Supplementary Materials for

Optochemical Control of Protein Localization and Activity Within Cell-Like Compartments

Reese M. Caldwell[1,ϕ], Jessica G. Bermudez[2,ϕ], David Thai[1], Chanat Aonbangkhen[3], Benjamin S. Schuster[2], Taylor Courtney[4], Alexander Deiters[4], Daniel A. Hammer[2], David M. Chenoweth[3] and Matthew C. Good[1,2*]

[1]Department of Cell and Developmental Biology, [2]Department of Bioengineering, [3]Department of Chemistry, University of Pennsylvania, PA 19104 USA. [4]Department of Chemistry, University of Pittsburgh, PA 15260

[ϕ] *Authors contributed equally to this work*

* *Corresponding Author: mattgood@pennmedicine.upenn.edu*

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SUPPLEMENAL METHODS

Molecular Cloning

Fusion constructs were cloned into bacterial expression vectors (Table 1). pETARA contains an N-terminal glutathione-S-transferase (GST) fusion tag and TEV cleavable linker, pRSETa was modified to contain an N-terminal 10-histidine, pMal contains an N-terminal maltose-binding protein (MBP) tag, and pETARm is a pET vector modified with an N-terminal MBP tag. Constructs generated for pulldown and localization experiments include His10-RFP-Haloenzyme, MBP-GFP-DHFR, and GST-GFP-DHFR; for split sfGFP reconstitution: His10-sfGFP.Strand10-Haloenzyme, GST-DHFR-sfGFP.Strand11, GST-sfSFGFP.Strands1-9, and GST-sfGFP; for split TEV reconstitution: GST-FKBP-TEV-C and MBP-FRB-TEV-N; for membraneless organelle formation: MBP-GFP-IDP. All constructs will be available on Addgene (Table S1).

Haloenzyme and dihydrofolate reductase (DHFR) fragments were amplified from pERB-254 and pERB-264 plasmids gifted by the Lampson lab (University of Pennsylvania). Split GFP fragments were amplified from a Gblock (Integrated DNA technologies) into fragments, as described previously[1]. Split GFP fusion proteins were designed with a 15-mer linker (GGGGSGGGGSGGAAA) between sfGFP strand 10 and Haloenzyme, and long 25–mer linker (GGGGGGTCAGAAGGTGGTGGTAGTG) between DHFR andsfGFP strand 11. Split TEV fragments were amplified from a TEV protease expression vector. TEV-N contains residues 1-118 of TEV protease, and TEV-C contains residues 119-236 followed by a polyarginine tag. FRB-TEV-N and FKBP-TEV-C constructs were designed with 10-mer linkers (GGGGSGGGGS)

between TEV and FRB or FKBP domains. FRB and FKBP domains were amplified from FRB-WASP and FKBP-Cdc42 (gifted by Michael Rosen, UT-Southwestern). The IDP, aa 1-168 of Laf-1, was cloned into a pET vector, with a TEV-cleavable MBP solubilization domain and GFP coupled to the IDP for fluorescent imaging.

Constructs were assembled through Phusion PCR (NEB) amplification and subsequent ligation-based (NEB) or InFusion cloning (Clontech). Constructs were cloned into XL1-Blue strains, selected by carbenicillin resistance, and verified by sequencing.

Protein Expression and Purification

Plasmid constructs were transformed into *E. coli* Rosetta 2 cells (Novagen) for protein expression. Cultures were grown in LB with carbenicillin and chloramphenicol at 37 °C to an OD_{600} of 0.4, then temperature shifted to 16 °C for 20 min, and induced using 0.5 mM IPTG at an OD_{600} between 0.5-0.7. Cultures were grown overnight at 16 °C, and cells were resuspended in lysis buffer (150 mM NaCl, 25 mM Tris-pH 7.8, 5% glycerol). Cells were lysed with three cycles of sonication and freeze-thaw, and clarified by centrifugation. The supernatants for His10-RFP-Haloenzyme and His10-sfGFP.Strand10-Haloenzyme were incubated with Ni-NTA agarose superflow resin (Qiagen) for 1 hr at 4°C. After extensive washing, proteins were eluted in 250 mM imidazole buffer (150 mM NaCl,25 mM Tris, 250 mM imidazole pH 8, 10% glycerol). Supernatants containing GST-GFP-DHFR, GST-DHFR-sfGFP.Strand11, GST-sfGFP.Strands1-9, GST-sfGFP, and GST-FKBP-TEV-C proteins were bound to glutathione agarose resin (Pierce). Proteins were eluted using glutathione buffer (15 mM reduced glutathione, 25 mM Tris-pH 7.8, 150 mM NaCl, 10% glycerol). Supernatants containing MBP-GFP-DHFR, MBP-FRB-TEV-N, and MBP-fluorescent IDP were bound to amylose agarose resin (NEB) and eluted using maltose buffer (10 mM maltose, 25 mM Tris pH 7.8, 150 mM NaCl, 5% glycerol). All proteins were then dialyzed overnight into a standard protein buffer (150 mM NaCl, 25 mM Tris-pH 7.8, 10% glycerol), followed by the addition of 2 mM tris(2-carboxyethyl)phosphine (TCEP) and storage at -80°C. GST-FKBP-TEV-C was further purified and buffer-exchanged by size exclusion chromatography (Superdex 75 10/300 GL) into final 'size exclusion buffer' (25 mM Tris pH 7.8, 300 mM NaCl, 10% glycerol) before addition of TCEP and storage at -80°C. All proteins were left with intact purification domains.

