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Engineering adenylate cyclases regulated by near-infrared window light

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Bacteriophytochromes sense light in the near-infrared window, the spectral region where absorption by mammalian tissues is minimal, and their chromophore, biliverdin IXα, is naturally present in animal cells. These properties make bacteriophytochromes particularly attractive for optogenetic applications. However, the lack of understanding of how light-induced conformational changes control output activities has hindered engineering of bacteriophytochrome-based optogenetic tools. Many bacteriophytochromes function as homodimeric enzymes, in which light-induced conformational changes are transferred via α-helical linkers to the rigid output domains. Here, we hypothesized that heterologous output domains requiring homodimerization can be fused to the photosensory modules of bacteriophytochromes to generate light-activated fusions. Bacteriophytochromes can be engineered to activate heterologous output domains that require homodimerization. We constructed an adenylate cyclase affected worm behavior in a light-dependent manner. The insights derived from this study can be applied to the engineering of other homodimeric bacteriophytochromes, which will further expand the optogenetic toolset.

Light has advantages over chemical means of regulating biological processes because it acts noninvasively and provides superior spatial and temporal resolution (1). Optogenetic approaches that rely on algal and archaeal channelrhodopsins controlling specific animal neurons opened up a new era in neurobiology (2, 3). Photoreceptors of several other types have been engineered to regulate biological processes and used in cell cultures and transparent animals (4, 5). However, application of optogenetic tools in heme-rich animal tissues has been hindered by high scattering and poor penetration of visible light. Light in the near-infrared window (NIRW), which encompasses the spectral region of 680–880 nm, penetrates animal tissues much better than light outside the NIRW (6). A significant fraction of NIRW light can pass through several centimeters of human tissues (7–9), which makes NIRW light a promising means of controlling biological processes in animals. Absence of photoreceptors of NIRW light in most animal tissues is an additional advantage that makes NIRW light harmless (10). This is in contrast to blue light, which is absorbed by flavins and porphyrins, and therefore promotes photooxidative damage (11).

Phytochromes are photoreceptors that absorb light in the NIRW of the spectrum (12–14). The photosensory modules of these photoreceptors covalently bind bilin chromophores. Plant and cyanobacterial phytochromes bind phycocyanobilins or phycocerythrobilins, whereas bacteriophytochromes bind biliverdin IXα. As the first product of heme turnover, biliverdin IXα is naturally present in animal cells, which makes bacteriophytochromes preferred over plant and cyanobacterial phytochromes, whose chromophore synthesis requires dedicated enzymes. Further, absorption wavelength maxima of bacteriophytochromes are red shifted compared with the absorption maxima of plant and cyanobacterial phytochromes. This results in a 2- to 10-fold gain in the penetration depth of light through mammalian tissues (7, 10). Up to now, bacteriophytochrome engineering for optogenetic applications has lagged behind the engineering of photoreceptors of other types (4, 5), including engineering of plant phytochromes (15, 16). The major obstacle to bacteriophytochrome engineering has been the lack of understanding of the mechanisms through which light-induced conformational changes are transduced to regulate output activities (12–14, 17).

Most or all bacteriophytochromes function as homodimeric enzymes, usually histidine kinases and, more rarely, diguanylate cyclases (DGCs). Enzymatic activities of both histidine kinases and DGCs require precise alignment of two monomers in a homodimer. In the case of DGCs, their product, cyclic dimeric GMP, affects cell behavior. A roundworm Caenorhabditis elegans, near-infrared light-activated adenylate cyclase affected worm behavior in a light-dependent manner.


