A red light-responsive photoswitch for deep tissue optogenetics

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Red light penetrates deep into mammalian tissues and has low phototoxicity, but few optogenetic tools that use red light have been developed. Here we present MagRed, a red light-activatable photoswitch that consists of a red light-absorbing bacterial phytochrome incorporating a mammalian endogenous chromophore, biliverdin, and a photo-state-specific binder that we developed using Affibody library selection. Red light illumination triggers the binding of the two components of MagRed and the assembly of split-proteins fused to them. Using MagRed, we developed a red light-activatable Cre recombinase, which enables light-activatable DNA recombination deep in mammalian tissues. We also created red light-inducible transcriptional regulators based on CRISPR–Cas9 that enable an up to 378-fold activation (average, 135-fold induction) of multiple endogenous target genes. MagRed will facilitate optogenetic applications deep in mammalian organisms in a variety of biological research areas.

Blue light–activatable photoswitches, such as CRY2-CIB1 (ref. 1), iLID-sspB1 and the Magnet system2, have emerged as a powerful core technology to directly manipulate protein activity using blue light with high spatiotemporal resolution in mammalian cells3–7. For example, using these photoswitches to recruit functional domains to the plasma membrane or the promoter region of a gene of interest, cellular functions, such as cellular dynamics8, signal transduction9 and gene expression10, can be manipulated by blue light. Beyond domain recruitment, a more robust strategy based on reassembling split-protein fragments using blue light–activatable photoswitches has been developed and widely applied in various protein classes, including nucleases11,12, recombinases11,12, proteases13, photoswitches has been developed and widely applied in various protein classes, including nucleases11,12, recombinases11,12, proteases13, photoswitches14, antibodies15 and neurotoxins16. Using blue light–activatable photoswitches to reassemble split-proteins has greatly expanded the range of optogenetically controllable molecular processes in the cell, allowing researchers to address otherwise intracellular biological questions. However, blue light is easily scattered and absorbed in mammalian tissues, greatly impairing the applicability of blue light–activatable photoswitches in deep tissues7.

Compared to blue light, red light through the near-infrared (NIR) transparency window (650–900 nm)17 is advantageous for optogenetic use in mammalian deep tissues due to its high tissue transparency, low invasiveness and low light scattering. Several red light–activatable photoswitches have been developed using Arabidopsis thaliana phytochrome B (PhyB) and cyanobacterial phytochrome (Cph1) and tested for optogenetic manipulation in living cells18–21. Recently, a small and highly sensitive red light–activatable photoswitch was developed based on an engineered A. thaliana phytochrome A (∆PhyA)22. However, the plant/cyanobacterial phytochrome-based photoswitches require the addition of exogenous chromophores, such as phytochromobilin and phycocyanobilin, to absorb red light and function23, which limits their use in mammalian systems. In contrast, bacterial phytochromes can function without exogenous chromophores by incorporating a mammalian endogenous chromophore, biliverdin (BV). One of the bacterial phytochromes, BphS, is activated by red light to convert guanylate triphosphate into cyclic diguanylate monophosphate (c-di-GMP). BphS allows for red light–inducible transcription of a target gene by using it together with additional modules, such as the c-di-GMP-responsive hybrid transactivator p65-VP64-NLS-BldD, the chimeric promoter P(FRLx) and YhjH phosphodiesterase24,25. However, the BphS-based system requiring many components is complicated, does not enable direct manipulation of protein activity and could cause adverse effects on mammalian cells from endogenous targets of c-di-GMP. Recently, a red light photoswitch based on a bacterial phytochrome from Rhodopseudomonas palustris (RpBphP1) and its binding partner has been developed26,27. RpBphP1 shows reversible photoconversion between a far-red light–absorbing (Pfr) dark-state and a red light–absorbing (Pr) photo-state. Upon red light illumination, RpBphP1 is converted to the Pr photo-state and forms a heterodimer with its binding partner PpsR2 or its downsized variant QPAS1, thereby being applicable to simply and directly manipulate protein activity.

Here we demonstrate that RpBphP1-PpsR2/QPAS1 has two serious weaknesses. First, although RpBphP1-PpsR2/QPAS1 is suitable for domain recruitment, these systems do not work well for split-protein reassembly, substantially limiting their use in optogenetic applications. Second, PpsR2 binds not only to the holo-protein of RpBphP1 upon red light illumination but also to its apo-protein irrespective of red light illumination. The light-independent binding of PpsR2 with the apo-protein of RpBphP1 causes spontaneous activation of the optogenetic tools in the dark condition and, thereby, greatly hampers their regulatability. These benchmarking

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studies show that a versatile, regulatable technology that enables optogenetic manipulation using red light remains to be developed.

To address these concerns, we present a red light–activatable, semi-synthetic photoswitch, named MagRed. MagRed is composed of a red light–absorbing, BV-binding bacterial phytochrome derived from *Deinococcus radiodurans* (DrBphP) and its photo-state–specific de novo synthetic binder. Compared to RpBphP1-PpsR2/QPAS1, MagRed enables split-protein reassembly with red light illumination. Using MagRed and split-Cre fragments, we developed a red light–activatable Cre recombinase, which enables DNA recombination upon red light illumination in mammalian deep tissues. Additionally, we applied MagRed to the domain recruitment strategy to develop a red light–activatable, highly efficient optogenetic gene expression system based on the CRISPR–Cas9 system, enabling high induction (up to 378-fold) of multiple user-defined endogenous gene targets. The MagRed-based optogenetic tools have robust and precise regulatability and exhibit minimal leak activity in the dark regardless of BV concentration in the cell, thereby overcoming the limitation of RpBphP1-PpsR2, which is hampered by leak activation in the dark. We also show that MagRed has reliable regulatability in terms of mutually independent ON/OFF switching using two-colored light illuminations and sustainable ON-switching even with pulsed illumination.

**Results**

**Development of MagRed.** To develop a red light photoswitch, we applied synthetic biological approaches to generate a photo-state–specific de novo binding partner of DrBphP, a bacterial phytochrome derived from *D. radiodurans* (Fig. 1a). DrBphP incorporates BV as a mammalian endogenous chromophore and reversibly photoconverts between a Pr dark-state (λ_{max} = 701 nm) and a Pfr photo-state (λ_{max} = 752 nm) with two-wavelength light illuminations (Supplementary Fig. 1a). In addition to chromophore availability in mammalian cells and controllability using two-colored light illuminations, previous studies have revealed that the photosensory core module of DrBphP hereafter referred to as DrBphP-PSM, undergoes a light-induced large conformational change between the Pr dark-state and the Pfr photo-state (Supplementary Fig. 1b). The large conformational change of DrBphP is an advantage for developing its de novo synthetic binder that selectively binds to the Pfr photo-state, hereafter referred to as DrBphP-PSM, undergoing a biexponential slow reversion kinetics with decay amplitudes of 24% for 7 minutes and 76% for the following 1,291 minutes (Supplementary Table 2), which is much slower than that of RpBphP1 (τ_{1/2} = 2.83 minutes) (Supplementary Fig. 2). The slow dark reversion kinetics of DrBphP is beneficial for keeping it associated with the photo-state–specific binder even after turning off red light illumination, thereby sustaining the activation of optogenetic tools even by single or pulsed illumination. In contrast, RpBphP1 does not have such a sustainability due to its fast dark reversion kinetics, which diminishes the effectiveness of single/pulsed illumination protocols.

To generate a binding partner of DrBphP, we applied Affibody, the Z domain of immunoglobulin-binding *staphylococcal* protein A. Thirteen residues in the first and second helices of Affibody were randomized to generate its ribosome–displayed library (Fig. 1b). We purified the DrBphP-PSM protein and immobilized it on magnetic beads and then performed in vitro selections using the Affibody library to obtain binders for DrBphP-PSM under the 660-nm and the 760-nm light illumination conditions, respectively. After six rounds of the selections, we sequenced the cDNA library and eliminated Affibody clones detected under both the 660-nm and the 760-nm light illumination conditions. Finally, we prioritized Affibody clones with high read counts detected only under the 660-nm condition (Supplementary Table 1).

