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Optical Control of Membrane Tethering and Interorganellar Communication at Nanoscales

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Experimental Procedures

Cell culture and transfection

HeLa and COS-7 cell lines used in this study were obtained from ATCC and grown in DMEM supplemented with 10% FBS (unless otherwise noted) at 37 ^{o}C and 5% CO_2. For transient transfection, cells were seeded onto 35-mm glass bottom dishes (MatTek) and a total of 0.1 to 0.2 μg plasmids were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

Plasmid construction

Based on our previously published backbone pTriEx-mCh-LOVSoc[1], the PB domains derived from different proteins were inserted downstream of $LOV2_{404-546}$ (*Avena sativa* phototropin 1) to replace a STIM1 cytosolic fragment (aa 336-486) by using the Polymerase Incomplete Extension (PIPE) method to make a series of mCh-LOV2-PB chimeras. Standard PCR was performed by using the KOD hot start DNA polymerase (EMD Millipore, Billerica, MA, USA) to amplify the PB fragments. The PB sequences used in this study were from human Rit (aa 193-219), Rin (aa 198-217), STIM1 (aa 666-685), STIM2 (aa 724-746), MARCKS (aa 152-176). The iSH2-OptoPB construct was prepared by subcloning inter-SH2 (iSH2) domain of p85α into the pTriEx-mCh-OptoPB (Rit-PB) vector using the restriction sites PacI and NcoI. Various versions of iSH_2-OptoPB V1.1-V1.6 (Figure S2b) with different linkers between LOV2 and Rit-PB were obtained by the PIPE method. To generate ER-tethered OptoPB (OptoPBer), a double-stranded DNA fragment encoding Rit-PB was synthesized as a gBlock by Integrated DNA Technologies (IDT) and then inserted into pcDNA3.1(+) between the NheI and XbaI sites. mRuby2 or GFP were amplified by standard PCR and subcloned between the ER signal peptide (SP) and transmembrane domain (STIM1, aa 195-240) of STIM1 using the restriction sites KpnI and NotI. Rit-PB was replaced by other PB domains using EcoRI and BamHI sites. To insert 2×, 4×, or 8× spacers into OptoPBer, annealed oligonucleotides or synthetic

fragments encoding the alpha-helical $(EAAAR)_4$ repeats were inserted into the single restriction site XhoI between the transmembrane domain and the LOV2 domain. pcDNA3-PH_{Akt}-GFP plasmid (for monitoring PIP_3) and GFP-C1-$PH_{PLC\delta}$ (for monitoring PIP_2) were purchased from Addgene (#18836, #21179). pENTR223-OSBPL5 was purchased from DNASU (HscD00513589) and incorporated into the pcDNA-DEST53 vector (Thermo Fisher Scientific, # 12288015) to make GFP-OSBPL5 by using the Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen, #11791-020). Mutagenesis of the OptoPB and OptoPBer variants were carried out by using a QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). For bacterial expression of OptoPB, the cDNA encoding LOV2-Rit-PB described above was amplified and inserted between the BamHI and XhoI sites of the vector pProEx-HTb (Life Technologies).

Fluorescence microscopy

Confocal microscopy studies were performed on a Nikon A1R with a 60x, NA 1.49 oil-immersion objective. Cells were imagined 16-24 h after transfection. Time-lapse imaging of fluorescence signal was recorded. Photostimulation was provided by an external blue light (470 nm, 4 mW/cm^2, ThorLabs Inc., Newton, NJ, USA) by continuous or repeated dark-light cycles. To examine if OptoPB overexpression perturbed PI localization, HeLa cells were co-transfected with mCh-OptoPB and the PIP_2 sensor (GFP-$PH_{PLC\delta}$) or PIP_3 sensor (PH_{Akt} –GFP). HeLa cells were starved in DMEM with only 0.5% FBS for up to 6 h (to deplete the basal level of PM-resident PIP_3) before imaging the iSH2-mch-OptoPB and PH_{Akt}-GFP constructs. To assess the cellular localization of mRuby2-OptoPBer, HeLa cells were co-transfected with an ER marker YFP-Sec61β or an ER-PM junction marker GFP-OSBPL5. ER-tetherd OptoPB (OptoPBer) accumulation at ER-PM junctions was monitored by a total internal reflection fluorescence (TIRF) microscope. TIRF images were acquired on a Nikon Eclipse Ti-E microscope customized with Nikon A1R+ confocal laser sources with a 60X, NA 1.49 oil-immersion TIRF objective (Nikon). A 488-nm laser source

was applied to activate the photoswitch LOV2. The images were acquired every 3 seconds for 1 minutes as one cycle. The cells were then kept in the dark for 5–10 minutes, followed by subsequent cycles of photostimulation.

