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Supplementary Information for

Directly light-regulated binding of RGS-LOV photoreceptors to anionic membrane phospholipids

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Supplementary text
Figs. S1 to S8
References for SI reference citations

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Supplementary Information Text

Supplementary Materials and Methods:

Unless stated otherwise: (i) water was Milli-Q water (ddH_2O, 18.2 MΩ • cm), (ii) Primers were synthesized by IDT, and (iii) genetic constructs were verified at the University of Pennsylvania's Cell Center or Genewiz by Sanger sequencing. Native-sequence and mammalian codon-optimized BcLOV4 plasmids are will be publicly available *via* Addgene.

I. GENETIC CONSTRUCTS AND PROTEIN EXPRESSION

[Bacterial genetic constructs]: Genes fragments encoding for BcLOV4 (GenBank ID CCD53251.1), *Cyphellophora europea* LOV (GenBank ID ETN36999.1), *Marsonnina brunnea* LOV (GenBank ID EKD19672.1), *Magnaporthe oryzae* LOV (GenBank ID EHA46884.1), and *Exophilia dermatitis* LOV (GenBank ID EHY60539.1) were ordered from Integrated DNA Technologies (IDT) as gBlocks® and if needed, assembled by Gibson cloning or PCR assembly. Transgenes were cloned by restriction digest into a pET21/28-derived BamUK bacterial expression vector (a kind gift from Dr. Ranganath Parthasarathy) with the high copy pUC origin of replication, kanamycin resistance, a T7/lacO promoter, and a multiple cloning site for in-frame fusion to a N-terminal $(His)_6$ tag. C-terminal mCherry fusions in BamUK were generated by Gibson cloning and feature a short, flexible $(GGGS)_2$ linker. Truncation variants were generated by PCR from the full-length template and subcloned into the mCherry-BamUK vector. All constructs were transformed into competent *E. coli* (NEB Turbo, C2984H).

Mutants were generated by laboratory methods based on Quickchange kits. Overlapping forward and reverse primers encoding the mutation of interest were designed with a melting temperature ≥78ºC. The designed mutation was introduced over 18 cycles of PCR with the high-fidelity Phusion polymerase (NEB M0531S), and template plasmid was digested with DpnI for 1 hour at 37 ºC prior to transformation into competent *E. coli* (NEB Turbo, C2984H).

[Mammalian genetic constructs]: DNA sequence of BcLOV4 was human codon-optimized the Genscript OptimumGene algorithm. The C-terminal mCherry fusion was created as described above. The mCherry-free variant with a C-terminal "3xFLAG®" tag (Sigma Aldrich) was genetically fused with a short GGGS flexible linker. Transgenes were cloned into the pcDNA3.1 mammalian expression vector under the CMV promoter (Invitrogen).

[Yeast genetic constructs]: BcLOV4-mCherry was cloned into a pRSII326 yeast expression vector with uracil auxotrophic marker (Addgene plasmid #35469, (*1*)), and transformed into *S. cerevisiae* (ATCC 201388 strain BY4741) competent cells prepared using a Zymo Research Frozen-EZ Yeast Transformation II Kit. Cells were cultured in uracil dropout medium (Sigma Aldrich).

[Recombinant protein expression, isolation, and purification] Bacterial expression plasmids were transformed into BL21(DE3) *E. coli* cells by mixing 10 ng of purified plasmid DNA into 10 uL of chemically competent cells (NEB C2527H), incubating at 4 ºC for 30 minutes, heat-shocking cells in a 42 ºC water bath for 30 seconds, placing heat-

shocked cells on ice for 2 minutes, and then incubating for 1 hour at 37 ºC in 100 uL S.O.C media. Transformed cells were grown on Luria Broth (LB) plates with 50 ug/mL kanamycin overnight at 37 ºC, and single colonies were picked and grown overnight to saturation in LB media with 50 ug/mL kanamycin. Cultures for protein production were initiated by diluting saturated overnight cultures 1:200 into fresh LB-kanamycin media in 1-2 L baffled flasks, and subsequently grown at 37 ºC with 250 rpm shaking to a mid-log phase of OD600 = 0.5-0.8. Protein production was induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG), and cells were grown for 18-22 hours at 18 ºC with 250 rpm shaking in a refrigerated incubator. Cells were then harvested in 250 mL centrifuge bottles by spinning at 3000 x *g* for 20 minutes, and subsequently frozen at -20 ºC for < 2 weeks prior to cell lysis and purification.

Frozen cells were thawed at room temperature for 5-10 minutes and then re-suspended in 50 mL ice-cold lysis buffer (50 mM sodium phosphate, 500 mM NaCl, 0.5% Triton-X-100, pH 6.5) per liter of harvested cell culture. All subsequent steps were carried out on ice or in a 4 ºC cold room. Re-suspended cells were homogenized with 3 passes through a 21-gauge syringe needle. 10 mL aliquots of lysate were each sonicated 5 times with a duty cycle of 15s ON, 30s OFF with a Fisher Scientific Series 60 Sonic Dismembranator at 100% power (60W). Individual aliquots were pooled and transferred to a 50 mL polycarbonate conical tube and clarified by centrifugation at 25,000 x *g* for 30 minutes at 4 ºC to remove insoluble fractions. The supernatant was decanted and kept at 4ºC prior to further purification.