For uncaging experiments, His10-RFP-Haloenzyme and His10-sfGFP.Strand10-Haloenzyme were pre-bound to 3X caged TMP-Haloligand (CTH) or non-caged TMP-Haloligand (TH). After incubation for 30 minutes on ice, excess CTH and TH dimerizer was removed using a PD10 desalting column (GE) or Amicon Ultra centrifugal filters (Sigma). CTH (Fig. S3D) and TH dimerizing compounds were synthesized as described previously[2].

Concentrations of purified proteins were measured using a Bradford assay. Purified proteins were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with coomassie before imaging.

Biochemical Assays

For pulldown assays, a saturating amount of MBP-GFP-DHFR or GST-GFP-DHFR (bait protein) was bound to 15 µl amylose or glutathione agarose resin. Excess bait protein was washed off beads using 1 ml of 'binding buffer' (100 mM NaCl, 25 mM Tris pH 8, 0.05% IGEPAL, 2 mM TCEP). Prey proteins tested included apo form of RFP-Haloenzyme, and RFP-Haloenzyme prebound to either non-caged TMP-Haloligand, or photocaged TMP-Haloligand (CTH). The RFP-Halo proteins,were placed in an imaging gasket and either left in the dark or exposed to

one of three light conditions (100 seconds of 405 nm laser light on a microscope, 5 minutes of 365 nM light from UV ballast, or 5 minutes of 365 nM light from LED on a microscope). 100 µl of 0.5 mg/ml of prey protein was bound to protein-coated beads at room temperature for 15 minutes. Unbound prey protein was washed away with 3 consecutive wash/spin cycles of 'GST wash buffer' (150 mM NaCl, 25 mM Tris pH 8. 10% glycerol, 2 mM TCEP). Proteins bound to beads were eluted using SDS-PAGE 1X loading buffer (diluted from 4X NuPage LDS Sample Buffer) and 2 min of heating at 95 °C. Samples were run on an SDS-PAGE gel and stained with coomassie before imaging at 700 nm on an Odyssey gel scanner (Li-Cor). Gels were quantified using ImageJ.

Cell-like compartments for localization experiments were generated by pipetting 1 µl of an aqueous phase into 70 µl of a continuous lipid and oil phase followed by repeated pipetting up and down to create water-in-oil emulsions. Droplets were placed in custom gaskets made of acrylic and glass for imaging. For localization of GFP-DHFR, 1 µM of His10-RFP-Haloenzyme and 0.1 µM GFP-DHFR were diluted in standard buffer and encapsulated with a lipid monolayer composed of 95% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 5% 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA(Ni)). Lipids were dissolved in decane oil at 25 mg/ml.

Bulk assays to measure activity for split GFP were carried out on a Tecan infinite 200Pro plate reader running Tecan i-control software. Wells contained 3 µM of DHFR-sfGFP.Strand11, 3 µM of sfGFP.Strand 10-Haloenzyme, and 24 µM sfGFPStrands 1-9 in 'high salt buffer' (500 mM NaCl, 50 mM Tris-pH 7.8, 10% glycerol). sfGFP.Strand10-Haloenzyme was either unbound, prebound to TH, or prebound to CTH. Split sfGFP samples, in a volume of 100 uL, were loaded into black 96-well half-area plates and measured with excitation 485 and emission 510 using gain 70 upon mixing and after 12 hours at room temperature. The difference in fluorescence of high salt buffer alone (negative control) was subtracted from the difference in fluorescence of split sfGFP samples between mixing and after 12 hours. Then, these buffer-subtracted change-in-fluorescence values were normalized to concentration of sfGFP by interpolation onto a standard curve generated from a dilution series of intact GST-sfGFP after 12 hour incubation (Fig. S3C).

