Molecular Therapy
Review

Controlling CRISPR with small molecule regulation for somatic cell genome editing

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Biomedical research has been revolutionized by the introduction of many CRISPR-Cas systems that induce programmable edits to nearly any gene in the human genome. Nuclease-based CRISPR-Cas editors can produce on-target genomic changes but can also generate unwanted genotoxicity and adverse events, in part by cleaving non-targeted sites in the genome. Additional translational challenges for *in vivo* somatic cell editing include limited packaging capacity of viral vectors and host immune responses. Altogether, these challenges motivate recent efforts to control the expression and activity of different Cas systems *in vivo*. Current strategies utilize small molecules, light, magnetism, and temperature to conditionally control Cas systems through various activation, inhibition, or degradation mechanisms. This review focuses on small molecules that can be incorporated as regulatory switches to control Cas genome editors. Additional development of CRISPR-Cas-based therapeutic approaches with small molecule regulation have high potential to increase editing efficiency with less adverse effects for somatic cell genome editing strategies *in vivo*.

INTRODUCTION

CRISPR-Cas systems are now being translated into several gene therapeutic candidates, with several promising results coming out of clinical trials.[1–5] Many therapeutic candidates to date have utilized *Streptococcus pyogenes* (Spy)Cas9 (referred to as Cas9 hereafter). Since 2012, when Cas9 was first implemented for genome editing,[6] CRISPR-Cas9 has been applied in *ex vivo* clinical trials related to cancers,[7–9] human immunodeficiency virus-1 (HIV) infection,[3] sickle cell disease (ClinicalTrials.gov: NCT03745287),[10] and β-thalassemia (ClinicalTrials.gov: NCT03655678) (with follow-up ClinicalTrials.gov: NCT04208529).[10] There are now 37 Cas9-related clinical trials with 11 phase 2 studies in the US (as of May 2021; no phase 3 or 4 studies). *In vivo* editing of somatic cells within patients has been lagging behind many of the *ex vivo* editing strategies, which in part can be attributed to challenges posed by persistent Cas9 activity after delivery. However, *in vivo* strategies are emerging: in March 2020, a phase 1/phase 2 clinical trial (ClinicalTrials.gov: NCT03872479) targeted the *CEP290* gene *in vivo* to treat a childhood blindness disease (Leber congenital amaurosis type 10), and another *in vivo* trial targeted the transthyretin (*TTR*) gene to treat hereditary transthyretin amyloidosis (ClincialTrials.gov: NCT04601051) using Cas9 delivered by lipid nanoparticles.

While traditional genome-editing systems such as zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) rely on protein-DNA interactions to promote sequence-specific DNA binding, Cas9 relies on short sequences of guide RNA to target the gene loci of interest.[11] The targeted DNA sequence is adjacent to the protospacer adjacent motif (PAM) site, a 5-NGG-3 (where N is any nucleotide) sequence that binds and activates the Cas9 enzyme to induce a DNA double-stranded break (i.e., "cut" site). Following the cut, endogenous cellular DNA repair pathways can fix the cut primarily either through (1) non-homologous end joining (NHEJ) that leads to insertions or deletions, or (2) homology-directed repair (HDR)—particularly when an exogenous donor repair template is proximal to the cut DNA. While the CRISPR-Cas9 nuclease can modify genes, concern arises with cleavage at non-targeted sites, which can generate lethal, undesirable,[12,13] unpredictable mutations, and toxicity in particular cells, such as neurons, from Cas9 activity.[14] Given that constitutive expression of Cas systems may result in more unintended on-target and off-target site modification, there is a need to control CRISPR activity to address the *in vivo* translational challenges.[15]

This review focuses on efforts that attempt to control CRISPR activity, most particularly strategies using small molecules, to turn Cas9 "on" or "off." Small molecules are of interest, as many can pass through the cell membrane, be administered orally, and pass the blood-brain barrier (BBB). Furthermore, established screening technologies can easily identify or repurpose small molecules that can target various cell machinery. Such small molecule regulation can be applicable to other CRISPR systems such as Cas12 (which generates staggered DNA breaks distal to the PAM site),[16] dCas9 (catalytically inactive Cas9 that can be used for gene regulation, epigenetic editing, chromatin engineering, and even imaging),[17,18] and base editors derived from Cas proteins.[19]

KEY CHALLENGES FOR *IN VIVO* EDITING

Even though the CRISPR-Cas system can modify DNA at high efficiencies up to 80%,[20] there are several key challenges that need to be

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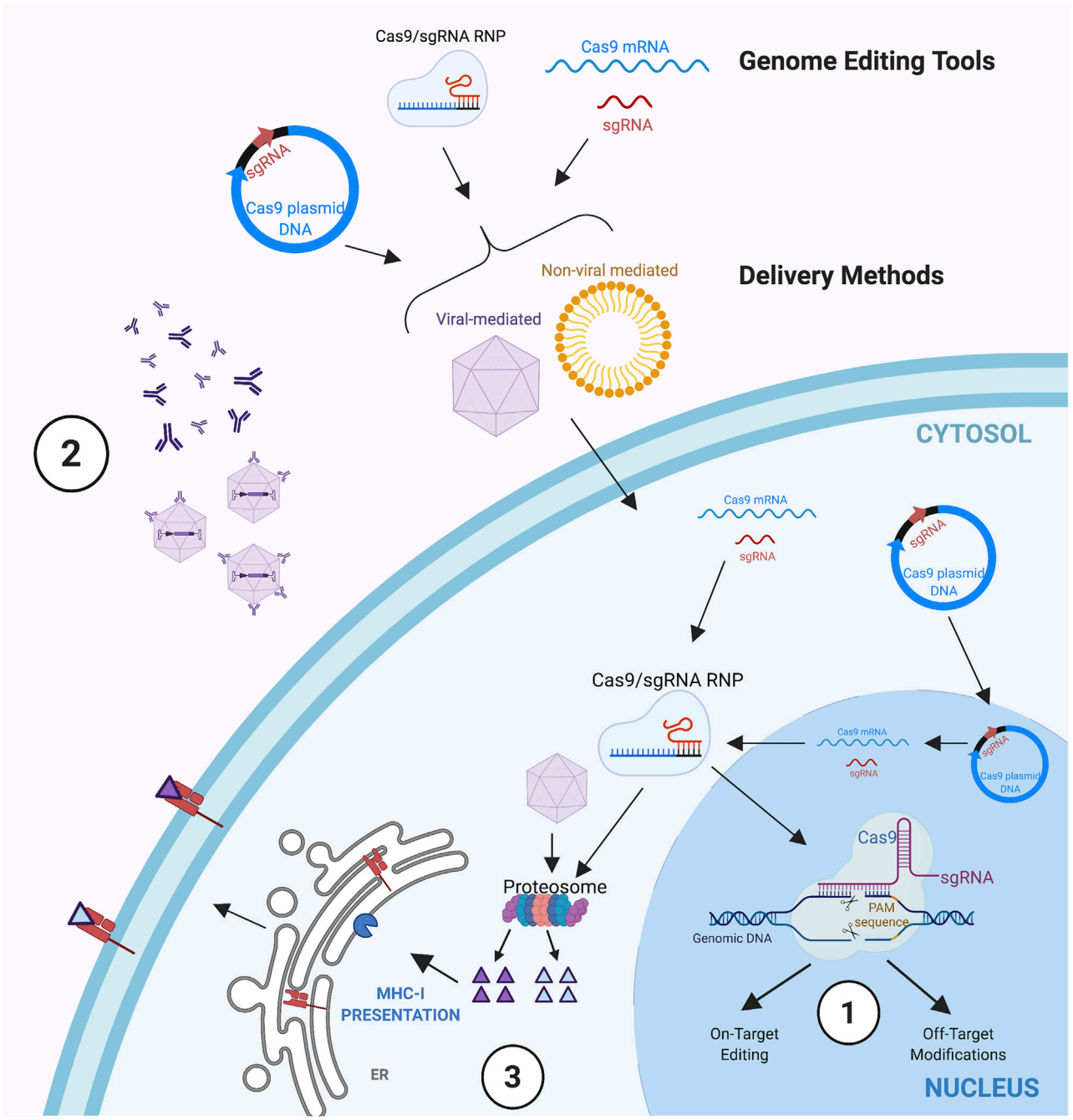

