Chapter 8

CRISPR/dCas9 Switch Systems for Temporal Transcriptional Control

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Abstract

In a swift revolution, CRISPR/Cas9 has reshaped the means and ease of interrogating biological questions. Particularly, mutants that result in a nuclease-deactivated Cas9 (dCas9) provide scientists with tools to modulate transcription of genomic loci at will by targeting transcriptional effector domains. To interrogate the temporal order of events during transcriptional regulation, rapidly inducible CRISPR/dCas9 systems provide previously unmet molecular tools. In only a few years of time, numerous light and chemical-inducible switches have been applied to CRISPR/dCas9 to generate dCas9 switches. As these inducible switch systems are able to modulate dCas9 directly at the protein level, they rapidly affect dCas9 stability, activity, or target binding and subsequently rapidly influence downstream transcriptional events. Here we review the current state of such biotechnological CRISPR/dCas9 enhancements. Specifically we provide details on their flaws and strengths and on the differences in molecular design between the switch systems. With this we aim to provide a selection guide for researchers with keen interest in rapid temporal control over transcriptional modulation through the CRISPR/dCas9 system.

Key words CRISPR/Cas9, Temporal regulation, Transcriptional modulation, Epigenome editing, Destabilizing domain, Dimerization domain, Optogenetics, ERT, Split protein, Intein

1 Introduction

Bacteria harbor an adaptive immune system formed by clustered regularly interspaced short palindromic repeats (CRISPR) and Cas nucleases to protect them against pathogenic consequences of phage infections. CRISPR/Cas systems consist of a CRISPR RNA that recruits a Cas nuclease to complementary DNA to subsequently cleave it. Target region recognition by Cas is mediated through the highly variable seed region of CRISPR RNA that forms RNA-DNA hybrids at homologous DNA sequences when in complex with Cas. Following these discoveries, the *Streptococcus pyogenes* CRISPR/Cas9 (*Sp*Cas9; from here on called Cas9) system was repurposed as a targetable nuclease accompanied with a programmable chimeric CRISPR single guide RNA (sgRNA) [1]. This development opened new possibilities to interrogate biological

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functions of genomic loci through targeted manipulation of genomic DNA in both eukaryotes as well as bacteria. Following the observation that nuclease-deactivated Cas9 mutants (dCas9) were still capable of target DNA binding [1], dCas9 was used as a vehicle for targeting effector domains (dCas9 effectors) to specific genomic loci in order to modulate their transcriptional activity. Among others, multiple copies of the *Herpes simplex* VP16 transcriptional activator such as VP64 (known as CRISPRa), the transcriptional repressor Krüppel-associated box (KRAB) domain (known as CRISPRi), and catalytic domains of several epigenetic enzymes have been targeted to a variety of genomic locations to modulate chromatin and transcription of endogenous genomic loci (discussed in detail elsewhere [2, 3]). In the majority of these reports, CRISPR/dCas9 was applied through constitutive (non-conditional) expression systems that do not allow for temporal control of induced transcriptional effects. Alternatively, doxycycline-inducible expression of sgRNA or dCas9 effectors has been applied to provide a certain degree of temporal control over the activity of dCas9 effectors, but the response time might be too slow to dissect fast transcriptional or epigenetic processes. Several recently developed switch systems that act at the protein level, instead, offer a more rapid temporal regulation of the activation or inactivation of dCas9 effectors. To date, several innovative approaches have been used to create CRISPR/dCas9 switches. In this review we discuss the current status of biotechnological adaptations of dCas9 effectors that were recently applied for temporal control of dCas9-induced epigenome editing and transcriptional modulation of target genes.

2 Inducible Reassembly of Split dCas9

The Cas9 crystal structure revealed a bilobed Cas9 architecture with a recognition lobe (α-helical/REC lobe) that is essential for binding sgRNA and DNA and the nuclease lobe containing the HNH and RuvC nuclease domains (Fig. 1a and b) [6, 7]. Interestingly, the interaction between the two lobes seems to be more dependent on nucleic acid (sgRNA) binding than on protein-protein contacts between the two lobes. This observation suggested that introducing an artificial split to separate the two lobes would allow control over Cas9 nuclease activity through regulating the reassembly of the two lobes. The Doudna lab was the first to report a functional split of Cas9 that was separated between the α-helical/REC lobe and the nuclease lobe and could be reassembled in vivo into a functional Cas9 through sgRNA-dependent recruitment of the two lobes [8]. However, during in vitro testing, the activity of the split Cas9 was about tenfold lower than that of wild-type Cas9, suggesting that reassembly of