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GMP (c-di-GMP), is synthesized from two GTP molecules at the interface between two Gly-Gly-Asp-Glu-Phe (GGDEF) domains ( Pfam database) (18) responsible for DGC activity. Each GGDEF domain brings a substrate molecule to the catalytic site (19, 20). In the inhibited state, the photosensory modules apparently prevent enzymatic domains from forming a properly aligned homodimer, whereas light-induced conformational changes restore enzymatically productive domain alignment. Although the exact nature of conformational changes is unknown, it is likely that they are mediated by the α-helical linkers that connect the photosensory modules to the output domains (21–23) (Fig. 1). Further, in all DGCs whose regulation has been studied at the structural level, enzyme activation has been shown or predicted to occur via alignment of the rigid GGDEF domains rather than via intradomain conformational changes (19, 20). Based on these considerations, we reasoned that bacteriophytochromes can regulate diverse output activities that depend on proper alignment of the two output domains (24).

Here we demonstrate that bacteriophytochrome photosensory modules can indeed regulate heterologous output domains. We engineered a series of photoactivated adenylate cyclases (ACs) designated NIRW light-activated AC (IlaC), by fusing a photosensory module from the Rhodobacter sphaeroides bacteriophytochrome DGC, BphG1 (25, 26), to an AC domain from the Nostoc sp. CyaB1 protein (27) (Fig. 1), which, like all bacterial type III ACs, works as a homodimer (28, 29). We chose to make IlaC because CyaB1 is a homodimeric AC. Details on 3D models are provided in SI Materials and Methods.

For the output AC domain, we looked for a protein (i) whose AC activity is confined to the AC domains, i.e., where regulatory domains are not required for basal activity, and (ii) whose AC activity in the dark can be detected in a bacterial screening system. CyaB1 from Nostoc sp. fit these requirements (27). The native CyaB1 protein has the following domain architecture, GAF–GAF–PAS–PAS–AC (where AC domain is responsible for AC activity (Fig. 1). The C-terminal AC domain of CyaB1 unangled from the regulatory domains possesses some AC activity (27). To monitor cAMP synthesis, we used Escherichia coli BL21[DE3] cya, which lacks the endogenous AC. Cya. In this strain, expression of the chromosomal lacZ gene encoding β-galactosidase is low because of the absence of activation by the cAMP-responsive protein (CRP) also known as catabolite activator protein (35). BL21[DE3] cya produces white colonies on agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (31). The C-terminal AC domain of CyaB1 (amino acids 585–857) re- stores lacZ expression, thus generating blue colonies. To endow this bacterial system with the ability to synthesize biliverdin IXα, we introduced the cyanobacterial heme oxygenase gene hol1 (36), whose product converts heme synthesized by E. coli into biliverdin IXα.

**Results**

**Components of the NIRW Light-Activated AC.** For the photosensory module of the NIRW light-activated AC, we chose the PAS–GAF–PHY module from BphG1 from R. sphaeroides, where PAS, GAF, and PHY (phytochrome) are protein domain names (Pfam database). The truncated derivative of BphG1, BphG, where the PAS–GAF–PHY module is linked to a GGDEF domain, functions as a light-activated DGC (25) (Fig. 1). BphG was particularly attractive because (i) absorption maxima of its dark [red-absorbing (Pₚ)] and lit [far-red–absorbing (Pᵣ)] forms, 712 and 756 nm, respectively, lie within the NIRW, and (ii) its DGC activity is activated by light by ~11-fold, the largest photodynamic range (fold activation) among bacteriophytochromes for which such a ratio has been quantified (26).

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**Engineering a NIRW Light-Activated AC.** We synthesized the DNA fragment encoding the AC domain of CyaB1 (amino acids 585–857) and fused it to amino acid 526 in the unstructured (loop) region of the GGDEF domain of BphG. Leu585 of CyaB1 is also in the loop region, 8 aa upstream of the first structural element, β-strand, of the AC domain (Fig. 1 and Fig. S1). The unstructured linkers were meant to prevent potential steric interference between the fusion partners. In accord with this intent, the chimeric protein, IlaC6, possessed AC activity, yet this activity was nonresponsive to light (Fig. 2A). Next, we determined the minimal AC domain size that retained enzymatic activity in the BphG–CyaB1 fusions. We fixed the fusion point in BphG at amino acid 526 and progressively shortened the AC domain, from Leu585 to Glu594 of CyaB1, where Glu594 is in the predicted β-strand of the AC domain (Fig. 2F). All fusions in this series, IlaC10–13, proved to be enzymatically active and nonresponsive to light (Fig. 2F). In the next round of engineering, the AC domain border was fixed at Glu594 but the unstructured region of the GGDEF domain and the α-helical linker extending from the PHY domain were subject to shortening. The fusion to Arg507 of BphG (IlaC30) and fusions containing shorter BphG fragments (IlaC26, –27, and –31) had no AC activity (Fig. 2A and B) likely because of the steric hindrance between the fusion components,