We assessed whether the top ten candidates of the prioritized Affibody clones could interact with DrBphP-PSM in mammalian cells using the tetR-tetO-based bioluminescent reporter gene (flucl/SEAP) expression system with VP16, a transcription activation domain (Fig. 1c). Of the tested clones, one Affibody clone named Aff6 displayed a high bioluminescence intensity upon red light illumination at 660 nm, which was similar to that induced by a direct fusion of tetR and VP16 (Fig. 1d,e and Supplementary Fig. 3). This result indicates that Aff6 binds to the Pfr photo-state of DrBphP-PSM with a high affinity. However, because it also showed a substantially high leakiness in the dark, its Light/Dark contrast was relatively low (1.2-fold induction). We found that the dark leak was significantly decreased by using the full-length DrBphP instead of DrBphP-PSM (Fig. 1e). However, the full-length DrBphP, hereafter referred to as just DrBphP, also produced decreased bioluminescence intensity upon red light illumination, resulting in low Light/Dark contrast (1.6-fold induction). We, thus, focused on a directed evolution of Aff6 to improve its interaction with DrBphP. First, we introduced individual alanine substitutions into the randomized 12 residues of Aff6. V18A substitution of Aff6 did not significantly alter the bioluminescence intensity compared to the wild-type, whereas the other substitutions remarkably decreased bioluminescence intensity (Supplementary Fig. 4). We conducted saturation mutagenesis at the V18 residue in Aff6 and found that V18F, V18W and V18H mutations significantly improved the Light/Dark contrast compared to the original Aff6 (Fig. 1d,e and Supplementary Fig. 5). Truncation of the N-terminal unstructured three residues from Aff6_V18F further enhanced the Light/Dark contrast (8.3-fold induction with 67% activity of tetR-VP16) (Fig. 1d,e), even though no significant change in the expression level was observed from the N-terminal truncation (Supplementary Fig. 6). We named the pair of DrBphP and Aff6_V18FAN ‘MagRed’.

**Photoswitching property of MagRed.** To investigate the photoswitching property of MagRed, the dissociation constants (K_d) of MagRed under 660-nm illumination and dark conditions were biochemically determined using a quartz crystal microbalance with dissipation monitoring (QCM-D). We found that the K_d under the 660-nm and dark conditions were 3.1 × 10^{-7} M and 1.5 × 10^{-8} M, respectively (Supplementary Fig. 7 and Supplementary Table 2). In addition, we also found that the rate constants of association (k_{on}) and dissociation (k_{off}) of MagRed under the 660-nm illumination conditions were 4.9-fold faster and 12-fold slower than those under the dark condition, respectively (Supplementary Table 2). To further characterize MagRed in mammalian cells, we performed a bioluminescence assay using a split-firefly luciferase (split-fluc) (Fig. 2a,b). In HEK293T cells expressing MagRed-fused split-fluc, repeated association and dissociation of MagRed was feasible using the 660-nm and the 800-nm pulsed illuminations (Fig. 2c). We also confirmed that the bioluminescence activity of full-length fluc as a control was almost unchanged by the 660-nm and 800-nm illuminations (Supplementary Fig. 8). These results demonstrate that the two-colored pulsed illuminations can independently and repeatedly control the association (switch-ON) and the dissociation (switch-OFF) of MagRed. Next, we measured the dissociation kinetics of MagRed in living cells. After the 660-nm illumination was turned off, MagRed-fused split-fluc maintained its bioluminescence with approximately 70% of the maximum intensity, after 21% decrease for the first 10 minutes (Fig. 2d). We also found that the biphasic slow decrease in the bioluminescence signal of MagRed-fused split-fluc is correlated with the bi-exponential slow dark reversion kinetics of DrBphP (Supplementary Fig. 9), showing that the slow dark reversion kinetics of DrBphP is an essential factor for developing MagRed with a highly stable controllability. Using full-length fluc as a control, we also confirmed that the consumption of d-luciferin can be ignored for the bioluminescence measurement (Supplementary Fig. 10).
Red light–activatable transcription system. To investigate the suitability of MagRed to domain recruitment applications, we applied MagRed to the CRISPR–Cas9–based photoactivatable transcription system (CPTS)\(^1\). In this system, MagRed plays a role in the red light–dependent recruitment of transcription activation domains, P65 and HSF1, to target loci, harboring inactive Cas9 (dCas9), single-guide RNA (sgRNA)-bearing MS2 aptamer and MS2 coat protein (Fig. 3a). We designed all configurations for the CPTS and examined their transcription activities using a luciferase reporter (NLS, nuclear localization signal; fluc, firefly luciferase; SEAP, secreted alkaline phosphatase; P\(_{\text{min}}\), minimal cytomegalovirus promoter). Fig. 1 | Development and characterization of MagRed. a, Schematic representation of DrBphP and its Pfr photo-state-specific binding partner. Upon 660-nm light illumination, a binding partner candidate fused with VP16 and NLS binds to the photoproduct of tetR-DrBphP anchored on the tetO element, thus activating transcription of the reporter gene (NLS, nuclear localization signal; fluc, firefly luciferase; SEAP, secreted alkaline phosphatase; P\(_{\text{min}}\), minimal cytomegalovirus promoter). b, Alignment of N-terminal amino acid sequence from Aff6 variants. The 13 residues diversified in the initial library are highlighted with red color, and the residues altered from Aff6 are marked in cyan color. c, Bioluminescence intensity in HeLa cells transfected with different transcription activators and a bioluminescence reporter plasmid. PSM, DrBphP-PSM; Full length, full-length DrBphP; V18F; Aff6_V18F, V18F mutated Aff6; Aff6_V18FΔN, ΔN terminus of Aff6_V18F. P values are indicated above the bars. (NS, not significant \(P>0.05\); \(*) P<0.05\); \(** P<0.01\); \(**** P<0.0001\); dark versus light using two-tailed unpaired t-test, from three biologically independent samples, mean ± s.d.). a.u., arbitrary units.
in an especially enhanced Light/Dark contrast (619-fold induction). Although we also tested configuration 3 with the tandem trimer and tetramer of Aff6_V18FΔN, the trimer and tetramer constructs did not exhibit further enhancement of transcription activity (Fig. 4a).

Therefore, we concluded that configuration 3, with the tandem dimer of Aff6_V18FΔN, was the best version of MagRed-based CPTS, called ‘Red-CPTS’. We confirmed that Red-CPTS works robustly in various mammalian cell lines (Supplementary Fig. 13). The time course of red light–dependent reporter gene expression shows that the transcription by Red-CPTS reaches its maximum 24 hours after the initiation of red light illumination and that only 1 hour of red light illumination can efficiently activate the reporter gene (∼9.3-fold induction) using Red-CPTS (Supplementary Fig. 14).

Next, we applied Red-CPTS to multiple endogenous genes by simultaneously delivering four sgRNAs separately targeting human gene promoters, as exemplified for ASCL1, HBGI, IL1R2 and MYOD1. Upon red light illumination, Red-CPTS significantly increased all the targeted gene transcriptions up to 378-fold with high Light/Dark contrasts (Fig. 4b). Notably, in all the targeted endogenous genes, each mRNA level of Red-CPTS-transfected cells in the dark was similar to those of mock-transfected cells (Fig. 4b), demonstrating that Red-CPTS has no obvious leak activity in the dark and, thereby, enables for robust regulation of multiplexed user-defined endogenous gene activation using red light illumination. Additionally, we also tested whether the 800-nm deactivation control could be applied to Red-CPTS using sgRNA targeting the human ASCL1 promoter. After the pre-activation of Red-CPTS by the 660-nm illumination for 12 hours, the cells were illuminated at 800 nm. We found that the ASCL1 mRNA level was decreased by the 800-nm illumination, which is significantly faster than the decrease in the dark conditions, and reached a minimum level in 1.5 hours (Extended Data Fig. 2). This result indicates that 800-nm light illumination can be used to actively switch Red-CPTS back from the activated state to the inactivated state. We also confirmed that the 800-nm light illumination has only minimal effect on the Red-CPTS activation (Supplementary Fig. 15), showing that the 800-nm deactivation light illumination can independently be used with the 660-nm activation light illumination.

Next, we investigated the relationship between the photoswitching efficiency of MagRed and various illumination conditions using Red-CPTS and found that the transcription activity of Red-CPTS reached a plateau at 1.0 W m⁻² of red light illumination (Extended Data Fig. 3a,d). We also found that the transcription activity of Red-CPTS can be finely tuned by changing the duration of red light illumination ON time (Extended Data Fig. 4) and OFF time (Extended Data Fig. 5). The illumination cycle consisting of 1 minute of 660-nm light, followed by 4 minutes in dark, maximally increased reporter gene expression with a 1,283-fold Light/Dark contrast (Extended Data Fig. 4a,d). Furthermore, transcription activity of Red-CPTS reached a plateau using the illumination cycle consisting of 1 minute of 660-nm light, followed by 9 minutes in dark (Extended Data Fig. 5a,d). As the comparison with Red-CPTS, we also tested the RpBphP1-PpsR2/QPAS1-based CPTS under various illumination conditions, because the illumination conditions under which the contrast between light and dark is maximized are likely to be different between DrBphP and RpBphP1 due to their different rates of dark reversion kinetics (Supplementary Fig. 2). However, due to the significantly high dark leakiness, the Light/Dark contrasts of RpBphP1-PpsR2/QPAS1-based CPTS were not improved in any of the tested light illumination conditions (Extended Data Figs. 3–5).