His6-tagged proteins were affinity-purified by fast protein liquid chromatogphy (FPLC, AKTA Basic) on Ni-NTA (GE HisTrap FF) columns. All exposed sample-containing FPLC segments were covered with aluminum foil to maintain darkness. After sample loading onto a 5 mL column at 1mL/min, the column was washed with 20 mM imidazole in buffer (50 mM sodium phosphate, 500 mM NaCl, 10% glycerol, pH 6.5) for 15 column volumes, followed by a linear gradient, from 20 to 200 mM imidazole, over 15 column volumes at 5 mL/minute. Proteins were eluted with 500 mL imidazole, and collected in 10 x 2 mL fractions. Samples were pooled based on purity assessed by SDS-PAGE and concentration assessed by absorbance spectroscopy (A280), and then buffer exchanged into 1x PBS using PD-10 desalting columns with Sephadex G-25 resin (GE 17085101). Buffer exchanged material was centrifuged at 4 ºC at 25,000 x *g* for 30 minutes to pellet insoluble protein debris. Buffer exchange was repeated twice more, and the column was re-equilibrated with 1x PBS prior to each usage. Purified protein was stored for < 2 weeks at 4 ºC.

[*In vitro* refolding] BcLOV4 DUF (∆386) was tagged with His6-Gb1 and expressed in BL21(DE3) E. coli. After lysing cells with a French Pressure Cell (Avestin EmulsiFlex-C5) and centrifuging, the supernatant was discarded and the pellet was re-suspended in protein solubilization buffer (50 mM Tris-HCl (pH 8), 500 mM NaCl, 0.5% Triton-X-100, 0.5 mM DTT, 6 M Guanidine HCl, 2 mM EDTA). After denaturation at 4°C for 5-10 minutes, supernatant was added drop-by-drop to 500 mL dilution buffer (50 mM Tris-HCl (pH 8), 500 mM NaCl) over a 2 hr period to refold the protein under dilute conditions. Protein was concentrated via Amicon stirred cell and then applied to a Superdex 75 or 200 size exclusion column.

II. EUKARYOTIC CELLULAR ASSAYS

[Mammalian cell culture and transduction]: HEK293T (ATCC CRL-3216) cells were cultured in D10 media composed of Dulbecco's Modified Eagle's Medium with GlutaMAX (Invitrogen 10566016), supplemented with 10% heat-inactivated fetal bovine serum

(FBS) and Penicillin-Streptomycin at 100 U/mL. Cells were maintained in a 5% CO_2 tissue-culture treated dishes in a water-jacketed incubator (Thermo/Forma 3110) at 37ºC. Cells were seeded onto collagen-treated glass bottom dishes or into 24 plates with glass-bottom wells (Cellvis P24-1.5H-N) and transfected at ~20-30% confluence using the TransIT-293 transfection reagent (Mirus Bio MIR2700) according manufacturer instructions. Cells were imaged 24-48 hours after transfection. HEK cell lineage and mycoplasma quality control testing was performed by Biosynthesis, Inc.

[Yeast sample preparation]: Yeast strains were immobilized on agarose pads prior to imaging, as reported by others (*2*). 500 uL cells (OD ~0.5) were plated on 35 mm, poly-D-lysine-coated glass-bottom dishes. Supernatant was aspirated off after 15 minutes. Agarose was overlaid to coat the entire dish (1300 uL of 1.2% low-melt agarose in dropout media, cooled at 37°C for five minutes), and then 550 uL liquid dropout medium was placed over the agarose. Dishes were equilibrated for 10 minutes at room temperature.

[Optical microscopy]: Fluorescence microscopy was performed on an automated Leica DMI6000B fluorescence microscope under MetaMorph control, equipped with a sCMOS camera (pco.edge), an LED illuminator (Lumencor Spectra-X), and 20X, 63X and 100X objectives. Aligned excitation was filtered at the Lumencor source for mCherry imaging (λ = 575/25 nm) and GFP imaging (λ = 470/24 nm), and BcLOV4 stimulation (λ = 440/20 nm). mCherry-fused proteins were imaged with filters from Chroma (T585lpxr dichroic and ET630/75m emission filter). Camera exposure times ranged from 0.1 to 2 seconds.

For membrane association measurements, isoprenylated GFP (*3*) was co-transfected as a plasma membrane marker. After a 5 s-long blue-light pulse (15 mW/cm^2), mCherry-tagged BcLOV4 images were collected every 200 ms to monitor membrane association, and every 5 s to monitor membrane dissociation. Localization kinetics were measured for single-cells by correlation analysis of line sections.

