

FRET-assisted photoactivation of flavoproteins for in vivo two-photon optogenetics

Tomoaki Kinjo^{1,2}, Kenta Terai^{3*}, Shoichiro Horita⁴, Norimichi Nomura⁴, Kenta Sumiyama⁵, Kaori Togashi², So Iwata^{4,6} and Michiyuki Matsuda^{1,3*}

Optical dimerizers have been developed to untangle signaling pathways, but they are of limited use in vivo, partly due to their inefficient activation under two-photon (2P) excitation. To overcome this problem, we developed Förster resonance energy transfer (FRET)-assisted photoactivation, or FRAPA. On 2P excitation, mTagBFP2 efficiently absorbs and transfers the energy to the chromophore of CRY2. Based on structure-guided engineering, a chimeric protein with 40% FRET efficiency was developed and named 2P-activatable CRY2, or 2paCRY2. 2paCRY2 was employed to develop a RAF1 activation system named 2paRAF. In three-dimensionally cultured cells expressing 2paRAF, extracellular signal-regulated kinase (ERK) was efficiently activated by 2P excitation at single-cell resolution. Photoactivation of ERK was also accomplished in the epidermal cells of 2paRAF-expressing mice. We further developed an mTFP1-fused LOV domain that exhibits efficient response to 2P excitation. Collectively, FRAPA will pave the way to single-cell optical control of signaling pathways in vivo.

A number of optogenetic methods that optically control protein–protein interactions have been developed to untangle signaling pathways with high spatiotemporal resolution^{1,2}. The light-sensing domains used as optical dimerizers include *Arabidopsis* ultraviolet-B resistance 8 (UVR8)³, the light-oxygen-voltage (LOV) domain of *Avena sativa* phototropin 1 (ref. ⁴), *Arabidopsis* cryptochrome 2 (CRY2)⁵, photodissociable dimeric variants of the Dronpa (pdDronpa)⁶ and *Arabidopsis* phytochrome B (PhyB)⁷. Among them, the CRY2 system is successfully used for optical control of intracellular signaling^{8–10}, lipid metabolism¹¹ and gene transcription^{12–14}. The photolyase homology region (PHR) of CRY2 contains a flavin adenine dinucleotide (FAD) molecule as the blue light-absorbing cofactor⁵. On photoactivation by blue light, CRY2 forms oligomer and binds to an interacting partner, CIB1 or CIBN¹⁵. By fusing a pair of signaling molecules to CRY2 and CIB1/CIBN, their protein–protein interaction can be controlled in a blue light-dependent manner^{8,16–18}.

2P absorption is a nonlinear optical process that depends on the square of the light intensity. Taking advantage of this property, Denk et al. invented 2P excitation microscopy (2PEM), in which fluorescence is confined to the vicinity of the z-focal plane with little excitation of fluorophores along the optical path¹⁹. 2PEM can also be applied to optogenetics. For instance, microbial opsins, light-gated ion channels, have been activated by 2P excitation to control neuronal activity at single-cell resolution in deep brain tissues^{20,21}. Although CRY2 was shown to be stimulated by 2P excitation previously⁵, there have been only a few reports on the application of optical dimerizer systems to 2PEM^{22,23}. In one example, the CRY2 was activated by 2P excitation in the early *Drosophila* embryo, but less effectively and more slowly than by 1P excitation²². To the best of our knowledge, there has been no study to improve this inefficient activation of CRY2 by 2P excitation.

The ERK is a serine/threonine kinase that serves as the output of the Raf-MEK-ERK signaling pathway²⁴. ERK regulates a variety

of biological processes, including cell proliferation, differentiation and tumorigenesis^{25,26}. Recent studies with a highly sensitive biosensor for ERK activity have revealed the complex spatiotemporal dynamics of ERK activity at single-cell resolution not only in tissue culture cells^{27–29}, but also in living tissues^{30–32}. For instance, in the mouse auricular epidermis, ERK activity pulses are fired spontaneously and propagated radially over 50 μm , a phenomenon called spatial propagation of radial ERK activity distribution (SPREAD)³⁰. However, little is known about the mechanism underlying SPREAD, due to the lack of tools to control ERK activity at single-cell resolution in living tissue.

Here, we developed a CRY2 variant, named 2paCRY2, that is efficiently activated by 2P excitation via FRET from blue fluorescent protein (BFP), which has higher 2P absorption than FAD, the chromophore of CRY2. We named this new technique FRET-assisted photoactivation, or FRAPA.

Results

Development of 2paCRY2. To examine the possibility of using the CRY2 system in conjunction with 2PEM, we first compared the efficiency of photoactivation of CRY2 between 1P and 2P excitation. The PHR domain of CRY2 (CRY2PHR) was fused to mCherry. CIBN was fused to enhanced green fluorescent protein (EGFP) and the CAAX motif (C terminus of K-ras)³³, so that photoactivation of CRY2PHR was expected to bring mCherry to the plasma membrane (Fig. 1a). Indeed, single-photon (1P) excitation efficiently translocated mCherry to the plasma membrane (Fig. 1b, right). However, 2P excitation did not change the localization of mCherry to a detectable level (Fig. 1b, middle). Note that the 2P excitation condition used in this experiment was equivalent to the one used routinely for EGFP imaging.

The inefficient photoactivation of CRY2 by 2P excitation appeared to reflect the low molar extinction coefficient (ϵ) of FAD,

¹Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan. ²Department of Diagnostic Imaging and Nuclear Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan. ³Research Center for Dynamic Living Systems, Graduate School of Biostudies, Kyoto University, Kyoto, Japan. ⁴Department of Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan. ⁵Laboratory for Mouse Genetic Engineering, RIKEN Center for Biosystems Dynamics Research, Osaka, Japan. ⁶RIKEN SPring-8 Center, Sayo, Hyogo, Japan.

*e-mail: terai.kenta.5m@kyoto-u.ac.jp; matsuda.michiyuki.2c@kyoto-u.ac.jp

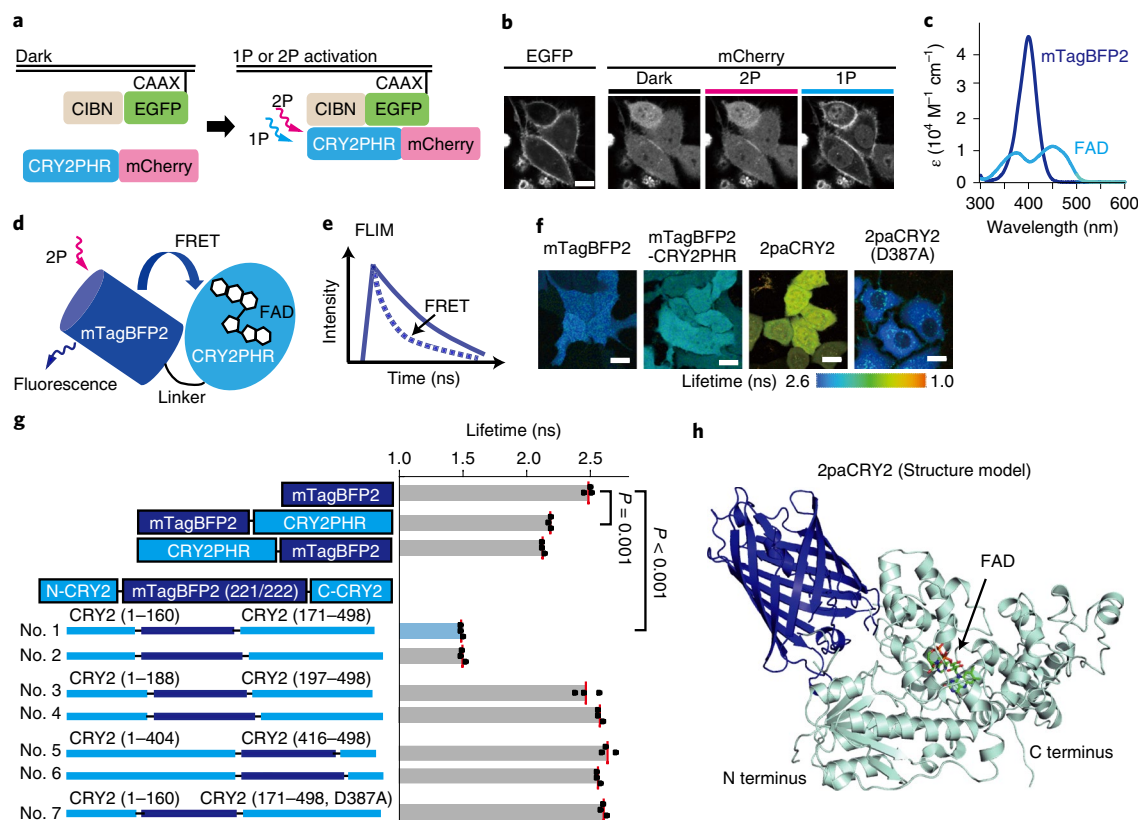


Fig. 1 | Development of 2paCRY2. **a**, Schematic representation of the CRY2-CIBN membrane-recruitment system. CIBN-EGFP-CAAX is localized at the plasma membrane. CRY2PHR-mCherry in the cytosol is translocated to the plasma membrane through binding to CIBN-EGFP-CAAX on excitation. **b**, 1P or 2P activation of CRY2PHR. HeLa cells expressing CRY2PHR-mCherry and CIBN-EGFP-CAAX were kept under a dark condition for 1 h and imaged for EGFP and mCherry signals. The cells were then activated by 1P or 2P excitation and imaged for mCherry signal. Representative images among $n = 3$ independent experiments; scale bars: 10 μm . **c**, Single-photon absorption spectra of recombinant mTagBFP2 and FAD. Representative data among $n = 2$ experiments. **d**, A schematic of FRAPA. CRY2PHR is fused to mTagBFP2 and activated by 2P excitation via FRET from mTagBFP2 to FAD. **e**, A schematic of the FLIM screening. **f**, Representative fluorescence lifetime images of HeLa cells expressing the indicated proteins. The pseudo-color indicates the lifetime in each pixel. Representative images among $n = 3$ independent experiments; scale bars: 10 μm . **g**, Fluorescence lifetime of mTagBFP2 or the indicated chimeric proteins in HeLa cells. mTagBFP2 (221/222) represents mTagBFP2 (a.a. 1-221 or a.a. 1-222); N-CRY2, the N terminus of CRY2PHR; C-CRY2, the C terminus of CRY2PHR; CRY2PHR (D387A) is the CRY2PHR mutant that disrupts FAD binding. The bar graphs and red lines show the mean values; $n = 3$ cells for each protein; two-tailed t-tests were used for comparison. **h**, Structure model of 2paCRY2 with FAD. The model is visualized with PyMOL. The residue from mTagBFP2 and CRY2PHR is shown as blue- and cyan-colored cartoon, respectively. The residues of FAD were shown as sticks representation with carbon, oxygen, phosphorus and nitrogen atoms. Green, carbon; red, oxygen; blue, nitrogen; orange, phosphorus.

because the 2P absorption cross section (σ_2) is typically proportional to the square of ϵ in non-centrosymmetric dipolar chromophores such as FAD^{34,35}. Therefore, to roughly estimate the σ_2 of FAD, we measured the ϵ of FAD in comparison with that of a BFP, mTagBFP2 (ref. 36). The molar extinction coefficients of FAD and mTagBFP2 are 11,300 $\text{M}^{-1} \text{cm}^{-1}$ at 450 nm and 45,700 $\text{M}^{-1} \text{cm}^{-1}$ at 400 nm, respectively (Fig. 1c), suggesting that the σ_2 of FAD could be about 16-fold lower than that of mTagBFP2. This measurement also inspired us to use a fluorescent protein as the donor to transfer energy to FAD by the principle of FRET (Fig. 1d). Since we demand that optogenetic tools for intravital imaging are compatible with FRET biosensors containing cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), we set four criteria for the donor fluorescent protein: a large 2P cross section, high quantum yield, large spectral overlap of the donor emission and the FAD absorption, and minimum spectral bleed-through to the CFP channel on CFP excitation. Among the four candidate fluorescent proteins we screened, we chose mTagBFP2 because it is a monomer with a long Förster radius (R_0) (Supplementary Table 1).

We fused full-length mTagBFP2 to either the N or C terminus of CRY2PHR and examined FRET efficiency by fluorescence

lifetime imaging microscopy (FLIM)^{37,38} (Fig. 1e–g). The fluorescence lifetimes of mTagBFP2-CRY2PHR and CRY2PHR-mTagBFP2 were 2.2 and 2.1 ns, indicating 12 and 16% FRET efficiency, respectively. To maximize the FRET efficiency from mTagBFP2 to FAD, we generated a series of chimeric proteins and measured the fluorescence lifetime. The FRET efficiency depends on the distance and relative orientation between the chromophores^{39,40}. Therefore, we generated truncated mutants of mTagBFP2 and CRY2PHR to decrease the distance between chromophores, and mutated the linker amino acid between mTagBFP2 and CRY2PHR to change the relative orientation of chromophores. However, the approximate FRET efficiency was about 20% at most in these fusion proteins (Supplementary Table 2b–g).

Structure-guided protein design has been successfully applied to the development of optogenetic tools such as PA-Rac1 (ref. 41) and psMEK⁶. Thus, we also followed the strategy in the design process. We generated three-dimensional (3D) models of mTagBFP2 and CRY2PHR from the crystal structure of the closely related TagBFP⁴² (Protein Database (PDB) code, 3M24) and *Arabidopsis thaliana* CRY1 (ref. 43) (PDB code, 1U3C) with homology modeling. Based on the structure model of CRY2PHR (Supplementary Fig. 1), we identified

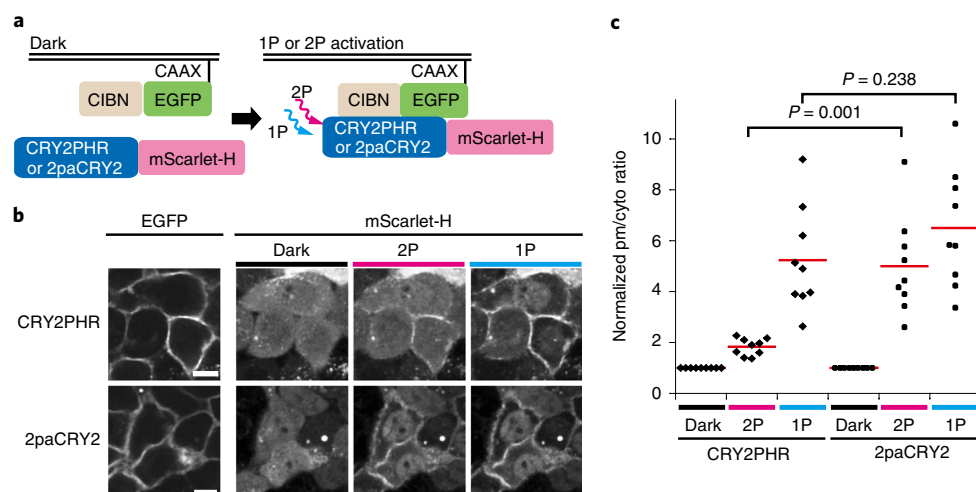


Fig. 2 | 2P activation of 2paCRY2. **a**, Schematic representation of the membrane-recruitment assay. CIBN-EGFP-CAAX is localized at the plasma membrane. CRY2PHR- or 2paCRY2-mScarlet-H in the cytosol (cyto) is translocated to the plasma membrane (pm) on 1P or 2P activation. **b,c**, Quantitative analysis of membrane recruitment on 2P activation. Scale bars: 10 μ m (**b**). Bee swarm plot shows the normalized pm/cyto ratios in each cell before and after 2P or 1P activation (**c**). Red lines represent the mean; nine cells obtained from two independent experiments; two-tailed *t*-tests were used for comparison.