Red light–activatable DNA recombination system. In addition to the domain recruitment strategy applied to CPTS, next we examined whether MagRed can be applied to the split-protein reassembly
with red light illumination. Of the existing optogenetic tools based on the split-protein reassembly system with blue light–activatable photoswitches, site-specific DNA recombine is one of the most attractive targets11,12,23–35. Principally, Cre recombinase is the most widely used DNA recombinase in biology, biotechnology and biomedical studies36–39. We fused MagRed to split-Cre fragments to develop a red light–activatable Cre recombinase applicable in mammalian systems (Fig. 5a). To test all configurations, we fused either DrBphP or Aff6_V18FΔN to the newly created N- and C-terminal ends of split-Cre fragments (CreN and CreC) as well as the original N-terminal end of CreN (Fig. 5b). We also tested two split positions (CreN59/CreC60 and CreN104/CreC106) for the split-Cre fragments. DNA recombination activities were examined using a Flocked-STOP luciferase reporter in HEK293T cells (Supplementary Fig. 16a). Most of the configurations using MagRed and split-Cre exhibited red light–dependent DNA recombination with significant Light/Dark contrasts (Fig. 5b–d and Supplementary Fig. 17a). Of the tested configurations, NLS-CreN104-Aff6_V18FΔN and NLS-DrBphP-CreC106 (configuration 1) gave the highest Light/Dark contrast (31-fold induction). This MagRed-based red light–activatable Cre recombinase was named RedPA-Cre. We confirmed that additional BV supplementation did not have significant effect on the DNA recombination activity of RedPA-Cre (Supplementary Fig. 18), revealing that RedPA-Cre works robustly at the endogenous BV concentration of living mammalian cells as Red-CPTS does.

We also tested RpBphP1-PpsR2/QPAS1 for the split-protein reassembly strategy with split-Cre and compared their DNA recombination activities with the MagRed version. Compared to MagRed, RpBphP1-PpsR2/QPAS1 displayed much lower Light/Dark contrasts in all the tested configurations fused with split-Cre despite additional BV supplementation for eliminating the apo-protein of RpBphP1 (Fig. 5b–d and Supplementary Fig. 17b). During RedPA-Cre development, new red light–inducible dimerization systems, composed of DrBphP-PSM and its nanobody-based binding partner LDB-3 and LDB-4, named nanoRed systems (nanoRed1 and nanoRed2, respectively), were reported40. We also examined whether nanoRed systems could be applied to the split-protein reassembly with split-Cre. We found that nanoRed systems also showed much lower Light/Dark contrasts in bioluminescence intensity under any configurations with split-Cre as RpBphP1-PpsR2/QPAS1 did (Fig. 5b-d and Supplementary Fig. 17c,d). The results reveal that MagRed enables split-protein reassembly with split-Cre for the development of a red light–activatable Cre recombinase, which is not achieved by RpBphP1-PpsR2/QPAS1 and nanoRed systems. As a benchmark study, we also compared RedPA-Cre with existing red light–responsive Cre recombinases, CreLite41 and L-SCREAblE (Light-Cre1 and Light-Cre2)42, which are based on PhyB, and the FISC system24, for DNA recombination activities with the MagRed version. Compared to MagRed, RedPA-Cre and FISC systems could be applied to the split-protein reassembly with split-Cre. We found that FISC systems also showed much lower Light/Dark contrasts in bioluminescence intensity under any configurations with split-Cre as RpBphP1-PpsR2/QPAS1 did (Fig. 5b-d and Supplementary Fig. 17c,d). The results reveal that MagRed enables split-protein reassembly with split-Cre for the development of a red light–activatable Cre recombinase, which is not achieved by RpBphP1-PpsR2/QPAS1 and nanoRed systems. As a benchmark study, we also compared RedPA-Cre with existing red light–responsive Cre recombinases, CreLite and L-SCREAblE (Light-Cre1 and Light-Cre2), which are based on PhyB, and the FISC system, which is based on BV-binding BphS, and found that RedPA-Cre outperformed these previously reported red light–dependent DNA recombination systems as well (Extended Data Fig. 6).
that induced by continuous red light illumination for 24 hours, RedPA-Cre can be efficiently activated by red light illumination for even short periods of illumination time. This appears to be due to the characteristic of RedPA-Cre, which maintains its DNA recombination activity even after turning off red light illumination because of the slow dark reversion kinetics of DrBphP in MagRed. To compare the DNA recombination activity of RedPA-Cre with that of full-length Cre (iCre), we labeled the NLS-CreN104-Aff6_V18FΔAN fragment of RedPA-Cre with the fluorescent protein Venus and then assessed its red light–inducible activity using a Floxed-STOP mCherry reporter (Supplementary Fig. 16b). Upon red light illumination for 24 hours, RedPA-Cre induced mCherry fluorescence with 55% efficiency of that induced by iCre (Fig. 5c).

Next, we examined whether RedPA-Cre could be applied to bicistronic designs. RedPA-Cre was able to be concatenated via an internal ribosome entry site (IRES) without compromising the red light–dependent DNA recombination and the low leak activity in the dark (Supplementary Fig. 19). As well as the case of Red-CPTS, the bicistronic RedPA-Cre had little responsiveness to the 800-nm light illumination (Extended Data Fig. 8). The bicistronic RedPA-Cre was further validated in vivo in living mice through intrahepatic gene delivery along with a Floxed-STOP fluc reporter. We found that the exposure of the mice to red light illumination at 660 nm from the ventral side induced marked bioluminescence signals by the DNA recombination reaction in their livers ($P = 0.011$ from $n = 3$ mice per group; Fig. 5f, Extended Data Fig. 9 and Supplementary Fig. 20). As observed in cultured cells in vitro, RedPA-Cre did not need additional BV supplementation to function in the liver of living mice. We also observed that the mice with RedPA-Cre exhibited little DNA recombination activity when kept in the dark. These results demonstrate that RedPA-Cre can efficiently induce DNA recombination in an internal organ in living mice even using external and non–invasive red light illumination. Additionally, we also revealed that Red-CPTS can be used to induce a red light–dependent gene transcription activation in vivo in living mice without additional BV supplementation (Extended Data Fig. 10 and Supplementary Fig. 20). Collectively, we confirm that MagRed-based optogenetic tools are functional and exhibit precise regulatability in vivo.

**Discussion**

After demonstration of optogenetic control of neurons with microbial opsins\(^4\), blue light–activatable photoswitches emerged as a core technology to control various protein activities and have facilitated biological applications in a variety of research fields beyond neuroscience. In the next generation of optogenetics, several red light–activatable photoswitches were developed as a core technology to optogenetically manipulate protein activities through the NIR transparency window. However, such a core technology with high versatility and regulatability for enabling the optogenetic manipulation using red light remains elusive, as demonstrated in this study. The lack of a powerful and useful technology is a major bottleneck hindering the development of red light optogenetic tools. To establish a better red light optogenetic technology, we applied synthetic biological approaches to develop a red light photoswitch named MagRed. MagRed can directly manipulate protein activity using red light with high spatiotemporal resolution, having two major advantages among the existing red light photoswitches in terms of versatility and regulatability.

Compared to RpBphP1-PpsR2/QPAS1 and nanoRed systems, MagRed has high versatility that enables for the split-protein reassembly with red light illumination. Based on MagRed and split-Cre, we developed RedPA-Cre, a red light–activatable Cre recombinase, allowing us to induce DNA recombination upon red light illumination in mammalian deep tissues, such as livers. In addition to split-Cre, we also applied MagRed to optical control of firefly luciferase based on split-fluc reassembly, demonstrating that MagRed is generally applicable to split-protein reassembly regardless of protein functions, sizes and structures. Split-proteins can be designed for every class of proteins in principle, and this has gained increased

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We tested various illumination conditions for the activation of RedPA-Cre using a Floxed-STOP fluc reporter. We found that all the tested different durations of red light illumination evoked significant bioluminescence induced by the DNA recombination (Extended Data Fig. 7). Red light illumination for only 30 seconds could induce DNA recombination with 38% efficiency of that induced by continuous red light illumination for 24 hours, indicating that RedPA-Cre can be efficiently activated by red light illumination for even short periods of illumination time. This appears to be due to the characteristic of RedPA-Cre, which maintains its DNA recombination activity even after turning off red light illumination because of the slow dark reversion kinetics of DrBphP in MagRed. To compare the DNA recombination activity of RedPA-Cre with that of full-length Cre (iCre), we labeled the NLS-CreN104-Aff6_V18FΔAN fragment of RedPA-Cre with the fluorescent protein Venus and then assessed its red light–inducible activity using a Floxed-STOP mCherry reporter (Supplementary Fig. 16b). Upon red light illumination for 24 hours, RedPA-Cre induced mCherry fluorescence with 55% efficiency of that induced by iCre (Fig. 5c).

