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2021-09

Manoilov , K Y , Verkhusha , V V & Shcherbakova , D M 2021 , ' A guide to the optogenetic regulation of endogenous molecules ' , Nature methods , vol. 18 , no. 9 , pp. 1027-1037 . https://doi.org/10.1038/s41

http://hdl.handle.net/10138/341048
https://doi.org/10.1038/s41592-021-01240-1

other
acceptedVersion

A guide to the optogenetic regulation of endogenous molecules

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Abstract

Genetically encoded tools for the regulation of endogenous molecules (RNAs, DNA elements and proteins) are needed to study and control biological processes with minimal interference caused by protein overexpression and overactivation of signaling pathways. Here, we focus on light-controlled optogenetic tools (OTs) that allow spatiotemporally precise regulation of gene expression and protein function. To control endogenous molecules, OTs combine light-sensing modules from natural photoreceptors with specific protein or nucleic acid binders. We discuss OT designs and group OTs according to principles of their regulation. We outline characteristics of OT performance, provide considerations for their use in vivo and review available OTs and their applications in cells and in vivo. Finally, we provide a brief outlook on the development of OTs.

Optogenetics enables light-dependent control of cellular processes with genetically encoded light-activated molecules. Widely known for its applications in neuroscience, where light-sensing channels and pumps are used to control neuronal activity, this technology now includes a wide variety of optogenetic tools (OTs) that can control a plethora of cellular functions[1-3]. High spatial and temporal precision and dose-dependent activation of molecules of interest can be achieved with light control. Furthermore, the precision of light-mediated control opens opportunities in biomedicine [4, 5]. Restricting biological activity, such as genome editing, to a specific time and place, is also needed to reduce off-target effects of constitutive activity and the associated cytotoxicity[6].

OTs have a modular organization: a light-sensing part, consisting of a photosensory module (PSM), typically derived from a plant or bacterial photoreceptor containing a chromophore, can be fused to an effector module (EM), which can be activated by the PSM upon illumination. In OTs designed to control endogenous molecules, specific protein binders or regulating peptides are used as EMs and define the tools' target, affinity, specificity, and mode of action.

Ideally, OTs should minimally interfere with endogenous pathways through non-physiological effects caused by overexpression of engineered enzymes, channels or receptors[7, 8]. Also, the targeted endogenous pathway should be regulated within the range of its natural signaling capacity. Recent years have seen progress in the development of OTs targeting endogenous molecules[8], which was facilitated by the availability of orthogonal recombinant protein binders, such as antibody-like intrabodies (iBs)[9], specific peptides[10], and universal and customizable DNA and RNA binders[11-15].

Here we aim to provide a guide to OTs for endogenous targets. For information on OTs serving other purposes, we refer readers to reviews discussing OTs in a general sense, including those regulating overexpressed or modified molecules[3, 5, 16-18] or those targeting signaling pathways through light-controlled receptors[4, 5]. We discuss genetically encoded OTs that require chromophores available in cells. We first outline general principles of OT design and OT regulation of endogenous targets and then describe available OTs, their main characteristics, and their applications to biological problems. We discuss possible ways to implement OTs in living animals and the challenges associated with such experiments. Finally, we provide a perspective for the engineering of OTs.

Modularity of optogenetic tools

Photosensory modules

The blue light-controlled Light-Oxygen-Voltage (LOV) family of protein domains is widely used as PSMs (**Fig. 1a**). LOV-domain PSMs rely on flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) chromophores and have the smallest size among PSMs. Light irradiation induces the formation of a transient bond between a cysteine residue in the LOV domain and the C4α position of non-covalently bound flavin. As a result, the conformation of the LOV domain changes, enabling the LOV domain to function as a photoswitch.

LOV domains from different organisms exhibit different mechanisms of action upon light illumination. *Rhodobacter sphaeroides Rs*LOV[11] domains form dimers in darkness and dissociate into monomers upon illumination. The second LOV domain of *Avena sativa* phototropin I (*As*LOV2) exhibits light-induced allosteric changes that can be effectively transduced to a coupled EM, changing the EM's conformation and function[19, 20]. A derivative system based on *As*LOV2 is the iLID-sspB, which displays light-induced heterodimerization[21]. Here, the SsrA peptide, which specifically binds SspB protein, is caged within the Jα-helix of the *As*LOV2. Another LOV-based heterodimerization pair used in OTs is the LOV domain of FKF1 and its interacting protein GIGANTEA[22].

The LOV domain from *Neurospora crassa* Vivid (VVD) protein incorporates FAD as a chromophore[23, 24]. Light induces the PSM's homodimeric association from monomers. To develop the Magnet tools[24], VVD was engineered into the two different proteins pMag and nMag, which undergo light-induced heterodimerization. In addition, VVD, which exhibits intrinsically slow dissociation in darkness, was tuned to have a faster dissociation kinetics.

Another blue light-sensing PSM is the cryptochrome 2 (CRY2) domain (**Fig. 1b**). CRY2 uses FAD as a chromophore and can both heterodimerize with its natural partner CIB1[25] and homo-oligomerize with itself upon light illumination[26, 27]. Variants of this system include CRY2PHR, in which CRY2 is truncated to its Photolyase-homology domain (PHR), and truncated CIB1 named CIBN. These vary in their signal-to-background ratio, kinetics and preference toward either homo- or hetero-dimerization[28].

Photodissociable dimeric pdDronpa protein[29] is a PSM that was derived from the green fluorescent protein (GFP) family and forms its chromophore from its amino acids (**Fig. 1c**).

Dimeric in darkness, pdDronpa monomerizes upon illumination with cyan light. Upon illumination with violet light, it reverts to its dimeric state.

Bacterial phytochromes (BphPs) are PSMs activated by near-infrared (NIR) light. BphPs incorporate biliverdin IXα as a chromophore, which is present in eukaryotic cells as an intermediate of heme catabolism[30]. Light causes isomerization of biliverdin resulting in photoswitching of BphPs between two states: the red-absorbingPr (660-700 nm) and the far-red absorbing Pfr (710-780 nm), either one of them being the ground state or the active state. Photoactivation of BphP induces either its enzymatic activity or protein-protein interaction with binding partners. *Rp*BphP1 (here called BphP1) interacts with its natural partner PpsR2[31] or with the smaller (17 kDa) engineered QPAS1 domain[32] (**Fig. 1d**). BphS is a BphP PSM with a different mechanism as it activates enzymatic synthesis of cyclic dimeric GMP (c-di-GMP)[33]. To function as an OT that induces transcription of a target gene, BphS was combined with several additional modules, including the c-di-GMP responsive hybrid transactivator p65-vp64-NLS-BldD protein which dimerizes upon c-di-GMP binding and recognizes its specific promoter P_{FRLX}. BphS system also needs another module, the YhjH phosphodiesterase, which breaks down c-di-GMP to maintain its negligible level in a cell[34].

Effector modules

EMs are used in OTs to control proteins and nucleic acids. Control of proteins is implemented via orthogonal recombinant protein binders, such as antibody-like intrabodies (iBs)[35] and specific peptides[10], whereas regulation of genes is achieved with CRISPR-Cas9 (CRISPR-associated protein 9), and other customizable binders, such as zinc finger proteins (ZFPs)[15] and transcription activator-like effector (TALE) proteins[36]. Two types of iBs are used in currently available OTs: nanobodies[37-39] and single-chain variable fragments (scFVs)[39] (**Fig. 1e**). Nanobodies are 15 kDa proteins derived from a single variable domain of camelid antibodies[40]. iBs can bind their targets either without interfering with their function or they can modulate protein function by inducing allosteric changes or direct protein inhibition. Databases such as sdAb-DB[41] and iCAN[42] are available for selecting iBs.

Synthetic non-immunoglobulin binders, which are also called antibody-mimetic proteins, are not widely explored as EMs, but are promising due to their high stability and small size. They include 10 kDa monobodies (adnectins) designed by molecular evolution of the fibronectin type

III domain[43], 20 kDa anticalins derived from human lipocalins, 6 kDa affibodies engineered based on a three-helical subdomain of a natural receptor for the Fc-portion of IgG, and 14-18 kDa DARPins derived from natural ankyrin repeat proteins (**Fig. 1f**)[44].

Short peptides (**Fig. 1g**) and small structural domains are useful EMs[10]. Peptides can exhibit a wide range of biological activities. Databases for peptides and their targets are available, e.g., BIOPEP-UWM[45], StraPep[46] and NeuroPep[47].