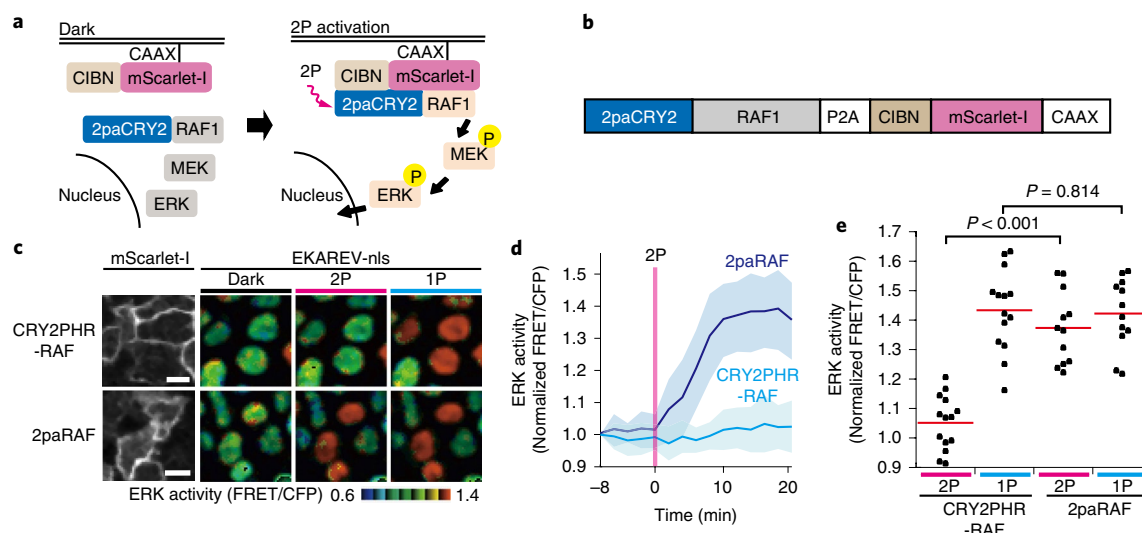


Fig. 3 | ERK activation by 2paRAF. **a**, Schematic representation of the 2paCRY2-RAF system. Recruitment of 2paCRY2-RAF1 to the plasma membrane by CRY2-CIBN interaction results in ERK activation. **b**, A schematic showing 2paRAF. **c-e**, The difference of ERK activation efficiency between CRY2PHR-RAF and 2paRAF. The representative images of ERK activity (FRET/CFP) in the HeLa cells expressing CRY2PHR-RAF or 2paRAF are shown in the IMD mode. Cells were activated by 1P or 2P excitation. Scale bars, 10 μ m (**c**). The cells were 2P activated at time 0 min. The average ERK activity (normalized FRET/CFP) was quantified and plotted as a function of time with the s.d.; $n = 14$ cells for CRY2PHR-RAF and 12 cells for 2paRAF (**d**). Bee swarm plot shows the ERK activity (FRET/CFP) in the HeLa cells expressing CRY2PHR-RAF1-P2A-CIBN-mScarlet-I-CAAX or 2paRAF before and 10 min after 2P or 1P activation (**e**); scale bars: 10 μ m. Red lines represent the mean; two-tailed *t*-tests were used for comparison.

six loops in CRY2PHR that may accept mTagBFP2 without disturbing the CRY2PHR structure. The lifetime of mTagBFP2 inserted into loop (amino acids (a.a.) 161–170) decreased from 2.5 to 1.5 ns (Fig. 1g, no. 1), achieving 40% FRET efficiency (Supplementary Table 2I). We named this product 2P-activatable CRY2, or 2paCRY2 (Fig. 1h). Note that the distance between the donor and acceptor chromophores calculated by using the Ab Initio Domain Assembly Server⁴⁴ predicted the FRET efficiency fairly well (Supplementary Fig. 2).

2P activation of 2paCRY2. Next, we used a membrane-recruitment assay to examine whether 2paCRY2 can be efficiently activated by 2P excitation. CRY2PHR-mScarlet-H and 2paCRY2-mScarlet-H

expressed in HeLa cells (Fig. 2a) were activated by 2P excitation for 30 scans. For two-photon activation, we used 800 and 840 nm for 2paCRY2 and CRY2PHR, respectively. As expected, 2paCRY2-mScarlet-H but not CRY2PHR-mScarlet-H was efficiently translocated to the plasma membrane after 2P excitation (Fig. 2b). We found that 2paCRY2 but not CRY2PHR could be activated by 2P excitation as efficiently as 1P excitation (Fig. 2c). In the dark condition, 2paCRY2 spontaneously returned to cytoplasm and this photo-activation/inactivation cycle was repeatable (Supplementary Fig. 3). Consistent with the peak absorption of TagBFP2, the 2P excitation was achieved most efficiently at 800 nm (Supplementary Fig. 4). Moreover, we confirmed that 2paCRY2 allows us to regulate

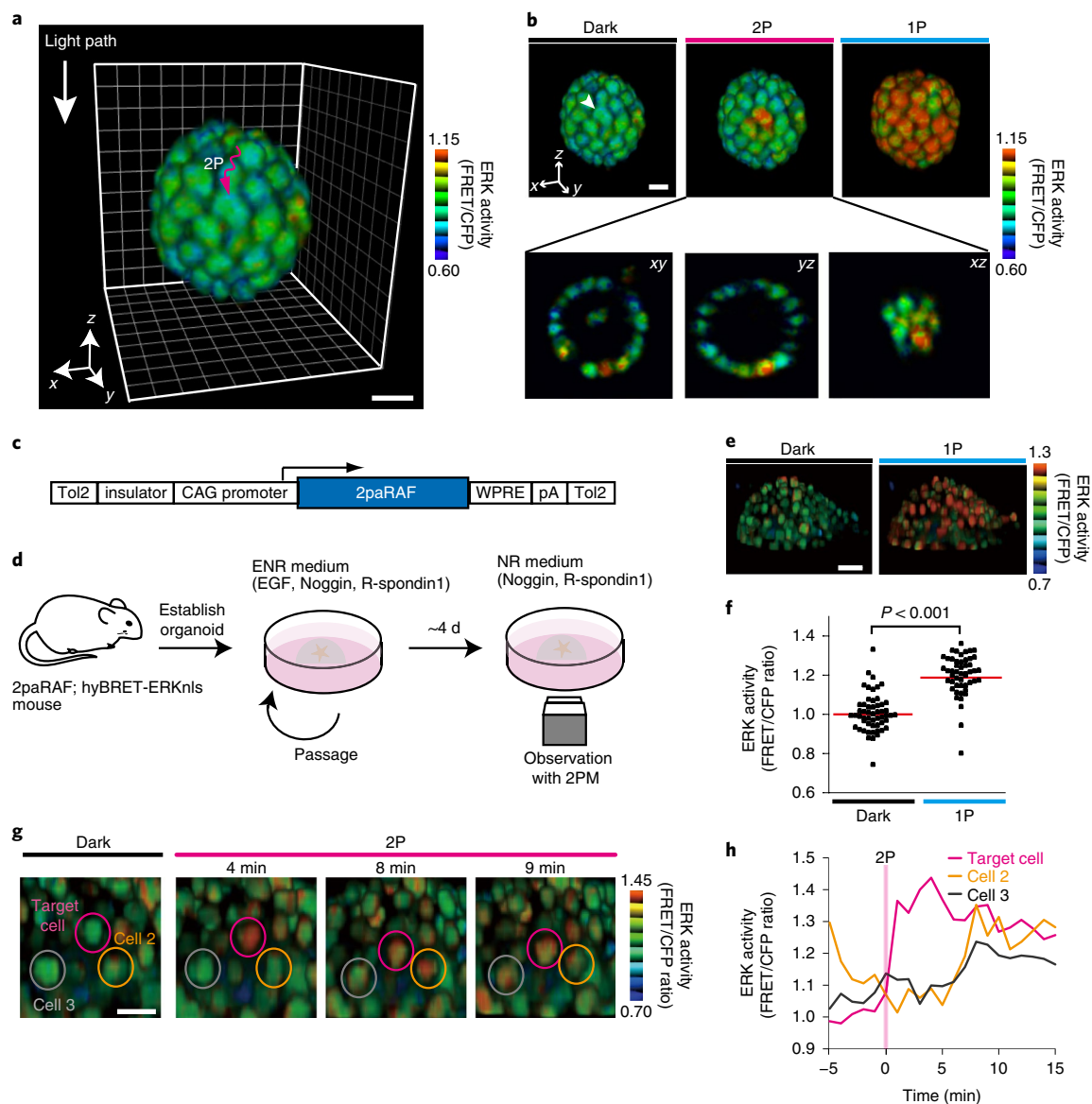


Fig. 4 | Single-cell control of ERK activity in the 3D MDCK cyst and organoids. **a**, A 3D reconstruction image of an MDCK cyst co-expressing 2paRAF and EKAREV-nls represented in the IMD mode. Representative data among $n=3$ independent experiments; scale bars, 10 μm . **b**, Representative 3D images of the MDCK cyst under a dark condition and 10 min after single-cell ERK activation by 2P excitation or whole-cell ERK activation by 1P excitation (upper panels). An arrowhead in the left panel indicates the target cell. The 2D section images (in the xy , yx and xz axis) of the MDCK cyst are also shown at 20 min after single-cell ERK activation by 2P (lower panels). Representative data among $n=3$ independent experiments. **c**, A schematic of the construct employed for generation of 2paRAF transgenic mice. **d**, Experimental setting for the imaging of intestinal organoids from 2paRAF mice crossed with hyBRET-ERKnl mice. Organoids were established from the small intestine of the mice, and cultured in media containing EGF, Noggin and R-spondin1 for a few days. Imaging was performed within 4 d of the last passage in the media from which EGF was removed. **e,f**, The efficiency of ERK activation under 1P excitation. The representative FRET/CFP images of the intestinal organoids before and after the 1P activation are shown in IMD mode. Representative data among $n=3$ independent experiments; $n=54$ cells in dark and 50 cells after 1P activation (**e**). Bee swarm plot shows the ERK activity (FRET/CFP) in each cell before and after the 1P activation (**f**). Red lines represent the mean; two-tailed t -tests were used for comparison. **g,h**, Representative time-lapse 3D images of organoids before and after single-cell ERK activation by 2P excitation. Target cell: the cell exposed to 2P excitation (**g**). Cells 2 and 3, the representative cells in contact with the target cell. Time courses of ERK activity were analyzed in three representative cells (**h**). Representative data among $n=3$ independent experiments.

membrane translocation at subcellular resolution (Supplementary Fig. 5). Of note, we did not find apparent signs of phototoxicity such as cellular swelling, blebbing or large vacuoles under these experimental conditions.

ERK activation by 2paRAF. Next, we applied 2paCRY2 for the optogenetic control of ERK activity^{28,45}. In this system, the 2paCRY2-fused RAF1 protein is recruited to the plasma membrane

through CRY2PHR binding to the membrane-tethered CIBN on 2P activation (Fig. 3a). We tested two polycistronic vectors—one carrying an internal ribosome entry site (IRES)⁴⁶ and the other carrying a P2A self-cleaving peptide⁴⁷—for the co-expression of 2paCRY2-RAF1 and CIBN-mScarlet-I-CAAX. In addition, we tested full-length RAF1 and the RAF1 catalytic domain (a.a. 307–648) (Supplementary Fig. 6). By analyzing the sensitivity and background signal, we employed the system with P2A and full-length

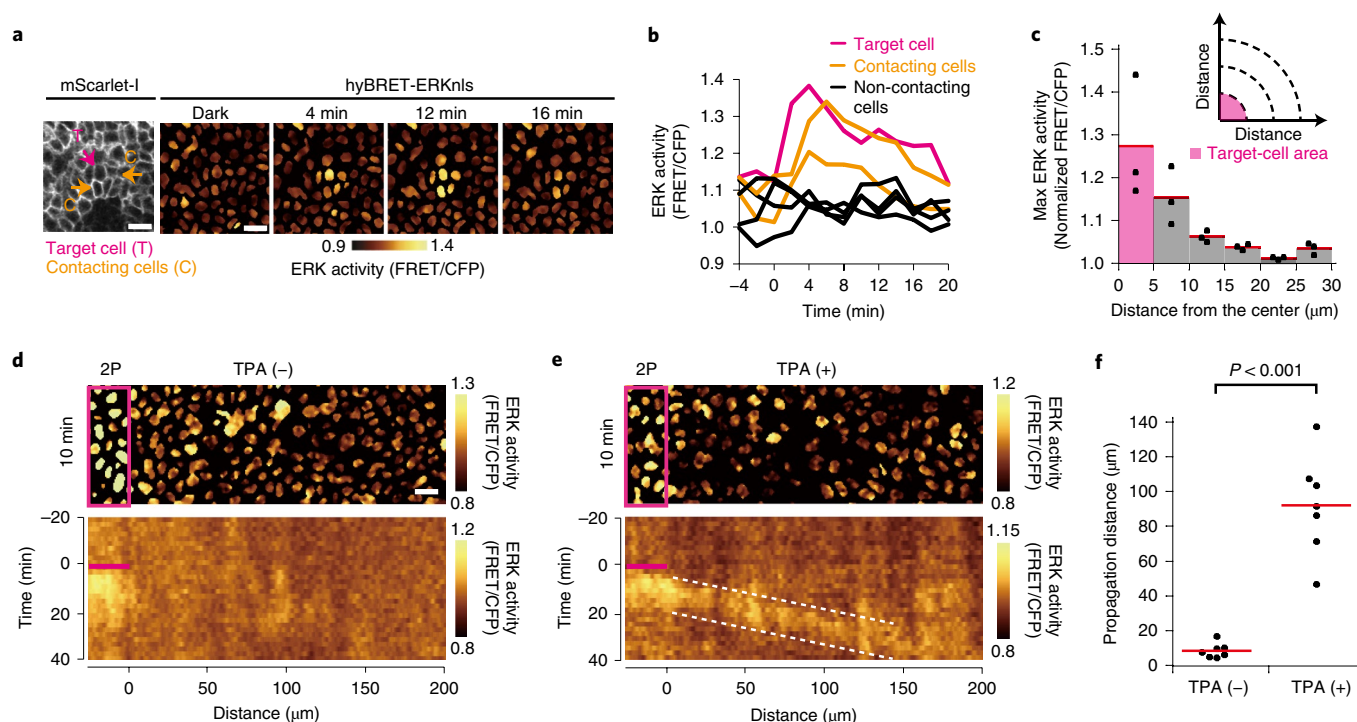


Fig. 5 | Induction of ERK activation waves in the mouse auricular epidermis. **a–c**, Single-cell ERK activation in the skin epidermis. The time-lapse images of ERK activity in the basal layer of the ear epidermis before and after single-cell ERK activation by 2P excitation are shown. The mScarlet-I signal represents the expression of 2paRAF. The epidermis of 2paRAF and hyBRET-ERKnl5 double-positive mice was imaged to analyze the ERK activity (FRET/CFP) after single-cell ERK activation. The FRET/CFP is represented in gold pseudo-colors. Magenta arrowhead, target cell; yellow arrowhead, representative contacting cells (**a**). Time courses of ERK activity after single-cell ERK activation in seven representative cells within each class of distance from the target cell (**b**). The maximum ERK activities (normalized FRET/CFP) of the target cells and those of the surrounding cells were measured and are shown with the distance from the center of the target cells (**c**). $n = 3$ cells from three independent mice. The bar graphs and red lines show the mean values; the magenta bar indicates the area where the target cell resided. **d–f**, Propagation distance of ERK activity with or without TPA treatment. The mouse ears were treated with or without double TPA treatment and labeled as TPA (+) and TPA (–), respectively. Representative images of ERK activity in the basal layer of the ear epidermis are shown in gold pseudo-color. The images are cropped from Supplementary Video 3; scale bar: 10 μm ; the square magenta region indicates the area of 2P activation. (**d,e**, upper). The time course of ERK activity is shown in kymographs. The 2P-activated area and time are indicated as magenta lines (**d,e**, lower). The dashed line highlights the propagating wave of ERK activity (**e**, lower). The maximum ERK propagation distances observed in 40 min were calculated from seven experiments from five independent mice and are shown in **f**. Scale bars: 10 μm . Red lines represent the mean; two-tailed *t*-tests were used for comparison.

RAF1, named as 2paRAF (Fig. 3b). We compared the 2paRAF and the classical CRY2PHR-RAF system (CRY2PHR-RAF1-P2A-CIBN-mScarlet-I-CAAX) in the response to 2P excitation in HeLa cells expressing EKAREV-nls. Note that the laser power for observation of EKAREV-nls was weak enough compared to the photoactivation, thereby minimizing the unintentional activation of CRY2PHR and 2paCRY2 (Fig. 3c–e). The FRET/CFP ratio in cells expressing 2paRAF was increased after 2P activation to the same extent as that after 1P excitation, but not in cells expressing CRY2PHR-RAF. These results demonstrate the superiority of 2paRAF to conventional CRY2PHR-RAF system.

Single-cell control of ERK activity in 3D Madin–Darby canine kidney (MDCK) cysts and the intestinal organoids. One of the unique advantages of 2PEM is that it permits excitation of the molecules in 3D space with little excitation of fluorophores along the optical path¹⁹. To demonstrate that 2paRAF activates ERK in 3D culture at single-cell resolution, we prepared MDCK cells stably expressing 2paRAF and EKAREV-nls. Because ERK activation is propagated to the neighboring cells in an EGF ligand- and EGF receptor-dependent manner^{27,28}, experiments were conducted in the presence of 1 μM PD153035 (EGFR inhibitor) and 10 μM TAPI-1 (MMPs inhibitor). MDCK cells form a monolayer cyst with a

central cavity. Z-stack images of the MDCK cell cysts were acquired by 2PEM, and ERK activation was not observed under this condition (Fig. 4a). On activation of a single cell, an increase in ERK activity (FRET/CFP) was only observed in the irradiated cell, indicating that ERK can be activated at single-cell resolution in 3D space (Fig. 4b, middle, and Supplementary Videos 1 and 2). The same cyst was further illuminated by blue light to confirm that almost all cells in the view field express sufficient levels of 2paRAF for photoactivation of ERK signal (Fig. 4b, right). These results proved that 2paRAF allows us to activate ERK at single-cell resolution in 3D space by 2PEM.

We then generated transgenic mice that ubiquitously expressed 2paRAF (Fig. 4c). Three mouse lines were established and named as 2paRAF mouse nos. 1, 2 and 3. There were some differences in the level and distribution of 2paRAF expression among the three mouse lines, but all mice were born at the expected Mendelian frequency. Notably, when 2paRAF mice were placed under room light, hyperplasia of the ear epidermis was observed, probably due to ERK activation. We therefore bred 2paRAF mice in light shaded cages. Under this condition, the mice displayed no overt phenotype.