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Compared to RpBphP1-PpsR2/QPAS1 and nanoRed systems, MagRed has high versatility that enables for the split-protein reassembly with red light illumination. Based on MagRed and split-Cre, we developed RedPA-Cre, a red light–activatable Cre recombinase, allowing us to induce DNA recombination upon red light illumination in mammalian deep tissues, such as livers. In addition to split-Cre, we also applied MagRed to optical control of firefly luciferase based on split-fluc reassembly, demonstrating that MagRed is generally applicable to split-protein reassembly regardless of protein functions, sizes and structures. Split-proteins can be designed for every class of proteins in principle, and this has gained increased
interest recently as computational approaches continue to identify new split positions in a diverse array of proteins\(^{43,46}\). These computational approaches to designing split-proteins and the present MagRed technology synergistically work to expand the range of optogenetic applications in mammalian deep tissues. In addition to its versatility, MagRed has high regulatability and shows only minimal leak activation in the dark, as opposed to RpBphP1-PpsR2/QPAS1. For an optogenetic tool, the leakage of protein activity in the dark is the most serious and unacceptable shortcoming because it causes unintentional protein function before light stimulation, which impairs the accuracy of optogenetic control and leads to misinterpretation\(^{35}\). MagRed offers tight regulatability, thereby enabling more precise optogenetic manipulation of protein activities. In addition, MagRed also has useful regulatability that can repeatedly control its association and dissociation by the 660-nm and 800-nm pulsed illuminations and can maintain the association form for a long period even after turning off the red light illumination. This regulatability of MagRed, derived from preferable switching properties of DrBphP, enables mutually independent ON/OFF switching with two-colored light illuminations and sustainable ON-switching even with pulsed illumination. Taking advantage of the useful regulatability of MagRed, we can manipulate protein activity more flexibly than with other existing tools. For example, we can terminate optogenetic manipulation at any time we intend. Additionally, we can reduce phototoxicity on biological samples using pulsed red light illuminations. Notably, these advantages can be obtained without additional chromophore supplementation. Overall, among the existing photoswitches, its high versatility and regulatability makes MagRed a unique optogenetic core technology.

Using MagRed, we developed RedPA-Cre and Red-CPTS for red light optogenetics. RedPA-Cre can efficiently induce DNA recombination with red light illumination, which is the first report on the red light–activatable site-specific DNA recombinase based on split-protein reassembly, which works in mammalian systems. The Cre-loxP system is widely used for gene insertion, deletion, inversion or cassette exchange in various animal models. Combined with current recombinase-based biological tools, such as cell lineage tracing\(^{44,45}\), genetic circuits\(^{46,47}\) and gene knock-out\(^{35}\) and knock-in\(^{35}\) analysis, RedPA-Cre with high spatiotemporal controllability could address more complicated biological questions and pathophysiological mechanisms for various diseases. Another MagRed-based optogenetic tool, Red-CPTS, enables activating the expression of multiplexed user-defined endogenous genes using red light illumination. In addition to transcription control, the Red-CPTS platform

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**Fig. 5 | A photoactivatable Cre-loxP recombination system based on the MagRed system.** a. Schematic representation of RedPA-Cre. Upon 660-nm light illumination, split-Cre fragment reassembly is induced by the light-inducible association of MagRed, leading to recombination of DNA sequences flanked by two loxP sites. b. Comprehensive RedPA-Cre designs with combination of four configurations and the two split positions of Cre. c, d. Mean Light/Dark contrasts of all tested combinations are plotted with real number scale in c and are shown as a heat map with log\(_2\) scale in d. These Light/Dark contrasts were calculated based on bioluminescence intensity of HEK293T cells transfected with different RedPA-Cre designs and a bioluminescence reporter plasmid. See Supplementary Fig. 17 for detailed results of each combination (mean from \(n = 4\) biological replicates). e. Fluorescence reporter (mCherry) assay with RedPA-Cre tagged with yellow fluorescent protein Venus. mCherry and Venus fluorescence were detected using mCherry channel and GFP channel of fluorescence microscopy, respectively. The percentage of mCherry-channel\(^{+}\) cells among GFP-channel\(^{+}\) cells (mCherry-channel\(^{+}\)/GFP-channel\(^{+}\)) was estimated from \(>1,000\) cells of three independent experiments. \(P\) values are indicated above the bars. (NS, not significant \(P > 0.05\); **** \(P < 0.0001\); dark versus light using two-tailed unpaired \(t\)-test, mean \(\pm\) s.d.). Scale bars, 20 \(\mu m\). f. Left, representative bioluminescence images of mice transfected with the bioluminescent reporter and RedPA-Cre of which configuration is NLS-DrBphP-CreC\(_{106}\)-IRES-NLS-Cre\(_{104}\)-Aff\(_{6}\_V\_18F\) at a 4:1 ratio. The mice were maintained in the dark and subjected to the illumination with 660-nm light. The flux expression was measured using 200 \(\mu l\) of 100 mM \(\beta\)-luciferin 25 hours after transfection. Right, gray and red bars represent the mean \(\pm\) s.d., and black dots represent the total bioluminescence intensity of each mouse (\(n = 3\) mice per group). No difference can be observed in the appearance between the mice maintained in the dark and the ones illuminated with red light at 660 nm for 16 hours. \(P\) values are indicated above the bars. (NS, not significant \(P > 0.05\); **** \(P < 0.0001\); two-way ANOVA with multiple comparisons, \(n = 3\) biologically independent samples, mean \(\pm\) s.d.). a.u., arbitrary units.
will also be extended to optogenetic control of other CRISPR applications based on the recruitment of effectors such as epigenetic modifications and base editing in mammalian deep tissues.

In conclusion, MagRed has the potential to regulate various cellular functions in a much more comprehensive range of biological samples, such as living cells, mammalian deep tissues and whole animals. The remarkable features of MagRed are valuable to systemically expand the optogenetic toolbox in the long-wavelength region, thereby opening new avenues in the field of optogenetics using red light.

Online content
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Methods

Cloning. All plasmids in this study are listed in Supplementary Table 3. Complete cDNA sequences of these plasmids, except for gifted and commercially available plasmids, are denoted in Supplementary Note. All constructs for RedFP-Cre and RedFP-CreP were inserted into the pCDA3.1 or pCDA3.1/V5-His3 vector (Invitrogen). All constructs for tetR-tetO system were cloned into the pSV40 vector, gifted from Wilfried Weber. All constructs encaging gRNAs were cloned into the pSpG4 vector (Addgene, 47108).

cDNA encoding codon-optimized Streptococcus pyogenes cas9 was amplified from pSpCas9-BB-2Amp (Addgene, 52901) and pSpCas9-BB-2Amp (Addgene, 42230). To eliminate the nuclear activity of cas9, we designed D10A and H840A mutations. cDNAs encoding the MS2 (N55K) mutant and p65–HSF1 were amplified from pMS2-P65–HSF1-GFP (Addgene, 61423). The EGFP-Cre gene was amplified from pENN.AAV.sMAC.ELIFCre.WPRE.SV40 (Addgene, 105540). cDNAs encoding PIP4C6Cen and PhyB-ArrCreC recur (Cr/Cre) were amplified from pCS2-PHP4C6Cen (Addgene, 131780) and pCS2-PhyB-ArrCreC (Addgene, 131781), respectively. cDNAs encoding PPyBNT-creN (Light-Cre1), PhyB-NLS-CreN (Light-Cre2) and PFF3-NLS-CreC (Light-Cre1 and Light-Cre2) were amplified from pLH_SC15 (Addgene, 100537) and pLH_SC16 (Addgene, 100538).
cDNAs encoding iCre, DrBphP, RpBphP1, PskR2, Zdk1, LDB-14 and LDB-3 were synthesized with optimized codons. cDNAs encoding VP16-NLS were amplified from pColdI-Avi-DrBphP-PSM. The cells were grown at 37 °C with shaking at 100 rpm in LB media (100 µg ml−1 ampicillin, 30 µg ml−1 chloramphenicol and 0.45 in 30 ml of LB media with ampicillin (100 mg ml−1). The culture was cooled to room temperature. Isopropyl-β-D-thiogalactoside (IPTG, Wako) and BV (Frontier Scientific) were added to the culture in final concentrations of 0.5 mM and 100 µM, respectively. The cells were cultured for 48 hours at 16 °C in the dark for protein expression. The cell suspension collected by centrifugation at 6,000 × g for 10 minutes (Thermo Fisher Scientific) containing 0.2 mg of lysozyme (Wako), 10 U of DNase I (Thermo Fisher Scientific) and protease inhibitor cocktail (Sigma-Aldrich) for 10 minutes at room temperature. The crude soluble extract was purified using a HisTrap FF column (GE Healthcare). Biotinylated DrBphP-PSM was performed using a nickel-affinity (Bio-Rad, A5800). The ribosome–protein fusion libraries were generated with the PURE system. The standard PURE translation mixture was prepared as described previously [2]. In vitro transcription and translation reaction was performed at 37 °C for 60 minutes, followed by stopping the reaction with 100 µl of TBS-Mg2+ buffer (50 mM Tris-HCl, 50 mM MgCl2 and 150 mM NaCl, pH 7.5). Meanwhile, 300 µl of 0.01 M of streptavidin beads (Dynabeads M-280 Streptavidin, Invitrogen) were washed once with 200 µl TBS-Mg2+ buffer. Biotinylated DrBphP-PSM was added to the washed beads and incubated at 4 °C for 30 minutes with gentle rotation. The beads were then washed once with 200 µl of TBS-Mg2+ buffer, followed by the addition of dilution reaction mixture. After 45-minute incubation at room temperature with gentle rotation, the beads were washed twice with 200 µl of TBS-Mg2+ buffer. To recover the enriched ribosome–protein fusion, 200 µl of elution buffer (TBS buffer + 50 mM EDTA) was added to the beads and incubated for 30 minutes with gentle rotation. The eluted solution was subjected to standard phenol–chloroform extraction and isopropanol precipitation to remove protein components. Purified RNAs were subjected to reverse transcription using primers O8 with SuperScript III (Thermo Fisher Scientific), and the enriched cDNA library was regerated by PCR using primers O5 and O6 and KOD -Multi & Epi- DNA polymerase. After six rounds of selection, the enriched DNA library was sequenced on a Illumina MiSeq machine using the MiSeq Reagent Kit version 3 (150 cycles, 130 bp and 45 bp, paired-end).