[HEK cell fixation and immunocytochemistry]: Cells were fixed in 4% paraformaldehyde in 1x PBS for 15 minutes at room temperature. Dark-adapted cells were fixed under a dim red light, while illuminated cells were irradiated with strobed illumination (λ = 455 nm, at ≥ 15 mW/cm^2, 5s ON / 25s OFF duty cycle) from a collimated LED (Mightex) for 30 s prior to and throughout the fixation. For immunocytochemistry analysis of 3xFLAG-tagged protein, cells were seeded and grown on poly-D-lysine treated #1.5 cover glass (Cellvis P24-1.5H-N), washed three times with 1x PBS + 0.1M glycine, and then blocked with 1% BSA (Thermo Fisher Blocker BSA 37525), 2% normal goat serum and 0.4% saponin in 1x PBS for 30 minutes at room temperature. Blocked cells were incubated overnight at 4ºC with mouse Alexa 488 conjugated anti-3xFLAG monoclonal antibody (Cell Signaling Technology #5407) at 1:250 dilution in blocking buffer, or with anti-3xFLAG antibody (Cell Signaling Technology #8146) followed by an Alexa 488 conjugated secondary antibody (Cell Signaling Technology #4408). Cells were washed three times with 1x PBS prior to imaging.

[Diffusion estimates]: The intracellular diffusion constant (D_{intra}) of BcLOV4-mCherry was calculated as 8.8E-8 cm^2/s, assuming an *in vitro* dark-adapted hydrodynamic radius of 10 nm measured by DLS and an intracellular viscosity of 2.5 centipoise in mammalian cells (*4*). The timescale for diffusion to the plasma membrane was considered the time to travel a length of a cell radius (r_{cell}) in two dimensions ($t \sim r_{cell}^2 / (4D_{intra})$ (*5*), assuming a 5 – 7.5 um radius for HEK cells. The diffusion timescale in HEK cells was estimated as ~

0.7 – 1.6 sec. Diffusion timescales in yeast (estimated as ~ 0.5 – 1.0 sec) were calculated similarly assuming a 2-3 um radius and cytoplasmic viscosity of 10 centipoise (*6*).

III. *IN VITRO* PROTEIN ANALYSIS: NON-LIPID INTERACTIONS

[Absorbance spectroscopy and photocycling measurements]: Absorbance scans were measured on an Ocean optics USB2000+ spectrophotometer with a deuterium/halogen light source. Full spectrum (λ = 250-700 nm range) absorbance scans were measured in quartz cuvettes (Starna Cells 16.100F-Q-10/Z15). Photocycle kinetics were measured by monitoring the absorbance at 450 nm (A450). After baseline measurements were made for 15 seconds at room temperature, the samples were stimulated with blue-light (10 s, λ = 455 nm, 15 mW/cm^2) delivered by a collimated LED (Mightex), and then dark-state recovery was monitored every 0.5s for an additional 2 minutes.

To make solid-phase fluorescence-based photocycling measurements, 40 ng of His6-tagged protein was mixed with 0.5 mg of magnetic Ni-NTA beads (ThermoFisher HIsPur Ni-NTA resin 88221) in a total reaction volume of 400 uL in 1x PBS and nutated at room temperature for 1 hour. After three x 5 minute-long washes with PBS + 20 mM imidazole, protein-bound bead samples were re-suspended in 200 uL of 1x PBS. Fluorescence scans were measured on a Tecan Infinite M200 plate reader and kinetics were measured by monitoring the fluorescence (ex 450, em 505) for 200 uL of protein-bound beads in a 96-well plate. After baseline measurements were made for 15 seconds at room temperature, the samples were stimulated with blue-light (10 s, λ = 455 nm, 15 mW/cm^2) delivered by a collimated LED (Mightex), and then thermal reversion in the dark was monitored continuously for an additional 2 minutes.

[Protein quantification and holo/apoprotein ratio estimates]: Concentration of flavin, and consequently flavin-bound holoprotein, was determined by A450 measurements based on a molar extinction coefficient of flavin of $\varepsilon_{FMN\text{-}450}$ = 12,500 M^{-1}*cm^{-1}. To estimate protein concentration from A280 measurements, the optical density loss contributions of flavin, mCherry, and photo-aggregate scattering were subtracted. To account for the cofactor contribution, the A450-derived flavin concentration was converted to $A280_{FMN}$ based on an extinction coefficient of $\varepsilon_{FMN\text{-}280}$ = 20,300 M^{-1}cm^{-1}. The A450/A587 ratio for purified His6-mCherry control protein (~0.01) was used to account for the mCherry contribution to A450 for mCherry-fused BcLOV4 protein. Scattering contributions were accounted for by the method according to reference (*7*). The apoprotein extinction coefficient of BcLOV4 variants were calculated using the ExPASy-ProtParam tool (*8*). Post-purification flavin incorporation rates were typically >80% for wild-type BcLOV4 and the constitutively active mutant, and ~95% for the photochemically inactive mutant. Unless stated otherwise, reported concentration is for holoprotein, not total protein.