Using a single guide RNA (sgRNA), the Cas9 DNA binder can be targeted to any endogenous genomic sequence that contains a protospacer-associated motif (PAM) (**Fig. 1h**). PAMs are 2-6 base pairs in length and differ for Cas9 molecules derived from different species. Multiple genes can be controlled by Cas9 or its catalytically "dead" mutant dCas9 simultaneously by providing multiple sgRNAs[14, 15]. The dCas9-sgRNA-DNA complex forms on a targeted DNA locus. When bound to a promoter, this complex inhibits gene expression. For alternative programmable ZFP binders (**Fig. 1i**), databases such as ZifBASE[48] and ZiFDB[49] are available.

For endogenous RNA regulation, catalytically inactive CRISPR-associated endoribonucleases of the Cas13 family (**Fig. 1j**), including Cas13a (C2c2) , Cas13b (C2c6) , and Cas13d nucleases, from various species are used as RNA binders[50].

Principles of OT regulation by light

OTs can control endogenous targets in several ways (**Fig. 1k-q**). EM binding properties can be regulated by illumination of the PSM. For example, a split protein can be assembled (**Fig. 1k**), EM active sites can be caged or uncaged (**Fig. 1m, n**), or EMs can change their oligomeric state (**Fig. 1p**). Furthermore, the availability of the EM for binding to its target can be regulated by the subcellular localization of the EM. Similarly, EMs can also serve as adapters to attach light-sensing proteins to endogenous targets for their regulation or their light-induced intracellular re-localization (**Fig. 1l**). Light-induced expression of EMs is also used (**Fig. 1q**).

OTs based on light-induced protein re-localization exploit the fact that endogenous proteins or their regulators can be active in one compartment or location and inactive in the other, such as the nucleus and the membrane. Here, light can control the re-localization of either the target or its effector, which can be an active EM regulating the target[37]. In the first case, the OT serves as an adapter that attaches the PSM via the EM to the target (**Fig. 1l**). When regulating genes or RNAs,

DNA or RNA binders serve as adapters for light-induced recruitment of transcriptional regulators (**Fig. 1l**).

Light-mediated control of EMs can be achieved through insertions of a LOV PSM. Light-induced conformational changes in *As*LOV2 inserted in surface-exposed loops of EMs (**Fig. 1m**) can affect the EM's binding site, thereby regulating binding affinity[38].

OTs using short peptides as EMs are frequently based on "caging" of the peptides by the *As*LOV2 domain (**Fig. 1n**). Here, the peptide is incorporated into the C-terminal J_α-helix of LOV2, and the peptide is sterically blocked as the J_α-helix is folded[51]. The light-induced unfolding of the J_α-helix exposes the peptide for interaction with its target.

Another "caging" strategy employs pdDronpa (**Fig. 1o**). Two pdDronpa1 domains are inserted in an EM molecule in such a way that their intramolecular dimerization blocks the active or binding site. Upon illumination, the pdDronpa1 domains are separated and the active or binding site is exposed[52]. For this type of caging, the light-dependent interaction between *As*LOV2 and Zdk protein can be used alternatively [53].

OTs acting through the light-induced expression of EMs targeting endogenous molecules (**Fig. 1q**) can be based on optogenetic systems for transcriptional activation, which include PSMs, such as BphPs-QPAS1 or CRY2-CIBN, in combination with Gal4-UAS or TetR-TetO modules[54].

Characteristics and limitations of optogenetic tools

The characteristics of an OT's performance include dynamic range, kinetics of light activation, background leakage in the darkness, reversibility, and photostability **(Supplementary Table 1)**. Frequently, there is a tradeoff between the background leakage and the dynamic range of response[13, 55].

There are no universal quantitative standards for OT characteristics, as they depend on experimental conditions, such as intensity and duration of activation light, expression levels of the OT and endogenous targets, reporter readout and other variables, such as stoichiometry between OT components and reporter. Therefore, OTs should only be compared in parallel experiments.

When setting up experiments to manipulate endogenous targets using light, researchers should first screen for optimal experimental conditions, such as expression levels and light intensities. Adequate control experiments are important to help reveal artifacts that may be caused

by background leakage and illumination. Finally, an optimal illumination regimen can minimize blue light cytotoxicity[56] and other possible side effects[57].

Optogenetic tools for light-mediated regulation of endogenous proteins

Regulation of binder affinity

Here we discuss OTs based on reconstitution of split iBs and allosteric regulation of iB affinity (**Fig. 2**). Split versions of nanobodies and scFvs were used in OTs called "optobodies"[39]. These optobodies incorporate VVD-based Magnet dimerization tools[24] or *As*LOV2-based iLID[21] as PSMs (**Fig. 1k, Fig. 2a**). Gelsolin (GSN) is an actin-binding protein and a regulator of actin assembly and disassembly. Light-mediated control of GSN via optobodies impaired the migration of NIH/3T3 cells[39]. GSN binding by the OT caused its inactivation and, consequently, destabilization of actin filaments, which impaired cellular motility (**Fig. 3a).** Another example involves an optobody controlling β2 adrenergic receptor (β2AR) that was used to inhibit isoprenaline-dependent activation of β2AR, its subsequent internalization, and cAMP-mediated Ca^{2+} influx into cells (**Fig. 3b**)[39]. Furthermore, split nanobodies were developed into a system for light-controlled protein degradation. The photoactivated deGraFP system included a split GFP nanobody fused to the F-box E3 domain[39]. The same strategy can be applied to endogenous proteins if specific nanobodies are used.

Allosteric regulation of nanobody affinity through light-induced conformation changes in an inserted *As*LOV2 is the main principle behind Opto-Nanobodies[38]. (**Fig. 1m, Fig. 2b**). Depending on the insertion position of *As*LOV2, light can trigger opposite effects: OT binding to or OT dissociation from the targeted protein epitope. Such an Opto-Nanobody[38] enabled light-dependent activation of Ras/Erk signaling by recruiting a SOS^{cat} domain to the plasma membrane for Erk activation via light-dependent binding of the OT to membrane-localized mCherry-CAAX (**Fig. 3c**). A similar system using *As*LOV2 insertion for allosteric regulation of affinity was developed based on a monobody (OptoMB)[58].

Caging of functional peptides and protein domains

An OT named PMI-LEXY[59] was developed by fusing the PMI peptide[60] (**Fig. 1f, Fig. 3d**), which inhibits nuclear Mdm2 and MdmX, to the light-inducible nuclear export system (LEXY)[51]. In

LEXY, a nuclear export signal (NES) is caged within the J_α-helix of the LOV domain. In the dark, PMI-LEXY was localized in the nucleus, where it inhibited the proteolytic activity of Mdm2 and MdmX towards p53, which is a tumor suppressor involved in the regulation of the cell cycle. Under blue light illumination, PMI-LEXY relocated to the cytoplasm releasing Mdm2 and MdmX for p53 degradation. The original LEXY was also used to control the endogenous target CRM1, which is a receptor responsible for NES-dependent export of NES-containing proteins, by seclusion of CRM1 in the nucleus[51].

OTs for regulating endogenous protein kinases were developed by caging of inhibitor peptides through *As*LOV2 domain (**Fig. 3e**). Examples include the PKI peptide for inhibition of cyclic-AMP dependent kinase (PKA)[61], the MKI peptide for inhibition of myosin light chain kinase (MLCK)[61], and the AMPK inhibitory peptide (AIP) for inhibition of AMP-activated protein kinase (AMPK)[62]. Activation of *As*LOV2-MKI led to light-dependent changes in plasma membrane protrusion dynamics. Optogenetic inhibition of AMPK arrested mitochondrial trafficking in migrating and invading cells.

paAIP2 is an OT for controlling Ca^{2+}/Calmodulin Kinase II (CaMKII)[63]. Autocamtide inhibitory peptide 2 (AIP2) was caged within the J_α helix of the *As*LOV2 domain (**Fig. 3e**). paAIP2 inhibited long-term potentiation (LTP), when activated during the induction of LTP in brain sections. In living mice, it inhibited memory formation when activated during a task.

Lumitoxins are OTs that are exposed on the cell surface. They consist of ion channel-blocking peptide toxins, such as *Dendroaspis angusticeps* α-dendrotoxin, which specifically binds to Kv1.1 and Kv1.2 channels, and they are caged with an *As*LOV2 domain (**Fig. 2c, Fig. 3e**)[64]. Peptide uncaging resulted in an increase in the peptide's distance from the plasma membrane and in a decrease of its local concentration and inhibition of K^+ channels.