Imaging and statistical analysis

The cytoplasmic intensities of mCh-tagged OptoPB were acquired and analyzed with the Nikon NIS-Elements imaging software. Data were normalized to the initial intensity of mCh-OptoPB when kept in the dark. The half-life values were determined by fitting the data points (from five independent measurements) with an exponential growth or decay function. To quantify ER-tethered OptoPB puncta intensities at ER-PM junctions in the confocal (Figure. 4b) and TIRF images (Figures. 4e, S3b and S4b), the images were background subtracted and converted to binary images to identify dim puncta and define the edges based on optimized thresholds using the NIS-Elements AR software (Nikon). All the graphs shown in this study were plotted by GraphPad Prism 5 software. Unless otherwise noted, quantitative data are expressed as the mean and standard deviation of the mean (s.e.m.; n = 5).

Cell viability assessment with trypan blue staining

HeLa cells were transfected with plasmids encoding mCherry (control) or mCherry-OptoPB. 24 h post-transfection, cells were exposed to pulsed blue light (10 s per min) for 0 h, 4 h and 12 h, respectively. Next, cells were harvested and stained with a Trypan Blue Solution (Thermo Fisher Scientific, #15250061), with the viable/dead cells counted by the TC20™ Automated Cell Counter (BIO-RAD).

Recombinant protein expression and purification

The plasmid encoding His_6-tagged OptoPB was transformed into the *E. coli* strain BL21 (DE3) cells. The transformed cells were grown at 37 °C in LB medium supplemented with 100 mg/L of ampicillin. IPTG (500 µM) was added to induce protein expression when OD_{600} of the culture reached between 0.6 and 0.8. The culture continued to grow overnight at 16 °C. Cultured cells were harvested after centrifugation. The cell pellets were resuspended in 1X Phosphate Buffered Saline (PBS, pH 7.4) and subjected to pulsed sonication. Cell lysates were clarified by centrifugation at 4 °C and supernatants were loaded onto Ni^{2+}-nitrilotriacetic acid (Ni-NTA)-agarose resin (Qiagen). After extensive washing with PBS buffer containing 25 mM imidazole for 3 times, the bound protein was eluted with PBS buffer containing 250 mM imidazole and 1 mM TCEP. Eluted fractions were further purified by gel filtration with a Superdex 200 10/300 GL column using the AKTApure fast protein liquid chromatography system (GE Healthcare).

UV-Vis spectra measurements

The UV-Vis spectra of purified His_6-tagged OptoPB protein (3 mg/ml) were recorded on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The absorbance from 410 nm to 540 nm was measured under dark. After blue light illumination (470 nm, 4 mW/cm^2, 2 min), the absorbance spectra were acquired every 25 sec till the OptoPB fully returned to its dark state.

Protein-lipid overlay assay

The phosphatidylinositol phosphate (PIP) strip^{TM} membranes (P6001; Echelon Biosciences) were blocked in 3% fatty acid-free BSA in PBS-T (0.1% v/v Tween 20) and gently agitated at room temperature for 1 hour. The strips were then incubated with 0.5 µg/ml recombinant OptoPB protein at room temperature for 2 hours, either kept in the dark or under constant illumination with a blue LED (470 nm, 4 mW/cm^2). The membranes were then washed five times in PBS-T and incubated for 1 hour with rabbit anti-His

antibody diluted at 1:1000. After extensive washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000) for 1 hour at room temperature with gentle agitation, followed by washing in PBS-T. The signals were detected by using ECL Western Blotting Substrate (Thermo Scientific).

Circular dichroism spectroscopy

Circular dichroism (CD) spectra for recombinant proteins were recorded in a Jasco-715 spectropolarimeter at 22 ^{o}C using a 1-mm path length quartz cell with the protein concentration at 20 μM in PBS containing 1 mM TCEP at pH 7.4. All spectra were obtained as the average of at least three scans with a scan rate of 50 nm min^{-1}. The CD signals was acquired in the range of 200 nm to 260 nm. To monitor the recovery process of OptoPB after blue light illumination, a time course measurement was employed with the parameters set as follows: wavelength at 222 nm; 5 min of measurement time; with time and data pitch set at 0.1 sec.

