The dual characteristics of light-induced cryptochrome 2 homo-oligomerization and hetero-dimerization for optogenetic manipulation in mammalian cells

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Keywords: optogenetics, light control, cryptochrome 2, oligomerization, CRY2-CIB1 dimerization

SUPPORTING INFORMATION

Plasmid construction. All the plasmids used in this study were cloned in the mammalian expression vector pEGFPN1-GFP-CIB1, pmCherryC1-mCh-CRY2, or pmCherryC1-CRY2-mCh using either DNA ligation, 2-step overlapping extension PCR or InFusion cloning technique. In this study we employed the photolyase homology region (PHR) of CRY2 (amino acids 1-498) and a truncated version of CIB1 (amino acids 1-170). CIB1-GFP-Caax, CIB1(1-335aa)-GFP-Caax and pmCherryC1-CRY2-mCh were gifts from Dr. Chandra Tucker, University of Colorado Denver. All other plasmids used in this study (see Supplementary table 1) were constructed in our lab using DNA ligation method and used as templates for additional plasmid constructs afterwards. The insertion sites, oligonucleotides and cloning methods used in plasmid construction are summarized in Supplementary Table 2.

Supplementary Table 1. Description of the plasmid constructs used in the paper

Construct Name	Description
CRY2-mCh	PHR domain of CRY2 (a.a. 1-498) fused to mCherry
CRY2-GFP	PHR domain of CRY2 (a.a. 1-498) fused to GFP
CIB1	A truncated version of the basic helix-loop-helix protein *Arabidopsis* CIB1 (a.a. 1-170)
CIB1-GFP	GFP fused to the C-terminus of CIB1
GFP-CIB1	GFP fused to the N-terminus of CIB1
CRY2(D387A)-mCh	Flavin adenine dinucleotide (FAD)-deficient mutant CRY2 (a.a. 1-498) fused to mCherry
CRY2-mCh-Sec61	The transmembrane domain of the ER targeting sequence Sec61 (a.a. 1-95) fused to the C-terminus of CRY2-mCh

CRY2(D387A)-mCh-Sec61	ER targeting sequence fused to the C-terminus of the light-insensitive mutant CRY2(D387A)-mCh
CRY2-mCh-Caax	Plasma membrane targeting sequence Caax (a.a. 1-15) fused to the C-terminus of CRY2-mCh
CIB1-GFP-Caax	Plasma membrane targeting sequence Caax (a.a. 1-15) fused to the C-terminus of CIB1-GFP
CIB1(1-335aa)-GFP-Caax	Plasma membrane targeting sequence Caax (a.a. 1-15) fused to the C-terminus of the full length construct CIB1-GFP
CRY2-mCh-Miro1	Mitochondrial outer membrane targeting sequence Miro1 (39 a.a.) fused to the C-terminus of CRY2-mCh
CIB1-GFP-Miro1	Mitochondrial outer membrane targeting sequence Miro1 (39 a.a.) fused to the C-terminus of CIB1-GFP
GFP-BICDN-CIB1	Cytoplasmic dynein-interacting protein BICDN (a.a. 1-594) fused to the N-terminus of CIB1
Raf-GFP-CIB1	Protein kinase Raf (a.a. 1-646) fused to the N-terminus of GFP-CIB1

Supplementary Table 2. Summary of the plasmid construction methods

Plasmid	Method	Template	Insertion sites	Forward primer	Reverse primer
GFP-CIB1	Ligation	pEGFPN1	Afl II Bsp1407I	n/a	n/a
CIB1-GFP	Ligation	CIB1-GFP-Caax	AgeI Bsp1407	n/a	n/a
CRY2-GFP	Ligation	CRY2-mCh	Crf9I NotI	n/a	n/a
CRY2(D387A)-mCh-Sec61	Ligation	CRY2(D387A)-mCh	BSP1407 NotI	n/a	n/a
Raf-GFP-CIB1	Ligation	GFP-CIB1	EcoRI AflII	n/a	n/a
CIB1-GFP-Miro	Overlap extension	CIB1-GFP-Caax	n/a	ATCACTCTCGGCATGGACGAGCTGTATAAGCCGCACGTGACACAAGCTGACCTCAAGAGC	CTCGAGATCTGAGTCCGGACTTGTACATCATCGCTGTTTCAATAATGCTTTGTACATAGC
CIB1	InFusion	CIB1-GFP-Miro	AgeI MfeI	ATATTCATGTACCGGTCTGATGTACAAGTCCGGA	TTAACAACAACAATTGCATTCATTTT
CRY2-mCh-Sec61	InFusion	CRY2-mCh	Bsp1407I NotI	ATGGACGAGCTGTACA	TAGACTCGAGCGGCCGCCTACGAACGAGTGTACTTGC
CRY2-mCh-Miro	InFusion	CRY2-mCh	MfeI BSP1407I	ATGGACGAGCTGTACAAGCCGCACGTGACACAAG	TTAACAACAACAATTGCATTCATTTTATGTT
GFP-BICDN-CIB1	InFusion	GFP-CIB1	Bsp1407I	ATGGACGAGCTGTACAAGTC	ATGGATCCCTTGTACAATTCTCGGCCCACCTCTG
CRY2-mCh-Caax	InFusion	CRY2-mCh	Bsp1407MfeI	ATGGACGAGCTGTACAAGGGTAAAAAGAAGAAAAAG	TTAACAACAACAATTGCATTCA

