Supporting Information

Engineering a Mi3$_{Chemout}$ System for Controlled Recruitment and Release of Proteins of Interest

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This PDF file includes:

Experimental section

Chemicals and reagents

PCR primers used in this study were synthesized from Sangon Biotech. Detailed information of the primers used is available in **Table S1**. PCR was performed by using Q5 High-Fidelity DNA polymerase (NEB) or EmeraldAmp Taq DNA polymerase (TaKaRa), depending on their fidelity requirements. PCR fragments were purified by a gel extraction kit or DNA cleanup Kit from Omega Bio-Tek. Restriction enzymes and T4 DNA ligase were ordered from Invitrogen. One-step isothermal assembly was carried out by kit from NEB. All constructs were sequenced by Sangon Biotech. Plasmid extraction kit was Omega Bio-Tek. Lysogeny broth (LB) was purchased from Invitrogen. Antibiotics were bought from BBI. HisTrap HP column, Superdex 200 increase 10/300 GL prepacked column for protein purification were product of GE Healthcare. Thrombin was bought from Solarbio Life Science.

Plasmid construction

Detailed information of the plasmids used is available in **Table S2**. The amino acid sequences of the protein fusions are listed in **Table S3**. *E. coli* DH5α was used for cloning. All the synthesized DNA fragments were codon optimized for *E. coli* expression. Plasmid pET28a-$Mi3_{Chem}$ encoding His-tagged FKBP-Mi3 and plasmid pFmCherry encoding His-tagged mCherry-FRB were stored by our laboratory. The DNA fragments encoding FRB-PhoCl was synthesized by Sangon Biotech, and cloned into plasmid expressing mCherry to give plasmids pFPmCherry. DNA fragments encoding His-tag free FRB-PhoCl-mCherry was amplified from plasmids pFPmCherry by PCR using primers and cloned into plasmid pET28a using one-step isothermal assembly, resulting in plasmids pET28a-hisfree FPmCherry. Plasmid pDV_1 encoding VioA and VioB is stored by our laboratory. Plasmid pDV_3 encoding FRB-PhoCl-VioC, FRB-PhoCl-VioE and $Mi3_{Chem}$ were synthesized by SynbIoB.

Protein purification

E. coli BL21(DE3) was used for protein expression. Protein expression vectors were transformed into homemade *E. coli* BL21(DE3) competent cells and cultured at 37°C on agar plates supplemented with 50 μg mL^{-1} kanamycin or 50 μg mL^{-1} spectinomycin. A single colony picked from the agar plate was inoculated into 5 mL LB medium and grown overnight at 37°C. The starter culture was then transferred into 1 L or 2 L of LB medium supplemented

with the appropriate antibiotics, and incubated at 37°C with shaking at 220 r.p.m. When the OD_{600} of the culture reached 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 0.3 mM was added into the cell culture to induce the overexpression of proteins. The cells were cultivated for 12 to 24 hours at 16°C and spun at 180 r.p.m. After collection of the cells by centrifugation at 5000 × g for 15 min at 4°C in a centrifuge (Thermo Fisher Scientific), cell pellets were resuspended with 20 mL binding buffer (25 mM Tris, 150 mM NaCl, 25 mM imidazole, 2 mM 2-mercaptoethanol, pH 7.8) and then lysed with a sonicator for 30 min in an ice bath. The cell lysate was centrifuged at 15000 × g for 20 min at 4°C. The supernatant was then filtered by 0.22 μm membrane and then loaded onto a HisTrap HP column. The sample-loaded HisTrap HP column was applied onto the AKTA fast protein liquid chromatography (FPLC) instrument (AKTA pure, Cytiva). A gradient of elution buffer (25 mM Tris, 250 mM NaCl, 500 mM imidazole, 2 mM 2-mercaptoethanol, pH 8.0), which was linearly increased to 100% in 50 mL, was applied to elute the target His-tagged protein. SEC was used to further purification of the protein using a Superdex 200 increase 10/300 GL column if needed. Protein was then desalted using FPLC equipped with HiTrap desalting column. After desalting, purified proteins were concentrated to a concentration between 2 mg mL^{-1} and 5 mg mL^{-1} using Merck Millipore Amicon Ultra-15 centrifuge and stored under −80°C in Tris buffer (pH 7.8) containing 150 mM NaCl and 10% (v/v) glycerol. To remove the upstream His-tag from FRB-PhoCl-mCherry, the purified His-tagged FRB-PhoCl-mCherry was subjected to thrombin cleavage. Specifically, 5U thrombin was added to the sample. The mixture was then incubated at 37°C for 3 hours. After that, Ni-NTA beads were added to remove the uncleaved proteins. The expression and purification of photosensitive proteins were carried out in the dark.