Figure 1. Three translational challenges for *in vivo* somatic cell genome editing with CRISPR-Cas9

(1) Off-target DNA double-strand break formation. The gene of interest is targeted by the single guide RNA (sgRNA) and cut by Cas9, and it results in double-stranded breaks that can subsequently be repaired to generate on-target edits. However, with prolonged exposure of Cas9 to the genome, there can be an increase in unwanted modifications at off-target sites. (2) Antibody neutralization to delivery vectors. Antigen-presenting cells may bind capsid proteins or other components from the delivery vector (e.g., polyethylene glycol [PEG]) to trigger an antibody-mediated immune response. (3) Immune response to Cas and vector proteins. MHC class I molecules may bind peptides from degradation of Cas9 and/or the viral vector and present them on the cell surface. These peptides could be presented to T cells and trigger an adaptive immune response.

addressed to make CRISPR-Cas systems more versatile for *in vivo* somatic cell therapeutic applications. One prominent safety concern involves the consequences of off-target DNA cleavage (Figure 1).[21–25] The single guide RNA (sgRNA) that targets a specific gene can tolerate up to five mismatches within the target site,[24,26] and hence there are typically hundreds of off-target sites in the human genome

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for a particular sgRNA. Decreasing the Cas9-sgRNA complex concentration has shown to improve the on-target/off-target ratio (cleavage specificity).[26] Tools to monitor off-target modifications have evolved quickly, and we refer the reader to several recent reviews on this topic.[27–29] The risk of off-target cutting can be exacerbated by prolonged expression of Cas9 and can be mitigated with methods to turn the CRISPR-Cas9 system off. Second, since Cas9 and other CRISPR-based systems are derived from bacteria, host immune responses may be an obstacle for CRISPR technology to go into the clinic (Figure 1). Major histocompatibility complex class I (MHC class I) immune responses by the host cells to Cas9 have been observed,[30] which can result in the elimination of Cas9-expressing cells. Hence, there is a need to develop methods to dispose of Cas9 immediately after editing has occurred; in this manner, the chance of Cas9 peptide presentation by MHC class I on cells would be lowered after delivery. Lower MHC class I presentation would reduce the chance that cytotoxic T cells would target these cells for elimination, thereby increasing the durability of any therapeutic effect from edited cells. In the extracellular space, intact Cas9 proteins can be recognized by pre-existing neutralizing antibodies and innate immune responses (Figure 1).[31] Non-viral vector methods, such as lipid-based vectors or nanoparticles with polyethylene glycol (PEG),[32,33] can be used to cloak Cas9 to potentially avoid these responses. For viral vectors, they can induce adaptive immune responses with repeated systemic administration.[34–37] Up to 50% of individuals are ineligible for AAV (adeno-associated virus) gene therapy through treatment or clinical trials because they have neutralizing antibodies to at least one of the AAV serotypes.[38–41] As a workaround in some cases, clinicians can immunosuppress a patient to avoid the initial immune response to AAV or other viral vectors.

CONTROLLING CAS9 VIA DELIVERY STRATEGIES

Inefficient delivery of therapeutic genome-editing payloads to defined cell types and tissues is still an outstanding challenge in the field,[42] but delivery strategies themselves can be exploited to establish control of Cas9 activity. The most common delivery strategy for limiting Cas9 activity and off-target editing involves transient delivery of either Cas9-encoding plasmid or messenger RNA (mRNA) or direct transient delivery of Cas9-sgRNA ribonucleoproteins (RNPs).[43] Transient delivery limits the levels of Cas9 proteins or active RNPs within cells to the lifetime of the protein or complex within the nucleus. For instance, mRNA encoding CRISPR base editors delivered to the livers of cynomolgus monkeys were cleared within 2 weeks but resulted in durable therapeutic responses for several months.[20] However, transient non-viral delivery for many applications is generally less efficient than viral delivery.[42] Adenoviruses (AdVs) and AAV vectors are the most prominent viral mode of Cas9 delivery. The packaging of CRISPR components into a single AAV vector is limited because the carrying capacity of AAV vectors is ~4.7 kb,[44] and the size of the Cas9 gene alone is ~4.3 kb;[30] additionally, in combination with the guide RNA and necessary elements (i.e., promoter, fluorescent proteins, and polyadenylation sequences), the size of the Cas9 system reaches more than 5 kb. Hence, the Cas9 system frequently needs to be split into two or more AAV vectors to be delivered.[45,46] The vector size problem can also be resolved by utilizing a smaller Cas protein that can fit AAV vectors for *in vivo* delivery such as Cas12a that uses a single nuclease domain to cleave complementary and non-complementary strands of DNA[47] or a "SauCas9" from *Staphylococcus aureus*.[21,48] Alternatively, a larger vector such as AdV or lentivirus may be used to deliver large transgenes.[49] The intein-mediated split-Cas9 is another way to reconstitute parts of Cas9.[50,51] Inteins are proteins that can splice translated polypeptide into a functional protein. Cas9 halves are fused to split inteins that are co-expressed in dual recombinant AAV vectors, and the full Cas9 is expressed once intein-mediated *trans*-splicing occurs.