Deep sequencing analysis. Only R1 reads of the paired-end reads were subjected to subsequent analysis. Analysis of the sequences was performed using custom Python scripts (Python 2.7). The sequences were filtered according to Phred quality scores (Q): reads were disregarded if more than half of the base calls were below Q20, and base calls with a quality score below Q20 were converted to N. For further quality assessment, sequences were searched for the two exact sequences at the constant region of an Affibody. It was assessed if the length between those two sites contained exactly 122 nucleotides. The sequences with expected length were considered to have a complete Affibody. Finally, we selected Affibody sequences that were enriched under 660-nm condition but not detectable under 760-nm condition.

Cell culture. HEK293T and Neuro 2a cells (American Type Culture Collection (ATCC)) were cultured at 37 °C under 5% CO2 in MEM (Sigma-Aldrich) with 10% FBS (HyClone). 100 U ml−1 of penicillin and 100 µg ml−1 of streptomycin (Gibco). HeLa cells (ATCC) were cultured at 37 °C under 5% CO2 in minimum essential medium (Media) (Sigma-Aldrich) with 10% FBS, 100 U ml−1 of penicillin and 100 µg ml−1 of streptomycin.

Light source. Except for split-flu reassembly assay, light illumination was performed inside a CO2 incubator using pre-assembled LED arrays (CCS). A regulated DC power supply (Kikusui Electronics) was used to control LED current flow. Temporal illumination pattern was generated by an Arduino microcontroller board.

TetR-tetO-based gene expression system using luciferase reporter. HeLa cells were plated at 1.0 × 105 cells per well in a 96-well black-wall plate (Greiner) and cultured for 24 hours. The cells were transfected with Lipofectamine 3000 reagent (Invitrogen). Plasmids encoding VP16-NLS, tetR and firefly luciferase reporter or SEAP reporter were transfected at a 1:1:1 ratio. The total amount of DNA was 0.1 µg per well. As a mock-control A66-VP16-NLS (−) condition, the A66 moiety of A66-VP16-NLS was replaced with irrelevant Affibody Zdk1 (ref. 17). Twenty-four hours after the transfection, the sample was added with 100 µl of media to a total volume of 300 µl and incubated under 660-nm pulsed light illumination (1 W m−2) that is repetitively switching on for 1 minute and then turning off for 4 minutes. For dark condition, the sample was wrapped in aluminum foil during incubation. After incubation for 24 hours, the culture medium was replaced with 100 µl of HBSS (Gibco) containing 0.1 mg ml−1 of mCherry CreN (Wako) and 0.45 in 30 ml of LB media with ampicillin (100 mg ml−1) and chloramphenicol (30 mg ml−1). The culture was cooled to room temperature.

Biochemical characterization using QCM-D. Dissociation constant (Kd) of MagRed was measured with a QCM-D instrument (QCell T (3T analytic)). His-Avi-DrBphP and His-Avi-VP16FAN-3xFLAG proteins were purified and characterized as described above, except that these were further purified by
size-exclusion chromatography (AKTAprime plus). His-Avi-DrBphP was biotinylated using the BirA enzyme. QCM sensor chips were cleaned for 60 seconds using a vacuum plasma apparatus (YHS-R, Sakagike Semiconductor). The cleaned sensor chips were biotinylated with a biotin–SAM formation reagent (Dojindo Laboratories) in ethanol for 1 hour. The biotinylated sensor chips were then rinsed with MilliQ for three times, dried with air flow and immersed in PBS (−) containing 1 mg/ml−1 of streptavidin for 1 hour. The streptavidin-coated sensor chips were rinsed by gentle shaking for 5 minutes with PBS (−) for three times and then attached to the QCM-D device. Stable baseline of the resonance frequency was monitored by flowing PBS (−) at a flow rate of 60 μl/min−1. Because DrBphP could be unintentionally activated by ambient light in the experimental process, we described the procedures as dark when such as bacteria expressing DrBphP (Cells are as follows: A5C, T5C, A5C10T5C10). Expression, purification, functionalization and fixation of the QCM sensor chip, we illuminated DrBphP on the sensor chip at 800 nm (5 W m−2) for 5 minutes to switch the activated DrBphP back to dark-state, according to the reference1. DrBphP on the sensor chip was then illuminated at 660 nm (2 W m−2) or kept in the dark for 5 minutes. The sample solution containing 10 μl of His-A66V18ΔN-3×FLAG in PBS (−) were introduced into the QCM device at a flow rate of 60 μl/min−1 for 10 minutes in the presence of 660-nm illumination (2 W m−2) or in the dark to measure the adsorption of His-A66V18ΔN-3×FLAG to the DrBphP-coated sensor chips. By analyzing the adsorption curves measured at the two different concentrations using first-order qGraph Viewer (3T analytik), we determine the k0, kq, and k− of MagRed in both photo-state and dark-state, according to the manufacturer’s protocol and the related reference2.

Split-fluc reassembly assay. HEK293T cells were plated in the presence of 25 μM BV at 4.0×104 cells in a 35-mm culture dish (Iwaki Glass) and cultured for 24 hours. Plasmids encoding Nic-fluc-DrBphP and 3xFLAG-A66V18ΔN-C128 were transfected as a 1:1 ratio. The total amount of DNA was 2.5 μg per dish. In case of full-length fluc as a control, the amount of DNA was 0.025 μg per dish. Twenty-four hours after transfection, the culture medium was replaced with 2 ml of HBSS with 200 μM d-luciferin. After incubation for 30 minutes at room temperature, the sample was illuminated at 800 nm (50 W m−2) for 5 minutes to achieve photoconversion of DrBphP from Pr to photo-state to Pr dark-state. Bioluminescence measurement was performed using a GloMax 20/20 Luminometer (Promega). The bioluminescence signals were integrated over 1 second and plotted every 1 second. We then illuminated the sample dish at 660 nm (10 W m−2) for 1 minute and performed the bioluminescence measurement.

Luciferase reporter assay for CPTs design. HEK293T and Neuro 2a cells were plated at 2.0×104 cells per well, and HeLa cells were plated at 1.0×104 cells per well, in a 96-well black-wall plate. The cells were cultured for 24 hours. Plasmids encoding NLS-cDs9-NLS, MS2, p65-HSF1, sgRNA and luciferase reporter were transfected at a 1:1:8 ratio. The total amount of DNA was 0.1 μg per well. In case of full-length fluc as a control, the amount of DNA was 0.025 μg per dish. Twenty-four hours after transfection, the culture medium was replaced with 2 ml of HBSS with 200 μM d-luciferin. After incubation for 30 minutes at room temperature, the sample was illuminated at 800 nm (50 W m−2) for 5 minutes to achieve photoconversion of DrBphP from Pr to photo-state to Pr dark-state. Bioluminescence measurement was performed using a GloMax 20/20 Luminometer (Promega). The bioluminescence signals were integrated over 1 second and plotted every 1 second. We then illuminated the sample dish at 660 nm (10 W m−2) for 1 minute and performed the bioluminescence measurement.

Endogenous gene activation by Red-CPTs. The procedures for plating and transfection were identical to those described above, except for using a mixture of sgRNAs separately targeting four endogenous genes. Forty-eight hours after transfection, total RNA extraction and reverse transcription PCR were performed using CellAmp Direct RNA Prep Kit (Takara Bio) with Superscript IV Vilo Master Mix (Thermo Fisher Scientific). Quantitative PCR was performed by StepOnePlus system (Thermo Fisher Scientific) using TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). TaqMan primers and probes were used to quantify the expression level of the target gene, and the GAPDH gene was measured as endogenous control (Life Technologies; Taqman Gene Expression Assay ID: Mm01545529_m1). In addition, the expression levels of the following genes were measured: HBG1: Hs00361131_g1; MYOD1: Hs02330075_g1; GAPDH: Hs99999905_m1). Expression fold changes were calculated using the ΔΔCT method and was shown to each relative mRNA level to that from non-transfected HEK293T cells as a negative control.