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**Fig. 1.** Domain architectures, and 3D models of the components used in IlaC engineering. *R. sphaeroides* BphG is bacteriophytochrome DGC, *Nostoc* sp. CyaB1 is homodimeric AC. Details on 3D models are provided in SI Materials and Methods.
thus the engineering space was limited to amino acids 508–526 of BphG.

One of the fusions in this region, IlaC18, produced a protein whose AC activity was higher in the dark than in the light, i.e., light-inactivated AC (Fig. 2A and B). This result showed that light induces conformational changes that are sufficient to misalign the enzymatically productive AC homodimer. Notably, light-inactivated fusions were also obtained at other fusion points, e.g., IlaC5 (Fig. 2A). Four fusions, IlaC29 (Arg516), IlaC17 (Leu512), IlaC22 (Arg509), and IlaC25 (Thr508) produced the desired, photoactivated enzymes. The AC activity of these fusions differed from each other as judged by colony color on X-Gal agar containing IPTG and X-Gal, either in the dark (Left of center) or in the red light (Right). Blue colony color indicates cAMP-CRP-induced lacZ expression. Each strain expressing an IlaC was plated in a sector of each of the four plates. The plating guide is in the Center. Dark-blue numbers, light-inactivated IlaC protein; red numbers, light-activated IlaC proteins. IlaC expression from pETIlaC# was induced at two IPTG levels: 10 μM (Upper plates) and 50 μM (Lower plates).

Fig. 2. IlaC engineering strategy. (A) A subset of BphG-CyaB1 protein fusion sequences, their AC activities, and responsiveness to light. The amino acid colors correspond to the colors of protein domains shown above protein sequences. Brown, PHY domain; blue, GGDEF domain; green, AC domain; and black, interdomain linkers. Predicted secondary structure elements, α-helix, and β-strand, are shown above sequences. AC activity: +, active; −, inactive, according to the lacZ plate assays (see B). Response to light: −, no response; +, activation; and −, inactivation. (B) Images of the lacZ plate assays of AC activity. Selected E. coli BL21(DE3) (pETIlaC# and pT7-ho1-1) strains from A were grown on LB agar containing X-Gal and IPTG, either in the dark (Left of center) or in the red light (Right). Blue colony color indicates cAMP-CRP-induced lacZ expression. Each strain expressing an IlaC was plated in a sector of each of the four plates. The plating guide is in the Center. Dark-blue numbers, light-inactivated IlaC protein; red numbers, light-activated IlaC proteins. IlaC expression from pETIlaC# was induced at two IPTG levels: 10 μM (Upper plates) and 50 μM (Lower plates).

Extending Lifetime of the Light-Activated State of an AC. Following photoactivation, bacteriophytochromes in the lit (P<sub>L</sub>) state spontaneously return to the ground, dark (P<sub>D</sub>), state via thermal reversion (12). The half-life of IlaC22 k27 in the P<sub>L</sub> state is 46 ± 3 s (Fig. 4A). A relatively short half-life is desirable for optogenetic applications that require short pulses of cAMP, whereas applications involving sustained light-induced increases in cAMP levels, will benefit from enzymes with more stable lit state.