Structural modeling and molecular dynamic (MD) simulation.

The primary model of the LOV2-Rit(PB) was built using I-TASSER[2] based on the crystal structure of LOV2 (PDB ID: 2V0U). The MD simulation was performed using the GROMACS-5.1.2 package[3] using the CHARMM27 force field[4]. The protein was first placed in a dodecahedron box, and the minimum distance between the solute and the box boundary was 1.2 nm. Then the box was filled with TIP3P water molecules[5]. Using the steepest descent method, the protein-water system was energy-minimized until the maximum force was smaller than 1000 kJ·mol^{-1}·nm^{-1}. Six Cl^- were added to neutralize the net charge of the protein by replacing the same number of solvent molecules at the position with the most favorable electrostatic potential. The system energy was minimized again using the steepest descent

followed by the conjugate gradient method with a force tolerance of 200 $kJ \cdot mol^{-1} \cdot nm^{-1}$. Two independent 100-ps equilibration simulations were performed using different random seeds to assign initial atomic velocities at 300 K, respectively. After adding positional restraint on heavy atoms, with a force constant of 1000 $kJ \cdot mol^{-1} \cdot nm^{-2}$, the final free MD was conducted with 40 ns. The protein structures were analyzed, aligned, and visualized using PyMol (DeLano Scientific LLC) and VMD[6].

Supplemental sequences

The OptoPB (LOV2-Rit-PB chimera) insert in pTriEx-mCh vector

TTGGCTACTACACTTGAACGTATTGAGAAGAACTTTGTCATTACTGACCCAAGATTGCCAGATAATCCCATTATAT
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ER tethered mRuby2-OptoPB (SP-mRuby2-TM-LOV2-STIM1-PB)

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Primer sequences for mutagenesis (5’ to 3’)

STIM1-K672A/K673A: GACTCCAGCCCAGGCCGGGCGGCGTTTCCCCTCAAAATC

STIM1-K677A: CGGAAGAAGTTTCCCCTCGCAATCTTTAAGAAGCCTCTTAAG

STIM1-K680A/K681A: GTTTCCCCTCAAAATCTTTGCGGCGCCTCTTAAGAAGTGATAAG

STIM1-K684A/K685A: CTTTAAGAAGCCTCTTGCGGCGTAGGCAGGATGGGGTGGCAG

STIM1-K684A: CTTTAAGAAGCCTCTTGCGAAGTAGGCAGGATGGGGTGGCAG

STIM1-K685A: CTTTAAGAAGCCTCTTAAGGCGTAGGCAGGATGGGGTGGCAG

STIM1-K684/K685A: CTTTAAGAAGCCTCTTGCGGCGTGATAAGGATCCTCTAGAG

STIM2-K743A/K744A: CAAAAATCAAAAGCCTTTTTGCGGCGAAATCTAAGTGAGGATCC

Rit-K205A/K208A: CAAAAACAGTGTATGGAGGAGGCTAAGATCACCATTCCGG

Rit-K214A/K215A: CACCATTCCGGAAGAGGAGAGATTCAGTAACTTGATAAGGATCC

Supplemental Figure 1.