For deactivation of Red-CPTs at 800 nm, the cells were illuminated with 660-nm pulsed light (1 minute ON and 4 minutes OFF) of 1 W m−2 for 12 hours to pre-activate Red-CPTs. The cells were incubated under the 660-nm pulsed illumination condition or switched to the dark condition or the 800-nm pulsed illumination condition (1 minute ON and 4 minutes OFF) of 10 W m−2.

Western blotting. For Supplementary Fig. 6, HEK293T cells were transiently transfected with plasmid encoding A66V18F-PV16-NSL-V5-His or A66V18ΔN-PV16-N5-V5-His. Twenty-four hours after transfection, the cells were lysed in M-PER reagent (Thermo Scientific). The cell extracts were adjusted to the same amount of total cellular protein (20 μg) and electrophoresed in a 15% polyacrylamide gel. After electrophoretic transfer to a nitrocellulose membrane, the membranes were blocked with Blocking One (Nacalai Tesque) for 1 hour. The membrane was analyzed by anti-β-actin (9F3) rabbit monoclonal antibody conjugated with HRP (Cell Signaling Technology) or anti-β-actin antibody conjugated with HRP (Santa Cruz Biotechnology). The membranes were subsequently transblotted using biotinylated HRP-conjugated secondary antibodies, washed in TBST, and incubated with streptavidin-HRP conjugated with HRP (Invitrogen, cat. no. R061-25, 1:5,000 dilution). The membranes were developed with SuperSignal West Femto Maximum Sensitivity (Thermo Scientific) according to the manufacturer’s protocol.

Luciferase reporter assay for RedP-A-Cre. HEK293T cells were plated in the presence of 25 μM BV at 2.0×104 cells per well in a 24-well black-wall plate. Plasmids encoding NLS-CreN104-A66V18ΔN-Venus, NLS-DrBphP-(FL)-CreC106 and floxed reporter were transfected at a 1:1:8 ratio. The total amount of DNA was 0.1 μg per well. Six hours after transfection, the sample was illuminated or kept in the dark. For the MagRed system and nanoRed1/2 systems, the illuminated samples were incubated under 660-nm pulsed light (1 minute ON and 4 minutes OFF) of 1 W m−2. For RppBphP1-PpsR2-QPAS1, the illuminated samples were incubated under 760-nm pulsed light (1 minute ON and 4 minutes OFF) of 10 W m−2. For incubation 18 hours, the culture medium was replaced with 100 μl of HBSS containing 200 μM d-luciferin as a substrate. After incubation for 30 minutes at room temperature, bioluminescence measurements were performed.

Fluorescence reporter assay for RedP-A-Cre. HEK293T cells were plated at 0.8×104 cells per well in a 24-well plate. Plasmids encoding NLS-CreN104-A66V18ΔN-Venus, NLS-DrBphP-(FL)-CreC106 and floxed reporter were transfected at a 1:1:8 ratio. The total amount of DNA was 0.1 μg per well. After incubation for 24 hours in the presence or the absence of 660-nm pulsed illumination, cells were fixed with 4% paraformaldehyde in PBS (Wako) for 15 minutes, followed by blocking with 5% BSA and 1% bovine serum albumin (Wako) for 1 hour. The cells were washed three times with PBS, and the cells were stained with 300 nM DAPI (Invitrogen) for 15 minutes. Images were acquired using an inverted microscope (DMi8000BR, Leica Microsystems) equipped with a x40 oil objective (Leica PLAN APO ×40/0.79 DCI) and a 16-bit camera (Cascade II:512, Photometrics) controlled by Metamorph software (Molecular Devices). The imaging of DAPI, GFP and mCherry channel was conducted with A5, L3 and TX2 filter cubes (Leica Microsystems).

Image processing. Obtained images were analyzed using ImageJ/FIJI® and CellProfiler®. The background of images was subtracted using the build-in ‘Subtract background’ tool in ImageJ. Nuclei were identified in the DAPI channel using an object diameter threshold of 5–20 pixel units. The mean intensity of Venus and mCherry fluorescence at each nucleus was measured to determine the population of GFP-positive (GFP+) cells and mCherry-positive (mCh+) cells. The intensity threshold was determined not to detect mCh+ cell in the mock-control condition. The number of mCh+ cells was then divided by the number of GFP+ cells and multiplied to obtain the percentage of mCh+/GFP+ cells.

In vivo RedP-A-Cre and Red-CPTs activation. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Tokyo and were conducted in accordance with the Guidelines for Care and Use of Laboratory Animals as stated by the University of Tokyo. Four-week-old female ICR mice and 6-week-old female BALB/c mice were purchased from Sankyo Labo Service. Housing conditions for the mice were 20–26 °C, 40–60% humidity and a 12-hour/12-hour light/dark cycle. The livers of 4-week-old female ICR mice were hydrodynamically transfected by injecting 10 μg of the pSVP40 plasmid, of which configuration is NLS-DrBphP-CreC106-IRE5-NLS-CreN104-A66V18FAN and 40 μg of the bioluminescent reporter plasmid with Transit-EF Hydromagnetic Delivery Solution (Mirus Bio). As a mock-control, pcDNA3.1 empty vector was

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used instead of the RedPA-Cre plasmid. Eight hours after injection, the abdominal surface fur of mice was removed using a deplaratory cream, and mice were randomly assigned to dark and red light groups. The person performing the hydrodynamic injections was blinded as to the assignment. Mice of the red light group were then illuminated with an LED light source (660 nm; 100 W m$^{-2}$, continuous). Every 8 hours, mice were returned back to home-cage and fed for 1 hour. Mice of the dark group were kept in the home-cage that received no light. Bioluminescence imaging of the mice was performed 25 hours after hydrodynamic injection. Before bioluminescence imaging, 200 μl of 100 mM d-luciferin was intraperitoneally injected into the mice. The mice were anesthetized with isoflurane (Wako). Five minutes after d-luciferin injection, bioluminescence images of the mice were obtained using the Lumазone bioluminescence imager (Nippon Roper) equipped with the Evolve 512 EMCCD camera (Photometrics) controlled by SlideBook software (Intelligent Imaging Innovations). In case of Red-CPTS for reporter gene activation, the ICR mice were hydrodynamically transfected with plasmids encoding NLS-dCas9-NLS, 2xAff6, V18FΔN-MS2-3xNLS, 3xNLS-DrBphP-p65-HSF1, sgRNA and luciferase reporter at a 1:1:1:1:1 ratio. The total amount of DNA was 150 μg per mouse. Bioluminescence imaging was performed as described above.

In case of Red-CPTS for endogenous gene activation, the BALB/c mice were hydrodynamically transfected with plasmids encoding NLS-dCas9-NLS, 2xAff6, V18FΔN-MS2-3xNLS, 3xNLS-DrBphP-p65-HSF1 and sgRNA at a 1:1:1:1 ratio. The total amount of DNA was 120 μg per mouse. Twenty-five hours after injection, the liver was collected in RNAlater solution (Invitrogen). Total RNA was extracted from the liver using a Preccelys Evolution tissue homogenizer (Bertin Instruments) with cooling system, Preccelys Lysing Kit CK28 and NucleoSpin RNA. After cDNA synthesis using SuperScript IV VILO Master Mix, reverse transcription PCR was conducted using Luna Universal Probe qPCR Master Mix (New England Biolabs). The sample was analyzed with the StepOnePlus system. TaqMan primers and probes were used to detect ASCLI and GAPDH genes (Life Technologies; TaqMan Gene Expression Assay IDs are as follows: ASCLI: Mm03058063_m1; Gapdh: Mm99999915_g1). The ΔΔCt method was applied to show each relative mRNA level to that from non-transfected mice as a negative control.

Reagent availability. The plasmids encoding MagRed and the related plasmids will be distributed by Addgene.

Statistical analysis. Microsoft Excel for Microsoft 365 and GraphPad Prism (version 9.0) were used for statistical analysis. For comparison between two groups, a two-tailed unpaired Student’s t-test was performed. For comparison among more than three groups, ordinary two-way ANOVA was performed. For determining P values between the matched-pairs groups, the Wilcoxon matched-pairs signed-rank test was performed. No sample size estimates were performed, and our sample sizes are consistent with those normally used in experiments for regulation of protein activity. No sample exclusion was carried out. A Life Sciences Reporting Summary for this paper is available.

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

Data availability
The data supporting the findings of this study are available within the article and its Supplementary Information. The source data for the main figures and extended data figures are provided as Source Data files. A crystal structure of Affibody (Protein Data Bank accession code 2M5A) was used to depict a schematic representation of a binding partner candidate in Fig. 1b. The read counts for all screening data are available on DDBJ Sequence Read Archive, accession numbers DRR243933 and DRR243934. Source data are provided with this paper.