In addition to controlling Cas9 via delivery, inducible systems can be implemented to stimulate Cas9 to edit the genome at specific times and sites, turning the system on and off. Switches for inducible CRISPR-Cas9 systems include small molecules,[52] light (including near infrared [IR]/UV),[46,53–56] magnetic fields,[57] and temperature.[58] These switches can easily be layered with other methods to control dose, timing, and localization of Cas9. Below, we focus on small molecule switches with CRISPR systems that have the potential to be implemented *in vivo* for therapeutic applications.

INDUCERS TO REGULATE CAS9 ACTIVITY AND ABUNDANCE

Switching on recombinases, promoters, and proteins fused to Cas9 are various ways to activate genome editing within cells. The Cre-recombinase system can give localized control over Cas9 expression via use of small molecules or tissue-specific promoters. In these strategies, the loxP-stop-loxP cassette is placed between the promoter of interest and the Cas9 coding sequence. Following administration of the small molecules, genomic recombination of the cassette within cells can result in the activation of Cas9. The bulk of research with Cre-dependent control of CRISPR-Cas9 has been to inactivate or knockout genes in various animal models.[59–62] The Cre-controlled CRISPR (3C) mutagenesis system uses a ligand-dependent chimeric Cre-recombinase, known as CreER recombinase.[59] The CreER recombinase is inactive until the synthetic estrogen receptor ligand 4-hydroxytamoxifen (4-OHT) is added to induce recombination and activate Cas9 activity. This method provides both spatial and temporal control: floxed chromosomal DNA is excised at specific promoters once 4-OHT is added. Some challenges in using recombinases and CRISPR-Cas9 together for somatic cell editing include the need to engineer recombination sites into the genome, toxicity of inducers, leakiness of gene expression in the absence of the inducer, and promoters not being available for every tissue.[63]

Cas9 activity can also be controlled by using small molecules to control sgRNA expression[64] through aptamers,[64–68] aptazymes,[69] or other sgRNA-controlling switches.[70] A doxycycline-inducible sgRNA expression system modulates the activity of the sgRNA rather than the Cas9 (Figure 2).[71] The sgRNA is driven by a U6 promoter that contains either one or two TetO operator sites. The tightly controlled double TetO system showed high cleavage efficiency after induction with negligible background activity in 11 human and mouse cell lines *in vitro* and a hematopoietic reconstitution mouse model.

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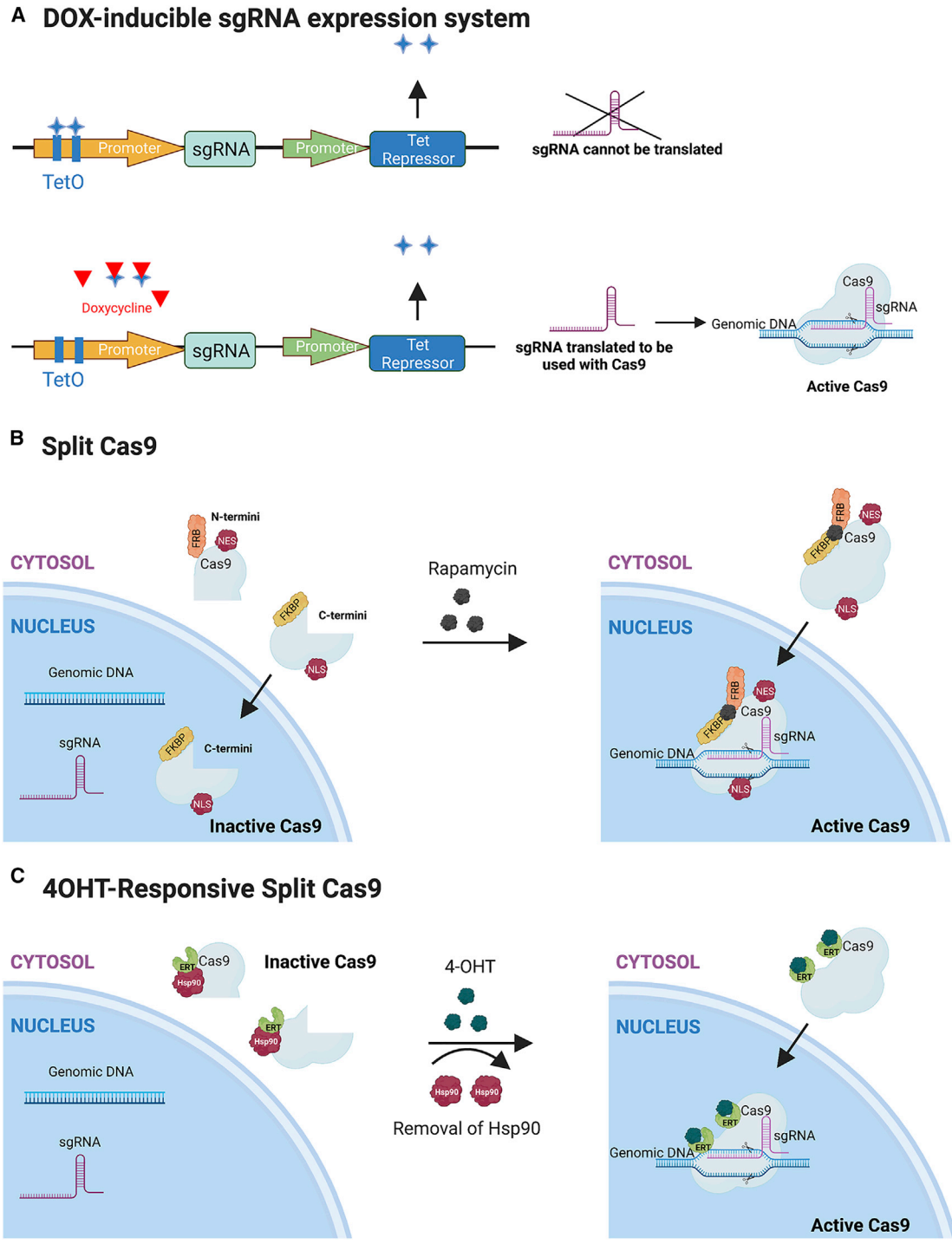

Figure 2. Small molecule inducible activation and split systems to control sgRNA or Cas9 expression

(A) The doxycycline-inducible sgRNA expression system[71] has a Tet repressor that keeps the sgRNA from being translated, so that Cas9 is not functional. However, once doxycycline is added, the repressors will no longer bind to the Tet-responsive sequence (TetO) and sgRNA will be expressed to use with Cas9. (B) The rapamycin-inducible Cas9[72] is split into two lobes, an N terminus lobe that has the FRB component and a nuclear export signal (NES), and the C terminus lobe that has FKBP and a nuclear localization signal (NLS). Without rapamycin, only the C terminus Cas9 goes into the nucleus and is not functional. With the addition of rapamycin, the lobes are fused via FRB and FKBP binding and the functional Cas9 is shuttled to the nucleus for activity. (C) The 4-hydroxytamoxifen (4-OHT)-responsive split Cas9[73] is sequestered in the cytoplasm and has multiple ligand domains of the estrogen receptor (ERT). Addition of 4-OHT releases the Cas9 fragments from a heat shock protein (Hsp90) and translocates the reconstituted Cas9 to the nucleus for genome editing.

