue (Fig. 4*A*). Indeed, cell lines expressing full-length cPAC were blue on X-Gal plates, revealing the production of endogenous cAMP and its subsequent activation of β-gal expression. By contrast, the *cya*$^-$ strain expressing the truncated cPAC(ΔRGP) construct failed to produce β-gal under identical conditions (Fig. S7). As expected from *in vitro* assay results, WT cPAC-expressing cultures grown under continuous blue light (Bc) possessed significantly more β-gal activity than cultures grown under continuous green light (Gc) (Fig. 4*B*). We expected that cPAC would modulate global cAMP levels in a heterologous system, measured via a direct cAMP assay method, as seen with previous PACs (28, 29, 31). Similar results were observed with *E. coli* liquid suspension cultures grown under Bc, which yielded significantly more cAMP (~2.5 times more) than cultures grown under G_c (Fig. 4*C*). Control measurements of the *cya*$^-$ strain lacking cPAC revealed insignificant amounts of cAMP production. These studies confirm that cPAC retains its photoregulatory properties *in vivo*.

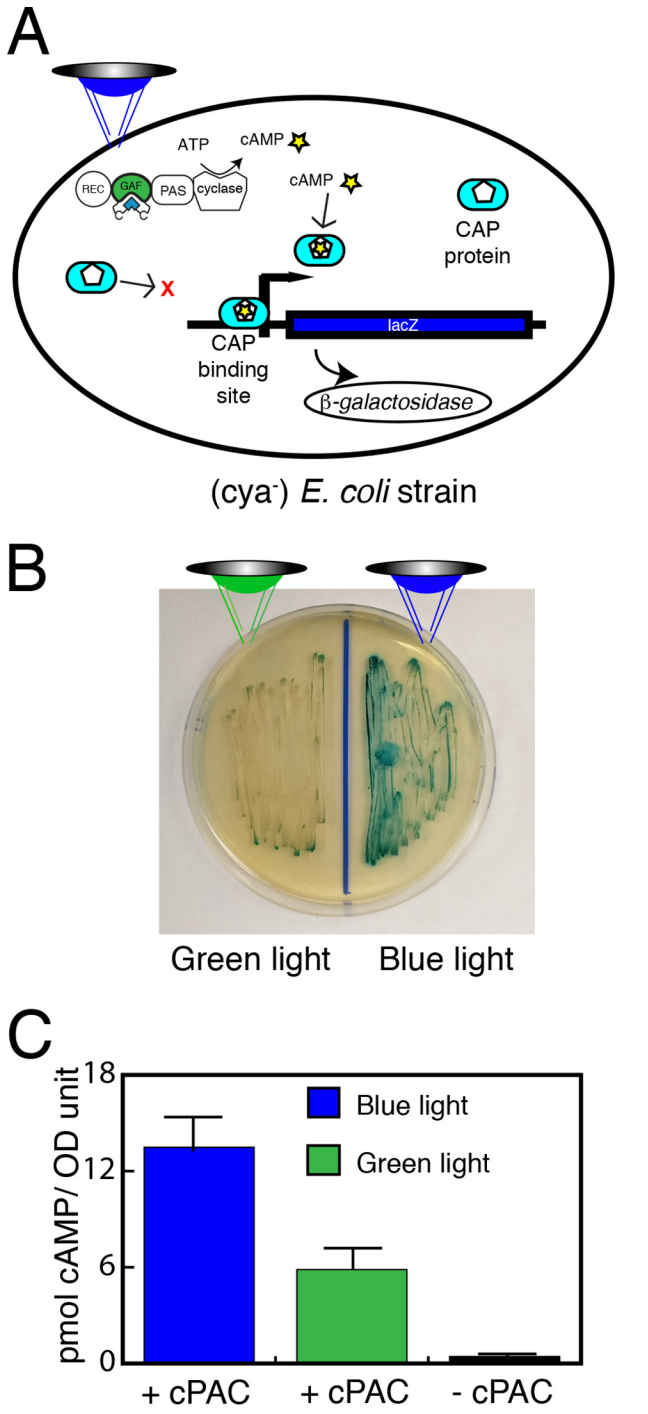

Figure 4. cPAC can modulate cAMP levels *in vivo*. *A,* schematic of cPAC mediating *lacZ* gene expression via modulation of cAMP levels in (*cya*$^-$) *E. coli* cells. *B,* LB (X-Gal/IPTG) plate displaying β-gal cleavage of X-Gal in *E. coli* expressing cPAC in the section of plate exposed to Bc, whereas Gc vastly reduced X-Gal cleavage in cells. *C, in vivo* levels of cAMP in *E. coli* liquid suspension cultures expressing cPAC and incubated under Bc or Gc. cAMP levels were normalized in each culture by O.D. (600 nm) value.

CBCR domain exchange yields cPAC variants with altered wavelength selectivity

CBCRs have been identified that can sense light from the near UV, throughout the visible spectra, and into the near IR (52). Because 3D structures of CBCRs so far examined are all quite similar, this suggests that substitution of different wavelength-sensing CBCRs for one another will be feasible to provide a broad array of cPAC reagents tailored for potentially any wavelength combination desired (53–55). We therefore sought to create new cPAC-based chimeras by exchanging the B/G CBCR GAF domain of cPAC with GAF domains from two other CBCR lineages. We chose the green/red CBCR RcaE from *Fremyella diplosiphon* (22) and a newly identified CBCR JSC1_58120g3 from *Leptolyngbya* sp. JSC1 (Fig. 5*A*). One of three GAF domains of a multi-GAF methyl-accepting chemotaxis transducer, *i.e.* JSC1_58120g3, is closely related to the canonical red/green family. JSC1_58120g3 is a previously unde-

