Optogenetic manipulation of cGMP in cells and animals by the tightly light-regulated guanylyl-cyclase opsin CyclOp

Shiqiang Gao1,*, Jatin Nagpal2,3,*, Martin W. Schneider2,3, Vera Kozjak-Pavlovic4, Georg Nagel1 & Alexander Gottschalk2,3,5

Cyclic GMP (cGMP) signalling regulates multiple biological functions through activation of protein kinase G and cyclic nucleotide-gated (CNG) channels. In sensory neurons, cGMP permits signal modulation, amplification and encoding, before depolarization. Here we implement a guanylyl cyclase rhodopsin from Blastocladiella emersonii as a new optogenetic tool (BeCyclOp), enabling rapid light-triggered cGMP increase in heterologous cells (Xenopus oocytes, HEK293T cells) and in Caenorhabditis elegans. Among five different fungal CyclOps, exhibiting unusual eight transmembrane topologies and cytosolic N-termini, BeCyclOp is the superior optogenetic tool (light/dark activity ratio: 5,000; no cAMP production; turnover (20 °C) ~17 cGMP s⁻¹). Via co-expressed CNG channels (OLF in oocytes, TAX-2/4 in C. elegans muscle), BeCyclOp photoactivation induces a rapid conductance increase and depolarization at very low light intensities. In O₂/CO₂ sensory neurons of C. elegans, BeCyclOp activation evokes behavioural responses consistent with their normal sensory function. BeCyclOp therefore enables precise and rapid optogenetic manipulation of cGMP levels in cells and animals.
Optogenetic tools developed in recent years opened new avenues in neuro- and cell biological research. These tools allow rapid, reversible and non-invasive modulation of membrane potential, protein–protein interaction, gene expression and second messenger signalling in live cells and whole animals, in a cell-specific manner. Many optogenetic tools are based on microbial rhodopsins. Rhodopsins are membrane proteins, responsible for light perception in the ultraviolet to visible spectral range. They are composed of a non-absorbing protein, opsin, to which a chromophore, retinal, is covalently linked. Rhodopsins from the animal visual system (type II or metazoan opsins) are G-protein-coupled receptors; they sense light and, in vertebrate rods and cones, via G, signalling-induced hydrolysis of cyclic GMP (cGMP), yield membrane hyperpolarization by closure of cyclic nucleotide-gated (CNG) cation channels. Also some opsins signalling via G or G have been described. In Drosophila eyes, rhodopsin leads to membrane depolarization via the activation of transient receptor potential (TRP) cation channels.

Several type I microbial rhodopsins were discovered and extensively studied, such as the light-activated H+ pump bacteriorhodopsin, the Cl− pump halorhodopsin, the sensory rhodopsins, and most recently the channelrhodopsins (ChRs). These light-activated channels and pumps were prominently established as optogenetic tools for membrane depolarization, respectively. In addition, large microbial rhodopsins were predicted from the Chlamydomonas reinhardtii genome sequence, consisting of opsins fused to signalling modules, like nucleotidyl cyclase domains; yet none of these opsins has been experimentally proven to be functional.

Other optogenetic tools utilize LOV or BLUF domains for light sensation and relay photon absorption to different protein domains, thus achieving cellular effects, for example, unmasking protein interaction modules, or triggering enzymatic activity. Optogenetic tools for light-driven cAMP generation exist, for example, photoactivated adenyl cyclases (PACs), which permit rapid production of cAMP in cells and animals, and can affect behaviour through modulation of specific cellular processes or signalling in the nervous system. Another second messenger widely utilized is cGMP, activating protein kinase G, or CNG channels, especially in sensory neurons in various animals. Here guanylyl cyclases act downstream of sensory receptors, or are modularity connected to them, signaling, for example, the detection of light, oxygen, temperature or chemicals. Guanylyl cyclases thus relay primary sensory signals to downstream cellular machinery that encodes this information for a signal output. As the output of a sensory neuron is transmitter release, this can be addressed by optogenetics using ChR2. Yet, intracellular encoding of the primary sensory signal via cGMP, which can be a control point for signal amplification or modulation, cannot be addressed using ChR2. Thus, an optogenetic tool affecting cGMP levels in such cells would enable control over intracellular encoding events in cGMP mediated signalling.

The existing PAC of Beggiatoa was converted into a guanylyl cyclase by mutating three amino acids (aa) relevant for nucleotide specificity; the respective protein (named bPGC, BlgC or EROS) has recently been used in male rats, to evoke penile erection, following transfection and blue illumination of the cavernosa. Yet, as bPGC was generated by mutation and did not evolve, its efficiency was low compared with the parental enzyme, and its productivity for GTP over ATP was only ~7:1 in vitro. Thus, naturally evolved light-triggered guanylyl cyclases may be more efficient optogenetic tools.

Recently, a new type I rhodopsin, fused with a guanylyl cyclase domain, was found in the fungus Blastocladiella emersonii. This rhodopsin guanylyl cyclase, BeGC1, was shown to be essential for phototaxis of zoospores in B. emersonii through green light-regulated cGMP production. Yet, the light-regulated guanylyl cyclase activity of BeGC1 was not directly demonstrated.

Here we show light-regulated guanylyl cyclase activity of BeGC1 by in vitro and in vivo assays using expression in Xenopus oocytes, HEK293T cells and muscle cells of the nematode Caenorhabditis elegans. We thus suggest renaming this protein to Cyclase Opsin (guanylyl cyclase opsin from B. emersonii, BeCyclOp). Light-activated BeCyclOp potently produces cGMP with high specificity (no cAMP production), fast kinetics and high turnover, and shows highest efficiency among four other fungal CyclOps tested. We co-express BeCyclOp in C. elegans muscle cells, together with the C. elegans CNG channel comprising TAX-2 and TAX-4 subunits, to demonstrate light-driven depolarization upon photoactivation of BeCyclOp. Last, in O2/CO2 sensory neurons of C. elegans, BeCyclOp light-activation triggers rapid tactic behavioural responses, which depend on the presence of all-trans retinal (ATR). Thus, BeCyclOp is a potent optogenetic tool for control or modulation of cGMP signalling in cells and animals.

