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Mini Review

Drug Inducible CRISPR/Cas Systems

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ABSTRACT

Clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems have been employed as a powerful versatile technology for programmable gene editing, transcriptional modulation, epigenetic modulation, and genome labeling, *etc.* Yet better control of their activity is important to accomplish greater precision and to reduce undesired outcomes such as off-target events. The use of small molecules to control CRISPR/Cas activity represents a promising direction. Here, we provide an updated review on multiple drug inducible CRISPR/Cas systems and discuss their distinct properties. We arbitrarily divided the emerging drug inducible CRISPR/Cas systems into two categories based on whether at transcription or protein level does chemical control occurs. The first category includes Tet-On/Off system and Cre-dependent system. The second category includes chemically induced proximity systems, intein splicing system, 4-Hydroxytamoxifen-Estrogen Receptor based nuclear localization systems, allosterically regulated Cas9 system, and destabilizing domain mediated protein degradation systems. Finally, the advantages and limitations of each system were summarized.

Contents

Abbreviations: 4-OHT, 4-Hydroxytamoxifen; ABA, abscisic acid; ADs, activation domains; arC9, allosterically regulated Cas9; Cas, CRISPR-associated protein; CIP, chemically induced proximity; CRISPR, clustered, regularly interspaced, short palindromic repeats; CrRNA, CRISPR RNA; dCas9, dead Cas9; DD, destabilizing domain; DHFR, dihydrofolate reductase; dCpf1, dead Cpf1; dLbCpf1, *Lachnospiraceae bacterium* dCpf1; dox, doxycycline; ER, Estrogen Receptor; FKBP, FK506-binding protein; FRB, FKBP-rapamycin-binding domain; GA, gibberellin; HIT, Hybrid drug Inducible CRISPR/Cas9 Technologies; Hsp90, heat shock protein 90; iPSCs, induced pluripotent stem cells; LBD, ligand binding domain; LSL, loxP-stop-loxP; MST, multiplex single transcript; NES, nuclear export sequence; NLS, nuclear localization sequence; P_{tet}, tetO-containing promoter; rtTA, reverse-tTA; Sa, *Staphylococcus areus*; Sp, *Streptococcus pyogenes*; sgRNA, single guide RNA; TetO, tet operator; TetR, Tet repressor protein; TMP, trimethoprim; TRE, tetracycline response element; TRE3G, Tet-On 3G protein; VPR, VP64-P65-Rta.

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1. Introduction

Over the past 6 years, clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and its derivatives have been discovered to accomplish a variety of sequence dependent genome modulation due to its RNA-guided DNA recognition capability across almost all species [1–3]. CRISPR/Cas9 derived from the adaptive immune system of bacterial and archaea, who use this system to protect themselves from foreign virus or plasmid invasion [4–6]. Cas9, in cooperation with a chimeric single guide RNA (sgRNA), binds and cleaves the genome DNA in a sequence specific manner according to RNA-DNA complementation [1]. The resulted double strand breaks can be repaired by precise homology directed repair in the presence of homology templates or the error prone non-homologous end-joining, which leads to small insertions or deletions [2]. Furthermore, catalytically inactive Cas9, also known as dead Cas9 (dCas9), which loses nuclease activity while maintaining the DNA binding capability, has been widely used to achieve transcription activation or repression, epigenetic editing, chromatin imaging and so on, based on fusion with different types of effectors [7–8]. In addition, CRISPR/Cas9 based technologies provide a convenient tool to target multiple loci simultaneously by introducing multiple sgRNAs.

Constitutive expression of Cas9 often results in undesirable outcomes, such as cellular toxicity and off-target effects. Moreover, precise investigation of complex biological processes and further programming them for therapeutic benefit requires fine spatio-temporal control of key regulatory events. This demands generation of inducible CRISPR/Cas9 systems for functional perturbation in a tunable way. Therefore, several inducible CRISPR/Cas9 systems have been generated in different laboratories based on varying strategies. Here, we provide an updated summary of these systems. Meanwhile, paralleled development of light-inducible CRISPR/Cas9 systems, although not covered in this review, enabled important spatial control of functional events by light [9–14]. The readers are encouraged to refer to these literatures if they are interested.

The emerging drug inducible CRISPR/Cas9 systems can be arbitrarily divided into two categories depending on whether at transcription or protein level does chemical control occurs. The first category includes Tet-On/Off system and Cre-dependent system, in which the transcription of Cas9 or sgRNA are subject to chemical control. The second category includes chemically induced proximity (CIP) systems, intein splicing system, 4-Hydroxytamoxifen (4-OHT)-Estrogen Receptor (ER) based nuclear localization systems, allosterically regulated Cas9 (arC9) system, and destabilizing domain (DD) mediated protein degradation systems. Finally, we will discuss the advantages and limitations of each system.