CRY2 cluster quantification using Matlab. A custom-written Matlab algorithm was built to automatically identify the number and intensity of CRY2 clusters in each cell. After activating CRY2 by a 2s blue light pulse, green fluorescence light was used to collect a time-lapse image sequence for each cell to track the formation and decay of CRY2 clusters in the cell over time (frame rate 0.2Hz). The program then processes each individual image frame in the following steps:

1. Image filtering: we perform Gaussian smoothing on the image using the built-in Matlab command *imfilter*, which convolves the image with a 5x5 pixel Gaussian kernel (1 pixel = 0.13μm). Gaussian smoothing is low-pass filtering, which means that it suppresses high-frequency detail (noise, but also edges), while preserving the low-frequency parts of the image (i.e. those that don't vary so much). In other words, this filtering technique suppresses noise in an image in order to enhance the detection of small features, i.e. CRY2 clusters.
2. Background subtraction: background of the image is calculated using a 10x10 pixel mean filter. The background image is then subtracted from the original image.
3. Cluster detection: the program identifies the local intensity maxima within the image, defined as the centers of the CRY2 clusters. The algorithm has been described in a previous publication.[1] In short, the algorithm pick the local maxima using the following criteria:
    a. The intensity of the pixel is within the top 2% percentile of brightness for the entire image
    b. The signal-to-noise ratio of a 7x7pixel region surrounding the local maximum is at least 2.5. The 7x7pixel region is chosen empirically as we found that this window size best represents the size of the majority of the clusters.
    c. There is no other pixel within a 7x7 pixel region surrounding the local maximum that is of greater or equal intensity.
    d. The intensity of the pixel is equal or greater than a cutoff value. This value is empirically chosen to be 100 and is kept consistent when processing all the images in this study.
4. Cluster intensity calculation: the intensity of the cluster is calculated by summing the intensity values of all the pixels in a 7x7 pixel region surrounding the center pixel.
5. Total cluster mass quantification: the CRY2 cluster mass in each image frame is calculated as the sum of all detected clusters intensities, normalized by the cell size (Equation 1).

$$\text{Total cluster mass} = \frac{\sum \text{Cluster intensity}}{\text{Cell size}} \qquad \text{(Eq. 1)}$$

The algorithm is very robust and can automatically detect at least 90% of the clusters in each image frame (Figure S5).

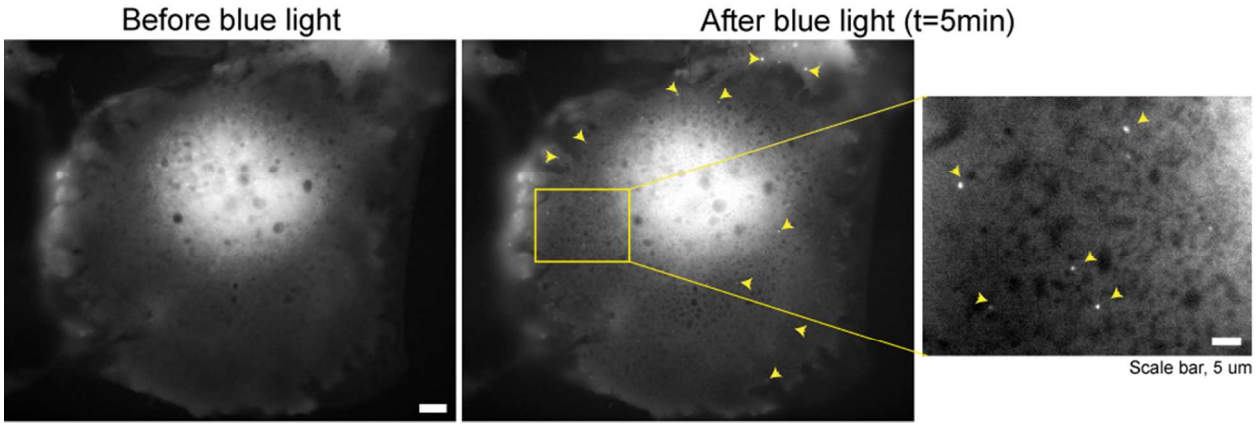

Figure S1. CRY2-GFP oligomerization under blue light stimulation in COS-7 cell. The cells was transfected with CRY2-GFP and were illuminated with 200ms blue light pulse every 5s ($9.7x10^3 mW/cm^2$, 460-480nm). Before blue light, CRY2-GFP is expressed in the cytoplasmic form. After 5 minutes of light activation, some small CRY2 clusters are visible as indicated by yellow arrowheads. Scale bar, 10μm.