Cell-like compartments for split sfGFP reconstitution were generated by pipetting 1 µL of the described sfGFP samples (3 µM of DHFR-sfGFP.Strand11, 3 µM of sfGFP.Strand 10-Haloenzyme, and 24 µM sfGFPStrands 1-9 in high salt buffer) into 70 µL of 25 mg/mL Cithrol-DPHS (Croda) dissolved in squalene (Sigma). Compartments were imaged 18 hours post-induction to allow chromophore maturation.

TEV protease assays used Abcam's TEV Protease Activity Assay Kit (Fluorimetric) (ab211109). Assays were run according to the manufacturer's instructions, with the exception of diluting proteins into standard buffer (150mM NaCl, 25mM Tris-pH 7.8, 10% glycerol) in place of buffer from the kit. Proteins were mixed at 2X in standard buffer, then diluted to a final concentration of 125 nM (for bulk assays) or 500 nM (for emulsions assays) in substrate mix (according to manufacturer instructions), with a final amount of 5 mM dithiothreitol (DTT). The assay functions through TEV cleavage of a peptide linker between a fluorophore and quencher, such that TEV cleavage leads to increased fluorescence of the sensor compound. Split TEV was mixed with 125 nM rapamycin (for bulk experiments), 500 nM rapamycin (for emulsion experiments), 73 nM dRap (for bulk experiments), 250 nM dRap (for emulsion experiments), or an equivalent volume of DMSO (Sigma). Rapamycin (Alfa Aesar, 99% pure) and dRap, synthesized as described

previously[3] (Fig. S4D), were dissolved in DMSO. For bulk experiments, 100 µL samples were loaded into white 96-well half-area plates and measured with excitation 490 nm and emission 560 nm using gain 48 on a Tecan infinite 200Pro plate reader running Tecan i-control software. Split TEV assays in bulk were run for 20 minutes at 37°C; activity represents the change in fluorescence during this period. A well containing substrate alone but not protein was used to subtract background fluorescence shifts.

Cell-like compartments for split TEV reconstitution were generated by pipetting 1 µL of the described split TEV samples (containing 500 nM FRB-TEV-N, 500 nM FKBP-TEV-C, and 500 nM rapamycin or 250 equivalent dRap with substrate and DTT) into 70 µL of 25 mg/mL Cithrol-DPHS) dissolved in squalene. Images were taken 20 and 80 minutes post-induction at room temperature.

For IDP experiments, cell-like compartments were generated by pipetting 1 µL of sample (containing 1 µM each split TEV protein, equimolar small molecule, 5 mM DTT, and 25% *Xenopus* egg extract) into 70 µL of 25 mg/mL Cithrol-DPHS in squalene. Egg extract was prepared fresh, as described previously[4]. Compartments were imaged 12 hours post-induction, however smaller protein droplets were visible sooner; droplet fusion is the rate limiting step towards formation of large membraneless organelles.

Microscopy, Illumination, and Image Analysis

For GFP-DHFR localization, water-in-oil emulsions were imaged immediately following photoactivation. Split TEV emulsions were imaged after 20 minutes and 80 minutes to measure the change in substrate fluorescence over time. For split sfGFP experiments, encapsulated proteins were imaged 18 hours after photoactivation. All water-in-oil emulsions were imaged in brightfield and using 488 nm laser illumination on an inverted confocal microscope (Olympus IX81) containing a spinning disk head (Yogogawa X1). Images were acquired using an EM-CCD (Andor iXon3) camera and MetaMorph acquisition software.

For GFP-DHFR localization and split sfGFP reconstitution in water-in-oil emulsions, CTH was uncaged using 1 to 100 seconds of 405 nm laser illumination at 100X magnification on a spinning disk confocal microscope. Split TEV proteins were photoactivated for 5 minutes (in bulk experiments) and 10 minutes (in emulsion experiments) using a UV lamp (Spectroline XX15A - 15W, 365 nm).

We used ImageJ to analyze fluorescent images of water-in-oil emulsions. For split sfGFP and split TEV experiments carried out in water-in-oil emulsions, we quantified multiple sets of images, $n > 20$. For light-inducible split sfGFP reconstitution, we first performed background subtraction to eliminate auto-fluorescence of the surrounding oil from our calculations; we subtracted average fluorescence intensity of the continuous oil phase from average fluorescence intensity inside the droplets. Endpoint fluorescence resulting from light-triggered split sfGFP reconstitution was measured and normalized to endpoint fluorescence achieved in positive control condition (TH) after 18 hours. For split TEV experiments, increase in sensor fluorescence values over a time range of 20-80 minutes were calculated for each experimental condition. For photoactivation of TEV, the increase in sensor fluorescence was normalized to the increase in fluorescence achieved from a positive control condition (+ rapamycin).

