

Engineering of photo-inducible binary interaction tools for biomedical applications

Corresponding Author: Professor Yubin Zhou

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, Lee and colleagues introduce two novel photo-inducible binary interaction tools (PhoBITs), which offer versatile control over a range of protein functions, including optogenetic modulation of GPCRs, ion channels, necroptosis, and innate immune signaling, among others. Below are a few suggestions for improvement:

1. In the section on “optical control of GPCR activation,” the authors utilize the Tango assay to monitor PAR activation. While this method offers quantitative amplification of relevant signals, it would be valuable to directly assess the specific signaling events triggered by PAR activation. Including this additional analysis would strengthen the overall findings.

2. The authors design PhoBIT for light-induced necroptosis, a strategy also explored in “Adv Sci, 2021 May 6; 8(13): 2100424” where MKLK is fused directly with CRY2. This construct appears simpler, and a direct comparison between the two approaches would be useful to highlight the advantages of the PhoBIT system. The same comparative experiments with previous work would be valuable for the calcium signaling activation shown in Figure 4.

3. Figure 7d shows observable aggregation of ssrA2(S2)-HA4-7c12 under light exposure; however, the co-localization changes in the merged image are not clearly evident. I suggest providing higher-quality representative images to clarify these findings. Additionally, the fluorescence images in the second row of Figure 3g should be improved for better resolution.

4. WT HA4-7c12 demonstrates inhibitory effects on BCR-ABL, however, in Figure 7f, the Western blot results indicate that the phosphorylation level of ABL in the A1+B group remains notably high, which is somewhat puzzling and warrants further clarification in the manuscript.

5. While the authors briefly mention blue light-induced dimerization and dissociation in the background, a more comprehensive discussion of other light-responsive tools, such as UV and red light-induced systems, would be beneficial. This addition would provide readers with a broader understanding of the optogenetic toolbox and its various applications.

Reviewer #2

(Remarks to the Author)

The authors developed 2 impressive tools in the manuscripts. These new optogenetic oligomerization and dissociation module can provide useful tools for biologists interested in various questions. The main target audience for such manuscript are either the potential users or protein engineers that wish to adapt such system to their biology and their systems and these results and findings are exciting, the studies do have some major shortcomings that should be addressed in the next revision for these potential users and adapters.

1. The study has a lot of qualitative result but surprising limited quantitative analysis of the data. There are often descriptions of ‘folds change’ in the text which is an important value but several values that are also critical are not presented or analysed. There should be statistical comparison of baseline values especially to no expression control (to compare background activity), statistical comparison of peak values (especially against existing tools or positive controls), in addition to folds change presented in the manuscript. There should also be also more description of values in the texts of the results

section.

2. There are insufficient negative controls in several experiments. There are no no-expression controls in the experiment in Fig 4, 6 and 7. Negative controls are important to compare the background activities associated with overexpression. Several experiments also show elevated background activities associated with the expression (e.g., Fig 2d, 4g and 6g) but there is no discussion, analysis of these differences.
3. There are no comparisons with existing tools. The dissociation tool should be compared to the LOV-TRAP system(s) for the background, peak values and folds change. For the second tool, it should be compared to Cry2olig with CIB1 in terms of performance.
4. Despite the first tool has one component that is smaller, there are no experiments that demonstrates that the small size is advantageous.
5. Figure 1K, the Y-axis is 'relative EGFP expression' – what is this relative to? There is very little information on this in the methods section.
6. Generally there is insufficient description of how the data are analyzed in the methods. The description of cells selected for analysis and whether any cells were eliminated and not analysed were also lacking. The description of how mitochondria and aggregation are selected for measurements, how
7. Fig 4c is unusual in that it does not show the individual data points as the other experiments. This is also one where it is important to show the statistical comparison of baseline (dark) values.
8. For the first tool it would be nice to see whether the tools still work well when expressed more than 24 hours. Does the imbalance of expression cause higher background 'leak' activity? Can this account for the elevated baseline shown in the Tango system?
9. It would be valuable and important to test whether there is any correlation between expression level and background 'leak' activities of both optogenetic modules. It would also be very important to test whether there is any importance to balance the expression of the 2 components in real experiments.

Minor point:

10. The panels in the figures 1 and 3 are hard to follow due to the inconsistent sizes and orders. The lack of regular 'grid' layout makes it confusing and laborious for the readers to follow. As several experiments are combined into one figure, the current layout where panels can either be below, to the right or to the right and below makes it difficult to match the panels to the legend text. The schematic diagram and results can be made into more consistent sizes between panels and the layout should be made into a 'grid' layout.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all my concerns. I have no further comments.

Reviewer #2

(Remarks to the Author)

The revised manuscript is much improved with additional control and more attempts of quantification. There are still some statistical analysis that can be improved or clarified. However, these corrections and clarifications are rather minor at this stage.

1. For the western blots, it will need quantification and statistical comparison. For Fig 6f, although the effects are obvious, the quantification is important to show that the control proteins are the same amount as they can appear to be saturated visually. For Fig. 7f, this is more important as there can be a small difference in the samples that are visually hard to distinguish.
2. For Fig 6g, an ANOVA should be used to compare the baselines of the samples and the peaks of the sample. In addition, poly I:C is not defined.
3. For Fig 7j, an ANOVA should be used with post-hoc test against the WT in the dark.
4. For Fig 4c, it is important to comment whether the different baselines are statistically different to the control.
5. For the multiple comparisons in Suppl Fig 1c, 3f/g, 6d & 7, the appropriate tests should be ANOVA with appropriate post-hoc tests. Student's t-test is only good for comparison of 2 samples and have no multiple-comparison correction.

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Point-to-point responses

We would like to express our gratitude to the editor and reviewers for your insightful and constructive feedback, which has significantly enhanced our manuscript. We have carefully addressed all the points raised and made necessary clarifications to the text and supplementary materials. All changes are highlighted in **blue font**. Additionally, a table summarizing the updated and revised Figures has been included below for your reference (**Table A1**).

Table A1. Major new data added in the revised manuscript.

Figure/ Panel Info	Original Figure No.	Response to Reviewer(s)	Remarks
1	1	R2.10	Reorganized panels
1d	1d	R2.3	Compared to existing tools
3	3	R2.10	Reorganized panels
3g	3g	R1.3	Replaced with high resolution confocal images
3h	3h	R2.3	Compared to existing tools
4c	4c	R1.2, R2.1, R2.2, R2.7	Added controls/ compared to existing tools
6	6	R2.2	Added negative controls
7d	7d	R1.3	Confocal images of ssrA2-monobody
7f	7f	R1.4, R2.2	Added negative controls/discussed caveats
S1c	New	R2.1, R2.3	Included raw data/compared to existing tools
S1e-f	New	R2.8, R2.9	Examined leaky activities
S3a-d	New	R1.1	Examined GPCR specific signaling
S3e-f	New	R2.4	Demonstrated advantage of small size of ssrA tag
S3g	New	R2.8, R2.9	Examined leaky activities
S4	S4	R2.3	Compared to the existing tool
S6	New	R2.2	Performed lower background activity assays
S7	New	R1.2	Compared to the existing tool
S8	S8	R2.2	Added negative controls

Reviewer #1 (Remarks to the Author):

We deeply appreciate the reviewer for the overall supportive remarks.

Response to R1 comments

1.1 *"In the section on "optical control of GPCR activation," the authors utilize the Tango assay to monitor PAR activation. While this method offers quantitative amplification of relevant signals, it would be valuable to directly assess the specific signaling events triggered by PAR activation. Including this additional analysis would strengthen the overall findings."*

Response: We appreciate the reviewer's valuable suggestion to assess the endogenous signaling pathways of protease-activated receptor G protein-coupled receptors (PAR-GPCRs). In response, we measured Ca^{2+} influx following light stimulation (**Fig. R1; new Supplementary Fig. 3a-d**). Specifically, we focused on Opto-PAR4, as it exhibited the most robust light-induced Tango activation (**Fig. 2d**).

Upon activation, PAR4 triggers two major signaling pathways: β -arrestin and G protein α_q ($\text{G}\alpha_q$). β -arrestin initiates various downstream cascades, including the activation of MAPK family members such as extracellular signal-regulated kinases (ERK), whereas $\text{G}\alpha_q$ activates phospholipase C β ($\text{PLC}\beta$), which catalyzes the hydrolysis of phosphatidylinositol to generate inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). These second messengers promote intracellular Ca^{2+} mobilization from internal stores and activate Ca^{2+} -regulated kinases and phosphatases (Price et al., 2021) (**Fig. R1a**). We chose to assess Ca^{2+} release using the ultra-sensitive Ca^{2+} indicator NEMOf (Li et al., 2023) for two reasons. First, the addition of the V2 vasopressin receptor carboxyl tail in the PAR-Tango system promotes the recruitment of β -arrestin, thereby masking the endogenous interaction between PAR4 and β -arrestin and downstream ERK1/2 activation. Thus, assessment of the β -arrestin-dependent pathway (**Fig. R1a**, right, red arrows) was not an optimal indicator for PAR activation. Second, measuring Ca^{2+} influx with a fluorescent reporter (**Fig. R1a**, left, blue arrows) provides a straightforward and robust assay that reflects the activation of $\text{G}\alpha_q$ signaling.

Confocal imaging revealed that Ca^{2+} influx occurred following Opto-PAR4 activation, with a half-life of 25.3 seconds (**Fig. R1b-c**) and a 4.7-fold increase in NEMOf signal (**Fig. R1d**). Collectively, these data demonstrate that the specific signaling pathway—in this case, Ca^{2+} influx—can be observed upon activation of Opto-PAR4.

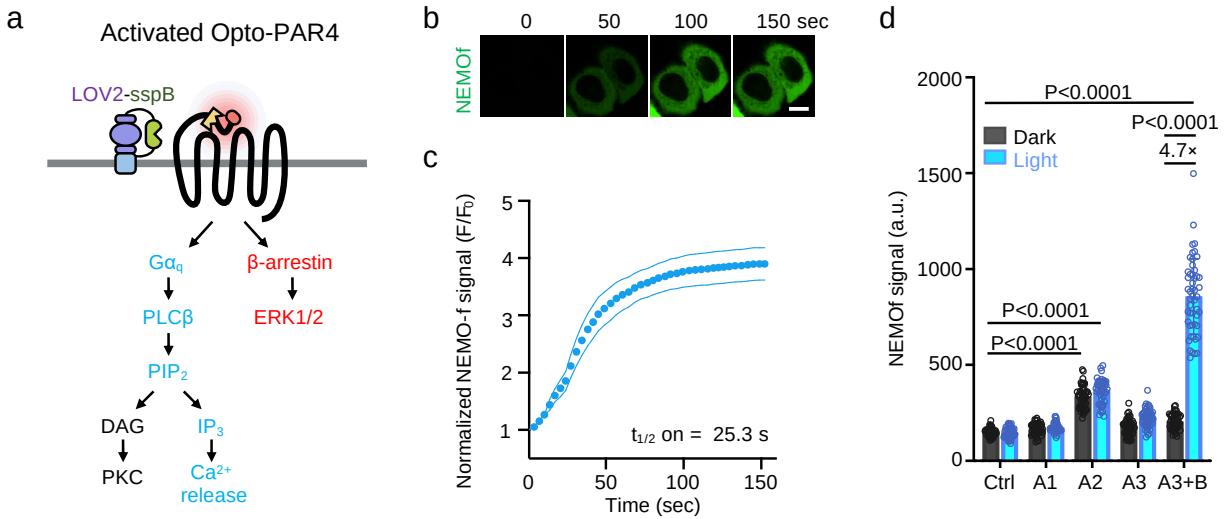


Fig. R1 | PhoBIT1 activates GPCR-mediated Ca^{2+} influx. (a) Schematic of PAR4-dependent signaling pathways. Upon activation, PAR4 triggers two distinct signaling pathways. Left: The $G\alpha_q$ - $PLC\beta$ signaling axis causes PIP_2 hydrolysis to generate two key second messengers: (1) IP_3 induces Ca^{2+} release from ER stores, while (2) DAG activates PKC . Blue font indicates the pathway assessed in this study using the Ca^{2+} indicator NEMOf. Right: The β -arrestin-dependent pathway (red font) was not suitable for assessment of Opto-PAR4 activation because the addition of V2 vasopressin receptor carboxyl tail masks the endogenous interaction between PAR4 and β -arrestin and downstream $ERK1/2$ activation. (b) Representative confocal images showing light-mediated Ca^{2+} influx in HeLa cells transfected with the A3+B combination in **Fig. 2c** at indicated time points. Ca^{2+} was visualized with NEMOf (green). Scale bar, 10 μ m. (c) Light-mediated Ca^{2+} influx in HeLa cells transfected with the A3+B combination as a function of time. Ca^{2+} influx was reported by NEMOf. The data were fit using a one-phase decay function, with the half-life determined to be 25.3 seconds. Data are shown as mean $\pm$ s.e.m. $n = 10$ cells from three independent biological replicates. (d) Quantification of NEMOf signal in HeLa cells expressing plasmid constructs shown in **Fig. 2c**. Ctrl: NEMOf only. Data are shown as mean $\pm$ SD. Statistical significance was determined with two-sided unpaired Student's t-test. $n = 45$ -50 cells from three independent biological replicates. Abbreviations: PIP_2 : Phosphatidylinositol 4,5-bisphosphate; IP_3 : Inositol 1,4,5-trisphosphate; $PLC\beta$: Phospholipase C beta; ER: Endoplasmic reticulum; DAG : Diacylglycerol; PKC : Protein kinase C; $ERK1/2$: Extracellular signal-regulated kinases 1/2.

We have added the following sentences to the Results section: “To determine whether the downstream pathways inherent to PAR4 activation can be triggered by light, we assessed Ca^{2+} influx mediated through the $G\alpha_q$ - $PLC\beta$ - PIP_2 - IP_3 axis using the

ultra-sensitive Ca^{2+} indicator NEMOf (**Supplementary Fig. 3a**). Upon blue light stimulation, we observed a 4.7-fold increase in NEMOf intensity with a half-life of 25.3 seconds, consistent with light-induced activation of $\text{G}\alpha_q$ signaling (**Supplementary Fig. 3b-d**)."

1.2 "The authors design PhoBIT for light-induced necroptosis, a strategy also explored in "Adv Sci, 2021 May 6; 8(13): 2100424" where MKLK is fused directly with CRY2. This construct appears simpler, and a direct comparison between the two approaches would be useful to highlight the advantages of the PhoBIT system. The same comparative experiments with previous work would be valuable for the calcium signaling activation shown in Figure 4."

Response: We agree that providing a direct comparison between PhoBITs-based tools and previously developed systems is helpful. Accordingly, we included the efficacy of light-induced necroptosis using the previously published LiPOP1 system in **Fig. 5** and assessed the light-induced Ca^{2+} signaling using the previously reported mCherry-CRY2-STIM1ct (233-685) (Ma et al., 2020) in **Fig. 4**.