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Timing and exposure of small molecules such as doxycycline have been shown to reduce toxicity and off-target effects that occur with constitutive expression of Cas9 complexes.[74] With a timed doxycycline system, in mouse embryonic stem cells (mESCs), there was only 1 out of 18 off-target sites that showed indels.[74] Doxycycline has also been utilized in the "self-inactivating CRISPR (SiC)" system,[75] which is composed of a sgRNA that can deactivate Cas9 in a timely manner through Tet-repressor based feedback.[76] This system reduced off-target effects (with the on-target/off-target editing raising from 0.8 to 1.3 after the addition of doxycycline).[75] Another recent study developed a Tet-On system called ObLiGaRe doxycycline inducible Cas9 (ODInCas9) that uses a doxycycline-inducible Cas9 to temporally regulate Cas9 in human induced pluripotent stem cells (iPSCs) and mESCs.[77] This ODInCas9 cassette can induce Cas9 expression *in vivo* with no background Cas9 activity and no leaky or sustained expression of Cas9.[77] Even though doxycycline-inducible promoters have been used to control CRISPR-Cas9 transcriptional activity,[71] these systems take several days to achieve maximum Cas9 activity and thus do not provide the high temporal resolution needed in some studies.[73] Regardless, tight control of Cas9 expression could be valuable for evading immune responses *in vivo*.

In an attempt to control the kinetics of genome editing, small molecules have also been used to drive the fusion between parts of Cas9.[72,78] The Cas9 system was split into N and C termini with the FKBP rapamycin binding (FRB) domain and the FK506 binding protein 12 (FKBP) fused to each terminus, respectively (Figure 2).[72] In the presence of the small molecule rapamycin, FKBP and FRB heterodimerize and reconstitute, making Cas9 functional. Without the addition of rapamycin, there was 10% indel frequency because of Cas9 auto-assembly.[72] The leaky Cas9 issue led the authors to localize the Cas9(C)-FKBP fragment to the nucleus with a nuclear localization sequence (NLS) and sequester the Cas9(N)-FRB portion in the cytoplasm with a nuclear export sequence (NES).[71,74,73,79,80] When rapamycin was present, there was inducible activation of Cas9. As a result, there were less off-target indels (5%–10%) as compared to wild-type (WT) Cas9 (27%) after half of Cas9 was nuclearized.[72] Nguyen et al.[73] added another layer of control by linking each half of Cas9 with the ligand-binding domain ERT from the estrogen receptor to sequester Cas9 in the cytoplasm; the synthetic ligand 4-OHT was needed to express the split Cas9 and translocate it to the nucleus (Figure 2). This method resulted in quick CRISPR activation with low background activity without rapamycin and high tunability. In this study, when rapamycin was administered, human embryonic kidney 293T (HEK293T) cells exhibited up to ~25% WT Cas9 activity.[73] By activating the system using 4-OHT and changing activation domains, background activity was reduced. The split Cas9s can be delivered in two viral or non-viral vectors, and therefore overcome the limitations imposed by the packaging capacity of a single vector. Even though there was minimal background activity for Cas9 genome editing in the study by Nguyen et al., the leakiness of the inducer could pose a challenge for translational somatic cell-editing applications.

As mentioned previously, intein-mediated split-Cas9 can also be useful in addressing vector size issues. In a study with Neuro-2a (N2a) cells, split Cas9 indels were similar to WT Cas9 (23.1% and 22.7%, respectively), showing that split inteins did not affect endonuclease activity.[50] Another study used a 4-OHT-responsive intein for Cas9 in HEK293 cells and found up to a 25-fold higher specificity (on-target/off-target indel frequency ratio) with low background activity in the absence of 4-OHT (Figure 3).[81] This intein-inducible system also bypasses the packaging limits using one vector, as the split components can be delivered separately. In contrast to other split systems, once splicing occurs, the system is irreversible. The iCas system (Figure 3) also tightly controls Cas9 through a mutated ligand-binding 4-OHT, but, unlike the 4-OHT-responsive intein and the split-Cas9 method, it is reversible.[82] Moreover, compared to the intein-Cas9 and split-Cas9, the iCas system has a higher editing efficiency (i.e., higher cleavage specificity),[82] but it had only 60% of WT Cas9 activity at most. Oakes et al.[83] added a ligand-binding domain of human estrogen receptor-α to Cas9, creating a 4-OHT-responsive Cas9 called allosterically regulated Cas9 (arC9). There was no background when 4-OHT was absent. arC9 exhibited only 30% of editing as compared to WT Cas9 once 4-OHT was added, and arC9 showed slow reversibility after removal of 4-OHT.[83]

INDUCIBLE DEGRADERS TO REMOVE CAS9

Inducible degradation strategies can be used to regulate Cas9. Degrons are typically short amino acid sequences located in the flexible regions of the protein with easy access to other proteins and transcriptional factors,[84] which can assist with degradation. Fusion of degrons to Cas9 would be able to eliminate the protein from the cell. Examples of such systems that include degrons are the auxin-induced degradation (AID) systems,[68,85] degradation tag (dTAG),[86] and the small molecule-assisted shut-off (SMASh) system (Figure 4).[87]

In the AID system, the auxin-binding receptor (osTIR1) from the rice plant and the plant hormone auxin are key components. In non-plant cells, TIR1 complexes with the conserved ubiquitin E3 ligase Skp1-Cul1-F box (SFC),[89–92] which is only activated in the presence of a natural or synthetic auxin termed indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) (Figure 4).[91] Kleinjan et al.[68] showed that a IAA17-dCas9 can efficiently be degraded with co-transfection of the auxiliary protein (osTIR1) and auxin. Expressing the osTIR1 gene under the control of a tetracycline-inducible promoter gives dCas9 tissue specific control.[68] However, this system requires a high dose of auxin and expression of osT1R1, which are not endogenous to humans, and currently, there is not sufficient research on the effects of these components on humans.