**Results**

**Expression of fungal CyclOp proteins in Xenopus oocytes.** The full-length cDNA and protein product of BeCyclOp has been confirmed in B. emersonii. BeCyclOp (626 residues, calculated 68 kDa) comprises a microbial (type I) opsin domain near the N-terminal and a guanylyl cyclase domain in the C-terminal part. Compared with other microbial opsins, BeCyclOp has a much longer N terminus (Supplementary Figs 1 and 2). Transmembrane helix prediction indicates that BeCyclOp might have eight transmembrane helices, while all other opsins known so far are seven transmembrane proteins with extracellular N and cytoplasmic C termini, respectively. The N terminus of BeCyclOp appears to contain no signal sequence, and should thus be cytosolic, like the predicted C terminus. The first transmembrane helix is predicted to span aa 147–169. The fourth helix (aa 249–270) in BeCyclOp shows high conservation with transmembrane helix 3 in other type I opsins (Supplementary Fig. 1, inset). The conserved lysine for binding retinal is in the last, the predicted eighth helix. Avelar et al. identified four additional predicted CyclOps in fungal genomes: three in Allomyces macrognosus (AmCyclOp1, 2 and 3) and CaCyclOp in Catenaria anguillulae (Supplementary Fig. 2). All of them exhibit the unusual transmembrane domain architecture, including the first transmembrane helix as a specific addition. We thus suggest numbering the CyclOp transmembrane helices from 0 to 7.

We obtained synthetic genes for all five CyclOps, codon optimized to mouse, and expressed them in Xenopus oocytes, as C-terminal yellow fluorescent protein (YFP) fusions (CyclOp-pYFP). All five CyclOp-pYFPs showed robust and similar expression in the dark 3 days post injection (dpi) of in vitro synthesized mRNA (cRNA), as judged by the observable YFP fluorescence in the plasma membrane (Fig. 1a). To assess these cells for light-mediated cGMP generation, the injected oocytes were illuminated for 2 min with green light (532 nm; 0.15 or 0.5 mW mm−2), followed by extract preparation and a colorimetric assay for cGMP content (Fig. 1b; Supplementary Table 1). Light-treatment led to a ~180-fold increased level of cGMP for BeCyclOp-pYFP, compared with the extract prepared from control oocytes kept in the dark (N = 2 experiments, n = 6 oocytes each). Addition of 1 μM ATR to the oocyte medium led to slightly increased BeCyclOp-pYFP expression (Fig. 1a); possibly, binding of ATR to BeCyclOp stabilizes the protein in the oocyte, as previously observed for ChR2 (ref. 29). Light-triggered cGMP production was also slightly higher with ATR (~1.5-fold; N = 2
Figure 1 | Assessing fungal CyclOps for light-regulated cGMP generation. (a) Plasma membrane fluorescence of Xenopus oocytes expressing BeCyclOp, AmCyclOp1, 2 and 3, and CaCyclOp, as C-terminal YFP fusions, with and without 1 μM ATR, as indicated. (b) cGMP production of different fungal CyclOps (as indicated), fused to YFP, was assessed in extracts prepared from oocytes expressing these proteins, that were either kept in the dark (D), or after 2 min green light (L; 532 nm, 0.15 mW mm$^{-2}$ – BeCyclOp) or 0.5 mW mm$^{-2}$ (all other CyclOps). ATR was added, unless noted. N = 2 experiments, mean of 5–6 oocytes each, error bars, s.d. (c) Light-induced cGMP production in membrane preparations from oocytes expressing various fungal CyclOps as YFP fusions (unless otherwise noted), or combinations thereof (see Table 1 for amounts of cRNAs injected). Membranes were kept in dark (D, black bars) or illuminated (L, green bars) with 532 nm light (0.5 mW mm$^{-2}$), and parallel reactions were quenched after 1 or 5 min, and cGMP measured. Note the split left y axis; logarithmic scale in lower part. On the right y axis, and shown in red, is the L/D ratio of the cGMP production rates. Average of N = 3 experiments; error bars, s.d. Statistically significant differences were determined by one-way analysis of variance: *P < 0.05; **P < 0.01; ***P < 0.001. (d) HEK293T cells were seeded on coverslips and transfected with a plasmid encoding BeCyclOp::YFP. Cells were fixed and analysed by confocal microscopy. Scale bar, 10 μm. (e) cGMP and cAMP assay of HEK293T cells transfected with BeCyclOp under different conditions. + ATR: 1 μM ATR added—ATR: no additional ATR. L, in light. D, in dark. Statistically significant differences determined by 1-way ANOVA: *P < 0.05; ***P < 0.001. n = 6, error bars, s.d.
The in vivo assay clearly demonstrated the light-activated guanylyl cyclase activity of BeCyclOp; however, due to variations of the oocytes, and as they contain phosphodiesterases (PDEs) that may degrade the newly generated cGMP, accurate quantification is difficult. We thus designed an in vitro assay based on isolated oocyte membranes. Measurements at long times after a light flash showed no decrease in cGMP, thus demonstrating a complete absence of PDE activity in our in vitro assay (Supplementary Fig. 3). To compare the different CyclOps:YFPs, we determined their expression levels in oocytes by calibrating the amount of the respective protein via its fluorescence, comparing it with a standard of purified YFP (Supplementary Fig. 4). Expression levels were essentially comparable for distinct batches of oocytes. Therefore, we could reliably determine guanylyl cyclase activities in saturating light (L) and in the dark (D), allowing to determine a L/D ratio of guanylyl cyclase activity (Fig. 1c; Table 1). At 5,000 ± 15-fold (N = 3), the L/D ratio of BeCyclOp (NO YFP) was highest. Interestingly, L/D appeared fourfold reduced for the fusion protein BeCyclOp::YFP, which showed the same high activity in the light, but a fourfold increased activity in the dark. To verify the enzyme specificity, we assayed BeCyclOp also for cAMP production in these samples; however, no obvious cAMP increase was detected (Table 1), suggesting that BeCyclOp is a highly specific guanylyl cyclase.