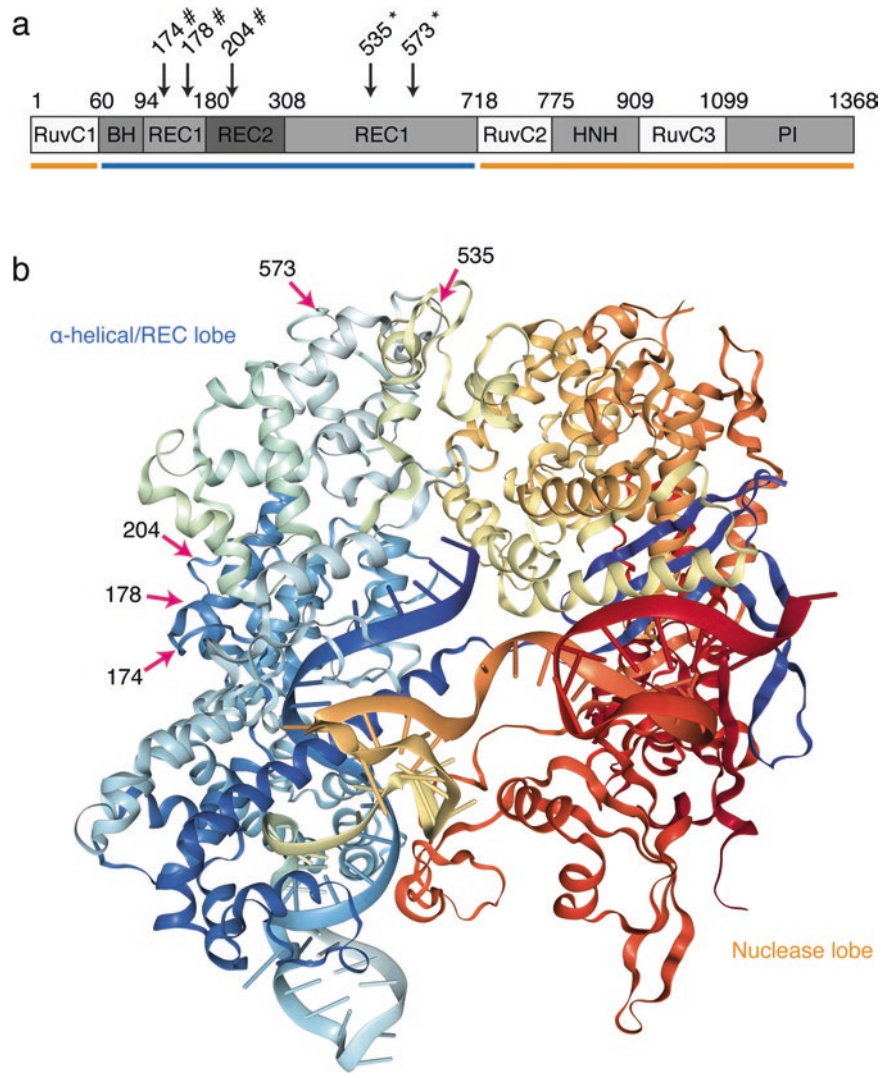

Fig. 1 Designs of split dCas9. (**a**) Schematic overview of the primary protein structure of dCas9 with locations of split sites as used by Zetsche et al. (*) and Nguyen et al. (#) for split dCas9 effectors indicated with arrows. The α-helical/REC lobe is indicated with a blue bar (BH = bridge helix), and the nuclease lobe is indicated with an orange bar (PI = PAM-interacting domain). (**b**) 3D protein structure of dCas9 in complex with a sgRNA molecule and target DNA [4], generated with NGL viewer [5]. The pink arrows indicate the various split locations as shown in (**a**)

the two lobes based on sgRNA-dependent recruitment alone was suboptimal. Following this observation several groups further improved the split Cas9 strategy in order to enforce reassembly of the two lobes by implementing inducible dimerization domains as switch systems. Dimerization domains are protein domains that rapidly bind together in response of a stimulus (light or chemical). When fused to the two Cas9 lobes, the dimerization domains in turn allow for enforced proximity and split Cas9 reassembly in response to the dimerization stimulus.

2.1 Rapamycin-Inducible Reassembly of Split (d)Cas9

Zetsche et al. were the first to build a chemical-inducible split Cas9 switch system [9]. Here they turned to the rapamycin-inducible dimerization system that is formed by a modified FK506-binding protein 12 (FKBP) and the FKB-rapamycin-binding domain of mTOR (FRB) [10]. In the presence of rapamycin, FKBP and FRB undergo dimerization within a few minutes [11]. Zetsche et al. fused FRB to the N-terminal dCas9 lobe (N-lobe) and FKBP to the C-terminal lobe, thereby creating a rapamycin-inducible split Cas9 switch system [9]. The authors tested various split positions within Cas9 for their ability to induce insertion/deletion mutations (indels) after rapamycin-induced split Cas9 reassembly. They found that splitting between amino acids 534 and 535 resulted in comparable activity to full-length (non-split) Cas9. However, the background activity in the uninduced state was as high as 38% of that of the rapamycin-induced indel frequency, indicating considerable split Cas9 reassembly (leakiness) in the absence of the inducer. Similar observations were made by Nihongaki et al. with another collection of FKBP/FRB-fused Cas9 split constructs [12]. Further testing revealed that the observed leakiness is independent of the dimerization domains but instead is caused by spontaneous reassembly in the presence of sgRNA as was observed before [8]. To solve this issue, Zetsche et al. enhanced the spatial separation of the two lobes by incorporating two nuclear export signals (NES) in the N-lobe to enforce its cytoplasmic sequestering, while including two nuclear localization signals (NLS) in the C-lobe to enforce its nuclear localization. This way, when treated with rapamycin, the newly synthesized C-lobes that have been translated in the cytoplasm will reassemble with the cytoplasmic sequestered N-lobes to form a functional Cas9. Through dominance of the multi-copy NLS on the C-lobe, the reconstituted Cas9 will undergo nuclear translocation. This spatial separation strategy was indeed successful to succumb the leaky nuclease activity in the uninduced state, without having adverse effects to the Cas9 activity in the presence of rapamycin.

Two versions of the split Cas9 system (Fig. 1a and b) were further adapted to generate a rapamycin-inducible transcriptional modulator by fusing VP64 to the C-terminus of a nuclease-deactivated Cas9 C-lobe (Fig. 2a). After administering rapamycin, expressions of

Fig. 2 (continued) enables transcriptional remodeling. (**b**) Transcription effector domains can be tethered to dCas9 when both components are fused to dimerization domains (dimerizer). After applying one of the dimerization-inducing signals (blue light, red light + phycocyanobilin (PCB), rapamycin, abscisic acid or gibberellic acid), the dimerization domains form a physical interaction resulting in functional dCas9 effectors. (**c**) dCas9 with an integrated intein self-splicing protein (red) prevents the correct folding of dCas9 effectors. After binding to 4-hydroxytamoxifen (4OHT), the intein induces self-splicing from dCas9. Simultaneously, dCas9 effectors auto-assemble into their mature and active form. (**d**) Fusions of dCas9 effectors with the destabilizing domain DHFR are naturally destabilized, which results in polyubiquitination and subsequent protease-mediated degradation. While upon binding to its ligand trimethoprim, the fusion protein is stabilized and thereby prevented from degradation