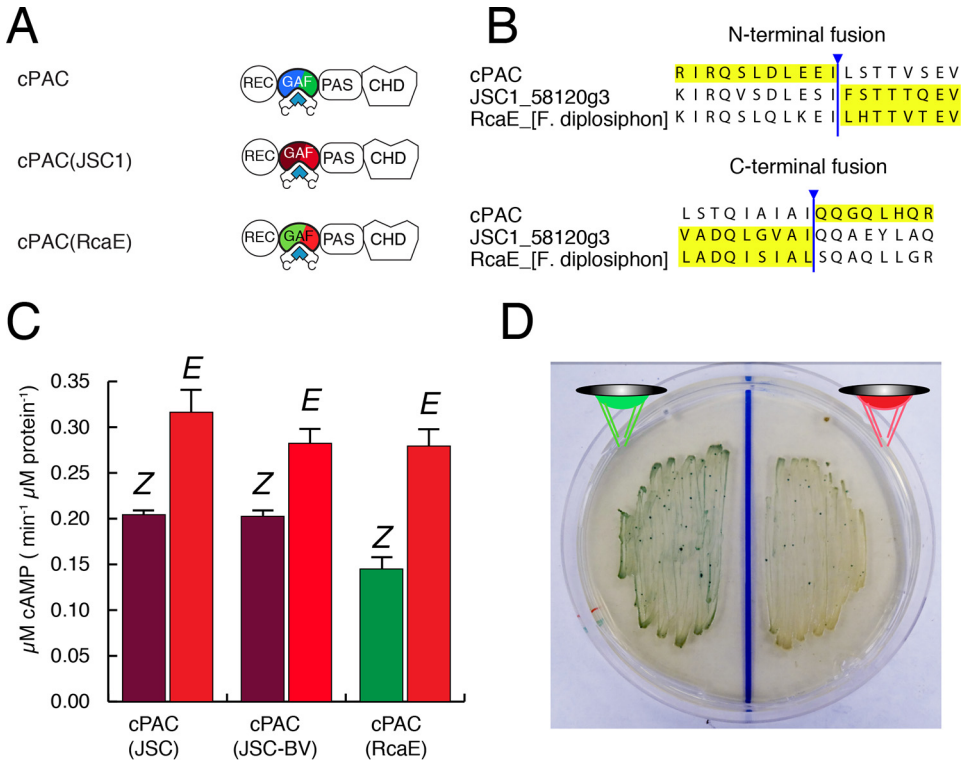

Figure 5. cPAC as a platform for multicolored fusion constructs. *A,* jellybean diagram of cPAC fusion constructs. *B,* alignment showing the N- and C-terminal fusion breakpoints for cPAC and GAF domains of JSC1_58120g3 and RcaE. *Yellow* highlight denotes residues present in the resultant fusion construct. *C,* bar graph of enzyme activity of fusion constructs in the 15*E* and 15*Z* photostates. *D,* LB plate (as described above) showing greater X-Gal cleavage and *blue* coloring in *E. coli* cells expressing cPAC(RcaE) that are exposed to Gc, as compared with cells exposed to Rc.

scribed CBCR found in the *Leptolyngbya* sp. JSC1 genome (56), so it was first expressed and purified as its PCB adduct for spectroscopic analysis. JSC1_58120g3 exhibited 15*Z* state peak absorbance of 712 nm and 15*E* state peak absorbance of 654 nm. Therefore, JSC1_58120g3 can be classified as a far-red/red CBCR similar, albeit unrelated to those recently described (26). JSC1_58120g3 can also utilize biliverdin (BV) as an alternative chromophore, although surprisingly, the spectral properties of the BV adduct of JSC1_58120g3 were quite similar to the PCB adduct (Fig. S8), peaking at 716 nm in its 15*Z* state and at 646 nm in its 15*E* state.

For selection of fusion junctions for these chimeras, we chose an N-terminal fusion point between charged residues in a conserved region within the first helix of the cPAC GAF domain, and a C-terminal fusion point in the C-terminal α helix in the GAF domain leading toward a highly conserved region at the N terminus of the downstream PAS domain (Fig. 5*B*). The resulting fusion proteins, designated cPAC(JSC1) and cPAC(RcaE), retained their donor parent CBCR photocycles (Fig. S8). Because JSC1_58120g3 binds BV as well and PCB, the BV adduct of cPAC (JSC1-BV) was examined spectrophotometrically. These measurements showed that both bilin adducts of cPAC retained the spectroscopic signature of the corresponding bilin-reconstituted JSC1_58120g3 parent, although their 15*E* photoproduct states of the former reverted to their 15*Z* dark states more rapidly than those of the parent JSC1_58120g3 constructs.