The dTAG system developed by Nabet et al.[86] avoids the usage of exogenous co-expression of degradation factors that are needed for the AID system. The dTag system has recently been shown to induce Cas9 degradation in HEK293T, U2OS, and *Drosophila*'s S2 cell lines.[88] The $FKBP12^{F36V}$ tag is fused to multiple parts of Cas9 (Figure 4).[88] When the heterobifunctional small molecule degrader dTAG is added, it binds to a E3 ubiquitin ligase on one side and

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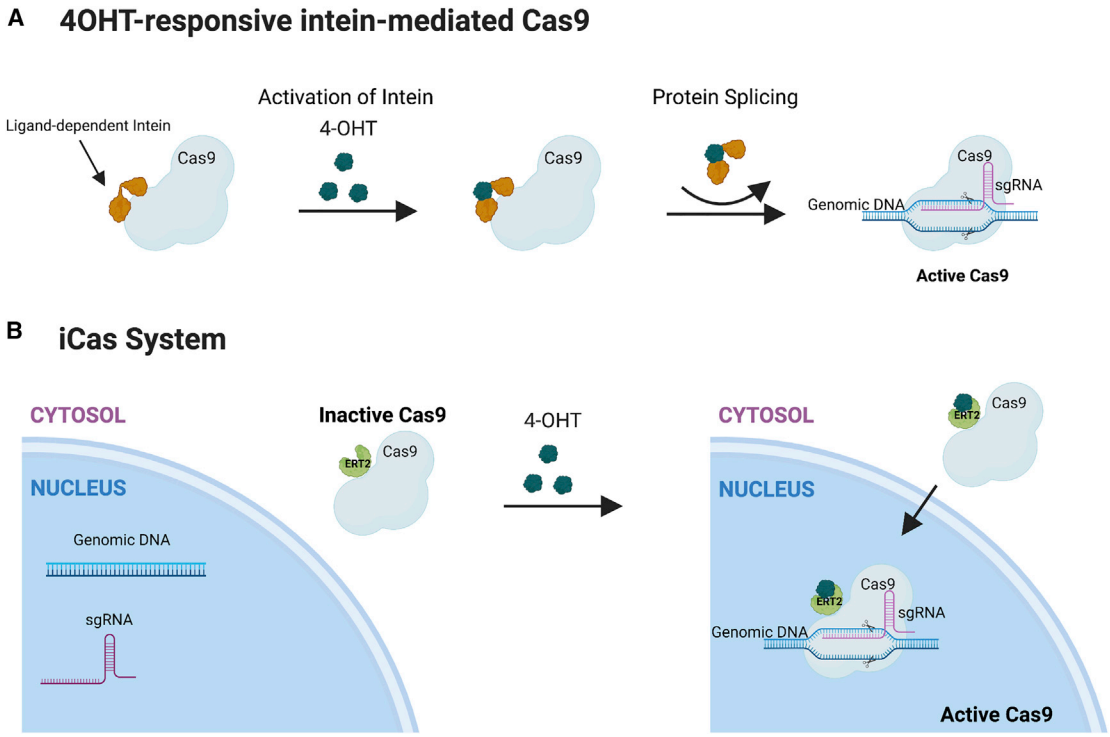

Figure 3. Small molecule inducible systems to control Cas9 expression

(A) The intein-mediated Cas9[81] has Cas9 fused to intein sequences. Once 4-OHT is added, the inteins are spliced out, leaving a functional Cas9. (B) The iCas system[82] consists of Cas9 fused to the hormone-binding domain of the estrogen receptor (ERT2). Upon addition of 4-OHT, which binds to ERT2, Cas9 is translocated to the nucleus to partake in gene-editing activity.

targets $FKBP12^{F36V}$ with the other. The E3 ligase hijacks the cell machinery and sends Cas9 for degradation. The FKBP-Cas9 fusion has shown a higher on-target/off- target ratio (cleavage specificity) than does WT Cas9. With the addition of the small molecule dTag-47, Cas9 shows 80%–90% degradation at doses as low as 12 nM.[88] Similar to that of the AID system, the immunogenicity of the dTAG components in humans has not been well characterized.

An alternative destabilizing degron is called the SMASh, with a degron domain from the hepatitis C virus (HCV) with a protease domain. A small molecule-controlled Cas9 repressible system was created with the fusion of the SMASh tag with Cas9.[87] Upon addition of a clinically approved HCV protease inhibitor called asunaprevir (ASV), Cas9 is degraded (Figure 4).[87] However, in the absence of this inhibitor, the SMASh tag self cleaves and removes itself to stabilize Cas9. Adding the SMASh tag to both the N and C termini of Cas9 yielded more than 50% degradation and 50% less indel formation with the addition of ASV as compared to having the SMASh tag on only the C termini. Moreover, because ASV only degrades newly synthesized Cas9, removal of ASV leads to restoration of gene editing activity. As with the dTag system, limiting Cas9 duration also enhances specificity of gene editing. Cas9 has also been fused to the FKBP12-derived destabilizing domain system that is stabilized with a synthetic ligand, Shield-1.[93,94] There are still many degrons and degrader systems that have not been tested in Cas9, and we refer the reader to a review on more degraders that can be used to control Cas9.[95,96]

SUITABILITY FOR SOMATIC CELL GENOME EDITING

Applying control strategies for *in vivo* somatic editing may be able to address the key challenges described in the first section concerning off-target adverse events, delivery, and immunogenicity. In Table 1, we compare and contrast how the inducible CRISPR-Cas9 systems can be evaluated across three key categories: (1) the degree of uninduced editing, (2) the degree of editing upon inducer addition, and (3) the animal model/cell type used to test the system.[71] Several activator strategies may be appropriate for translational studies. The doxycycline-inducible, Cre-recombinase system has been tested in mouse models on a variety of genes, but only now are doxycycline-inducible Cas9 systems being introduced into the field for therapeutic purposes. While there is high control over the timing of Cas9-mediated editing with doxycycline in Cre-recombinase systems without a significant reduction in editing activity, this strategy is usually irreversible upon activation. Additionally, diffusion of doxycycline into and out of cells can lead to genotoxicity related to off-target effects/recombinase.[77,97] Moreover, doxycycline at higher concentrations has been shown to cause a cytotoxic effect or a decrease in mitotic activity in human cell lines.[98,99] In general, diffusion and transport into various tissues is a concern for establishing broad control over genome editing inside the body, especially