Formation and purification of $Mi3_{Chem}$/FRB-PhoCl-mCherry assemblies

To construct $Mi3_{Chem}$/FRB-PhoCl-mCherry assemblies, purified $Mi3_{Chem}$ protein cages and molar excess FRB-PhoCl-mCherry (FRB-PhoCl-mCherry: FKBP-Mi3 = 2:1) were mixed in a buffer containing 2 μM rapamycin, 50 mM Tris (pH 7.8) and 150 mM NaCl, and incubated for 1 h at room temperature. The final concentrations of $Mi3_{Chem}$ protein cage and FRB-PhoCl-mCherry were 0.2 mg mL^{-1} and 0.75 mg mL^{-1}, respectively. The mixture was filtered with 0.22 μm membrane and then purified by AKTA FPLC system equipped with Superdex 200

increase 10/300 GL at a flow rate of 0.5 mL min^{-1} in the same Tris buffer. The collected assemblies were concentrated by using Merck Millipore Amicon Ultra-15 and stored with 10% (v/v) glycerol under -80°C for further experiments.

Negative staining transmission electron microscopy (TEM)

Protein cages were purified and checked using negative staining TEM. 10 μL sample with a concentration of about 0.2 mg mL^{-1} was loaded onto the copper grid and blotted with filter paper. The grid was then negatively stained using 10 μL of 3% uranyl acetate for about 3 min and excess staining solution was blotted off with filter paper. Images were taken at a magnification of 40,000 with a Hitachi HT7700 electron microscope operating at 100 kV.

Confocal imaging

For the observation of agarose beads, the beads were suspended in a protein mixture and observed in a glass-bottom Petri dish. For the observation of engineered *E. coli* cells, 1 mL of cells expressing fluorescent proteins was collected by centrifugation at 2000 × g. Cell pellets were washed three times with PBS and resuspended in 0.2 mL of minimal medium. Then, 1 μl sample was added onto a glass slide for imaging. Images were obtained on an Olympus FV1000 inverted confocal microscope or on a Zeiss LSM 980 inverted confocal microscope equipped with a laser line module containing 405, 488, and 594 nm lasers and a ×63 oil immersion objective lens, according to experimental requirements. For PhoCl fluorescence signal, a 488 nm laser was provided and the fluorescent signal was detected through a bandpass of 530 nm. For mCherry signal, fluorescence was excited with a 594 nm laser, and fluorescence signal was collected from 610-680 nm. The images were analyzed using the ImageJ software.

Table S1. Primers used in this study

Primers	Sequence 5'-3' (restriction sites are underlined)
pet28a-FRB-F	TAAGAAGGAGATATACCATGCGTGTGGCAATTCTGTGGCA
pet28a-mCherry-R	GAGCTCttaCTTGTACAGCTCGTCCATGCCG
mCherry-pet28a-F	GACGAGCTGTACAAGtaaGAGCTCCGTCGACAAGCT
FRB-pet28a-R	GCCACACGCATGGTATATCTCCTTCTTAAAGTTAAACAAAATT

Table S2. Plasmids used in this study

Plasmids	Backbone	Description	Source
pET28a-Mi3$_{Chem}$	pET28a(+)	N-terminal His-tagged FKBP, $(GGS)_4$ linker and C-terminal C-tagged Mi3, Kan^+	laboratory stock plasmid
pFPmCherry	pCDFDuet1	N-terminal His-tagged FRB, $(GGGGS)_3$ linker, PhoCl, $(GGS)_4$ linker and mCherry, $Spec/Strep^+$	this study
pFmCherry	pCDFDuet1	N-terminal His-tagged mCherry, $(GGS)_4$ linker and C-terminal His-tagged FRB, $Spec/Strep^+$	laboratory stock plasmid
pDV$_1$	pACYCDuet 1	VioA and VioB, Cm^+	laboratory stock plasmid
pDV$_3$	pCDFDuet1	FRB-PhoCl-VioC, FRB-PhoCl-VioE and Mi3$_{Chem}$, $Spec/Strep^+$	this study
pET28a-hisfree FPmCherry	pET28a(+)	FRB, $(GGGGS)_3$ linker, PhoCl, $(GGS)_4$ linker and mCherry, Kan^+	this study