[Dynamic Light Scattering (DLS) and turbidity imaging]: Particle size analysis was performed using a Zetasizer Nano Series (Malvern Instruments, λ = 633 nm) for 5 µM protein in 1x PBS buffer. Three 10 s-long scans were averaged. After establishing baseline values in the dark, samples were illuminated by a collimated LED (Mightex, 5 s, λ = 455 nm, 15 mW/cm^2), and then returned to the dark DLS chamber for post-illumination measurements for up to 60 minutes. Macroscale turbidity images were taken with a Canon G12 camera.

[Thin-Layer Chromatography (TLC) / Cofactor identification]: Protein (10 nmol) was boiled in 70% ethanol for 2 min, chilled on ice for 2 min, and then centrifuged at 16,000 *x* g for 10 min. (according to reference (*9*)). Standards were dissolved in water at 1 µM (FMN, Sigma F6750; FAD, Sigma F6625; Riboflavin, Sigma R4500). Thin-layer chromatography was performed on glass silica gel plates (Sigma Z292974) using standard methods with *n*-butanol : acetic acid : water (3:1:1 vol:vol). Plates were dried and imaged on a UV transilluminator. R_f values: BcLOV4 cofactor (0.26), FMN (0.26), FAD (0.14), Riboflavin (0.61).

[Size-Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)]: FPLC-purified BcLOV4 was buffer-exchanged into a SEC-MALS-compatible buffer (50 mM sodium phosphate, 500 mM sodium chloride) using PD-10 desalting columns with Sephadex G-25 resin (GE17085101). The protein was analyzed by SEC-MALS using an in-line HPLC (Agilent Technologies 1200), and MALS system (Wyatt DAWN HELEOS II and OPTILAB T-rEX). SEC purified protein was loaded onto a Superdex 75 agarose and dextran-based column (GE) in 50 mM sodium phosphate and 500 mM sodium chloride. A 100 µL sample of BcLOV4 at 0.4 mg/mL was injected at a flowrate of 0.5 mL/min, over a total 53 minute-long profile. Data was collected and analyzed using Astra chromatography software (Wyatt).

[Denaturing gel analysis and Western blots]: Protein samples in 1x NuPAGE® lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific, NP0007) were heated to 70 ºC for 10 minutes and loaded on 4-12% Bis-Tris NuPAGE® SDS-PAGE gels, along with Mark12 unstained standard (ThermoFisher Scientific, LC5677). Gels were run in 1x MOPS running buffer for 45 minutes at 200V. Gels were visualized by staining with InstantBlue Coomassie stain (Expedeon ISB1L) and imaging on a digital scanner. For Western blotting, proteins were run with MagicMark XP Western Protein Standard (ThermoFisher Scientific, LC5602), transferred to PVDF membranes (ThermoFisher Scientific, LC2005) at 30V for 1 hour in 1x NuPAGE® transfer buffer (ThermoFisher Scientific, NP0006), probed with mouse monoclonal antibodies to the antigen of interest, and then probed with IRDye 680RD Goat anti-Mouse IgG (Li-Cor 925-68070). Western blots were imaged on an Odyssey CLx Infrared Imaging System (Li-Cor Biosciences, Model 9140).

IV. *IN VITRO* PROTEIN ANALYSIS: PROTEIN-LIPID INTERACTIONS

[Surface Plasmon Resonance (SPR)]: SPR measurements were performed using a Biacore T200 (GE Healthcare) instrument at 25 °C. Small unilamellar vesicles (SUV) were generated by initially hydrating 1.5 mM phospholipids (phosphatidylcholine, Aldrich P3556 and Avanti 840051C; 18:1 (Δ9-Cis) phosphatidylcholine (DOPC), Avanti 850375; phosphatidylserine, Aldrich P7769 and Avanti 840032C; 18:1 phosphatidylserine (DOPS), Avanti 840035; phosphatidylglycerol Aldrich, P8318; phosphatidic acid, Aldrich P9511; cardiolipin, Aldrich C0563; PIP2, Cell Signals, #902; PIP3, Cell Signals, #908) of a given composition in HBS-N buffer (25 mM HEPES, 150 mM NaCl, pH 7.4), then sonication followed by 8 freeze/thaw cycles in a dry ice/ethanol bath, and finally 15 passes through an Avanti Lipid extruder with a 0.05 um membrane. SUVs were immobilized by flowing them over the surface of a carboxymethylated dextran chip with covalently attached lipophilic groups (Sensor chip L1) at 2 uL/min for 30 min. Typically, 4000-10000 RU (resonance units) of liposomes were immobilized. His6-BcLOV4-mCherry variants were buffer exchanged three times into HBS-N and passed over the chip surface at a flow rate of 30 uL/min for 10 minutes. The chip was regenerated after

each binding experiment by the injection of 100 mM NaOH at 50 uL/min for 1 min. SUV coated chips were used for a maximum of ~12 hours before they were stripped with 40 mM octyl-B-glucoside and 0.5% SDS and re-coated. Steady-state equilibrium binding values were fit and analyzed with the curve fitting toolbox in MATLAB.