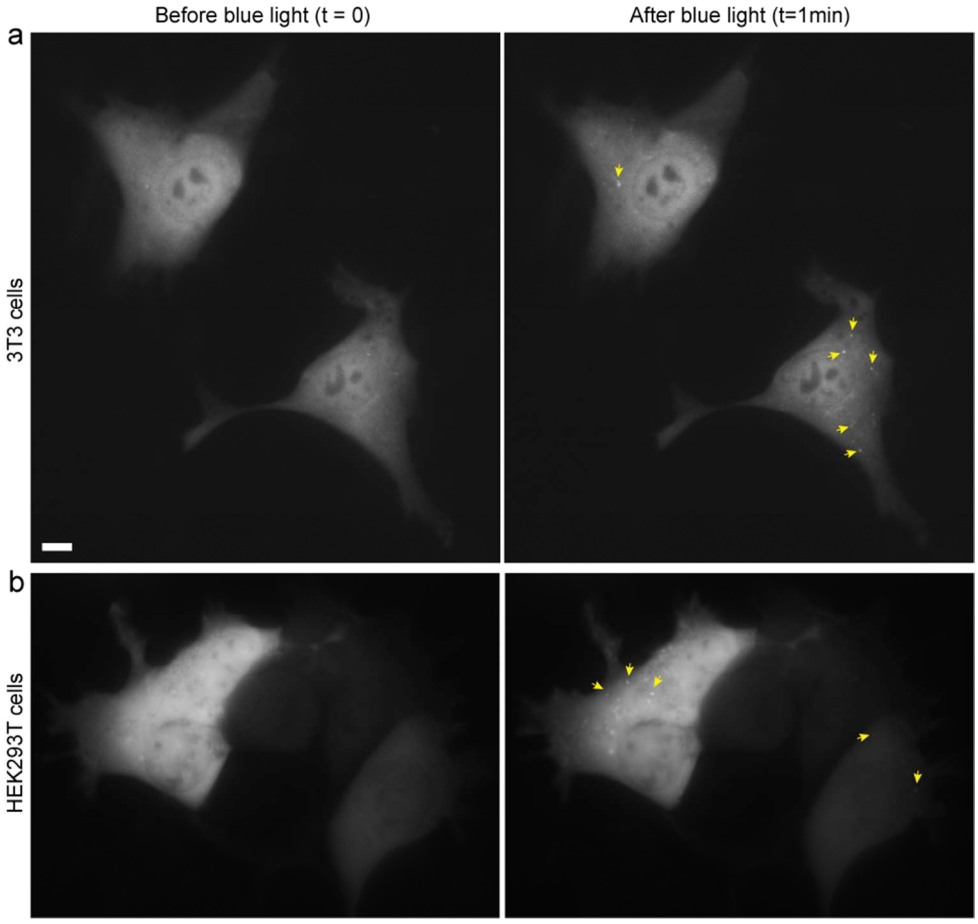

Figure S2. Cytoplasmic CRY2-mCh has low oligomerization propensity under blue light activation in 3T3 cells (a) and HEK293T cells (b). Cells were transfected with CRY2-mCh and activated with 200ms blue light pulse for every 5s. A few small CRY2 clusters are visible in each cell as indicated by yellow arrowheads. Scale bar, 10μm.

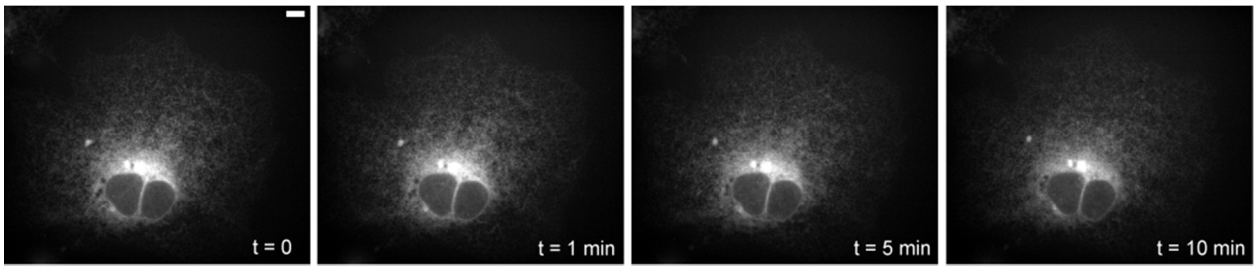

Figure S3. Green light illumination does not cause CRY2 oligomerization. Cell transfected with CRY2-mCh-Sec61 was exposed to green light (~550nm, $9.7x10^3 mW/cm^2$) every 5s at 200ms exposure for a total imaging time of 10 min.