Diaphanous-related formins (DRFs) nucleate and promote long unbranched actin filaments. An intramolecular interaction between the N-terminal diaphanous inhibitory domain (DID) and C-terminal diaphanous auto-regulatory domain (DAD) keeps DRFs inactive. To manipulate DRFs such as mDia1/2, *As*LOV2-based OTs were developed[65]. For instance, the DAD is caged by *As*LOV2 in Nuc.LOV-DAD[66] and PA-DAD[65] (**Fig. 3e**). In the uncaged state, the autoinhibition of mDia1/2 is released, which leads to actin polymerization.

Caging through *As*LOV2 was also used in OTs regulating the endogenous RE1-silencing transcription factor (REST)[67], which is a transcriptional repressor participating in nervous system

development (**Fig. 3e**). In *As*LOV2-PAH1, the *As*LOV2 domain is fused to the REST-interacting portion of the mSin3a corepressor. Activated *As*LOV2-PAH1 competes with endogenous mSin3a for binding with REST. In an alternative OT called *As*LOV2-RILP N313, the active domain of the REST inhibitor REST-interacting LIM domain protein (RILP) is appended to the C-terminus of the *As*LOV2. Activated *As*LOV2-RILP N313 interacts with REST, thereby preventing its DNA binding. In neurons, OT activation resulted in increased Na^+-channel 1.2 activity, elevated transcription of brain-derived neurotrophic factor, and boosted Na^+ currents leading to neuronal firing[67].

Intracellular calcium is regulated by Ca^{2+} release-activated Ca^{2+} (CRAC) channels consisting of stromal interaction molecule 1 (STIM1) and ORAI Ca^{2+} channels. To manipulate intracellular calcium levels, the Opto-CRAC tool can be used to activate ORAI1 by LOV domain-based uncaging of STIM1 regulatory fragments[68] (**Fig. 1n, Fig. 2d**). Opto-CRAC was applied to regulate gene expression in cultured cells and to activate various calcium-dependent signaling pathways in immune cells, including effector T cells, macrophages and dendritic cells. However, opto-CRAC does not elicit a sufficiently strong response and is not fast enough to manipulate calcium in neurons.

Allosteric regulation through oligomerization

An alternative strategy for regulating CRAC channels is exemplified by OTs called OptoSTIM1 and monSTIM1. These tools consist of STIM1 C-terminal fragments that are fused to CRY2 PHR[69, 70]. Light-dependent homoassociation of CRY2 PHR results in oligomerization of the STIM1 fragments, which in turn activate ORAI1 (**Fig. 1p, Fig. 2f**). OptoSTIM1 triggered Ca^{2+} influx in cultured neurons upon activation with pulsed light. In mice, activation of OptoSTIM1 resulted in improved contextual memory formation. The more recently developed monSTIM1 makes use of a CRY2 PHR version that is ultrasensitive to light, allowing non-invasive light delivery in vivo[69]. Its activation in mice resulted in neuronal stimulation, which caused c-Fos immediate-early gene expression. monSTIM1 allowed demonstrating that light-controlled calcium increase can change fear behavior phenotypes[69] when expressed in pyramidal neurons in the anterior cingulate cortex, which is responsible for socially transmitted fear responses[71].

In a comparison of different OTs regulating ORAI-STIM1[55], fast on and off kinetics were observed with an oligomerization-based variant that consisted of LOV-derived iLID/sspB fused to STIM1ct, while a larger dynamic range of response was achieved with light-dependent oligomerization via CRY2 PHR.

Binders as adapters

A system for shuttling target proteins between the cytoplasm and the nucleus harnessed the NIR light-controlled BphP1-QPAS1 pair[37]. Here, iB binders served as adapters and were fused either to BphP1 or to its interacting partner QPAS1 (**Fig. 1l**). Nuclear export or localization signals (NES or NLS, respectively) were added to the BphP1-QPAS1 complex, shifting its nuclear-to-cytoplasm localization ratio in the desired way. For example, Ras and subsequent Raf1/MEK/ERK signaling was controlled in a light-dependent manner with an OT based on the NS1 monobody inhibiting Ras (**Fig. 3d**). In this system**,** the shuttling OT is mostly localized to the nucleus in darkness. Upon illumination, the OT and Ras were trapped in the cytoplasm through interaction with cytoplasmic BphP1.

The iRIS-B shuttle (**Fig. 2e**) is based on the previously developed NIR-blue-light inducible shuttle iRIS[72, 73] and combines the BphP1-QPAS1 module fused with nanobodies with a NES signal, and an *As*LOV2-caged NLS[74]. iRIS-B can relocate between three distinct subcellular compartments depending on the illumination wavelength. In combination with a non-interfering nanobody targeting endogenous β-actin, iRIS-B could cause rearrangements of the actin cytoskeleton (**Figs. 3f, 3g**).

To degrade a protein of interest upon illumination, a RING-type E3 ubiquitin ligase domain can be brought to the target[75]. Specifically, a fusion of a nanobody and the CRY2-PHR domain "tagged" a protein of interest for light-dependent interaction with a CIBN-E3 fusion.

Optogenetic tools for light-mediated control of endogenous genes

Allosteric regulation through dissociation

Several OT designs enabled control of genes: gene editing, transcription activation or inhibition, epigenetic modification or chromatine re-arrangement (**Fig. 4**). paRC9 is an early proof-of-

principle OT for the regulation of dCas9. In darkness, dCas9 fused with *Rs*LOV formed an inactive dimer. Upon illumination, the dimer dissociated, allowing dCas9 to bind DNA[11] (**Fig. 4a**).

Another example of a single-component OT is photoswitchable Cas9 (ps-Cas9), in which the DNA-binding site of Cas9 or dCas9 was caged by tandem pdDronpa domains[52] (**Fig. 1o, Fig. 4b**). Light-induced photodissociation of the pdDronpa dimer resulted in DNA binding and editing (with Cas9) or transcriptional regulation (with dCas9).

Regulation of CRISPR binders

Instead of direct regulation of CRISPR binders, an alternative approach relies on the control of (d)Cas9 by anti-CRISPR (Acr) proteins. In the CASANOVA system[76], the AcrIIA4 Cas9 inhibitor protein is allosterically regulated by an inserted *As*LOV2 domain (**Fig. 1m**, **Fig. 4c**). In darkness dCas9 is inactive. Blue light deactivates the *As*LOV2-AcrIIA4 fusion, thereby activating dCas9. In addition to transcriptional regulation, this OT was shown to regulate the epigenetic state of chromatin by acting on a dCas9-histone acetyltransferase core domain fusion (**Fig. 5a**). The CASANOVA-C3 is a similar system for controlling *Neisseria meningitidis Nme*Cas9 through its inhibitor[77].

Light-induced reconstitution of split (d)Cas9 via the VVD-based pMag and nMag photodimerizers[24] forms the basis of photoactivatable Cas9 (paCas9)[13] (**Fig. 1k**). The catalytically inactive padCas9 mediates light-induced inhibition of transcription (**Fig. 4e**). Since split (d)Cas9 is prone to a low level of spontaneous complementation[78] causing dark background, paCas9-2 based on low-affinity Magnets producing lower background was created[13].

The Split-CPTS2.0 tool combines split dCas9[13] with the recruitment of a transactivator[79]. Split-CPTS2.0 was applied to regulate transcription in cultured cells (**Fig. 5a**). In contrast to many dCas9-based OTs, Split-CPTS2.0 produced sufficiently strong upregulation of gene expression to result in biologically relevant effects. For instance, upregulation of *NEUROD1* with this OT drove neuronal differentiation in induced pluripotent stem cells (iPSCs)[79] (**Fig. 5b**).

Adapters connecting light-controlled proteins to endogenous genes

Similar to Split-CPTS2.0, the Light-Activated CRISPR/Cas9 Effector system (LACE)[15] employs light-induced interactions between Cas9 and transcriptional regulators for optogenetic control of transcription (**Fig. 1l**), with the difference that LACE involves intact rather than split dCas9. In the

LACE system[15], dCas9 is coupled to VP64 via a CIB1-CRY2-based PSM (**Fig. 4d**). This system was applied in cultured cells[14, 15] (**Fig. 5a**).