First, we compared our light-induced necroptosis system with previously established tool LiPOP1 (He et al., 2021). Our results indicate that the A2+B group shows comparable levels of light-induced necroptosis versus LiPOP1, but with slightly lower background activation and higher overall necroptosis following 6 hours of pulsed illumination (**Fig. R2**; new **Supplementary Fig. 7**).

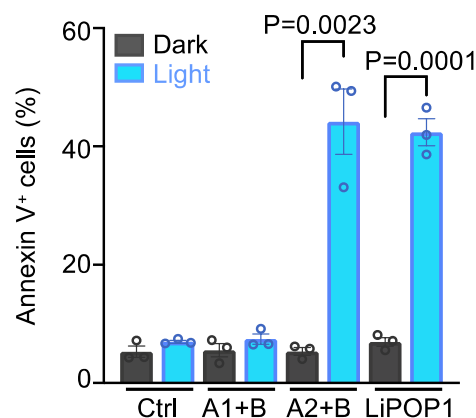


Fig. R2 | Quantification of light-induced necroptosis. Annexin V staining was applied to evaluate necroptosis in HeLa cells expressing the indicated constructs (as described in **Fig. 5b**), in the absence (gray bar) or presence (blue bar) of photostimulation. Ctrl: cells transfected with the empty vector; LiPOP1: mCh-CRY2-MKL-NT(3A). Data are shown as mean $\pm$ s.e.m. $n = 70$ -100 cells from three

independent biological replicates. Statistical significance was determined with two-sided unpaired Student's t-test.

We have included the following sentences to the Results section: “Additionally, MLKL-NT-ssrA2-mCherry exhibited comparable levels of light-induced necroptosis to those of the established tool LiPOP1 following light illumination (**Supplementary Fig. 7**).”

We have added the sentences to the related Method section: “For the cell viability assay, LiPOP1 made of mCherry-CRY2-MLKL-NT(3A) was used as the positive control (He et al., 2021). Confocal imaging was performed 4 hours after stimulation with pulsed 470 nm blue light cycles (30 s ON, 3 min OFF).”

Second, we compared the light-induced Ca^{2+} influx of our PhoBIT2-STIM1ct construct with that of mCherry-CRY2-STIM1ct (233–685), a tool previously made by our lab known to exhibit robust light-induced Ca^{2+} influx via CRY2-mediated oligomerization (Ma et al., 2020). As a control, we expressed only the Ca^{2+} indicator NEMOf in HeLa cells (**Fig. R3; modified Fig. 4c**). Our results showed that PhoBIT2-STIM1ct, created by inserting ssrA2 at site 685, exhibited comparable Ca^{2+} influx in response to blue light stimulation. Specifically, PhoBIT2-STIM1ct showed a 7.4-fold increase in NEMOf intensity upon blue light stimulation, compared to a 6.8-fold increase observed with mCherry-CRY2-STIM1ct (233–685).

We also observed elevated basal Ca^{2+} level in several STIM1ct variants with ssrA2 insertions, with the most pronounced increase at sites 276 and 463. In contrast, insertion at site 343 and mCherry-CRY2-STIM1ct (233–685) exhibited only minimal basal Ca^{2+} level under the dark condition. Notably, the activation level of ssrA2-STIM1ct (site 685) was comparable to that of mCherry-CRY2-STIM1ct (233–685), with mean NEMOf intensities of 1240.1 and 1302.8, respectively. However, ssrA2-STIM1ct (site 685) exhibited lower basal activity than mCherry-CRY2-STIM1ct (233–685), with mean NEMOf intensities of 168.7 and 192.4, respectively.

In conclusion, we demonstrated that the PhoBIT2 system effectively activates Ca^{2+} influx at levels comparable to the established tool mCherry-CRY2-STIM1ct (233–685) and yet exhibits lower basal activity in the dark.

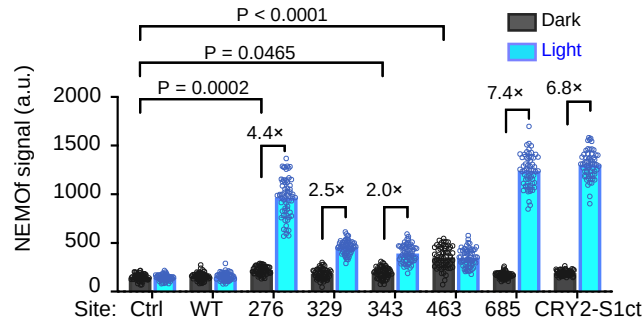


Fig. R3 | Quantification of light-induced changes of intracellular Ca^{2+} levels (indicated by NEMOf) in HeLa cells expressing the indicated *ssrA2*-inserted STIM1ct variants and mCh-CRY2-sspB2. *ssrA2*-STIM1ct (site 685) showed the most notable changes and was used in experiments shown in d-g. **Ctrl:** HeLa cells expressing only the calcium indicator NEMOf. **WT:** STIM1ct (233-685) without *ssrA2* insertion. **CRY2-STIM1ct:** mCherry-CRY2-STIM1ct (233-685) construct previously reported by us. Data are shown as mean $\pm$ SD. $n = 55$ -60 cells from three independent biological replicates. Statistical significance was determined with two-way ANOVA.

We have added the following sentences to the Results section: “Notably, the light-induced activation is comparable to that of the previously established tool, mCherry-CRY2-STIM1ct (233-685) (**Fig. 4c**) (Ma et al., 2020).”

1.3 “Figure 7d shows observable aggregation of *ssrA2*(S2)-HA4-7c12 under light exposure; however, the co-localization changes in the merged image are not clearly evident. I suggest providing higher-quality representative images to clarify these findings. Additionally, the fluorescence images in the second row of Figure 3g should be improved for better resolution.”

Response: We thank the reviewer for bringing this critical point that could potentially confuse readers.

First, we have provided a new set of high-quality zoomed-in images to replace the previous **Fig. 7d**. These new images clearly show the increased mCherry fluorescence intensity on mitochondria (**Fig. R4; modified Fig. 7d**).

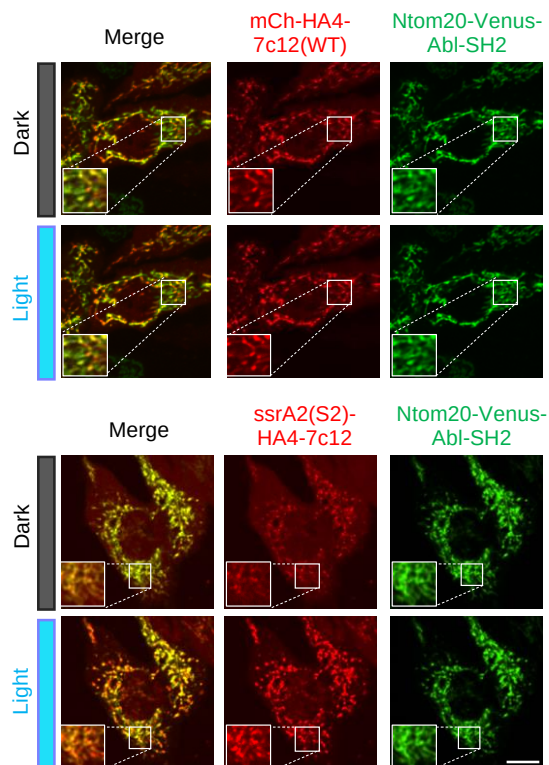


Fig. R4 | Light-induced enhanced binding of ssrA2-inserted monobody HA4-7c12. Confocal images of HeLa cells co-expressing the fusion monobody HA4-7c12 with or without ssrA2 insertion (top panel, WT; bottom panel, S2 variant) and Ntom20-Venus-Abl-SH2 upon blue light stimulation. **Enlarged mitochondria regions are shown in white boxed insets.** Scale bar, 10 μ m.

Second, to address the reviewer's concern related to potential confusion caused by the second row of **Fig. 3g**, we have updated these images (**Fig. R5; modified Fig. 3g**) to better represent the cytoplasmic distribution pattern of mCh-CRY2-sspB-WT, resulted from the disrupted interaction due to ssrA mutation A2W. Additionally, we have provided further explanations in the results section to clarify this point.

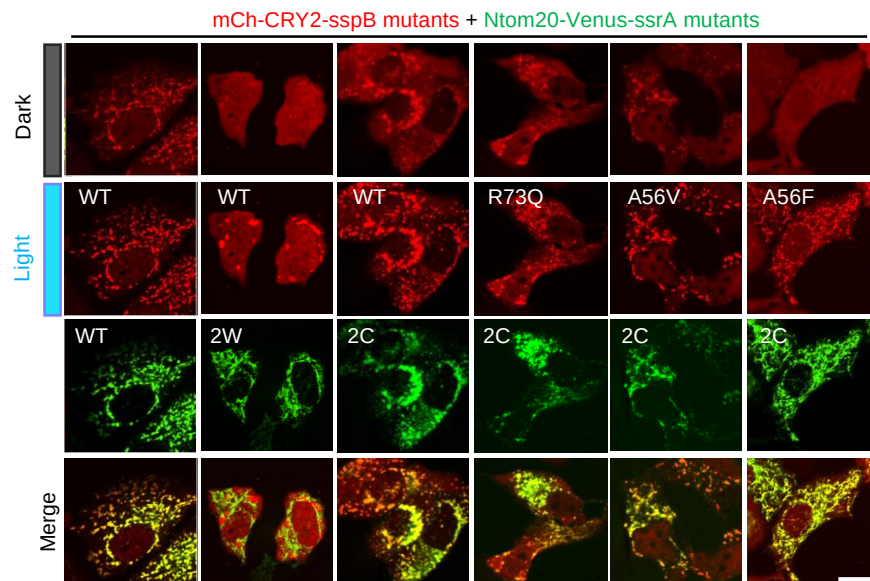


Fig. R5 | Comparison of *ssrA* and *sspB* mutations. Representative confocal images of HeLa cells co-expressing the indicated combinations of mitochondria-anchored *ssrA* mutants (Venus-tagged; green) and CRY2-fused *sspB* mutants (mCherry-tagged; red), before and after photostimulation. Scale bar, 10 μ m.

Additional sentences in the Results section: “In contrast, the *ssrA*-A2W variant markedly disrupted the interaction with mCh-CRY2-*sspB*-WT, leading to a diffuse cytoplasmic distribution of mCh-CRY2-*sspB*-WT in HeLa cells in the dark (Fig. 3g). Upon blue light stimulation, mCh-CRY2-*sspB*-WT formed clusters that failed to colocalize with mitochondrial-anchored *ssrA*-A2W (Fig. 3g).

1.4 “WT HA4-7c12 demonstrates inhibitory effects on BCR-ABL, however, in Figure 7f, the Western blot results indicate that the phosphorylation level of ABL in the A1+B group remains notably high, which is somewhat puzzling and warrants further clarification in the manuscript.”

Response: We sincerely appreciate the reviewer’s insightful comment. In response, we have repeated the Western blot experiment and incorporated the negative control: lysate of wild-type K562 cells (K562WT) (Fig. R6; modified Fig. 7f). The updated results clearly demonstrate that K562WT exhibits substantially elevated Bcr-Abl phosphorylation, whereas groups expressing the tandem monobody HA4-7c12 (A1+B and A2+B) show a marked reduction in Bcr-Abl phosphorylation, suggesting that HA4-7c12 overexpression strongly inhibits this phosphorylation. Additionally, consistent with our previous observation that *ssrA*2-HA4-7c12 exhibits lower basal binding affinity

compared to HA4-7c12(WT) (**Fig. 7d**), the A2+B group displays higher Bcr-Abl phosphorylation levels without light stimulation (A2+B; dark). However, upon light stimulation, ssrA2-HA4-7c12 effectively suppresses Bcr-Abl phosphorylation, as indicated by the reduced band intensity (**Fig. R6; modified Fig. 7f**). In summary, these results firmly validate the light-induced inhibition of Bcr-Abl phosphorylation. Compared to K562WT, both HA4-7c12 groups (A1+B and A2+B) exhibit reduced Bcr-Abl phosphorylation, with A1+B showing stronger, light-independent inhibition and A2+B demonstrating robust, light-dependent inhibition.

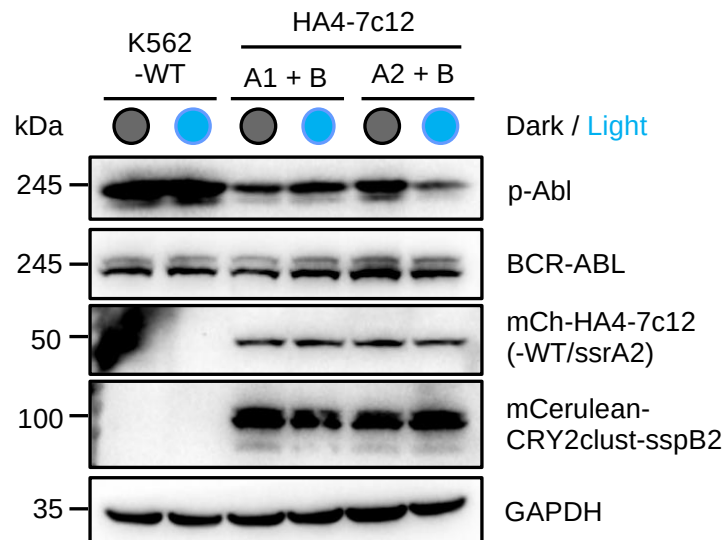


Fig. R6 | WB analysis of inhibition of Bcr-Abl phosphorylation. Western blot detecting transduced constructs and the non-expression control (K562WT), endogenous BCR-ABL and its phosphorylation state upon dark (grey circle) or blue light stimulation (blue circle) in K562 cells.

We have updated the Results section as follows: “Western blot analysis revealed light-dependent inhibition of Y412 phosphorylation in K562 cells co-expressing the A2+B constructs (**Fig. 7f**), whereas cells co-expressing A1+B (lacking ssrA2) showed minimal change upon light stimulation. This is consistent with the weaker pre-binding observed with ssrA2-HA4-7c12 in the dark (**Fig. 7d**). The inclusion of a K562WT control (**Fig. 7f**, left two lanes) confirmed high basal Bcr-Abl phosphorylation levels.”

1.5 “While the authors briefly mention blue light-induced dimerization and dissociation in the background, a more comprehensive discussion of other light-responsive tools, such as UV and red light-induced systems, would be beneficial. This addition would provide readers with a broader understanding of the optogenetic toolbox and its various applications.”

Response: We sincerely appreciate the reviewer's insightful suggestion to discuss alternative light-responsive systems. In response, we have expanded our discussion to include the potential integration of red-light- and UV-sensitive modules (e.g., BphP1 or UVR8) into the PhoBIT platform. These additions emphasize the versatility of our engineered ssrA/sspB pairs and outline future directions for achieving precise spatiotemporal control in complex tissues.