Table S3. Amino acid sequences of the proteins

Proteins	Amino Acids Sequence
$Mi3_{Chem}$	MGSSHHHHHHGGSGVQVETISPGDGRTFPKRGQTCVVHYTG MLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSV GQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGGSG GSGGSGGSMKMEELFKKHKIVAVLRANSVEEAKKKALAVFL GGVHLIEITFTVPDADTVIKELSFLKEMGAIIGAGTVTSVEQAR KAVESGAEFIVSPHLDEEISQFAKEKGVFYMPGVMTPTELVK AMKLGHTILKLFPGEVVGPQFVKAMKGPFPNVKFVPTGGVN LDNVCEWFKAGVLAVGVGSALVKGTPVEVAEKAKAFVEKIR GCTEGSGEPEA*
FRB-PhoCl-mCherry	MGSSHHHHHHSSGLVPRGSSQDPRVAILWHEMWHEGLEEAS RLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISGGG GSGGGGSGGGGSMVIPDYFKQSFPEGYSWERSMTYEDGGICI ATNDITMEGDSFINKIHFQGTNFPPNGPVMQKRTVGWEASTE KMYERDGVLKGDVKMKLLLKGGGHYRCDYRTTYKVKQKP VKLPDYHFVDHRIEILSHDKDYNKVKLYEHAVARNSTDSMD ELYKGGSGGMVSKGEETITSVIKPDMKNKLRMEGNVNGHAF VIEGEGSGKPFEGIQTIDLEVKEGAPLPFAYDILTTAFHYGNRV FTKYPRGGSGGSGGSGGSVSKGEEDNMAIIKEFMRFKVHME GSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDIL SPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDG GVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGW EASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAK KPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGM DELYK*
His-tag free FRB-PhoCl-mCherry	MRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHA MMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVK DLTQAWDLYYHVFRRISGGGGSGGGGSGGGGSMVIPDYFKQ SFPEGYSWERSMTYEDGGICIATNDITMEGDSFINKIHFQGTN FPPNGPVMQKRTVGWEASTEKMYERDGVLKGDVKMKLLLK GGGHYRCDYRTTYKVKQKPVKLPDYHFVDHRIEILSHDKDY NKVKLYEHAVARNSTDSMDELYKGGSGGMVSKGEETITSVI KPDMKNKLRMEGNVNGHAFVIEGEGSGKPFEGIQTIDLEVKE GAPLPFAYDILTTAFHYGNRVFTKYPRGGSGGSGGSGGSVSK GEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGT QTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYL KLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKL RGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRL KLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNE DYTIVEQYERAEGRHSTGGMDELYK*
VioA	MGSSHHHHHHSSGLVPRGSHMKHSSDICIVGAGISGLTCASH