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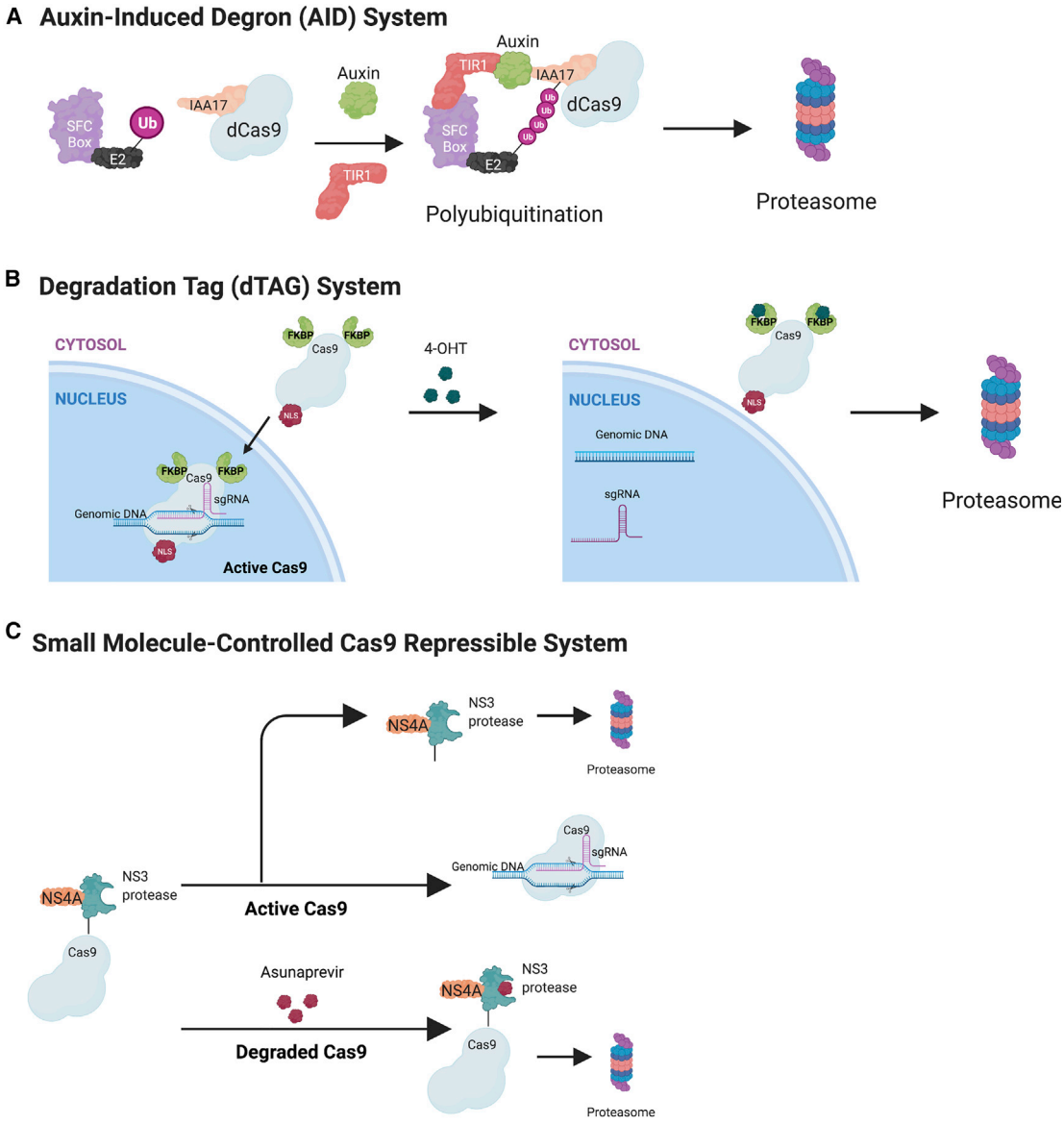

Figure 4. Inducible degradation systems to control CRISPR-Cas9 genome editing

(A) Auxin-induced degron (AID) system.[68] dCas9 can be fused with the degron IAA17. T1R1 is the auxin-binding receptor from the rice plant, and auxin is a plant hormone. In non-plant cells, T1R1 complexes with the conserved ubiquitin E3 ligase and Skp1-Cul1-F (SFC) box. dCas9 has been shown to be degraded with the AID system but has not been demonstrated with the Cas9 nuclease. (B) Degradation tag (dTAG) system.[88] Cas9 can be fused to multiple mutant FKBP12 (i.e. $FKBP12^{F36V}$). Once $FKBP12^{F36V}$ is polyubiquitinated, Cas9 will be degraded along with the $FKBP12^{F36V}$. (C) Small molecule-controlled Cas9 repressible system.[87] Cas9 is fused to a SMASh tag. Upon the addition of the inhibitor asunaprevir (ASV) that attaches to the NS3 protease and NS4A degron, Cas9 is degraded. When asunaprevir is removed, the NS3 protease self-cleaves and is degraded with the NS4A, while Cas9 is fully functional.

for the brain and spinal cord (Table 2). Both the SiC system and ODIn-Cas9 have the ability to turn Cas9 on and off directly instead of constitutively expressing Cas9 such as the 3C system.[75,77] The disadvantage to methods such as these is that the system takes days to achieve maximum Cas9 activity because the Tet-inducible system uses repressor-based feedback regulation of Cas9. Moreover, the SiC method only inactivates Cas9 and keeps the Cas9 protein still expressed in the system, which may lead to host immune responses.

The split systems are useful in terms of bypassing packaging limits, since multiple vectors deliver Cas9 components. The rapamycin-inducible split Cas9 could be a win-win for control and immunogenicity.[72] Because rapamycin is an immunosuppressive drug,[112] it can alter the immune response to the CRISPR-Cas9 editor during editing. However, with the initial FRB/FKBP and intein-mediated split Cas9,[50,72] the systems were irreversible: once activated, Cas9 would remain constitutively active. Within a year, researchers overcame

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Table 1. Potential inducible CRISPR-Cas9 systems for somatic cell genome editing

Inducer/small molecule	"Leakiness" (degree of uninduced editing)	Degree of editing upon inducer addition	Animal model(s)/cell type(s) used to test system	Advantages	Disadvantages	Reference
Cre-controlled CRISPR (3C) mutagenesis system	not reported	not reported	zebrafish embryos (RPE cells or in neural crest-derived melanocytes)	• heat treatment is also needed to display gene editing • zebrafish line can be used for conditionally controlled tissue-specific biallelic gene inactivation	• has not been tested in human cells • cannot be used for somatic cell editing in humans due to their inability to produce cre-recombinase	Hans et al.[59]
2xTetO DOX-inducible sgRNA system	low (0%–14%)	20%–80% (high cleavage activity) (activity score >0.5 for most cell types)	L-363, MC-38, A-498, LL/2, LP-1, 786-0, NCI-H1299, CT26, 4 TI, HEK293T, mouse (spleen cells, bone marrow cells, blood cells)	• can be used for high-throughput genetic screening • mouse model available for this method • DOX can be paired with IPTG-inducible systems to enable modulation of two genes independently	• variable editing efficiency in different cells	Sun et al.[71]
Cre-recombinase doxycycline	not reported	not reported	mouse/mESCs	• can be light activated • temporal advantage since system is regulated by doxycycline	• non-reversible • diffusion of small molecules in and out of cells causes off-target recombination • DOX at higher concentrations has been shown to cause cytotoxic effect in human cell lines • cannot be used for somatic cell editing in humans due to their inability to produce cre-recombinase	Dow et al.[74]
Self-inactivating CRISPR (SiC) with doxycycline	not reported	on-target/off-target editing 0.8 to 1.3 after addition of doxycycline	myeloid and lymphoid cells *in vivo*, both in mouse peripheral blood and bone marrow as well as *in vitro* human lymphocytes (HL-60s)	• Cas9 on and off method exists	• system takes days to achieve max Cas9 activity, so there is a longer time for not only on-target editing but off-target cleavages as well	Kelkar et al.[75]
ObLiGaRe doxycycline-inducible SpCas9 (OdInCas9)	no Cas9 background activity	not reported	hiPSC, HCT116, HEK293, HepG2, A549, OVCAR8, N2a cells	• inducible Cas9 expression • reversibility with DOX withdrawal • low immune response with use of AAV to deliver sgRNA	• off-target recombination • not able to use for somatic cell editing in humans due to Tet systems not being able to be reproduced in humans	Lundin et al.[77]
Split FK506 binding protein 12/FKBP rapamycin binding (FKBP/FRB) system	10%	fewer off-target indels (5%–10%) as compared to WTCas9 (27%)	HEK293FT	• inducible Cas9 expression • rapamycin is crucial to provide temporary immunity against Cas9	• auto-assembly problematic when half of Cas9 not nuclearized • non-covalent protein dimerization	Zetsche et al.[72]