We have added the following sentences in the Introduction section: “Beyond these blue-light- systems, additional optogenetic modules responsive to other wavelengths, such as UV and red light, have been developed. The UVR8-COP1 system utilizes UV-B light to regulate protein-protein interactions. Moreover, red and near-infrared (NIR) responsive systems, such as PhyB-PIF and BphP1-QPAS1, offer greater tissue penetration depth, making them particularly advantageous for in vivo applications (Anselm et al. 2009, Taras et al. 2017, Tan et al., 2022; Zhou et al., 2025). Incorporating these alternative wavelength-responsive optogenetic modules enables multiplexed, wavelength-specific control of distinct cellular functions.”

More sentences have been added to the Discussion section as well: “While PhoBITs primarily utilize blue-light-responsive photoswitches (LOV2 and CRY2), optogenetic systems operated by alternative wavelengths offer complementary advantages. For instance, the UV-B-responsive UVR8-COP1 system provides confined spatial control due to the limited penetration depth of UV light. Conversely, red and near-infrared (NIR) light-responsive systems, such as PhyB-PIF and BphP1-QPAS1, enable deeper tissue penetration and are particularly useful for in vivo applications (Tan et al., 2022, Qiao et al., 2024). Integrating these alternative modules into future PhoBITs design could significantly expand the optogenetic toolbox, enabling multiplexed regulation of protein interactions. By leveraging different wavelengths, future optogenetic control could achieve even greater precise and orthogonality in complex biological systems.”

Reviewer #2 (Remarks to the Author):

The authors developed 2 impressive tools in the manuscripts. These new optogenetic oligomerization and dissociation module can provide useful tools for biologists interested in various questions. The main target audience for such manuscript are either the potential users or protein engineers that wish to adapt such system to their biology and their systems and these results and findings are exciting, the studies do have some major shortcomings that should be addressed in the next revision for these potential users and adapters.

We sincerely thank reviewer for the supportive remarks from R2 that “*The authors developed 2 impressive tools in the manuscripts. These new optogenetic oligomerization and dissociation module can provide useful tools for biologists interested in various questions.*”

Response to R2 comments

2.1 “*The study has a lot of qualitative result but surprising limited quantitative analysis of the data. There are often descriptions of ‘folds change’ in the text which is an important value but several values that are also critical are not presented or analysed. There should be statistical comparison of baseline values especially to no expression control (to compare background activity), statistical comparison of peak values (especially against existing tools or positive controls), in addition to folds change presented in the manuscript. There should also be also more description of values in the texts of the results section.*”

Response: We thank the reviewer for the great advice. We have followed the valuable suggestion to improve the rigor of this study. We have included the following data:

First, we have included the raw data corresponding to **Fig. 1d**, which illustrate the light-induced dissociation of mitochondria across various ssrA insertion sites in PhoBIT1 (see **Fig. R7; Supplementary Fig. 1c**). The figure legend and source data have been updated accordingly.

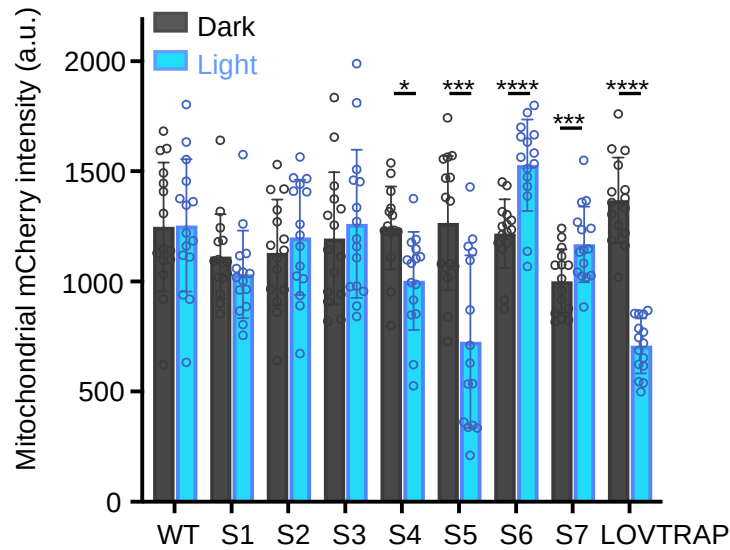


Fig. R7 | Quantification of light-induced mitochondria dissociation of mCh-sspB (LOV2) variants. Quantification of light-induced changes in mitochondrial fluorescence signals arising from the indicated mCherry (mCh)-tagged sspB (LOV2) variants upon photostimulation (470-nm laser with 30% input) in HeLa cells. LOVTRAP control: HeLa cells co-expressing NTOM20-Venus-LOV2 and mCherry-Zdk1 subjected to the identical light stimulation. Data are shown as mean $\pm$ SD. $n = 15$ cells from three independent biological replicates. Statistical significance was determined with two-sided unpaired Student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Second, we have replotted the panel in **Fig. 4c** to show individual data points (please see **Fig. R3** for details). This updated figure also includes the non-expression control, along with statistical comparisons of peak values against the previously published tool, mCh-CRY2-STIM1ct (233-685), developed by our group. For a more detailed discussion of these results, please refer to our response to **Comment 1.2**.

2.2 “There are insufficient negative controls in several experiments. There are no no-expression controls in the experiment in Fig 4, 6 and 7. Negative controls are important to compare the background activities associated with overexpression. Several experiments also show elevated background activities associated with the expression (e.g., Fig 2d, 4g and 6g) but there is no discussion, analysis of these differences.”

Response: We thank the reviewer for the thoughtful suggestions. In response, we have repeated the following experiments to address the concerns raised.

First, for **Fig. 4**, we have repeated the experiments and included a non-expression control (Ctrl), in which only the calcium indicator NEMOf was transfected into HeLa cells

(**Fig. R3**, please also refer to our response to **Comment 1.2** for additional discussion). The results showed that the non-expression control exhibited no Ca^{2+} influx upon blue light stimulation, confirming that the light-induced Ca^{2+} influx was specific to the expressed constructs. Similarly, STIM1ct (233-685) (WT) did not induce Ca^{2+} influx without blue light stimulation. Compared to the additional controls (Ctrl, WT), certain *ssrA2*-STIM1ct insertion variants, including sites 276, 343, and 463, showed significant background activities in the dark. In contrast, the WT control, insertion sites 329 and 685, and the previously reported tool mCh-CRY2-STIM1ct (233-685) (CRY2-S1ct) exhibited minimal background activity in the dark state.

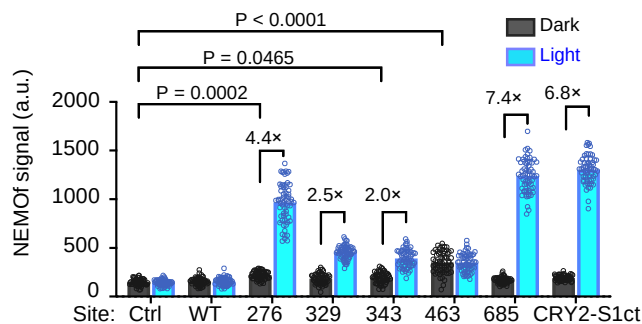


Fig. R3 | Quantification of light-induced changes of intracellular Ca^{2+} levels (indicated by NEMOf) in HeLa cells expressing the indicated *ssrA2*-inserted STIM1ct variants and mCh-CRY2-sspB2. *ssrA2*-STIM1ct (site 685) showed the most notable changes and was used in experiments shown in d-g. **Ctrl:** HeLa cells expressing only the calcium indicator NEMOf. **WT:** STIM1ct (233-685) without *ssrA2* insertion. **CRY2-STIM1ct:** mCherry-CRY2-STIM1ct (233-685) construct previously reported by us. Data are shown as mean $\pm$ SD. $n = 55$ -60 cells from three independent biological replicates. Statistical significance was determined with two-way ANOVA.

Second, for **Fig. 6**, we have included the GFP-only control as a negative control in **Fig. R8b-c** (new **Fig. 6b-c**) and **Fig. R9** (new **Supplementary Fig. 8a**). The Figure legends, results, and source data have been updated accordingly. As shown in **Fig. R8c** and **Fig. R9a**, the GFP-only control showed no co-clustering with mCherry-CRY2-sspB2(B1) or sspB2-mCherry-CRY2(B2), indicating minimal or no basal activity. However, while the western blot results in **Fig. R8f** did not show basal activation related to the transient phosphorylation of the downstream signaling proteins TBK and IRF3 in the absence of light, a slight increase in IFN- β secretion (statistically non-significant) was observed with prolonged overexpression of the cytosolic STING (cSTING) fragment (**Fig. R8g**). We

reason that this low-level background activity may be attributed to overexpression of the cSTING fragment.

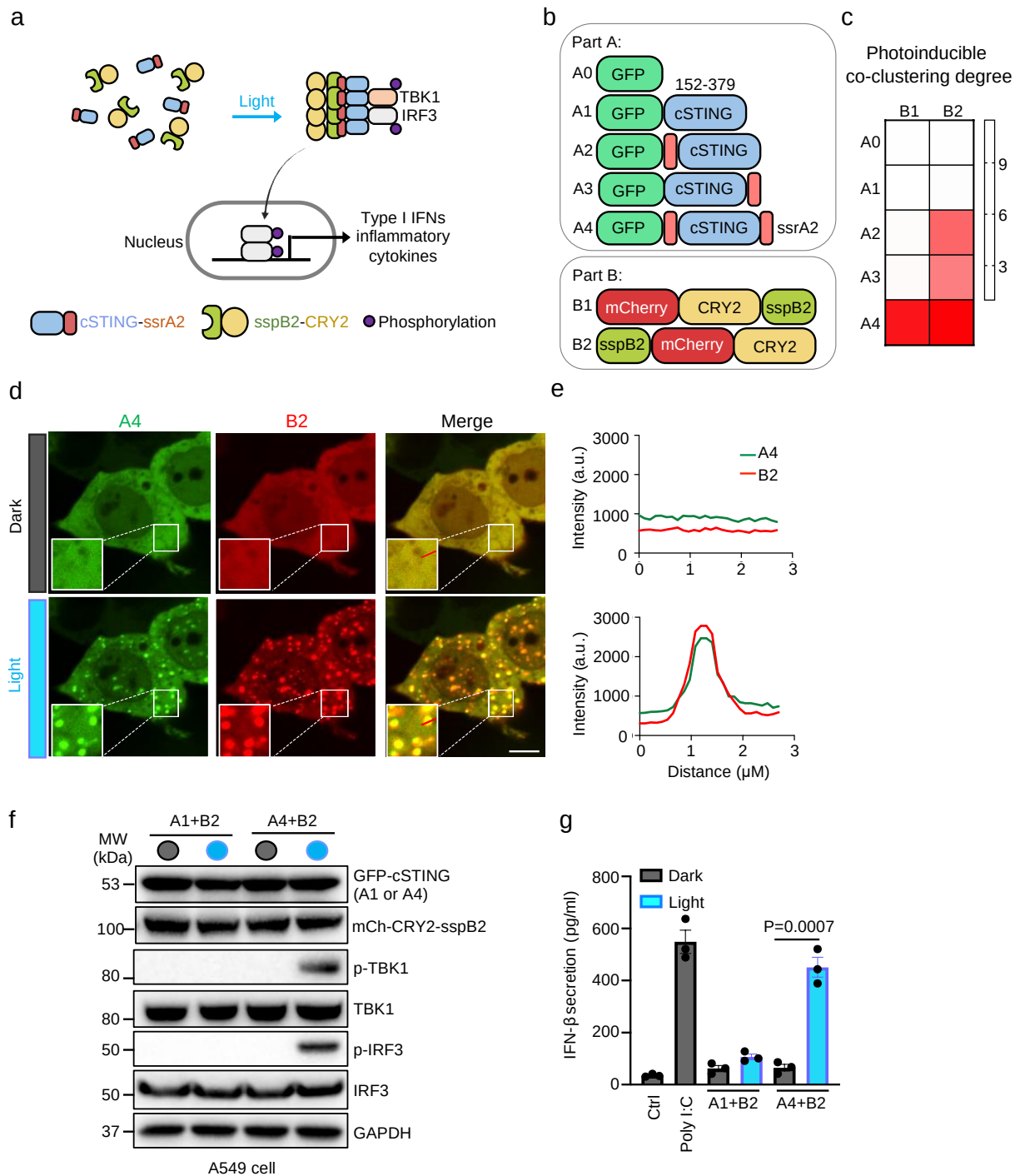


Fig. R8 | ssrA2-tagging of STING to photo-manipulate innate immune response.

(a) Schematic demonstrating light-dependent activation of STING signaling via coupling

PhoBIT2 with cytosolic STING (cSTING; aa 152-379). Light-dependent oligomerization of sspB2-CRY2 leads to co-clustering of ssrA2-cSTING, which in turn activates downstream TBK1 and IRF3 phosphorylation. Next, phosphorylated IRF3 (p-IRF3) undergoes nuclear translocation to trigger type I interferon responses. (b) Domain architectures of PhoBIT2-cSTING variants used in the study. Part A consists of [GFP control](#), GFP-cSTING, either tagged or untagged with ssrA at the N and/or C termini. Part B features mCherry, CRY2, and sspB2 arranged in two different configurations. (c) Heatmap showing the degree of oligomerization by measuring the fold change in GFP intensity (Part A) relative to mCherry (Part B) following light stimulation. (d) Confocal images of HeLa cells co-expressing the indicated constructs before and after photostimulation. Scale bar, 10 μ m. (e) The intensity profiles of GFP-ssrA2-cSTINGct-ssrA2 ([A4](#); green) and sspB2-mCh-CRY2 (B2; red; across the red line) in response to photostimulation. (f) Western blot analysis of TBK1, IRF3, and their phosphorylated forms, in A549 cells expressing the indicated constructs before and after photostimulation. GAPDH is used as the loading control. (g) ELISA measurements of secreted IFN- β . A549 cells expressing the indicated constructs were either kept in the dark or exposed to pulsed blue light (20 s ON and 30 s OFF for 8 h). Data show three biological replicates (mean $\pm$ s.e.m; two-sided unpaired Student's t-test).