2. Systems of Drug Induction at the Transcription Level

This category is based upon drug control of Cas9 or gRNA transcription through a drug inducible promoter, as seen in the doxycycline (dox)-induced Tet system, or indirectly through drug control of Cre recombinase activity (Fig. 1).

2.1. Tet System

The Tet system is one of the most wildly used drug inducible transgene expression system. It is based on releasing the *Escherichia coli* Tet repressor protein (TetR) from its bound tet operator (TetO) sequence upon addition of tetracycline or its derivative dox [15]. Fusion of the VP16 activation domain to TetR results in a transcriptional activator tTA. In the Tet-Off system, dox binds to tTA and triggers its release from the tetO-containing promoter (P_{tet}), thus switching off its driven transgene. The reverse-tTA (rtTA) is derived from tTA mutant with a reverse activity, which only binds to TetO in the presence of dox. Therefore, in the rtTA based Tet-On system, addition of dox triggers rtTA binding to the P_{tet} and switches on the target gene. M2rtTA2 is an alternative version of rtTA showing reduced basal expression [16]. The Tet-On 3G protein (TRE3G), which contains 5 amino acid distinctions from M2rtTA2, further increases the sensitively to dox and decreases the background expression when used in combination with optimized tetracycline response element (TRE) repeats [15].

Tet-On system has been harnessed to regulate either Cas9 or sgRNA expression to generate the inducible CRISPR/Cas9 systems (Fig. 1A). In the dox-inducible Cas9 system, the authors used rtTA and TRE, which consists of 7 repeats of the 19 bp TetO sequence (TCCCTATCAGTGATAGAGA) separated by spacers to control Cas9 expression [17]. Further, an optimized M2rtTA2-TRE system was adopted to generate an inducible Cas9 for less background activity in human pluripotent stem cells, named iCRISPR [18]. TRE3G has also been applied to generate inducible CRISPR transgenic mice [19]. TRE3G driven Cas9 or Cas9D10A (Cas9n) implements the temporal control of gene modification. Replacing Cas9 with dCas9 fused with transcription activation domains such as VP64-P65-Rta (VPR), results in inducible target gene activation [20]. Introducing this system into human induced pluripotent stem cells (iPSCs) demonstrated a controllable iPSC neuronal differentiation.

sgRNA expression can also be regulated by Tet system [21–22]. sgRNA is driven by the RNA promoter, such as H1. TetR, bound to TetO repeats in between H1 promoter and sgRNA, serves as a block for transcription, thus leading to suppression of sgRNA expression. Dox binding to TetR triggers its release from TetO, thus relieving the suppression of sgRNA expression. Using this system, researchers established an inducible lentiviral guide RNA platform for gene editing in murine and human cells [21].

2.2. Cre Dependent System

Cre recombinase can efficiently excise DNA fragments flanked by loxP sites in mammalian cells [23]. A loxP-stop-loxP (LSL) cassette can be placed in between a promoter and Cas9 coding sequence. Cre mediated loxP recombination removes stop signal, thus activating Cas9 expression (Fig. 1B). A mouse line containing a LSL-Cas9 cassette knocked in the Rosa26 locus has been generated [24]. Tissue specific expression of Cas9 can be achieved by crossing with mice harboring Cre recombinase driven by tissue specific promoters. When combined with sgRNA *in situ* delivery, this mouse line provided a useful tool for tissue specific genome editing *in vivo* and modeling diseases such as cancer [24]. Also, Cre recombinase can be driven by small molecule sensitive promoters, such as the TRE repeats [25–26]. In addition, Cre activity can be regulated by fusing with the ligand binding domain (LBD) of the ER or its mutant, rendering a tamoxifen-dependent Cre action [27–29], or by fusing with the LBD of a mutated progesterone receptor which responds to the synthetic steroid RU486 but not endogenous progesterone [30–31]. These drug inducible Cre systems have been used to control Cas9 activity through recombination of the LSL cassette [32–33].