CPTS is a CRISPR–Cas9-based photoactivatable transcription system (CPTS) [14]. In a second iteration, CPTS was evolved into the CPTS2.0 system (**Fig. 4d**) and the split CPTS2.0. In these systems, p65 and HSF1 transcriptional activators are fused with MCP-protein, which binds sgRNAs containing inserted MS2 loops. These OTs were used in cultured cells (**Fig. 5a**). Both CPTS2.0 and split CPTS2.0 had a larger dynamic range than CPTS. CPTS2.0 exhibited the highest response, but it had the largest dark background.

The LITE system[36] (**Fig. 4f**) is based on light-mediated recruitment of transactivator via heterodimerization of the CRY2-CIB1 and uses DNA-binding *Xanthomonas sp.* TALE proteins (**Fig. 1i**). Similarly, in the LITEZ system (**Fig. 4f**) a ZFP is coupled to a transactivator domain via light-induced heterodimerization of the GIGANTEA-LOV domain from FKF1[80]. The LITE system was applied to the regulation of *NEUROG2* gene expression in Neuro2a neuroblastoma cells[36]. In addition, LITE was used in primary neuronal culture and the brain of live mice for regulation of the *GRM2* gene encoding the metabotropic glutamate receptor mGluR2.

OTs targeting distal regulatory elements were developed for light-induced chromatin rearrangements. The Light-activated-dynamic-looping (LADL) system[81] (**Fig. 4g**) based on CRY2-CIB1 redirected the stretch enhancer (SE) away from the *KLF4* gene and positioned it close to the *ZFP462* promoter. This rearrangement increased the number of *ZFP462* mRNA transcripts per cell and nascent *ZFP462* transcripts per allele in mouse embryonic stem cells.

Light-mediated control of EM expression

In addition to the direct control of PSMs over EMs, another strategy is to place the expression of EMs under light control, such as in the FACE system[34]. This NIR-activated CRISPR-dCas9 effector system (**Fig. 4h**) utilizes a BphS PSM and additional modules such as the transactivator p65–VP64–NLS–BldD, the chimeric promoter P_{FRLx} driving the FGTA4 transactivator, a sgRNA–dCas9 complex, and the YhjH phosphodiesterase. FACE was applied in vitro and in vivo (**Fig. 5c**). For example, the *NEUROG2* gene was upregulated in murine iPSCs to induce neuronal differentiation (**Fig. 5b**).

FACE was developed further into the NIR light (FRL)-activated split-Cas9 (FAST) system[82]. In FAST, the chimeric promoter P_{FRL} drives expression of NLS-Cas9(N)-Coh2, which

is a part of split Cas9 fused to the cohesion domain Coh2. A DocS-Cas9(C)-NES fusion protein is driven by a constitutive promoter P_{hCMV}. Coh2 and DocS are *Clostridium thermocellum* proteins that interact with high affinity to reconstitute Cas9. FAST was applied for editing of the *PLK1* oncogene in a mouse xenograft tumor model (**Fig. 5d**).

Controlling endogenous RNAs

PAMEC is an OT for light-controlled modification of endogenous RNA. The tool is based on CRY2-CIBN heterodimerization[83]. Here, the RNA binder *Porphyromonas gulae* dCas13 is fused to CIBN, whereas an RNA N6-methyladenosine editing (m^6A) effector is fused to CRY2PHR (**Fig. 4i**). PAMEC enabled spatiotemporal control of m^6A editing in response to blue light. The more efficient $PAMEC^R$ recruits additional m^6A effectors through MCP-CIBN binding to MS2 loops in engineered guide RNAs. Alternative approaches for light-controlled RNA regulation used light-induced expression of Cas13b (**Fig. 1q**)[84,85].

Recently, chimeric regulatory RNAs were generated that interact reversibly and in a light-dependent manner with a LOV photoreceptor called PAL through an inserted aptamer[86]. A regulatory RNA bound to PAL is caged and cannot bind cellular RNAs. Through light-regulated interaction of short hairpin (sh)RNAs with PAL in mammalian cells, endogenous RNAs can be controlled.

Optogenetics in vivo

Recombinant AAVs are attractive tools for OT delivery in mammals, since they exhibit low immunogenicity, infect dividing and non-dividing cells, and allow transient but long-term transgene expression[87]. However, their low packaging capacity of ~4.5 kb limits their use for delivering OTs. Integration-deficient lentiviruses that allow packaging of up to 8 kb are a promising alternative[88]. Larger DNA fragments of >8 kb can be delivered using adenoviruses, which do not integrate in the genome[89]. However, they only provide transient gene expression and are highly immunogenic.

NIR OTs are preferable for use in mammals, since NIR light can penetrate deeply into the tissue[31, 90]. Blue OTs are effective for small translucent organisms, like tadpoles, fishes and fruit flies. To apply blue OTs in mice, lanthanide-doped upconversion nanoparticles, which act as nano-

transducers converting NIR light into visible light emission, can be used[68]. Also, ultra-photosensitive blue OTs are available[69]. Moreover, bioluminescence can be used to activate OTs via bioluminescence resonance energy transfer (BRET)[91-93] . Although promising, these tools require supplying luciferase substrate, are relatively slow, and should be optimized for efficient BRET coupling. Lastly, a light guide can be installed to deliver blue light[36] (**Fig. 5e**), while bearable wireless light sources (**Fig. 5e**)[94] do not restrict animal movements and are safer.

Outlook

The modular structure of OTs and structural similarity between EM binders facilitate further OT development. To develop additional binders, advanced methods for rapid and effective selection of nanobodies are available[95]. Binding specificity and affinity of binders can be customized and fine-tuned by such approaches as the phage, bacterial and yeast displays, as well as RNA and ribosomal displays for peptide selection[96]. Non-immunoglobulin protein binders[44] (**Fig. 1f**) are promising for OT engineering, since they have a simpler structure, reduced size, and better stability in cells.

OTs can be improved in their dynamic range and leakage in darkness by selecting PSMs that are optimal for the desired application. For example, one can use either low-affinity pMag-nMag or high-affinity pMag-nMagHigh1 heterodimerizing pairs[13]. Separation of OT parts in different subcellular compartments and decrease in OT expression levels help to reduce background[31].

For applications in vivo, the BphP1-QPAS1 system has advantages due to its sensitivity to NIR light. A recent single-component, low-background NIR transcriptional regulation system, iLight, can be used for the development of OTs for endogenous gene expression or repression[97].

In conclusion, OTs controlling endogenous molecules have opened up the possibility to study biological processes with minimal interference and maximal spatiotemporal precision. By modulating the function of an endogenous protein target, changing its subcellular location, tuning the expression of a gene, or regulating chromatin, it should be possible to further our understanding of biological processes and possibly to develop next-generation therapies.

Acknowledgments

This work was supported by the EY030705 (to D.M.S.) and GM122567 from the US National Institutes of Health, 322226 from the Academy of Finland, and 21-64-00025 from the Russian Science Foundation (all to V.V.V.). We apologize to researchers whose work we could not quote here due to space limitations and the focus of this review on endogenous targets.

Competing interests

The authors declare no competing interests.

Figure legends

Figure 1. Structural domains in optogenetic tools (OTs) and principles of OT regulation. ***Photosensory modules (PSMs):*** (**a**) LOV domains: *R. sphaeroides* RsLOV (4HJ6) and *A. sativa* LOV2 (2V0U), FMN chromophore. (**b**) *A. thaliana* CRY2 PHR (1U3D), FAD chromophore. (**c**) pdDronpa (6D38) and its chromophore. (**d**) *R. palustris* BphP1 (4EHO), biliverdin IXa (BV) chromophore. Truncated *R. sphaeroides* PpsR2 (4HH2) is shown as QPAS1. ***Effector modules (EMs): protein binders.*** (**e**) anti-GFP nanobody (3OGO), scFV fragment of anti-ErbB2 antibody chA21 (3H3B). (**f**) Non-immunoglobulin binders: monobody S9 to fluoride channel Fluc Ec2 (6BX4), affibody to protein Z (1LP1), anticalin P3D11 to protein CD98 (6S8V), anti-IL-13 DARPIN 6G9 (5KNG). (**g**) p53-Mdm2-MdmX inhibitory (PMI) peptide[60]. ***EMs: DNA binders.*** (**h**) *S. pyogenes* dCas9 with sgRNA and PAM region of DNA (6K57), sgRNA bound to DNA. (**i**) C2H2 zinc finger protein bound to DNA (1MEY),TALE-DNA complex from *X. oryzae* (3UGM). ***EMs: RNA binders.*** (**j**) *L. shahii* Cas13a with RNA (5WTK)[97]. ***Principles of optogenetic regulation****:* (**k**) Split protein binder reassembles upon illumination and binds target protein[39] (top) or DNA[13] (bottom). (**l**) Light-induced heterodimerization. EM binders serve as adaptors connecting the PSM to its target. Illumination can result in target relocalization or recruitment of functional proteins[37] (top), such as transcription activator, inhibitor or epigenetic modifier[14, 15, 36]. (**m**) Light-induced changes in *As*LOV2 domain's PSM are transduced to the EM, resulting in binding to or dissociation from its targeted protein[38] (top) or DNA binder[76] (bottom). (**n**) *As*LOV2-caged peptide or small domain is released for protein binding after photo-uncaging[61, 63, 64]. (**o**) An EM binding site is caged via an inserted dimeric pdDronpa. (**p**) CRY2-based OTs are activated upon light-induced oligomerization[70]. (**q**) OTs based on light-induced expression of EMs.