	LLDSPACRGLSLRIFDMQQEAGGRIRSKMLDGKASIELGAGR YSPQLHPHFQSAMQHYSQKSEVYPFTQLKFKSHVQQKLKRA MNELSPRLKEHGKESFLQFVSRYQGHDSAVGMIRSMGYDAL FLPDISAEMAYDIVGKHPEIQSVTDNDANQWFAAETGFAGLI QGIKAKVKAAGARFSLGYRLLSVRTDGDGYLLQLAGDDGW KLEHRTRHLILAIPPSAMAGLNVDFPEAWSGARYGSLPLFKG FLTYGEPWWLDYKLDDQVLIVDNPLRKIYFKGDKYLFFYTDS EMANYWRGCVAEGEDGYLEQIRTHLASALGIVRERIPQPLAH VHKYWAHGVEFCRDSDIDHPSALSHRDSGIIACSDAYTEHAG WMEGGLLSAREASRLLLQRIAAKLAAALEHHHHHH*
VioB	MSILDFPRIHFRGWARVNAPTANRDPHGHIDMASNTVAMAG EPFDLARHPTEFHRHLRSLGPRFGLDGRADPEGPFSLAEGYN AAGNNHFSWESATVSHVQWDGGEADRGDGLVGARLALWG HYNDYLRTTFNRARWVDSDPTRRDAAQIYAGQFTISPAGAGP GTPWLFTADIDDSHGARWTRGGHIAERGGHFLDEEFGLARLF QFSVPKDHPHFLFHPGPFDSEAWRRLQLALEDDDVLGLTVQY ALFNMSTPPQPNSPVFHDMVGVVGLWRRGELASYPAGRLLR PRQPGLGDLTLRVSGGRVALNLACAIPFSTRAAQPSAPDRLTP DLGAKLPLGDLLLRDEDGALLARVPQALYQDYWTNHGIVDL PLLREPRGSLTLSSELAEWREQDWVTQSDASNLYLEAPDRRH GRFFPESIALRSYFRGEARARPDIPHRIEGMGLVGVESRQDGD AAEWRLTGLRPGPARIVLDDGAEAIPLRVLPDDWALDDATV EEVDYAFLYRHVMAYYELVYPFMSDKVFSLADRCKCETYAR LMWQMCDPQNRNKSYYMPSTRELSAPKARLFLKYLAHVEG QARLQAPPPAGPARIESKAQLAAELRKAVDLELSVMLQYLY AAYSIPNYAQGQQRVRDGAWTAEQLQLACGSGDRRRDGGIR AALLEIAHEEMIHYLVVNNLLMALGEPFYAGVPLMGEAARQ AFGLDTEFALEPFSESTLARFVRLEWPHFIPAPGKSIADCYAAI RQAFLDLPDLFGGEAGKRGGEHHLFLNELTNRAHPGYQLEV FDRDSALFGIAFVTDQGEGGALDSPHYEHSHFQRLREMSARI MAQSAPFEPALPALRNPVLDESPGCQRVADGRARALMALYQ GVYELMFAMMAQHFAVKPLGSLRRSRLMNAAIDLMTGLLR PLSCALMNLPSGIAGRTAGPPLPGPVDTRSYDDYALGCRMLA RRCERLLEQASMLEPGWLPDAQMELLDFYRRQMLDLACGK LSREAGSSHHHHHH*
VioE- PhoCl-FRB	MGSSHHHHHHSSGMENREPPLLPARWSSAYVSYWSPMLPDD QLTSGYCWFDYERDICRIDGLFNPWSERDTGYRLWMSEVGN AASGRTWKQKVAYGRERTALGEQLCERPLDDETGPFAELFL PRDVLRRLGARHIGRRVVLGREADGWRYQRPGKGPSTLYLD AASGTPLRMVTGDEASRASLRDFPNVSEAEIPDAVFAAKRGT GGGGSGGGGSGGGGSVIPDYFKQSFPEGYSWERSMTYEDGGI CIATNDITMEGDSFINKIHFKGTNFPPNGPVMQKRTVGWEAS TEKMYERDGVLKGDVKMKLLLKGGGHYRCDYRTTYKVKQ KPVKLPDYHFVDHRIEILSHDKDYNKVKLYEHAVARNSTDS

	MDELYKGGSGGMVSKGEETITSVIKPDMKNKLRMEGNVNG HAFVIEGEGSGKPFEGIQTIDLEVKEGAPLPFAYDILTTAFHYG NRVFTKYPRGGSGGSGGSGGSRVAILWHEMWHEGLEEASRL YFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDL MEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIS*
VioC-PhoCl-FRB	MMKRAIIVGGGLAGGLTAIYLAKRGYEVHVVEKRGDPLQDL SSYVDVVSSRAIGVSMTVRGIKSVLAAGIPRAELDACGEPIVA MAFSVGGQYRMRELKPLEDFRPLSLNRAAFQKLLNKYANLA GVRYYFEHKCLDVDLDGKSVLIQGKDGQPQRLQGDMIIGAD GAHSAVRQAMQSGLRRFEFQQTFFRHGYKTLVLPDAQALGY RKDTLYFFGMDSGGLFAGRAATIPDGSVSIAVCLPYSGSPSLT TTDEPTMRAFFDRYFGGLPRDARDEMLRQFLAKPSNDLINVR SSTFHYKGNVLLLGDAAHATAPFLGQGMNMALEDARTFVEL LDRHQGDQDKAFPEFTELRKVQADAMQDMARANYDVLSCS NPIFFMRARYTRYMHSKFPGLYPPDMAEKLYFTSEPYDRLQQ IQRKQNVWYKIGRVNGGGGSGGGGSGGGGSVIPDYFKQSFP EGYSWERSMTYEDGGICIATNDITMEGDSFINKIHFKGTNFPP NGPVMQKRTVGWEASTEKMYERDGVLKGDVKMKLLLKGG GHYRCDYRTTYKVKQKPVKLPDYHFVDHRIEILSHDKDYNK VKLYEHAVARNSTDSMDELYKGGSGGMVSKGEETITSVIKP DMKNKLRMEGNVNGHAFVIEGEGSGKPFEGIQTIDLEVKEGA PLPFAYDILTTAFHYGNRVFTKYPRGGSGGSGGSGGSRVAIL WHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGP QTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAW DLYYHVFRRISGGSHHHHHH*
mCherry-FRB	MGSSHHHHHHSSGVSKGEEDNMAIIKEFMRFKVHMEGSVNG HEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFM YGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTV TQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSE RMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELY KGGSGGSGGSGGSRVAILWHEMWHEGLEEASRLYFGERNVK GMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWC RKYMKSGNVKDLTQAWDLYYHVFRRISGGSHHHHHH*