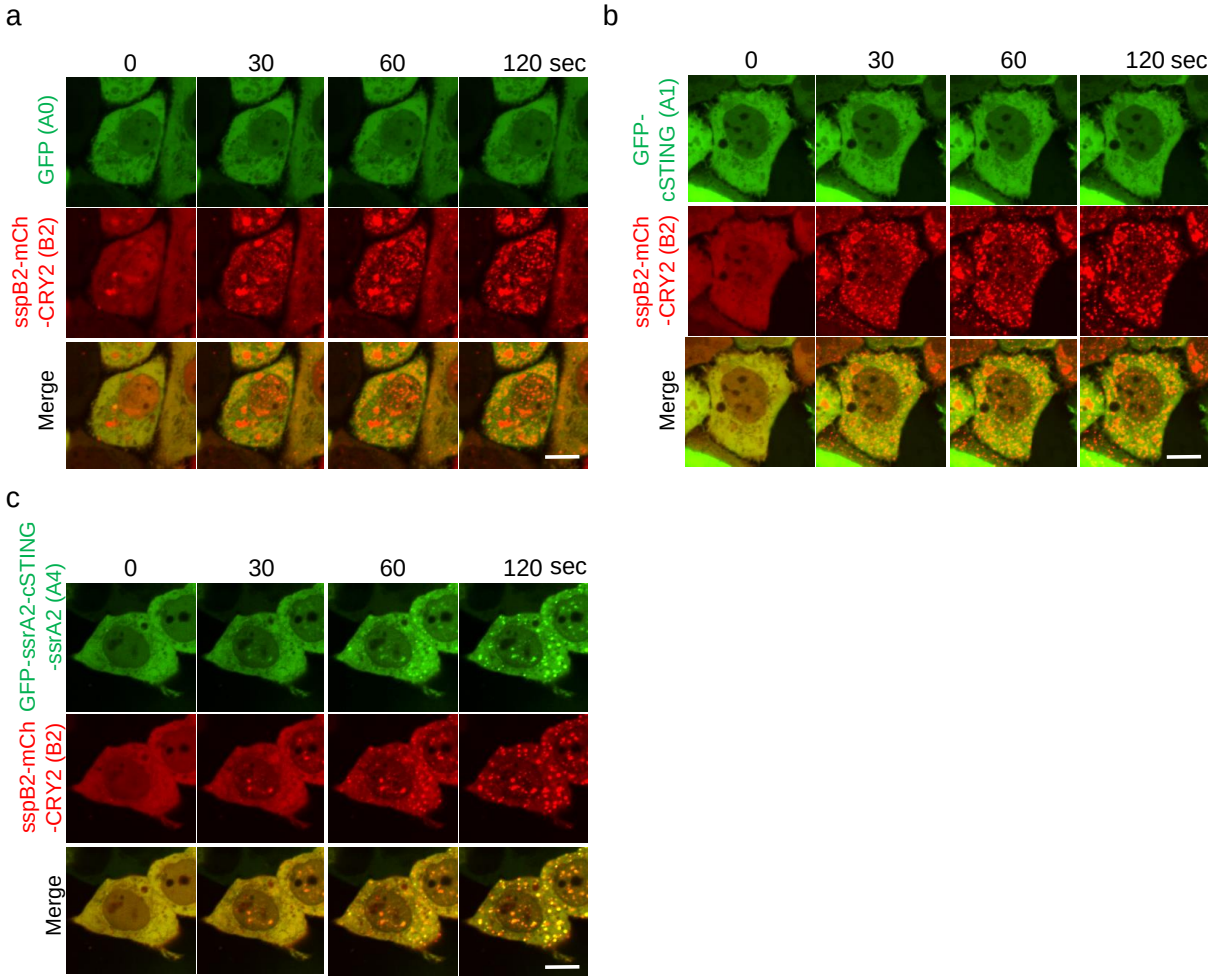


Fig. R9 | Light-inducible clustering of cSTING enabled by PhoBIT2. (Related to Fig. 6) Shown are time-lapsed images of HeLa cells expressing GFP alone or sspB2-CRY2-mCh (combination A0 + B2 in Fig. 6; a), GFP-cSTING and sspB2-CRY2-mCh (combination A1 + B2 in Fig. 6; b), or GFP-ssrA2-cSTING-ssrA2 and sspB2-CRY2-mCh without (combination A4 + B2; c) upon blue light stimulation at the indicated time points. Scale bar, 10 μ m.

We have added the following sentences to the Results section: “Though not statistically significant, we did observe a slight elevation in IFN β secretion from the A4 + B2 group without light stimulation. We speculate that the background activity may arise from overexpression of the cSTING fragment. This limitation could potentially be addressed by optimizing the minimal activation domain through truncation of the STING fragment, which is a feasible strategy that has proven effective in several previous studies (Dou et al. 2023).”

Third. for **Fig. 7**, we repeated the WB analysis and included lysate from wildtype (WT) K562 cells as a non-expression control, as shown in **Fig. R6**; **modified Fig. 7f**. Please refer to our response to **Comment 1.4** for additional details.

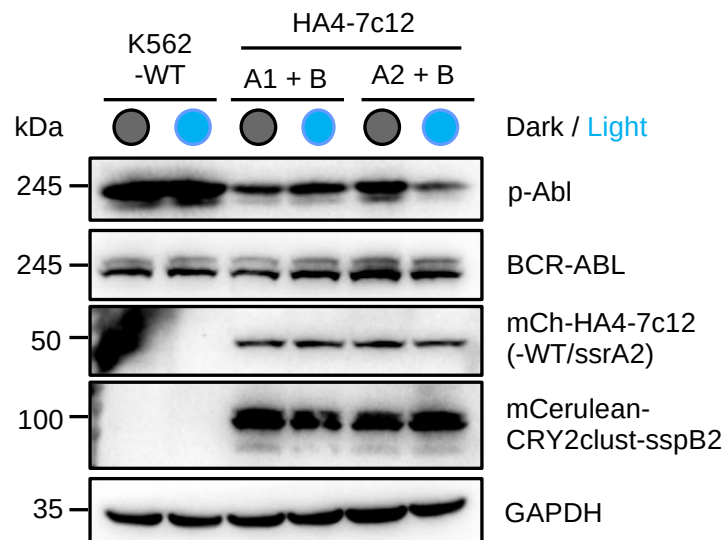


Fig. R6 | WB analysis of inhibition of Bcr-Abl phosphorylation. Western blot detecting transduced constructs and non-expression control (K562WT), endogenous BCR-ABL and its phosphorylation state upon dark (grey circle) or blue light stimulation (blue circle) in K562 cells.

Forth. for **Fig. 4g**, we conducted an additional experiment to investigate the source of background activity. We hypothesized that the elevated background observed in ssrA2-STIM1ct (site 685) was due to the presence of three NFAT response elements (RE) upstream of the luciferase gene in the pGL4.30[luc2P/NFAT RE/Hygro] reporter plasmid. To bypass this, we measured endogenous IL-2 secretion in Jurkat cells transduced with ssrA2-STIM1ct (site 685) and mCh-CRY2-sspB2, as Jurkat cells are a well-established model for T cell signaling with native NFAT binding sites across the genome (**Fig. R10**, new **Supplementary Fig. 6**). Expression of ssrA2-STIMct (site 685)-mCerulean and mCh-CRY2-sspB2 was confirmed by Western blot (**Fig. R10c**). Upon blue light stimulation, IL-2 secretion increased significantly, while background levels in the dark are rather limited.

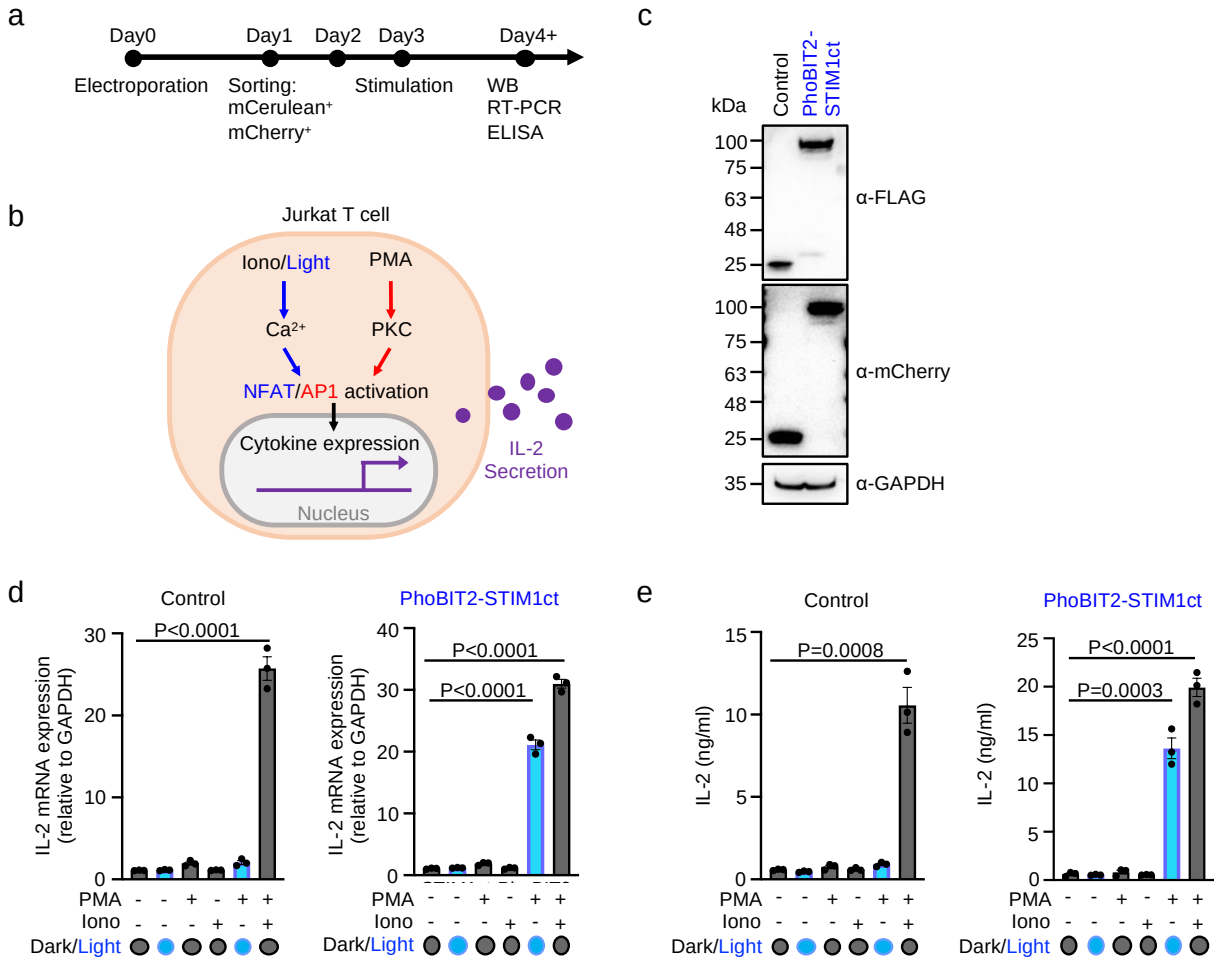


Fig. R10 | Light-induced IL-2 secretion in Jurkat T cells. (a-b) Schematic illustrations of the experimental design and the signaling pathway for cytokine production in Jurkat T cells. (c) Western blot analysis of cell lysates from Jurkat T cells electroporated with either a control plasmid mixture (mCerulean-FLAG and mCherry) or a PhoBIT2-STIM1ct mixture (ssrA2-STIM1ct (site 685)-mCerulean-FLAG and mCh-CRY2-sspB2). (d-e) Quantification of IL-2 expression by qRT-PCR (d) and secreted IL-2 by ELISA (e) in sorted mCerulean⁺ mCherry⁺ Jurkat T cells electroporated with the indicated plasmids under the specified conditions. Data are shown as mean $\pm$ s.e.m. Statistical significance was determined with two-sided unpaired Student's t-test. n = 3 independent biological replicates. Abbreviations: Iono, Ionomycin; PMA, phorbol myristate acetate; PKC, protein kinase C; NFAT, Nuclear factor of activated T-cells; AP1, the activator protein complex 1; IL-2, Interleukin-2.

Modified method sections:

Chemicals and reagents

Place 1: For cell culture, the HeLa, HEK293T, A549, [Jurkat](#) and K562 cell lines were purchased from ATCC.

Place 2: For ELISA assays, [Ionomycin was purchased from Sigma-Aldrich, USA.](#) [Human IL-2 ELISA kit was purchased from Thermo Fisher Scientific, USA.](#) hIFN- β ELISA assay (luex-hifnbv2) was purchased from InvivoGen, USA.

Cell culture and transfection

HeLa, HEK293T, and A549 cells were cultured in DMEM, [while K562 and Jurkat cells were cultured in RPMI 1640.](#) [For Jurkat cells, 2 mM L-glutamine \(Sigma-Aldrich\) was supplemented.](#) Both DMEM and RPMI 1640 media were maintained at 37°C with 5% CO₂ and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin cocktail. For fluorescence imaging experiments, cells were seeded in four-well 35-mm glass-bottom dishes one day before transfection. The plasmids were transfected using Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. Cells were imaged 18-24 h after transfection.

[Electroporation and treatment of Jurkat cells](#)

[Jurkat T cells were electroporated using the Neon Transfection System \(Invitrogen\) according to the manufacturer's instructions at day 0. In brief, 1 million Jurkat T cells were mixed with a total of 10 \$\mu\$ g plasmids encoding ssrA2-STIMct \(site 685\)-mCerulean and mCh-CRY2-sspB2 or mCerulean and mCherry as control. Cells are subjected to electroporation and incubated in fresh RPMI 1640 in a 6-well plate overnight for recovery. On day 1, mCerulean and mCherry double positive cells were sorted and transferred into a new 6 well plate supplemented with fresh medium for growth. On day 3, cells were subjected to indicated treatments, including 1 \$\mu\$ M ionomycin, blue light \(30 s ON, 1 min OFF, 10–40 mW/mm²\), 10 nM PMA for 8 hours. Supernatants were collected by centrifugation at 300 \$\times\$ g for 5 minutes and stored at -20°C until analysis. Pellets were collected for western blot analysis and RNA isolation for real-time PCR analysis.](#)

[ELISA measurements of human IL-2](#)

Supernatant collected from Jurkat cells was prediluted at a ratio of 1:25 (20 μ L supernatant + 480 μ L sample diluent). Then, 50 μ L of the diluted supernatant was added to pre-coated anti-IL-2 antibody microwells of the Human IL-2 ELISA Kit (BMS221-2, Thermo Fisher Scientific), along with 50 μ L of biotin-conjugated anti-IL-2 antibody (1:100 dilution in 1X Assay Buffer), and incubated at room temperature for 2 hours. The plate was washed 6 times with Wash Buffer, and 100 μ L of streptavidin-HRP (1:100 dilution) was added, followed by a 1-hour incubation at RT and another 6 washes. Then, 100 μ L of TMB substrate solution was added and incubated for 30 minutes at room temperature. The reaction was stopped with 100 μ L of Stop Solution, and absorbance was measured at 450 nm using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek). IL-2 concentrations were calculated using a standard curve (range: 18.8–1200 pg/mL) and multiplied by the total dilution factor ($25 \times 2 = 50$, accounting for the 1:25 predilution and the additional 1:2 dilution in the assay) to obtain the final concentrations. Statistical analysis was performed using a one-way ANOVA.