Figure 2. Examples of optogenetic tools for the regulation of endogenous proteins. ***Recruitment to the membrane for activation:*** (**a**) *Optobody.* Light-induced reconstitution of split iB results in its reconstitution and binding to the membrane-localized target[39]. (**b**) *Opto-Nanobody.* Activated through inserted *As*LOV2, the OT is brought to the plasma membrane[38]. ***Inhibition or activation by caged functional peptide or domain.*** (**c**) *Lumitoxins.* In the darkness, α-dendrotoxin peptide inhibits voltage-dependent K^+ channels. Upon illumination, the LOV2 domain unwinds resulting in a change of the toxin's spatial position relative to the channel, reducing the channel's

inhibition [64]. (**d**) *Opto-CRAC system*. Upon illumination, STIM1 is uncaged and activates the ORAI Ca^{2+} channel [68]. (**e**) ***Tridirectional transport***. *iRIS-B shuttle*. In darkness, NES-iB(GFP)-mCherry-QPAS1-LOV2 with caged NLS is located in the cytoplasm. 460 nm light uncages the NLS and the system enters the nucleus, whereas 780 nm light targets it to the membrane via BphP1-QPAS1 interaction[37]. ***Light-induced oligomerization.*** (**f**) *Opto-STIM1 system.* Upon illumination, Cry2 PHR domains undergo homoassociation resulting in oligomerization of STIM-CT fragments. Oligomeric OTs effectively activate ORAI1 channels triggering calcium influx [70].

Figure 3. Selected applications of optogenetic tools for regulation of endogenous proteins. (**a**) Gelsolin (GSN) is blocked by the assembled optobody upon illumination, which results in destabilization of actin filaments and reduction of cell motility [39]. (**b**) Activated optobody to β2-adrenergic receptor (β2AR) prevents internalization and signal transduction of β2AR[39]. (**c**) BphP1-QPAS1-based OT for inhibition of the small GTPase Ras via regulator iB [37] (left). Ras is activated by light-induced targeting of Ras-activating SOS^{CAT} domain to the membrane (right)[38]. (**d**) Regulation of Mdm2-MdmX-mediated p53 proteolysis in cells by PMI-LEXY [59], which contains the PMI peptide inhibitor and a NES caged through *As*LOV2[51], which cause PMI re-localization to the cytoplasm under light. (**e**) Peptide and small domain-regulating OTs acting by LOV2-mediated photocaging [61, 63-65, 68]. Downregulation: LOV2-PKI and LOV2-MKI [61], PA-AIP [62], LOV2-AIP2 [63], nuc.LOV-DAD [66] and PA-DAD [65] regulating diaphanous related formins, *As*LOV2-PAH1 and *As*LOV2-RILP N313 regulating RE1-silencing transcription factor (REST) [67]. Upregulation: LOV2-α-dendrotoxin [64] and LOV2-STIM domain [68]. (**f**) Translocation of monomeric actin to the plasma membrane by iRIS-B. Reduction of cell motility and filopodia formation occurs [37]. (**g**) Re-localization of monomeric actin from the cytoplasm to the nucleus using iRIS-B causes relocalization of the myocardin-related transcription factor A (MRFT-A) to the cytoplasm [37].

Figure 4. Examples of optogenetic tools for the regulation of endogenous genes. (**a**) *Single-component paRC9.* Under illumination, a dimer of VVD domains dissociates, exposing dCas9 DNA binding sites, which results in transcriptional repression[11]. (**b**) *Single-component photoswitchable (d)Cas9.* Gene transcription activation using dCas9 with a pdDronpa-caged DNA-binding site. In the dark, the pdDronpa dimer hinders the dCas9 active site. (**c**) *Anti-CRISPR*

CASANOVA system. Anti-CRISPR protein (AcrIIA4) is regulated through *As*LOV2. AcrIIA4 regulates dCas9 fused to transcription regulator or epigenetic modifier domain [76]. (**d**) *Recruitment of transcriptional activators or repressors.* Cry2-CIB1 mediate recruitment of either the transcriptional activators p65 and HSF1 in the *CPTS 2 system* [79] (left) or the transcriptional activator VP64 in the *LACE system* [15] (right) to the dCas9-targeted DNA locus. (**e**) *Split-based pa(d)Cas9.* Light induces reconstitution of split dCas9. (**f**) *Non-dCas9 approaches. LITE system* (left) uses Cry2-CIB1 heterodimerization to recruit the VP64 transcriptional activator (or histone modification enzyme) to the DNA-bound TALE[36]. The *LITEZ system* (right) uses interaction of GIGANTEA with LOV domain from the FKF1 to bring VP64 to DNA-bound zinc finger protein [80]. **(g)** *Chromatin regulation LADL system.* Oligomerization of Cry2 brings together two dCas9 molecules with distinct sgRNAs via fused CIB1. This results in DNA loop formation[81]. **(h)** *FACE system.* The system is triggered by BphS, which produces c-di-GMP upon illumination. Excess of c-di-GMP is degraded by YhjH. C-di-GMP controls the transcriptional activator p65-VP64-BLS-BldD. Synthesized MS2-p65-HSF1 acts as a transactivator of dCas9[34]. **(i)** *RNA modification.* In the PAMEC system [83], dCas13b binds RNA using a sgRNA molecule. Light-induced CRY2-CIBN heterodimerization recruits an effector for RNA m^6A methylation.

Figure 5. **Selected applications of optogenetic tools for regulation of endogenous genes. *In vitro.*** (**a**) Transcriptional activation demonstrated with the LACE, CPTS, CPTS2, split-CPTS2, CASANOVA and FACE OTs. The genes are: *NEUROD1*, *ASCL1* (Achaete-scute complex-like transcription factor 1), *MYOD1* (myoblast determination protein 1), *IL1RN* (interleukin-1 receptor antagonist protein), *IL1R2* (interleukin-1 receptor type 2), *HBG1* (hemoglobin subunit gamma 1), *HBG2* (hemoglobin subunit gamma 2), *NANOG* (homeobox transcription factor Nanog), *NEUROG2*, *TTN* (titin), and *RHOXF2* (Rhox homeobox family member 2) [14, 15, 34, 76, 79]. (**b**) *Light-induced pluripotent stem cells (iPSCs) differentiation into neurons.* For FACE targeting *NEUROG2* (neurogenin-2), murine iPSCs were stably transfected with lentivirus [34]. For Split-CPTS2 targeting *NEUROD1* (neuronal differentiation 1), human iPSCs were transfected with plasmids [79].***In vivo.*** **(c)** *CPTS2 and FACE systems applied in vivo.* Muscle tissue was electroporated with plasmid mixes for regulation of *LAMA1* (laminin subunit alpha 1) and *FST* (follistatin) [34]. **(d)** *Gene regulation by FAST system.* Plasmid mix was injected in developing xenograft tumors of A549 cells. Upon illumination, the tumors reduced in size[82]. (**e**) AAV-based

gene delivery. Delivery of blue light by a light guide (upper panel) or a wearable light source (lower panel) in the mouse brain.