BeCyclOp has the highest homology to BeCyclOp, and it also showed by far the highest activity, after BeCyclOp, that is, ~28–54% of BeCyclOp cGMP production in the light (depending on the assay, N = 3), together with a slightly increased dark activity (Fig. 1c). BeCyclOp thus has a L/D ratio of 230, which is significantly less than the L/D of >1,000 for BeCyclOp or BeCyclOp::YFP. CaCyclOp is the only CyclOp for which we could measure an adenyl cyclase (AC) activity, however, it was only detectable in light and was <0.1% of guanylyl cyclase activity (N = 3; Table 1). In contrast, AmCyclOp1 showed very weak, and AmCyclOp2 showed virtually no photoactivated guanylyl cyclase activity, in either assay. AmCyclOp3 led to ~15- to 16-fold cGMP production compared with the dark (with or without addition of ATR; Fig. 1e), while for control cells (light and dark condition), all samples had a cGMP concentration of ~0.02 µM (+ ATR light: 0.024 µM; + ATR dark: 0.02 µM; – ATR light: 0.016 µM; – ATR dark 0.02 µM, all N = 6). No obvious cAMP production was found. In C. elegans extracts, from animals expressing BeCyclOp in muscle cells, likewise a ~12-fold increase in cGMP production was observed, with no significant cAMP increase (N = 6; Fig. 4c). We thus characterized the BeCyclOp protein further.

### Turnover of BeCyclOp

We measured the turnover number of BeCyclOp by determining the amount of BeCyclOp::YFP in membranes, based on the YFP fluorescence, and relating it to the amount of cGMP generated in the light (Supplementary Fig. 4). For this experiment, on average, we obtained about 1 pmol of BeCyclOp::YFP per oocyte. In vitro assays with these membranes, from 2 different batches of 20 and 21 oocytes (n = 6), yielded an average 17 ± 4 pmol cGMP per second and per oocyte-derived membrane (equivalent to 1 pmol BeCyclOp; Supplementary Fig. 4). The turnover of BeCyclOp (in light, at 20°C) is thus ~17 cGMP s⁻¹.

### Action spectrum of BeCyclOp

To obtain an action spectrum, aliquots of the BeCyclOp-containing membrane preparation were illuminated with light of different wavelengths, but equal amount

---

**Table 1 | BeCyclOp, AmCyclOps, and CaCyclOp membrane extract activity (cGMP and cAMP generation) under dark (D) and 0.5 mM Wm⁻² 532 nm green laser illumination (L).**

| Membranes from oocytes expressing (nanograms of cRNA injected) | cGMP (pmol min⁻¹) | cAMP (pmol min⁻¹) |
|---|---|---|
| **Dark** | **Green** | **L/D** | **Dark** | **Green** |
| Control oocytes (0) | NA | NA | NA | NA |
| AmCyclOp1 (28) | 0.04 ± 0.003 | 3.7 ± 0.7 | 93 | NA | NA |
| AmCyclOp2 (28) | NA | NA | NA | NA |
| AmCyclOp3 (28) | 0.7 ± 0.03 | 0.7 ± 0.03 | 1 | NA | NA |
| AmCyclOp1 + 3 (14 + 14) | 0.09 ± 0.006 | 3.3 ± 0.04 | 37 | NA | NA |
| AmCyclOp1 + 2 (14 + 14) | 0.008 ± 0.007 | 3.2 ± 0.01 | 400 | NA | NA |
| AmCyclOp2 + 3 (14 + 14) | 0.02 ± 0.001 | 0.02 ± 0.02 | 1 | NA | NA |
| AmCyclOp1 + 2 + 3 (7 + 7 + 14) | 0.01 ± 0.001 | 2.6 ± 0.2 | 260 | NA | NA |
| CaCyclOp (28) | 1.2 ± 0.1 | 280 ± 2 | 230 | NA | 0.2 ± 0.04 |

**Different oocyte batch**

| BeCyclOp no YFP (28) | 0.2 ± 0.1 | 1,000 ± 150 | 5,000 | NA | NA |
| BeCyclOp (28) | 0.8 ± 0.08 | 920 ± 200 | 1,100 | NA | NA |

AmCyclOp, guanylyl cyclase rhodopsin from Allomyces macrogynus; BeCyclOp, guanylyl cyclase rhodopsin from Blastocladiella emersonii; CaCyclOp, guanylyl cyclase rhodopsin from Catenaria anguillicida; cRNA, coding RNA; L/D, light and dark ratio; NA, no obvious activity detectable.

cRNA injection amounts as indicated. Final activity refers to the activity of membrane extract from 1 oocyte. All constructs except ‘BeCyclOp no YFP’ are with C-terminal YFP tag. N = 3 experiments; errors: s.d.
of photons, and cGMP production was quantified (N = 3; Fig. 2a). The action spectral peak was around 530 nm (green light), while 660 nm (red) and 385 nm (ultraviolet) light could still activate BeCyclOp, albeit much less efficiently. This is in good agreement with the effects of different light wavelengths in promoting phototaxis of B. emersonii zoospores\(^3\). 

**Light intensity dependence of BeCyclOp function.** Next, we measured light intensity dependence of BeCyclOp using a green laser (532 nm), near the action spectrum peak. Half-maximal activation (\(K_{0.5}\)) was observed at 0.055 mW mm\(^{-2}\) (N = 3; Fig. 2b). For some previously analysed PACs, cyclase activity continues in the dark after a light flash because of a slow photocycle. For bPAC from Beggiatoa, cAMP production levels off with a time constant \(\tau = 23 \pm 2\) s at pH 7.4 after a 4 s light flash\(^2\). The slow photocycle of bPAC is in accordance with a high light sensitivity (\(K_{0.5} = 4\) \(\mu\)W mm\(^{-2}\)). Similarly, Microcoleus mPAC (\(K_{0.5} = 6\) \(\mu\)W mm\(^{-2}\)) has a slow photocycle (\(\tau \approx 14\) s; ref. 32). The larger \(K_{0.5}\) value for BeCyclOp suggests a faster photocycle.

**Time dependence of light-activated BeCyclOp activity.** To measure the life time of the light-induced intermediate of BeCyclOp that possesses the increased guanylyl cyclase activity, we built a computer-controlled system where cyclase activity was initiated by a 20 ms laser flash (532 nm), and stopped after different time intervals (20 ms to 2.6 s) by quenching with 0.1 M HCl. Figure 2c shows that the cGMP concentration keeps increasing in the dark after the light flash with \(\tau = 320 \pm 20\) ms \((N = 3)\) at 21°C, reaching saturation after \(\sim 1.5\) s. Further measurements were extended up to 10 min (Supplementary Fig. 3); however, no ongoing increase or decrease of cGMP concentration in the dark was observed in our *in vitro* assay, thus indicating that indeed the active photointermediate of BeCyclOp is rather short lived, which makes this a well-controllable tool for optogenetics. In continuous light, BeCyclOp catalysed cGMP at a constant rate, showing no deactivation even after 20 min (Fig. 2d).