[Water-in-oil emulsions]: Lipids (see SPR methods for chemical sources) were re-suspended in chloroform in a glass test tube or round-bottom flask. Chloroform was evaporated under a stream of nitrogen and the remaining lipid film was dissolved into decane (Aldrich D901) at 2.5-25 mg/mL, based on the solubility of each individual phospholipid. To facilitate suspension in decane and to remove excess chlorofrom, solutions were heated at 50 ºC for 3 hours and sonicated in a water bath for 30 minutes. Lipids were stored in glass vials with teflon caps (Thomas Scientific 1234R80) at -20 ºC. In experiments, 30 uL of 20 mM lipids (total molarity) was mixed vigorously with 1.28 uL of purified mCherry-tagged protein in PBS by pipetting up and down until a cloudy suspension formed. 20 uL of the water-oil emulsion was transferred to microwells and imaged at 20x (see Optical Microscopy for hardware description).

Automated MATLAB scripts were used to identify droplets, segment either just the outer ring interface or the aqueous dispersed phase and to calculate integrated fluorescence intensity and area over these regions. The ratio of membrane inner leaflet-emulating interface fluorescence intensity per unit area to cytoplasm-emulating dispersed phase fluorescence intensity per unit area was considered a measure of membrane-binding, and normalized to the max ratio for illuminated wildtype protein.

[Protein-lipid overlay assay]: Phospholipids at 3 mM in 1:1 chloroform:methanol (with 0.1% HCl) were spotted (1 uL) onto 0.2 um pore size nitrocellulose blotting membranes (ThermoFisher Scientific, LC2000). After drying for 1 hour at room temperature, blots were either stored at 4 ºC, or blocked for 1-4 hours in PBS containing 3% BSA (no detergent). 0.5 µM of His6-tagged BcLOV4 variants were then added to PBS / 3% BSA (GST-tagged positive control at 23.6 nM) and this solution was incubated with a BSA-blocked, lipid-spotted nitrocellulose membrane for 1 hour at room temperature. Membranes were washed 4 times with PBS-T (PBS + 0.1% Tween20) and then probed with mouse anti-His6 primary antibody at 1:2000 dilution (Cell Signaling Technology, 2366) in PBS + 3% BSA for either 1 hour at room temperature or overnight at 4 °C. After an additional 4 washes with PBS-T, membranes were probed with IRDye 680RD Goat (polyclonal) Anti-Mouse IgG (Licor 925-68070) at 1:15000 dilution in PBS-T + 3% BSA for 1 hour at room temperature. Blots were washed an additional 4 times in PBS-T and then in 1x PBS. Blots were imaged on an Odyssey Infrared Imaging System in the λ = 700 nm channel at Intensity 5.

V. BIOINFORMATICS

[Maximum-likelihood phylogenetic tree construction]: The tree was constructed for all candidate sequences by aligning sequences with MUSCLE, building a phylogenetic tree with PhyML and rendering a tree with TreeDyn, using all default settings in an automated “one-click” mode pipeline through the phylogeny.fr webserver (http://www.phylogeny.fr) (*10*). Taxonomic class assignments were made with the Interactive Tree of Life (iTOL) server (http://itol.embl.de) (*11*).

[Secondary structure modeling and consensus annotation]: Candidate amino acid sequences were submitted individually to 4 secondary-structural prediction algorithms: iTASSER (*12*), Jpred (*13*), Phyre (*14*), and PSIPRED (*15*). A consensus secondary-

structure prediction was generated by equally weighting alpha-helix and beta-sheet predictions from the 4 algorithms at every amino acid residue and requiring 2 of 4 programs to agree on any given structural element. Amphipathic helices were predicted with the HeliQuest webserver using all default settings (*16*).

[*De novo* energy minimization modeling in Rosetta]: *De novo* structural predictions were made with Rosetta v 3.8 on 100 Intel E5-2665 2.4Ghz Xeon Processors using the Abinitio Relax protocol (*17*). The consensus secondary structure prediction was used throughout the process to filter out trajectories that were unlikely to converge to the supplied secondary structure. Near-native topologies were identified by determining the most frequently sampled conformations using clustering with RMSD as the distance metric. The lowest energy trajectory of the largest cluster was hypothesized to be the closest approximation of the native structure.