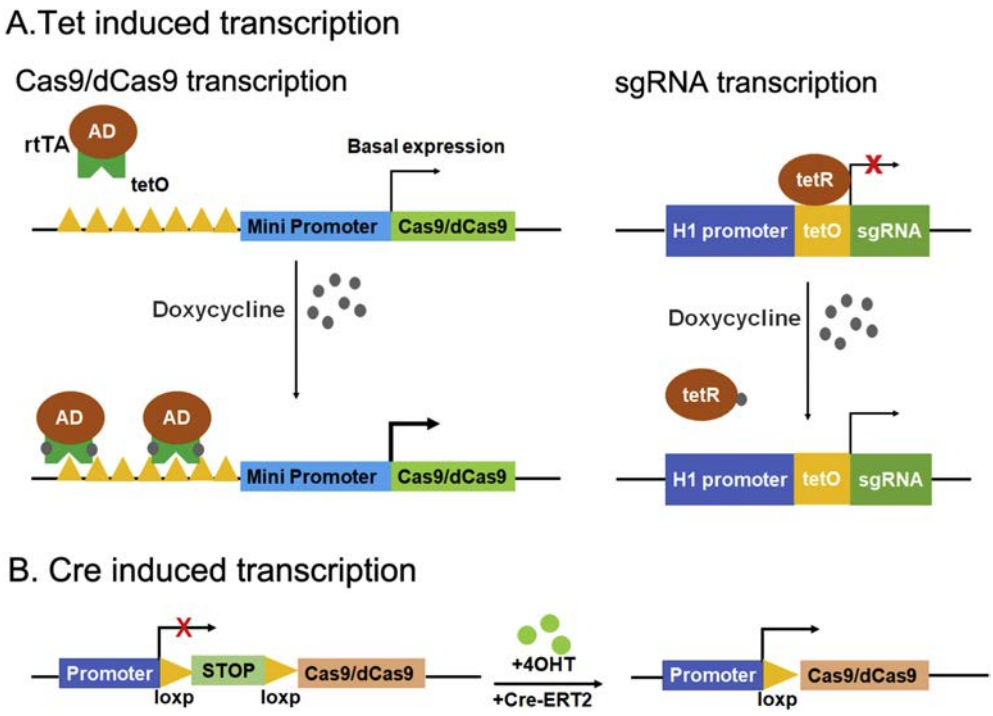

Fig. 1. CRISPR/Cas9 systems of drug induction at the transcriptional level. A. Tet based CRISPR/Cas9 systems for doxycycline regulation of Cas9 (left) or sgRNA (right) expression. AD, activation domain. B. Cre-ER^{T2}-dependent CRISPR/Cas9 system. Cre-ER^{T2} removes the loxp-stop-loxp cassette and drives Cas9 expression dependent on 4-OHT regulated nuclear translocation.

3. Systems of Drug Induction at the Posttranslational Level

CRISPR/Cas9 activity can also be controlled by a drug at the post-translational level. Multiple mechanisms of action, including drug induced dimerization, conformation recovery, nuclear localization, and protein degradation, have been employed (Fig. 2). It is worth noting that posttranslational drug induction, in theory, acts faster than transcriptional drug modulation, because the former is ready to go while transcription and translation takes extra time in the latter.

3.1. CIP Systems

CIP utilizes small molecules or membrane permeable proteins to induce physical association of two binding partners and to concomitantly drive the interaction between proteins of interest fused to them in an inducible, rapid and specific manner [34]. The most widely used CIP system is rapamycin and its derivatives [35], expanded to the recent developed systems including S-(+)-abscisic acid (ABA)-inducible ABI-PYL1 [36] and gibberellin (GA)-inducible GAI-GID1 [37], both of which were derived from plant hormone signaling pathways. For further information about CIP, please refer to [38–39]. In this part, we will discuss the strategies developed recently that harness CIP systems to control CRISPR/Cas9 action of genome editing, transcription modulation, and chromosomal remodeling [40] (Fig. 2A).

Rapamycin, an inhibitor of mammalian target of rapamycin, induces heterodimerization of FK506-binding protein (FKBP)12 and the FKBP-rapamycin-binding domain (FRB) [41]. To generate an inducible CRISPR/Cas9 system, Bernd Zetche et al. splitted *Streptococcus pyogenes* Cas9 (SpCas9) into two parts, Cas9(N) and Cas9(C) based on information from its crystal structure, and fused them with FRB and FKBP, respectively [42]. The two split parts can be reconstituted to the full and functional Cas9 in the presence of rapamycin (Fig. 2A, upper panel). However, the Cas9 background activity was high due to auto-assembly in the absence of rapamycin. To damp the background activity, the authors compartmentalized the two split partners by fusing a nuclear export sequence (NES) to Cas9(N)-FRB fragment and two nuclear localization sequences (NLS) to Cas9(C)-FKBP. Notably, the split Cas9 system showed reduced off-target activity measured in surveyor assays and deep sequencing, possibly due to limited exposure of genomic DNA to intact Cas9. Duy Nguyen et al. tested three different split sites other than the design above, all locating on the flexible loops of the REC2 domain [43]. This domain is evolutionarily divergent among many orthologous Cas9 proteins, thus suggesting flexibility in modifications. By fusing Cas9(N) and Cas9(C) with FRB and FKBP respectively, they also demonstrated rapamycin inducible genome editing.