Recently, we observed that ERK activation is propagated to the neighboring cells in the intestinal epithelium and organoids therefrom³¹. To prove that ERK activation in a single intestinal epithelial

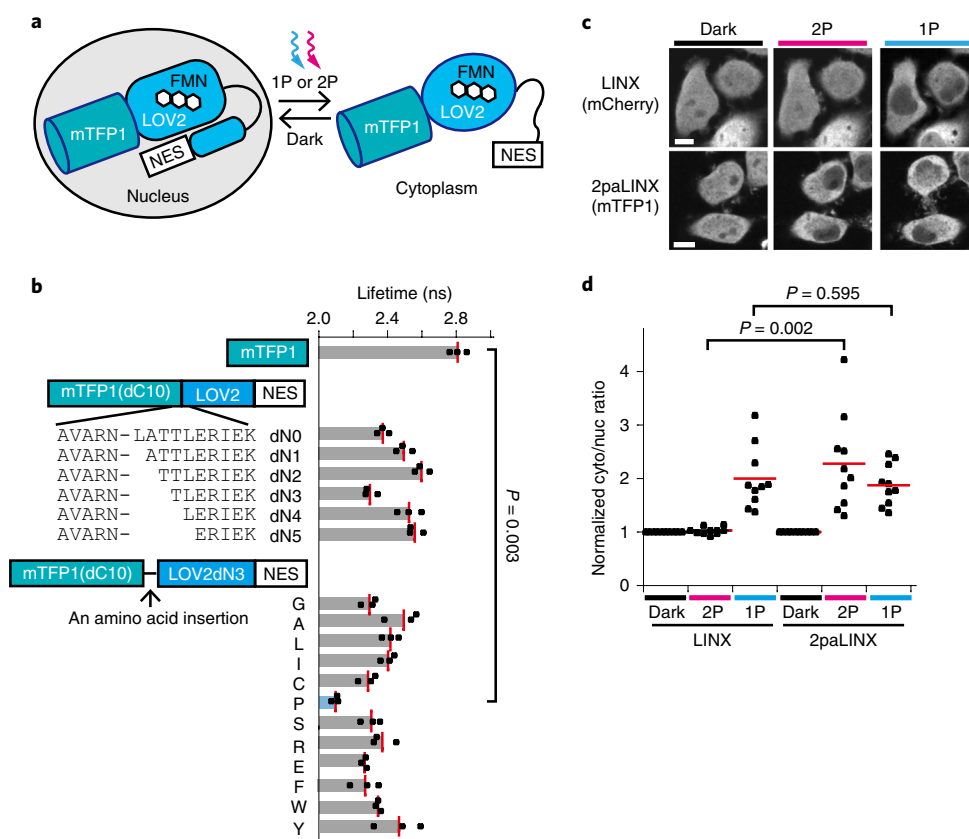


Fig. 6 | 2P activation of 2paLINX. **a**, Schematic representation of the 2paLINX. Photoactivation of LOV2 exposes a NES and translocates 2paLINX from nucleus to cytoplasm. **b**, Fluorescence lifetimes of mTFP1 wildtype and fusion proteins. The bar graphs and red lines show the mean values; $n = 3$ cells for each protein; two-tailed t -tests were used for comparison. **c**, Nucleocytoplasmic shuttling on 1P or 2P activation. Scale bars, 10 μm . **d**, The bee swarm plot shows the normalized intensity ratio between cytoplasm and nucleus (cyto/nuc) in each cell before and after photoactivation. Red lines represent the mean; $n = 10$ cells from $n = 2$ independent experiments; two-tailed t -tests were used for comparison.

cell can cause ERK activation in the neighboring cells, we generated intestinal organoid from the offspring from a mating between 2paRAF mice and hyBRET-ERKnl mice⁴⁸, which is the latest mouse line ubiquitously-expressing the FRET biosensor for ERK activity (Fig. 4d). Hereafter, for monitoring ERK activity, CFP was excited at 880 nm, where 2paCRY2 is less excited than 840 nm. First, the intestinal organoid was imaged by 2PEM, before and after the 1P excitation with blue light. As expected, most cells exhibited an increase in the FRET/CFP ratio, indicating efficient ERK activation (Fig. 4e,f). Next, a single cell in the organoid was activated by 2P excitation. The target cell showed ERK activation, followed by ERK activation in some neighboring cells (Fig. 4g). There was an approximately 5 min delay between the ERK activation in the target cell and that in adjacent cells (cells 2 and 3) (Fig. 4h), which resembles the time course of ERK activation propagation previously reported in a 2D culture system²⁸.

Induction of ERK activation waves in the mouse auricular epidermis. The propagation waves of ERK activation in vivo were originally discovered in the mouse auricular epidermis and named SPREAD³⁰. In SPREAD, from a few initiator cells, ERK activation is radially propagated to some 100 neighboring cells within 100 μm diameter and disappears within about 30 min. Here, we asked whether SPREAD could be reproduced by the induction of ERK activation in 2paRAF mice.

First, a single cell in the basal layer of the auricular epidermis of a 2paRAF and hyBRET-ERKnl double-positive mouse was activated by 2P excitation. ERK activation was successfully induced in the

target cell (Fig. 5a,b). However, propagation of ERK activation was limited to cells in contact with the target cell (Fig. 5a–c). Next, therefore, we increased the number of stimulated cells by expanding the stimulation area to $26 \times 66 \mu\text{m}^2$ (Fig. 5d). To detect the propagation of ERK activation, ERK activity was averaged along the y axis of the imaged area and is shown as a kymograph. Again, the propagation of ERK activation was limited only to the cells in contact with the photoactivated cells (Fig. 5d and Supplementary Video 3, upper).

Because the frequency of SPREAD is markedly higher in S/G2/M phase than in G1/G0 phase, we speculated that the propagation of ERK activation may depend on the cell cycle. The 12-O-tetradecanoylphorbol 13-acetate (TPA) is a potent tumor promoter and an activator of protein kinase C⁴⁹. Thus, to see the cell cycle effect, we topically applied TPA to the mouse ear skin before the experiments. In contrast to the epidermis without TPA application, ERK activation was propagated to $91.9 \pm 28.5 \mu\text{m}$, a value comparable to that in SPREAD, in the TPA-treated mice (Fig. 5e,f and Supplementary Video 3, lower). These results provide a useful insight into why SPREAD is mostly observed in S/G2/M phase. Although we cannot rule out the possibility that the initial firing frequency is also suppressed in G1/G0 phase, at least one primary reason that SPREAD was mostly observed in S/G2/M phase is that the propagation of ERK activation was suppressed in G1/G0 phase.

2P activation of LOV2. Last, we asked whether FRAPA can be applicable to other flavoproteins. Since the LOV domain also contains flavin derivative as the chromophore, we applied FRAPA to the LOV domain-based light-inducible nuclear exporter (LINX)⁵⁰. We

employed mTFP1 as the donor because of its large 2P absorption cross section⁵¹. mTFP1 was fused to the N terminus of a LINX variant, LINXa4 (RLR), which comprises an optimized LOV domain and a nuclear exporting signal (NES) (Fig. 6a). We optimized the linker and obtained a proline mutant named 2paLINX, which exhibited 25% FRET efficiency (Fig. 6b). Next, we compared the efficiency of 2P activation between the mCherry-tagged prototype LINX and 2paLINX (Fig. 6c,d). Cells expressing the prototype LINX and 2paLINX were activated by 30 scans, for the optimal 2P activation. As expected, 2paLINX but not LINX was exported from the nucleus to the cytoplasm after 2P excitation. We confirmed that prototype LINX could also be exported to the cytoplasm by 1P excitation as efficiently as 2paLINX. Collectively, these results strongly support the applicability of FRAPA to various photoreceptors.

Discussion

Here, we present a technique named FRAPA. The idea came from the notion that the ϵ of FAD, the chromophore of CRY2, is only one-quarter that of mTagBFP2 (Fig. 1c). Because, based on the two-level approximation^{34,35}, the estimated σ_2 is typically proportional to the square of ϵ in non-centrosymmetric dipolar chromophore, the difference in σ_2 could be about 16-fold. Therefore, the 40% FRET efficiency in 2paCRY2 enabled CRY2 to be efficiently activated by 2PEM. Other photoactivatable proteins, such as LOV, BLUF, VVD and Magnets, are also known to contain either FMN or FAD². Even in the case of the improved LOV for 2PEM, the value of σ_2 ($S_0 \rightarrow S_1$ transition) is less than one Goepfert–Mayer unit⁵². Because the σ_2 value of mTFP1 is more than 70-fold larger than that of LOV (Supplementary Table 1), 25% FRET efficiency between mTFP1 and LOV was sufficient to increase the sensitivity of LOV (Fig. 6). Moreover, fluorescent dyes that have large 2P cross sections⁵³ would also serve as better donors for FRAPA, although their use may be limited to in vitro studies. Meanwhile, molar extinction coefficients of red-shifted optogenetic proteins such as PhyB are larger than those of flavin-containing optical dimerizers⁵⁴. In agreement with this property, in our preliminary experiments, we have found that PhyB could be stimulated at 1,200 nm with laser intensity that is used routinely for the observation of far-red fluorescent proteins. Therefore, 2paCRY2 also enables simultaneous operation of two orthogonal systems.

Induced ERK activation in 2paRAF mice revealed two important constraints for the occurrence of SPREAD. First, in quiescent epidermal cells, ERK activation in single or multiple cells can induce ERK activation only in the adjacent cells within about 10 μ m. Second, in the TPA-treated skin, induced ERK activation can propagate the ERK activation wave to the distance that we observed in SPREAD (Fig. 5f). Then what is the effect of TPA application? One possibility is that propagation of ERK activation may be specific to cells in the S/G2 phase. The frequency of SPREAD has been highly correlated with the fraction of cells in the S/G2 phase in the epidermis³⁰. It is also reported that the pheromone-induced MAPK response in yeast is incomplete in G1 phase⁵⁵. It will be important to further pinpoint which molecule in the EGF-EGFR-ERK MAPK pathway is critically regulated during the cell cycle. Another possibility in regard to the requirement for TPA is related to the inflammatory response. In agreement with previous reports showing that TPA treatment induces infiltration of inflammatory cells such as neutrophils, macrophages and lymphocytes^{56,57}, we often encountered inflammatory cells invading the basal layer of ear skin during imaging. It is possible that the propagation of ERK activity is mediated by cytokines from the inflammatory cells. In fact, epidermal wounds, which inevitably accompany inflammatory response, generate large waves of ERK activation³⁰. Thus the effect of inflammatory cells may not be neglected.

In conclusion, we have developed 2paCRY2 and 2paLINX as proofs-of-concept of FRAPA. Our approach will provide a guideline

for the design of 2P-activatable optogenetic tools to control protein–protein interactions at single-cell resolution in vitro and in vivo, and will shed new light on signal transduction research in living tissues.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at <https://doi.org/10.1038/s41592-019-0541-5>.

Received: 18 April 2019; Accepted: 26 July 2019;

Published online: 9 September 2019

References

1. Fan, L. Z. & Lin, M. Z. Optical control of biological processes by light-switchable proteins. *Wiley Interdiscip. Rev. Dev. Biol.* **4**, 545–554 (2015).
2. Spiltoir, J. I. & Tucker, C. L. Photodimerization systems for regulating protein–protein interactions with light. *Curr. Opin. Struct. Biol.* **57**, 1–8 (2019).
3. Chen, D., Gibson, E. S. & Kennedy, M. J. A light-triggered protein secretion system. *J. Cell Biol.* **201**, 631–640 (2013).
4. Lungu, O. I. et al. Designing photoswitchable peptides using the AsLOV2 domain. *Chem. Biol.* **19**, 507–517 (2012).
5. Kennedy, M. J. et al. Rapid blue-light-mediated induction of protein interactions in living cells. *Nat. Methods* **7**, 973–975 (2010).
6. Zhou, X. X., Fan, L. Z., Li, P., Shen, K. & Lin, M. Z. Optical control of cell signaling by single-chain photoswitchable kinases. *Science* **355**, 836–842 (2017).
7. Levskaya, A., Weiner, O. D., Lim, W. A. & Voigt, C. A. Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature* **461**, 997 (2009).
8. Lee, S. et al. Reversible protein inactivation by optogenetic trapping in cells. *Nat. Methods* **11**, 633–636 (2014).
9. Nguyen, M. K. et al. Optogenetic oligomerization of Rab GTPases regulates intracellular membrane trafficking. *Nat. Chem. Biol.* **12**, 431 (2016).
10. Valon, L., Marín-Llauradó, A., Wyatt, T., Charras, G. & Treppe, X. Optogenetic control of cellular forces and mechanotransduction. *Nat. Commun.* **8**, 14396 (2017).
11. Idevall-Hagren, O., Dickson, E. J., Hille, B., Toomre, D. K. & De Camilli, P. Optogenetic control of phosphoinositide metabolism. *Proc. Natl Acad. Sci. USA* **109**, E2316–E2323 (2012).
12. Zhou, X. X. et al. A single-chain photoswitchable CRISPR-Cas9 architecture for light-inducible gene editing and transcription. *ACS Chem. Biol.* **13**, 443–448 (2018).
13. Konermann, S. et al. Optical control of mammalian endogenous transcription and epigenetic states. *Nature* **500**, 472–476 (2013).
14. Polstein, L. R. & Gersbach, C. A. A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. *Nat. Chem. Biol.* **11**, 198–200 (2015).
15. Hallett, R. A., Zimmerman, S. P., Yumerefendi, H., Bear, J. E. & Kuhlman, B. Correlating in vitro and in vivo activities of light-inducible dimers: a cellular optogenetics guide. *ACS Synth. Biol.* **5**, 53–64 (2016).
16. Hughes, R. M. et al. Optogenetic apoptosis: light-triggered cell death. *Angew. Chem. Int. Ed. Engl.* **54**, 12064–12068 (2015).
17. Katsura, Y. et al. An optogenetic system for interrogating the temporal dynamics of Akt. *Sci. Rep.* **5**, 14589 (2015).
18. O'Banion, C. P. et al. Design and profiling of a subcellular targeted optogenetic cAMP-dependent protein kinase. *Cell Chem. Biol.* **25**, 100–109.e108 (2018).
19. Denk, W., Strickler, J. H. & Webb, W. W. Two-photon laser scanning fluorescence microscopy. *Science* **248**, 73–76 (1990).
20. Prakash, R. et al. Two-photon optogenetic toolbox for fast inhibition, excitation and bistable modulation. *Nat. Methods* **9**, 1171–1179 (2012).
21. Andrasfalvy, B. K., Zemelman, B. V., Tang, J. & Vaziri, A. Two-photon single-cell optogenetic control of neuronal activity by sculpted light. *Proc. Natl Acad. Sci. USA* **107**, 11981–11986 (2010).
22. Guglielmi, G., Barry, J. D., Huber, W. & De Renzis, S. An optogenetic method to modulate cell contractility during tissue morphogenesis. *Dev. Cell* **35**, 646–660 (2015).
23. Schindler, S. E. et al. Photo-activatable Cre recombinase regulates gene expression in vivo. *Sci. Rep.* **5**, 13627 (2015).
24. Nishida, E. & Gotoh, Y. The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**, 128–131 (1993).
25. Shaul, Y. D. & Seger, R. The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim. Biophys. Acta* **1773**, 1213–1226 (2007).
26. Dhillon, A. S., Hagan, S., Rath, O. & Kolch, W. MAP kinase signalling pathways in cancer. *Oncogene* **26**, 3279–3290 (2007).