Real-time PCR analysis

Total RNA was isolated from electroporated Jurkat cells using TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Concentration was determined using a Nanodrop. cDNA was synthesized from the RNA using the Superscript III First-Strand cDNA Synthesis Kit (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. Real-time PCR was then conducted on the ABI Prism 7000 analyzer (Applied Biosystems) with the SYBR Green ER qPCR Super Mix Universal kit (Invitrogen) and specific primers. The resulting data were presented as the accumulation index ($2^{\Delta\Delta C_t}$). The primer sequences are listed in **Supplementary Table 3**. IL-2 mRNA expression level was normalized to *GAPDH*.

We have added the following sentences to the Results section: “Additionally, we validated the endogenous IL-2 mRNA expression and secretion upon blue light stimulation in Jurkat cells (**Supplementary Fig. 6a-b**). Jurkat cells co-expressing ssrA2-STIM1ct (site 685)-mCerulean and mCh-CRY2-sspB2 exhibited elevated IL-2 mRNA levels and IL-2 secretion following blue light illumination (**Supplementary Fig. 6c-e**). In contrast to the NFAT luciferase assay (**Fig.4g**), where the 3x NFAT response element (RE) may have contributed to elevated background activity, assessment of endogenous IL-2 showed minimal background activation, providing a more physiologically-relevant assessment of signaling specificity.

Finally, we have strengthened the scientific rigor of our study by including non-expression controls for **Figs. 4, 6 and 7**, now shown in **Figs. R3, R6, R8, R9, and R10**. Additionally, we focused on addressing concerns regarding elevated background activities observed in **Figs. 2d, 4g and 6g**. For **Fig. 2d**, we have investigated the source of background activity. Based on results presented in **Figs. R15 and R16** and discussion in the response to **Comment 2.8**, we conclude that the primary cause was not prolonged overexpression of PhoBIT1 components but rather the stoichiometric ratios between *ssrA* and *sspB*(LOV2) constructs. Our data suggest that an optimized expression ratio of 2.5× to 5× (*sspB*(LOV2) over *ssrA*) minimizes the background activity. For **Fig. 4g**, we evaluated endogenous IL2 mRNA expression and secretion in Jurkat cell (**Fig. R10**) as an alternative to the luciferase assay. Our results indicated that the elevated background in the luciferase assay likely stemmed from the use of multiple repeated NFAT REs, whereas the endogenous IL-2 production (relying on the endogenous NFAT binding sites upstream of the *IL2* gene) assay showed reduced background and provided a more physiologically-relevant readout. For **Fig. 6g**, we reasoned that the background activity may result from overexpression of the cSTING fragment. We propose that this could be mitigated by identifying a minimal activation domain of the signaling component, which is a feasible strategy that has proven effective in several previous studies. Collectively, our results demonstrated that background activities could arise from various sources, and these issues can be addressed.

2.3 “There are no comparisons with existing tools. The dissociation tool should be compared to the LOV-TRAP system(s) for the background, peak values and folds change. For the second tool, it should be compared to Cry2olig with CIB1 in terms of performance.”

Response: We thank the reviewer for the helpful suggestion. In response, we have conducted a comparative analysis of PhoBIT1 versus LOVTRAP, and PhoBIT2 versus CRY2olig/CIB1, to evaluate the relative performance of our systems.

First, we have carefully compared our PhoBIT1 system to the established LOVTRAP system by co-expressing pTriEx-Ntom20-Venus-LOV2 and pTriEx-mCherry-Zdk1 (Addgene # 81057) in HeLa cells under the same stimulation conditions, as described in the original study (Wang et al., 2016). Quantitative data shown in **Fig. R11 (modified Fig. 1d)** and **Fig. R7 (new Supplementary Fig. 1c)**; also see our response to **Comment 2.1** indicate that *sspB* (LOV2) with insertion at site 5 (S5) exhibits light-induced dissociation comparable to the LOVTRAP system (Wang et al., 2016).

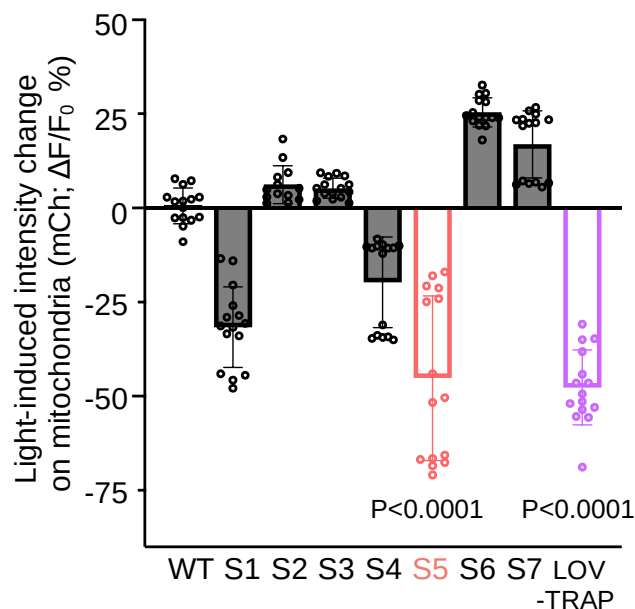


Fig. R11 | Quantification of light-induced changes in mitochondrial fluorescence signals arising from the indicated mCherry (mCh)-tagged *sspB*(LOV2) variants. The S5 variant exhibits the strongest dissociation effect upon photostimulation (470-nm laser with 30% input). **LOVTRAP control:** HeLa cells co-expressing Ntom20-Venus-LOV2 and mCherry-Zdk1 (Addgene # 81057) under the identical light stimulation condition. Data are shown as mean $\pm$ SD. $n = 13$ -15 cells from three independent biological replicates. Statistical significance was determined with two-sided unpaired Student's t-test.

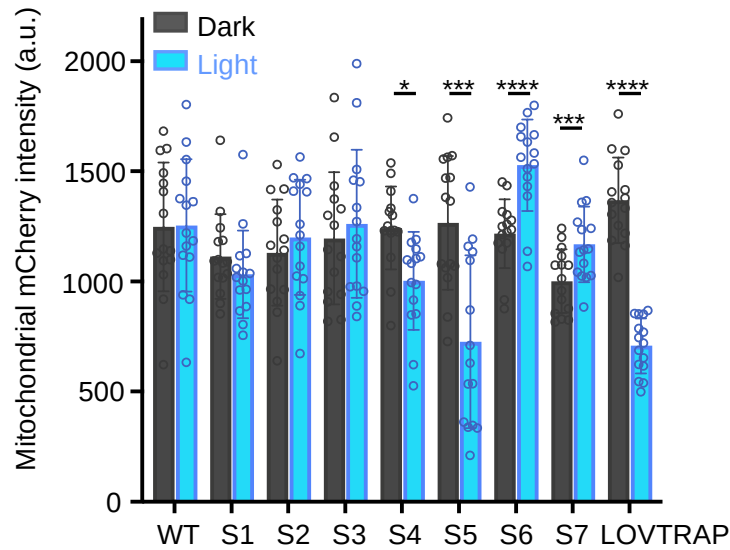


Fig. R7 | Quantification of mitochondrial fluorescence signals arising from the indicated mCherry (mCh)-tagged *sspB* (LOV2) variants before and after photostimulation (470-nm laser with 30% input) in HeLa cells. LOVTRAP control: HeLa cells co-expressing Ntom20-Venus-LOV2 and mCherry-Zdk1 under identical light stimulation condition. Data are shown as mean $\pm$ SD. $n = 15$ cells from three independent biological replicates. Statistical significance was determined with two-sided unpaired Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

We have added the following sentences to the Results section: “Compared to the established LOVTRAP system (Wang et al., 2016), the *sspB*(LOV2) variant S5 showed comparable degree of dissociation upon blue light stimulation (**Fig. 1d**).”

Second, we conducted a detailed comparison between the CRY2oligo/CIB1 system and our PhoBIT2 platform by evaluating the background binding activity, peak fluorescence intensity, and fold change upon light stimulation. In our experimental setup, the smaller CIB1 fragment was anchored to mitochondria, while the larger CRY2oligo was fused to a mCherry tag and allowed to diffuse freely in the cytoplasm. Quantification data shown in **Fig. R12 (modified Supplementary Fig. 4a)** indicate that the CRY2oligo/CIB1 system exhibited low background activity (0.24 mCherry basal binding) in the dark and a substantial light-induced increase in signal intensity, with a 6.43-fold change, visualized as a grass-green dot in **Fig. R15**. In contrast, further optimization of the *ssrA* and *sspB* pair led to the development of an improved system (PhoBIT2), which exhibited even lower background binding activity and a higher fold change upon blue light stimulation (**Fig. R13, modified Fig. 3h**). Notably, previous attempts to fuse LOVTRAP components directly into PAR-family GPCRs failed to elicit

blue-light-dependent receptor activation, implying that component size is a critical factor for inter-/intra-domain insertion without disrupting protein structure and function. While the CRY2oligo/CIB1 system achieves robust light-induced binding, it is best suited for N- or C-terminal fusions rather than intra-/internal-domain insertions. Taken together, PhoBIT2 offers distinct advantages, including smaller component size, lower basal activity, and enhanced dynamic change, making it more suitable for flexible and precise optogenetic control within complex protein architectures.

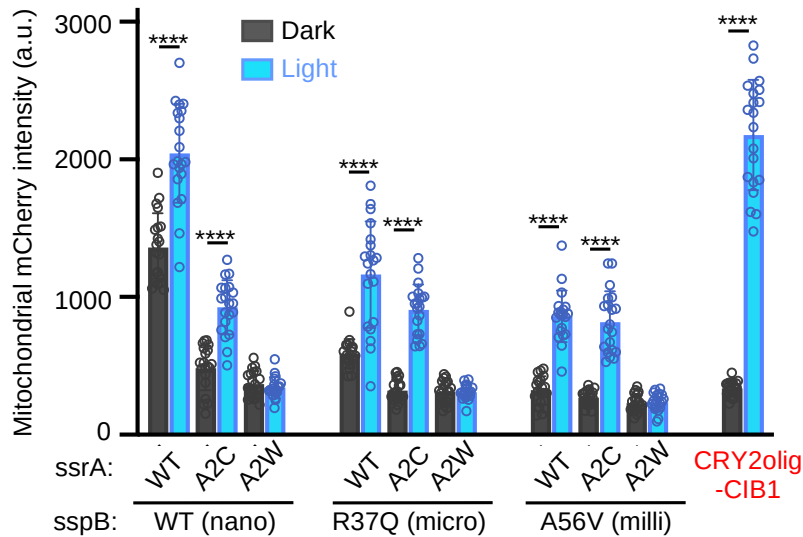


Fig. R12 | Performance of sspB variants during two rounds of PhoBIT2 optimization. (Related to Fig. 3) (a) First-round sspB optimization. Quantification of light-dependent binding of mCh-CRY2-sspB mutants toward mitochondria-anchored ssrA variants (WT, A2C, or A2W), alongside a CRY2oligo-CIB1 control (Ntom20-Venus-CIB1 and mCh-CRY2oligo). Data are shown as mean $\pm$ SD. $n = 20$ cells from three independent biological replicates. Statistical significance was determined with two-sided unpaired Student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

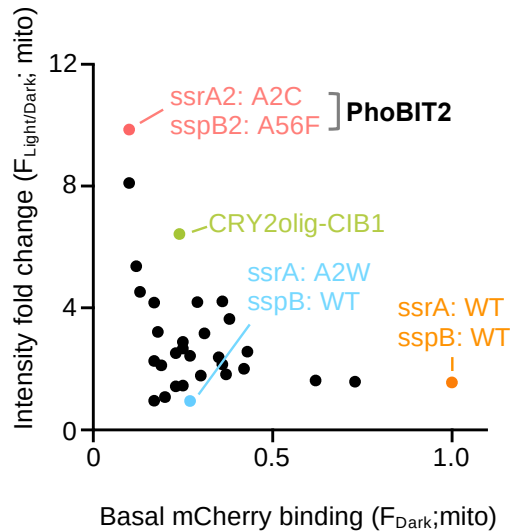


Fig. R13 | Quantification of light-induced binding dynamics during ssrA–sspB screening. Scatter plot showing basal mitochondrial mCherry signal in the dark state (F_{Dark} ; X-axis) versus the fold-change in mitochondrial mCherry intensity upon light stimulation ($F_{\text{Light/Dark}}$; Y-axis). **CRY2olig-CIB1:** HeLa cells co-expressing Ntom20-Venus-CIB1 and mCh-CRY2olig.

We have added sentences to the results section: “Notably, compared to the established light-inducible heterodimerization system CRY2olig/CIB1, the optimized ssrA-A2C and sspB-A56V pair exhibited lower basal binding and higher light-induced association, indicating improved overall performance (**Fig. 3h** and **Supplementary Fig. 4a**).”

2.4 “Despite the first tool has one component that is smaller, there are no experiments that demonstrates that the small size is advantageous.”

Response: We thank the reviewer for raising this critical point. In response, we have carefully compared our engineered systems with existing tools; please refer to **comment 2.3** for more details. To further evaluate the advantage of PhoBIT1’s compact size, we replaced the light-sensitive component of optoPAR4 (**Fig. 2c-d**) with Zdk1 and LOV2 from the LOVTRAP system. We focused on PAR4 for this comparison because it demonstrated the highest light-induced activation among engineered PAR variants. Considering the size of each component, we fused the smaller Zdk1 to the N-terminus of cleaved PAR4 (cPAR4) and displayed the larger LOV2 extracellularly (**Fig. R14a; Supplementary Fig. 3e, left**). Although this configuration did not disrupt protein

localization (**Fig. R14a; Supplementary Fig. 3e, right**), the LOVTRAP-based construct failed to elicit light-dependent PAR4 activation, as evidenced by the lack of tdTomato expression (**Fig. R14b; Supplementary Fig. 3f**). We reasoned that Zdk1, despite its modest size (59 amino acids, **Supplementary Table 1**), may impose steric hinderance on the agonist-embedded N-terminus of cPAR4, preventing effective interaction with the receptor binding pocket and thereby impairing receptor activation. In contrast, fusion of the ssrA component (7 amino acids) with the N-terminus of cleaved PAR4 did not abolish receptor activation, clearly speaking for the advantage of PhoBIT1's minimal size for applications requiring intradomain insertion.