To increase lifetime of the lit state of IlaC22 k27, we relied on success in extending this parameter in the Arabidopsis thaliana phytochrome PhyB (37, 38). Four residues in the proximity of the chromophore involved in controlling dark recovery of PhyB are conserved in BphG (Fig. S4). We introduced in IlaC22 k27 the same mutations as those that prolonged the half-life of PhyB. Three mutants (R205A, G450E, and R468A) resulted in the loss of AC activity and were discarded (Fig. 4B). The fourth mutant, IlaC22 k27 Y259F, was purified and characterized in vitro. Its absorption maxima were slightly shifted compared with IlaC22 k27 R509W, two of which were in the BphG photosensory module (D120W and Q371H) and one in the AC domain (Q670R of CyaB1) (Fig. S3). Because the photodynamic range of IlaC22 k27 was within twofold of the photodynamic range of the BphG, we did not attempt to increase it further. Instead, we focused on modifying another important parameter, i.e., stability of the photoactivated state.

Improving Photodynamic Range of the Photoactivated ACs. Because the photodynamic range of the first-generation NIRW light-activated ACs were significantly lower than the photodynamic range of BphG, we intended to improve this parameter by random PCR-based mutagenesis. Here, we focused on IlaC22 as a template because of its low dark activity. In the light, IlaC22 produced blue colonies only when expressed at high, 50 μM, IPTG concentration. We therefore screened the library of the PCR-mutagenized IlaC22 gene for blue colony appearance at low, 10 μM, IPTG concentration. After screening ~10<sup>5</sup> mutant clones, we found mutants with significantly increased AC activity. The best one had an R509W (BphG numbering) substitution, right at the junction between the PHY and AC domains (Fig. 2A).

The photodynamic range of AC activity of the purified IlaC22 R509W was approximately fourfold (Fig. 3). To further improve the photodynamic range, we mutagenized the ilac<sub>22</sub> R509W gene at high mutation frequency. Our primary goal was to decrease the dark AC activity of IlaC22 R509W. We therefore searched for white colonies at high, 100 μM, IPTG, in the dark. The strategy used in this screen is illustrated in Fig. 2B. After screening of >10<sup>5</sup> mutant clones, we identified a derivative, IlaC22 k27, that had significantly lower activity in the dark but only slightly lower activity in the light, compared with IlaC22 R509W (Fig. 3), which resulted in the photodynamic range of sixfold. IlaC22 k27 was found to contain three new mutations, compared with IlaC22 R509W, two of which were in the BphG photosensory module (D120W and Q371H) and one in the AC domain (Q670R of CyaB1) (Fig. S3). Because the photodynamic range of IlaC22 k27 was within twofold of the photodynamic range of the BphG, we did not attempt to increase it further. Instead, we focused on modifying another important parameter, i.e., stability of the photoactivated state.

NIRW Light-Activated Control of Caenorhabditis elegans Behavior. To test performance of a NIRW light-activated AC in an animal model, we expressed IlaC k27 in cholinergic neurons of the roundworm C. elegans. Previously, a blue-light-activated AC, PACα, has been used in C. elegans as a tool for optogenetic manipulation of behavior (33). Expression of PACα in the cholinergic
neurons, using the promoter for the vesicular acetylcholine transporter, *unc-17*, followed by stimulation with blue light, resulted in increased locomotory activity (33). However, blue light activates a *C. elegans* avoidance response and is toxic upon prolonged irradiation (33, 39), thus confounding the interpretation of the behavioral response to the optogenetic manipulation. Therefore, *IlaC* could be advantageous for behavioral analyses in *C. elegans*.

We generated transgenic animals expressing *IlaC22 k27* in cholinergic neurons using the *unc-17* promoter. *IlaC22 k27* transgenic animals cultivated on an agar surface and exposed to daily light from the environment were more active than wild-type animals, as evident by the higher frequency of their body bends (Fig. 5A). Hyperactivity is characteristic of animals with increased activity of cAMP-dependent protein kinase A (PKA), such as mutants for the gene *kin-2*, which encodes for the regulatory subunit of PKA (40). To test the effect of red light, we grew wild-type and *IlaC* animals in the dark for a single generation. Individual animals were transferred to an agar surface that did not contain food. Animals were monitored under monochromatic light-emitting diode (LED) irradiation for 90 s. During this time, body bends were counted during the following light regimen: green light 0–30 s, red 31–60 s, and green 61–90 s. Whereas control animals did not alter their locomotory activity in response to red light (Fig. 5B), *Punc-17:ilaC* animals performed more body bends in the presence of red light (Fig. 5B and Movie S1).