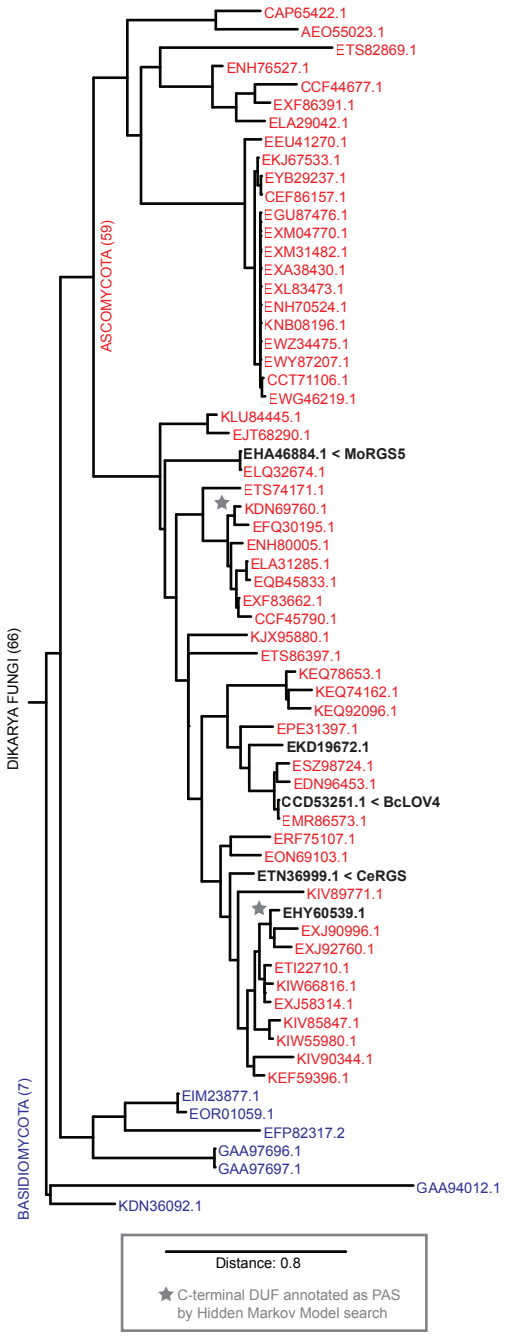


Fig. S1. Maximum likelihood phylogenetic tree constructed by aligning 66 full-length fungal RGS-LOVs. The tree is overlaid with taxonomic distinctions at the phylum level. Tree was rendered via the Phylogeny.fr automated pipeline. Black = variants tested for solubility based on literature precedent (BcLOV4, MoRGS5) or homology to BcLOV4 with short length. Numbers refer to GenBank IDs.