1 FKBP and FRB are shown in red; Flexible linkers are shown in blue; PhoCl, mCherry, and Mi3
2 are shown in green; Epitope tags are underlined; The thrombin recognition sites are indicated
3 by dashed lines.
4

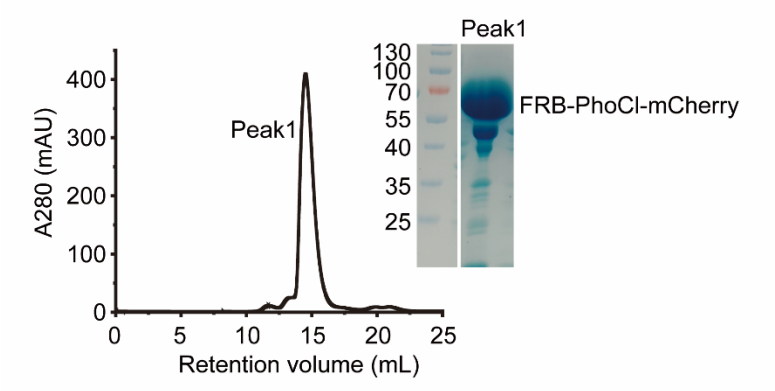

Figure S1. SEC profile of purified FRB-PhoCl-mCherry. Collected peaks were further analyzed by SDS-PAGE.

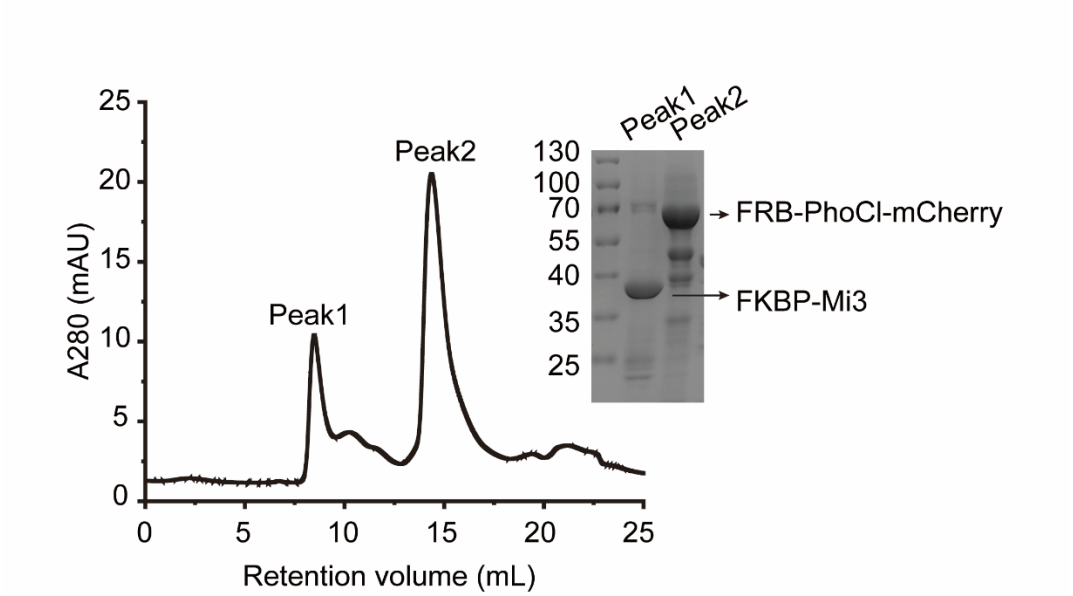

Figure S2. SEC profile of protein mixture of $Mi3_{Chem}$ protein cage and FRB-PhoCl-mCherry in the absence of rapamycin. Collected peaks were further analyzed by SDS-PAGE**.**