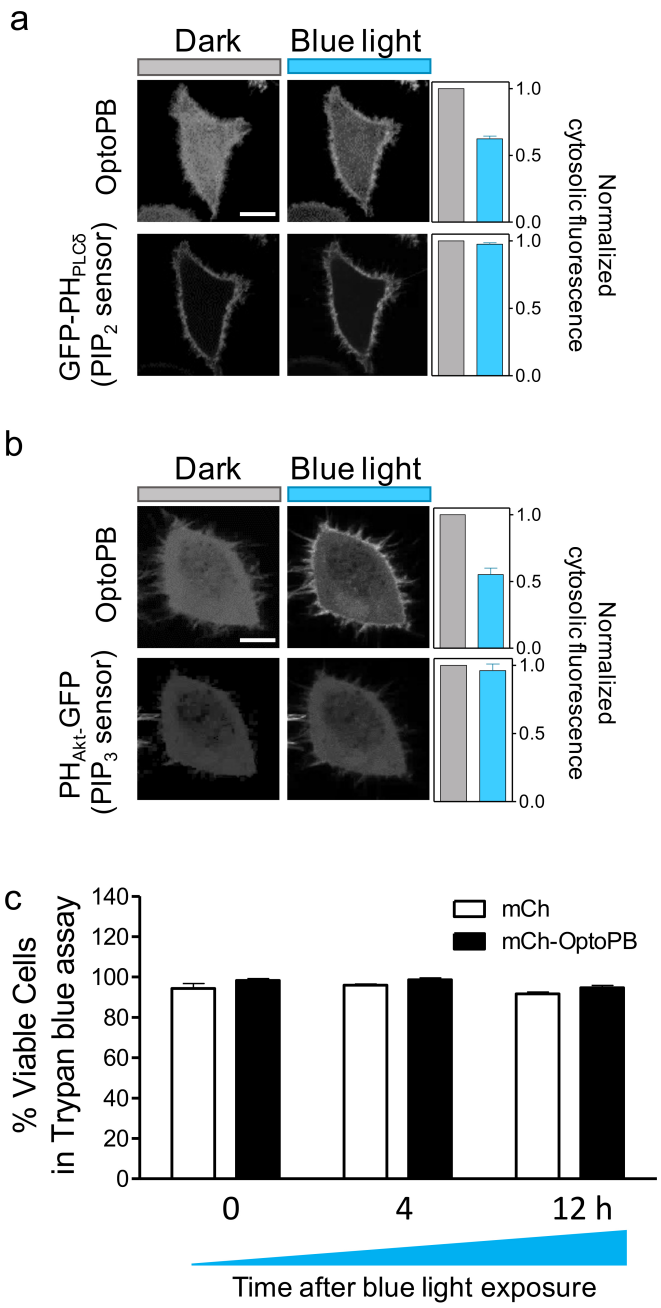

Figure S1. OptoPB overexpression did not cause significant changes in overall distribution of major phosphoinositides and cell viability**.** OptoPB was co-transfected with (a) GFP-PH$_{PLC\delta}$ (PI(4,5)P$_2$ sensor) or (b) PH$_{Akt}$-GFP (PI(3,4,5)P$_3$ sensor) into HeLa cells. Before imaging for condition (b), the cells were pre-incubated in serum-reduced DMEM culture medium for at least 4 h. After blue light stimulation, the accumulation of OptoPB did not perturb the overall distribution of both PI markers. (c) Quantification of cell viability by trypan blue staining. Cells were either shielded (0 h) or exposed to pulsed blue light (10 s per min for 4 h or 12 h). Data were presented as mean ± s.e.m. (n=5).

Supplemental Figure 2.