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Table 1. *Continued*

Inducer/small molecule	"Leakiness" (degree of uninduced editing)	Degree of editing upon inducer addition	Animal model(s)/cell type(s) used to test system	Advantages	Disadvantages	Reference
4-Hydroxytamoxifen (4-OHT) with split FKBP/FRB system	none to low background activity	25% Cas9 activity	HEK293T	• high tunability • split systems can easily be delivered in multiple plasmids for efficient delivery • rapamycin could reduce the immune response against Cas9	• no reversibility • Cas9 remains constitutively active	Nguyen et al.[73]
4-OHT-responsive intein for Cas9	low	25-fold higher specificity (on-target/off-target indel frequency)	HEK293 cells	• improved specificity	• irreversible • split proteins can form aggregates due to exposed hydrophobic core	Davis et al.[81]
iCas system	low (but higher than split-Cas9 and comparable to intein-Cas9)	38%–60% editing	HEK293	• higher editing efficiency (as compared to split Cas9 and 4-OHT-responsive intein-mediated Cas9 at multiple loci) • reversible	• high background activity	Liu et al.[82]
Allosterically regulated Cas9 (arC9)	no background	30% editing	*E. coli*, HEK293T, murine BNL CL.2 cells	• reversible	• slow reversibility (took 2 days)	Oakes et al.[83]
Degradation tag (dTAG) system	low	on-target/off-target editing is enhanced (depending on the site, 1.5-fold to 4-fold as much compared to WT Cas9)	HEK293T, U2OS.eGFP.PEST, *Drosophila* S2 cells, mESC cell line	• small molecule activation provides temporal control • Cas9 target specificity is enhanced by addition of dTag • degradation of Cas9 allows for control of editing outcome • reversible	• while said to be reversible, there is no data on reversibility with Cas9 • tested in only a few organisms	Sreekanth et al.[88]
Small molecule-Controlled Cas9 repressible system	not reported	on-target/off-target editing is enhanced	HEK293T cells	• reversible • no structural modification from tagging • easy to measure half-life kinetics	• existing Cas9 cannot be degraded • exact mechanism is unknown	Wu et al.[87]
Auxin-induced degron (AID) system	low background with auxin absent	not reported	HEK293FT, CHO-K1	• makes tissue-specific activity controllable • reversibility with auxin removal when using miniAID system • minimal basal degradation	• receptor (osTIR1) might be immunogenic • high dosages of auxin osTIR1 needed to attain degradation • IAA has been shown to cause toxicity at high amounts[100]	Kleinjan et al.[68] Li et al.[85]

Selected genome editing studies that have been conducted using small molecule regulation are compared here. Leakiness, degree of editing upon inducer addition, and animal model/cell type used to test the system are summarized, with the reference indicated on the right. Leakiness is the degree of editing that occurs when the small molecule is not added to activate the system. Animal/cell models: RPE, retinal pigment epithelium; L-363; human plasma cell leukemia cell line; MC-38, C57BL/6 murine colon adenocarcinoma cell line; A-498, *Homo sapiens* kidney carcinoma cell line; LL/2, murine Lewis lung carcinoma cell line; LP-1, human myeloma cell line; 786-0, kidney adenocarcinoma cell line; NCI-H1299, human non-small cell lung carcinoma cell line; CT26, undifferentiated colon carcinoma cell line; 4T1, murine breast cancer cell line; HEK293T/FT, human embryonic kidney cell line; HL-60, human lymphocyte line; hiPSC, human-induced pluripotent stem cell; HCT116, human colon cancer cell line; HepG2, human liver cancer cell line; A549, adenocarcinoma human alveolar basal epithelial cell line; OVCAR8, human ovarian carcinoma cell line; N2a, mouse neuroblastoma cell line; U2OS, *Homo sapiens* bone osteosarcoma; CHO-K1, Chinese hamster ovary cell line.

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Table 2. Small molecules with potential to cross blood-brain barrier (BBB)

Small Molecule	System(s)	Crosses BBB?	References
4-Hydroxytamoxifen (4-OHT)	Cre-controlled CRISPR (3C), 4-OHT with split FKBP/FRB system, 4-OHT-responsive intein for Cas9, ERT2-based iCas system, allosterically regulated Cas9 (arC9)	yes	Rotheneichner et al.[101]
Doxycycline (DOX)	2xTetO DOX-inducible sgRNA system, self-inactivating CRISPR (SiC), ObLiGaRe doxycycline inducible Cas9 (ODInCas9)	yes	Norrby et al.[102]
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	2xTetO DOX-inducible sgRNA system	yes	Morton et al.[103] and Ryan and Scrable[104]
Rapamycin	split FK506 binding protein 12/FKBP rapamycin binding (FKBP/FRB) system	yes	Majumder et al.[105]
Auxin	auxin-induced degron (AID) system	possibly, shown to work in *Drosophila melanogaster*	McClure et al.[106]
Indole-3-acetic acid (IAA)	AID system	possibly (but highly unlikely to use because of toxicity)	Lin et al.,[107] Chen et al.,[108] Hąc-Wydro and Flasiński,[100] and Puurunen et al.[109]
1-Naphthaleneacetic acid (NAA)	AID system	possibly, shown to work in *Drosophila melanogaster*	Lin et al.[107] and Chen et al.[108]
dTag-47	dTag System	unknown	N/A
Asunaprevir	SMASh	no	Koduri et al.[110]
Shield-1	mutated FKBP12-derived destabilization domain	yes	Froschauer et al.[111]

The ability for small molecules to penetrate the BBB could be helpful for controlling genome editing in the central nervous system.

this issue by fusing estrogen receptors to split and intein-mediated Cas9,[73,81] and they developed the iCas and arC9 to harness system reversibility by using 4-OHT.[82,83]