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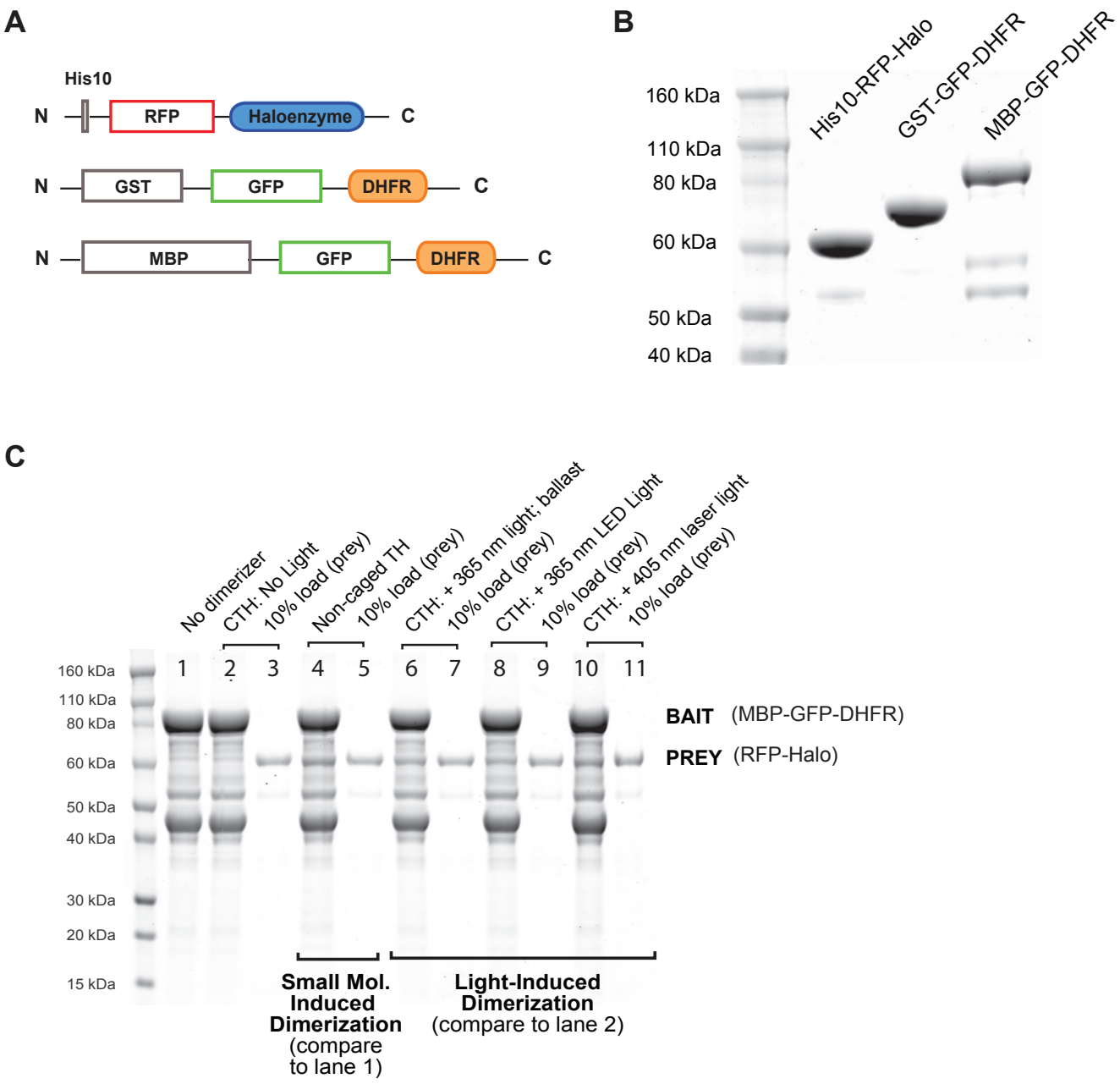