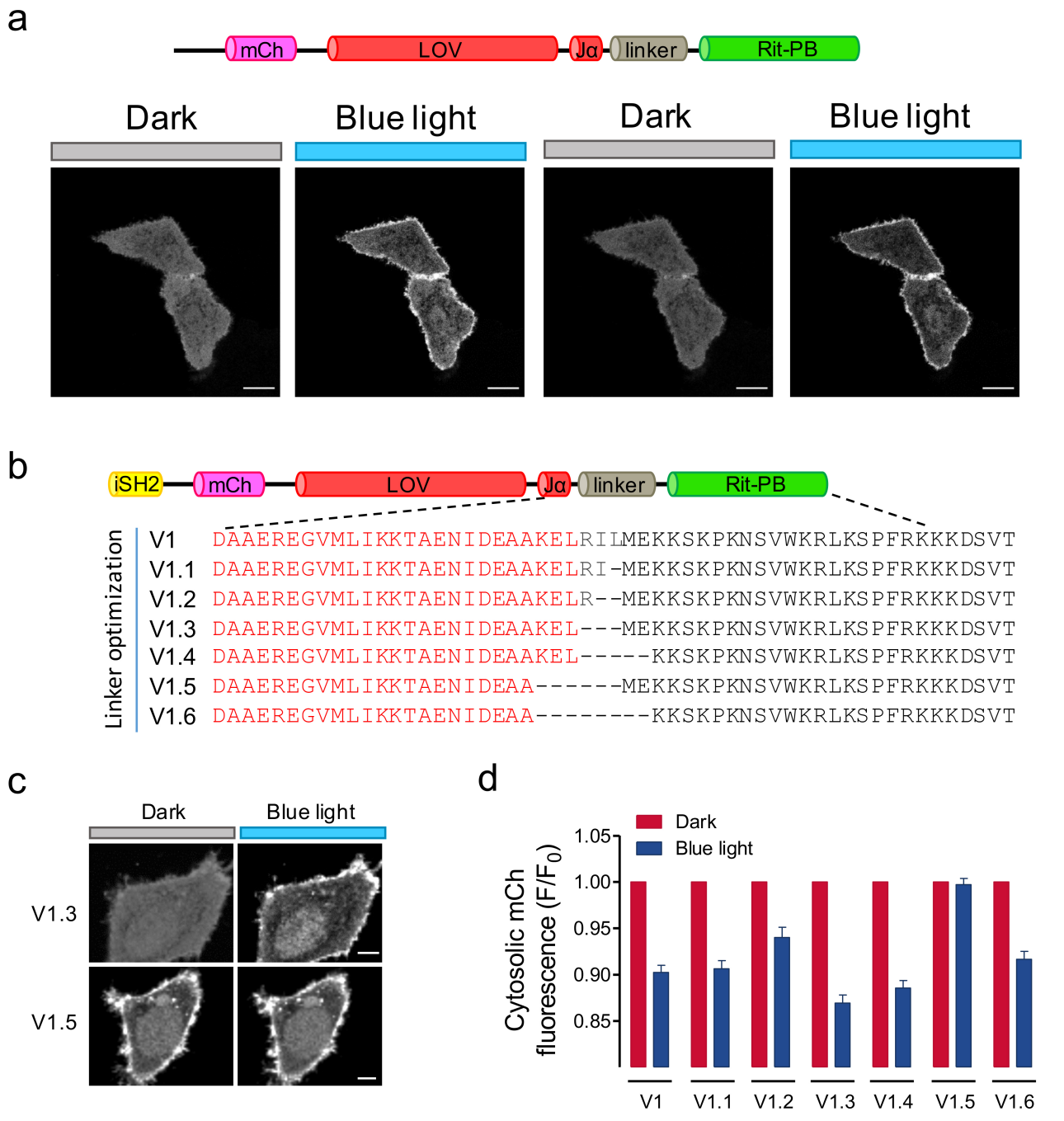

Figure S2. Reversible cytosol-to-PM translocation of OptoPB (a) and optimization of iSH2-OptoPB chimeras (b-d). (a) Confocal images showing the reversible translocation of mCherry fused OptoPB between the cytosol and PM during two dark-light cycles (blue bar: 470 nm for 1 min at 4 mW/cm^2). (b) Design of iSH2-mCherry-OptoPB variants by varying the J-alpha helix and linker regions. (c) Representative confocal images of HeLa cells expressing iSH2-mCherry-LOV2-Rit-PB variants before and after blue light illumination (1 min at a power density of 4 mW/cm^2). Scale bar, 5 µm. (d) Quantification of normalized cytosolic intensities of iSH2-mCherry-OptoPB before and after photostimulation (n=5).

Supplemental Figure 3.