In addition, Bernd Zetche et al. applied this strategy to dCas9 and fused VP64 activation domain to one split fragment to achieve rapamycin inducible transcription activation [42]. However, this split dCas9 system caused a permanently gene activation even when the rapamycin was removed. Similarly, Duy Nguyen et al. also expanded their design to dCas9 and generated inducible transactivation system by fusing VP64 or VPR to Cas9(C) fragment. However, the background activity was also high due to sgRNA induced auto-assembly in the absence of rapamycin [42,44].

Similarly, fusing dCas9 and the transactivation domain with two inducer-binding-proteins respectively can generate an inducible CRISPR/Cas9 activator (Fig. 2A, lower panel). Yuchen Gao et al. assessed 6 previously reported systems including 3 light-inducible and 3 CIP systems [45]. Using a reporter assay, the authors demonstrated high efficiency of ABA and GA induced dimerization systems in contrast to 3 light- and rapamycin-inducible systems. ABA induces heterodimerization of ABI1 and PYL1, which are fused with dCas9 and activation domains (ADs) respectively. GA, on the other hand, introduces dimerizaiton of GAI and GID1. The authors also replaced activation domains VPR to a repression domain, the Krüppel-associated box [46], which can be used for drug inducible knockdown. The ABA and GA inducible systems can be further adopted to orthogonal *Staphylococcus areus* (Sa)dCas9, which allows simultaneous modulation of multiple genes in an orthogonal manner (Fig. 3A) [45]. Importantly, ABA- and GA-inducible systems were demonstrated to be reversible and dose responsive.

Other type II Cas enzymes, such as Cpf1, have also been used to generate CIP-dependent systems. In comparison with SpCas9, the CRISPR