**The long cytosolic N terminus of BeCyclOp.** Based on the primary sequence and derived structural model (Supplementary Fig. 1), BeCyclOp (like the other CyclOps; Supplementary Fig. 2) has a cytoplasmic N terminus of 146 aa and a non-canonical transmembrane 0 helix, followed by the canonical seven trans-membrane helices of microbial rhodopsins. This is a striking difference to all other type I opsins studied to date. We generated a terminally truncated version of BeCyclOp by cutting the first 90 aa, using the second methionine as translational start. The truncated version (BeCyclOpS) expressed well in *Xenopus* oocytes (Fig. 3b) and exhibited potent light-activated guanylyl cyclase activity (Fig. 3c). However, compared with full-length BeCyclOp, BeCyclOpS shows higher dark activity and lower light-induced cGMP production, suggesting that the BeCyclOp N terminus is required for tight light regulation of guanylyl cyclase activity (Fig. 3c). Further indicating its cytoplasmic localization. To probe the cytoplasmic localization of the N terminus, we used Bimolecular Fluorescence Complementation (BiFC)\(^3\). To this end, we fused the C-terminal 85 aa (positions 155–239) of YFP (YC) to the N terminus of BeCyclOp and the N-terminal 155 aa of YFP (YN) to its C terminus (BeCyclOp::BiFC; Fig. 3a). Strong fluorescence was observed (Fig. 3b), indicating YC and YN reconstitution of YFP, thus proving cytoplasmic localization of both BeCyclOp termini.

---

The figure shows the action spectrum of BeCyclOp, with a peak around 530 nm, and the kinetics of cGMP production in response to different light intensities. The text explains the methods used to measure the activity and localization of BeCyclOp, highlighting its potential as a tool for optogenetics.
Figure 3 | Cytosolic localization and regulation of cyclase activity by the BeCyclOp N terminus. (a) BeCyclOp BiFC construct: aa 1–155 of YFP (YC) fused to C-terminal, and aa 155–239 of YFP (YN) fused to the N-terminal ends of BeCyclOp, respectively. (b) Oocyte membrane fluorescence images, resulting from expression of different BeCyclOp YFP/BiFC fusion constructs. BeCyclOpS refers to deletion of aa 1–90 in the N terminus. (c) cGMP production in Xenopus oocyte expressing different BeCyclOp constructs in dark (D) and after illumination (L; 532 nm, 0.2 mW mm⁻², 2 min). N = 2 experiments, mean value of six oocytes each; error bars, s.d. Statistically significant differences determined by one-way analysis of variance: **P < 0.01; ***P < 0.001.

Unlike for BeCyclOpS, guanylyl cyclase activity in the dark was not increased in BeCyclOp::BiFC and was only slightly weaker than for BeCyclOp::YFP during illumination (Fig. 3c).

Activation of a CNG channel after BeCyclOp photostimulation. We previously measured the activity of light-activated cyclases indirectly by the activation of CNG channels21,22,32. Olfactory (OLF)/T537S is a bovine CNG channel (CNGA2; ref. 34) which is highly sensitive to cAMP (K₀,₅ ≈ 14 μM) and even more to cGMP (K₀,₅ ≈ 0.7 μM). We co-expressed BeCyclOp::YFP and the OLF channel in oocytes, and used the two-electrode voltage-clamp technique to monitor light-induced cGMP production via cGMP-activated currents (Supplementary Fig. 5A). Indeed, a brief green laser flash (100 ms, 532 nm) induced ~5 μA of inward current (at −60 mV) in oocytes, 3 dpi of 0.6 ng BeCyclOp::YFP and 6 ng OLF cRNA each (Supplementary Fig. 5B). Light-induced currents became obvious already 100 ms after illumination. As shown by our in vitro assay, the photocycle of BeCyclOp is relatively fast with a decay time of 320 ms for the light-activated guanylyl cyclase activity (Fig. 2c); thus the photocurrent starts decreasing ~1 s after activation, presumably due to cGMP diffusion and PDE activity (Supplementary Fig. 5C).

BeCyclOp is a more effective GC than bPGC (BlgC) and EROS. A soluble photoactivated guanylyl cyclase (bPGC or BlgC) was generated from bPAC by three point mutations23, but only very recently (during revision of this manuscript) tested in animal cells35. To compare BeCyclOp::YFP and bPGC::YFP, we expressed them in oocytes (Supplementary Fig. 6A), and measured the cGMP concentrations obtained 3 dpi, in the dark, or after 2 min of illumination with blue light (464 nm, 10 μW mm⁻²; Supplementary Fig. 6B). These illumination conditions favoured bPGC with its action spectrum maximum in the blue and its higher light sensitivity. cGMP concentrations of bPGC-expressing oocytes, kept in dark, and those of control (that is, non-expressing) oocytes were similar. However, blue light yielded >50 times more cGMP in BeCyclOp-expressing oocytes (N = 3) than in bPGC-expressing oocytes. Further, when illuminating for 20 min, bPGC-expressing oocytes contained significantly more cAMP than controls, while BeCyclOp-expressing oocytes did not exceed the cAMP level of controls (Supplementary Fig. 6C).