We also monitored the effects of AC activation in cholinergic neurons on the frequency of thrashing movements in liquid medium. Swimming animals, which were reared in the dark, were video monitored for a total of 2 min during the following light regimen: green 0–30 s, red 31–60 s, green 61–120 s. Whereas control animals did not alter their thrashing rate in response to red light, *Punc-17:ilaC* animals increased their thrashing rates when exposed to red light (Fig. 5C). Interestingly, their thrashing rates significantly decreased during the second exposure to green light (Fig. 5C), suggesting poststimulatory fatigue. Thus, our results indicate that the NIRW light-activated AC can be used as a tool for optogenetic manipulation of cAMP levels in animals.

**Discussion**

Unique photochemical properties of bacteriophytochromes, i.e., (i) light absorbance in the NIRW, optimal for use in red-blooded animals, (ii) naturally available in animal cells, and (iii) innocuous nature of NIRW light, position them as superior photoreceptors for optogenetic applications in animals (10). However, bacteriophytochrome engineering for optogenetic applications has been hindered by the lack of understanding of mechanisms through which light-induced conformational changes induce changes in output domains. The two most common bacteriophytochrome types, histidine kinases and DGCs, operate as homodimeric enzymes, where proper alignment of two monomeric kinase or GGDEF domains is essential for enzymatic activity. We postulated that the *α*-helices extending from the PAS–GAF–PHY photosensory modules are primarily responsible for alignment of the output domains (Fig. 1) and hypothesized that the light-induced movements mediated by the *α*-helices can regulate alignment of unrelated, heterologous domains whose activity requires properly aligned homodimers. The NIRW light-activated ACs, IlaCs, constructed in this study, are consistent with our assumption. The recently solved structures of the photosensory module of the *Deinococcus* bacteriophytochrome showed that the distance between the tips of the signaling *α*-helices changes by as much as ~30 Å in response to light (41).

What lessons can we draw for future homodimeric bacteriophytochrome engineering? First, the choice of the output domains and screening scheme are important. For example, the ability of the AC domains of CyaB1 to spontaneously homodimerize helped us to distinguish between enzymatically active

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**Fig. 3.** Kinetics of cAMP accumulation by the purified *IlaC* proteins in the dark and light. (Upper row) First-generation light-responsive proteins: *IlaC18*, light-inactivated AC; *IlaC17*, 22 and 25, light-activated AC. (Lower row) *IlaC22* mutants with improved photodynamic ranges: *IlaC22 R309W* and *IlaC k27*. AC activity was measured at room temperature. cAMP was quantified by high-performance liquid chromatography (HPLC). Black traces, dark (dim green light); red traces, irradiation with 700-nm light.