Figure S1. Components for light-inducible dimerization and boundary localization within cell-like compartments.(A) Schematic of fusion constructs. (B) SDS-PAGE gel of His10-RFP-Halo (expected MW: 64.6 kDa), GST-GFP-DHFR (expected MW: 74.4 kDa), and MBP-GFP-DHFR (expected MW: 91.1 kDa) proteins. (C) Coomassie-stained SDS-PAGE gel for pulldown assay using amylose beads to bind bait. MBP-GFP-DHFR ('Bait') and His10-RFP-Halo ('Prey') do not interact in the absence of dimerizer (lane 1) but bind to one another in the presence of non-caged TMP-Halo (TH) dimerizer (lane 4). 10% load is shown for each prey protein, which were separately loaded with TH or CTH and exposed to dark or light. When prey is bound to caged TMP-Halo (CTH) compound in the dark, it does not interact (lane 2). Upon illumination prey protein binds to bait. Light uncaging with 100 seconds of 405 nm laser light on a microscope (lane 10), or 5 minutes of 365 nM light from UV ballast (lane 6) or 5 minutes of 365 nM light from LED on a microscope (lane 8).

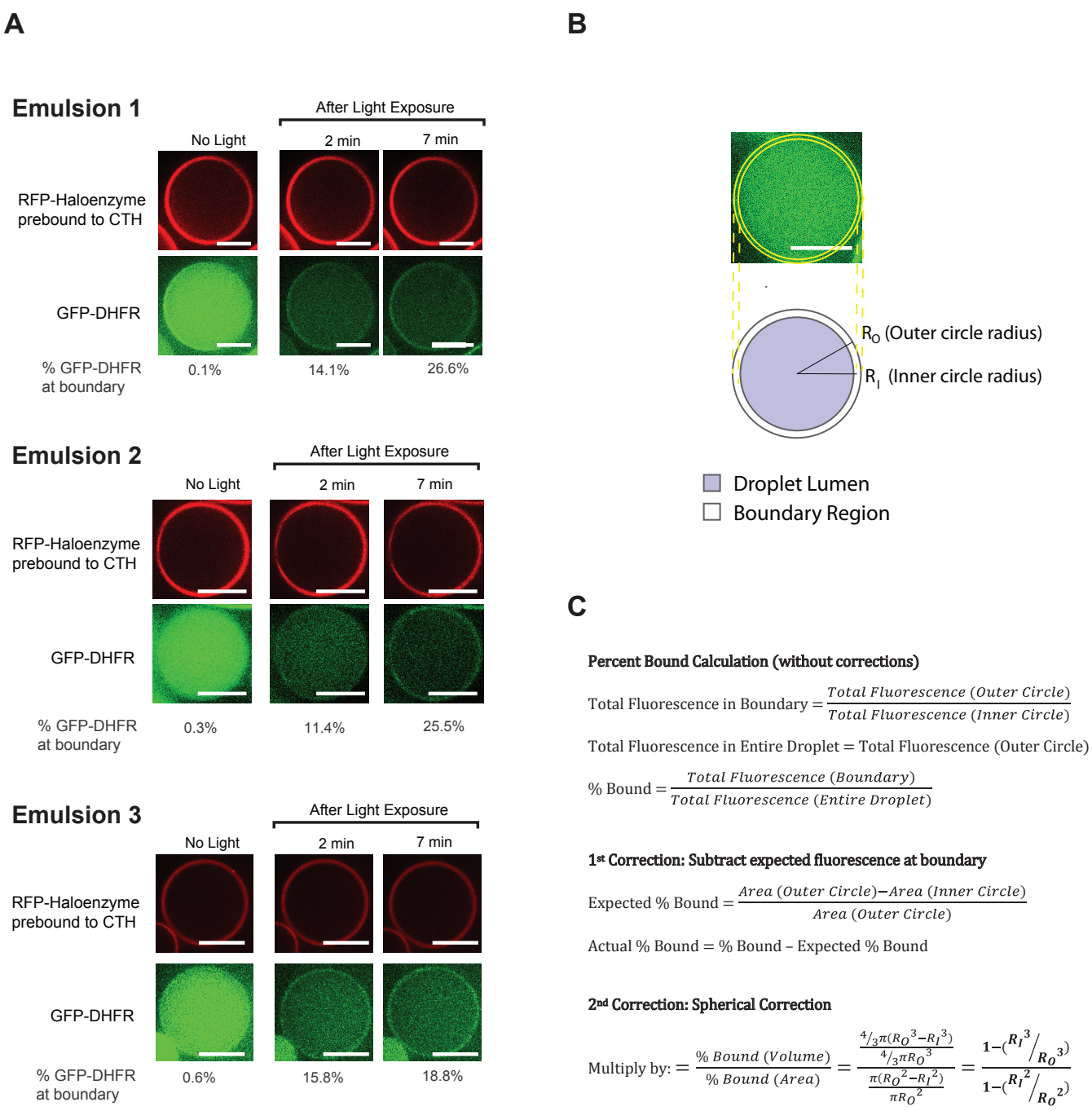

Figure S2. Light-inducible recruitment to compartments boundaries. (A) Demonstration of light-dependent recruitment of GST-GFP-DHFR to the emulsion boundary. His10-RFP-Halo binds to DGS-NTA(Ni) lipid in the droplet boundary. Recruitment of GST-GFP-DHFR to the boundary is triggered by 405 nM laser illumination, which uncages CTH and promotes dimerization of Haloenzyme and DHFR. Shown here: 3 additional emulsions, separate from that shown in Fig 1E. (B) Schematic: quantifying fluorescence at the compartment boundary. (C) Calculation of percent of total fluorescence present at the boundary region. Scale bar: 10 μm