Finally, the cPAC chimeras were analyzed for adenylyl cyclase activity under similar *in vitro* assay conditions used for WT cPAC. Although the overall cyclase activity was lower in each construct when compared with WT cPAC, both chimeras displayed differential AC activity for their 15*Z* and 15*E* states. For cPAC(RcaE), the 15*E* state was twice as active as its 15*Z* state, whereas cPAC(JSC) exhibited only a ~33% enhancement in its 15*E* state (Fig. 5*C*). A similar result was also observed for cPAC(JSC-BV). In addition, when transformed into the VJS711 *cya*$^-$ strain, cPAC(RcaE) was able to activate β-gal activity under green light, whereas red light suppressed β-gal activity on agar plates (Fig. 5*D*). Taken together, these studies document that re-engineering new color specificity for cPACs by CBCR exchange is a viable and surprisingly facile approach. Such first-generation constructs represent promising subjects for further optimization of spectrally diverse cPAC tools for *in vivo* regulation of cAMP signaling with light.

Discussion

Cyanobacteria rely on the ability to sense environmental light conditions and therefore utilize photosensory proteins to optimize photosynthesis and regulate motility (37), processes that are influenced by levels of the second messenger cAMP. *Microcoleus* sp. *PCC 7113* has one of the largest complement of adenylyl cyclase enzymes for cyanobacteria; among them is the novel cPAC light sensor described here. The many distinct sensory modules in the *Microcoleus* adenylyl cyclase family suggest that cAMP signaling participates in many processes critical for adaptation to the complex intertidal and soil sediment environments where *Microcoleus* is found (57–59). Flavin-

and retinal-based light-sensing adenylyl cyclases are notably widespread in cyanobacteria and algae (12, 30, 37, 60–62), but cPAC is the first example of an adenylyl cyclase with a CBCR input domain.