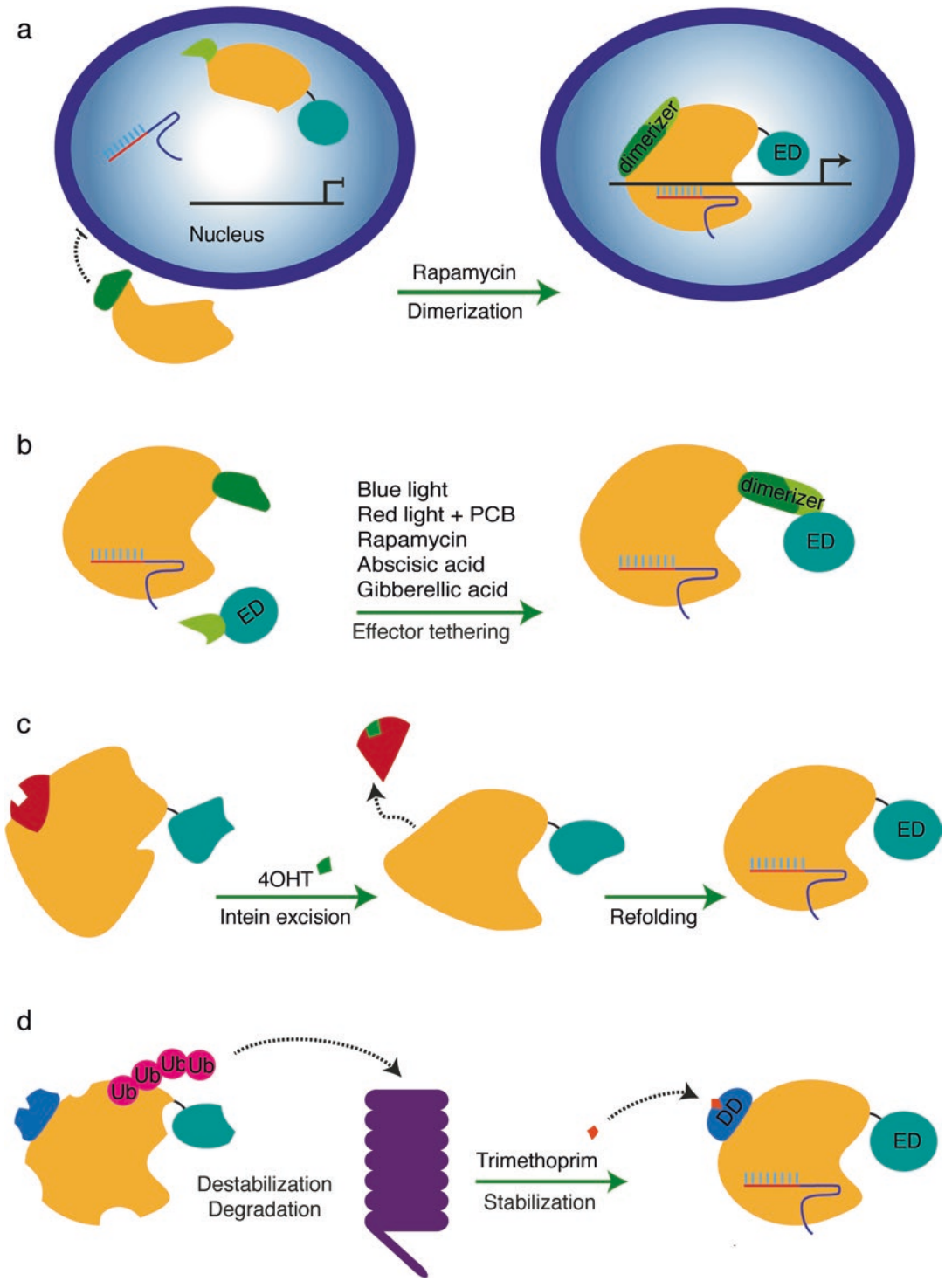

Fig. 2 CRISPR/dCas9 switch systems for temporal transcriptional control. (**a**) Each dCas9 split is fused to one component of the rapamycin-inducible dimerization system (dimerizer). The dCas9 splits remain physically separated in their ground state and reassemble and translocate after addition of rapamycin, which subsequently

several human (*ASCL1*, *IL1RN*) and mouse (*Neurog2*) genes were highly induced by the reassembled FKBP/FRB.dCas9-VP64 split (Table 1) and were similar to the levels induced by the full-length (non-split) dCas9-VP64 version. However, in contrast to the Cas9 split constructs, high leakiness of up to 50-fold background transcription in absence of rapamycin was observed. This suggests either that despite spatial separation the lobes are still able to spontaneously reassemble or sgRNA is able to recruit the C-lobe-VP64 alone to target loci. Another possible caveat of this dCas9 switch system is that up to 70 h after withdrawal of rapamycin transcriptional activation of target loci kept increasing. This could be related to the fact that rapamycin has a high affinity and low dissociated constant for FKBP/FRB in vitro and in vivo [20], which could result in a slow dissociation of FKBP/FRB.dCas9 split constructs after rapamycin withdrawal. This dCas9 switch system would thus be more effective for an experimental setting that solely demands rapid control over induction and not cessation. However, the limited or slow reversibility could also be caused by induction of a stable active chromatin state (epigenetic memory) at the target locus.

2.2 Combining Inducible Split dCas9 Reassembly and Nuclear Translocation

In order to improve the leaky target gene activation seen for the split FKBP/FRB.dCas9-VP64 in uninduced cells, Nguyen et al. added an additional switch based on the ligand-binding domain of the estrogen receptor (ERT) to allow exogenous control over nuclear translocation of the split dCas9 [13]. ERT is routinely used to trigger rapid nuclear translocation of fusion proteins, which through binding to tamoxifen or 4-hydroxytamoxifen (4OHT) are released from HSP90-mediated cytoplasmic sequestering [21, 22]. Nguyen et al. used a similar approach as Zetsche et al. by fusing FRB to the N-lobe and FKBP to the C-lobe of split dCas9 but used a different split Cas9 layout (Fig. 1a and b). In addition, both the N- and C-lobes were fused to a single copy of ERT at their N- and C-termini that allowed for tight spatiotemporal control over cellular localization and reassembly through addition of 4OHT and rapamycin [13]. Indeed the inclusion of ERT to their FKBP-FRB.dCas9 split system improved the performance of the split dCas9 by drastically attenuating leaky transcriptional activation. To further enhance target gene induction, the authors exchanged VP64 for the potent transcriptional activator domain VPR, which is composed of VP64, p65, and Rta activation domains [23]. This ERT-FKBP/FRB-ERT.dCas9-VPR fusion construct was up to fivefold more efficient in activating a reporter target gene than the VP64 edition and also was up to 1.5-fold more effective than a direct dCas9-VPR fusion. The authors also compared their system to the design from Zetsche et al., which was split between amino acids 534 and 535 and where translocation was solely controlled constitutively by NES/NLS sequences. They found that the ERT-FKBP/FRB-ERT.dCas9 split between amino acids 204 and 205

Table 1
Efficiencies of switch systems

Switch dCas9 system	Switch inducer	Effector domain	Fold expression modulation	Fold leakiness	Target locus	Ref.
Split dCas9						
FKBP-FRB	Rapamycin	VP64	~70× ~200× ~1000× ~25,000× ~10×	~2.5× ~7× ~20× ~50× ~2×	*MYOD1* *Neurog2* *ASCL1* *IL1RN* Reporter	[9] [13]
ERT/FKBP-FRB	4OHT + rapamycin	VPR	~22× ~40/100×	None None	*OCT4* Reporter	
GR/FKBP-FRB	Dexamethasone + rapamycin	VPR	~15×	~2×	Reporter	[13]
Effector domain tethering						
CRY2-CIB1	Blue light	P65 VP64	~1000× ~51× ~4.5× ~3× ~40×	None None None None ~2×	*IL1RN* *ASCL1* *MYOD1* *NANOG* Reporter	[14]
CRY2-$CIBN_{\times 2}$ (LACE)	Blue light	VP64	~1000× ~6× ~2.5×	None None None	*IL1RN* *HBG1/2* *ASCL1*	[15]
FKF1-GI	Blue light	VPR	~2.3×	None	Reporter	[16]
PHYB-PIF	Red light + phycocyanobilin	VPR	Up to 5.2×	~1.5×	Reporter	[16]
FKBP-FRB	Rapamycin	VPR	Up to 5.6×	Up to 2×	Reporter	[16]
$FKBP_{3\times}$-FRB	Rapamycin	VPR	~25× ~22×	~2× ~5×	*TTN* *RHOXF2*	[17]
PYL1-ABI		VPR	Up to 38×	None	*CXCR4*	[16]
			Up to 165×	None	Reporter	
		KRAB	Down to 0.2×	Not tested	Reporter	
		SunTag-VP64	8.9×	None	*CXCR4*	
GID1-GAI	Gibberellic acid (GA)	VPR	Up to 4.9×	None	*CD95*	[16]
			Up to 94×	None	Reporter	
		KRAB	Down to 0.3×	Not tested	Reporter	