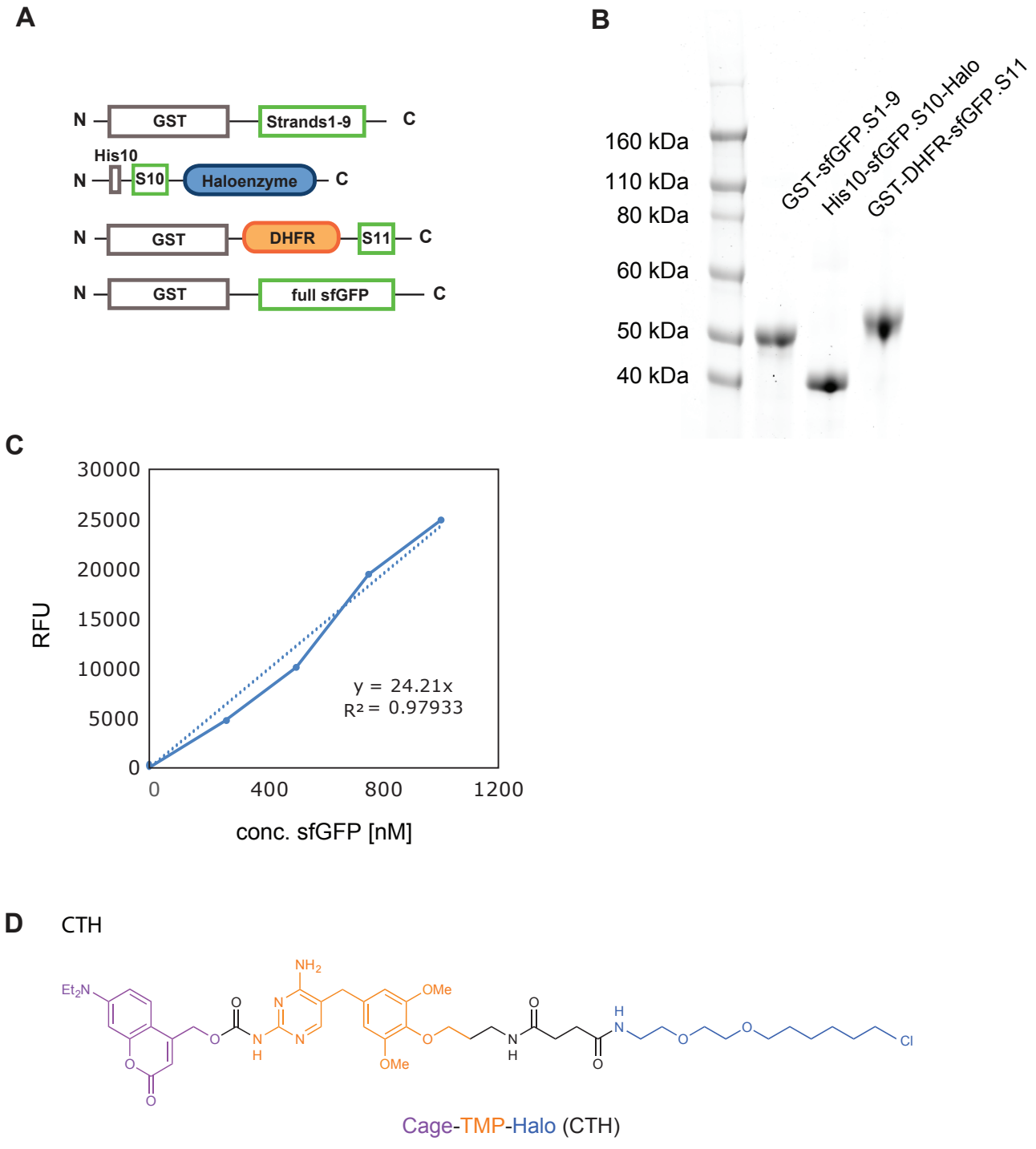

Figure S3. Proteins used for tripartite sfGFP reconstitution. (A) Schematic representation of fusion constructs used for three-component sfGFP reconstitution. (B) SDS-PAGE gel of GST-sfGFP.Strands1-9 (expected MW: 49.7 kDa), His10-sfGFP.Strand10-Halo (expected MW: 42.2 kDa), and GST-DHFR-sfGFP.Strand11 (expected MW: 51.0 kDa). (C) Dilution series of GST- sfGFP fluorescence used for interpolating amount of split sfGFP reconstitution using plate reader assay. (D) Chemical structure of coumarin-cage-TMP-Halo molecule