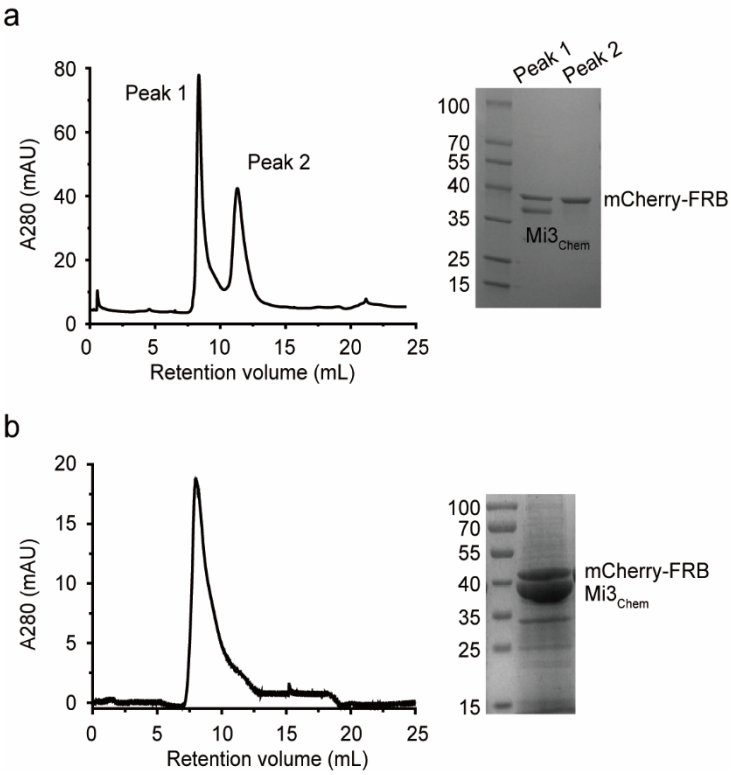

Figure S3. SEC and SDS-PAGE analyses demonstrating the absence of PhoCl domain prevented the photocleavage-induced release of target protein. (**a**) The cargo protein mCherry-FRB coeluted with $Mi3_{Chem}$ protein cage at void volume in the presence of rapamycin. (**b**) The recruited mCherry-FRB continued to bind with $Mi3_{Chem}$ protein cage even upon violet illumination, as demonstrated by SEC and SDS-PAGE.

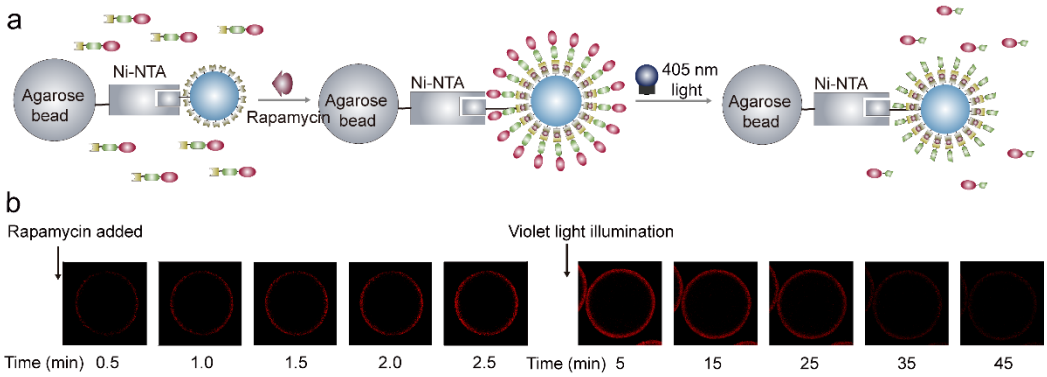

Figure S4. In vitro characterization of Mi3$_{Chemout}$ system using agarose beads. (**a**) Schematic of rapamycin-induced recruitment and photocleavage-triggered release of fluorescent proteins by Mi3$_{Chem}$ protein cage-coated agarose beads. (**b**) Time course fluorescent images of FRB-PhoCl-mCherry binding to Mi3$_{Chem}$ protein cage-coated agarose beads.