(continued)

Table 1 (continued)

Switch dCas9 system	Switch inducer	Effector domain	Fold expression modulation	Fold leakiness	Target locus	Ref.
GID1-$GAI_{3\times}$	Gibberellic acid (GA)	VPR	~16× ~29×	None ~2×	*ASCL1* *IL1RN*	[17]
Structural interference						
	4OHT	VPR	~95×	~10×	Reporter	[13]
Destabilizing domains						
DHFR (#)	Trimethoprim	VP192	~100×	~5×	*OCT4*	[18]
			~1000× (stable cells)	~100×	*OCT4*	
			~20×	~5×	*SOX2*	
			~100×	~2×	*NANOG*	
			~1.5×	None	*LIN28*	
			~400×	~200×	*ASCL1*	[19]
DHFR ($)	Trimethoprim	PCP-VP64	Up to ~120×	~2×	*IL1RN*	[19]
ER50 ($)	4OHT	MCP-P65-HSF1	~70×	None	*ASCL1*	[19]

Overview of the four main switch strategies for dCas9 effectors with their individual induction (VP64, VP192, VPR, p65) or repression (KRAB) levels of target loci and their leakiness. Fold expression modulation and leakiness are either relative to mock-transfected control or uninduced control. For the destabilization domains, either a first-generation dCas9 system (#; an effector domain fused to dCas9) or second-generation system ($; an effector domain tethered to sgRNA by PP7 or MS2 aptamer-coat proteins PCP or MCP, respectively) has been used. The inducer for systems containing ERT or ER50 is 4-hydroxytamoxifen (4OHT)

was threefold more efficient to induce transcription of a genomically integrated reporter, suggesting that the ERT system is superior to passive nuclear translocation of cytoplasmic reassembled dCas9. Also the different split positions used by the two reports might contribute to the increased efficiency, but a direct comparison of these different split positions is still missing.

In addition to ERT, Nguyen et al. replaced the ERT for the glucocorticoid receptor α (GR), which is a nuclear receptor protein that translocates to the nucleus upon binding to dexamethasone. This fusion approach, however, was 2.5-fold less efficient in activating the reporter gene than the ERT-FKBP/FRB-ERT.

dCas9-VPR split constructs. Despite this lower efficiency, varying the nuclear receptors of such a split system does enable the possibility for orthogonal gene targeting.

Taken together, the ERT-FKBP/FRB-ERT.dCas9-VPR system allows tight temporal control by addition of 4OHT/rapamycin with little background activity and therefore would offer an effective dCas9 switch system for temporal transcriptional regulation. However, due to the implementation of the FKBP/FRB dimerization domains, the reversibility upon washout of the inducers is still very slow. Below we will discuss alternative approaches that allow more rapid switching in both directions.

3 Inducible Effector Domain Tethering

In the previous section, we discussed several approaches where split dCas9 effectors can be activated through induced reassembly. In an alternative strategy, dCas9 effector activity is controlled through inducible tethering of effector domains to a full-length copy of dCas9. Mechanistically these systems are comprised of two dimerization domains, such as the FKBP/FRB system described above, that bind to each other only in the presence of an external stimulus (chemical or light). By fusing dCas9 and an effector domain each to one of the dimerization domains, inducible effector domain tethering is achieved (Fig. 2b).

3.1 Light-Induced Tethering of Effector Domains

The first application of inducible effector domain tethering to designer DNA-binding domains was done with a blue-light-inducible optogenetic system [24, 25]. The core of this so-called LITE system is composed of the *Arabidopsis thaliana* photolyase-like blue-light receptors cryptochrome 2 (CRY2) and cryptochrome-interacting basic-helix-loop-helix 1 (CIB1), which are both fused to a protein of interest (POI). In the inactive (dark) state, flavin adenine dinucleotide (FAD) non-covalently binds to the N-terminal photolyase homology region of CRY2 (CRY2PHR), thereby blocking binding of CIB1. However, upon blue-light (390–480 nm) illumination, FAD undergoes reduction and allows CIB1 to bind CRY2, thereby bringing both fused POIs in close proximity [26, 27]. Nihongaki et al. adapted the LITE system to the CRISPR/dCas9 platform and tested several CIB1 variants fused to dCas9 and CRY2PHR fused to either the VP64 or p65 transcriptional activators [14]. They found that the combination of a C-terminal truncated variant of CIB1 (trCIB1, also known as CIBN) fused to dCas9 and CRY2PHR fused to p65 to be most effective in activating target genes induced by blue-light irradiation. With this system the *IL1RN* target gene could be induced up to 1000-fold above the empty vector control without background induction in darkness. Although impressive, whether the inducible

CRY2PHR-p65/CIBN.dCas9 effector is as efficient in activating target gene expression as a direct fusion of dCas9-p65 was not assessed.

Shortly after, Polstein et al. presented an analogous optogenetic adaptation of dCas9 that instead involved blue-light-inducible tethering of two CRY2 (full length)-VP64 copies to dCas9 fused with CIBN at its N- and C-terminus (CIBN-dCas9-CIBN) [15]. This approach, entitled LACE, was more effective to activate the *IL1RN* target locus (~1100-fold) than when tethering a single VP64 (CRY2-VP64) (up to ~60-fold), while it showed no detectable leaky induction compared to untransfected cells. Moreover, the inducible tethering through blue light does not reduce the activation potential of VP64 since target gene activation was as effective as a direct dCas9-VP64 fusion. When using CRY2PHR-VP64 instead of CRY2-VPR, the same level of target gene activation was achieved, but ~5-fold increased leaky induction in the dark was observed. This suggests that CRY2 is superior in the LACE system, while in contrast the CRY2PHR was superior in the LITE system [14]. Interestingly, the induced gene activation could be quickly reversed after cessation of blue-light illumination with a $t_{1/2}$ of about 5 h, pointing the potential use of the system as a rapid bidirectional dCas9 switch.