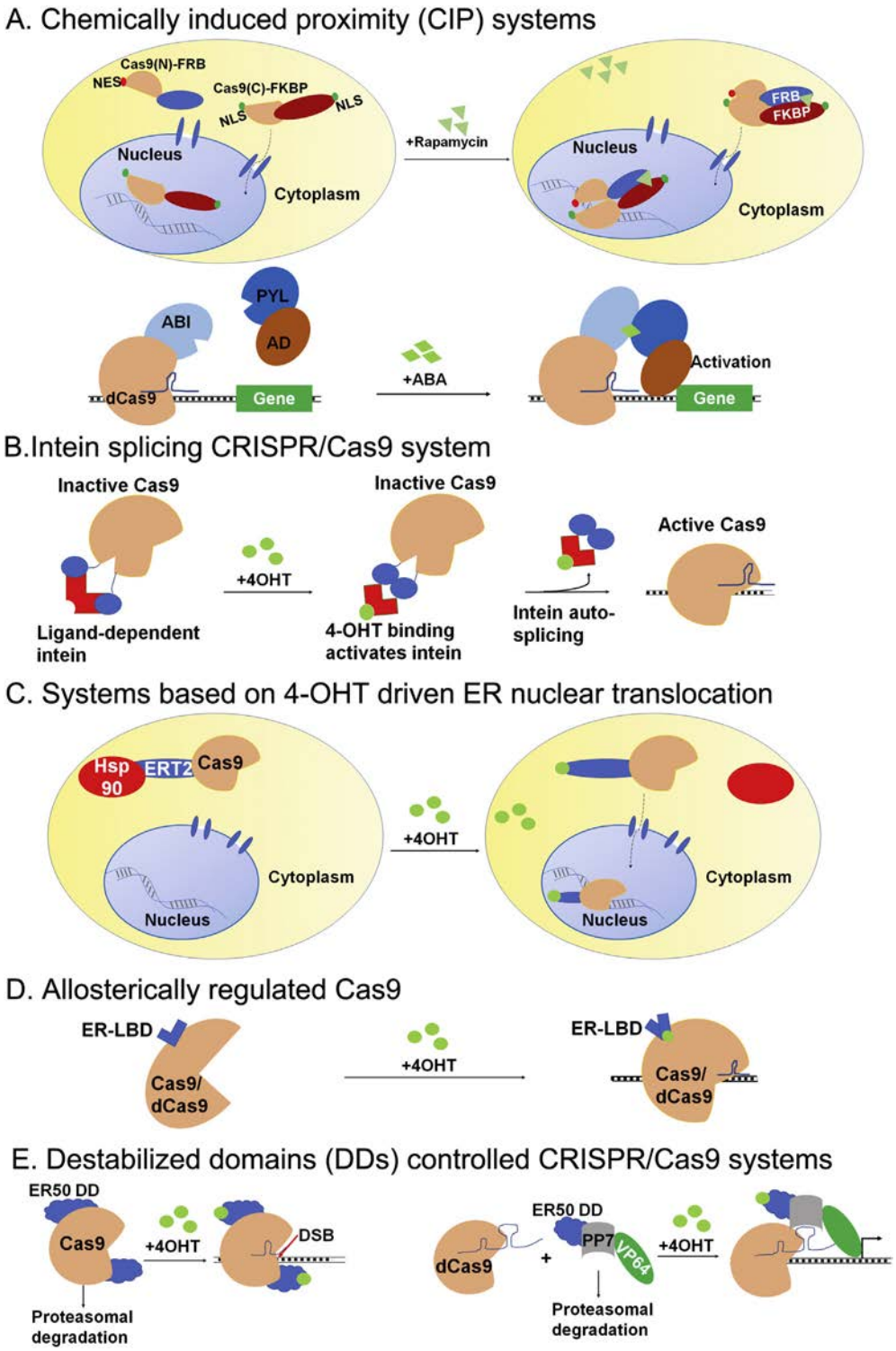

Fig. 2. CRISPR/Cas9 systems of drug induction at the posttranslational level. A. Chemically induced proximity (CIP) systems. Drug induces split Cas9 dimerization and forming complete and functional Cas9 (upper panel); similarly, drug induces dimerization of dCas9 and transcriptional activation domains (ADs) for gene activation (lower panel). NES, nuclear export sequence; NLS, nuclear localization sequence. B. Intein splicing CRISPR/Cas9 system. C. Systems based on 4-OHT driven ER nuclear translocation. D. Allosterically regulated Cas9. E. Destabilized domains (DDs) controlled CRISPR/Cas9 systems. Small molecule stabilizes DD-Cas9 (left) or DD-PP7-ADs (right) and renders them drug inducible. DSB, double strand break.

RNA (crRNA) for Cpf1 is shorter in length. Cpf1 utilizes a T-rich PAM and contains RNase activity, auto-processing multiple crRNAs in a multiplex single transcript (MST) [47–49]. Dead Cpf1 (dCpf1) fused with transactivation domain VPR was demonstrated to activate endogenous genes at a comparable level to dCas9-based activators [50–51]. To render this system drug inducible, Y Esther Tak et al. fused *Lachnospiraceae bacterium* dCpf1 (dLbCpf1) and VPR with DmrA and DmrC respectively, which form a heterodimer in the presence of a rapamycin analog, the A/C heterodimerizer [52]. Further, the authors demonstrated that the drug inducible transactivation potency dramatically increased when dLbCpf1 was fused with 4 tandem copies of the DmrA domain. Synergistic activation of different endogenous genes was also demonstrated using MST. In addition, kinetic experiments showed this drug inducible system reached its maximum activation 25-35 h after drug addition and