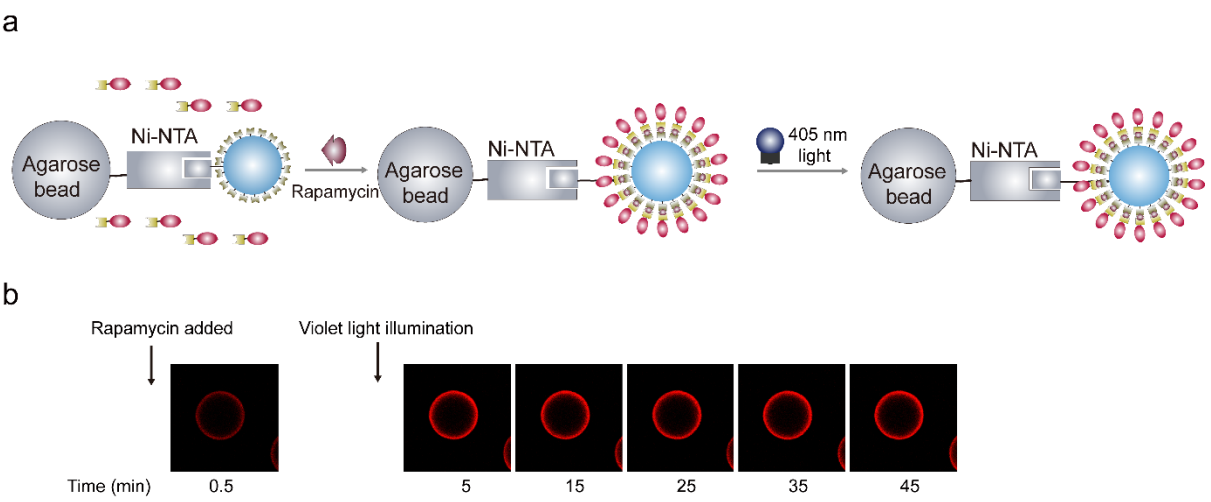

Figure S5. mCherry-FRB without PhoCl domain cannot be released from $Mi3_{Chem}$ targeted agarose beads upon violet light illumination. (**a**) Schematic of rapamycin induced recruitment and photocleavage triggered release of mCherry-FRB by $Mi3_{Chem}$ protein cages targeted agarose beads. (**b**) Time course fluorescent images of mCherry-FRB binding to Ni-NTA agarose beads conjugated with $Mi3_{Chem}$ protein cages. The mCherry signal continued to remain on the beads under violet light illumination.

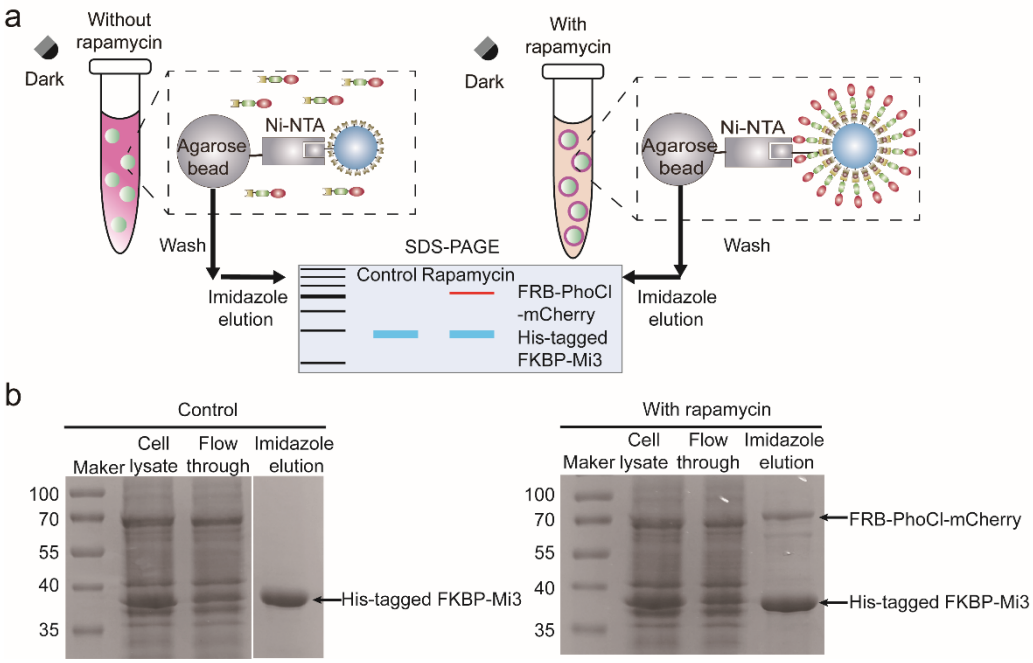

Figure S6. (a) Schematic of pull-down analysis used to investigate the rapamycin induced-recruitment of POIs to protein cage inside *E. coli*. His-tagged $Mi3_{Chem}$ protein cages and His-tag-free FRB-PhoCl-mCherry were co-expressed in *E. coli*. His-tagged $Mi3_{Chem}$ protein cages were purified from the cell lysate by using Ni-NTA agarose beads, with or without rapamycin. The beads were washed under the same rapamycin conditions, followed by imidazole elution. (b) SDS-PAGE analysis of eluents showing the rapamycin induced interaction between FRB-PhoCl-mCherry and $Mi3_{Chem}$ protein cage.