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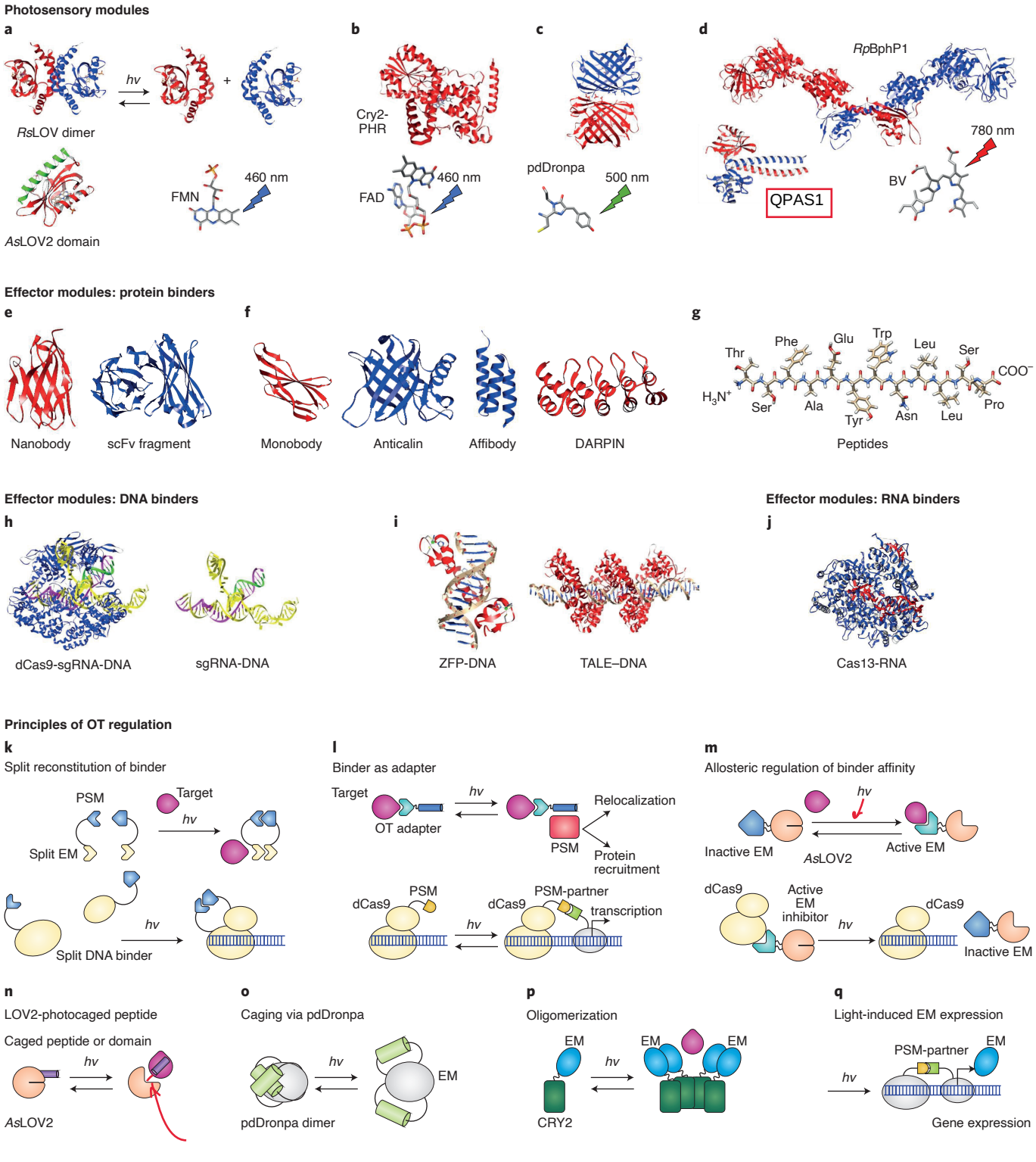
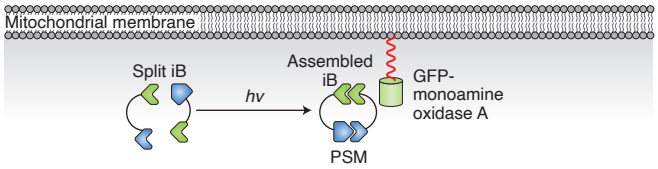

Photosensory modules
a
hv
+
RsLOV dimer
AsLOV2 domain
FMN
460 nm
b
Cry2-
PHR
FAD
460 nm
c
pdDronpa
500 nm
d
RpBphP1
780 nm
BV
QPAS1
Effector modules: protein binders
e
Nanobody
scFv fragment
f
Monobody
Anticalin
Affibody
DARPIN
g
Thr
Phe
Glu
Trp
Leu
Ser
COO⁻
H3N+
Ser
Ala
Tyr
Asn
Leu
Pro
Peptides
Effector modules: DNA binders
h
dCas9-sgRNA-DNA
sgRNA-DNA
i
ZFP-DNA
TALE–DNA
Effector modules: RNA binders
j
Cas13-RNA
Principles of OT regulation
k
Split reconstitution of binder
PSM
Target
hv
Split EM
Split DNA binder
l
Binder as adapter
Target
OT adapter
Relocalization
PSM
Protein
recruitment
dCas9
PSM
PSM-partner
transcription
m
Allosteric regulation of binder affinity
Inactive EM
AsLOV2
Active EM
dCas9
Active
EM
inhibitor
Inactive EM
n
LOV2-photocaged peptide
Caged peptide or domain
AsLOV2
o
Caging via pdDronpa
pdDronpa dimer
EM
p
Oligomerization
EM
CRY2
q
Light-induced EM expression
PSM-partner
Gene expression
add this small linker between
these two, as I indicated in red