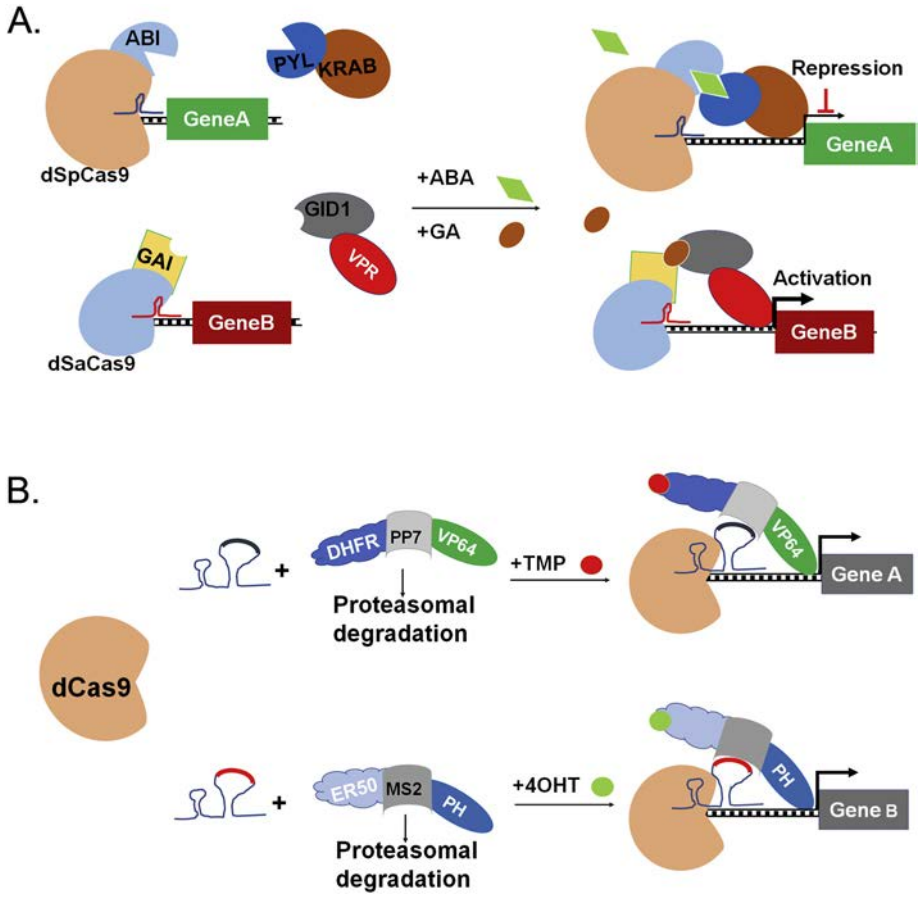

Fig. 3. Schematic presentation of two orthogonal CRISPR/Cas9 systems. A. Two independent chemically induced proximity (CIP) systems in combination with spCas9 and saCas9 respectively form an orthogonal CRISPR/Cas9 system for independent regulation of two genes. KRAB, Krüppel-associated box; VPR, VP64-P65-Rta. B. Two independent destabilized domain (DD) systems in combination with distinct aptamers constitute another orthogonal system. PH, P65-HSF1.

returned to base line after drug withdrawal, a demonstration of reversible drug control.

3.2. Intein Splicing System

Inteins are similar to self-splicing introns, however, they are transcribed and translated together with their host proteins. After translation, Inteins catalyze auto-excitation of themselves and concomitantly join the flanking peptides without perturbing their biological function [53–54]. Inteins exist only in unicellular organisms including archaea, bacteria and eukarya, as well as virus and phages. There are more than one hundred inteins in nature. Inteins virtually can be harnessed to any polypeptide backbone, therefore serving as a useful molecular switch to control target proteins. To bypass the packaging limit of adenovirus-associated virus, several groups attempted to fuse split-Cas9 with intein fragments [55–58]. Upon co-expression, two split-intein fragments are capable of auto-splicing, which results in the recovery of full length Cas9. To further render this molecular switch inducible, modified inteins were generated that are sensitive to either temperature [59], light [60] or ligands [61–62].

The 4-OHT-responsive intein were created by insertion of the human LBD of ER into the *M. tuberculosis* RecA intein, and an evolved clone, 37R3–2 with higher splicing efficiency were identified [62–63]. To generate an optimally recovered Cas9 upon splicing, 37R3–2 were inserted in Cas9 at specific sites (S219 or C574) after testing 15 candidate sites (Fig. 2B) [64]. The authors further demonstrated that this conditionally active Cas9 induced a comparable genome editing efficiency as wild-type Cas9 at several endogenous genomic loci. Meanwhile, a much lower off-target effect was observed when compared with constitutively active Cas9. To decrease undesired background activation induced by an endogenous hormone β-estradiol, a point mutation (G521R) was introduced in the ER LBD, rendering selective response to the synthetic exogenous 4-OHT [65]. Notably, intein splicing was detected as early as 4 h after 4-OHT treatment. Additionally, unlike split Cas9, the recovered Cas9 was almost identical to wild-type Cas9 upon splicing. However, a major limitation of the intein-based system is that it is irreversible.

3.3. Systems Based on 4-OHT-ER Mediated Nuclear Translocation

The ER works as a transcription factor whose activity is regulated by the hormone estrogen. In the absence of estrogen, the ER is sequestered by heat shock protein 90 (Hsp90) in the cytoplasm. Upon ligand binding, it disassociates from Hsp90 and translocates to the nucleus, acting as a transcription factor [65]. The LDB of ER has been widely used as a drug inducible tool, the best known example of which is the generation of an inducible Cre recombinase upon ER fusion [29]. Distinct ER mutants with selective affinity to the synthetic 4-OHT over the endogenous β-estradiol were identified, which are critical for reducing undesired background activity, especially *in vivo* [66]. Till now, there are three ER mutants available: (1) mouse ER™ with a G525R mutation [67–68], (2) human ER^{T} with a G521R mutation [27,69] and (3) human ER^{T2} with G400 V/M543A/L544A triple mutations [28,66]. Among them, ER^{T2} has been widely used because of its high selectivity [28]. Multiple 4-OHT inducible CRISPR/Cas9 systems based on ER mediated nucleus translocation have been reported [32–33].

Table 1
Summary of drug inducible CRISPR systems.