In a more recent study by Gao et al., a series of inducible dCas9 tethering systems were compared [16]. In stark contrast to the reports described above, this study did not observe any light-inducible expression for the CRY2-CIBN blue-light system, maybe because the cells were illuminated with a much lower light intensity (50 μW versus 15 mW per cm^2). The authors also tested another pair of blue-light-inducible dimerization domains based on the *Arabidopsis thaliana* FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) and GIGANTEA (GI) system [28]. Also for this system no light-inducible induction was observed. Slightly better results were obtained for a red-light-inducible system, based on *Arabidopsis thaliana* phytochrome B (PHYB) and the N-terminal part of phytochrome-interacting factor (PIF) 6. Here dimerization is induced by a combination of red light (650 nm) and addition of the exogenous chromophore phycocyanobilin (PCB) [29]. Mechanistically red light induces photoisomerization of covalent bonds between PCB and PHYB that in turn changes the conformational state of PHYB so it can bind to PIF. Following transient expression of the PIF-VPR and PHYB-dCas9 constructs and 48-h illumination with red light and treatment with PCB, the dCas9 effector was able to induce an EGFP reporter up to 5.2-fold without observing leaky induction in the dark [16]. Although the PIF-PHYB system thus appears to be the most effective among the light-inducible systems tested in the study by Gao et al., this finding might also be attributable to the suboptimal illumination conditions for the blue-light systems.

Moreover, these three light-inducible systems were not tested at endogenous loci such as *IL1RN* or compared to direct fusions of dCas9-VPR, so their relative induction potentials remain elusive. However, a particularly interesting feature of the PHYB-PIF optogenetics system is that the induced dimerization can be reversed within seconds after exposure to far-red (>750 nm) light but is otherwise stable for hours in the dark [30]. These properties could thus allow interesting applications for highly tunable dCas9 effectors after further optimization.

3.2 Chemically Induced Tethering of Effector Domains

Apart from the light-inducible systems discussed above, Gao et al. also tested three chemical-inducible dimerization systems to control the activity of dCas9-tethered VPR through addition of the respective activating molecules [16]. Apart from the rapamycin-inducible FKBP-FRB system, the authors fused the components of two *A. thaliana*-derived phytohormone-sensitive dimerization domains to dCas9 and an effector domain. In the first system, pyrabactin resistance-like 1 (PYL1) and abscisic acid-insensitive 1 (ABI) dimerize upon addition of the phytohormone *S*-(+)-abscisic acid (ABA). Mechanistically, upon binding to ABA, PYL1 undergoes a conformational change that generates a hydrophobic binding pocket, which functions as an ABI binding site [31, 32]. In the other phytohormone-responsive system tested, a derivative of gibberellic acid (GA) that can pass the plasma membrane (GA-acetoxymethyl) is used to promote binding of the gibberellin-insensitive dwarf1 (GID1) receptor to gibberellin-insensitive (GAI) [33, 34]. After transient delivery of each dCas9 switch system, rapamycin-induced tethering of VPR to dCas9 only weakly activated an EGFP reporter, whereas application of the ABA- and GA-inducible switch systems was far more effective to activate the same reporter gene through enforced VPR tethering to dCas9 (*see* Table 1) [16]. Furthermore, the ABA- and GA-inducible systems achieved activation of endogenous loci up to 4.9- to 38-fold, respectively, which is technically not as impressive as some of the other systems (Table 1), but a direct comparison between them is hindered by the fact that each study uses different cell lines and loci to evaluate performance. Interestingly, ABA- and GI-inducible systems showed no leaky activation in the absence of the stimulus during transient delivery or stable integration, whereas the FKBP-FRB system only showed some weak background activation upon stable integration in absence of rapamycin [16]. Noteworthy is that the application of rapamycin-inducible FKBP-FRB dimerization domains in inducible effector domain tethering appears to allow a much tighter control over background activity than compared to its application in the split dCas9 approach. However, ABA- and GA-induced tethering of VPR allowed for much higher target gene induction (Table 1) and was, depending on the target locus, similar or even more effective in gene activation than a direct

dCas9-VPR fusion, which is comparable to the LACE system and the split dCas9 systems.

To generate an inducible CRISPRi switch system for targeted gene silencing, Gao et al. exchanged the VPR domain in their ABA- and GA-inducible dCas9 switch systems for a KRAB repressor, which allowed 5.6-fold (0.18×) and 3.2-fold (0.3×) repression, respectively. Binding of the non-tethered dCas9 only had a weak repressive effect (up to 1.2-fold), which could either be related to leaky recruitment of KRAB domain or due to dCas9 binding itself. Comparison to a direct fusion of KRAB-dCas9 revealed the inducible constructs as less effective in attenuating target gene expression, suggesting that further optimization of the ABA- and GA-inducible CRISPRi system would be needed.

In another study the gene activation potential by chemically inducible tethering was further enhanced by increasing the copy number of a dimerization domain fused to dCas9 [17]. In this way several copies of an effector domain can be recruited to a single dCas9 protein. The authors found that fusing three copies of GAI to dCas9 increased target gene activation through a GID1-VPR fusion by almost tenfold compared to a single GAI domain [17]. Interestingly, for the FKBP-FRB system, fusing more than two copies of FKBP to dCas9 did not further enhance the activation potential of rapamycin-inducible effector domain tethering.

Taken together, several switch systems have been developed where effector domains can be recruited to dCas9 either upon light exposure or through addition of a small molecule. Of these the LACE, LITE, and ABA- and GA-inducible systems can upregulate target genes with high efficiency and low background and are equally or even more effective than their non-inducible dCas9 variant. Therefore the selection of a specific switch system to regulate effector domain tethering should not solely be based on these parameters alone. In the next section, we discuss additional factors that should be taken into account as well.

3.3 Choosing between Light- or Chemical-Inducible Tethering of Effector Domains

Although the focus of inducible effector domain tethering lies mostly on its efficiency to induce transcriptional modulation (*see* Table 1), other more practical issues should not be overlooked. For instance, when opting for light- or chemical-inducible dCas9 switch systems, the working costs of either should be taken into account as well. The light-inducible systems need costly apparatuses to generate the optimal light intensity while preventing overheating of cultured cells through powerful LEDs or lasers. In contrast, the ligand-inducible systems only require the presence of mostly inexpensive chemicals or hormones. Another downside of optogenetic systems in controlling gene expression is that during long-term exposure, phototoxicity might impair the viability of cells. Of course, toxic effects of the applied chemicals must also be tested beforehand as well. Furthermore, when interested in apply-

ing different orthogonal dCas9 effectors simultaneously, the light-inducible systems currently give several options that are limited to only two wavelengths (red and blue). In contrast, the ligands of chemical-inducible systems would easily be supplemented together in the same culture conditions after careful testing of potential toxic effects at the cellular level.