Function of BeCyclOp in muscle cells of live C. elegans. Guanylyl cyclase function is abundantly found in sensory neurons in many animals. One widely used animal model in the neurosciences is C. elegans. To assess BeCyclOp’s utility in animal optogenetics, we expressed a synthetic cDNA, codon optimized for C. elegans, in body wall muscle cells (BWMs), along with an intrinsic cGMP-gated heteromeric CNG channel found in many C. elegans sensory neurons, consisting of TAX-2 and TAX-4 subunits (Fig. 4a). TAX-2/4 form an unspecific cation channel both in vitro35 and in vivo36. Activation of this channel is expected to cause muscle depolarization and contraction. As BWMs are large, they can evoke macroscopic contraction of the animal. Cultivation of animals expressing BeCyclOp/TAX-2::GFP/TAX-4::GFP in BWMs (Fig. 4b) in the presence of ATR, and 100 ms illumination with blue (450–490 nm) or green (520–550 nm) light caused longitudinal body contractions of about 9–10% of the initial length within 1.5 s (rise time constant ~0.36–0.38 s (n = 10–13 animals); Fig. 4d,e). This was comparable to effects evoked by ChR2 expression and photostimulation in the same tissue18,37,38. As a control, we raised animals without ATR. Since the chromophore is absent in C. elegans, opsin optogenetic tools are expressed but non-functional in such animals. Also for BeCyclOp/TAX-2/4, no contraction could be observed without ATR addition. Light evoked a ~12-fold increase in cGMP concentration, as determined from crude whole-animal extracts, compared with the dark condition (N = 6; Fig. 4c). No evidence for BeCyclOp-mediated cAMP generation was found (N = 4), although illuminated samples showed a trend to increased cAMP. This was independent of ATR (and thus, functional BeCyclOp), and...
may be due to a C. elegans-specific blue-light avoidance response. Contractions evoked by photoactivated BeCyclOp/TAX-2/4 (with ATR) were long lasting and recovered to a plateau of about 3% within ~30 s ($\tau_{\text{off}} \approx 8.1$ s, $n = 13$ animals; blue light); for green light, the plateau was about 6% contraction ($\tau_{\text{off}} \approx 10.8$ s, $n = 10$ animals). This indicates more pronounced
cGMP generation when BeCyclOp is stimulated at its action spectral peak (Fig. 4e). In comparison, contractions evoked by ChR2 recover within 1–1.5 s ($t_{off} \approx 0.58$ s; ref. 38). Thus, in muscle, cGMP levels may be persistently increased by BeCyclOp activation, likely due to low PDE activity. Also, the TAX-2/4 channel, while it may partially desensitize, appears to not inactivate upon prolonged cGMP increase. Light-evoked contractions were repeatable: three consecutive light pulses over 3 min evoked comparable effects (Fig. 4f). Contractions more or less reached similar values, showing that BeCyclOp can be repetitively activated. No light-induced contractions were observed when BeCyclOp alone was expressed in BWMs, that is, without the TAX-2/4 channel (Fig. 4g). We could not measure photocurrents by electrophysiology, likely due to persistent cGMP and TAX-2/4 currents following animal dissection in white light.

**Light sensitivity of BeCyclOp in *C. elegans***. We used the behavioural effects of BeCyclOp/TAX-2/4 stimulation to determine the light sensitivity in *C. elegans*. This indirect measure depends on BeCyclOp and TAX-2/4 expression levels, duration and wavelength of the light pulse, on- and off-rate of BeCyclOp’s guanylyl cyclase activity, photocycle kinetics and expression of PDEs in BWMs. Illumination for 100 ms was sufficient to evoke contractions. Shorter light pulses also had effects, but these were less robust and variable. To determine the light dose required to evoke full effects, we used 1 s, 530 nm light pulses, at intensities between 0.02–1.52 μW mm$^{-2}$ ($n = 10$ animals each; Fig. 5a). The peak contractions showed a negative linear correlation with the log of the light intensity (Fig. 5b). Illumination of animals raised without ATR (1.52 μW mm$^{-2}$) evoked no contraction. The light intensity required to evoke robust contraction is ~200-fold less than what is typically needed to evoke muscle contractions using ChR2 (refs 18,38), thus BeCyclOp appears to be much more light sensitive; however, this may be an apparently higher light sensitivity, as BeCyclOp/TAX-2/4 uses second messenger amplification, and a high-conductance channel, unlike ChR2 photocurrents that need to trigger muscle contraction directly.

**Activating BeCyclOp in O$_2$ sensors slows down locomotion**. Cell body in anterior ganglion (BAG) neurons of *C. elegans* are O$_2$/CO$_2$ sensors that detect a drop in O$_2$ as well as increases in CO$_2$ and evoke a transient slowing response$^{40–43}$. To detect O$_2$, BAG neurons use O$_2$-binding soluble receptor guanylyl cyclases, GCY-31 and GCY-33, as well as the TAX-2/4 channel. We expressed BeCyclOp in the BAG neurons using the *flp-17* promoter (Fig. 6a; ref. 42). Locomotion speed was measured using a multiworm tracker$^{44}$, equipped with a ring of blue light-emitting diodes, thus enabling to apply light of sufficient intensity to a whole-culture dish. Photostimulation of BeCyclOp in BAG caused transient slowing responses (Fig. 6b; Supplementary Movie 1). Stimuli of increasing duration (500 ms, 1, 2, 5 and 10 s; 30 s intervals; $N = 5$ (4 for no ATR) experiments; $n = 15–20$ animals each) evoked swollen responses of increasing duration, though there was always a transient minimum, following which the animals gradually increased their speed again, despite ongoing light stimulation (see also insets in Fig. 6, as well as Supplementary Fig. 7 for analysis of speed changes within 1 s before and during light stimuli). These behaviours were similarly observed for animals expressing ChR2 in BAG ($N = 3$ experiments; $n = 15–20$ animals each; Fig. 6c; ref. 42). For both optogenetic tools, stimulus protocols could be repeated, leading to similar results. In control experiments with animals raised without ATR, light stimuli >2 s evoked a transient in locomotion speed. This is attributable to the function of the *C. elegans* ultraviolet/blue-light sensor, LITE-1, which evoked escape behavior$^{39}$. Light-evoked slowing responses due to BAG activation, both in response to BeCyclOp or ChR2 activation, apparently overcame light-evoked photophobic responses. When the prolonged blue-light stimuli ended, animals showed a rebound speed increase that was more pronounced for longer stimuli; locomotion speed then fell back to initial speeds within ~20–30 s. In sum, BeCyclOp enables triggering cGMP signalling in animals, either in sensory cells using cGMP, or in other cells ectopically expressing CNG ion channels.

**Discussion**

Here we molecularly characterized a new class of type I opsins, the cyclase opsins (Cyclops) from several fungal species, for photoactivation, action spectrum and light sensitivity, after heterologous expression, in part with CNG channels. We show enzymatic specificity, high turnover, virtually non-existing dark activity and unusual topology of *Blastocladiella* BeCyclOp. Compared with other Cyclops, BeCyclOp was most effective. To assess its potential in animal optogenetics, we expressed it in *C. elegans* muscles, together with the TAX-2/4 CNG channel, as well as in oxygen sensory neurons that intrinsically use cGMP for signalling. Both cell types could be photoactivated, and macroscopic behaviours were rapidly induced and turned off, or were long-lasting, depending on the tissue. BeCyclOp thus qualifies as a new class of type I opsins.
mean speeds, as a moving average, using a sliding window bin corresponding to 1 s for filtering the data. Black bars, 30 s.

b increase in animals without ATR is due to photophobic behavior.