Class	Design	Application	Reversible?	Drug	Model system	Drug property	References
Tet system	TRE-Cas9 H1-TetO-sgRNA	GE/TR	Y	Dox	HEK293T, HeLa, SKBR3, MCF 10A, mESC, hESC	FDA approved	[17–20] [21]
Cre-ERT2 system	LSL-Cas9:Cre-ER	GE	N	4OHT	Mouse	FDA approved	[32,33]
CIP systems	FRB/FKBP-split Cas9	GE/TR	N	Rapamycin	HEK293FT, N2A	FDA approved	[42,43]
	ABI/PYL1-dCas9/ADs	TR	Y	ABA	HEK293T	Experimental plant hormone	[45]
	GAI/GID1-dCas9/ADs	TR	Y	GA	HEK293T	Experimental plant hormone	[45]
	DmrA/DmrC-dCpf1/ADs	TR	Y	A/C hetero-dimerizer	HEK293, U2OS	Experimental	[52]
Intein splicing	intein-Cas9	GE	N	4OHT	HEK293	FDA approved	[64]
Nucleus translocation regulation	iCas	GE	Y	4OHT	HEK293T, HepG2, MSC, hESC	FDA approved	[70–72]
	HIT	GE/TR					
Allosteric regulation	ER-LBD insertion	GE/TR	Y	4OHT	HEK293T, BNL CL.2	FDA approved	[74]
Destabilized domain regulation	DHFR-Cas9/dCas9-ADs/PP7-ADs	GE/TR	Y	TMP	U2OS, HEK293T, hESC	FDA approved	[81,83]
	ER50-Cas9/MS2-ADs	GE/TR	Y	4OHT/CMP8	U2OS, HEK293T	FDA approved	[83]
	FKBP12-Cas9	GE	Y	Shield1	A549	Studied in clinical trials.	[84]

Abbreviations: GE: gene editing; TR: transcriptional regulation; Y: yes; N: no; mESC: mouse embryonic stem cell; hESC: human embryonic stem cell; MSC: mesenchymal stem cell.

3.3.1. HIT/iCas Systems

Our laboratory has developed an ER^{T2} based genome editing tool named Hybrid drug Inducible CRISPR/Cas9 Technologies (HIT)-Cas9, in which 2 NES and 2 ER^{T2} domains are fused sequentially at the C terminal of Cas9 to deliver efficient activity upon 4-OHT induction without introducing significant background in its absence (Fig. 2C) [70]. In iCas design, 4 ER^{T2} domains are fused with Cas9, 2 to each terminus [71]. In side by side experiments [70], HIT-Cas9 showed high efficiency, low background, and selective response to exogenous 4-OHT when compared with iCas [71], 4-OHT inducible-intein [64] and split-Cas9 [42]. In addition, our laboratory also developed HIT systems for inducible transcription activation by grafting ER^{T2} to previously existing dCas9 based transactivation devices, including direction fusion, SAM and SunTag [72]. After comprehensive optimization within each category and head-to-head comparison among the best performers, we concluded HIT-SunTag as the most efficient drug inducible activator, which includes three constructs, dCas9-NLS-GCN4, scFv-2E-VP64, and scFv-2E-PH. Furthermore, we generated HIT2 system for simultaneous genome editing and transcription activation by changing dCas9 with Cas9 in the HIT-SunTag system and altering sgRNA lengths for ramification of Cas9's DNA binding and cutting activities [72].

3.3.2. ER^{T2}- Split Cas9-FRB/FKBP

As previously discussed, Duy Nguyen et al. generated a rapamycin inducible split Cas9 system for genome editing [43]. To damp its background activity, they fused ER^{T2} to both the splitted Cas9-FRB/FKBP partners and found that this design confer a tighter drug control of both genome editing and transactivation, consistent with findings from HIT and iCas systems [70–72]. However, it required two small molecules rapamycin and 4-OHT to reach the highest efficiency. Finally, the authors successfully adopted similar design to a previously reported split SaCas9 [73], as well as the LBD of another nuclear hormone receptor, the glucocorticoid receptor α. This effort promised orthogonal regulation of different genomic loci under control of distinct drugs.

3.4. Systems Based on ER Conformational Switch

Instead of fusion, Benjamin Oakes et al. inserted ER-LBD within Cas9 to generate a different drug inducible system [74] (Fig. 2D). To identify suitable insertion sites, they generated transposon-based domain insertion libraries and identified that the optimal insertion sites are located at flexible loops, helix end and solvent-exposed residues. Motif insertion at these hotspots tend not to interrupt Cas9 activity. Further, they generated an ER insertion library. From paralleled screen with 4-OHT and counter-screen without 4-OHT, they obtained an arC9 with the ER-LBD insertion at the residue 231. This design was also expanded to dCas9 to accomplish drug inducible gene activation. The arC9:231 and darC9:231 both showed drug inducible activity in prokaryotic and eukaryotic cells, consistent with its mechanism of action based on conformational switch, not nuclear translocation. The authors also demonstrated that the nuclease activity of arC9 is reversible, which can be reactivated after 2 days recovery.