Our studies show that cPAC is differentially modulated by blue (B) and green (G) lights. Blue light-activated CBCR-based diguanylate cyclases, which trigger rapid settling to avoid photodamage of the photosynthetic apparatus at high fluences, are notably more common in cyanobacteria (23, 63). In addition, the need for B light–regulated adenylyl cyclase activity is underscored by the observation that all known PAC sensors are activated by B light. Unlike the bPAC family of BLUF-, LOV-, and retinal-based sensors, the activity of cPAC can be rapidly adjusted/reversed by G light, providing greater temporal control of cAMP signaling. Our *in vitro* studies document the bistability of cPAC and thus implicate the potential to trigger and reverse cAMP signaling *in vivo*. Beyond the scope of the present investigation, such studies necessitate construction of cell lines such as mammalian neurons or other eukaryotic cells engineered with more sophisticated reporter systems.

cPAC: An integrator of multiple signal inputs?

Well suited to play a role in light-regulated cAMP production in cyanobacteria, the complex architecture of cPAC suggests that it integrates information from multiple input signals. Indeed, our studies implicate the N-terminal REC domain as an input domain that is also required for the adenylyl cyclase activity of cPAC. cPAC was most active when its REC domain was modified to mimic its phosphorylated state, and was least active in an unphosphorylable REC domain variant. These observations suggest that the REC domain stabilizes the homodimeric quaternary structure of full-length cPAC to enhance formation of the interdomain transition state of Type III adenylyl cyclase enzymes (5). The enhanced activity of the phosphomimic mutant may be due to further constraints on the REC domain that favor catalysis. Because the truncated constructs are essentially inactive, we propose that the REC domain may also minimize conformational sampling of the GAF, PAS, and/or CHD domains possibly by reducing unproductive conformational sampling of the homodimer. Future studies will be needed to address these hypotheses.

The cPAC gene is located immediately downstream of an ORF that encodes a histidine kinase, MIC7113_RS10675, annotated as a PAS-SsrA sensor kinase. SsrA proteins are known components of two-component systems (64), thus we speculate that MIC7113_RS10675 may be responsible for the regulation of the phosphorylation status of cPAC. This locus also has apparent orthologs in other cyanobacteria. It will be interesting to test whether cPAC phosphorylation plays a more dramatic regulatory role of the adenylyl cyclase activity compared with the phosphomimetic variant. With respect to other input signals to cPAC, bicarbonate does not appear to be a major agonist in contrast with its known regulatory role of other cyanobacterial adenylyl cyclase proteins (65–69). cPAC was greatly stimulated by the presence of calcium ions (Ca^{2+}). Because Ca^{2+} has been shown to help mediate ATP binding in adenylyl cyclase enzymes (69), changes in Ca^{2+} concentrations may also trigger increased cAMP production by cPAC. The Ca^{2+} sensitivity of cPAC remains an important consideration for optogenetic applications going forward (see below).

cPAC as a optogenetic tool

The activity of cPAC was sufficient to trigger light-dependent differences in cAMP levels *in vivo*, as well as trigger cAMP-regulated gene expression (*lacZ*) in *E. coli* in a B/G-dependent fashion. The successful manipulation of cAMP *in vivo* by cPAC shows that this protein is a viable platform for optogenetic tool applications in a wide variety of photosynthetic species where reduced bilins, also known as phytobilins, are present. These not only includes cyanobacteria, but also eukaryotic algae and plants (70). We envisage considerable utility of this tool for light-dependent reprogramming of plastid metabolic pathways in chlorophyte algae such as *Chlamydomonas reinhardtii*, due to its ability to produce PCB despite its lack of bilin-based sensors in the phytochrome family (71).