Code availability
The code for analysis of the read counts for all screening data has been deposited on GitHub (https://github.com/Kazushi40/NGS_analysis).

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Author contributions
M.S. conceived the project and provided supervision. G.Y. developed the synthetic binder, with support from Y.S., and performed preliminary tetR-tetO experiments. R.N., K.M., K.F. and Y. Kuwasaki measured absorption spectra of DrBphP and RpBphP1. Y. Kuwasaki, S.Y. and K.M. performed biochemical study using QCM. Y. Kuwasaki and M.N. performed split-thc reassembly assays. Y. Kakihara and Y. Kuwasaki performed experiments of Red-CPTS, with help from T.N. Y. Kuwasaki, T.O. and Y. Kakihara performed experiments of Red-CPTS for reporter gene activation, the ICR mice were hydrodynamically transfected with plasmids encoding NLS-dCas9-NLS, 2xAff6, V18FΔN-MS2-3xNLS, 3xNLS-DrBphP-p65-HSF1 and sgRNA at a 1:1:1:1 ratio. The total amount of DNA was 120 μg per mouse. Twenty-five hours after injection, the liver was collected in RNAlater solution (Invitrogen). Total RNA was extracted from the liver using a Preccelys Evolution tissue homogenizer (Bertin Instruments) with cooling system, Preccelys Lysing Kit CK28 and NucleoSpin RNA. After cDNA synthesis using SuperScript IV VILO Master Mix, reverse transcription PCR was conducted using Luna Universal Probe qPCR Master Mix (New England Biolabs). The sample was analyzed with the StepOnePlus system. TaqMan primers and probes were used to detect ASCLI and GAPDH genes (Life Technologies; TaqMan Gene Expression Assay IDs are as follows: ASCLI: Mm03058063_m1; Gapdh: Mm99999915_g1). The ΔΔCt method was applied to show each relative mRNA level to that from non-transfected mice as a negative control.

Reagent availability. The plasmids encoding MagRed and the related plasmids will be distributed by Addgene.

Statistical analysis. Microsoft Excel for Microsoft 365 and GraphPad Prism (version 9.0) were used for statistical analysis. For comparison between two groups, a two-tailed unpaired Student’s t-test was performed. For comparison among more than three groups, ordinary two-way ANOVA was performed. For determining P values between the matched-pairs groups, the Wilcoxon matched-pairs signed-rank test was performed. No sample size estimates were performed, and our sample sizes are consistent with those normally used in experiments for regulation of protein activity. No sample exclusion was carried out. A Life Sciences Reporting Summary for this paper is available.

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

Data availability
The data supporting the findings of this study are available within the article and its Supplementary Information. The source data for the main figures and extended data figures are provided as Source Data files. A crystal structure of Affibody (Protein Data Bank accession code 2M5A) was used to depict a schematic representation of a binding partner candidate in Fig. 1b. The read counts for all screening data are available on DDBJ Sequence Read Archive, accession numbers DRR243933 and DRR243934. Source data are provided with this paper.

Author contributions
M.S. conceived the project and provided supervision. G.Y. developed the synthetic binder, with support from Y.S., and performed preliminary tetR-tetO experiments. R.N., K.M., K.F. and Y. Kuwasaki measured absorption spectra of DrBphP and RpBphP1. Y. Kuwasaki, S.Y. and K.M. performed biochemical study using QCM. Y. Kuwasaki and M.N. performed split-thc reassembly assays. Y. Kakihara and Y. Kuwasaki performed experiments of Red-CPTS, with help from T.N. Y. Kuwasaki, M.N., K.S., R.B. and M.Y. performed experiments of RedPA-Cre. Y. Kuwasaki, K.S. and M.S. wrote the manuscript and prepared the figures. R.B. and M.Y. edited the manuscript. All authors checked and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to Moritoshi Sato.
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Extended Data Fig. 1 | Effect of additional BV supplementation on the three different CPTS designs. (a-c) Mean bioluminescence intensities (from three independent biological samples) of CPTS designs based on RpBphP1-PpsR2 (a), RpBphP1-QPAS1 (b), and MagRed (c) were plotted. The designs of each configuration (#1-8) were shown in Fig. 3b, c. *P* values are indicated above the bars. (N.S., not significant *P* > 0.05; **P* < 0.01; BV minus vs. plus using two-tailed Wilcoxon matched-pairs signed rank test).
Extended Data Fig. 2 | Red-CPTS can be actively switched off using 800-nm light illumination. (a-d) After the pre-activation of Red-CPTS by the 660-nm illumination for 12 h, the cells were further incubated under the 660-nm illumination condition (a) or switched to the dark condition (b) or the 800-nm illumination condition (c). As a control, the sample was incubated under the dark condition throughout the experiment (d). ASCL1 mRNA levels were measured at 0 h, 0.75 h, 1.5 h and 3.0 h as shown in the figure. Box plots show the median (center line), first and third quartiles (box edges), 1× the SD (whiskers), and individual data points. (n = 6 biologically independent samples, mean ± s.d.).
Extended Data Fig. 3 | Comparison of Red-CPTS and RpBphP1-PpsR2/QPAS1-based CPTS at different illumination intensities. (a–c) Fluc reporter gene activation by Red-CPTS (a), RpBphP1-PpsR2-based CPTS (b), and RpBphP1-QPAS1-based CPTS (c) at various illumination intensities. RpBphP1-PpsR2/QPAS1-based CPTS has configuration #3 shown in Fig. 3b, c. Experimental conditions are same as those in Fig. 3c, d except for the illumination intensities. Ratios of the mean bioluminescence intensity under the red light condition (red bar) to that under the dark condition (gray bar) are depicted above the bars. Bar data are shown as the mean ± s.d. from four biological replicates. Dots represent individual data points. (d) Left: Comparison of the Light/Dark contrasts between Red-CPTS and RpBphP1-PpsR2/QPAS1-based CPTS at different illumination intensities. Right: The cropped data with the mean Light/Dark contrast of 0 to 3.
Extended Data Fig. 4 | Dependence of Red-CPTS activity on the duration of ON time of the red light illumination. (a–c) Fluc reporter gene activation by Red-CPTS (a), RpBphP1-PpsR2 (b), and RpBphP1-QPAS1 (c) with various durations of ON time of the red light illumination. RpBphP1-PpsR2/QPAS1-based CPTS has configuration #3 shown in Fig. 3b, c. Experimental conditions are the same as those in Fig. 3c, d except for the illumination cycle. Ratios of the mean bioluminescence intensity under the red light condition (red bar) to that under the dark condition (gray bar) are depicted above the bars. Bar data are shown as the mean ± s.d. from four biological replicates. Dots represent individual data points. (d) Left: Comparison of the Light/Dark contrasts between Red-CPTS and RpBphP1-PpsR2/QPAS1-based CPTS at various durations of ON time of the red light illumination. Right: The cropped data with the mean Light/Dark contrast of 0 to 3.
Extended Data Fig. 5 | Dependence of Red-CPTS activity on the duration of OFF time of the red light illumination. (a–c) Fluc reporter gene activation by Red-CPTS (a), RpBphP1-PpsR2 (b), and RpBphP1-QPAS1 (c) with various durations of OFF time of the red light illumination. RpBphP1-PpsR2/QPAS1-based CPTS has configuration #3 shown in Fig. 3b, c. Experimental conditions were the same as those in Fig. 3c, d except for the illumination cycle. Ratios of the mean bioluminescence intensity under the red light condition (red bar) to that under the dark condition (gray bar) are depicted above the bars. Bar data are shown as the mean ± s.d. from four biological replicates. Dots represent individual data points. (d) Left: Comparison of the Light/Dark contrasts between Red-CPTS and RpBphP1-PpsR2/QPAS1-based CPTS at various durations of OFF time of the red light illumination. Right: The cropped data with the mean Light/Dark contrast of 0 to 3.
Extended Data Fig. 6 | Comparison of RedPA-Cre with the existing red light-responsive recombinase systems. For the BV/PCB (+) conditions, HEK 293T cells were plated at 2.0 × 10⁴ cells per well in a 96-well black-wall plate in the presence of 25 μM BV (for RedPA-Cre and FISC system) and 20 μM PCB (for CreLite and L-SCRaMbLE), respectively. For the BV/PCB (−) conditions, the procedures for plating were identical to those described above except for the chromophore supplementation. Plasmid amount used for each experiment is described below the graph. Especially, because a previous study has revealed that FISC system shows the highest recombination efficiency when Cre N-fragment (pXY169), Cre C-fragment (pXY177) and red light-responsive activator (pXY137) were transfected at 1:1:10 ratio, we additionally tested this transfection condition for FISC system. Following experimental procedures are the same as those in Fig. 5c, d. Ratios of the mean bioluminescence intensity under the red light condition (red bar) to that under the dark condition (gray bar) are depicted above the bars. Bar data are shown as the mean ± s.d. from four biological replicates. Dots represent individual data points. P values are indicated above the bars. (N.S., not significant P > 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; dark vs. light using two-tailed unpaired t-test).
**Extended Data Fig. 7 | RedPA-Cre activation with various red-light illumination conditions.** HEK 293T cells were transfected with plasmids encoding RedPA-Cre and the bioluminescence reporter without additional BV supplementation. Twenty-four hours after the transfection, the cells were illuminated with red light at different illumination durations and intensities. In the left panels showing the illumination conditions, gray corresponds to the dark condition and red with asterisk corresponds to the red light condition with denoted light intensities. Ratios of the mean bioluminescence intensity under the red light condition (red bar) to that under the dark condition (gray bar) are depicted above the bars. Bar data are shown as the mean ± s.d. from four biological replicates. Dots represent individual data points. *P* values are indicated above the bars. (** *P* < 0.0001; one-way ANOVA with multiple comparisons).
Extended Data Fig. 8 | RedPA-cre under dark, 800-nm illumination, and 660-nm illumination conditions. DNA recombination activities of RedPA-cre were compared among the dark, the 800-nm illumination and the 660-nm illumination conditions. Experimental conditions are the same as those in Fig. 5c, d except for that the 800-nm samples are incubated under 800-nm pulsed light (1 min ON and 4 min OFF) of 10 W m\(^{-2}\). Bar data are shown as the mean ± s.d. from four biological replicates. Dots represent individual data points. \(P\) values are indicated above the bars. (N.S., not significant \(P > 0.05\); ****\(P < 0.0001\); dark vs. 660 nm, dark vs. 800 nm and 800 nm vs. 660 nm using two-tailed unpaired t-test).
Extended Data Fig. 9 | RedPA-Cre enables for DNA recombination reaction upon noninvasive red light illumination in living mice. (a, b) ICR mice were transfected with a bioluminescent reporter plasmid together with pcDNA3.1 empty plasmid as a control (a) or with plasmid encoding RedPA-Cre of which configuration is NLS-DrBphP-CreC106-IRES-NLS-CreN104-Aff6_V18FΔN (b). Twenty-five hours after the transfection, bioluminescence images were obtained. No difference can be observed in the appearance between the mice maintained in the dark and the ones illuminated with red light at 660 nm for 16 h. The total bioluminescence intensities were shown in Fig. 5f (n = 3 mice per group).
Extended Data Fig. 10  |  In vivo gene activation by Red-CPTS upon noninvasive red light illumination. (a, b) ICR mice were transfected with plasmids encoding Red-CPTS and luciferase reporter together with a plasmid encoding unrelated sgRNA as a negative control (Empty) or sgRNA targeting GAL4UAS (GAL4UAS). After the transfection, the mice were noninvasively illuminated at 660 nm or kept in the dark as shown in Supplementary Figure 28, and then bioluminescence imaging of the mice was performed. (c) Total bioluminescence intensities of the mice shown in a and b. Gray and red bars represent the mean ± s.d., and dots represent the total bioluminescence intensity of each mouse (n = 4 mice per group). (N.S., not significant; ****P < 0.0001; using two-way ANOVA with multiple comparisons). (d) Red light-dependent endogenous gene activation by Red-CPTS with unrelated sgRNA as a negative control (Empty) or sgRNA targeting mouse ASCL1 (mASCL1) in vivo in living BALB/c mice. Data are represented as the relative mRNA level to the non-transfected negative control (n = 6 mice per group). Gray and red bars represent the mean ± s.d., and dots represent individual data points. No difference can be observed in the appearance between the mice maintained in the dark and the ones illuminated with red light at 660 nm for 16 h. P values are indicated above the bars. (N.S., not significant P > 0.05; ****P < 0.0001; using two-way ANOVA with multiple comparisons).
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
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- 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 MikroWin2000, version 4.41; Metamorph, version 7.7.6.0; Slide book, version 4.2; StepOne Software, version 2.3; Miseq Control Software, version 2.6.2.1; LAS-3000 UVmini, version 2.2; GLOMAX SIS, version 1.7.0; UVPProbe, Ver. 2.43; qCell T, version 1.8.58.35793