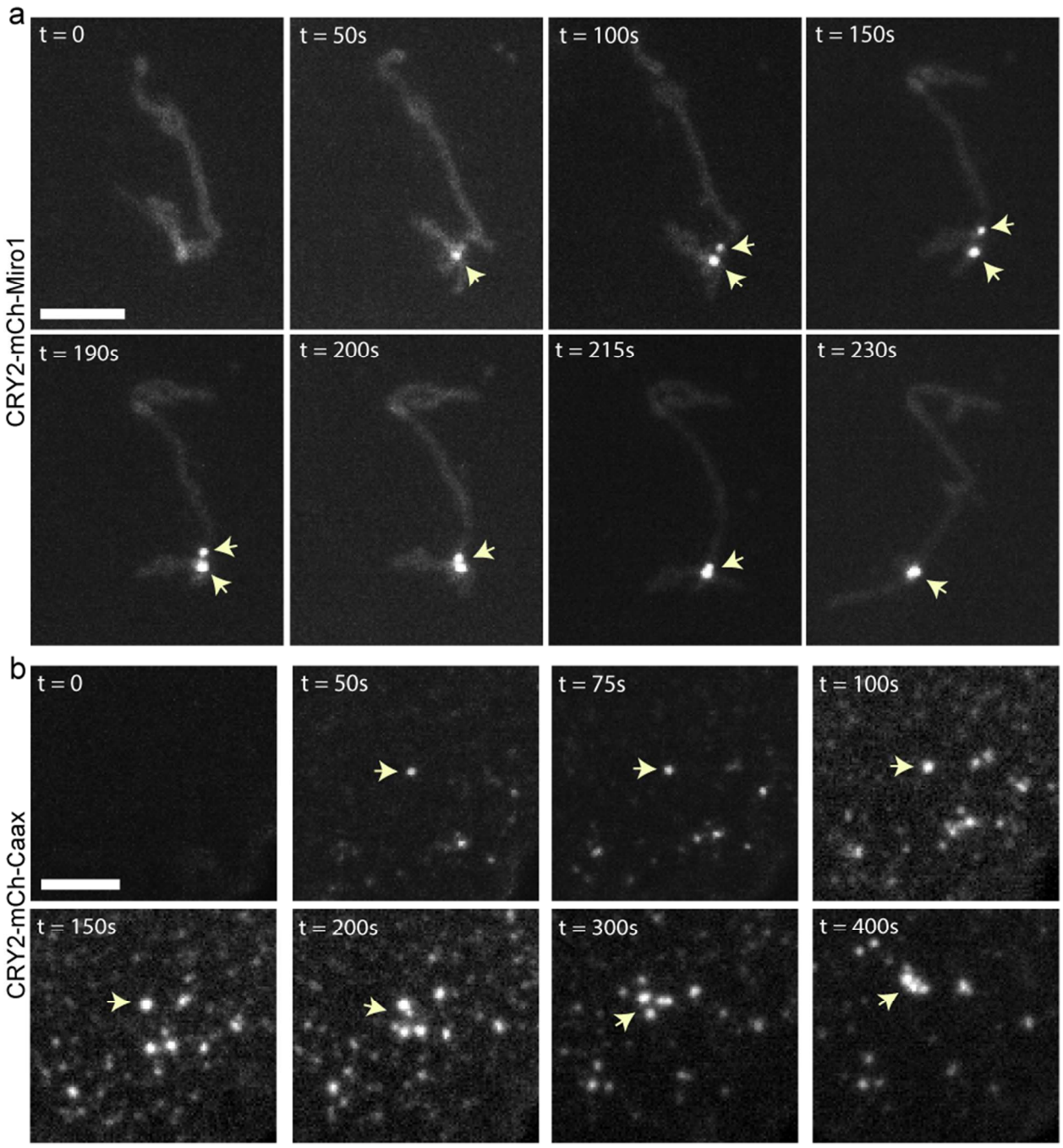

Figure S4. CRY2 bound to mitochondria membrane and plasma membrane show similar dynamic behavior compared to CRY2 bound to ER membrane. (a) CRY2 bound to the outer membrane of mitochondria form clusters seconds after blue light stimulation. The clusters grow in number and intensity and could fuse together to form larger clusters. (b) CRY2 bound to the plasma membrane show similar dynamic behavior upon blue light stimulation. CRY2 clusters increase in size and merged together to grow into larger oligomers on the plasma membrane (yellow arrow bars). Scale bars, 5μm.

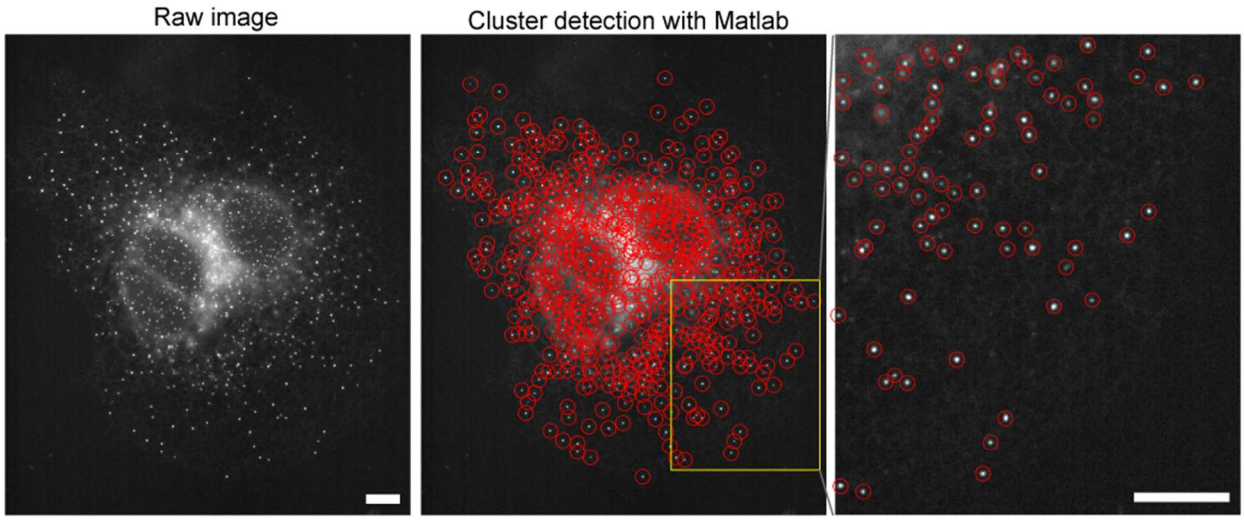

Figure S5. Automatic CRY2 cluster quantification using a custom-written Matlab program. The detected clusters in the cell (n=715) are marked by the red circles. Scale bars, 10μm.