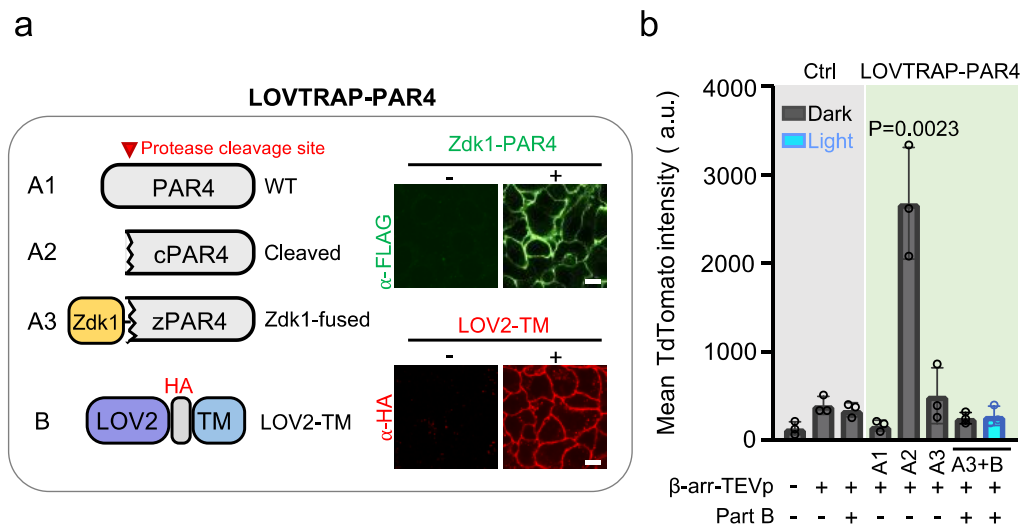


Fig. R14 | Zdk-fused PAR4 fails to respond to light stimulation. (a) Left: Design of the LOVTRAP-fused PAR4 constructs. An HA tag is inserted between LOV2 and the transmembrane domain (TM) of the platelet-derived growth factor receptor (PDGFR) β . Right: Surface expression of FLAG-tagged PAR4 and HA-tagged surface-displayed LOV2 assessed using immunostaining with indicated primary antibodies. Scale bar = 10 μ m. (b) Quantification of tdTomato expression in HeLa cells transfected with the indicated constructs, with and without light stimulation. $n = 50$ -100 cells from three independent biological replicates. Data are presented as means $\pm$ SD. Statistical significance was determined with two-sided unpaired Student's t-test.

We have added the following sentences to the Results section: “To further evaluate the advantage of the compact size of PhoBIT1, we replaced the light-sensitive component of optoPAR4, which showed the strongest light-induced activation among engineered optoPARs, with Zdk1 and LOV2 (**Supplementary Fig. 3e**) from the LOVTRAP system. We fused the smaller Zdk1 (59 amino acids) to the N-terminus of cleaved PAR4 (cPAR4) and displayed the larger LOV2 extracellularly. Although this

configuration did not significantly affect protein localization (**Supplementary Fig. 3e**, right), the LOVTRAP-based construct failed to elicit light-dependent PAR4 activation, as evidenced by the lack of tdTomato reporter expression (**Supplementary Fig. 3f**). We reasoned that Zdk1, despite its modest size (**Supplementary Table 1**), may impose steric hinderance on the agonist-embedded N-terminus of cPAR4, preventing effective interaction with the receptor binding pocket and thereby impairing receptor activation. In contrast, fusion of the ssrA tag (7 amino acids) with the N-terminus of cleaved PAR4 did not abolish receptor activation, thus indicating the advantage of PhoBIT1 for applications requiring intradomain insertion.”

We also added the sentences to the Discussion section: “For example, replacing the ssrA tag (7 amino acids) in optoPAR4 with Zdk1 (59 amino acids) resulted in a loss of light-dependent PAR4 activation, possibly due to the steric hindrance imposed by the bulky Zdk1 on the ligand binding of the agonist-embedded N-terminus of cPAR4 (**Supplementary Fig. 3e-f**). In contrast, the minimal size of ssrA tag enabled flexible fusion to the N-terminus of PAR4 without compromising receptor function, underscoring the practical advantage of its compact design for applications requiring minimal structural perturbation.”

2.5 “Figure 1K, the Y-axis is ‘relative EGFP expression’ – what is this relative to? There is very little information on this in the methods section.”

Response: We thank the reviewer for pointing out the need for clarification regarding the Y-axis label in **Fig. 1K**. In response, we have added a detailed explanation regarding “relative EGFP expression” (i.e., the normalization method) both in the figure legend and at the end of the “CRISPRi assay” section in the Method section.

We have added sentence to methods section: The relative EGFP expression was calculated by normalizing the EGFP fluorescence intensity to the mean intensity of the dark group lacking sgNT1.

We have also revised the related figure legend: (k) Quantification of relative EGFP expression in HeLa cells under the indicated conditions. HeLa cells were co-transfected with sspB(LOV2)-BFP-KRAB, dCas9-ssrA, and SV40-EGFP, with or without sgNT1. Cells were either maintained in the dark (black bars) or exposed to blue light (blue bars). EGFP expression levels were normalized to the mean fluorescence intensity of HeLa cells co-transfected with the same constructs but lacking sgNT1 in the dark condition. n = 45 cells from three independent biological replicates. Data are presented

as means $\pm$ SD. Statistical significance was determined with two-sided unpaired Student's t-test. *** $P < 0.000001$.

2.6 “Generally there is insufficient description of how the data are analyzed in the methods. The description of cells selected for analysis and whether any cells were eliminated and not analysed were also lacking. The description of how mitochondria and aggregation are selected for measurements, how”

Response: We appreciate the reviewer's comment regarding the need for greater clarity in our data analysis procedures. In response, we have added a detailed description of how the fluorescent intensity on mitochondria was quantified in the “Time-lapsed confocal imaging and data analysis” in the method section.

We have added the following sentences to the Methods section: For analysis of mitochondrial fluorescence intensities, cells exhibiting aggregated or morphologically abnormal mitochondria were excluded from analysis. Regions of interest (ROIs) were then selected based on the expression of the mitochondria-anchored plasmid. Specifically, in experiments using NTOM20-Venus fused to ssrA, green fluorescence was used to identify mitochondria. Subsequently, the fluorescence intensity of the target protein within the ROI was quantified for further analysis.

2.7 “Fig 4c is unusual in that it does not show the individual data points as the other experiments. This is also one where it is important to show the statistical comparison of baseline (dark) values.”

Response: We sincerely thank the reviewer for this helpful advice to improve the transparency and rigor of our data presentation. In response, we have updated **Fig. R3** to display individual data points and include statistical comparisons of baseline(dark) values, specifically in reference to the previously reported tool mCh-CRY2-STIM1ct (233–685). For additional details, please refer to our response to **Comment 1.2**.

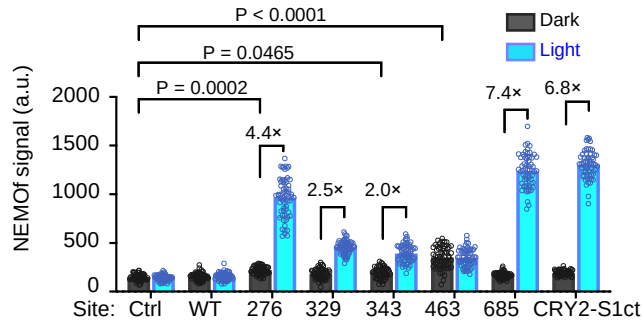


Fig. R3 | Quantification of light-induced changes of intracellular Ca^{2+} levels (indicated by NEMOf) in HeLa cells expressing the indicated *ssrA2*-inserted STIM1ct variants and mCh-CRY2-sspB2. *ssrA2*-STIM1ct (site 685) showed the most notable changes and was used in experiments shown in d-g. **Ctrl:** HeLa cells expressing only the calcium indicator NEMOf. **WT:** STIM1ct (233-685) without *ssrA2* insertion. **CRY2-STIM1ct:** mCherry-CRY2-STIM1ct (233-685) construct previously reported by us. Data are shown as mean $\pm$ SD. $n = 55$ -60 cells from three independent biological replicates. Statistical significance was determined with two-way ANOVA.

2.8 “For the first tool it would be nice to see whether the tools still work well when expressed more than 24 hours. Does the imbalance of expression cause higher background ‘leak’ activity? Can this account for the elevated baseline shown in the Tango system?”

Response: We thank the reviewer for this insightful question. To address whether prolonged expression or imbalanced stoichiometry contributes to elevated background activity, we examined light-induced dissociation at two different time points (24 h and 48 h). As shown in **Fig. R15a-b**, no significant difference was observed in dissociation performance, expression levels, or fold change upon light stimulation (0.58-fold at 24 h versus 0.6-fold at 48 h), indicating that prolonged expression does not impair PhoBIT1 functionality. We next examined whether the leaky activity resulted from the imbalanced stoichiometry between Part A (*ssrA*-PAR4) and Part B (*sspB*(LOV2)). To test this, we co-transfected varying amounts of Part B relative to Part A, ranging from zero to five times the amount of Part A, as shown in **Fig. R16**. Notably, higher relative expression of Part B reduced background activity in the dark. However, at a five times excess group, the light-induced activation also declined, likely due to reduced plasmid overload, which may have limited Part A expression. Compared to **Fig. 2d**, where the A3+B combination induced a 5.7-fold change, optimizing the plasmid ratio reduced leaky activity in the dark state and improved light-induced activation.

Fig. R15 | Prolonged expression of PhoBIT1 does not cause increased background activity. (a) Representative confocal images of HeLa cells co-expressing the indicated constructs at 24 and 48 hours after transfection. Scale bar, 10µm. (b) Quantification of light-induced changes in mitochondrial fluorescence intensity upon photostimulation. Fold-change values (light/dark) are shown above each bar. Data are shown as mean $\pm$ s.e.m. n = 25 cells from three independent biological replicates. Statistical significance was determined with two-sided unpaired Student's t-test. Ns, not significant.



Fig. R16 | Modulation of OptoPAR performance through stoichiometric optimization of plasmid transfection ratios. Quantification of tdTomato expression in HeLa cells transfected with the indicated constructs, with and without light stimulation. The transfection ratios of Part B (sspB(LOV2)) to Part A3 (ssrA-fused PAR4) are indicated below each group. Fold-change values are shown above the bars. n = 50-100 cells from three independent biological replicates. Data are presented as means $\pm$ SD.

We have added the following sentences to the Results section:

1. “To assess the performance of PhoBIT1 over prolonged expression, we evaluated light-induced dissociation at 24 and 48 hours post-transfection (**Supplementary Fig. 1e**). No significant differences were detected in dissociation efficiency or expression levels, with light-induced dissociation fold changes of 0.58 at 24 hours and 0.60 at 48 hours (**Supplementary Fig. 1f**).”
2. “To investigate the elevated basal activity observed in the Tango assay (**Fig. 2d**, PAR4 A3+B group), we adjusted the stoichiometry of Part A (ssrA-PAR4) and Part B (sspB(LOV2)-TM) by co-transfecting cells with varying amounts of Part B plasmid (**Supplementary Fig. 3g**). Increasing Part B expression reduced leaky tdTomato expression in the dark, suggesting that excess sspB(LOV2)-TM enhances sequestration of ssrA-PAR4. However, at a 5:1 ratio, light-induced activation diminished, likely due to plasmid overload impairing Part A expression. By fine-tuning the ratio of Part B to Part A, we achieved a greater light-induced fold change than the 5.7-fold observed with sPAR4 (**Fig. 2d**), while simultaneously minimizing basal activity (**Supplementary Fig. 3g**).”

2.9 “It would be valuable and important to test whether there is any correlation between expression level and background ‘leak’ activities of both optogenetic modules. It would also be very important to test whether there is any importance to balance the expression of the 2 components in real experiments.”

Response: We have carefully examined the correlation between components of PhoBIT1 system, and our results support the conclusion that stoichiometry plays a critical role for background activity. For a detailed discussion and supporting data, please refer to our response to **Comment 2.8**.

2.10 “The panels in the Figures 1 and 3 are hard to follow due to the inconsistent sizes and orders. The lack of regular ‘grid’ layout makes it confusing and laborious for the

readers to follow. As several experiments are combined into one Figure, the current layout where panels can either be below, to the right or to the right and below makes it difficult to match the panels to the legend text. The schematic diagram and results can be made into more consistent sizes between panels and the layout should be made into a 'grid' layout."

Response: We sincerely appreciate the reviewer's valuable feedback regarding figure organization and clarity. In response, we have revised the layout of **Fig. R17 (modified Fig. 1)** and **Fig. R18 (modified Fig. 3)** to improve readability and visual consistency, while preserving the original order and content of the panels.

First, in **Fig. R17 (modified Fig. 1)**, panels (e, f, g) have been repositioned in the second row in a left-to-right order, while panels (h, i, j, k) have been rearranged in the third row, also in a left-to-right order. In addition, we have adjusted the sizes of the panels and standardized font sizes to fit within a consistent grid layout.

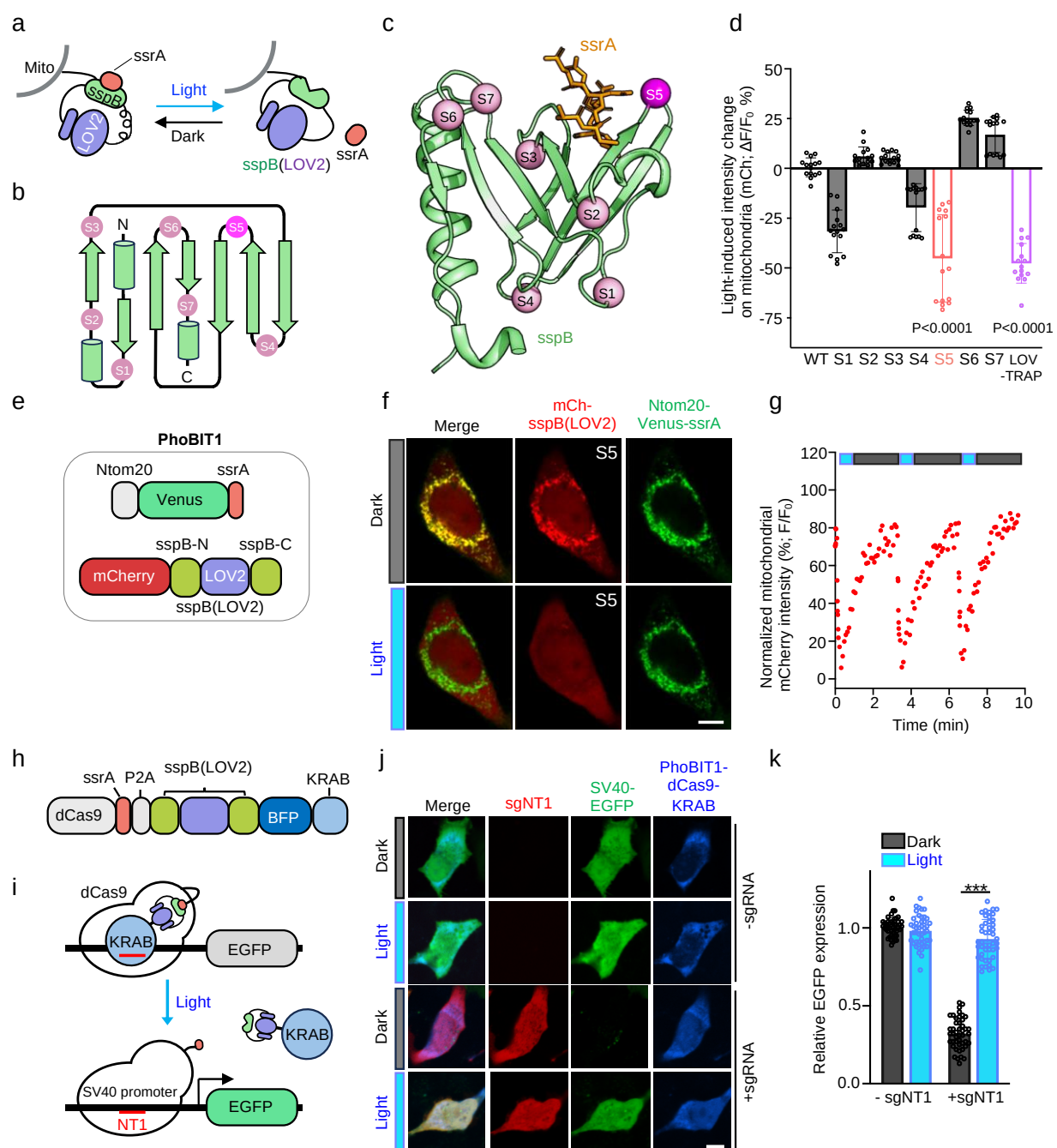


Fig. R17 | Design of PhoBIT1 as a light-OFF switch and its application to control gene expression. Figure legend remained unchanged.