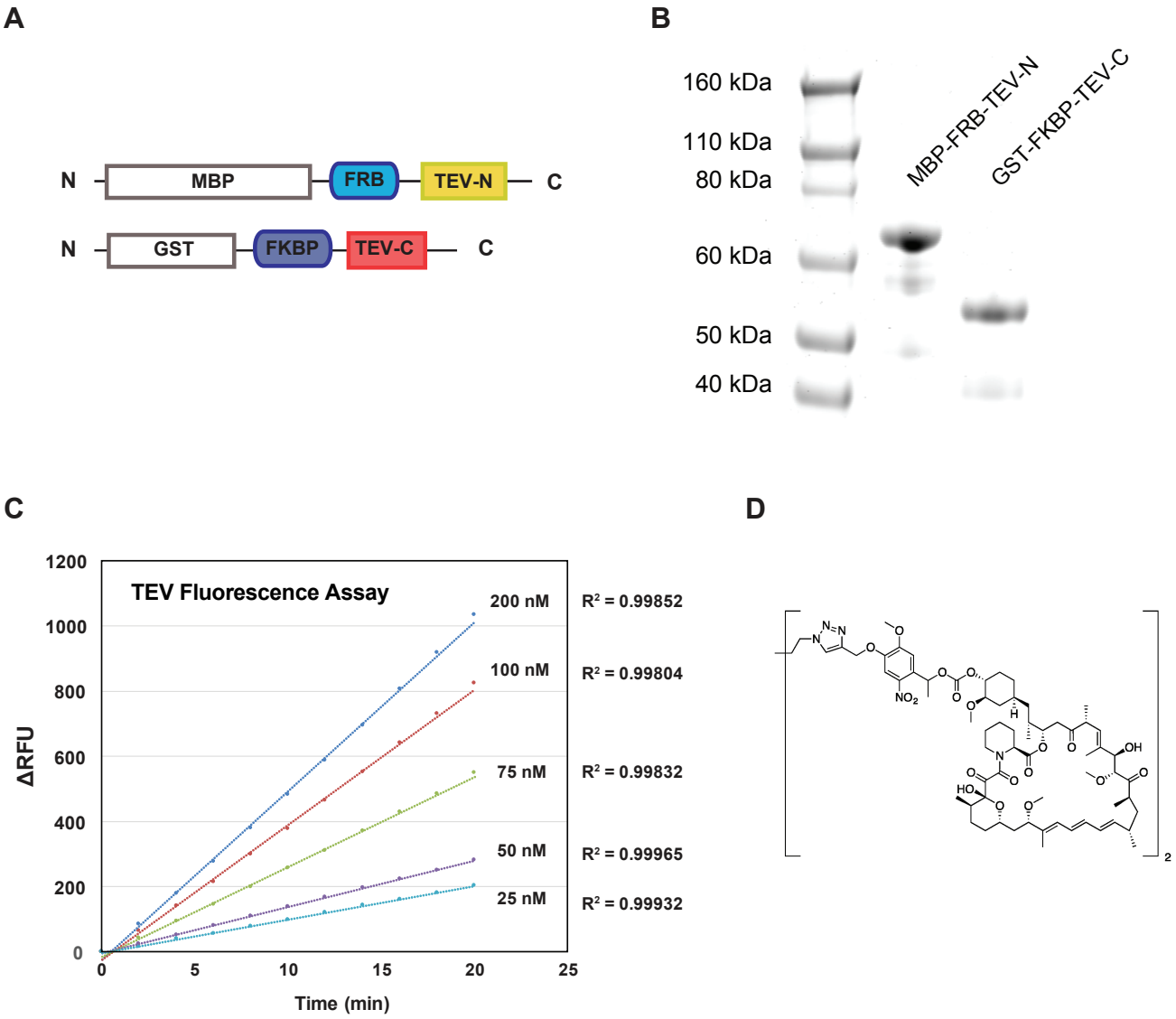

Figure S4. Proteins used for split-TEV protease reconstitution. (A) Schematic of fusion constructs used for split-TEV reconstitution. (B) SDS-PAGE of MBP-FRB-TEV-N (expected MW: 69.7 kDa) and GST-FKBP-TEV-C (expected MW: 54.5 kDa). (C) Timecourse of TEV protease fluorescence assay using varying amounts of rapamycin and 150 nM split TEV proteins. Assay performed as described by manufacturer. Data is the average from triplicate experiments. R^2 > 0.99 for all series, demonstrating linearity of the assay over time. (D) Chemical structure of dRap molecule.

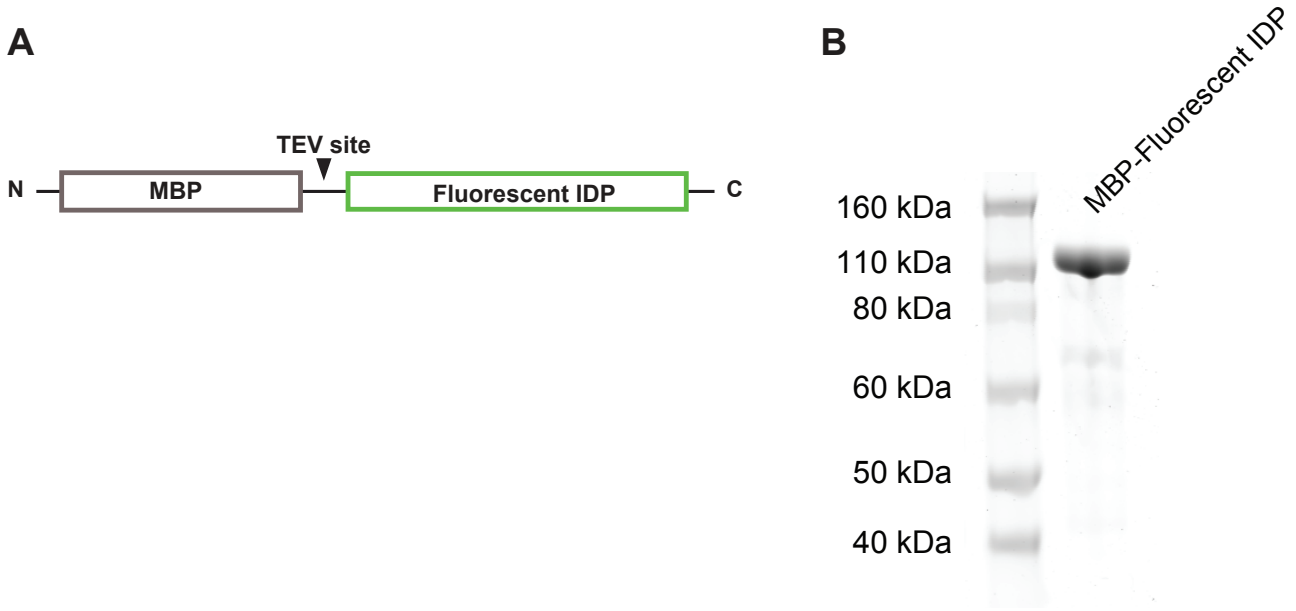

Figure S5. Fluorescent IDP fusion protein. (A) Schematic of IDP fusion construct for generation of membraneless organelles. (B) SDS-PAGE of MBP-Fluorescent IDP (expected MW: 106.2 kDa).

Table S1. Constructs used in this study

Plasmid	Parent Vector	Gene	N-term. fusion
pJB01	pRSETa	RFP-Haloenzyme	His10
pJB02	pETARA	GFP-DHFR	GST
pJB03	pMal	GFP-DHFR	MBP
pRC01	pETARA	sfGFP.Strands1-9	GST
pRC02	pRSETa	sfGFP.Strand10-Haloenzyme	His10
pRC03	pETARA	DHFR-sfGFP.Strand11	GST
pRC04	pMal	FRB-TEV-N	MBP
pRC05	pETARA	FKBP-TEV-C	GST
pRC06	pETARm	Fluorescent IDP	MBP

pETARA is a pET derived bacterial expression vector for an N-terminal GST fusion protein.

pRSETa is a pRSET bacterial expression vector modified for an N-terminal His10 fusion protein.

pMal is a bacterial expression vector for an N-terminal MBP fusion protein.

pETARm is a pET derived bacterial expression vector for an N-terminal MBP fusion protein.