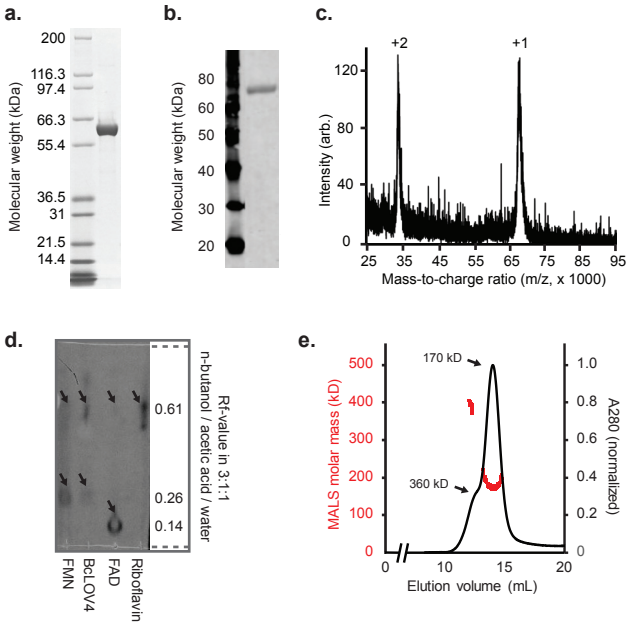


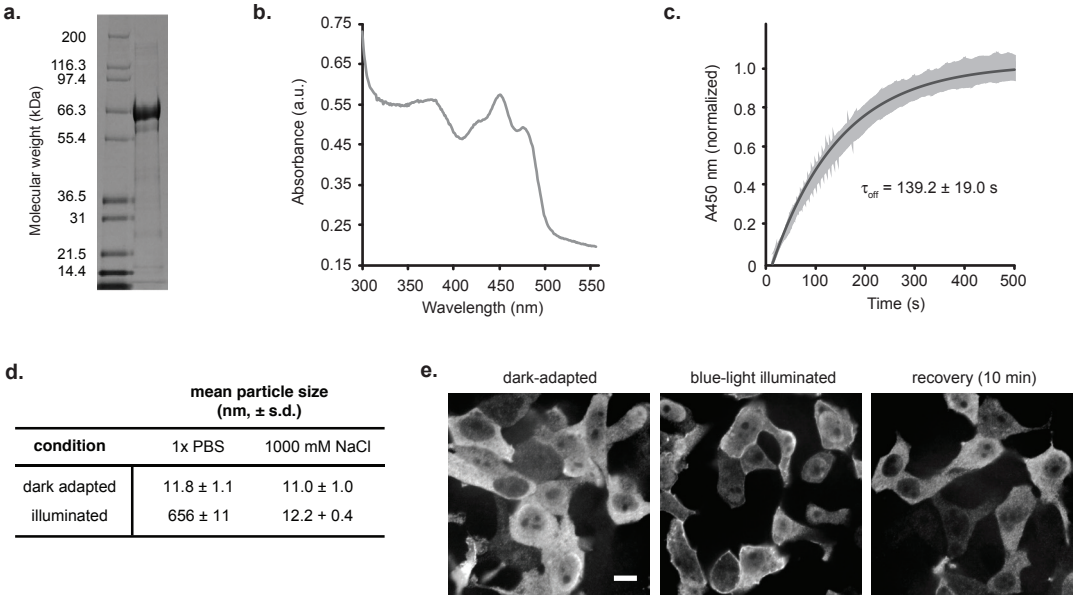
Fig. S2. BcLOV4 dark-adapted holoprotein characterization. (a) BcLOV4 purity analysis by SDS-PAGE denaturing gel (left: Mark 12 ladder, right: protein). The protein runs fast with respect to the ladder. **(b)** Molecular weight characterization by Western blot of His6-BcLOV4. Calculated molecular weight = 67 kDa including tag (left: Magic Mark XP ladder, right: protein). **(c)** Molecular weight characterization by MALDI mass spectrometry (Measured MW for +1 state = 67,587 Da). **(d)** Cofactor identification. Silica-plate TLC of pure references (FMN = flavin mononucleotide, FAD = flavin adenine dinucleotide, and Riboflavin) and cofactor extracted from BcLOV4 in 3:1:1 n-butanol:acetic acid:water co-solvent. Arrows are visualization guides for the spots. BcLOV4 binds FMN. All lanes show riboflavin impurities or breakdown products. **(e)** SEC-MALS analysis of FPLC-purified His6-tagged BcLOV4 is a dark-adapted oligomer (dimer/trimer mixture).

Protein	Purification source	Description of condition	Spectroscopy	Tau (s)
His6_BcLOV4	E Coli	In solution, in 1M NaCl + 10% glycerol	Absorbance	18.5 ± 2.0
His6_BcLOV4	E Coli	In solution, in 1M NaCl	Absorbance	20.4 ± 1.8
His6_BcLOV4_AH1mut	E Coli	In solution, in 1M NaCl	Absorbance	31.4 ± 16.4
His6_BcLOV4_mCherry	E Coli	In solution, in 1M NaCl	Absorbance	17.12 ± 0.7
His6_BcLOV4	E Coli	Solid-phase, in PBS	Fluorescence	47.4 ± 15.6
EL222	E Coli	In solution, in PBS	Absorbance	27.3 ± 5.3
EL222	E Coli	In solution, in PBS	Fluorescence	29.6 ± 3.4
EL222	E Coli	Solid-phase, in PBS	Fluorescence	51.1 ± 3.8

Fig. S3. Table of photocycling kinetics. Absorbance = Derived from A(450). Fluorescence = Derived from λem = 505 nm (λex = 450 nm). Solid-phase = Derived from His6-tagged protein immobilized on Ni-NTA beads. Blue light stimulation: λ = 455 nm @ 15 mW/cm2 for 5 seconds.

	mean particle size (nm, ± s.e.m.)		
condition	250 mM NaCl	500 mM NaCl	1000 mM NaCl
dark adapted	9.2 ± 1.7	9.2 ± 2.1	8.3 ± 1.8
illuminated	1059 ± 296	388 ± 106	8.7 ± 1.7
recovery in dark (5 min)	1016 ± 243	8.9 ± 1.7	9.7 ± 1.5
recovery in dark (10 min)	946 ± 308	10.4 ± 1.4	8.9 ± 2.1

Fig. S4. In vitro BcLOV4 photo-aggregation by dynamic light-scattering (DLS). The phenomenon is reversible in the dark at high-salinity, which also limits aggregate size. Data at 1000mM NaCl also shown in main text Figure 1.



	mean particle size (nm, ± s.d.)	
condition	1x PBS	1000 mM NaCl
dark adapted	11.8 ± 1.1	11.0 ± 1.0
illuminated	656 ± 11	12.2 + 0.4

Fig. S5. Characterization of CeRGS from Cyphellophora europaea in vitro and in mammalian cells. CeRGS recapitulates the in vitro photo-aggregation and light-dependent membrane localization observed with BcLOV4, confirming the generality of the phenomenon amongst RGS-LOV proteins. **(a)** SDS-PAGE denaturing gel of purified CeRGS (left: Mark 12 ladder, right: protein). **(b)** UV-Vis spectrum. **(c)** Recovery kinetics of protein stabilized by 1M NaCl and 10% glycerol to prevent photo-induced aggregation, monitored at λ = 450 nm absorbance (A450). Black = Exponential fit. Gray = Mean ± s.d. (N = 3). **(d)** Dynamic light scattering data with and without electrostatic stabilization. Illuminated = Immediately after 5 second illumination (15 mW/cm2, λ = 455 nm) (N = 2-3). **(e)** Spinning disk confocal images of HEK cells expressing 3xFLAG-tagged CeRGS, probed with Anti-FLAG M2 monoclonal antibody and stained with Anti-mouse IgG Alexa 488 conjugated secondary antibody. Scale bar = 10 um. Images are from separate fixed samples.