Second, in **Fig. R18 (modified Fig. 3)**, panel (a) has been reorganized from a vertical to a horizontal layout for improved visual flow. Additionally, the label “Optimized ssrA–sspB” has been updated to “PhoBIT2” to better reflect the final system name. Panels (c–e), (f), and (g–h) have also been rearranged horizontally to maintain a consistent and intuitive grid structure throughout the figure.

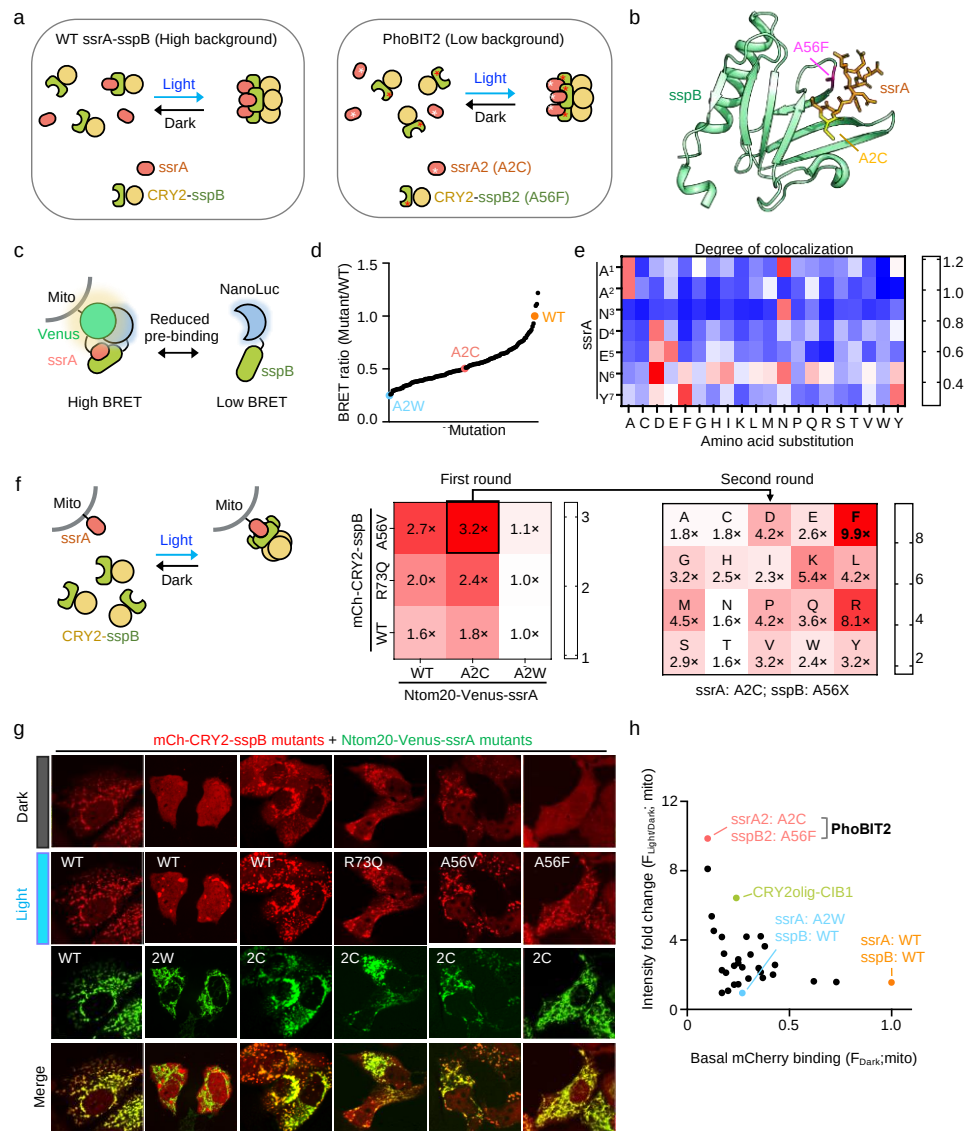


Fig. R18 | Design and optimization of PhoBIT2 as a light-ON switch. (f) **Left**, cartoon illustration of a mitochondria translocation assay used to assess the relative strength of ssrA-sspB variant interaction in living cells. **Right**, heatmaps showing the fold-change of mitochondrial mCherry intensity for the indicated sspB variants following photostimulation.

[Point-to-point responses](#)

We sincerely thank the editor and reviewers for your insightful and constructive feedback, which has significantly enhanced the rigor of our manuscript. We have carefully addressed all the points raised and made necessary clarifications to the text and supplementary materials. All changes are highlighted in [blue font](#). A table summarizing the updated and revised Figures has been included below for your reference (**Table A1**).

Table A1. Data added/updated in the revised manuscript.

Figure/ Panel Info	Original Figure No.	Response to Reviewer(s)	Remarks
4c	4c	R2.4	Updated statistical analysis
6g	6g	R2.2	Updated statistical analysis
7j	7j	R2.3	Updated statistical analysis
S1c	S1c	R2.5	Updated statistical analysis
S4b-c	S3f-g	R2.5	Updated statistical analysis
S7d-e	S6d-e	R2.5	Updated statistical analysis
S8	S7	R2.5	Updated statistical analysis
S9d	New	R2.1	Quantified WB blot intensity
S10c	New	R2.1	Quantified WB blot intensity

Reviewer #1 (Remarks to the Author):

The authors have addressed all my concerns. I have no further comments.

[Response to R1 comments](#)

We sincerely thank the reviewer for all the supportive remarks to accept the revised manuscript.

Reviewer #2 (Remarks to the Author):

The revised manuscript is much improved with additional control and more attempts of quantification. There are still some statistical analysis that can be improved or clarified. However, these corrections and clarifications are rather minor at this stage.

[Response to R2 comments](#)

We would like to express our gratitude for the reviewer's advice and positive remark that *"The revised manuscript is much improved with additional control and more attempts of quantification."*

2.1. *"For the western blots, it will need quantification and statistical comparison. For Fig 6f, although the effects are obvious, the quantification is important to show that the control proteins are the same amount as they can appear to be saturated visually. For Fig. 7f, this is more important as there can be a small difference in the samples that are visually hard to distinguish."*

Response: We thank the reviewer for the comment. *First*, we have quantified the band intensities using ImageJ and performed statistical analysis for **Fig. 6f**, shown in **Fig. R1** (new **Supplementary Fig. 9d**).

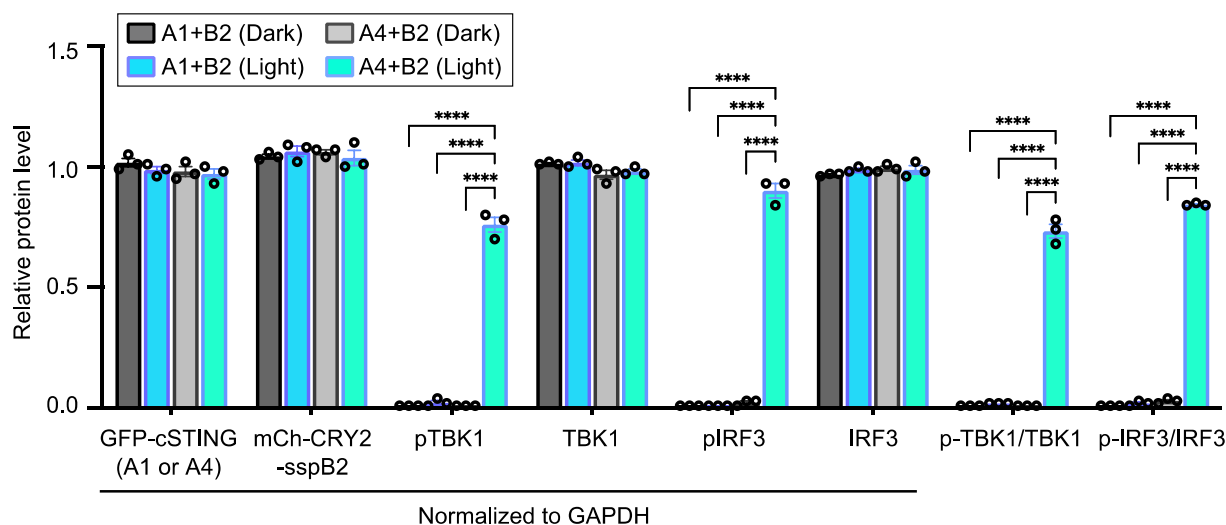


Fig. R1 | Quantification of protein expression levels in A549 cells. Densitometric analysis of Western blot bands for TBK1, IRF3, and their phosphorylated forms in A549 cells expressing the indicated constructs, before and after photostimulation (as shown in **Fig. 6f**). Protein levels were normalized to the indicated control. Data represent mean $\pm$ s.e.m. from three independent biological replicates. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's post hoc test. **** $P \leq 0.0001$.

We have included the following sentences to the Results section:

After 30 min of blue light stimulation, robust phosphorylation of both TBK1 and IRF3 was observed in the A4 + B2 group but not in the A1 + B2 group (**Fig. 6f** and **Supplementary Fig. 9d**).

Second, we have quantified the band intensities using imageJ and performed statistical analysis for **Fig. 7f**, shown in **Fig.R2 (new Supplementary Fig. 10c)**.

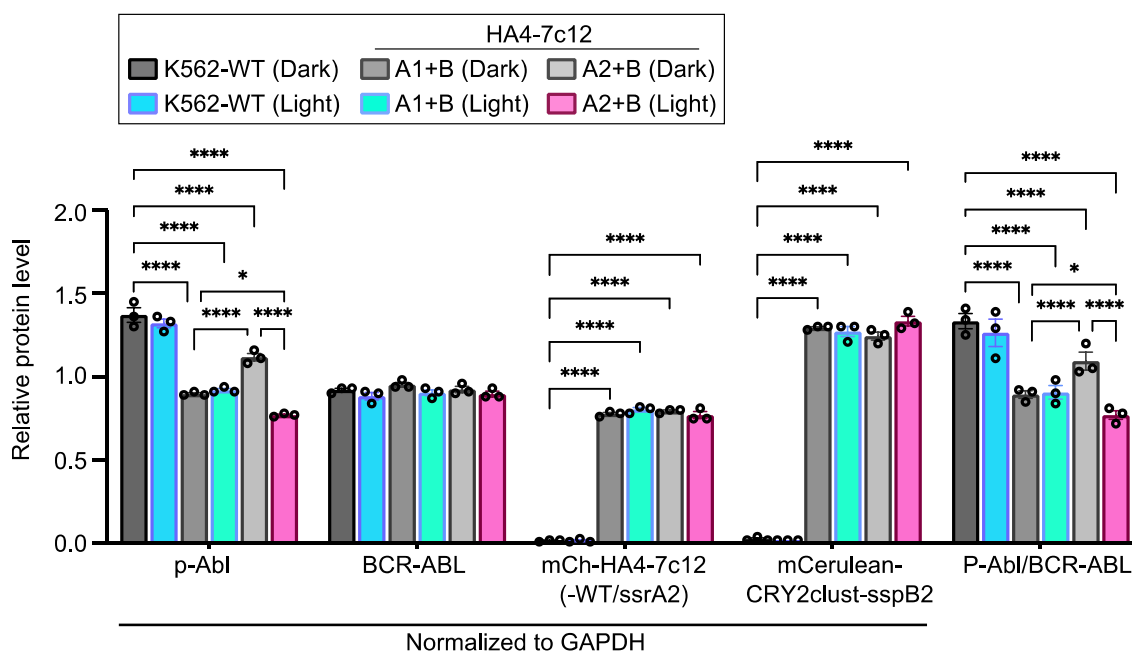


Fig. R2 | Quantification of protein expression levels in K562 cells. Densitometric analysis of Western blot band intensities for BCR-ABL and p-Abl in K562 cells with or without expression of the indicated constructs, before and after photostimulation (as shown in **Fig. 7f**). Protein levels were normalized to the indicated control. Data are shown as mean $\pm$ s.e.m. from three independent biological replicates. Statistical significance was determined by ordinary one-way ANOVA followed by Dunnett's post hoc test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

We have included the following sentences to the Results section:

"Western blot analysis revealed light-dependent inhibition of Y412 phosphorylation in K562 cells co-expressing the A2+B constructs (Fig. 7f, and **Supplementary Fig, 10c**),"

We have added the following sentences to the Methods section:

"Densitometric analysis of Western blot bands was performed using the Gel Analysis function in Image J (NIH) (version 2.16.0/1.54p). Net intensities were calculated by subtracting background signal from the plot profiles and normalized to GAPDH or the indicated reference proteins."

2.2. “For Fig 6g, an ANOVA should be used to compare the baselines of the samples and the peaks of the sample. In addition, poly I:C is not defined.”

Response: We appreciate the reviewer’s advice. We have reanalyzed the results using a two-way ANOVA followed by Turkey’s test, with only significant P values displayed accordingly. Detailed information regarding the poly I:C control used in **Fig. 6g** has been updated accordingly.