3.5. Ligand-DD Mediated Cas9 Stabilization

Thomas Wandless's laboratory generated a ligand dependent DD system. DD is an engineered unstable LBD that degraded in the absence of ligand. When DD fusion to a protein of interest, the stability of the fusion protein will be under small molecule control [75]. In the absence of small molecule ligand, DD directs the fusion protein to proteosome dependant degradation. Ligand binding to DD shields the fusion protein from degradation. Several ligand-DD pairs have been generated through mutant library screen, including Shield1-FKBP12 mutant [75–76], trimethoprim (TMP)-*Escherichia coli* dihydrofolate reductase (DHFR) mutant [77–81] and CMP8/4-OHT-Estrogen receptor destabilized domain (ER50 DD) [82].

FKBP12, ER50 and DHFR DDs have been applied to Cas9 system for inducible gene editing and activation [81,83–84] (Fig. 2E). As for drug inducible genome editing, DHFR or ER50 DDs were fused to both N- and C-terminals of Cas9 [83], while FKBP12 DD was fused to the N-terminal of Cas9 [84] (Fig. 2E left panel). To generate a drug inducible dCas9 activator, DHFR or ER50 DDs were fused to the N-terminal of PP7-ADs to render gene activation inducible based on the dCas9/sgRNA-aptamer/PP7-ADs design [85] (Fig. 2E right panel). Using different small molecule-DD pairs combined with different aptamers, for example 4-OHT:ER50 DD:MS2-ADs and TMP:DHFR DD:PP7-ADs, orthogonal regulation can also be achieved (Fig. 3B) [83]. Moreover, DHFR DD can be fused to the N-terminus of dCas9 activator directly to accomplish drug inducible gene activation [81].

4. Conclusion and Perspective

In this review, we discussed a variety of published drug inducible CRISPR/Cas9 systems. Key properties of each system were summarized in Table 1. Analyzing these systems altogether provides interesting observations: First, it appears that subjecting CRISPR/Cas9 to drug control commonly enables greater precision and less off-target activity, regardless of its working mechanism [70]. This promises an important

advantage of such approaches for scenarios, such as somatic gene therapy, where precision and safety are essential. Second, some of the drugs used in these systems are clinically relevant, including dox, 4-OHT, and rapamycin, *etc*., all approved drugs used in human being. Others are tool compounds whose application is currently limited in laboratory studies. Further translational studies using these systems would require optimization of these compounds to a clinical quality. Third, similar to any other drug inducible systems, those for CRISPR/Cas9 constantly face the dilemma between drug induced efficiency and background in its absence. It was observed multiple drug inducible systems commonly showed lower efficiency in comparison to a constitutive system [39,42,64,70–72,74,83]. It remains a challenge to less sacrifice signal in expense of noise activity. Fourth, reversibility is important in certain applications. Avoiding prolonged exposure of genomic DNA to Cas9 or Cpf1 might be important in reducing off-target effects. As for transcription regulation, a reversible control is a must for dynamic modulation. In this regard, Tet systems, systems based on ERT2 nucleus translocation and DD degradation, and CIP systems should be reversible either from prior knowledge or empirical testing [17,45,70–72,83–84]. On the contrary, split and intein systems are irreversible [42–43,64]. Fifth, the speed of response determines the temporal resolution of drug induction of a system. iCas was demonstrated to act faster than Tet-on, split, and intein systems, while HIT-Cas9 showed a similar speed of action with iCas [70–72]. Sixth, almost all systems were built for editing, while some were used for transcriptional regulation. Examination of all these systems for multiple purposes would further our understanding of their properties. Seventh, most of the drug inducible systems were based on SpCas9, while some have been adopted to Cpf1, SaCas9, and even TALE and TALEN [43,45,52,70,72,86]. Further expansion of these designs to other orthologous species will broaden their genomic coverage. Eighth, most of the systems have only been used in common tool cell lines such as HEK293T. Further application in animal models and more clinically relevant cell types such as human stem cells will add significant value for these drug inducible CRISPR systems.

Declaration of Competing Interest

We declare a conflict of interest associated with this paper: J.Z. and Y. W. have pursued a patent position on the invention of multiple HIT systems of drug inducible CRISPR or TALE(N).

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