Data analysis Protein structure graphics were obtained using PyMOL(TM) Molecular Graphics System, Version 2.1.0. Deep sequence analysis was performed using custom python scripts (python 2.7). The code for analysis of these data has been deposited on Github (https://github.com/Kazushi40/NGS_analysis). Obtained images were analyzed using imageJ (version 2.0.0-rc-69/1.52p) and CellProfiler (version 3.2). The calculation of biochemical parameters in the QCM experiment was performed on a software qGraph Viewer, version 1.8.58.35793. Microsoft Excel for Microsoft 365 and GraphPad Prism (version 9.0) were used for the statistics analysis.

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 and 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 supporting the findings of this study are available within the article and its Supplementary Information. The source data for the main Figures and Extended Data Figures are provided as Source Data files. A crystal structure of Affibody (PDB accession code 2M5A) was used to depict a schematic representation of a binding partner candidate in Fig. 1b. Crystal structures of DvBphP-PSM (PDB accession code 400P and 400I) were used to depict a schematic representation of
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Life sciences study design

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

Sample size
No sample-size calculation was performed. Following standards of the field, sample sizes were estimated which were capable of detecting statistically relevant differences.

Data exclusions
No sample exclusion was carried out.

Replication
All in vitro and in vivo experiments included biological replicate. At least three independent experiments were performed for each experiments. All attempts at replication were successful.

Randomization
Mice used for in vivo RedPA-Cre and Red-CPTS activation experiments were randomly assigned to dark and red light group after HTV injection. In the other experiments of this study, there was no need for randomization.

Blinding
The persons performing the hydrodynamic injections were blinded as to the assignment. In the other experiments of this study, blinding was not possible because the same investigator processed the experiment and analyzed the data.

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 |
| ☑ Paleontology and archaeology |
| ☑ Animals and other organisms |
| ☑ Human research participants |
| ☑ Clinical data |
| ☑ Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑ ChIP-seq |
| ☑ Flow cytometry |
| ☑ MRI-based neuroimaging |

Antibodies

**Antibodies used**
- anti-V5 Tag mouse monoclonal antibody conjugated with HRP: Invitrogen, Cat#R961-25, Lot. 2158033
- anti-mCherry (1607) rat monoclonal antibody conjugated with Alexa Fluor 594: Invitrogen, Cat#M11240, Clone 1607, Lot. 2159126
- anti-β-tubulin (9F3) rabbit monoclonal antibody conjugated with HRP: Cell Signaling Technology, Cat# 5346, Clone 9F3, Lot. 4
- anti-GAPDH (G-9) mouse monoclonal antibody conjugated with HRP: Santa Cruz Biotechnology inc., Cat# sc-365602, Clone G-9, Lot. F1219

**Validation**

All the antibodies used were validated by the manufacturer as follows:
- anti-V5 Tag mouse monoclonal antibody conjugated with HRP: Species reactivity, Tag; Application, Western Blotting, Immunohistochemistry, Immunocytochemistry, Flow cytometry, ELISA, Immunoprecipitation, and ChIP assay (https://www.thermosphere.com/antibody/product/V5-Tag-Antibody-Monoclonal/R961-25).
- anti-mCherry (1607) rat monoclonal antibody conjugated with Alexa Fluor 594: Species reactivity, Tag; Application, Immunohistochemistry, Immunocytochemistry, and Flow cytometry (https://www.thermosphere.com/antibody/product/mCherry-Antibody-clone-1607-Monoclonal/M11240).
- anti-β-tubulin (9F3) rabbit monoclonal antibody conjugated with HRP: Species reactivity, Human, Mouse, Rat, Monkey, Zebrafish, Bovine; Application, Western Blotting (https://www.cellsignal.jp/products/antibody-conjugates/b-tubulin-9f3-rabbit-mab-hrp-conjugate/5346).
- anti-GAPDH (G-9) mouse monoclonal antibody: Species reactivity, Human, Mouse, Rat. Application; Western Blotting, Immunoprecipitation, Immunofluorescence, Immunohistochemistry, and ELISA (https://www.scbt.com/p/gapdh-antibody-g-9). Furthermore, the V5 Tag Monoclonal Antibody was extensively used in our laboratory.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  HEK 293T, HeLa and Neuro-2a cells [ATCC].

Authentication  These cell lines have not been authenticated.

Mycoplasma contamination  None of the cell lines have been tested routinely for Mycoplasma contamination.

Commonly misidentified lines (See ICCLAC register)  Cell lines used in this paper are not listed in the database of cross-contaminated or misidentified cell lines by ICCLAC.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  Four-week-old female ICR mice and 6-week-old female BALB/c mice were purchased from Sankyo Labo Service.

Wild animals  This study did not involve wild animals.

Field-collected samples  The study did not involve samples collected from the field.

Ethics oversight  All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Tokyo and were conducted in accordance with the Guidelines for Care and Use of Laboratory Animals as stated by the University of Tokyo.

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