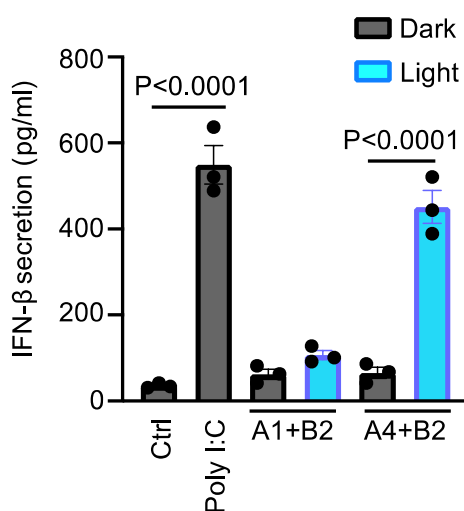


Fig. R3 | ELISA measurements of secreted IFN-β. A549 cells expressing the indicated constructs were either kept in the dark or exposed to pulsed blue light (20 s ON and 30 s OFF for 8 h). Data are shown as mean ± s.e.m from three biological replicates. Statistical analysis was performed using two-way ANOVA followed by Turkey’s post hoc test. Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic double-stranded RNA (dsRNA) analog that acts as an immunostimulant in the assay.

We have included the following sentences to the Method section:

Chemicals and reagents

“The hIFN-β ELISA assay (luex-hifnbv2) was purchased from InvivoGen, USA. Poly I:C was purchased from Sigma-Aldrich, USA.”

ELISA quantification of human IFNβ

“On day 2, cells were stimulated by cells with 10 µg/ml poly I:C or pulsed 470 nm blue light (20 s ON and 5 min OFF) for 24 h.”

2.3. “For Fig 7j, an ANOVA should be used with post-hoc test against the WT in the dark.”

Response: We performed a two-way ANOVA analysis for **Fig. 7j** and updated the P value in the figure accordingly in **Fig.R3 (modified Fig. 7j)**.

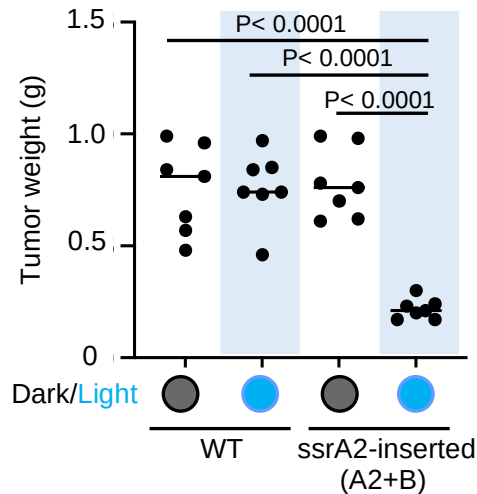


Fig. R4 | Quantification of xenograft tumor weights. N = 7 mice (mean $\pm$ s.e.m.); line at median for the scatter plot shown in panel j. **n = 7 mice (mean $\pm$ s.e.m.).** Line at median for the scatter plot. Statistical analysis was performed using two-way ANOVA followed by Turkey's post hoc test.

2.4. “For Fig 4c, it is important to comment whether the different baselines are statistically different to the control.”

Response: We appreciate the reviewer's valuable advice. To address this, we performed a two-way ANOVA analysis to compare all groups to the control (Ctrl) under both Dark and Light conditions, followed by multiple comparisons using Dunnett's test (**Fig. R4**). The results indicate that only positions 276, 343 (*P* value updated from 0.0465 to 0.0378), and 463 showed statistically significant differences compared to the Ctrl group in the Dark state, as reflected in the figure. Other positions in the Dark state did not exhibit statistically significant differences. In the Lit state, all groups except WT were significantly different from Ctrl (*P* < 0.0001); for clarity, the figure displays *P* values only for the dark state and fold changes for the light state. The updated statistical results have been incorporated into the latest version of the figure.

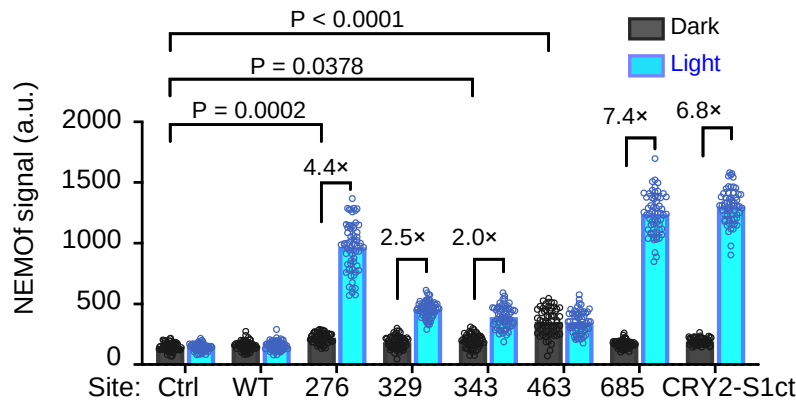


Fig. R5 | Quantification of light-induced changes of intracellular Ca^{2+} levels (indicated by NEMOf) in HeLa cells expressing the indicated ssrA2-inserted STIM1ct variants and mCh-CRY2-sspB2. ssrA2-STIM1ct (site 685) showed the most notable changes and was used in experiments shown in d-g. Ctrl: HeLa cells expressing only the calcium indicator NEMOf. WT: STIM1ct (233-685) without ssrA2 insertion. CRY2-STIM1ct: mCherry-CRY2-STIM1ct (233-685) construct previously reported by us. Data are shown as mean $\pm$ SD. $n = 55$, 59 or 60 cells from three independent biological replicates. Statistical significance was determined with two-way ANOVA followed by post-hoc Turkey's test.

We have included the following sentences to the Results section:

“In contrast, insertion at other positions resulted in either negligible or moderate Ca^{2+} responses (**Fig. 4b-c**). In the dark, insertions at positions 276, 343, and 463 showed significantly different responses compared to the control, while others did not. Under light stimulation, all variants except the WT differed significantly from the control ($P < 0.0001$).”

2.5. For the multiple comparisons in Suppl Fig 1c, 3f/g, 6d & 7, the appropriate tests should be ANOVA with appropriate post-hoc tests. Student's t-test is only good for comparison of 2 samples and have no multiple-comparison correction.

Response: Following the reviewer's valuable suggestion, we have reanalyzed all figures mentioned in the comment, including **Supplementary Fig. 1c (Fig.R5)**, **Supplementary Fig. 4b (Fig. R6)**, **Supplementary Fig. 4c (Fig. R7)**, **Supplementary Fig. 7d and 7e (Fig. R8)**, and **Supplementary Fig. 8 (Fig. R9)**. Figure legends and P values have been updated accordingly.

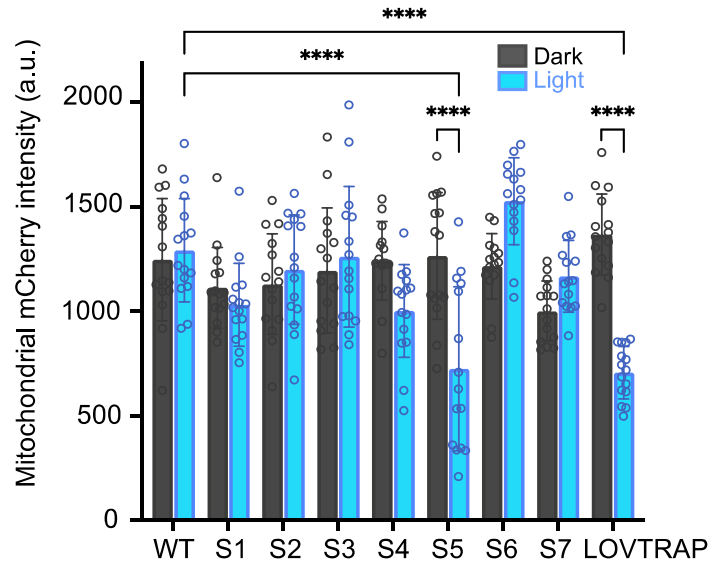


Fig. R6 | Quantification of light-induced mitochondria dissociation of mCh-sspB (LOV2) variants. Quantification of light-induced changes in mitochondrial fluorescence signals arising from the indicated mCherry (mCh)-tagged sspB (LOV2) variants upon photostimulation (470-nm laser with 30% input) in HeLa cells. LOVTRAP control: HeLa cells co-expressing NTOM20-Venus-LOV2 and mCherry-Zdk1 subjected to the identical light stimulation. Data are shown as mean $\pm$ SD. $n = 15$ cells from three independent biological replicates. Statistical significance was determined with [two-way ANOVA followed by post-hoc Turkey's test](#). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

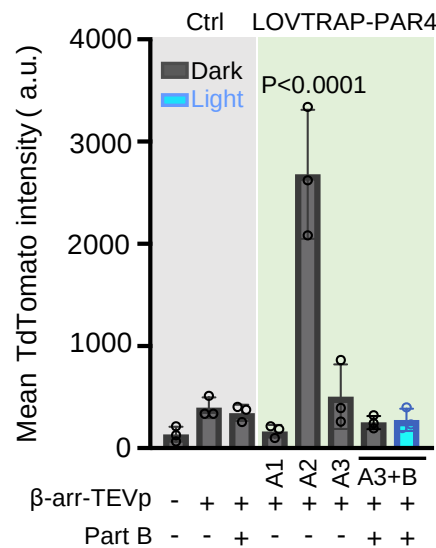


Fig. R7 | Zdk-fused PAR4 fails to respond to light stimulation. Quantification of tdTomato expression in HeLa cells transfected with the indicated constructs, with and without light stimulation. $n = 100$ cells from three independent biological replicates. Data are presented as

means $\pm$ SD. Statistical significance was determined with [ordinary one-way ANOVA](#) followed by [post-hoc Dunnett's test](#).

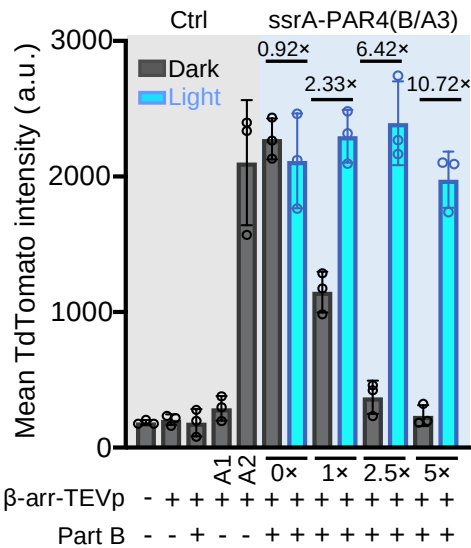


Fig. R8 | Modulation of OptoPAR performance through stoichiometric optimization of plasmid transfection ratios. Quantification of tdTomato expression in HeLa cells transfected with the indicated constructs, with and without light stimulation. The transfection ratios of Part B (sspB(LOV2)) to Part A3 (ssrA-fused PAR4) are indicated below each group. Fold-change values are shown above the bars. n = 100 cells from three independent biological replicates. Data are presented as means $\pm$ SD. [Statistical significance was determined using two-way ANOVA](#) followed by [post-hoc Tukey's test](#). All groups, both under dark and light conditions, were significantly different from Ctrl1 (far left; $P < 0.0001$), except for Ctrl2, Ctrl3, A1, and 1x-dark. The comparison of Ctrl1 vs. 1x-dark yielded a P -value of 0.0006. Significant differences were observed between dark and light conditions for the 1x, 2.5x, and 5x groups ($P < 0.0001$), while other groups were not statistically significant.

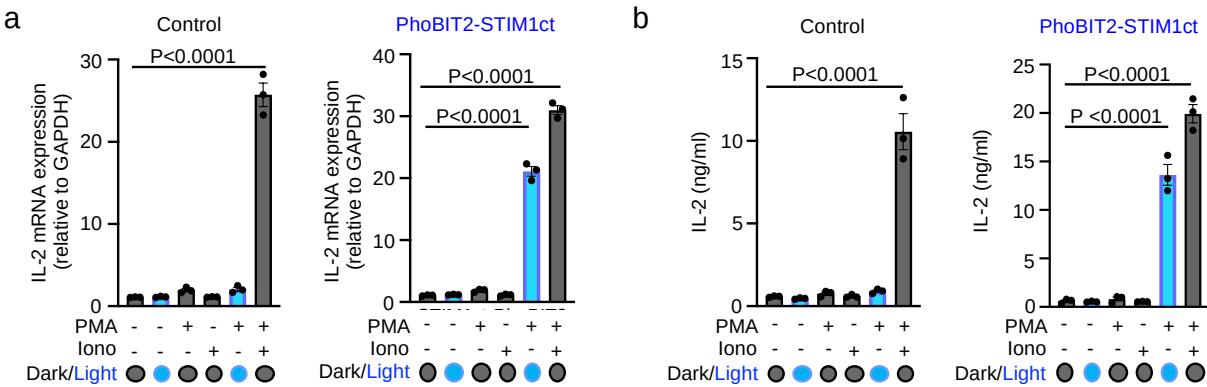


Fig. R9 | Light-induced IL-2 secretion in Jurkat T cells. (a-b) Quantification of IL-2 expression by qRT-PCR (a) and secreted IL-2 by ELISA (b) in sorted mCerulean+ mCherry+ Jurkat T cells electroporated with the indicated plasmids under the specified conditions. Data are shown as mean $\pm$ s.e.m. Statistical significance was determined with [two-way ANOVA followed by post-hoc Turkey's test against the negative control \(far left\)](#). n = 3 independent biological replicates. Abbreviations: Iono, Ionomycin; PMA, phorbol myristate acetate; PKC, protein kinase C; NFAT, Nuclear factor of activated T-cells; AP1, the activator protein complex 1; IL-2, Interleukin-2.

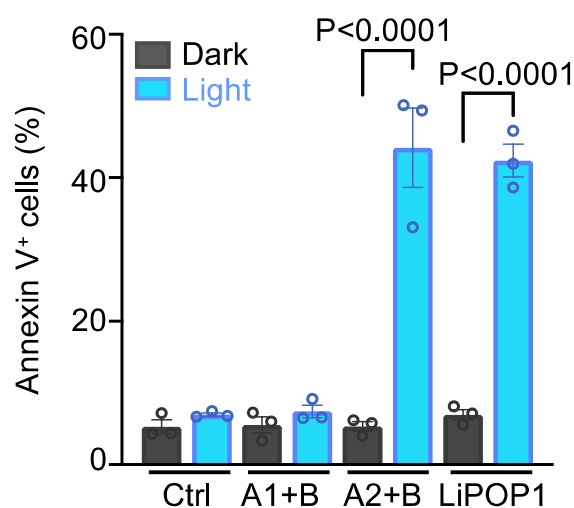


Fig. R10 | Quantification of light-induced necroptosis. Annexin V staining was applied to evaluate necroptosis in HeLa cells expressing the indicated constructs (as described in Fig. 5b), in the absence (gray bar) or presence (blue bar) of photostimulation. Ctrl: cells transfected with the empty vector; LiPOP1: mCh-CRY2-MLKL-NT(3A). Data are shown as mean $\pm$ s.e.m. n = 100 cells from three independent biological replicates. Statistical significance was determined with [two-way ANOVA followed by post-hoc Turkey's test](#).