Figure 6 | BeCyclOp triggers O₂ sensory BAG neurons that intrinsically use cGMP signalling. (a) Confocal image of expression of BeCyclOp:SL2::mCherry bicistronic construct in the BAG neuron pair in the head of C. elegans. Cell bodies, nerve ring processes and sensory dendrites reaching the nose are visible. Outline of the animal indicated by a dashed white line, anterior is up. Green fluorescence: GFP co-expression marker localized to intestinal cell nuclei. Scale bar, 20 μm. (b) Mean absolute locomotion speed of the indicated numbers of animals expressing BeCyclOp in BAG neurons, during repeated blue light exposure (470 nm, 70 μW mm⁻²) for the indicated periods (blue bars), with 30 s interstimulus intervals, as deduced from video analysis using a multishrimp tracker. Animals in the absence (orange curves) or presence of ATR (green curves) in the culture media are compared. Speed increase in animals without ATR is due to photophobic behavior. N = 5 (4 for no ATR) experiments with n = 15–20 animals each; error bars, s.e.m. (c) As in b, but animals expressing ChR2 in BAG neurons were used instead. N = 3 experiments with n = 15–20 animals each; error bars, s.e.m. The insets show mean speeds, as a moving average, using a sliding window bin corresponding to 1 s for filtering the data. Black bars, 30 s.

a new optogenetic tool for specific cGMP manipulation in live animals.

Microbial rhodopsins attracted much attention in recent years because of their impact in optogenetics. The first type I rhodopsin described was bacteriorhodopsin12, a light-activated H⁺ pump. Bacteriorhodopsin resembles animal rhodopsins in the common seven transmembrane structure and in its Schiff-base binding of the retinal chromophore to a lysine in transmembrane helix 7. This overall structure is preserved in all subsequently discovered microbial rhodopsins (Cl⁻ pumps, the phototaxis sensory rhodopsins, the channelrhodopsins and so on), and type I rhodopsins share homology in transmembrane helices 3 and 7, and in amino acids of the retinal-binding pocket. Fungal CycOps now add a novel aspect to the diversity of type I rhodopsins by the way of their additional N-terminal transmembrane helix 0 (Supplementary Fig. 2). Based on the absent signal peptide and transmembrane helix prediction, the N terminus was expected to be cytosolic. We confirmed this by our BiFC experiment. To our knowledge, this is the first finding of an N terminus of any type I rhodopsin being intracellular. Compared with the full-length BeCyclOp, the N-terminally shortened CycOp showed higher dark activity and lower light-induced cGMP production, suggesting that the N terminus is involved in tight light regulation of the cytosolic guanylyl cyclase activity.

We show by expression in Xenopus oocytes and in muscle and nerve cells of C. elegans that BeCyclOp 'behaves' well and that its activity is under tight light control. Other fungal CycOps we tested were much less efficient, and/or less specific. The preparation of CycOp-containing membranes from oocytes enabled biophysical characterization of these proteins without interference by PDEs. The very high ratio of light versus dark activity (L/D) of 5,000 observed for BeCyclOp was reduced to 1,100 by fusing YFP to the C terminus, mainly due to a fourfold increased dark activity. Previously the highest L/D ratio (300) was reported for bPAC from Beggiatoa22.

The action spectrum of BeCyclOp is a typically broad rhodopsin spectrum with a 530 nm maximum. Though BeCyclOp is most sensitive to green light, it still responds to violet and red light; the latter may further facilitate its use in animals, deep within tissue. To avoid pre-activation, we worked under far-red light (650–720 nm) for experiments with BeCyclOp membranes, as well as with transgenic C. elegans, as activity here was indistinguishable from dark activity. The time constant for light-activation of the guanylyl cyclase is expected to be faster than 1 ms if BeCyclOp is similar to other type I opsins; in fact it is too fast to be measured by our methods. Also inactivation upon light-off (τ ~ 320 ms) is relatively fast for BeCyclOp, contrasting previously described flavoproteins and ACs bPAC and mPAC (τ ~ 20 and 10 s, respectively22,32). Provided PDEs are expressed in the cells addressed by BeCyclOp, this enables not only good optogenetic ON, but also OFF control. The faster photocycle of BeCyclOp resulted in light sensitivity lower than that of bPAC and mPAC, but even more light-sensitive mutants may be obtainable. The only PGC available until now is the mutated PAC from Beggiatoa (termed bPAC22, or BlaC23), which was first engineered by Ryu et al.23, to a PGC and named BlgC. This PGC was recently renamed EROS, and shown to mediate light-inducible penile erection in rats35. Here we mutated a human
cGMP s

bPGC (aka BlgC or EROS), we estimate a turnover of 0.2 light-activated nucleotidyl cyclase to date. For the cytosolic
/C176 assay for BeCyclOp PGC activity yielded
B cGMP in
C. elegans range for any photoactivated nucleotidyl cyclase with a very high production, and it has the highest so far reported dynamic example, in sperm swimming, erectile dysfunction, NO signalling also in other systems where cGMP signalling plays a role, for consequences of their activation. But BeCyclOp will prove useful shape the response of the respective neurons and the behavioural
C. elegans function in
possibly involving modulation of cGMP signalling, whether it is indistinguishable if this desensitization is evoked in BAG, BAG stimulation led to slowing of locomotion speed. However, BeCyclOp is highly specific for cGMP production, since we
in vitro conditions23. Our in vitro assay for BeCyclOp PGC activity yielded ~17 cGMP s−1 at 20 °C, the highest reported turnover for any characterized light-activated nucleotidyl cyclase to date. For the cytosolic bPGC (aka BlgC or EROS), we estimate a turnover of 0.2 cGMP s−1 as its activity seems 100-fold lower than that of BeCyclOp or of pBC. Summarizing the advantages of BeCyclOp, we found that it is highly active and specific for cGMP production, and it has the highest so far reported dynamic range for any photoactivated nucleotidyl cyclase with a very high ratio of light versus dark activity (L/D) of 5,000.