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**Fig. 4.** Photochemical characterization of *IlaC* variants. (A) Kinetics of the dark recovery of *IlaC* derivatives from the lit (*Pfr*) state. Plotted are changes in absorbance at 755 nm over time following 5-min excitation with 700-nm light. Blue trace, *IlaC22 k27*; green trace, *IlaC22 k27 Y259F*. Half of the *Pfr* form of *IlaC22 k27* decayed after 46 ± 3 s and half of the *Pfr* form of the *IlaC22 k27 Y259F* mutant decayed after 197 ± 9 s. (B) Plate assays of the *IlaC k22* dark recovery mutants. (C) Light-induced spectral changes in *IlaC22* derivatives. Black (*Pfr*), before irradiation; red (*Pfr*), after irradiation with 700-nm light; Δ, difference (*Pfr* − *Pfr*) spectra. *IlaC22 k27* (Left); *IlaC22 k27 Y259F* (Right).
and inactive fusions (Fig. 2A and B). The high sensitivity of the screening system used here was useful for detecting relatively low (twofold) photodynamic ranges (Fig. 2B). An engineering strategy that involves (i) construction of permissive fusions (involving unstructured linkers) as the first step, (ii) subsequent minimization of the output domain size, and finally, (iii) shortening (or possibly, extending) signaling α-helices, may also be universally applicable. Another lesson learned is that photovactivation ratios of the first-generation light-responsive fusions can be significantly improved by extensive mutagenesis. Are there limitations of homodimeric bacteriophytochrome engineering? First, it is imperative that energy associated with the light-induced conformational changes is sufficient to manipulate the output domain alignment. For overly tight homodimers, monomer interactions may have to be weakened. Another important issue is reasonable correspondence in distances between fusion points in the signaling and output components. We estimated that the distances between the α-helical residues of BphG that resulted in photoactivated fusions were in the range of 11–22 Å, whereas the distances between the N-terminal β-strands of the catalytically inactive CyaB1 AC domain homodimer were ~40 Å (Fig. S1). These distances turned out to fit nicely in the 10–40 Å spread between the α-helices of the Deinococcus BphP in the dark and lit states (41). Whether proteins whose homodimers are separated by larger distances can be made light-responsive is unclear.

We found not one but several photoactivated IlaC fusions. Interestingly, they differed from each other by either 3 or 4 residues (IlaC29 = IlaC17 + 4 aa, IlaC25 = IlaC17 − 4 aa, and IlaC22 = IlaC17 − 3 aa) (Fig. 2A), which places their AC domains roughly in the same helical phase but separated by one or two α-helical turns (1 turn = 3.6 aa) (42). This result is consistent with the model where α-helices act as rods involved in aligning rigid output domains located at their tips. It is therefore unsurprising that the 1 or 2-aa shifts out of helical phase destroy proper domain alignment and result in the loss of light responsiveness (Fig. 2A). The involvement of α-helices in mediating intramolecular signal transduction is not unique to bacteriophytochromes, and other researchers have used such helices for heterologous domain replacements (43–49).

IlaC expressed in neurons of the roundworm C. elegans affected behavior in response to light. In the case of C. elegans, the main advantages of IlaCs over blue-light activated ACs are the absence of the photoavoidance response and the lack of phototoxicity associated with prolonged exposure to blue light (33, 39). However, we anticipate that IlaCs will prove most useful in applications in deep mammalian tissues inaccessible by blue light. Our demonstration that homodimeric bacteriophytochromes are amenable to protein engineering (24) and recent progress in structural understanding of dark-to-light conformational changes (41) should encourage design of new NIRW light-activated proteins. Because activities of many signal transduction components depend on homodimerization, including membrane receptors, cyclic nucleotide phosphodiesterases, certain protein phosphatases, proteases, nucleases, and transcription factors, we expect significant expansion of the optogenetic toolset involving NIRW light.

Materials and Methods
Microbiological Methods. E. coli BL21(DE3) cya lacking endogenous adenylate cyclase CyaA (26) and containing two plasmids, pT7-ho1-1 (25) that expresses the Synechocystis sp. heme oxygenase ho1 (36) and pETIlaC (expressing IlaC proteins) were used for IlaC screening. Strains were grown at 30 °C in LB supplemented with X-Gal (40 μg/mL), ampicillin (50 μg/mL), and kanamycin (25 μg/mL). IPTG was added for induction of IlaC proteins. For light-sensitive experiments, Petri dishes were placed onto All-Red (660 nm) LED grow light panels (30.5 × 30.5 cm; LED Wholesalers). Light irradiance was ~0.2 mW cm⁻². For growth in the dark, Petri dishes were wrapped in aluminum foil.