27. Albeck, J. G., Mills, G. B. & Brugge, J. S. Frequency-modulated pulses of ERK activity transmit quantitative proliferation signals. *Mol. Cell* **49**, 249–261 (2013).
28. Aoki, K. et al. Stochastic ERK activation induced by noise and cell-to-cell propagation regulates cell density-dependent proliferation. *Mol. Cell* **52**, 529–540 (2013).
29. Toettcher, J. E., Weiner, O. D. & Lim, W. A. Using optogenetics to interrogate the dynamic control of signal transmission by the Ras/Erk module. *Cell* **155**, 1422–1434 (2013).
30. Hiratsuka, T. et al. Intercellular propagation of extracellular signal-regulated kinase activation revealed by in vivo imaging of mouse skin. *eLife* **4**, e05178 (2015).
31. Muta, Y. et al. Composite regulation of ERK activity dynamics underlying tumour-specific traits in the intestine. *Nat. Commun.* **9**, 2174 (2018).
32. Ogura, Y., Wen, F. L., Sami, M. M., Shibata, T. & Hayashi, S. A switch-like activation relay of EGFR-ERK signaling regulates a wave of cellular contractility for epithelial invagination. *Dev. Cell* **46**, 162–172.e165 (2018).
33. Hancock, J. F., Paterson, H. & Marshall, C. J. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* **63**, 133–139 (1990).
34. Drobizhev, M. et al. Strong two-photon absorption in new asymmetrically substituted porphyrins: interference between charge-transfer and intermediate-resonance pathways. *J. Phys. Chem. B* **110**, 9802–9814 (2006).
35. Rebane, A. et al. New all-optical method for measuring molecular permanent dipole moment difference using two-photon absorption spectroscopy. *J. Lumin.* **130**, 1619–1623 (2010).
36. Subach, O. M., Cranfill, P. J., Davidson, M. W. & Verkhusha, V. V. An enhanced monomeric blue fluorescent protein with the high chemical stability of the chromophore. *PLoS ONE* **6**, e28674 (2011).
37. Yasuda, R. et al. Supersensitive Ras activation in dendrites and spines revealed by two-photon fluorescence lifetime imaging. *Nat. Neurosci.* **9**, 283–291 (2006).
38. Gratton, E., Breusegem, S., Sutin, J., Ruan, Q. & Barry, N. Fluorescence lifetime imaging for the two-photon microscope: time-domain and frequency-domain methods. *J. Biomed. Opt.* **8**, 381–390 (2003).
39. Clegg, R. M. Fluorescence resonance energy transfer. *Curr. Opin. Biotechnol.* **6**, 103–110 (1995).
40. Jares-Erijman, E. A. & Jovin, T. M. FRET imaging. *Nat. Biotechnol.* **21**, 1387 (2003).
41. Wu, Y. I. et al. A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature* **461**, 104 (2009).
42. Subach, O. M. et al. Structural characterization of acylimine-containing blue and red chromophores in mTagBFP and TagRFP fluorescent proteins. *Chem. Biol.* **17**, 333–341 (2010).
43. Brautigam, C. A. et al. Structure of the photolyase-like domain of cryptochrome 1 from *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **101**, 12142–12147 (2004).
44. Xu, D., Jaroszewski, L., Li, Z. & Godzik, A. AIDA: ab initio domain assembly for automated multi-domain protein structure prediction and domain-domain interaction prediction. *Bioinformatics* **31**, 2098–2105 (2015).
45. Zhang, K. et al. Light-mediated kinetic control reveals the temporal effect of the Raf/MEK/ERK pathway in PC12 cell neurite outgrowth. *PLoS ONE* **9**, e92917 (2014).
46. Akagi, T., Sasai, K. & Hanafusa, H. Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. *Proc. Natl Acad. Sci. USA* **100**, 13567–13572 (2003).
47. Lo, C. A. et al. Quantification of protein levels in single living cells. *Cell Rep.* **13**, 2634–2644 (2015).
48. Komatsu, N. et al. A platform of BRET-FRET hybrid biosensors for optogenetics, chemical screening, and in vivo imaging. *Sci. Rep.* **8**, 8984 (2018).
49. Nishizuka, Y. Turnover of inositol phospholipids and signal transduction. *Science* **225**, 1365–1370 (1984).
50. Lerner, A. M., Yumerefendi, H., Goudy, O. J., Strahl, B. D. & Kuhlman, B. Engineering improved photoswitches for the control of nucleocytoplasmic distribution. *ACS Synth. Biol.* **7**, 2898–2907 (2018).
51. Drobizhev, M., Makarov, N. S., Tillo, S. E., Hughes, T. E. & Rebane, A. Two-photon absorption properties of fluorescent proteins. *Nat. Methods* **8**, 393–399 (2011).
52. Homans, R. J. et al. Two photon spectroscopy and microscopy of the fluorescent flavoprotein, iLOV. *Phys. Chem. Chem. Phys.* **20**, 16949–16955 (2018).
53. Hayek, A. et al. Cell-permeant cytoplasmic blue fluorophores optimized for in vivo two-photon microscopy with low-power excitation. *Microsc. Res. Tech.* **70**, 880–885 (2007).
54. Cornejo, J., Beale, S. L., Terry, M. J. & Lagarias, J. C. Phytochrome assembly. The structure and biological activity of 2(R),3(E)-phytochromobilin derived from phycobiliproteins. *J. Biol. Chem.* **267**, 14790–14798 (1992).
55. Conlon, P., Gelin-Licht, R., Ganesan, A., Zhang, J. & Levchenko, A. Single-cell dynamics and variability of MAPK activity in a yeast differentiation pathway. *Proc. Natl Acad. Sci. USA* **113**, E5896–E5905 (2016).
56. Stanley, P. L., Steiner, S., Havens, M. & Trampusch, K. M. Mouse skin inflammation induced by multiple topical applications of 12-O-tetradecanoylphorbol-13-acetate. *Ski. Pharmacol. Physiol.* **4**, 262–271 (1991).
57. Lee, S. H. et al. Suppression of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in mice by transduced Tat-Annexin protein. *BMB Rep.* **45**, 354–359 (2012).

Acknowledgements

We thank J. Miyazaki, T. Nagai (Osaka University), A. Hotta (Kyoto University), K. Kawakami (National Institute of Genetics), H. Miyoshi (Keio University) and C. Tucker (University of Colorado, Denver) for the plasmids. We also thank Y. Suzuki, J. Kawamata (Yamaguchi University) and T. Furuta (Toho University) for helpful discussion. We are grateful to the members of the Matsuda Laboratory for their helpful input; K. Hirano, K. Takakura, A. Kawagishi and Y. Takeshita for their technical assistance and the Medical Research Support Center of Kyoto University for in vivo imaging. This research was partially supported by the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research from AMED under Grant No. JP18am0101079 (to S.I.)). This work was also supported by Japan Society for the Promotion of Science (JSPS) grant nos. KAKENHI17J07950 (to T.K.), KAKENHI18K07066, AMED16gm0610010h004 (to K.Terai), KAKENHI15H02397, KAKENHI15H05949 'Resonance Bio', KAKENHI16H06280 'ABiS', CRESTJPMJCR1654 and the Nakatani Foundation (to M.M.).

Author contributions

T.K., K. Terai and M.M. conceptualized the study. T.K., S.H., K. Terai, N.N., K.S., S.I. and M.M. designed the methodology. T.K., K. Terai and M.M. performed validation. T.K., K. Terai, S.H., N.N., S.I. and M.M. conducted the formal analysis. The investigation was conducted by T.K., K. Terai and M.M. The data curation was done by T.K., K. Terai and M.M. Resources were collected by T.K., K. Terai and M.M. The original draft was written by T.K. Writing, review and editing was done by K. Terai, N.N., K.S., K. Togashi, S.I. and M.M. K. Terai and M.M. supervised the work. Project administration was done by K. Terai and M.M. T.K., K. Terai, S.I. and M.M. were responsible for funding acquisition.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41592-019-0541-5>.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to K.T. or M.M.

Peer review information: Rita Strack was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2019

Methods

Plasmids. To develop optogenetic tools, we employed the PHR domain of *A. thaliana* CRY2 (a.a. 1–498) and a C terminal truncated version of CIB1 (a.a. 1–170) called CIBN. The complementary DNA of CRY2PHR was synthesized with codon optimization for mammalian cells by GenScript. The cDNA of CIBN was derived from pCIBN(deltaNLS)-pmGFP (Addgene plasmid no. 26867)⁵. As donors for FRAPA, the cDNAs of mTagBFP2 (ref. ³⁶) and EBFP2 (ref. ⁵⁸) were synthesized with codon optimization by GeneArt (Thermo Fisher Scientific). The plasmid containing the cDNA of Sirius was a gift from T. Nagai⁵⁹.

To analyze the FRET efficiency of mTagBFP2-CRY2PHR chimeric proteins by FLIM, plasmids containing the CAG promoter were generated by an In-Fusion system (Clontech). To generate pCAGGS-2paCRY2, cDNA encoding CRY2PHR (a.a. 1–160), a truncated mTagBFP2 (a.a. 1–221) with flexible GSG linkers, and CRY2PHR (a.a. 171–498) were PCR-amplified and assembled using In-Fusion into pCAGGS vector⁶⁰. The detailed DNA sequence of 2paCRY2 is described in Supplementary Note 1. Some mutants including CRY2PHR (D387A) were generated using overlap PCR extension. All other plasmids encoding a series of CRY2 variants are listed in Supplementary Table 2.

For the membrane-recruitment assay, pCAGGS-CRY2PHR-mScarlet-H and pCAGGS-2paCRY2-mScarlet-H was generated from pmScarlet-C1 that was obtained from Addgene (plasmid no. 85042)⁶¹, using overlap PCR extension. The cDNA encoding CIBN-EGFP-CAAX was PCR-amplified from pCX4neo-CIBN-EGFPx²⁸ and subcloned into pCAGGS vector.

For mTagBFP2 purification, the cDNA of mTagBFP2 was introduced into pBAD/Myc-His vector (Thermo Fisher Scientific). mTagBFP2 (a.a. 1–221) was fused with a His8 tag at the C terminus. The construct was inserted downstream of the pBAD promoter. *Escherichia coli* TOP10 cells (Thermo Fisher Scientific) were transformed with the resulting plasmid.

To generate transgenic mice, pT2ADW-2paRAF coding 2paCRY2, GSG linker, full-length human RAF1, self-cleaving P2A peptide⁶², CIBN, GSG linker, mScarlet-I and the CAAX domain of the KRas protein (a.a. 170–189) were assembled and subcloned into pT2ADW vector⁴⁸ by In-Fusion. To establish stable cell lines, pT2ADWpuro-2paRAF, including the internal ribosome entry site (IRES) and puromycin-resistant gene, were assembled using In-Fusion. The detailed DNA sequence of 2paRAF is described in Supplementary Note 1.

To develop 2paLINX, cDNA of mTFP1 was purchased from Allele Biotechnology. pNIA-CEN-FLAG-LINXa4 (RLR)-Bre1 was a gift from B. Kuhlman (Addgene plasmid no. 116884). All chimeric mutants of mTFP1 and LINX were PCR-amplified and assembled using In-Fusion into pCAGGS vector. The detailed DNA sequence of 2paLINX is described in Supplementary Note 2.

Reagents. TPA and PD153035 were purchased from Calbiochem. TAPI-1 was purchased from Merck. Noggin was obtained from PeproTech. R-spondin1 was purchased from R&D Systems. EGF was purchased from Sigma-Aldrich.

Cell culture and establishment of cell lines. HeLa and Lenti-X 293T cells were purchased from the Human Science Research Resources Bank and Clontech, respectively. HeLa and Lenti-X 293T cells were maintained in DMEM (Wako Pure Chemical Industries) with 10% fetal bovine serum (SAFC Biosciences) and penicillin/streptomycin (Nacalai Tesque). MDCK cells were purchased from the RIKEN BioResource Center (no. RCB0995) and maintained in MEM (Life Technologies) with 10% fetal bovine serum (SAFC Biosciences), 1% non-essential amino acids (Life Technologies), 1% GlutaMAX Supplement (Life Technologies), 1 mM sodium pyruvate (Life Technologies) and penicillin/streptomycin (Nacalai Tesque).

MDCK cells stably expressing EKAREV-nls⁶², a FRET biosensor for ERK activity, were established through lentiviral infection. For lentiviral production, Lenti-X 293T cells were co-transfected with plasmids of FRET biosensors encoded in the pCII-EF vector, psPAX2, which was obtained from Addgene (plasmid no. 12260), and pCMV-VSV-G-RSV-Rev, which was a kind gift from H. Miyoshi (RIKEN BioResource Center), by lipofection using Polyethyleneimine 'Max' MW 40,000 (Polyscience Inc.). Virus-containing media were collected at 48 h after transfection, filtered and applied to target cells with 10 µg ml⁻¹ polybrene (Nacalai Tesque). Two days after infection, the infected cells were selected with 10 µg ml⁻¹ blasticidin S (InvivoGen) for at least 1 week. To obtain MDCK cells expressing EKAREV-nls and 2paRAF, MDCK cells expressing EKAREV-nls were co-transfected with a pT2ADWpuro-2paRAF and pCAGGS-T2TP by electroporation with a NEPA21 electroporator (Nepa Gene) and then selected in the presence of 2 µg ml⁻¹ puromycin (InvivoGen).

Transgenic mice. The transgenic mice expressing 2paRAF were generated by Tol2-mediated gene transfer as previously described^{63,64}. Briefly, fertilized eggs derived from C57BL/6N mice were microinjected with a mixture of Tol2 transposase messenger RNA and the pT2ADW-2paRAF. Mice of the resulting lines, named 2paRAF mice, were then backcrossed with B6N-Tyrc-Brd/BrdrCrCr mice, also known as B6 Albino mice (Charles River), for more than four generations. Offspring were genotyped by PCR with the primers 5'-GGCATCAGTGTGCAGTCATACAAC-3' and 5'-GCTGCCGTAGAGGAAGCTAGTAGC-3' for the 2paCRY2 protein, yielding 321-base-pair products.

The tunable range of activity is always a key issue in the development of optogenetic tools. We made a particular effort to minimize the basal ERK activity by the expression of CRY2-RAF1 fusion proteins and chose 2paRAF suitable for the in vivo study (Fig. 3). The 2paRAF mice do not show any overt lesions for 3–4 months after birth; however, after 4–6 months hypertrophy or papilloma-like lesion was observed in the skin of some mice, probably due to ERK activation by room light exposure or basal 2paCRY2-CIBN binding under a dark state. These results are consistent with the phenotype of RAF1 transgenic mice⁶⁵. Recently, a new set of CRY2-CIB system was shown to lower the basal CRY2-CIB binding under a dark condition⁶⁶. This may alleviate the skin lesion, if applied to 2paCRY2.

For in vivo FRET imaging hyBRET-ERKns mice were crossed with 2paRAF mice. The transgenic mice expressing the hyBRET biosensor for ERK activity, called hyBRET-ERKns mice, have been reported previously⁴⁸. These mice were backcrossed for more than ten generations with C57BL/6 mice. Newborn mice were illuminated with a blue flashlight LEDGFP-3W (Optocode) and inspected for yellow fluorescence through yellow-colored glasses (Optocode). Mice were housed in a specific-pathogen-free facility in temperature-controlled rooms with a 14 h light/10 h dark cycle, and received a routine chow diet and water ad libitum. 2paRAF mice were housed under dark conditions. For in vivo imaging, 8–24-week-old male or female mice were used. At the end of the experiments, euthanasia was performed by anesthetic overdose. The animal protocols were reviewed and approved by the Animal Care and Use Committee of Kyoto University Graduate School of Medicine (nos. 10584, 14079, 15064, 16038 and 17539) and the methods were carried out in accordance with the relevant guidelines and regulations.

Absorbance spectra determination for FAD and purified mTagBFP2. FAD disodium salt hydrate was purchased from Sigma-Aldrich. mTagBFP2 was purified according to the manufacturer's protocol. Briefly, *E. coli* TOP10 cells harboring the expression plasmid for TagBFP2-His8 were grown overnight at 37 °C and 250 r.p.m. in Luria-Bertani (LB) medium. The culture was diluted 1:100 into 100 ml of LB supplemented with 0.2% arabinose and grown overnight at 37 °C. The cells were collected by centrifugation at 6,000g for 15 min and re-suspended in buffer S1 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM imidazole, 0.1% Triton X-100, Roche EDTA-free protease inhibitor tablet). Cells were disrupted by sonication. The lysate was clarified via centrifugation and loaded onto a HisTrap Crude column (GE Healthcare) equilibrated with buffer S2 (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM imidazole). Bound proteins were eluted with buffer S3 (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 250 mM imidazole), concentrated with Amicon Ultra-15 (molecular weight cut-off 10 K) and loaded onto a Superdex200 Increase 10/300GL column (GE Healthcare) equilibrated with TBS buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl). The peak fractions were pooled, concentrated, flash frozen in liquid nitrogen and stored at –80 °C. Absorbance spectra were scanned at wavelengths from 300 to 600 nm using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech).

Calculation of Förster distance. Förster distance R_0 (in Å) was calculated from the equation⁶⁷

$$R_0 = 9.78 \times 10^3 (Q_D J n^{-4} \kappa^2)^{1/6}$$

where κ^2 is the orientation factor (the rotationally averaged value 2/3 was used), $n = 1.33$ is the refractive index of water, Q_D is the quantum yield of the donor and J is the spectral overlap integral calculated as

$$J = \frac{\int f_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda}{\int f_D(\lambda) d\lambda}$$

where λ is the wavelength, f_D is the normalized donor emission spectrum and ϵ_A is the acceptor molar extinction coefficient.

Design and modeling of engineered CRY2PHR proteins. The structural coordinates of the TagBFP (PDB entry, 3M24) and PHR domain of cryptochrome 1 from *A. thaliana* (PDB entry, 1U3C) were used as templates to generate homology models of the mTagBFP2 and CRY2PHR, respectively. The models were built using the program MODELLER⁶⁸ and visualized with PyMOL (<http://www.pymol.org/>). Tertiary structures and relative spatial arrangements of fusion constructs between the mTagBFP2 and CRY2PHR were predicted using the AIDA (Ab Initio Domain Assembly) server (<http://ffas.godziklab.org/AIDA/>)⁶⁴. The AIDA server provides an iterative method for automated domain assignment, modeling and assembly, which is useful for structure prediction of artificially created multi-domain proteins containing discontinuous domains (that is, domains with 'inserted' domains) such as 2paCRY2.