BeCyclOp is highly useful for animal optogenetics. It generated cGMP in
C. elegans muscle cells, which could be photo-depolarized when the TAX-2/C4 NG channel was co-expressed. This combination, due to its ability to signal through light amplification through second messenger generation, may even be used as a new optogenetic tool for efficient cellular depolarization with low light, though the need for co-expression of three genes (or two, if the OLF channel is used) makes this less versatile than ChR2. In the absence of PDEs in the respective cell type, the signal turns off slowly; thus, combination with the recently described red-light-activated PDE (LAPD) may be beneficial45.

However, BeCyclOp is most useful as an optogenetic tool in cells that intrinsically signal via cGMP, and is of particular interest for functional analysis of sensory neurons. In the
C. elegans BAG neurons that sense ambient O2 and CO2, photo-stimulation of ChR2 led to a transient decrease in locomotion speed (ref. 42, and Fig. 6c), and the same effects
photostimulation of ChR2 led to a transient decrease in oxygen (ref. 42, and Fig. 6c), and the same effects on cGMP were observed in animals in which we expressed and stimulated BeCyclOp in BAG neurons. Unlike ChR2, which merely depolarizes the BAG neurons, BeCyclOp evokes this outcome by triggering the cGMP second messenger upstream of the intrinsic CNG channel TAX-2/4. BAG neurons may use the intermediate cGMP signal also for modulation of the sensory output, or for plasticity. For both BeCyclOp and ChR2, prolonged BAG stimulation led to slowing of locomotion speed. However, the slowing was transient, despite ongoing light stimulus. This may represent desensitization or even habituation. Using ChR2, it is indistinguishable if this desensitization is evoked in BAG, possibly involving modulation of cGMP signalling, whether it is affected at BAG output synapses, or even in downstream neuronal networks that cause and execute slowing. Using BeCyclOp, we can now likely exclude that prolonged stimulation of BAG leads to modulation of intrinsic cGMP signalling and that the desensitization response must occur downstream of BAG depolarization.

BeCyclOp will permit detailed studies of sensory neuron function in
C. elegans and other animals. In C. elegans, it is particularly useful for study of environmental cues like odorants, tastes, gases or temperature, and how cGMP signalling may shape the response of the respective neurons and the behavioural consequences of their activation. But BeCyclOp will prove useful also in other systems where cGMP signalling plays a role, for example, in sperm swimming, erectile dysfunction, NO signalling and smooth muscle relaxation, or even in animal vision, where cGMP is degraded upon illumination. For the latter, BeCyclOp would have to be combined with a PDE; conversely, BeCyclOp may become an important optogenetic tool to model (that is, 'acutely' induce) retinal degeneration as observed in PDE6 mutants.

Methods

Xenopus oocyte expression. The five fungal CyclOp sequences, including BeCyclOp (= BeGC1) were synthesized by GeneArt Strings DNA Fragments (Life technologies, Thermofisher Scientific). According to their sequence20, with codon usage optimized to Mus musculus. The DNA was inserted into the oocyte expression vector pGEMHE within N-terminal BamHI and C-terminal Xhol restriction sites; YFP was attached to its C-terminal end. The CyclOp BiFC construct was made by ligating the C-terminal 85 aa of YFP (aa 153–239; Y) to the N-terminal 155 aa of YFP (N) which present at the BeCyclOp C terminus of BeCyclOp construct and shortened by a present Pkt nucleotide restriction site. The two fragments for ligation were amplified from BeCyclOp::YFP with primers BeCyclOpKpn5F (5′-CGGCGGATCCATTTGTTCTCTCCTAGCT-3′) and oJN51 (5′-TGATACATACCGCTGCACTGCTTTGATGTC-3′). This was ligated into pyc-g:8-tax:2-GFP (pBY3117 in ref. 46), digested with SpHl and Xmal (resulting in a 6,790 bp long DNA fragment) using ‘in fusion cloning’ (Clontech). For pln58 [pyro-3:tax:4-GFP], the strategy was as for pln55, but here we used 8,689 bp long fragment obtained upon digestion of pBY2974 (in ref. 46) with SpHl and Xmal used as the vector backbone. We also removed a mutation, T724A, which we found in the parent plasmid pBY2974, using the Q5 site-directed mutagenesis (New England Biolabs Inc.) kit and primers oJN53 (5′-CTACTGACACCGGGAGAACCTGA-3′) and oJN55 (5′-ATTGGTTTGTGTGGGAGAC-3′). For pln63 [pyro-3:BeCyclOp::SL2::mCherry], cDNA encoding BeGC1 (B. emersonii rhodopsin/guanylyl cyclase 1, GenBank: KF309949;1) was used to optimize expression in C. elegans using the web tool: http://worm-srv3.mpi.cbg.de/codons/cgi-bin/ optimize.py45. Three artificial introns were added to the sequence for mRNA stabilization. The resulting gene was synthesized by Eurofins Genomics (Ebersberg, Germany) in a pUC57 vector. BeCyclOp was sub-cloned into pmyo-3::BeCyclOp::SL2::mCherry vector backbone using BglII and XbaI. For pln66 [pflp-17::BeCyclOp::SL2::mCherry], the strategy was as for pJN53, but here we used 8,689 bp long fragment obtained upon digestion of pBY2974 (in ref. 46) with SpHl and Xmal used as the vector backbone. We also removed a mutation, T724A, which we found in the parent plasmid pBY2974, using the Q5 site-directed mutagenesis (New England Biolabs Inc.) kit and primers oJN53 (5′-CTACTGACACCGGGAGAACCTGA-3′) and oJN55 (5′-ATTGGTTTGTGTGGGAGAC-3′). The PCR fragment was cloned into pmyo-3::BeCyclOp::SL2::mCherry following digestion with XbaI and PstI, thus replacing pmyo-3 with pflp-17 using ‘in fusion’ cloning.