Recombinant DNA Techniques. The DNA fragment of bphG1 gene (RSP4191) encoding the photosensory PAS–GAF–PHY module was amplified by PCR from the R. sphaeroides 2.4.1 genome. The DNA fragment of cyaB1 from Nostoc (formerly Anabaena) sp. PCC 7120 was synthesized by BioBasics with the codon use optimized for R. sphaeroides. Two fragments were joined by fusion PCR using GoTag (Promega) and subsequently cloned into the Xbal and KpnI sites of pET22a(+) (Invitrogen) to yield a series of plasmids, pETIlaC. Each of these plasmids encodes a unique BphC-CyaB1:His₆ fusion protein. Site-directed mutagenesis was performed using a QuikChange kit (Stratagene). Error-prone PCR mutagenesis was carried out using GeneMorph II Random Mutagenesis kit (Agilent Technologies).

The Punc-17::ilaC plasmid, pNQ149, was constructed using the MultiSite Gateway Three-Fragment Vector Construction kit (Invitrogen). pNQ149 combines the unc-17 promoter (obtained from the Promoeome library in the pdonP4-P1r exit vector), the ilaC22 k27 DNA, and the unc-54 3’ UTR in the pdonP27K-P3 entry vector.

Protein Purification and AC Assays. The IlaC proteins were purified as C-terminal His₆-tagged fusions using Ni-affinity chromatography (Novagen). AC assays were performed using freshly purified proteins at room temperature, essentially as described earlier (50). Detailed protocols are available in SI Materials and Methods.

Spectroscopy. Electronic absorption spectra were recorded with a UV-1601 spectrophotometer (Shimadzu) at room temperature. Protein solution (100 μL) in a 10-mm light path quartz cuvette was irradiated by 1-W (700 nm) LED directly in the spectrophotometer from the top of the cuvette.

C. elegans Cultivation and Transgenesis. Animals were cultivated on nematode growth medium (NGM) agar and were fed E. coli DBA37 derived from strain OP50 (51). All experiments were performed on hermaphrodites. The following strains were used in this study: N2 (Bristol), N717 qnEx386[Punc-17::Iac; Pmyo-2::mCherry], and N721 qnEx388[Punc-17::Iac; Pmyo-2::mCherry]. Transgenic animals were created by microinjection (52) using a Leica DMIRB inverted differential interference contrast microscope equipped with an Eppendorf
Femtojet microinjection system. N2 animals were injected with 25 ng/μL of pNQ149 in combination with 5 ng/μL of pCP930 (Pyro−2mCherry) and 120 ng/μL 1-kb molecular weight ladder (New England Biolabs).

C. elegans Behavioral Assays. Animals were grown in the dark for one generation on NGM agar seeded with DBA37t bacteria. For the assays performed on agar, L4 larvae were transferred to NGM plates seeded with DBA37t and grown to early adulthood overnight. Individual adult animals were transferred in the presence of green light onto an NGM plate without bacteria and left undisturbed. Animals were observed using a Leica MZ16 stereo microscope. During this time, the animals were exposed to green light for 30 s, red light for 30 s, and green light for 30 s. Body bends were counted by the observer. Irradiation was provided by a LED Color Changing kit IP66 (LED Wholesalers). No biliverdin IXα was added to the agar.

For the swimming assays, we followed with minor modifications the protocols described by Weisenseifer et al. (33). L4 larvae were transferred to NGK plates seeded with DBA37t bacteria supplemented with 1 mM biliverdin hydrochloride (Sigma) and grown to early adulthood for 1 d. Individual early adult animals were transferred in the presence of green light into a 10-μL drop of NGM and M9 in a 1:1 ratio supplemented with 1 mM biliverdin hydrochloride. Animals were video monitored for 2 min using a USB 2.0 monochrome camera (ImagingSource, model DNK-72AU02), mounted on a Leica MZ16 stereo microscope. During the 2-min recording, the animals were exposed to green light for 30 s, red light for 30 s, and green light again for 60 s. Body bend frequency was counted by observing the video recordings.

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