Fluorescence lifetime imaging. HeLa cells were transfected with each BFP variant and a series of BFP-CRY2 fusion-protein variants. The 2P fluorescence lifetime imaging was performed with an FV1000MPE inverted microscope, equipped with a ×30/1.05 numerical aperture (NA) silicon oil-immersion objective lens (UPLSAPO 30XS; Olympus), Mai Tai DeepSee Ti:sapphire laser (Spectra-Physics), an infrared (IR) cut filter, RDM690 (Olympus) and an emission filter, FF01-

479/40–25 (Semrock). The excitation wavelength was tuned to 700 nm for Sirius, 760 nm for EBFP2 and 800 nm for mTagBFP2, respectively. Average laser power was set at 1 to 3 mW as measured under the objective lens with the Model 407 A Portable Laser Power Meter (Spectra-Physics). FLIM images were obtained using a time-correlated single-photon counting board (SPC-150, Becker and Hickl). Images (512 × 512 pixels) were acquired at 524 ms per frame and averaged over 60–180 frames (total acquisition time, 30–90 s). Fluorescence lifetimes were measured in manually selected regions of interest (ROI) by using SPCImage software (Becker and Hickl) according to the manufacturer's protocol.

Fluorescent imaging and optical control of cultured cells. For the membrane-recruitment assay, HeLa cells were seeded onto 35-mm glass-based dishes (AGC Inc.). At 12 h after seeding, the cells were co-transfected with pCAGGS-CIBN-EGFP-CAAX and pCAGGS-CRY2PHR-mScarlet-H, or with pCAGGS-CIBN-EGFP-CAAX and pCAGGS-2paCRY2-mScarlet-H. At 48 h after transfection, cells were imaged with a FV1200MPE-IX83 inverted microscope equipped with a ×30/1.05 NA silicon oil-immersion objective lens (UPLSAPO 30XS, Olympus), InSight DeepSee Ultrafast laser (Spectra-Physics), IR cut filter, RDM690 (Olympus), two dichroic mirrors, DM505 (Olympus) and DM570 (Olympus), and two emission filters (BA495–540 for EGFP and BA575–630 for mScarlet-H) (Olympus). For EGFP and mScarlet-H detection, the excitation wavelengths were set to 930 nm and 1,040 nm, respectively. To compare the 1P- and 2P-activation efficiency of CRY2, HeLa cells expressing CRY2PHR-mCherry and CIBN-EGFP-CAAX were kept under dark conditions for 1 h and imaged for EGFP and mCherry signals. The cells were then activated by 1P (480 nm, 5 mW cm⁻², 1 s) or 2P (840 nm, 5 mW, 0.276 μm per pixel, 2 μs per pixel, 512 × 512 pixels, a single scan) excitation and imaged for mCherry signal. To compare the 2P-activation efficiency of CRY2 and 2paCRY2, the laser power was set at 3 mW for observation of EGFP, 6 mW for observation of mScarlet-H and 5 mW for 2P activation of CRY2 and 2paCRY2, as measured under the objective lens. HeLa cells expressing CIBN-EGFP-CAAX and CRY2PHR- or 2paCRY2-mScarlet-H were kept under dark conditions for 1 h. Then, the cells were exposed to the 2P activation (840 nm for CRY2PHR and 800 nm for 2paCRY2, 5 mW, 0.207 μm per pixel, 2 μs per pixel, 512 × 512 pixels, every 5 s, 30 scans) or 1P activation (480 nm, 5 mW cm⁻², for 1 s after 2P activation).

Membrane recruitment of CRY2 and 2paCRY2 in HeLa cells was quantified by measuring the ratio of plasma membrane to cytosolic mScarlet-H intensities. The ROIs were placed in the membrane area of CIBN-EGFP-CAAX images manually and transferred to the CRY2PHR- or 2paCRY2-mScarlet-H images. The ROIs were also placed in the cytosolic area of CRY2PHR- or 2paCRY2-mScarlet-H images.

For FRET imaging, HeLa cells expressing the hyBRET biosensor⁴⁸ were seeded onto 35-mm glass-based dishes, and transfected with the plasmids encoding CRY2PHR-RAF system (CRY2PHR-RAF1-P2A-CIBN-mScarlet-I-CAAX) or 2paRAF. At 36 h after transfection, the cells were serum-starved for 12 h with DMEM/F12 supplemented with 0.1% BSA. At 48 h after transfection, the cells were imaged with a FV1200MPE-IX83 inverted microscope equipped with a ×30/1.05 NA silicon oil-immersion objective lens, InSight DeepSee Ultrafast laser, IR-cut filter (BA685RIF-3), two dichroic mirrors (DM505 and DM570) and two emission filters (BA460–500 for CFP and BA520–560 for YFP) (Olympus).

To compare the 2paRAF variants (using P2A, IRES), cells were imaged and photoactivated simultaneously by 2P excitation (840 nm, 10 mW, 0.828 μm per pixel, 2 μs per pixel, 20 scans).

To compare the 2paRAF and the prototype CRY2PHR-RAF system, the laser power was set at 3 mW for observation and 7.5 mW for photoactivation. Images of the cells expressing EKAREV-nls, ERK FRET biosensor, were acquired (840 nm, 3 mW, 0.828 μm per pixel, 2 μs per pixel, 512 × 512 pixels, every 2 min) for 40 min. For photoactivation, the cells were scanned iteratively with three consecutive Z-stacks separated by 1 μm (840 nm for CRY2PHR and 800 nm for 2paCRY2, 7.5 mW, 0.414 μm per pixel, 2 μs per pixel, three Z-stacks, 10 times) just before the time-lapse imaging.

To compare mCherry-tagged LINX and 2paLINX, HeLa cells expressing mCherry-tagged LINX or 2paLINX were kept under a dark condition for 1 h. The cells were then activated by 2P (870 nm, 5 mW, 0.207 μm per pixel, 2 μs per pixel, every 1 min, 30 times) and imaged for either mCherry or mTFP1. Then, the cells were kept again under dark conditions and activated by 1P (480 nm, 3 mW cm⁻², for 1 s, every 1 min, 30 times).

Images were processed and analyzed with MetaMorph (Universal Imaging Corporation) software²⁸. Briefly, FRET efficiency was visualized as FRET/CFP ratio images shown in the intensity-modulated display (IMD) mode, in which eight colors from red to blue represent the FRET/CFP ratio and the 32 grades of color intensity represent the fluorescence intensity of CFP according to the color scale shown in each figure.

Two-photon activation and time-lapse imaging of 3D MDCK cysts and intestinal organoids.

To generate MDCK cysts in 3D culture, 7.5 × 10³ MDCK cells expressing EKAREV-nls and 2paRAF were centrifuged and suspended in 30 μl of Matrigel (BD Biosciences) and placed into a 35-mm diameter glass-bottom dish. Matrigel was allowed to polymerize at 37 °C for 20 min. After polymerization

of Matrigel, dishes were supplied with Advanced DMEM/F12 Medium (Thermo Fisher Scientific) containing 10% FBS, and incubated at 37 °C for 7–14 d.

MDCK cysts were imaged with an LCV110-MPE incubator microscope equipped with a ×25/1.05 NA water-immersion objective lens (XLPLN 25XWMP2, Olympus) and InSight DeepSee Ultrafast laser, an IR-cut filter (BA685RIF-3), two dichroic mirrors (DM505 and DM570) and two emission filters (BA460–500 for CFP and BA520–560 for YFP). The laser power was set at 3 mW for observation of EKAREV-nls and 12.5 mW for 2P activation of 2paRAF. For time-lapse FRET imaging, images were acquired (840 nm, 3 mW, 0.795 μm per pixel, 2 μs per pixel, 512 × 512 pixels, every 5 min) for 40 min (15 min before photoactivation to 20 min after photoactivation). Z-stack images were acquired at 1 μm intervals for a total of 70 μm. For photoactivation, a single cell was scanned iteratively (840 nm, 12.5 mW laser power, 0.397 μm per pixel, 2 μs per pixel, 30 scans, a single plane). Images were processed and analyzed with MetaMorph software.

Small intestinal organoids were generated according to the published protocol^{31,69}. Briefly, intestinal crypts were isolated from the small intestine of hyBRET-ERKnl and 2paRAF double-positive mice by incubation for 30 min at 4 °C in PBS containing 2 mM EDTA, and cultured in Advanced DMEM/F12 Medium (Thermo Fisher Scientific) supplemented with EGF (50 ng ml⁻¹, PeproTech), Noggin (100 ng ml⁻¹, PeproTech) and R-spondin1 (500 ng ml⁻¹, R&D Systems). For EGF starvation, organoids were cultured in media without EGF for 12 to 24 h. Organoids were embedded in Growth Factor Reduced Matrigel (Corning) and maintained by serial passaging. Within 4 d of the last passage, organoids were imaged to minimize the autofluorescence emitted from the cell debris accumulating in their central cavities. All organoids were generated from mice housed in a specific pathogen-free facility, and assumed to be free from mycoplasma contamination.

Small intestinal organoids were imaged with an LCV110-MPE incubator microscope equipped with a ×25/1.05 water-immersion objective lens (XLPLN 25XWMP2, Olympus) and InSight DeepSee Ultrafast laser, an IR-cut filter (BA685RIF-3), two dichroic mirrors (DM505 and DM570), and two emission filters (BA460–500 for CFP and BA520–560 for YFP). The excitation wavelengths for CFP and 2paRAF were tuned to 880 and 810 nm, respectively. The laser power was set at 6.5 mW for observation and 17 mW for 2P activation. For time-lapse FRET imaging, images were acquired (880 nm, 6.5 mW, 0.53 μm per pixel, 2 μs per pixel, 320 × 320 pixels, every 1 min) for 20 min (5 min before photoactivation and 15 min after photoactivation). For photoactivation, a single cell was scanned iteratively (810 nm, 17 mW, 0.265 μm per pixel, 2 μs per pixel, 10 scans at time 0). Z-stack images were acquired at 2 μm intervals for a total of 80 μm. Images were processed and analyzed with MetaMorph software. The 3D image reconstruction and fluorescence intensity measurements was performed by using Volocity software (PerkinElmer).

In vivo two-photon imaging and two-photon activation in the skin epidermis.

Mice expressing hyBRET-ERKnl and 2paRAF were typically treated with 25 μM TPA in 20 μl of acetone at 48 h and just before experiments. Hairs were removed by depilation cream 12 h before experiments. Mice were anesthetized with 1.5% isoflurane (Abbot) inhalation and placed in the supine position on an electric heat pad maintained at 37 °C. Ear skin was immobilized on a custom-made vacuum-stabilized imaging window (Olympus Engineering Corporation), which was connected to a vacuum aspirator⁷⁰ and designed to continuously pull on the ear skin. The suction force was set to approximately 30 kPa. The bowl of the imaging window was warmed to 37 °C with a lens heater (TP-LH, Tokai Hit).

Then, 2P excitation microscopy was performed with FV1200MPE-BX61WI upright microscopes, equipped with a ×25/1.05 water-immersion objective lens (XLPLN 25XWMP, Olympus) and an InSight DeepSee Ultrafast laser, IR-cut filter (BA685RIF-3), two dichroic mirrors (DM505 and DM570) and two emission filters (BA460–500 and BA520–560 for CFP and YFP). The excitation wavelengths for CFP and 2paRAF were tuned to 880 and 810 nm, respectively. For single-cell ERK activation, the region of interest was scanned iteratively (50–60 μm², 810 nm, 7.5 mW, 0.198 μm per pixel, 2 μs per pixel, 30 times). For multiple-cell ERK activation, the laser power was set at 2 mW for observation and 7.5–15 mW for photoactivation. For time-lapse FRET imaging, images were acquired (880 nm, 2 mW, 0.397 μm per pixel, 2 μm per pixel, 512 × 512 pixels) every 2 min for 60 min (20 min before photoactivation and 40 min after photoactivation) and were represented by a gold pseudo-color image for better presentation of the propagation of ERK activity. For photoactivation, the region of interest was scanned iteratively (26 × 66 μm², 810 nm, 7.5–15 mW, 0.662 μm per pixel, 2 μs per pixel, 40 × 100 pixels, 10 scans) in the basal layer of the ear skin.

For the measurement of ERK propagation after single-cell ERK activation, circles with increasing radius were drawn from the center of target cell and the FRET/CFP ratio in the pixels therein showing the top 20% of values in each time point was measured and averaged as using a custom MATLAB script (MathWorks). Kymographs of ERK activity were also generated using a custom MATLAB script. The propagation distance was manually measured from the kymographs.

Statistical analysis. All statistical analyses were carried out using KaleidaGraph software (Synergy Software). No statistical analysis was used to predetermine the sample size. An unpaired Welch's *t*-test was used to evaluate statistically

significant differences. Data are expressed as the mean $\pm$ s.d. *P* values were shown in figure legends.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available within the article and its Supplementary Information or from the corresponding author upon reasonable request. Some plasmids encoding 2paCRY2, 2paRAF and 2paLINX are available through AddGene (IDs: 129651 to 129655). The 2paRAF mouse strain is available through the Center for Animal Resources and Development, Kumamoto University (ID: 2853).

Code availability

All analysis code used in this study is available upon reasonable request.

References

58. Ai, H. W., Shaner, N. C., Cheng, Z., Tsien, R. Y. & Campbell, R. E. Exploration of new chromophore structures leads to the identification of improved blue fluorescent proteins. *Biochemistry* **46**, 5904–5910 (2007).
59. Tomosugi, W. et al. An ultramarine fluorescent protein with increased photostability and pH insensitivity. *Nat. Methods* **6**, 351–353 (2009).
60. Niwa, H., Yamamura, K. & Miyazaki, J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199 (1991).
61. Bindels, D. S. et al. mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nat. Methods* **14**, 53–56 (2017).
62. Komatsu, N. et al. Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol. Biol. Cell* **22**, 4647–4656 (2011).
63. Urasaki, A., Morvan, G. & Kawakami, K. Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics* **174**, 639–649 (2006).
64. Sumiyama, K., Kawakami, K. & Yagita, K. A simple and highly efficient transgenesis method in mice with the Tol2 transposon system and cytoplasmic microinjection. *Genomics* **95**, 306–311 (2010).
65. Tarutani, M., Cai, T., Dajee, M. & Khavari, P. A. Inducible activation of Ras and Raf in adult epidermis. *Cancer Res.* **63**, 319–323 (2003).
66. Taslimi, A. et al. Optimized second-generation CRY2-CIB dimerizers and photoactivatable Cre recombinase. *Nat. Chem. Biol.* **12**, 425–430 (2016).
67. Lillo, M. P., Beechem, J. M., Szpikowska, B. K., Sherman, M. A. & Mas, M. T. Design and characterization of a multisite fluorescence energy-transfer system for protein folding studies: a steady-state and time-resolved study of yeast phosphoglycerate kinase. *Biochemistry* **36**, 11261–11272 (1997).
68. Sali, A. & Blundell, T. L. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815 (1993).
69. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262–265 (2009).
70. Sano, T., Kobayashi, T., Ogawa, O. & Matsuda, M. Gliding basal cell migration of the urothelium during wound healing. *Am. J. Pathol.* **188**, 2564–2573 (2018).

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☐ ☒ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☐ ☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☐ ☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☐ ☒ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	Fluorescent images were obtained using Fluoview (version 3.0.2.4). Fluorescent lifetime images were obtained using SPC-150 (ver. 9.6.3).
Data analysis	The homology models were built using the program MODELLER 9.21 and visualized with PyMOL (v1.6.0.0). Tertiary structures and relative spatial arrangements of fusion constructs were predicted using the AIDA server (http://ffas.godziklab.org/AIDA/) and visualized with PyMOL (v1.6.0.0). Fluorescent lifetime images were analysed using SPCImage 5.4. For membrane-translocation assay and FRET imaging, images were processed and analyzed with ImageJ (version 1.51k) and MetaMorph (version 7.8.13.0). Three-dimensional (3D) image reconstruction and fluorescence intensity measurements was performed by using Volocity (Version 6.2.0). The measurement of ERK propagation and kymographs analysis of ERK activity were performed using a MATLAB R2018b. Statistical analyses were performed using KaleidaGraph (version 4.5.2).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available within the article and its Supplementary Information or from the corresponding author upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size was not predetermined with any statistical test. Sufficient sample sizes were estimated based on previous studies with similar systems. Experiments were repeated to achieve consistent results and permit statistical analysis when appropriate. For the fluorescent lifetime analysis, we aimed to measure at least 3 cells because these experiments are early stage of the screening. For the final products such as mTagBFP2, 2paCRY2, and 2paLINX we performed at least 2 independent experiments. For mouse studies, we designed our experiments to ensure that minimum number of mice are used to obtain biologically significant results and aimed to get at least 3 sample sizes in each group in each independent experiment.
Data exclusions	No data were excluded from the analyses.
Replication	We conducted the same experiments with culture cells or several animals to check the reproducibility of our results. All attempts at replication, that did not encounter technical issues during the experiment, are successful and results are reported in the manuscript.
Randomization	All cells and animals were allocated to experimental groups randomly, but no formal randomization were performed.
Blinding	No blinding was performed because these experiments were performed to optimize and characterize a molecular toolset, not test a hypothesis.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	HeLa cells, Lenti-X 293T cells, MDCK cells
Authentication	HeLa cells were purchased from the Human Science Research Resources Bank. MDCK cells were purchased from the RIKEN BioResource Center (no. RCB0995). Lenti-X 293T cells were purchased from the Clontech.
Mycoplasma contamination	The cell lines were not tested for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified lines were used in the study.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals	For generation of transgenic mice, fertilized eggs derived from C57BL/6N mice were microinjected. Founder animals were bred with Jcl:ICR mice to produce stable lines. 2paRAF transgenic mice were backcrossed with B6N-Tyrc-Brd/BrdCrCl mice for more
--------------------	--

than four generations. hyBRET-ERKnl^s transgenic mice were backcrossed with B6N-Tyrc-Brd/BrdCrCl mice for more than ten generations. For in vivo imaging, 8 to 24-week-old male or female mice were used. Details on the mice used in this study have been included in the Methods section of this manuscript.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.