Known PACs (with BLUF and LOV domains) have previously been shown to be effective in regulating a variety of cAMP-related processes in heterologous organisms with B light (10, 11, 29, 31, 72). An advantage of cPAC, and its variants, lies with the extensive color palette of CBCRs and the ability to interchange CBCR GAF domains that sense light from the near UV to the near IR; this contrasts with the more limited color-sensing properties of canonical phytochromes (14). Based on these attributes, CBCRs have garnered considerable interest for use as optogenetic tools in live cells (53–55, 73). The ability of CBCRs to switch rapidly between stable photostates (15*E/Z*) without relying on a dark or thermal reversion to reset between active and inactive states is another key feature of the cPAC family. This property enables switching between active/inactive states with supplemental light of the appropriate wavelength to trigger rapid changes in cAMP-dependent processes *in situ*.

Finally, our studies demonstrate that cPAC is a good lead compound for creation of a suite of adenylyl cyclase enzymes that respond to a variety of wavelengths. The successful fusion constructs tested here, especially cPAC(RcaE), establishes the plug-and-play modularity of the cPAC regulator that presage our ability to make robust light-responsive adenylyl cyclases that span the near UV to the NIR. Although intriguing as proof-of-concept enzymes, these proteins, along with native cPAC, are good targets for further engineering efforts. We expect that our growing understanding of CBCR structure (22, 74) will inform structure-based approaches to design new cPACs with improved signaling dynamics and dynamic range.

Applications beyond photosynthetic species

As various CBCRs bind several types of bilin chromophores, constructs can conceivably be tailored to utilize natural precursors that may be more abundant in the organism of choice. For example, although PCB is not naturally produced in nonphotosynthetic species, BV is widely distributed in nature including yeast and mammals. For this reason, biosynthesis of PCB has also been engineered into eukaryotic "chassis" organisms that naturally do not produce these compounds (75–78). Increased expression of heme oxygenases has been used to enhance the

synthesis of BV in bacterial and mammalian cells (79, 80). Indeed, bilin availability remains a critical issue to resolve for optogenetic applications because, in all cases tested, chromophore binding is critical for optimizing the dynamic range of two-color regulation by phytochrome and CBCR systems (39, 54, 81).

As shown previously, CBCRs can also be engineered to alter the dark reversion rate of the photosensory domain, which can be used to create tools that are active at a particular wavelength, and inactive in the dark (55). When combined with light-activated phosphodiesterase proteins (82), the levels of cAMP also can be tailored to precise levels, or to eliminate basal activity, in the 15*Z* state of cPAC and variants. We envisage a bright future for combination of these enzymes and spectrally diverse PACs for the fine-tuned control of cAMP-regulated processes in living cells using a variety of colors of light.

Experimental procedures

Protein expression and spectral analysis

The gene Mic7113_2205 (cPAC) was purchased as synthetic DNA from GenScript®. cPAC and variants were expressed as intein–chitin-binding domain fusion proteins in *E. coli* cells engineered to produce PCB using a dual plasmid system, as described previously (27, 40). Proteins were purified from *E. coli* on a chitin column (New England Biolabs) in accordance with the manufacturer's directions and characterized by SDS-PAGE (Fig. S9). Absorption spectra and dark reversion measurements for cPAC and variants were acquired as previously described (27). Photoconversion of cPAC and variants during *in vitro* calculations was verified in this manner.