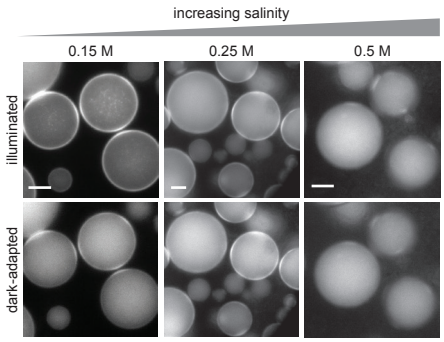


Fig. S6. Electrostatic shielding of light-induced membrane localization. Increasing salinity in the dispersed or aqueous phase of the lipid-stabilized water-in-oil emulsions reduces BcLOV4 binding to 20% phosphatidylserine / 80% phosphatidylcholine interfaces in blue light. Scale bar = 25 um. Illumination = 15 mW/cm2, λ = 440/20 nm.

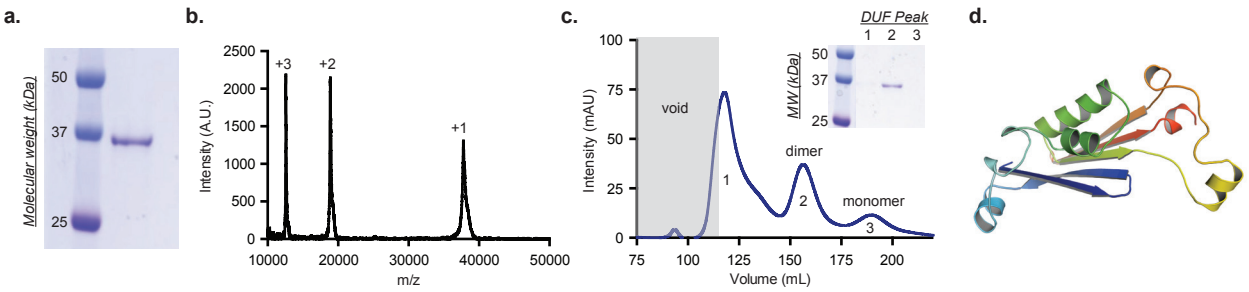


Fig. S7. Isolation and characterization of BcLOV4 DUF proteins. (a) SDS-PAGE gel of bacterially expressed, refolded His6-GB1-DUF (predicted MW = 37.8 kDa). **(b)** Thin-Layer MALDI-TOF mass spectrometry, charge states listed for each peak (His6-GB1-DUF; measured MW = 37,764 Da for +1 state vs. predicted MW = 37,749 Da). **(c)** Superdex S75 size exclusion chromatography shows the individual DUF is a mix of dimer and monomeric states (His6-GB1-DUF). **(d)** PYMOL rendering of Rosetta de novo energy minimization model of the DUF suggests it adopts a mixed α/β-fold consistent with CD spectroscopy, and possibly adopts a PAS-like fold.

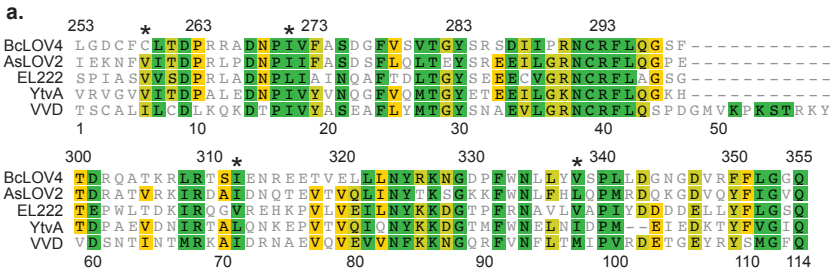


Fig. S8. Kinetic tuning of BcLOV4 membrane dissociation. (a) Sequence alignment of BcLOV4, AsLOV2 from Avena sativa, EL222 from Erythobacter litoralis HTCC2594, YtvA from Bacillus subtilis, and VVD from Neurospora crassa LOV domains (top number = BcLOV4 residue), with asterisks denoting mutated residues in this study. **(b)** Table of relative membrane dissociation times of mCherry-tagged mutants screened in HEK cells in parallel, using time constants determined from relative fraction of membrane-bound BcLOV4 variants from cellular line sections. C258I had the most persistent inducible localization. **(c)** Example fluorescence micrographs of mCherry-tagged wildtype protein and C258I mutant. Scale bar = 10 um. **(d)** Time constant for the C258I mutant determined from relative fraction of membrane-bound BcLOV4 variants from cellular line sections over extended imaging time. 95% CI = 490.2 – 853.2 s. **(e)** Photocycling of purified bacteriallly expressed C258I in 1M NaCl + 10% glycerol is also lengthened vs. wildtype.

Supplementary References: *[Numbering reset from Main]*

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