C. elegans strains. Cultivation was on nematode growth medium (NGM) in the presence of the Escherichia coli strain OP50–1 according to standard methods46. The following strains were used or generated: CX10923: kyEx2416 [pflp-17::ChR2: mCherry; un2-122::gfp]; Z1743: light-1(ce314); zdx831[pyro-3::BeCyclOp::SL2::mCherry; pyro-3:tax:2-GFP; pyro-3:tax:3-GFP] XZ1745: zeEx852[plp-17::BeCyclOp::SL2::mCherry; pelt-2::GFP-nls]; Z1746: lite-1(ce314); zeEx853[pyro-3::BeCyclOp::SL2::mCherry]. Transgenic C. elegans were obtained by microinjection of DNA into the gonads of nematodes by standard procedures47. For Z1743, 6×T10, 17 ng µl−1 pyro-3::tax:2-GFP; 6×T10, 17 ng µl−1 pyro-3::tax:3-GFP; 2×T10, 17 ng µl−1 pyro-3:tax:2-GFP; and 5 ng µl−1 pyro-3:tax:3-GFP were microinjected into lite-1(ce314) background. For Z1745/zeEx852, 1.5 ng µl−1 plp-17::BeCyclOp::SL2::mCherry was microinjected into N2 worms to obtain the transgenic line. For Z1746/ zdx831, 1.5 ng µl−1 pyro-3::BeCyclOp::SL2::mCherry was microinjected into lite-1(ce314) background.

Oocyte extract preparation. For expression in oocytes and extraction for the cyclase assay, cRNAs (~ 35 mg) from different CyclOp sequences were injected into Xenopus oocytes, which were allowed to be formed for 3 days. For six oocytes were pooled as one sample and homogenized (in dark or after illumination) in
were rinsed off the plates with M9 buffer, subsequently washed with M9 buffer
0.1 N HCl by pipetting using a 10–100
NATURE COMMUNICATIONS | DOI: 10.1038/ncomms9046 ARTICLE
Immunoassay Kit (Arbor assays). For
picked onto NGM plates supplemented with OP50-1 with or without 100
30 L4 larvae (strain ZX1746, worms expressing BeCyclOp only in BWMs) were
each. The plates were kept in a 20
Oocyte electrophysiology
were sedimented at 800
Mixtures (75 mM Tris/HCl, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol, 1 mM ATP,
2 mM GTP) for one reaction. 180 m
1. Fenno, L., Yizhar, O. & Deisseroth, K. The development and application of
Briefly, 15–20 young adults were transferred to an NGM plate and starved for 1 h.

References
1. Mao, C., Yizhar, O. & Deisseroth, K. The development and application of
Bioinformatics. Sequence alignments were done using ClustalX 2.1 (ref. 52) and
coloured with BoxShade (http://www.ch.embnet.org/software/BOX_form.html).
For the amino acid representation of type 1 rhodopsin transmembrane helix 3, a
transmembrane helix prediction was done using the TMHMM 2.0 web-based tool (http://www.cbs.
dtu.dk/services/TMHMM/)49. The snake plot of BeCyclOp was done using
Protter (http://wlab.ethz.ch/protter/start.html), utilizing the Phobius algorithm58.
Sample size was chosen so that statistical significance could be achieved within the
accuracy of the respective experiment.

C. elegans behavioural assays. Transgenic strains were kept in dark on standard
NGM plates (5.5 cm diameter, 8 ml NGM) with OP50-1 bacteria without or with 100
Gentamycin, pH 7.4). Transmission light was used. Blue light illumination was provided with a 50 W HBO lamp was channelled through excitation bandpass filters of
470 ± 20 nm (blue illumination) and 535 ± 15 nm (green illumination). Both the
and green illumination had an intensity of 0.9 mW mm
2. For the intensity
profile, 530 ± 7.5 nm (green illumination) from a monochromator (Polychrom V)
was used. Intensities were individually adjusted using the neutral density filters
(Zeiss, Germany); intensity was measured using an S120UV Sensor with PM 100
power metre (Thorlabs, Dachau, Germany). Duration of illumination was defined by a computer-controlled shutter (Sutter Instruments, USA)
using a custom written LabView script50,51. Consecutive frames were extracted from video micrographs of behaving worms for body length analysis using a custom written LabView script50,51. Measured length of individual animals was normalized by the mean length (averaged over 20 frames) before the
photostimulation and followed over hundreds of consecutive movie frames at 10 Hz.
Length chronograms of multiple worms were then averaged. Displayed are means ± s.e.m. For the speed assay, 14 stage animals were picked onto NGM agar plates supplemented with 100
M of ATR. After 10–24 h of feeding in the dark, 13–15 young adults were transferred to an NGM plate and starved for 1 h.
A multivorm tracker, which can analyse ~ 100 animals simultaneously45 was used to follow behavior by video tracking. Blue light illumination was provided with a custom-made ring of high power 470 nm blue light-emitting diodes. The transmission light source was covered with a red film that cuts off all wavelengths below 590 nm. The NGM plate was covered with a lid, on top of which a neutral density filter was placed such that the blue light intensity incident on worms was 70 µW mm
2. Speed was computed using ‘Choreography’, an additional programme for off-line analysis of different behavioural parameters43.

Bioinformatics. Sequence alignments were done using ClustalX 2.1 (ref. 52) and
coloured with BoxShade (http://www.ch.embnet.org/software/BOX_form.html).
For the amino acid representation of type 1 rhodopsin transmembrane helix 3, a
transmembrane helix prediction was done using the TMHMM 2.0 web-based tool (http://www.cbs.
dtu.dk/services/TMHMM/)49. The snake plot of BeCyclOp was done using
Protter (http://wlab.ethz.ch/protter/start.html), utilizing the Phobius algorithm58.
Sample size was chosen so that statistical significance could be achieved within the
accuracy of the respective experiment.

References
1. Mao, C., Yizhar, O. & Deisseroth, K. The development and application of
optogenetics. Annu. Rev. Neurosci. 34, 389–412 (2011).
2. Kramer, R. H., Mourot, A. & Adesnik, H. Optogenetic pharmacology for
control of native neuronal signaling proteins. Nat. Neurosci. 16, 816–823
(2013).
3. Dugue, G. P., Akemann, W. & Knopfel, T. A comprehensive concept of
optogenetics. Prog. Brain. Res. 196, 1–28 (2012).
4. Knopfel, T. et al. Toward the second generation of optogenetic tools.
J. Neurosci. 30, 14998–15004 (2010).
5. Mei, Y. & Zhang, F. Molecular tools and approaches for optogenetics. Biol.
Psychiatry 71, 1033–1038 (2012).
6. Bamann, C., Nagel, B. & Bamberg, E. Microbial rhodopsins in the spotlight.
Cell 120, 610–616 (2010).