4 Blocking dCas9 Function Through Structural Interference

Another strategy to control (d)Cas9 activation at the posttranslational level is to block the folding of its mature conformation. Davis et al. were the first to apply this strategy by introducing an inducible self-splicing protein intron (intein) into specific sites of the Cas9 REC1 and REC2 domains [35]. Here they used a modified RecA intein that contains a 4OHT-binding domain, which upon binding to 4OHT undergoes self-excision from Cas9 and thereby augments complete maturation of the Cas9 protein [36, 37]. After testing several insertion sites for the RecA intein, S219 was identified as the most promising candidate, allowing Cas9 activation within 4 h of 4OHT treatment. However, compared to WT Cas9, the intein-Cas9 was half as efficient in creating indels but at the same time displaying only very low leakiness (~1.5-fold) [35]. The same intein design has also successfully been used to control activity of a dCas9-VPR fusion (Fig. 2c), where it was nearly as effective in activating a reporter gene as the efficient ERT-FKBP/FRB-ERT.dCas9-VPR switch system (Table 1) [13]. However, ~10-fold leaky activation was observed in the absence of the stimulus, which is considerably high compared to several other dCas9 switch systems that have been discussed above. Another limitation of the intein approach is that activation of the dCas9 effector is irreversible once the intein is released from dCas9. Therefore, for the intein system to support transient induction with tight temporal control, it must be combined with other approaches that regulate (d)Cas9 inactivation or degradation, which we will discuss in the following section.

5 Regulating CRISPR/dCas9 Effectors Through Destabilizing Domains

So far, we have discussed various approaches that allow external control over the formation of functional dCas9 effectors. In this section we will introduce attempts to modulate the steady-state levels of dCas9 fusion proteins by controlling their degradation. Destabilizing domains (DDs) are small-molecule-binding proteins that when fused to a POI can control its stability [38]. Two classes of DDs exist: the first class destabilizes the fusion protein in the presence of the small molecule that in turn leads to degradation by

the proteasome, while the second class represents DDs that destabilize fusion proteins in absence of the small molecule, while upon addition they are rescued from degradation. This latter class has recently been applied to dCas9 effectors to regulate their activity (Fig. 2d).

Balboa et al. were the first to show a proof of principle for regulating dCas9-induced transcriptional regulation through controlling degradation of a dCas9 effector by using the dihydrofolate reductase (DHFR)-derived DD [18]. This modified domain, originating from *Escherichia coli*, is rapidly and reversibly stabilized through binding to the small-molecule trimethoprim (TMP) [39]. By fusing DHFR to dCas9-VP192 (12 copies of the VP16 activator), Balboa et al. aimed to construct a tool that allowed TMP-dependent conditional induction of endogenous genes through regulating dCas9 effector protein levels [18]. Indeed, transient transfection of HEK293 cells with a dCas9-VP192 containing an N-terminal DHFR fusion construct (DHFR-dCas9-VP192) was able to efficiently induce endogenous target gene expression (*OCT4*, *SOX2*, *NANOG*, and *LIN28*) of up to ~1000-fold in presence of TMP (Table 1). However, in the absence of TMP, up to ~100-fold leaky gene induction was observed which was likely related to inefficient degradation of DHFR-dCas9-VP192. Another study by Maji et al. confirmed these issues when targeting the *ASCL1* gene in HEK293 cells which showed leaky expression up to ~50% of that of TPM-stabilized target gene induction [19]. Since it appeared to be difficult to destabilize dCas9, maybe due to its large size, the authors also tested a second-generation CRISPRa system, where dCas9 and the effector domain are delivered separately such that the effector domain alone can be targeted for degradation [19]. In a second-generation CRISPRa system, the sgRNAs are tagged with an aptamer, such as MS2 or PP7, which can recruit effector domains that are fused to their respective aptamer-coat protein (MCP and PCP) [40]. By expressing DHFR fused to PCP-VP64, Maji et al. were able to induce the *IL1RN* gene ~120-fold in presence of TMP within 8–12 h with only ~2-fold leaky induction in mock-treated cells [19]. Interestingly, PCP-VP64-induced target gene expression could be reversed with a $t_{1/2}$ around 4–8 h by replacing the TMP-containing medium, suggesting this switch system would be a particular useful tool when rapid bidirectional control over transcription is required.

In the same study by Maji et al., the authors applied another destabilizing domain named ER50 to a second-generation CRISPRa system [19]. The ER50 DD is an adaptation of the estrogen receptor ligand-binding domain (residues 305–549) from the estrogen receptor alpha (ERS1), which is protected from proteasome-mediated degradation upon binding of 4OHT and thus stabilized [41]. By fusing ER50 to MCP and the transcriptional activator p65/HSF1, Maji et al. generated a 4OHT-inducible

transcriptional activation domain. After supplementing cells expressing ER50-MCP-p65/HSF1 with 4OHT, the fusion protein was rapidly stabilized, while as expected dCas9 levels remained unaffected, and target genes (*IL1RN* and *ASCL1*) were rapidly induced to comparable levels as the DHFR variant (Table 1). The authors, however, did not compare gene induction to the performance in absence of the DDs, making it difficult to judge whether the benefits of acquiring switch control over second-generation CRISPRa systems come with the cost of a lower target gene induction. Taken together, the control of effector domain protein levels through destabilizing domains seems to be a promising strategy to induce transcription, though when applied to second-generation CRISPRa systems.

6 Anti-CRISPR Proteins

Since CRISPR/Cas systems are part of the bacterial adaptive immune system that protect against phage invasion, one could envision that phages would have developed mechanisms over time to bypass these antiphage armaments. Indeed, a recent screen for CRISPR/Cas inhibitors from *Listeria monocytogenes* prophages identified two small anti-CRISPR proteins AcrIIA2 and AcrIIA4, which can inactivate the nuclease activity of CRISPR/Cas9 from *L. monocytogenes* Cas9 (LmCas9) and SpCas9 in vivo [42]. Furthermore, the authors show that AcrIIA4 can block dCas9-induced gene repression (CRISPRi) in *Escherichia co*li and Cas9-mediated gene editing of a reporter locus in human cells. Structural comparison revealed that AcrIIA2 and AcrIIA4 interact with SpCas9 in a sgRNA-dependent manner and mainly interfere with Cas9 DNA substrate recognition through blocking the PAM-interacting site located in the PI domain [43]. As such the delivery of recombinant anti-CRISPR proteins could offer another mode of regulation for CRISPR/dCas9-mediated transcriptional modulation by allowing temporal control over its binding to genomic target sites by blocking dCas9 binding to sgRNA.