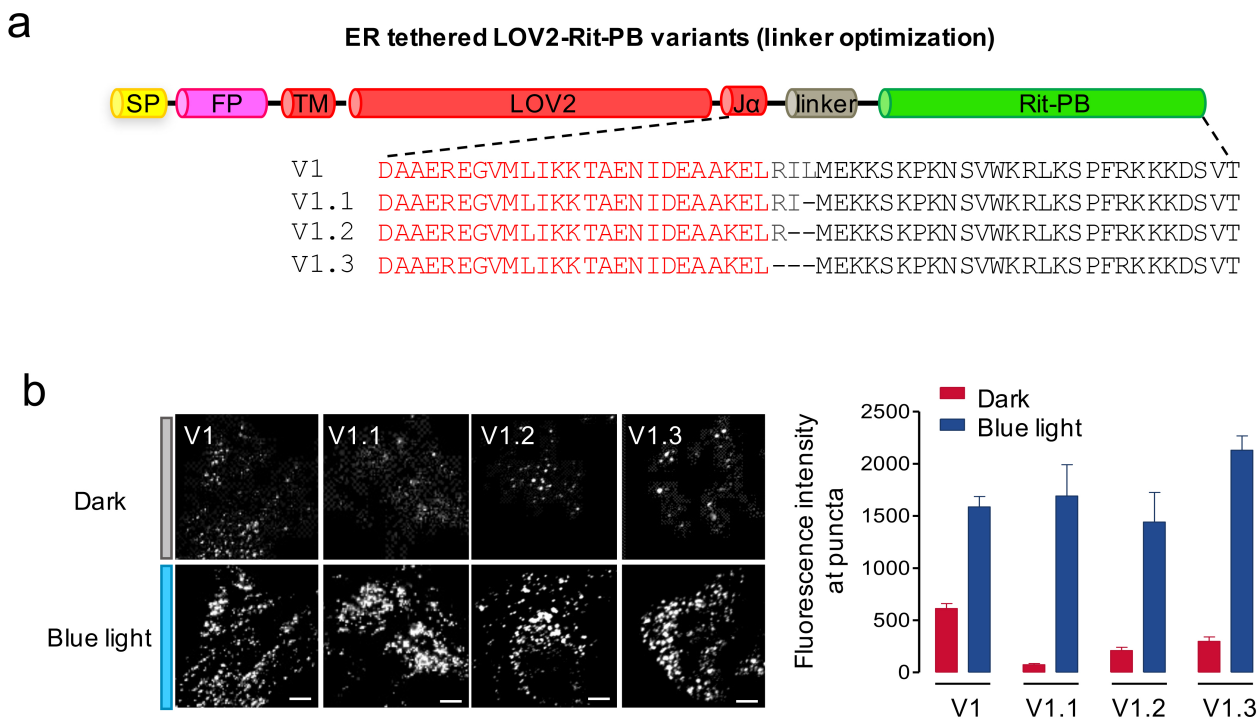

Figure S3. Optimization of ER-tethered LOV2-Rit-PB chimeras by varying the linker regions. (a) Design and sequences of ER tethered OptoPB fused with the Rit polybasic domain. (b) TIRF images of indicated variants of ER tethered LOV2-Rit-PB when expressed in HeLa cell before and after blue light illumination. The bar graph showed the quantification of the fluorescence signals of puncta (n=5). Note the appearance of puncta in the dark that suggested dark activation. Optimizing the linker regions reduced the degree of dark activation, as best illustrated by V1.1.

Supplemental Figure 4.