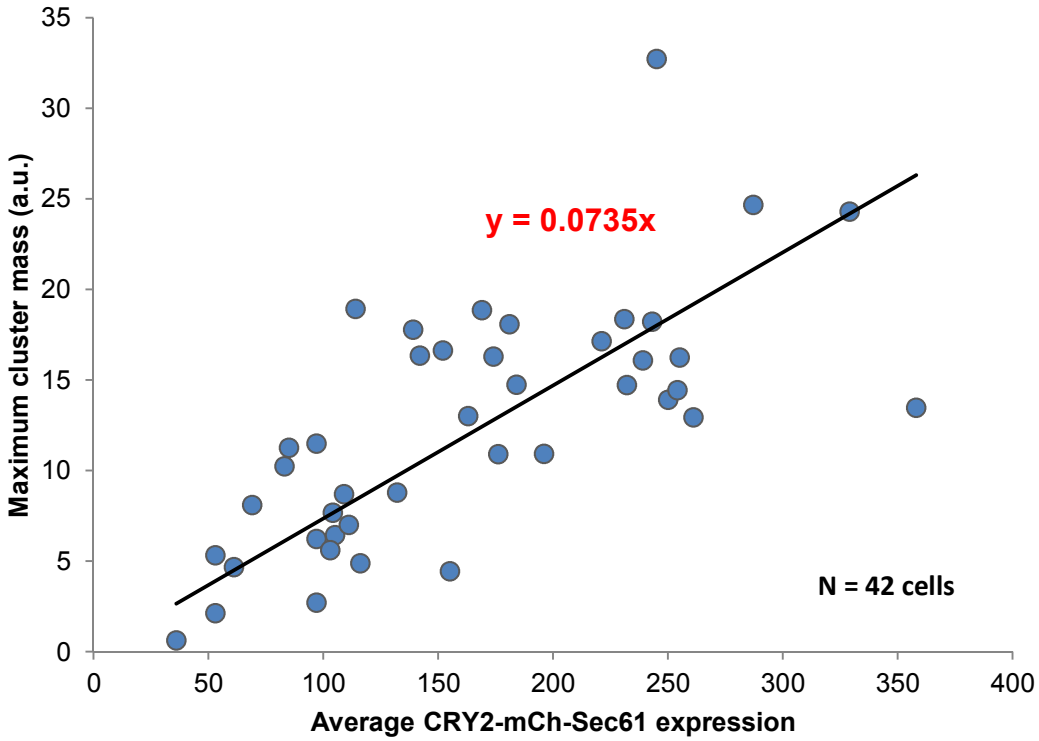

Figure S6. CRY2 oligomerization as indicated by the maximum cluster mass is quantified for 42 cells. A plot of the maximum cluster mass vs. the CRY2 expression level of individual transfected cells shows a linear relationship – higher CRY2 expression level results in higher CRY2 oligomerization.

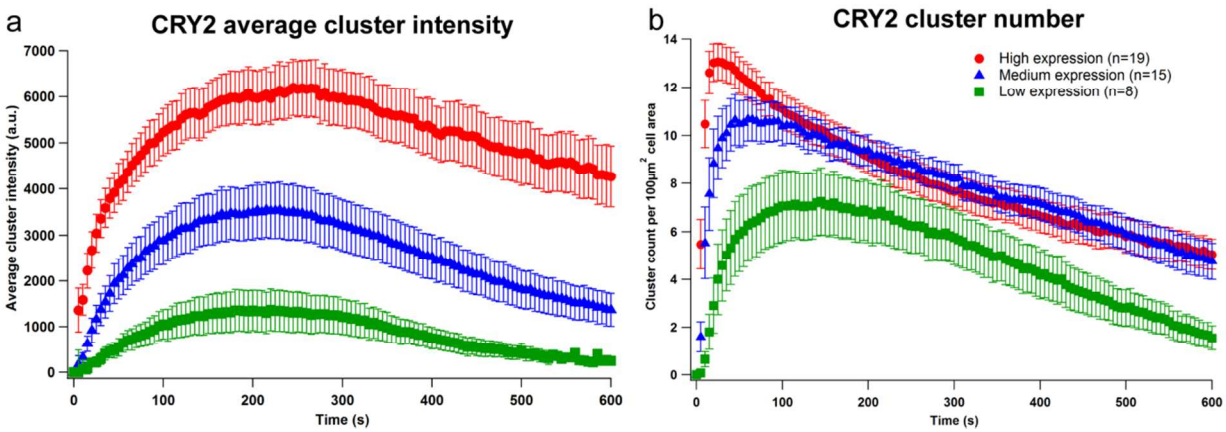

Figure S7. Both the number and the average intensity of CRY2 clusters formed on the ER membrane depend on the expression level of CRY2-mCh-Sec61. Cells were activated with a single 2s blue light pulse. CRY2 cluster detection and quantification were performed using an automatic Matlab algorithm as described earlier. (a) Average CRY2 cluster intensity in the cell. (b). Average CRY2 cluster count per 100µm^2 cell area. Plots show mean ± 1 s.e.m.