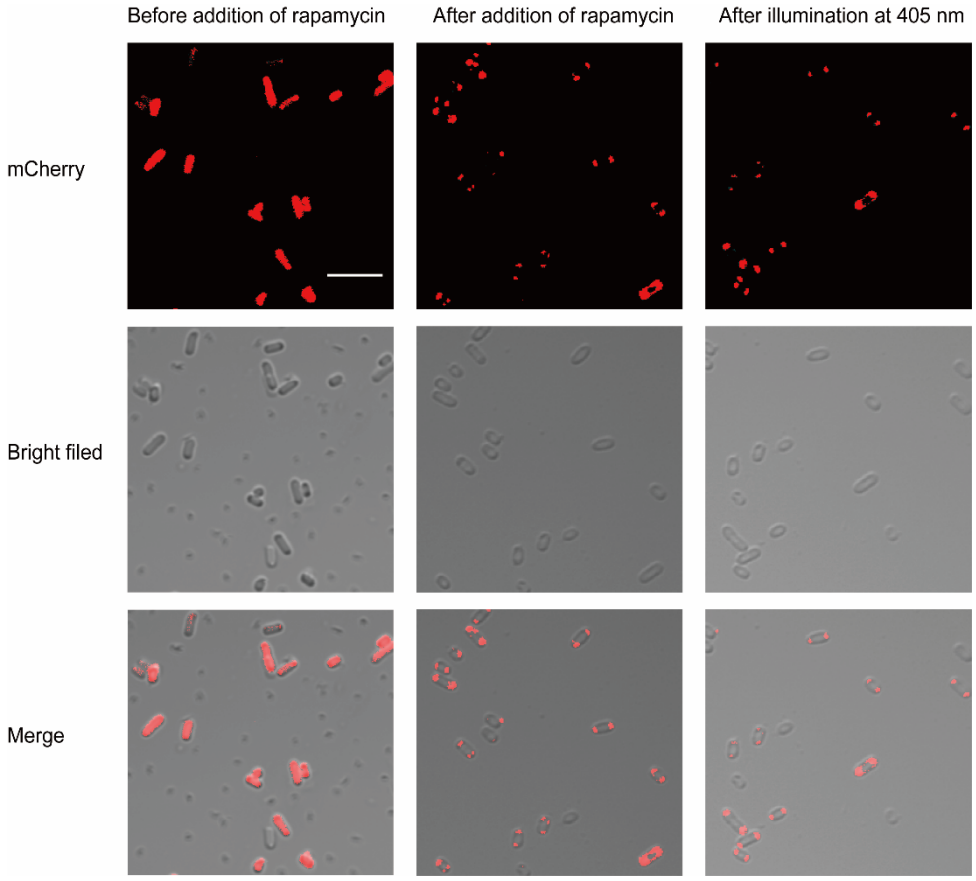

Figure S7. Confocal images of cells expressing $Mi3_{Chem}$ protein cage and mCherry-FRB in the presence of rapamycin before and 15 minutes after violet light illumination with 10 s pulses every 10 s, for 10 min. The cells maintained punctate fluorescent signals even after explosion to violet light. Scale bar, 5 μm.

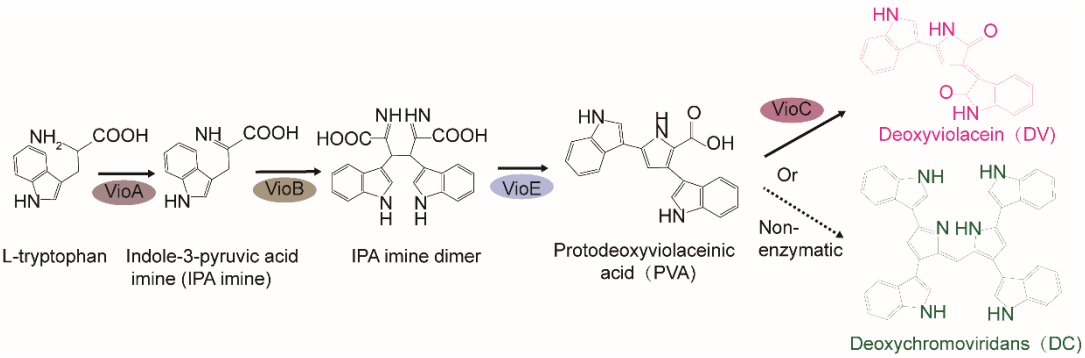

Figure S8. Schematic diagram of the deoxyviolacein (DV) biosynthetic pathway.