In vitro cyclase assay

The BCA assay (Pierce) was used to determine protein concentration for all assays with BSA as a protein standard. The standard cyclase assay buffer was composed of 0.5 M Tris-HCl, pH 7.6, 0.05 M NaCl, 0.01 M $MgCl_2$, 0.5 mM EDTA. $CaCl_2$ was added to buffer at a final concentration of 5 mM, whereas $NaHCO_3$ was added at a final concentration of 50 mM. The desired concentration of GTP or ATP was added to buffer prior to reaction initiation. Purified protein (10 mM final concentration) in the 15*E* or 15*Z* states was added to initiate reaction in the dark. Photostates of protein were achieved using LEE filters (number 071 Tokyo blue and number 090 dark yellow green) as described previously (83), and confirmed via spectroscopic analysis. Assays were terminated after 1 h in the dark with addition of EDTA, pH 8.0, to a final concentration of 0.1 M. An aliquot (15 μl) of each reaction solution was injected into an Agilent 1100 series HPLC and passed through a Phenomenex® Synergi 4 μM Fusion-RP 80 Å column and Security Guard Cartridge (C18, 4 × 2 mm), under conditions previously described (83). The internal standard of cAMP (Sigma) eluted at 12.1 min. A standard curve of cAMP levels using area under peak values correlated with cAMP concentrations of 2.0 to 400 μM. All area calculations were performed using Agilent ChemStation software. All cyclase assays are performed in triplicate at room temperature (20 °C).

HPLC-SEC

Buffer used for HLPC-SEC assays was 40 mM Tris acetate, pH 8.3, 1 mM EDTA, 0.15 M NaCl. Run time was 40 min with an isocratic flow rate of 0.6 ml/min, using ThermoScientific Ultimate 3000 HPLC and a GE Superdex^{TM} 200 Increase 10/300 GL column. Aliquots (50 μl) of purified cPAC and variants (in either 15*E* or 15*Z* states) were injected in a 100-μl loop. 15*E* and 15*Z* states were achieved as described above, and confirmed via spectroscopic analysis. Elution traces and peaks were analyzed using Chromeleon 7 software (Dionex). All runs were performed in darkness to avoid photoconversion prior to elution. Molecular weight calculations were performed via standard curve preparation, seen in supporting Fig. S4. Samples were analyzed in duplicate ($n = 2$) and the molecular weight measurements were averaged and the plus/minus spread was described.

In vivo cPAC expression in a cya$^-$ E. coli strain

cPAC and variants in PCB-producing *E. coli* K12 strain VJS711 that lacked the endogenous adenylyl cyclase gene (referred to in this paper as strain *cya*$^-$) were grown overnight in LB medium containing 0.2 mM kanamycin and 0.2 mM chloramphenicol in darkness at 30 °C before suspension culture or plate assays. For suspension culture assay, aliquots (3 ml) of the overnight suspension cultures were then transferred to NUNC 12-well cell culture plates (catalog number 150628). To generate Bc, Gc, or continuous red light (Rc) conditions, half of each plate was covered with filters (number 071 Tokyo blue (maximum absorbance: 440 nm) or number 090 dark yellow (maximum absorbance: 525 nm) or number 164 flame red (maximum absorbance: 625 nm) respectively; LEE filters, Burbank, CA) and allowed to incubate further for 6 h. The light fluence rate for light incubation was 4–5 μmol m^{-2} s^{-1}. *E. coli* liquid suspension cultures were then analyzed for global cAMP levels with the cAMP Direct EIA kit (Arbor Assays LLC, catalog number K019-H1) as directed by manufacturer. All experiments were performed in triplicate with two technical replicates. The limit of detection of the EIA kit was 0.2 pmol/ml with sensitivity of 0.64 pmol/ml. For plate assays, overnight grown cells were plated on LB plates containing 1 mM IPTG, 84 mg/liter of amino levulinic acid, 0.2 mM kanamycin, and 0.2 mM chloramphenicol to which X-Gal (40 μl) was freshly added. Plates were incubated at 30 °C overnight with light conditions acquired as stated above.

Construction of fusion proteins and site-directed mutagenesis

Synthetic fusion proteins were constructed using a modified protocol for the sequence and ligation-independent cloning method (84). Site-directed mutagenesis was performed using a protocol modified from the QuikChange Site-directed Mutagenesis kit (Agilent Technologies®). Proteins were expressed and analyzed as described above.

Alignment of sequences

Alignments for the cPAC enzyme active site and GAF region were analyzed using MUSCLE (default settings), visualized via JalView, and manually edited in Adobe Illustrator.

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