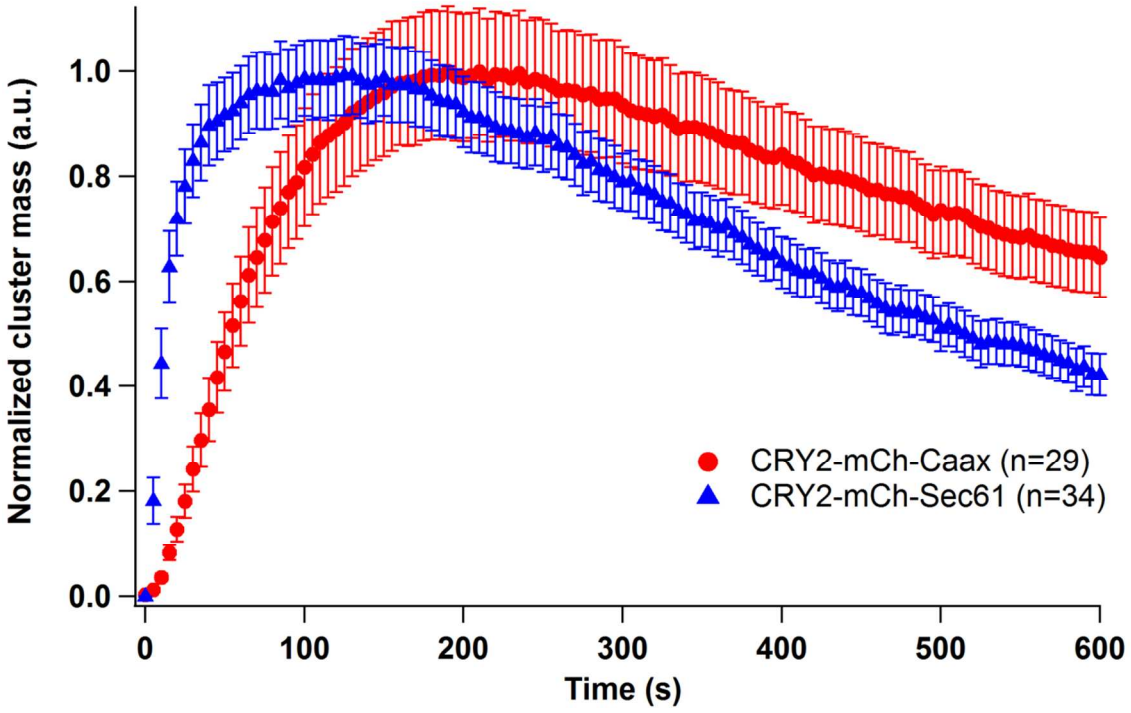

Figure S8. Comparing CRY2 cluster formation on the plasma membrane (CRY2-mCh-Caax) vs. on the ER membrane (CRY2-mCh-Sec61). Cells were activated with a single 2s blue light pulse. The cluster mass value in each cell was normalized to 1. Plot shows mean ± 1 s.e.m.

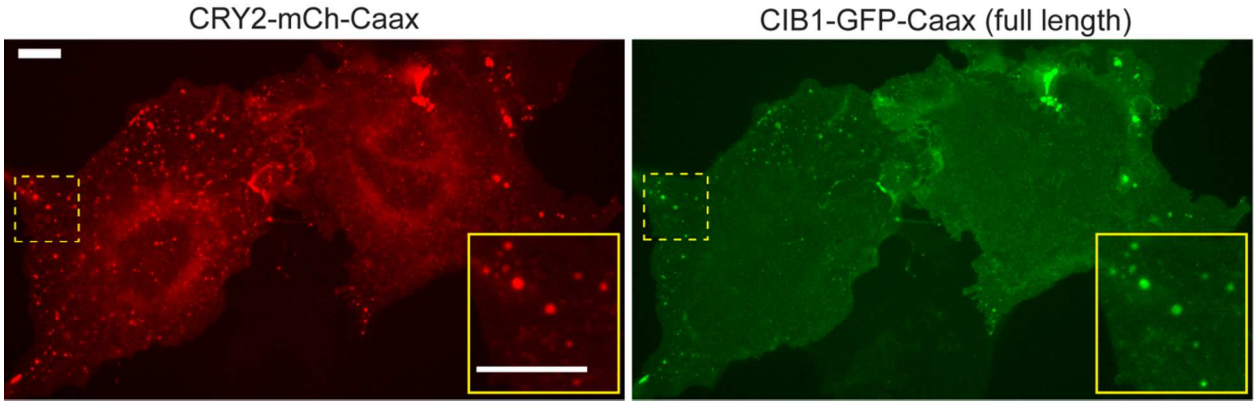

Figure S9. For full length CIB1 (1-335aa), CRY2 oligomerization and CRY2-CIB1 hetero-dimerization co-exist in the same system, very similar to the truncated CIB1 (1-170aa). Cells were co-transfected with CRY2-mCh-Caax and the full length CIB1(1-335aa)-GFP-Caax. Both CRY2 and CIB1 (1-335aa) were tethered to the plasma membrane via Caax motif. Upon blue light illumination (200ms blue light pulse every 5s), CRY2 form numerous bright clusters on the membrane. Full length CIB1 are also found to accumulate in the same clusters. Images were taken at 5min after blue light activation. Insets show enlarged regions marked in the dotted lines. Scale bars, 10μm.