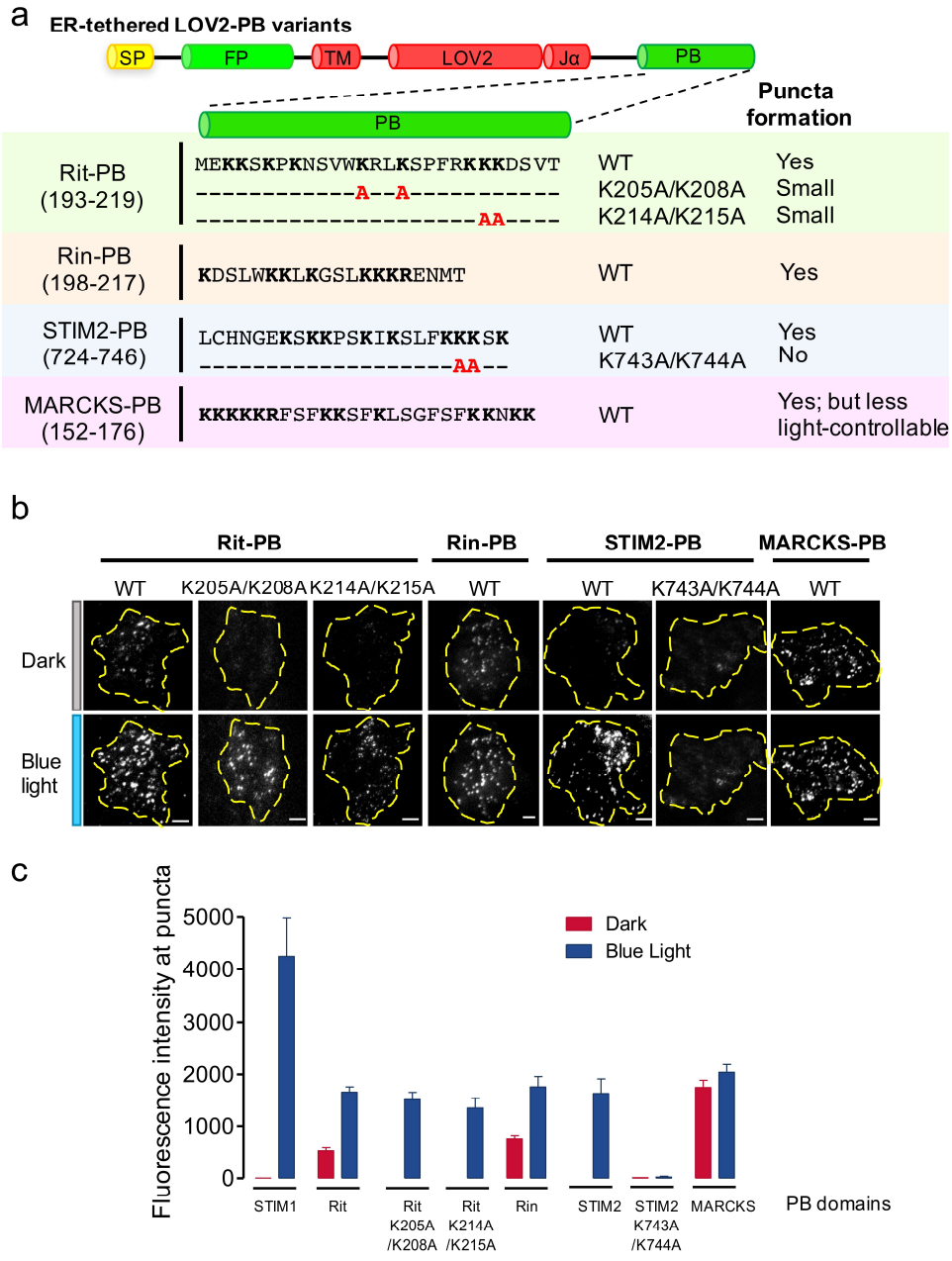

Figure S4. Grafting the PB domains derived from different proteins into the OptoPBer scaffold to determine critical residues involved in protein-PIs/PM interactions in living cells. (a) Schematic design of ER-tethered OptoPB variants fused with PB domains from Rit, Rin, STIM2, or MARCKS, as well as the indicated K-to-A mutants. (b-c) TIRF images (b) and quantification of fluorescent OptoPBer puncta signals at ER-PM junctions (c) for each of indicated constructs before and after photo-illumination at 470 nm for 5 min (4 mW/cm^2). The quantification data representing STIM1-PB in panel c was also shown in Figure 4f. Scale bar 5 μm.

Supplemental Figure 5.