Recruitment to membrane for activation/inhibition

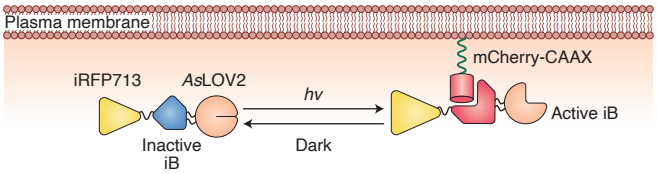
a

Optobody



b

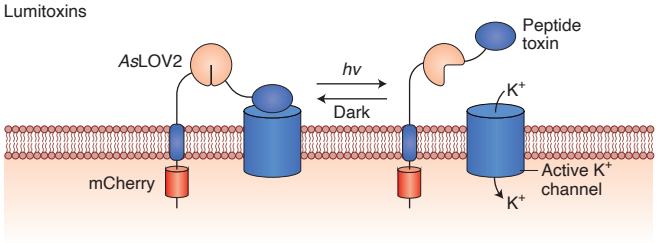
Opto-Nanobody



Inhibition or activation by caged functional peptide or domain

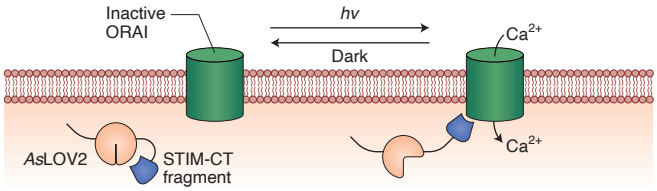
c

Lumitoxins



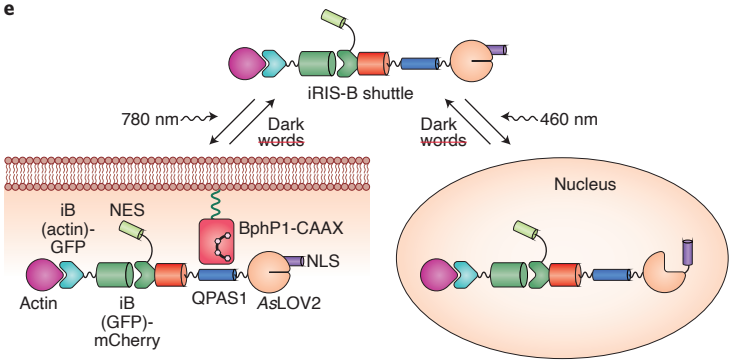
d

Opto-CRAC



Tridirectional transport

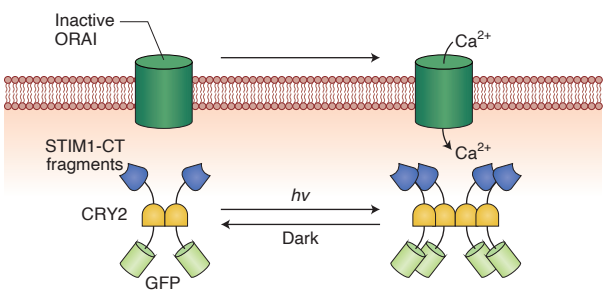
e

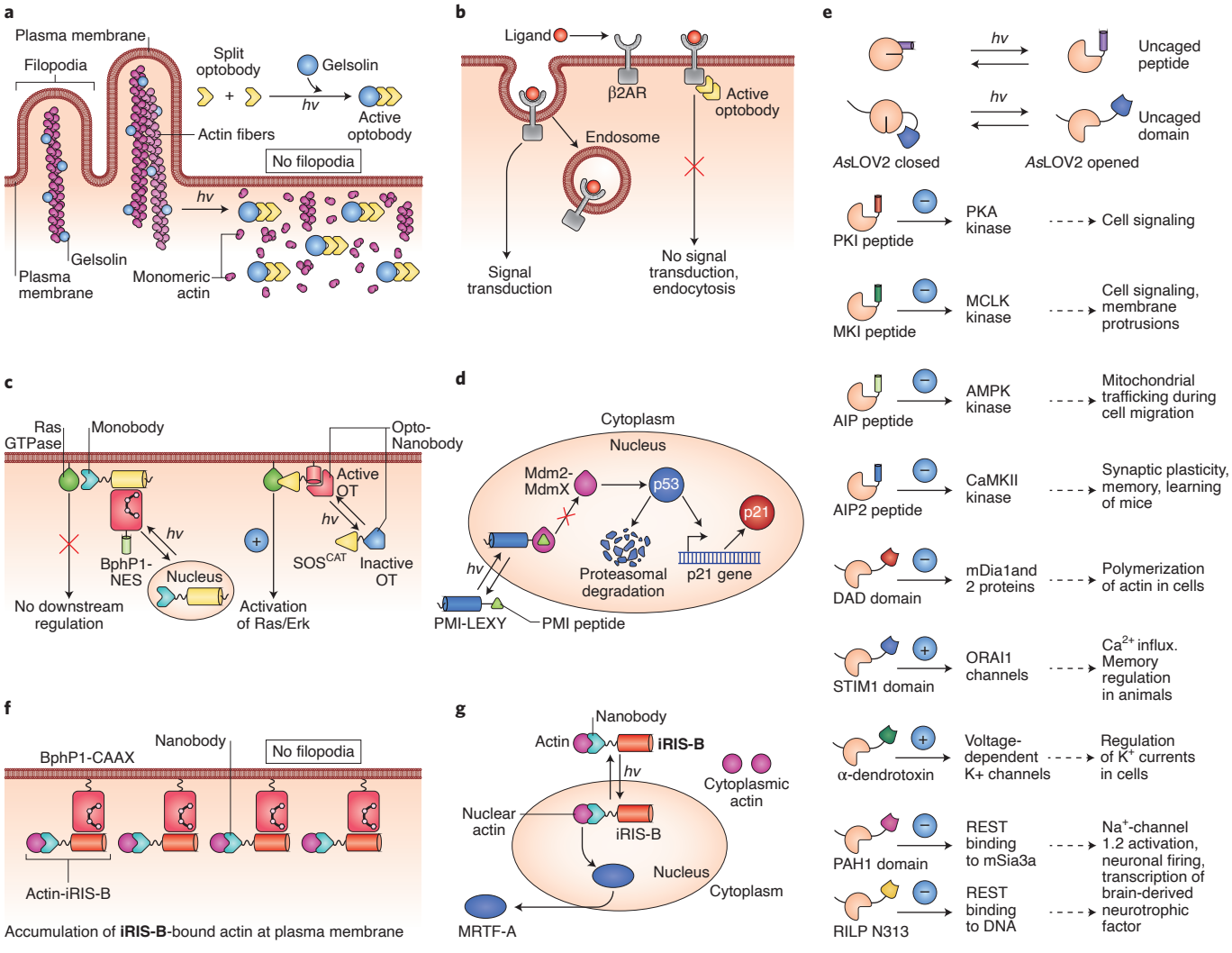


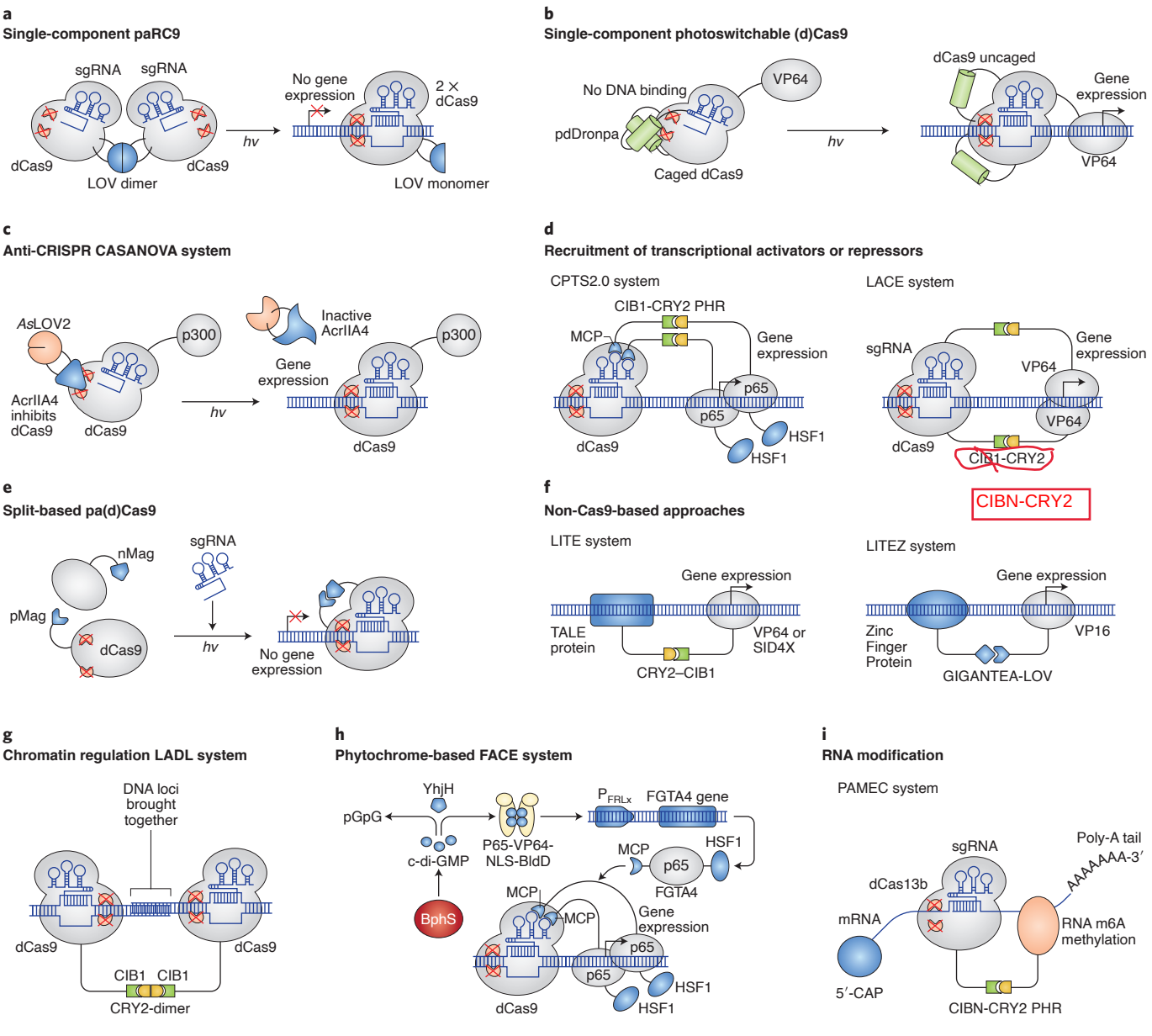
Light-induced oligomerization

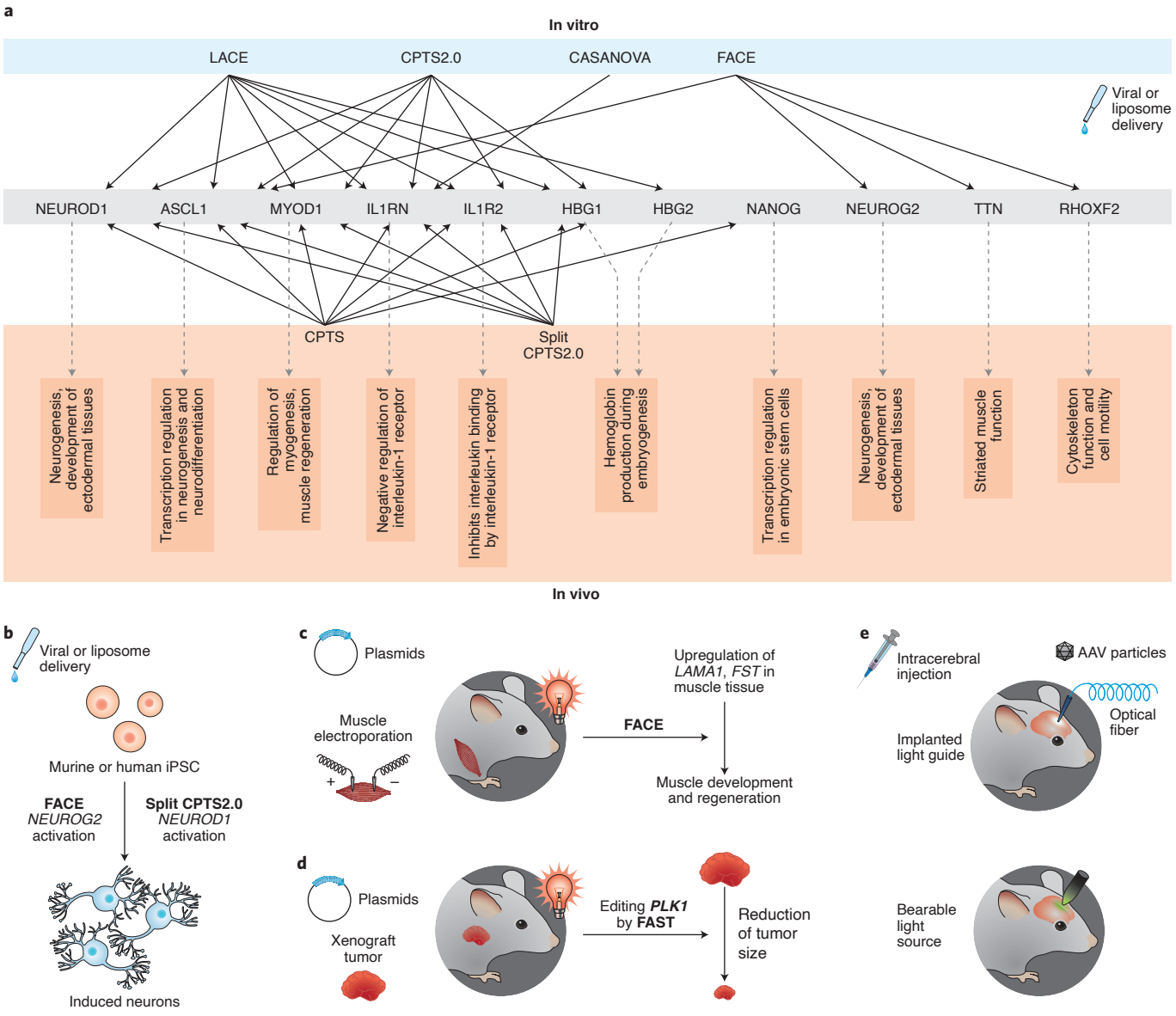
f

Opto-STIM1




a
Plasma membrane
Filopodia
Split optobody
Gelsolin
hv
Active optobody
Actin fibers
No filopodia
hv
Gelsolin
Plasma membrane
Monomeric actin
b
Ligand
β2AR
Active optobody
Endosome
Signal transduction
No signal transduction, endocytosis
c
Ras GTPase
Monobody
Opto-Nanobody
Active OT
hv
hv
BphP1-NES
Nucleus
SOSCAT
Inactive OT
No downstream regulation
Activation of Ras/Erk
d
Cytoplasm
Nucleus
Mdm2-MdmX
p53
p21
Proteasomal degradation
p21 gene
hv
PMI-LEXY
PMI peptide
e
hv
Uncaged peptide
hv
Uncaged domain
AsLOV2 closed
AsLOV2 opened
PKI peptide
PKA kinase
Cell signaling
MKI peptide
MCLK kinase
Cell signaling, membrane protrusions
AIP peptide
AMPK kinase
Mitochondrial trafficking during cell migration
AIP2 peptide
CaMKII kinase
Synaptic plasticity, memory, learning of mice
DAD domain
mDia1and 2 proteins
Polymerization of actin in cells
STIM1 domain
ORAI1 channels
Ca2+ influx. Memory regulation in animals
α-dendrotoxin
Voltage-dependent K+ channels
Regulation of K+ currents in cells
PAH1 domain
REST binding to mSia3a
Na+-channel 1.2 activation, neuronal firing, transcription of brain-derived neurotrophic factor
RILP N313
REST binding to DNA
f
BphP1-CAAX
Nanobody
No filopodia
Actin-iRIS-B
Accumulation of iRIS-B-bound actin at plasma membrane
g
Nanobody
Actin
iRIS-B
hv
Cytoplasmic actin
Nuclear actin
iRIS-B
Nucleus
Cytoplasm
MRTF-A


a
Single-component paRC9
sgRNA
sgRNA
dCas9
LOV dimer
dCas9
hv
No gene expression
2 × dCas9
LOV monomer
b