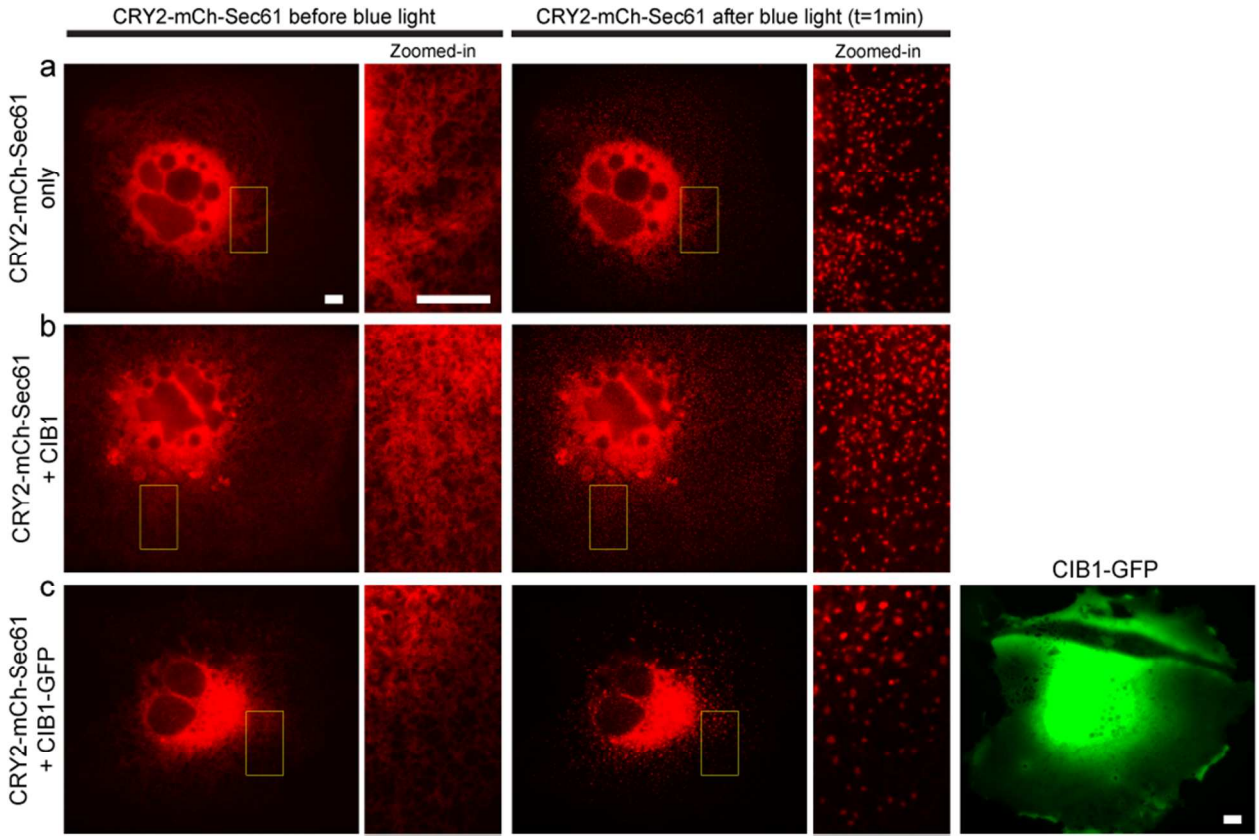

Figure S10. CRY2 oligomerization on the ER membrane readily occurs in the presence of CIB1 fusion proteins. Cells were illuminated with a single blue light pulse of 2s. (a) CRY2 clustering in a cell singly transfected with CRY2-mCh-Sec61. In cells co-transfected with CIB1 (b) or CIB1-GFP (c), the oligomerization is not visibly affected. Scale bars, 10um.

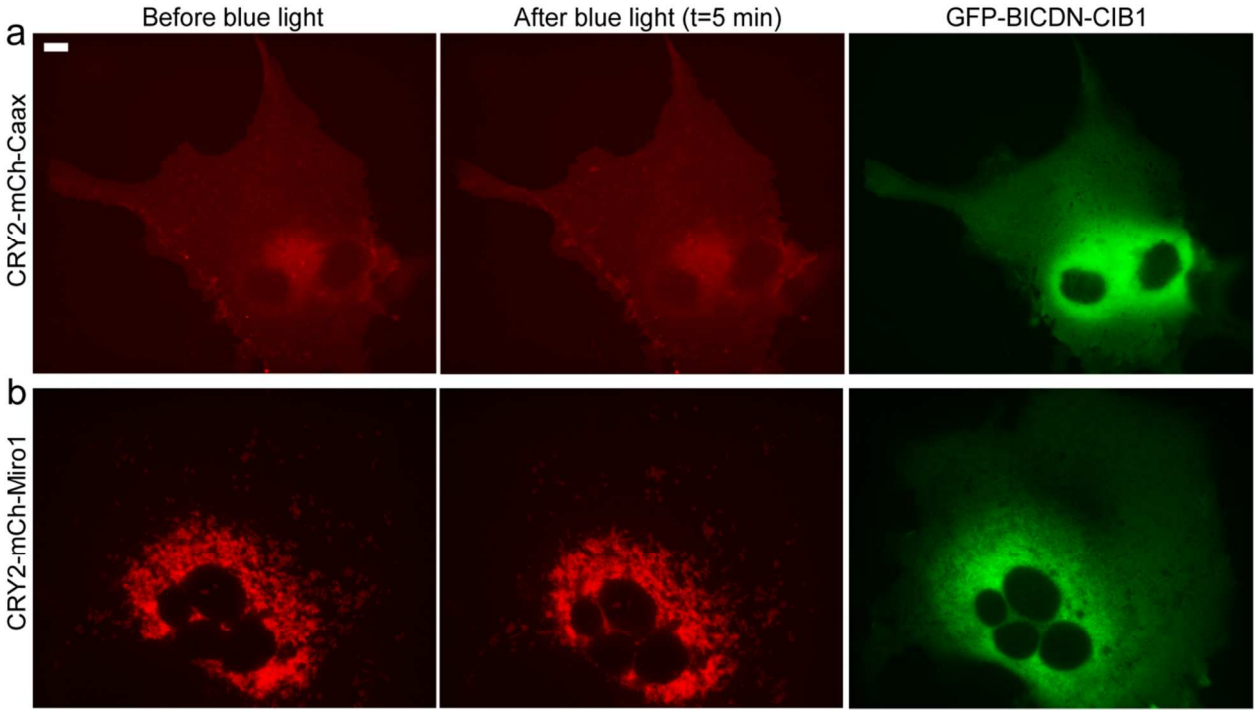

Figure S11. Co-expression of GFP-BICDN-CIB1 with either CRY2-mCh-Caax or CRY2-mCh-Miro1 completely blocks CRY2 cluster formation on the plasma membrane or the mitochondria membrane. COS-7 cells were illuminated with 200ms blue light pulse every 5s for 5min. (a) A COS7 cell co-transfected with CRY2-mCh-Caax and GFP-BICDN-CIB1 shows no CRY2 cluster after blue light stimulation. (b) A COS7 cell co-transfected with CRY2-mCh-Miro1 and GFP-BICDN-CIB1 shows no CRY2 cluster after blue light stimulation. Scale bar, 10μm.