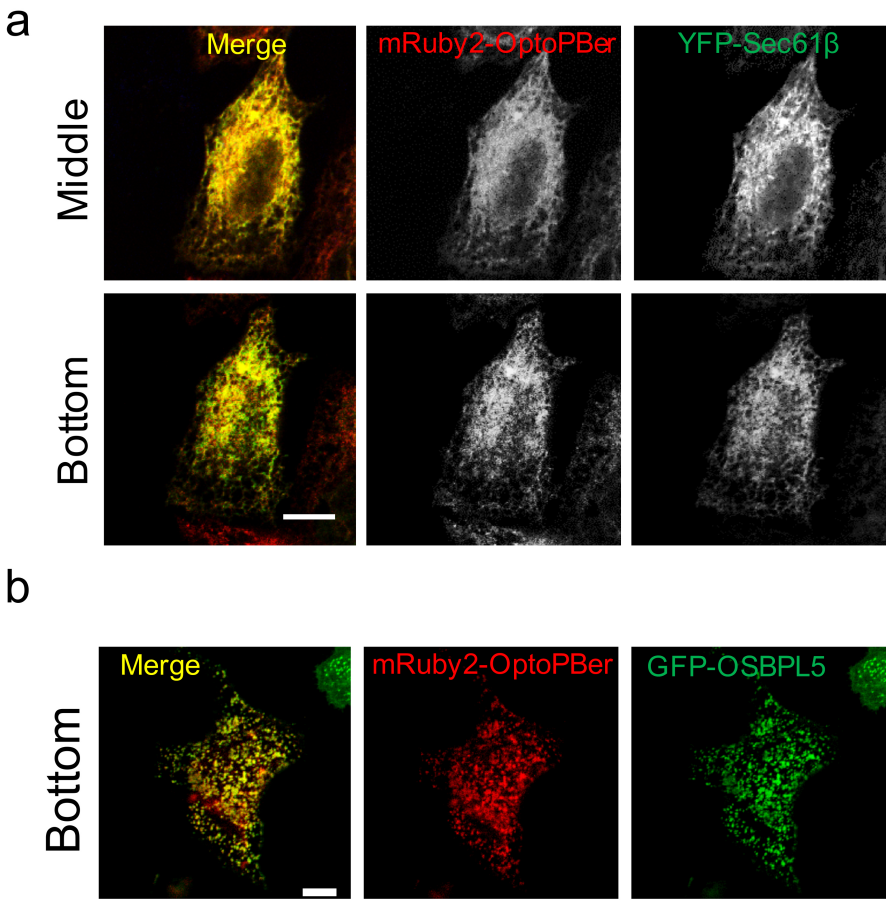

Figure S5. OptoPBer localized to ER and the ER-PM junction in HeLa cells. (a) HeLa cells transfected with YFP-Sec61β (a well-established ER marker; green) and mRuby2-OptoPBer (red) before blue light stimulation. The middle and bottom layers were shown. (b) Confocal imaging showing the localization of mRuby2-OptoPBer (red) and a recently-reported ER-PM junction marker GFP-OSBPL5 (green) after blue light stimulation (1 min, 4 mW/cm^2) and recovery to the dark.

Supplemental Figure 6.

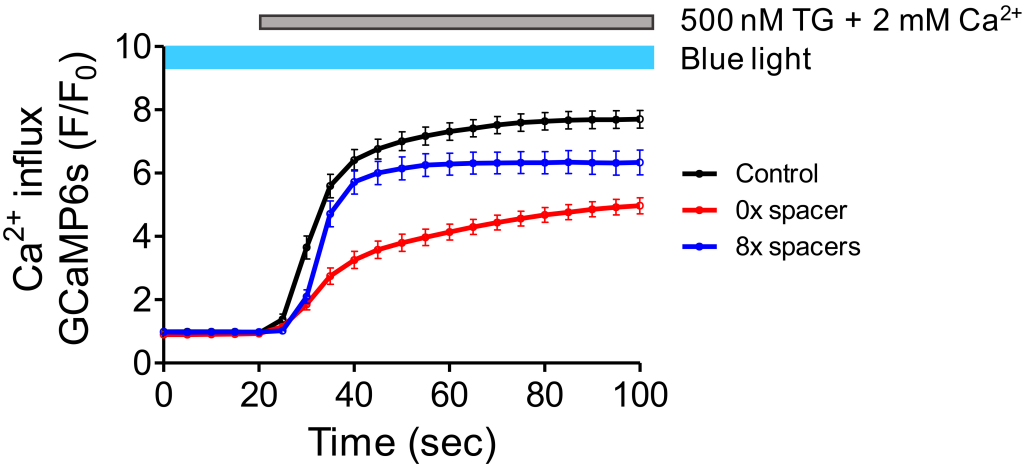

Figure S6. Store-operated calcium entry (SOCE) in HeLa cells following the photo-inducible manipulation of the gap distances at ER-PM contact sites. HeLa cells were either transfected with mRuby2 (control; black), mRuby2-OptoPBer with 0x (red) or 8x helical spacers (blue). Following store depletion with TG (500 nM), the intracellular Ca^{2+} levels (SOCE) were monitored by GCaMP6s. Transfected HeLa cells were exposed to blue light illumination as indicated (4 mW/cm^2).

Supporting movie 1:

Reversible recruitment of mCh-OptoPB to the plasma membrane of HeLa cells by switching on and off blue light stimulation (470 nm; 4 mW/cm^2) for two cycles.

Supporting movie 2:

Light-controllable reversible labeling and accumulation of mRuby2-OptoPBer at ER-PM contact sites monitored by a confocal microscope. Two light-dark cycles were applied (470 nm; 4 mW/cm^2).

Supporting movie 3:

TIRF imaging of reversible puncta formation at ER-PM junctions in HeLa cells expressing ER-tethered LOV2-STIM1-PB (left) or LOV2-Rit-PB (right). HeLa cells expressing the indicated proteins were subjected to two light-dark cycles (470 nm; 4 mW/cm^2; 5 min ON for each cycle with a 15-min interval in the dark: 0-300 sec and 1200-1500 sec).

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