7 Conclusions and Future Perspectives

In just a few years of time, CRISPR/dCas9 effectors have sparked great interest throughout the scientific community as flexible tools to modulate transcription from genomic loci. Although the conventional CRISPR/dCas9 system is effective for most laboratory applications, switch systems that allow rapid inducible control over CRISPR/dCas9 and their transcriptional effects offer an additional treasure of possibilities to dissect transcriptional and epigenetic processes in much detail. Compared to conventional

dCas9 effectors, CRISPR/dCas9 switch systems, particularly the split dCas9 and inducible effector domain tethering, are equally effective in modulating target gene expression as their conventional counterparts, while in addition having the benefit of allowing rapid temporal control over induction or cessation of transcriptional modulation.

Overall, several dCas9 effector switch systems have been developed to date that allow efficient induction of target genes with very low background activity (Table 1). However, since the conditions under which each of the listed switch system has been tested differ greatly (e.g., cell type, target gene, induction time, delivery method, expression system), it is difficult to identify the most efficient switch system at this point. However, there seems to be a tendency that the FKBP-FRB.dCas9-VP64 split system, the intein-dCas9-VPR, and the DHFR-dCas9-VP192 exhibit increased leakiness in absence of their respective chemical inducer. However, these issues could possibly be solved by combining multiple approaches, as discussed throughout this review. Several groups have shown that inducible tethering of effector domains to dCas9 allows efficient gene activation with low background activity. However, a possible disadvantage of this approach is that even without tethering, dCas9 is able to bind to target DNA in the presence of a sgRNA and compete with transcription factor binding or inhibit transcriptional elongation [1].

For transient gene activation, reversibility of activated dCas9 effectors can be useful, which has however only been addressed for a small subset of the switch systems described in this review. In general light-inducible systems respond very rapidly and reversibly in the order of (milli)seconds to several minutes. Some ligand-inducible systems, such as the PYL1-ABI systems, can be turned off on the time scale of hours after retrieval of the activation signal (ABA), while others such as the FKBR-FRB system seem to dissociate only very slowly following several days. For most dCas9 switch systems, the time scale for which they can be turned off again remains to be determined.

As we summarize in this review, most advances have been made in generating switch systems with low background activity in the uninduced state. However, further improvements will be required to allow rapid quantitative control of target gene transcriptional activity. For instance, for none of the switch systems, it is currently known how fast and how synchronous a population of cells will respond to the stimulus. Moreover, for several systems it has not been tested whether modulation of the stimulus dose allows quantitative control over transcriptional output. Similarly, the dissociation kinetics and thus reversibility of these dCas9 split constructs and tethered effector domains are poorly characterized.

The majority of experiments discussed in this review were performed in HEK293 cells either through transient transfection or AAV delivery of CRISPR/dCas9 constructs. Observed differences in leakiness and activation potential between these transient methods and constitutive expression after stable integration could be another important feature when considering their application in a given experimental setting or cell type. Moreover, leakiness often depends on the target gene, making the choice of the best switch system even more complicated. Genome-wide CRISPR/dCas9 screens that include various switch systems could potentially provide a clearer answer to whether a certain system offers a more robust performance. In the end, answering these questions would drive forward an impressive molecular toolbox of dCas9 effectors that have the power to significantly propel our understanding of not only transcription but also its biological consequences.

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References

1. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152(5):1173–1183. https://doi.org/10.1016/j.cell.2013.02.022
2. Thakore PI, Black JB, Hilton IB, Gersbach CA (2016) Editing the epigenome: technologies for programmable transcription and epigenetic modulation. Nat Methods 13(2):127–137. https://doi.org/10.1038/nmeth.3733
3. Kungulovski G, Jeltsch A (2016) Epigenome editing: state of the art, concepts, and perspectives. Trends Genet 32(2):101–113. https://doi.org/10.1016/j.tig.2015.12.001
4. Jiang F, Zhou K, Ma L, Gressel S, Doudna JA (2015) STRUCTURAL BIOLOGY. A Cas9-guide RNA complex preorganized for target DNA recognition. Science 348(6242):1477–1481. https://doi.org/10.1126/science.aab1452
5. Rose AS, Hildebrand PW (2015) NGL viewer: a web application for molecular visualization. Nucleic Acids Res 43(W1):W576–W579. https://doi.org/10.1093/nar/gkv402
6. Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E, Doudna JA (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 343(6176):1247997. https://doi.org/10.1126/science.1247997
7. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 156(5):935–949. https://doi.org/10.1016/j.cell.2014.02.001
8. Wright AV, Sternberg SH, Taylor DW, Staahl BT, Bardales JA, Kornfeld JE, Doudna JA (2015) Rational design of a split-Cas9 enzyme complex. Proc Natl Acad Sci U S A 112(10):2984–2989. https://doi.org/10.1073/pnas.1501698112
9. Zetsche B, Volz SE, Zhang F (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. Nat Biotechnol 33(2):139–142. https://doi.org/10.1038/nbt.3149

10. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ (2006) A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell 126(5):995–1004. https://doi.org/10.1016/j.cell.2006.07.025
11. Inoue T, Heo WD, Grimley JS, Wandless TJ, Meyer T (2005) An inducible translocation strategy to rapidly activate and inhibit small GTPase signaling pathways. Nat Methods 2(6):415–418. https://doi.org/10.1038/nmeth763
12. Nihongaki Y, Kawano F, Nakajima T, Sato M (2015) Photoactivatable CRISPR-Cas9 for optogenetic genome editing. Nat Biotechnol 33(7):755–760. https://doi.org/10.1038/nbt.3245
13. Nguyen DP, Miyaoka Y, Gilbert LA, Mayerl SJ, Lee BH, Weissman JS, Conklin BR, Wells JA (2016) Ligand-binding domains of nuclear receptors facilitate tight control of split CRISPR activity. Nat Commun 7:12009. https://doi.org/10.1038/ncomms12009
14. Nihongaki Y, Yamamoto S, Kawano F, Suzuki H, Sato M (2015) CRISPR-Cas9-based photoactivatable transcription system. Chem Biol 22(2):169–174. https://doi.org/10.1016/j.chembiol.2014.12.011
15. Polstein LR, Gersbach CA (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat Chem Biol 11(3):198–200. https://doi.org/10.1038/nchembio.1753
16. Gao Y, Xiong X, Wong S, Charles EJ, Lim WA, Qi LS (2016) Complex transcriptional modulation with orthogonal and inducible dCas9 regulators. Nat Methods 13(12):1043–1049. https://doi.org/10.1038/nmeth.4042
17. Bao Z, Jain S, Jaroenpuntaruk V, Zhao H (2017) Orthogonal genetic regulation in human cells using chemically induced CRISPR/Cas9 activators. ACS Synth Biol 6(4):686–693. https://doi.org/10.1021/acssynbio.6b00313
18. Balboa D, Weltner J, Eurola S, Trokovic R, Wartiovaara K, Otonkoski T (2015) Conditionally stabilized dCas9 activator for controlling gene expression in human cell reprogramming and differentiation. Stem Cell Rep 5(3):448–459. https://doi.org/10.1016/j.stemcr.2015.08.001
19. Maji B, Moore CL, Zetsche B, Volz SE, Zhang F, Shoulders MD, Choudhary A (2017) Multidimensional chemical control of CRISPR-Cas9. Nat Chem Biol 13(1):9–11. https://doi.org/10.1038/nchembio.2224
20. Putyrski M, Schultz C (2012) Protein translocation as a tool: the current rapamycin story. FEBS Lett 586(15):2097–2105. https://doi.org/10.1016/j.febslet.2012.04.061
21. Feil R, Wagner J, Metzger D, Chambon P (1997) Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun 237(3):752–757. https://doi.org/10.1006/bbrc.1997.7124
22. Redeuilh G, Moncharmont B, Secco C, Baulieu EE (1987) Subunit composition of the molybdate-stabilized "8-9 S" nontransformed estradiol receptor purified from calf uterus. J Biol Chem 262(15):6969–6975
23. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, PRI E, Lin S, Kiani S, Guzman CD, Wiegand DJ, Ter-Ovanesyan D, Braff JL, Davidsohn N, Housden BE, Perrimon N, Weiss R, Aach J, Collins JJ, Church GM (2015) Highly efficient Cas9-mediated transcriptional programming. Nat Methods 12(4):326–328. https://doi.org/10.1038/nmeth.3312
24. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F (2013) Optical control of mammalian endogenous transcription and epigenetic states. Nature 500(7463):472–476. https://doi.org/10.1038/nature12466
25. Polstein LR, Gersbach CA (2012) Light-inducible spatiotemporal control of gene activation by customizable zinc finger transcription factors. J Am Chem Soc 134(40):16480–16483. https://doi.org/10.1021/ja3065667
26. Liu H, Yu X, Li K, Klejnot J, Yang H, Lisiero D, Lin C (2008) Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. Science 322(5907):1535–1539. https://doi.org/10.1126/science.1163927
27. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker CL (2010) Rapid blue-light-mediated induction of protein interactions in living cells. Nat Methods 7(12):973–975. https://doi.org/10.1038/nmeth.1524
28. Yazawa M, Sadaghiani AM, Hsueh B, Dolmetsch RE (2009) Induction of protein-protein interactions in live cells using light. Nat Biotechnol 27(10):941–945. https://doi.org/10.1038/nbt.1569
29. Levskaya A, Weiner OD, Lim WA, Voigt CA (2009) Spatiotemporal control of cell signalling using a light-switchable protein interaction. Nature 461(7266):997–1001. https://doi.org/10.1038/nature08446

30. Ni M, Tepperman JM, Quail PH (1999) Binding of phytochrome B to its nuclear signalling partner PIF3 is reversibly induced by light. Nature 400(6746):781–784. https://doi.org/10.1038/23500
31. Miyazono K, Miyakawa T, Sawano Y, Kubota K, Kang HJ, Asano A, Miyauchi Y, Takahashi M, Zhi Y, Fujita Y, Yoshida T, Kodaira KS, Yamaguchi-Shinozaki K, Tanokura M (2009) Structural basis of abscisic acid signalling. Nature 462(7273):609–614. https://doi.org/10.1038/nature08583
32. Liang FS, Ho WQ, Crabtree GR (2011) Engineering the ABA plant stress pathway for regulation of induced proximity. Sci Signal 4(164):rs2. https://doi.org/10.1126/scisignal.2001449
33. Ueguchi-Tanaka M, Nakajima M, Katoh E, Ohmiya H, Asano K, Saji S, Hongyu X, Ashikari M, Kitano H, Yamaguchi I, Matsuoka M (2007) Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin. Plant Cell 19(7):2140–2155. https://doi.org/10.1105/tpc.106.043729
34. Miyamoto T, DeRose R, Suarez A, Ueno T, Chen M, Sun TP, Wolfgang MJ, Mukherjee C, Meyers DJ, Inoue T (2012) Rapid and orthogonal logic gating with a gibberellin-induced dimerization system. Nat Chem Biol 8(5):465–470. https://doi.org/10.1038/nchembio.922
35. Davis KM, Pattanayak V, Thompson DB, Zuris JA, Liu DR (2015) Small molecule-triggered Cas9 protein with improved genome-editing specificity. Nat Chem Biol 11(5):316–318. https://doi.org/10.1038/nchembio.1793
36. Buskirk AR, Ong YC, Gartner ZJ, Liu DR (2004) Directed evolution of ligand dependence: small-molecule-activated protein splicing. Proc Natl Acad Sci U S A 101(29):10505–10510. https://doi.org/10.1073/pnas.0402762101
37. Peck SH, Chen I, Liu DR (2011) Directed evolution of a small-molecule-triggered intein with improved splicing properties in mammalian cells. Chem Biol 18(5):619–630. https://doi.org/10.1016/j.chembiol.2011.02.014
38. Lucas X, Ciulli A (2017) Recognition of substrate degrons by E3 ubiquitin ligases and modulation by small-molecule mimicry strategies. Curr Opin Struct Biol 44:101–110. https://doi.org/10.1016/j.sbi.2016.12.015
39. Iwamoto M, Bjorklund T, Lundberg C, Kirik D, Wandless TJ (2010) A general chemical method to regulate protein stability in the mammalian central nervous system. Chem Biol 17(9):981–988. https://doi.org/10.1016/j.chembiol.2010.07.009
40. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517(7536):583–588. https://doi.org/10.1038/nature14136
41. Miyazaki Y, Imoto H, Chen LC, Wandless TJ (2012) Destabilizing domains derived from the human estrogen receptor. J Am Chem Soc 134(9):3942–3945. https://doi.org/10.1021/ja209933r
42. Rauch BJ, Silvis MR, Hultquist JF, Waters CS, McGregor MJ, Krogan NJ, Bondy-Denomy J (2017) Inhibition of CRISPR-Cas9 with bacteriophage proteins. Cell 168(1–2):150–158. e110. https://doi.org/10.1016/j.cell.2016.12.009
43. Dong, Guo M, Wang S, Zhu Y, Wang S, Xiong Z, Yang J, Xu Z, Huang Z (2017) Structural basis of CRISPR-SpyCas9 inhibition by an anti-CRISPR protein. Nature. https://doi.org/10.1038/nature22377