Ethics oversight

The animal protocols were reviewed and approved by the Animal Care and Use Committee of Kyoto University Graduate School of Medicine (No. 10584, 14079, 15064, 16038, and 17539) and the methods were carried out in accordance with the relevant guidelines and regulations.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

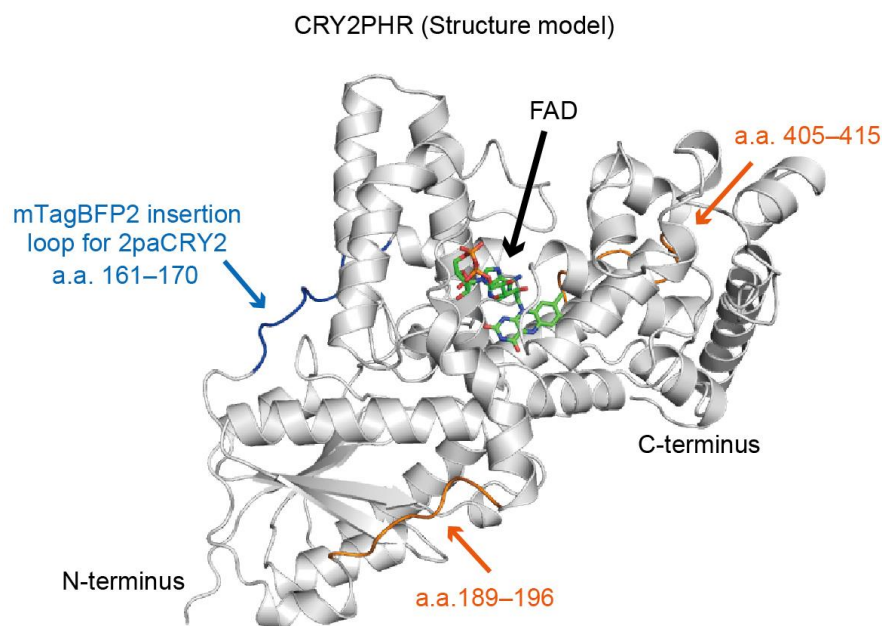
In the format provided by the authors and unedited.

FRET-assisted photoactivation of flavoproteins for in vivo two-photon optogenetics

Tomoaki Kinjo^{1,2}, Kenta Terai^{3*}, Shoichiro Horita⁴, Norimichi Nomura⁴, Kenta Sumiyama⁵, Kaori Togashi², So Iwata^{4,6} and Michiyuki Matsuda^{1,3*}

¹Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan. ²Department of Diagnostic Imaging and Nuclear Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan. ³Research Center for Dynamic Living Systems, Graduate School of Biostudies, Kyoto University, Kyoto, Japan. ⁴Department of Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan. ⁵Laboratory for Mouse Genetic Engineering, RIKEN Center for Biosystems Dynamics Research, Osaka, Japan. ⁶RIKEN SPring-8 Center, Sayo, Hyogo, Japan.

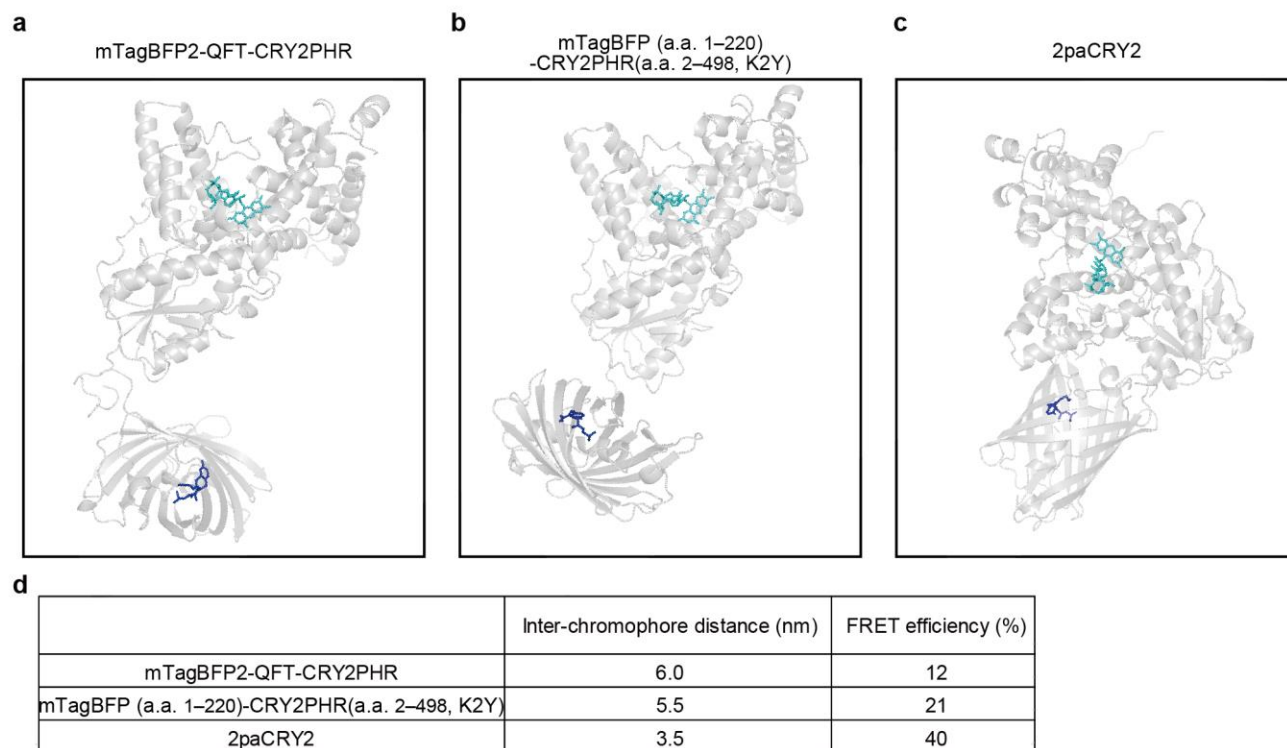
*e-mail: terai.kenta.5m@kyoto-u.ac.jp; matsuda.michiyuki.2c@kyoto-u.ac.jp



Supplementary Figure 1

Structure model of CRY2PHR with FAD.

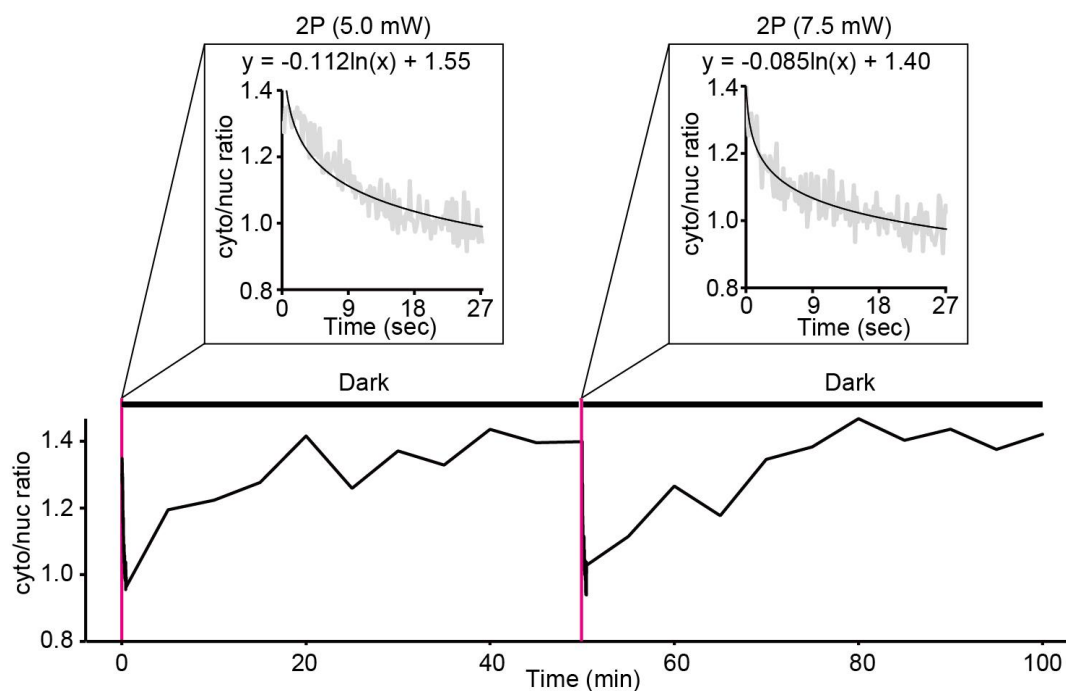
The model is visualized with PyMOL. The structure model of CRY2PHR is shown as gray-colored cartoon. The mTagBFP2 insertion loop for 2paCRY2 (a.a. 161-170) is shown in blue. Other representative loops for insertion are shown in orange. Residues of FAD were shown as sticks representation with carbon, oxygen, phosphorus, and nitrogen atoms; Green: Carbon, Red: Oxygen, Blue: Nitrogen, Orange: phosphorus.



Supplementary Figure 2

Inter-chromophore distances predicted by the structure model.

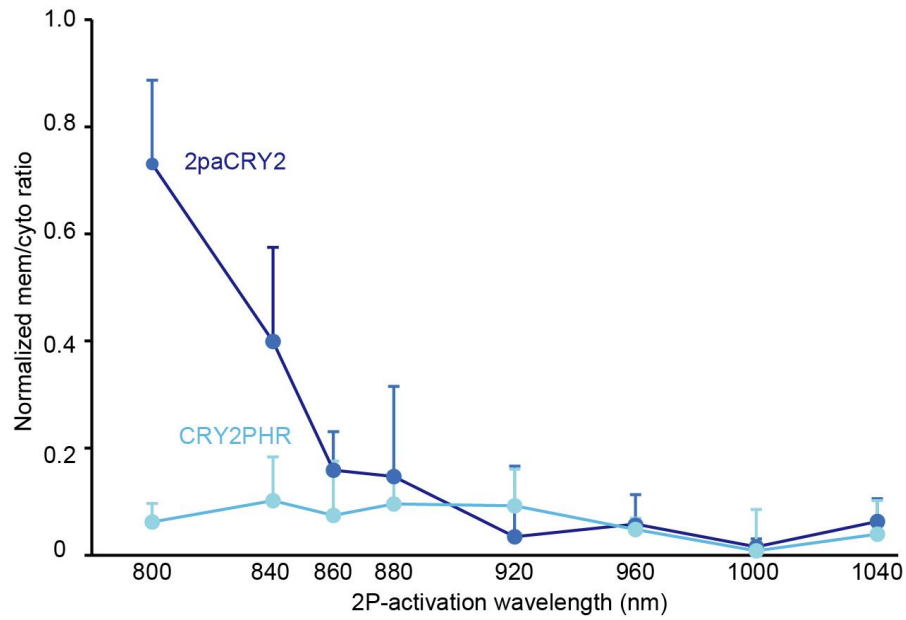
a–c, Structure models of representative mTagBFP2-CRY2PHR chimeric proteins: mTagBFP2-QFT-CRY2PHR, mTagBFP (a.a. 1–220)-CRY2PHR (a.a. 2–498, K2Y) and 2paCRY2. The chromophores of mTagBFP2 (Leu63-Tyr64-Gly65) are shown in blue. FADs are shown in cyan. **d**, Shown here are inter-chromophore distance measured in each structure model and the FRET efficiency quantified by FLIM (Figure 1).



Supplementary Figure 3

Kinetics and repeatability of 2paCRY2 activation.

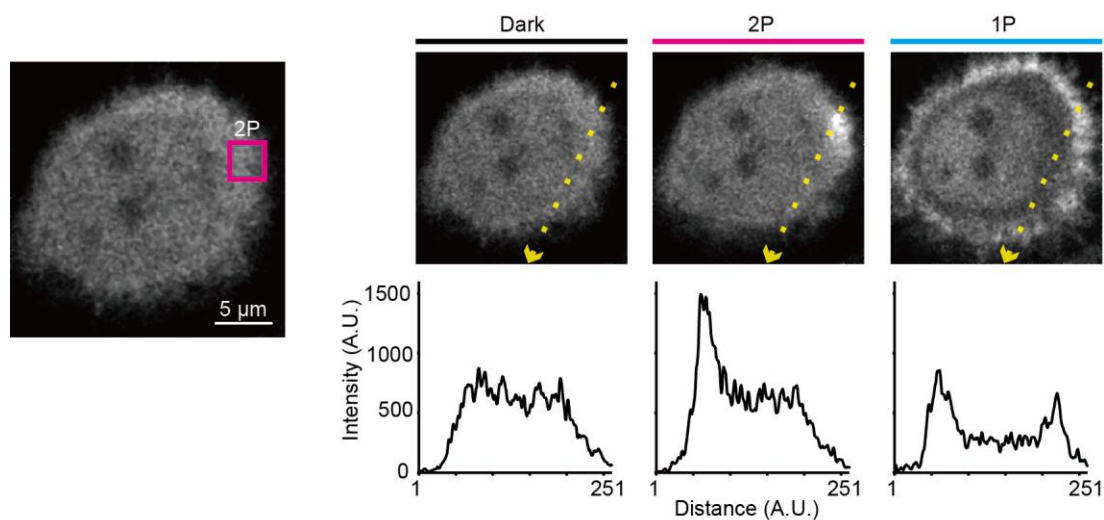
HeLa cells expressing CIBN-EGFP-CAAX and 2paCRY2-mScarlet-H were kept under a dark condition for at least 1 h. Then, the cells were exposed to the 2P activation (810 nm, 5.0/7.5 mW, 0.207 $\mu\text{m}/\text{pixel}$, 2 $\mu\text{s}/\text{pixel}$, 128 $\times$ 128 pixels, 150 scans), which took approximately 30 sec. The plasma membrane translocation of 2paCRY2-mScarlet-H was monitored at 1040 nm and quantified by the intensity ratio of the cytosol versus nucleus. The gray and black lines in squared boxes represent the raw and the fitted data, respectively. In this experiment, the nuclear mScarlet-H intensity was used as the reference, because the kinetics of the plasma membrane translocation of 2paCRY2-mScarlet-H was markedly faster than that of the nucleocytoplasmic shuttling. Representative data among $n = 3$ independent experiments.



Supplementary Figure 4

2P activation spectra of CRY2PHR and 2paCRY2.

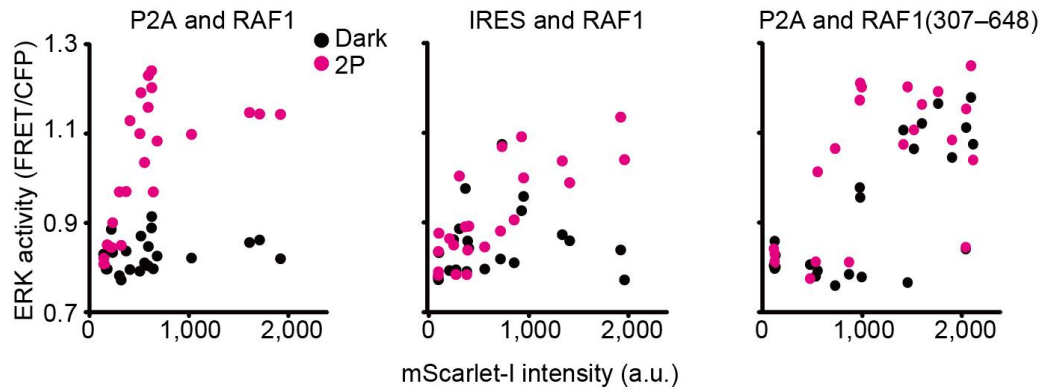
HeLa cells expressing CIBN-EGFP-CAAX and CRY2PHR- or 2paCRY2-mScarlet-H were kept under a dark condition for at least 1 h. Then, the cells were exposed to the 2P activation at the indicated wavelength (5 mW, 0.207 $\mu\text{m}^2/\text{pixel}$, 2 $\mu\text{s}/\text{pixel}$, 512 $\times$ 512 pixels, every 5 sec, 30 scans) or 1P activation (480 nm, 5 mW/cm², for 1 sec after 2P activation). Membrane versus cytosol ratio (mem/cyto ratio) of mScarlet-H intensity was quantified at each wavelength. The increase in mem/cyto ratio after 2P activation was normalized by that after 1P activation. n = 6 cells; Representative data among n = 2 independent experiments. Blue and cyan dots represent mean; error bars, s.d..



Supplementary Figure 5

Optical control of 2paCRY2 localization at subcellular resolution

A HeLa cell expressing CIBN-EGFP-CAAX and 2paCRY2-mScarlet-H was kept under a dark condition for at least 1 h. Then, the red squared region including plasma membrane was exposed to the 2P activation (810 nm, 7.5 mW, 0.103 $\mu\text{m}/\text{pixel}$, 2 $\mu\text{s}/\text{pixel}$, 30 $\times$ 30 pixels, 50 scans). The mScarlet-H intensities along the yellow broken lines are shown in the lower panels. Representative data among $n = 3$ independent experiments.