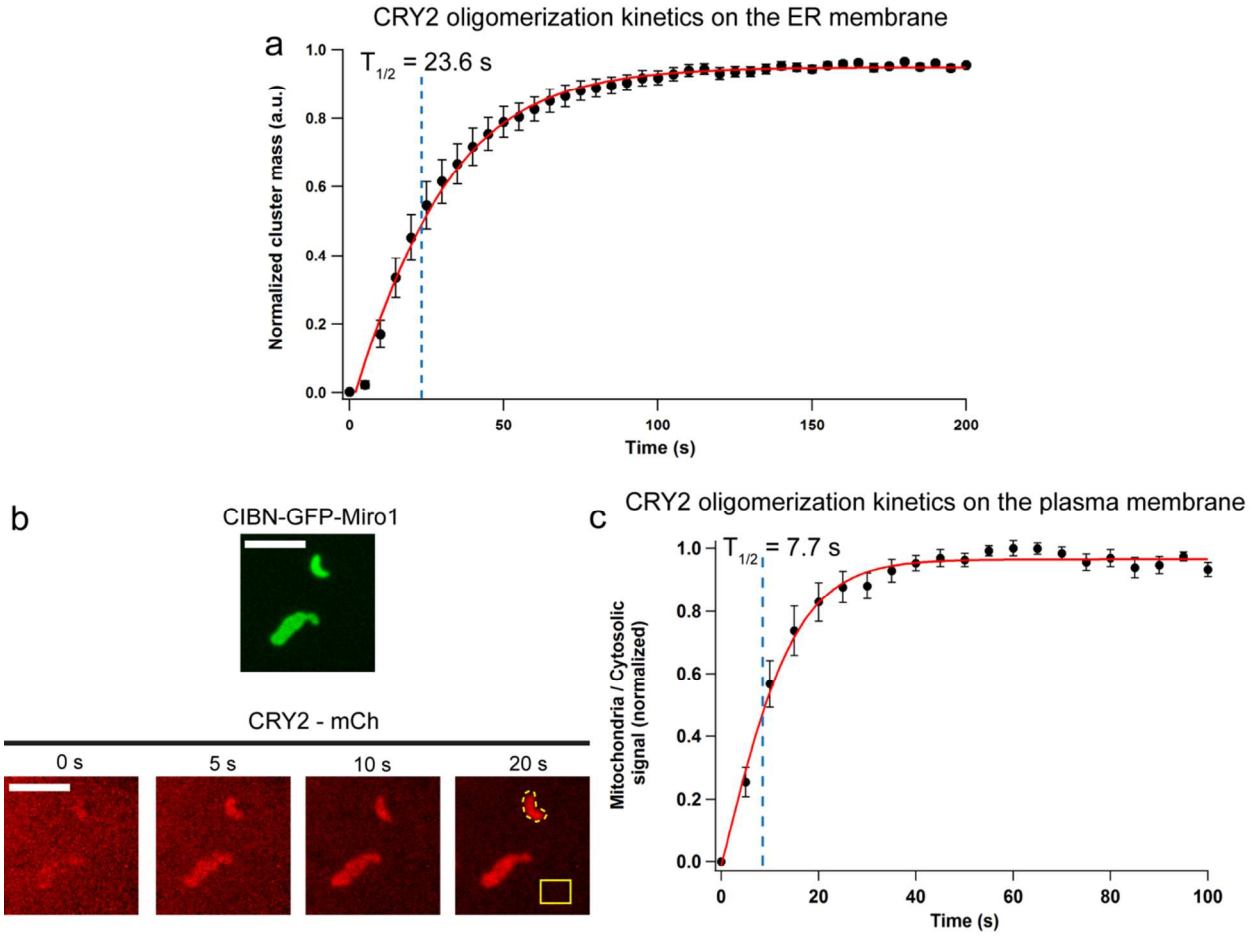

Figure S12. Quantifying the kinetics of CRY2-CRY2 and CRY2-CIBN interaction. COS-7 cells were exposed to intermittent blue light illumination (200ms blue light pulse at every 5s). (a) Measuring CRY2-CRY2 binding kinetics. Cells were singly transfected with CRY2-mCh-Sec61. The total cluster mass of all the visible CRY2 clusters in a cell increased sigmoidally over time upon blue light activation. Fitting this relationship to a sigmoidal function (IGOR, WaveMetrics) allowed us to extract $T_{1/2}$, the time at which the CRY2 cluster mass reached the half-maximal amount. The average $T_{1/2}$ value for CRY2 homo-oligomerization on the ER membrane is 23.6s. Plot shows mean ± s.e.m., n=13. (b-c) Measuring CRY2-CIB1 ibinding kinetics. Cell was co-transfected with CIBN-GFP-Miro1 and CRY2-mCh. (b) Upon blue light activation, cytoplasmic CRY2 was recruited to mitochondria. (c) Extracting $T_{1/2}$ value for CRY2-CIBN interaction by plotting the ratio of CRY2 mitochondria signal (region shown by dotted line) over the CRY2 cytosolic signal (region shown by solid rectangle). $T_{1/2}$ was extracted by fitting the data to a sigmoidal function (IGOR, WaveMetrics). Plot shows mean ± s.e.m. Data were averaged from measurement of 7 mitochondria in two different cells. Scale bars, 5μm.

References

(1) Zhang, K., Osakada, Y., Xie, W., and Cui, B. (2011) Automated Image Analysis for Tracking Cargo Transport in Axons, *Microscopy Research and Technique 74*, 605-613.