Single-component photoswitchable (d)Cas9
No DNA binding
VP64
pdDronpa
Caged dCas9
hv
dCas9 uncaged
Gene expression
VP64
c
Anti-CRISPR CASANOVA system
AsLOV2
p300
AcrIIA4 inhibits dCas9
dCas9
hv
Inactive AcrIIA4
p300
Gene expression
dCas9
d
Recruitment of transcriptional activators or repressors
CPTS2.0 system
CIB1-CRY2 PHR
MCP
Gene expression
p65
p65
HSF1
HSF1
dCas9
LACE system
sgRNA
Gene expression
VP64
VP64
dCas9
CIB1-CRY2
CIBN-CRY2
e
Split-based pa(d)Cas9
nMag
pMag
dCas9
sgRNA
hv
No gene expression
f
Non-Cas9-based approaches
LITE system
Gene expression
TALE protein
VP64 or SID4X
CRY2–CIB1
LITEZ system
Gene expression
Zinc Finger Protein
VP16
GIGANTEA-LOV
g
Chromatin regulation LADL system
DNA loci brought together
dCas9
dCas9
CIB1 CIB1
CRY2-dimer
h
Phytochrome-based FACE system
YhjH
pGpG
c-di-GMP
BphS
P65-VP64-NLS-BldD
P FRLx
FGTA4 gene
MCP
HSF1
p65
FGTA4
MCP
MCP
Gene expression
p65
p65
HSF1
HSF1
dCas9
i
RNA modification
PAMEC system
sgRNA
dCas13b
mRNA
5′-CAP
Poly-A tail
AAAAAAA-3′
RNA m6A methylation
CIBN-CRY2 PHR


a
In vitro
LACE
CPTS2.0
CASANOVA
FACE
Viral or liposome delivery
NEUROD1
ASCL1
MYOD1
IL1RN
IL1R2
HBG1
HBG2
NANOG
NEUROG2
TTN
RHOXF2
CPTS
Split CPTS2.0
Neurogenesis, development of ectodermal tissues
Transcription regulation in neurogenesis and neurodifferentiation
Regulation of myogenesis, muscle regeneration
Negative regulation of interleukin-1 receptor
Inhibits interleukin binding by interleukin-1 receptor
Hemoglobin production during embryogenesis
Transcription regulation in embryonic stem cells
Neurogenesis, development of ectodermal tissues
Striated muscle function
Cytoskeleton function and cell motility
In vivo
b
Viral or liposome delivery
Murine or human iPSC
FACE
NEUROG2
activation
Split CPTS2.0
NEUROD1
activation
Induced neurons
c
Plasmids
Muscle electroporation
FACE
Upregulation of LAMA1, FST in muscle tissue
Muscle development and regeneration
d
Plasmids
Xenograft tumor
Editing PLK1 by FAST
Reduction of tumor size
e
Intracerebral injection
AAV particles
Optical fiber
Implanted light guide
Bearable light source