Supplementary Figure 6

Quantification of the ERK activity (FRET/CFP) in HeLa cells expressing 2paRAF variants with P2A and full-length RAF1, IRES and full-length RAF1, or P2A and the RAF1 catalytic domain (a.a. 307-648) before and after the 2P activation.

HeLa cells stably expressing EKAREV-nls, an ERK FRET biosensor, were transfected with the plasmids encoding 2paRAF connected with full-length RAF1 or the RAF1 catalytic domain (a.a. 307-648) via P2A or IRES. Cells were photoactivated and imaged by 2P excitation (840 nm, 10mW, 0.828 $\mu\text{m}/\text{pixel}$, 2 $\mu\text{s}/\text{pixel}$, 20 scans). The ERK activity was plotted against mScarlet-I intensity to examine the correlation between the expression level of the plasmid and ERK activity. The ERK activity (FRET/CFP) in each cell before (black dots) and after (magenta dots) 2P activation (840 nm, 10 mW, 0.828 $\mu\text{m}/\text{pixel}$, 2 $\mu\text{s}/\text{pixel}$, 20 scans) was quantified and plotted as a function of mScarlet-I intensity; n = 21 cells; Representative data among n = 2 independent experiments. a.u., arbitrary units.

Supplementary Table 1. Fluorescence property of blue fluorescent proteins

Protein	λ_{ex} (nm)	λ_{em} (nm)	ε (M ⁻¹ cm ⁻¹)	$\lambda_{2\text{P}}$ (nm)	σ_2 (GM)	QY	R_0 (nm)	Reference
SIRIUS	355	424	15,000	NA	NA	0.24	3.18	Tomosugi, 2009
EBFP2	383	448	32,000	750	13	0.56	3.88	Ai, 2007
TagBFP	402	457	52,000	807	12	0.63	4.01	Subach, 2011
mTagBFP2	399	454	50,600	NA	NA	0.64	4.02	Subach, 2011
mTFP1	462	492	64,000	875	70	0.85	3.74	Drobizhev, 2011

Abbreviations: λ_{ex} , fluorescence excitation maximum; λ_{em} , fluorescence emission maximum; ε , extinction coefficient; $\lambda_{2\text{P}}$, two-photon absorption maximum; σ_2 , 2P cross section; GM, Goeppert-Mayer; QY, quantum yield; R_0 , Förster distance between FP and FAD; NA, Not available.

Supplementary Table 2. Fluorescent lifetime of chimeric proteins.

Protein variants	Fluorescent lifetime (ns)
A. Tandem fusion of mTagBFP2 and CRY2PHR with a QFT short linker	
mTagBFP2	2.5
mTagBFP2-QFT-CRY2PHR	2.2
mTagBFP2-QFT-CRY2PHR(D387A)	2.3
CRY2PHR-QFT-mTagBFP2	2.1
CRY2PHR (D387A)-QFT-mTagBFP2	2.3
mTagBFP2-QFT-CRY2PHR-2aa-mTagBFP2	2.2
B. Tandem fusion of the C-terminal deletion mutant of mTagBFP2 and CRY2PHR with a QFT short linker	
mTagBFP2 (a.a. 1–232)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–231)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–230)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–229)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–228)-QFT-CRY2PHR	2.3
mTagBFP2 (a.a. 1–227)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–226)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–225)-QFT-CRY2PHR	2.1
mTagBFP2 (a.a. 1–224)-QFT-CRY2PHR	2.1
mTagBFP2 (a.a. 1–223)-QFT-CRY2PHR	2.1
mTagBFP2 (a.a. 1–222)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–221)-QFT-CRY2PHR	2.3
mTagBFP2 (a.a. 1–220)-QFT-CRY2PHR	2.3
mTagBFP2 (a.a. 1–219)-QFT-CRY2PHR	2.2
mTagBFP2 (a.a. 1–213)-QFT-CRY2PHR	ND
C. Tandem fusion of the C-terminal deletion mutant of mTagBFP2 and CRY2PHR with a GS flexible linker	
mTagBFP2 (a.a. 1–222)-GSGSGS-CRY2PHR	2.2
mTagBFP2 (a.a. 1–222)-SSGGSGGGSGGGGS-CRY2PHR	2.3
D. Tandem fusion of the C-terminal deletion mutant of mTagBFP2 and CRY2PHR with a mutation in the linker	
mTagBFP2 (a.a. 1–221)-A-CRY2PHR	2.2
mTagBFP2 (a.a. 1–221)-L-CRY2PHR	2.2
mTagBFP2 (a.a. 1–221)-F-CRY2PHR	2.2
mTagBFP2 (a.a. 1–221)-W-CRY2PHR	2.2
mTagBFP2 (a.a. 1–221)-P-CRY2PHR	2.2
mTagBFP2 (a.a. 1–220)-G-CRY2PHR	2.2
mTagBFP2 (a.a. 1–220)-I-CRY2PHR	2.2
mTagBFP2 (a.a. 1–220)-R-CRY2PHR	2.2
mTagBFP2 (a.a. 1–220)-W-CRY2PHR	2.3
mTagBFP2 (a.a. 1–220)-E-CRY2PHR	ND
mTagBFP2 (a.a. 1–220)-P-CRY2PHR	2.2
mTagBFP2 (a.a. 1–220)-PAP-CRY2PHR	2.2
mTagBFP2 (a.a. 1–220)-PPAP-CRY2PHR	2.1
E. Tandem fusion of mTagBFP2 and the N-terminal deletion mutant of CRY2PHR without a linker	
mTagBFP2-QFT-CRY2PHR (a.a. 6–498)	2.2
mTagBFP2-QFT-CRY2PHR (a.a.11–498)	2.4
mTagBFP2-QFT-CRY2PHR (a.a.16–498)	2.4
mTagBFP2-QFT-CRY2PHR (a.a.21–498)	2.4
mTagBFP2-QFT-CRY2PHR (a.a.31–498)	2.4
mTagBFP2-QFT-CRY2PHR (a.a.41–498)	2.4
mTagBFP2-QFT-CRY2PHR (a.a.51–498)	ND

Protein variants	Fluorescent lifetime (ns)
F. Tandem fusion of the C-terminal deletion mutant of mTagBFP2 and N-terminal deletion mutant of CRY2PHR without a linker	
mTagBFP2 (a.a. 1–220)-CRY2PHR	2.1
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 2–498)	2.0
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 2–498, D387A)	2.5
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 3–498)	2.2
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 4–498)	2.2
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 5–498)	2.6
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 6–498)	2.6
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 7–498)	2.5
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 8–498)	2.4
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 9–498)	2.4
mTagBFP2 (a.a. 1–220)-CRY2PHR (a.a. 10–498)	2.5
G. Tandem fusion of the C-terminal deletion mutant of mTagBFP2 and N-terminal deletion mutant of CRY2PHR with mutation in the linker	
mTagBFP2 (a.a. 1–220)-G-CRY2PHR (a.a. 3–498)	2.1
mTagBFP2 (a.a. 1–220)-L-CRY2PHR (a.a. 3–498)	2.4
mTagBFP2 (a.a. 1–220)-M-CRY2PHR (a.a. 3–498)	2.0
mTagBFP2 (a.a. 1–220)-F-CRY2PHR (a.a. 3–498)	2.0
mTagBFP2 (a.a. 1–220)-Y-CRY2PHR (a.a. 3–498)	2.0
mTagBFP2 (a.a. 1–220)-W-CRY2PHR (a.a. 3–498)	2.0
mTagBFP2 (a.a. 1–220)-Q-CRY2PHR (a.a. 3–498)	2.0
mTagBFP2 (a.a. 1–220)-R-CRY2PHR (a.a. 3–498)	2.0
mTagBFP2 (a.a. 1–220)-H-CRY2PHR (a.a. 3–498)	2.0
mTagBFP2 (a.a. 1–220)-P-CRY2PHR (a.a. 3–498)	2.0
H. Insertion of truncated mTagBFP2 into the loops of CRY2PHR without a linker	
CRY2PHR (a.a. 1–130)-mTagBFP2 (a.a. 1–221)-CRY2PHR (a.a. 150–498)	2.3
CRY2PHR (a.a. 1–130)-mTagBFP2 (a.a. 1–222)-CRY2PHR (a.a. 150–498)	ND
CRY2PHR (a.a. 1–160)-mTagBFP2 (a.a. 1–221)-CRY2PHR (a.a. 171–498)	2.5
CRY2PHR (a.a. 1–160)-mTagBFP2 (a.a. 1–222)-CRY2PHR (a.a. 171–498)	2.3
CRY2PHR (a.a. 1–160)-mTagBFP2 (a.a. 1–221)-CRY2PHR (a.a. 197–498)	2.6
CRY2PHR (a.a. 1–188)-mTagBFP2 (a.a. 1–222)-CRY2PHR (a.a. 197–498)	2.6
CRY2PHR (a.a. 1–404)-mTagBFP2 (a.a. 1–221)-CRY2PHR (a.a. 416–498)	2.5
CRY2PHR (a.a. 1–404)-mTagBFP2 (a.a. 1–222)-CRY2PHR (a.a. 416–498)	2.6
I. Insertion of truncated mTagBFP2 into the loops of CRY2PHR with GSG flexible linkers	
CRY2PHR (a.a. 1–130)-GSG-mTagBFP2 (a.a. 1–221)-GSG-CRY2PHR (a.a. 150–498)	ND
CRY2PHR (a.a. 1–130)-GSG-mTagBFP2 (a.a. 1–222)-GSG-CRY2PHR (a.a. 150–498)	ND
CRY2PHR (a.a. 1–160)-GSG-mTagBFP2 (a.a. 1–221)-GSG-CRY2PHR (a.a. 171–498): 2paCRY2	1.5
CRY2PHR (a.a. 1–160)-GSG-mTagBFP2 (a.a. 1–222)-GSG-CRY2PHR (a.a. 171–498)	1.5
CRY2PHR (a.a. 1–160)-GSG-mTagBFP2 (a.a. 1–221)-GSG-CRY2PHR (a.a. 171–498, D387A)	2.6
CRY2PHR (a.a. 1–160)-GSG-mTagBFP2 (a.a. 1–222)-GSG-CRY2PHR (a.a. 171–498, D387A)	2.6
CRY2PHR (a.a. 1–188)-GSG-mTagBFP2 (a.a. 1–221)-GSG-CRY2PHR (a.a. 197–498)	2.5
CRY2PHR (a.a. 1–188)-GSG-mTagBFP2 (a.a. 1–222)-GSG-CRY2PHR (a.a. 197–498)	2.6
CRY2PHR (a.a. 1–231)-GSG-mTagBFP2 (a.a. 1–227)-SGS-CRY2PHR (a.a. 244–498)	2.5
CRY2PHR (a.a. 1–303)-GSG-mTagBFP2 (a.a. 1–222)-SGS-CRY2PHR (a.a. 315–498)	2.3
CRY2PHR (a.a. 1–404)-GSG-mTagBFP2 (a.a. 1–221)-GSG-CRY2PHR (a.a. 416–498)	2.6
CRY2PHR (a.a. 1–404)-GSG-mTagBFP2 (a.a. 1–222)-GSG-CRY2PHR (a.a. 416–498)	2.6
J. Insertion of mTagBFP2 into the loops of CRY2PHR with EAAAK rigid linekrs	
CRY2PHR (a.a. 1–130)-EAAAK-mTagBFP2 (a.a. 1–221)-EAAAK-CRY2PHR (a.a. 150–498)	ND
CRY2PHR (a.a. 1–404)-EAAAK-mTagBFP2 (a.a. 1–221)-EAAAK-CRY2PHR (a.a. 416–498)	2.6

Protein variants	Fluorescent lifetime (ns)
K. Tandem fusion of EBFP2 and CRY2PHR	
EBFP2	2.8
EBFP2-CRY2PHR	2.3
L. Tandem fusion of the C-terminal deletion mutant of EBFP2 and N-terminal deletion mutant of CRY2PHR without a linker	
EBFP2 (a.a. 1–229)-CRY2PHR	2.4
EBFP2 (a.a. 1–229)-CRY2PHR (a.a. 2–498)	2.3
EBFP2 (a.a. 1–229)-CRY2PHR (a.a. 3–498)	2.4
EBFP2 (a.a. 1–228)-CRY2PHR	2.0
EBFP2 (a.a. 1–228)-CRY2PHR (a.a. 2–498)	2.3
EBFP2 (a.a. 1–228)-CRY2PHR (a.a. 3–498)	2.3
M. Insertion of EBFP2 into the loops of CRY2PHR with GSG flexible linkers	
CRY2PHR (a.a. 1–129)-GSG-EBFP2 (a.a. 1–229)-GSG-CRY2PHR (a.a. 149–498)	2.6
CRY2PHR (a.a. 1–160)-GSG-EBFP2-GSG-CRY2PHR (a.a. 170–498)	ND
CRY2PHR (a.a. 1–161)-GSG-EBFP2 (a.a. 1–229)-GSG-CRY2PHR (a.a. 171–498)	2.4
CRY2PHR (a.a. 1–187)-GSG-EBFP2 (a.a. 1–229)-GSG-CRY2PHR (a.a. 197–498)	2.5
CRY2PHR (a.a. 1–233)-GSG-EBFP2 (a.a. 1–229)-GSG-CRY2PHR (a.a. 241–498)	2.4
CRY2PHR (a.a. 1–304)-GSG-EBFP2 (a.a. 1–229)-GSG-CRY2PHR (a.a. 317–498)	2.2
CRY2PHR (a.a. 1–404)-GSG-EBFP2 (a.a. 1–229)-GSG-CRY2PHR (a.a. 416–498)	2.1
N. Insertion of EBFP2 into the loops of CRY2PHR with GGSGG flexible linkers	
CRY2PHR (a.a. 1–129)-GGSGG-EBFP2 (a.a. 1–229)-GGSGG-CRY2PHR (a.a. 149–498)	ND
CRY2PHR (a.a. 1–161)-GGSGG-EBFP2 (a.a. 1–229)-GGSGG-CRY2PHR (a.a. 171–498)	ND
CRY2PHR (a.a. 1–187)-GGSGG-EBFP2 (a.a. 1–229)-GGSGG-CRY2PHR (a.a. 197–498)	2.3
CRY2PHR (a.a. 1–233)-GGSGG-EBFP2 (a.a. 1–229)-GGSGG-CRY2PHR (a.a. 241–498)	ND
CRY2PHR (a.a. 1–304)-GGSGG-EBFP2 (a.a. 1–229)-GGSGG-CRY2PHR (a.a. 317–498)	ND
CRY2PHR (a.a. 1–404)-GGSGG-EBFP2 (a.a. 1–229)-GGSGG-CRY2PHR (a.a. 416–498)	2.4
O. Tandem fusion of SIRIUS and CRY2PHR	
SIRIUS	1.5
SIRIUS-CRY2PHR	1.3

※ ND: Not detectable (fluorescence)

Supplementary Note 1

All nucleic acid sequences of 2paCRY2 and 2paRAF

2paCRY2:

	0	30	60
	ATGAAGATGGACAAGAAAACCATCGTGTGGTTTTCGGAGAGATCTGAGGATTGAGGACAAC		
	CCAGCCCTGGCCGCTGCCGCTCACGAAGGCAGCGTGTTCAGTGTTTCATCTGGTGCCCC		
	GAGGAAGAGGGACAGTTCTACCCAGGAAGGGCCAGCCGCTGGTGGATGAAGCAGTCCCTG		
CRY2PHR (a.a. 1–160)	GCTCACCTGTCTCAGAGTCTGAAGGCCCTGGGGTCAGATCTGACCCTGATCAAAACCCAT		
	AACACAATCAGCGCCATTCTGGACTGTATTGCGGTGACTGGAGCCACCAAAGTGTTT		
	AATCACCTGTATGATCCCGTGAGCCTGGTGCGGGACCATACAGTGAAGGAGAAACTGGTG		
	GAAAGAGGCATCAGTGTGCAGTCATACAACGGGGATCTGCTGTATGAGCCATGGGAAATC		
	TACTGCGAGAAGGGGAAACCCTTTACCAGCTTCAATTCTACTGGAAGAAATGTCTGGAC		
GSG linker	ggatctggaATGAGCGAGCTGATTAAGGAGAACATGCACATGAAGCTGTACATGGAGGGC		
	ACCGTGGACAACCATCACTTCAAGTGCACATCCGAGGGCGAAGGCAAGCCCTACGAGGGC		
	ACCCAGACCATGAGAATCAAGGTGGTCGAGGGCGGCCCTCTCCCTTCGCCTTCGACATC		
	CTGGCTACTAGCTTCTCTACGGCAGCAAGACCTTCATCAACCACACCCAGGGCATCCCC		
	GACTTCTTCAAGCAGTCCTTCCCTGAGGGCTTCACATGGGAGAGAGTCACCACATACGAA		
mTagBFP2 (a.a. 1–221)	GACGGGGGCGTGCTGACCGCTACCCAGGACACCAAGCCTCCAGGACGGCTGCCTCATCTAC		
	AACGTCAAGATCAGAGGGGTGAACCTCACATCCAACGGCCCTGTGATGCAAGAAGAAAACA		
	CTCGGCTGGGAGGCATTACCGAGACGCTGTACCCCGCTGACGGCGGCCTGGAAGGCAGA		
	AACGACATGGCCCTGAAGCTCGTGGCGGGAGCCATCTGATCGCAAACGCCAAGACCACA		
	TATAGATCCAAGAAACCCGCTAAGAACCTCAAGATGCCTGGCGTCTACTATGTGGACTAC		
	AGACTGGAAAGAATCAAGGAGGCCAACACGAGACCTACGTCGAGCAGCACGAGGTGGCA		
GSG linker	GTGGCCAGATACggttagcggTCCATGGAGGCTGTATGCCTATTACAGCCGCTGCCGAAGCT		
	ATCTGGGCCCTGCTCCATTGAAGAGCTGGGACTGGAAAACGAGGCCGAAAAGCCTTCTAAT		
	GCTCTGTGACTCGCGCCTGGTCCCCAGGATGGTCTAACGCTGATAAACTGCTGAATGAG		
	TTCATCGAAAAGCAGCTGATTGACTATGCCAAAACTCTAAGAAAGTGGTGGGCAACAGC		
	ACCAGCCTGCTGTCTCCATACCTGCACCTTGGAGAGATCAGTGTGCGGCACGTGTTCCAG		
	TGCCTAGAAATGAAGCAGATCATTTGGGCCAGGGATAAAAAAGTGAGGGCGAAGAGTCA		
	GCCGACCTGTTTCTGAGGGGAATCGGACTGAGGGAGTACTCCCGCTATATTTGTTTCAAT		
CRY2PHR (a.a. 171–498)	TTTCCCTTCACTCACGAACAGTCACTGCTGAGCCATCTGCGGTTCTTTCTTGGGACGCT		
	GATGTGGACAAGTTCAAAGCCTGGCGGCAGGGAAGAACAGGCTATCCCTGGTGGATGCT		
	GGAATGAGAGAGCTGTGGGCCACTGGCTGGATGCACAATAGGATCCGCGTGATTGTGAGC		
	TCCTTTGCTGTGAAGTTCTGCTGCTGCCTTGGAAAGTGGGGCATGAAATATTTTGGGAT		
	ACACTGCTGGATGCCGACCTGGAGTGTGACATCCTGGGGTGGCAGTACATCTCCGGATCT		
	ATTCCAGATGGCCATGAACTGGATAGGCTGGACAACCCGCTCTGCAGGGCGCTAAGTAT		
	GACCCTGAGGGAGAATACATCAGGCAGTGGCTGCCTGAGCTGGCTAGGCTGCCAACTGAA		
	TGGATTACCATCCCTGGGACGCTCCTCTGACAGTGCTGAAGGCCAGTGGGGTGGAGCTG		
	GGAACATAATTACGCTAAACCTATCGTGGATATTGACACCGCCAGAGAGCTGCTGGCTAAG		
	GCCATCTCCCGACCCAGAGAAGCCAGATCATGATTGGCGCTGCC		

2paRAF:

0	30	60
ATGAAGATGGACAAGAAAACCATCGTGTGGTTTCGGAGAGATCTGAGGATTGAGGACAAC		
CCAGCCCTGGCCGCTGCCGCTCACGAAGGCAGCGTGTTTCCAGTGTTTCATCTGGTGCCCC		
GAGGAAGAGGGACAGTTCTACCCAGGAAGGGCCAGCCGCTGGTGATGAAGCAGTCCCTG		
GCTCACCTGTCTCAGAGTCTGAAGGCCCTGGGGTCAGATCTGACCCTGATCAAAACCCAT		
AACACAATCAGCGCCATTCTGGACTGTATTCGGGTGACTGGAGCCACCAAAGTGGTGTTT		
AATCACCTGTATGATCCCGTGAGCCTGGTGCGGGACCATACAGTGAAGGAGAACTGGTG		
GAAAGAGGCATCAGTGTGCAGTCATACAACGGGGATCTGCTGTATGAGCCATGGGAAATC		
TACTGCGAGAAGGGGAAACCCTTTACCAGCTTCAATTCCCTACTGGAAGAAATGTCTGGAC		
ggatctggaATGAGCGAGCTGATTAAGGAGAACATGCACATGAAGCTGTACATGGAGGGC		
ACCGTGGACAACCATCACTTCAAGTGCACATCCGAGGGCGAAGGCAAGCCCTACGAGGGC		
ACCCAGACCATGAGAATCAAGGTGGTCGAGGGCGGCCCTCTCCCCTTCGCCTTCGACATC		
CTGGCTACTAGCTTCCTCTACGGCAGCAAGACCTTCATCAACCACACCCAGGGCATCCCC		
GACTTCTTCAAGCAGTCCTTCCCTGAGGGCTTCACATGGGAGAGAGTCACCACATACGAA		
GACGGGGGCGTGCTGACCGCTACCCAGGACACCAGCCTCCAGGACGGCTGCCTCATCTAC		
AACGTCAAGATCAGAGGGGTGAACCTTCACATCCAACGGCCCTGTGATGCAGAAGAAAACA		
CTCGGCTGGGAGGCATTACCGAGACGCTGTACCCCGCTGACGGCGGCCTGGAAGGCAGA		
2paCRY2	AACGACATGGCCCTGAAGCTCGTGGGCGGGAGCCATCTGATCGCAAACGCCAAGACCACA	
	TATAGATCCAAGAAACCCGCTAAGAACCTCAAGATGCCTGGCGTCTACTATGTGGACTAC	
	AGACTGGAAAGAATCAAGGAGGCCAACACGAGACCTACGTGAGCAGCACGAGGTGGCA	
	GTGGCCAGATACggtagcggTCCATGGAGGCTGATGCCTATTACAGCCGCTGCCGAAGCT	
	ATCTGGGCCTGCTCCATTGAAGAGCTGGGACTGGAAAACGAGGCCGAAAAGCCTTCTAAT	
	GCTCTGCTGACTCGCGCTGGTCCCCAGGATGGTCTAACGCTGATAAACTGCTGAATGAG	
	TTCATCGAAAAGCAGCTGATTGACTATGCCAAAACTCTAAGAAAGTGGTGGGCAACAGC	
	ACCAGCCTGCTGTCTCCATACCTGCACCTTGGAGAGATCAGTGTGCGGCACGTGTTCCAG	
	TGCGCTAGAATGAAGCAGATCATTTGGGCCAGGGATAAAAACAGTGAGGGCGAAGAGTCA	
	GCCGACCTGTTTCTGAGGGGAATCGGACTGAGGGAGTACTCCCGCTATATTTGTTTCAAT	
	TTTCCCTTCACTCACGAACAGTCACTGCTGAGCCATCTGCGGTTCTTTCCTTGGGACGCT	
	GATGTGGACAAGTTCAAAGCCTGGCGGCAGGGAAGAACAGGCTATCCCCTGGTGGATGCT	
	GGAATGAGAGAGCTGTGGGCCACTGGCTGGATGCACAATAGGATCCGCGTGATTGTGAGC	
	TCCTTTGCTGTGAAGTTCCTGCTGCTGCCTTGGAAGTGGGGCATGAAATATTTTTGGGAT	
	ACACTGCTGGATGCCGACCTGGAGTGTGACATCCTGGGGTGGCAGTACATCTCCGGATCT	
	ATTCCAGATGGCCATGAACTGGATAGGCTGGACAACCCCGCTCTGCAGGGCGCTAAGTAT	
	GACCCTGAGGGAGAATACATCAGGCAGTGGCTGCCTGAGCTGGCTAGGCTGCCAACTGAA	
	TGGATTACCATCCCTGGGACGCTCCTCTGACAGTGCTGAAGGCCAGTGGGGTGGAGCTG	
	GGAACTAATTACGCTAAACCTATCGTGGATATTGACACCGCCAGAGAGCTGCTGGCTAAG	
	GCCATCTCCCGGACCAGAGAAGCCAGATCATGATTGGCGCTGCCATGGAGCACATACAG	

	0	30	60
	GGAGCTTGAAGACGATCAGCAATGGTTTTGGATTCAAAGATGCCGTGTTTGATGGCTCC AGCTGCATCTCTCCTACAATAGTTCAGCAGTTTGGCTATCAGCGCCGGGCATCAGATGAT GGCAAACCTCACAGATCCTTCTAAGACAAGCAACACTATCCGTGTTTTCTTGCCGAACAAG CAAAGAACAGTGGTCAATGTGCGAAATGGAATGAGCTTGCATGACTGCCTTATGAAAGCA CTCAAGGTGAGGGGCTGCAACCAGAGTGCTGTGCAGTGTTGAGACTTCTCCACGAACAC AAAGGTAAAAAGCACGCTTAGATTGGAATACTGATGCTGCGTCTTTGATTGGAGAAGAA CTTCAAGTAGATTTCTGGATCATGTTCCCTCACAACACACAACCTTTGCTCGGAAGACG TTCCTGAAGCTTGCCTTCTGTGACATCTGTGAGAAATTCCTGCTCAATGGATTTCTGATG CAGACTTGTGGCTACAAATTTTCATGAGCACTGTAGCACCAAAGTACCTACTATGTGTGTG GACTGGAGTAACATCAGACAACCTTATTGTTTCCAAATTCCTACTATTGGTGATAGTGGA GTCCCAGCACTACCTTCTTTGACTATGCGTCGTATGCGAGAGTCTGTTTCCAGGATGCCT GTTAGTTCTCAGCACAGATATTCTACACCTCACGCCTTCACCTTTAACACCTCCAGTCCC TCATCTGAAGGTTCCCTCTCCAGAGGCAGAGGTCGACATCCACACCTAATGTCCACATG GTCAGCACCACGCTGCCTGTGGACAGCAGGATGATTGAGGATGCAATTCGAAGTCACAGC GAATCAGCCTCACCTTCAGCCCTGTCCAGTAGCCCCAACAACTCTGAGCCCAACAGGCTGG TCACAGCCGAAAACCCCGTGCCAGCACAAAGAGAGCGGGCACCAGTATCTGGGACCCAG 		
RAF1	GAGAAAAACAAAATTAGGCCTCGTGGACAGAGAGATTCAAGCTATTATTGGGAAATAGAA GCCAGTGAAGTGATGCTGTCCACTCGGATTGGGTCAGGCTCTTTTGGAACTGTTTATAAG GGTAAATGGCACGGAGATGTTGCAGTAAAGATCCTAAAGGTTGTCGACCCAACCCAGAG CAATTCAGGCCTTCAGGAATGAGGTGGCTGTTCTGCGCAAAACACGGCATGTGAACATT CTGCTTTTCATGGGGTACATGACAAAGGACAACCTGGCAATTGTGACCCAGTGGTGCGAG GGCAGCAGCCTCTACAAACACCTGCATGTCCAGGAGACCAAGTTTCAGATGTTCCAGCTA ATTGACATTGCCCGGCAGACGGCTCAGGGAATGGACTATTTGCATGCAAAGAACATCATC CATAGAGACATGAAATCCAACAATATATTTCTCCATGAAGGCTTAACAGTGAAAATTGGA GATTTTGGTTTGGCAACAGTAAAGTCACGCTGGAGTGGTTCTCAGCAGGTTGAACAACCT ACTGGCTCTGTCTCTGGATGGCCCCAGAGGTGATCCGAATGCAGGATAACAACCCATTC AGTTTCCAGTCGGATGTCTACTCCTATGGCATCGTATTGTATGAACTGATGACGGGGGAG CTTCCTTATTCTCACATCAACAACCGAGATCAGATCATCTTCATGGTGGGCCGAGGATAT GCCTCCCAGATCTTAGTAAGCTATATAAGAACTGCCCCAAAGCAATGAAGAGGCTGGTA GCTGACTGTGTGAAGAAAGTAAAGGAAGAGAGGCCCTCTTTTCCCCAGATCTGTCTTCC ATTGAGCTGCTCCAACACTCTCTACCGAAGATCAACCGGAGCGCTTCCGAGCCATCCTTG CATCGGGCAGCCCACTGAGGATATCAATGCTTGACGCTGACCACGTCCCCGAGGCTG 		
P2A	CCTGTCTTCctcgagGGAAGCGGAAGCGACGAATTTTAGTCTACTGAAACAAGCGGGAGAC GTGGAGGAAAACCTGGACCTATGAATGGAGCTATAGGAGGTGACCTTTTGCTCAATTTT 		
CIBN	CCTGACATGTCGGTCTAGAGCGCCAAAGGGCTCACCTCAAGTACCTCAATCCCACCTTT GATTCTCCTCTCGCCGGCTTCTTTGCCGATTCTTCAATGATTACCGGCGGCGAGATGGAC 		

	0	30	60
	AGCTATCTTTCGACTGCCGGTTTGAATCTTCCGATGATGTACGGTGAGACGACGGTGGA		
	GGTGATTCAAGACTCTCAATTTGCGCGGAAACGACGCTTGGGACTGGAAAATTTCAAGGCA		
CIBN	GCGAAGTTTGATACAGAGACTAAGGATTGTAATGAGGCGGCGAAGAAGATGACGATGAAC		
	AGAGATGACCTAGTAGAAGAAGGAGAAGAAGAGAAGTCGAAAATAACAGAGCAAAACAAT		
	GGGAGCACAAAAAGCATCAAGAAGATGAAACACAAAGCCAAGAAAGAAGAGAACAATTTCT		
	TCTAATGATTCATCTAAAGTGACGAAGGAATTGGAGAAAACGGATTATATTCATctcgag		
	GGATCTGGAATGGTGAGCAAGGGCGAGGCAGTGATCAAGGAGTTCATGCGGTTCAAGGTG		
	CACATGGAGGGCTCCATGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGC		
	CCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTC		
	TCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAGGGCCTTCATCAAGCACCCC		
	GCCGACATCCCCGACTACTATAAGCAGTCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTG		
mScarlet-I	ATGAACTTCGAGGACGGCGGGCGCCGTGACCGTGACCCAGGACACCTCCCTGGAGGACGGC		
	ACCCTGATCTACAAGGTGAAGCTCCGCGGCACCAACTTCCCTCCTGACGGCCCCGTAATG		
	CAGAAGAAGACAATGGGCTGGGAAGCGTCCACCGAGCGGTTGTACCCGAGGACGGCGTG		
	CTGAAGGGCGACATTAAGATGGCCCTGCGCCTGAAGGACGGAGGCCGCTACCTGGCGGAC		
	TTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGATGCCGGGCGCCTACAACGTCGAC		
	CGCAAGTTGGACATCACCTCCCACAACGAGGACTACACCGTGGTGGAACAGTACGAACGC		
CAAX	TCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGtctagaAAGATGAGC		
	AAAGATGGTAAAAAGAAGAAAAAGAAGTCAAAGACAAAGTGTGTAATTATG		

Supplementary Note 2

All nucleic acid sequences of 2paLINX

2paLINX:

	0	30	60
mTFP1 (dC10)	ATGGTGAGCAAGGGCGAGGAGACCACAATGGGCGTAATCAAGCCCGACATGAAGATCAAG		
	CTGAAGATGGAGGGCAACGTGAATGGCCACGCCTTCGTGATCGAGGGCGAGGGCGAGGGC		
	AAGCCCTACGACGGCACCAACACCATCAACCTGGAGGTGAAGGAGGGAGCCCCCTGCC		
	TTCTCCTACGACATTCTGACCACCGCGTTCGCCTACGGCAACAGGGCCTTCACCAAGTAC		
	CCCGACGACATCCCCAACTACTTCAAGCAGTCCTTCCCGAGGGCTACTCTTGGGAGCGC		
	ACCATGACCTTCGAGGACAAGGGCATCGTGAAGGTGAAGTCCGACATCTCCATGGAGGAG		
	GACTCCTTCATCTACGAGATACACCTCAAGGGCGAGAACTTCCCCCAACGGCCCCGTG		
	ATGCAGAAGAAGACCACCGCTGGGACGCCTCACCAGAGAGGATGTACGTGCGCGACGGC		
	GTGCTGAAGGGCGACGTCAAGCACAAGCTGCTGCTGGAGGGCGGCGGCCACCACCGCGTT		
	GACTTCAAGACCATCTACAGGGCCAAGAAGGCGGTGAAGCTGCCCGACTATCACTTTGTG		
	GACCACCGCATCGAGATCCTGAACCACGACAAGGACTACAACAAGGTGACCGTTTACGAG		
	P	AGCGCCGTGGCCCGCAAC	CCTACACTTGAACGTATTGAGAAGAACTTTGTCTATTACTGAC
	CCAAGATTGCCAGATAATCCCATTATATTCGCGTCCGATAGTTTCTTGCAATTGACAGAA		
	LOV2 (dN3)	TATAGCCGTGAAGAAATTTTGGGAAGAACTGCAGGTTTCTACAAGGTCCTGAACTGAT	
		CGCGCGACAGTGAGAAAAATTAGAGATGCCATAGATAACCAAACAGAGGTCACTGTTTCA	
		CTGATTAATTATACAAAGAGTGGTAAAAAGTTCTGGAACCTCTTCACTTGCAGCCTATG	
		CGAGATCAGAAGGGAGATGTCCAGTACTTTATTGGGGTTTCAGTTGGATGGAAGTGAAGCA	
	NES	CTGCGAGATGCTGCAGAGAGAGAGGGAGTCATGCTGATTAAGAAAAGTGCAGAAAATATT	
		GATGAAGCGGCAAAAGAGCTGGCCGGGCTGGATCTG	