Advantages of a degrader, such as the dTAG system, are that it can be regulated via small molecules and/or light, does not need any exogenous elements like the AID system does, and has a cell-permeable small heterofunctional degrader. The AID system includes a receptor that cannot be found in the human body, but it can associate with the ubiquitin machinery within human cells. By placing the osTIR1 gene under tissue-specific promoters, Cas9 activity can be spatially controlled. However, even when condensing the IAA17 to mini-IAA7,[91,113] the tag is still 7.4 kDa and fusion to Cas9 may hinder Cas9's editing ability and stability. The small molecules in both the AID and dTag systems have not been very well characterized in humans as compared to the SMASh system that uses a clinically approved drug. ASV cannot cross the BBB, so it would not be useful for control in the central nervous system, but the small molecule Shield-1 can cross the BBB (Table 2).[110,111] Using the FKBP destabilizing domains fusion with Cas9 enables conditional and temporal control of Cas9 via Shield-1 that is necessary for Cas9 activity.[93]

LAYERED AND COMBINATORIAL CONTROL

All of the above studies that use small molecules to control Cas9 or its sgRNA could be combined or layered with additional control strategies for CRISPR that do not rely on small molecule application. First, inhibitor proteins can be used to inactivate the functional Cas9, which would reduce off-targets related to prolonged Cas9 activity. Bacterial phages express anti-CRISPR (Acr) proteins to inhibit immune functionality.[114–118] These natural Acrs can be useful in regulating Cas9 activity and acting as "off switches" for the CRISPR-Cas9 system.[115,117] Most Acr proteins have been tested with dCas9. AcrIIA2 and AcrIIA4 inhibit dCas9, with AcrIIA2 blocking ~25% of dCas9 function and AcrIIA4 blocking 85% of dCas9.[115] In HEK293T cells, co-expression of either Acr protein reduced Cas9-based gene editing.[115] In human K562 cells, AcrIIA4 shows nearly complete inhibition of Cas9 at three different target loci.[119] Methods have also been developed to control the activity of Acrs spatiotemporally using light. AcrIIC3 has been engineered to be light-dependent to control *Neisseria meningitidis* Cas9,[46] and AcrIIC4 has been engineered to be light-dependent to inhibit *Spy*Cas9.[53] Strategies where constitutively active Cas9 is functionally deactivated using inhibitors have high translational potential. However, photoactive Cas9 methods require laboratories to have specialized illumination devices, and penetration of light into tissue is limited.[120] To avoid the use of illumination devices, inhibitors may be able to work in combination with one of the other small molecules mentioned previously or delivered in AAV vectors after efficient editing has occurred via Cas9. Furthermore, there could be immune responses to the Acr inhibitory proteins as well as to Cas9 because Acr proteins only inactivate Cas9 and do not repress the expression.

Second, translational control could be combined to avoid editing in nontarget cells/tissues.[121,122] MicroRNAs (miRNAs) are short, single-stranded non-coding RNA molecules that can regulate gene expression post-transcriptionally by either inhibiting the translational

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pathway and/or targeting particular mRNAs for degradation.[123,124] Since the activity of miRNAs differ among cell types, it makes miRNAs effective markers to track in targeted cells.[121] The miR-Cas9 switch represses Cas9 when the target miRNA is expressed (OFF-state) and activates Cas9 when the target miRNA is absent (ON-state).[125] The initial miRNA-Cas9 switch study by Hirosawa et al.[125] was in HeLa cells, human iPSCs, and iPSC-derived differentiated neuronal cells. The authors initially found an ON-state that had leaky Cas9. To fix this issue, the researchers regulated Cas9 by using Acr protein that responds to miRNA. AcrIIA4 activity was regulated by miRNA to turn the system ON to achieve cell type-specific editing and activation in HeLa cells.[121] Similarly, a miRNA-responsive AcrIIA4 system for cell-specific genome editing was developed by Hoffman et al.[122] and tested in hepatocytes, cardiomyocytes, human hepatocellular carcinoma cells (Huh-7), human cervix carcinoma cells (HeLa), and human embryonic kidney cells (HEK293T).[122] Unlike Hirosawa et al., the more recent Cas-ON-switch design had a post-translational negative feedback loop based on Acr proteins. This strategy was tried on variants of Cas9, including dCas9-effector fusions and *Nme*Cas9. Developing systems that combine synthetic RNA switches that respond to internal endogenous signals or miRNAs with the CRISPR-Cas system are ideal as they circumscribe editing to target cells and, in theory, do not affect other cell types.

Lastly, all of the above studies use small molecules to control Cas9 or its sgRNA could also be layered upon additional control strategies that utilize endogenous or self-deleting/restrictive mechanisms. First, Oakes et al.[126] use endogenous proteases to trigger activation of Cas9. Circular permutation was used to reengineer Cas9 for a diverse range of protease-sensing Cas9s (ProCas9s). Data showed that there was no background activity for the ProCas9s prior to proteolytic cleavage for two cell lines and up to 35% genome editing. Second, the self-deleting system was tested *in vitro* (HEK293T cells) as well as *in vivo* (male C57BL/6J mice) by Li et al.[127] An AAV vector expressing the self-deleting guide RNA (gRNA) was co-injected with the AAV-Cas9 or delayed by 5 days. With the delayed injection, the vector was blocked from entering the murine liver because of host immune responses. When tested at other endogenous targets, there was similar editing efficiency between the WT AAV-Cas9 and self-deleting system.[127] The self-deleting system decreased Cas9 levels by 70%–84%. However, the decrease occurred during a period of several weeks; a high amount of Cas9 was still present 4–6 weeks after AAV administration.[127] Li et al.[127] were unsuccessful in developing a single vector system for the self-deleting system so that the same gRNA sequence could destroy the target gene and prevent expression of Cas9. Third, Wang et al.[128] were able to encode the self-restricting system within a single plasmid, avoiding the issue of an extra gRNA and observed no additional off-target modifications. Using the self-restricting system, Cas9 is reduced to 10% of peak levels 60 hours after transfection in HEK293T cells while editing efficiency is at 50%, with off-target formation down by 76.7%.[128] Wang et al. did not systematically sequence for additional off-targets that may have been introduced by the extra self-deleting gRNA, but Li et al. found no modification of several additional off-target sites. Moreover, the self-restricting system may be easily loaded into viral vectors or nanoparticles for *in vivo* delivery since it is a single vector system.

OUTLOOK

Controlling CRISPR-Cas9 activity is critical for these genome editing tools to have an impact in the clinic, and small molecule strategies may be able to address several *in vivo* translational challenges for somatic cell editing.[129] Delivery of not only the inducer, but also the editor, is a challenge, because AAV, the most popular gene therapy delivery vector, is only 0.4 kb larger than Cas9[30,130] and many other editors, including base editors. Moreover, Cas9 shows immunogenicity in several studies,[131,132] and any system that involves expression of a non-endogenous human protein is a major concern for gene therapy; thus, obtaining regulatory approval to test split or degradable Cas9 systems is a crucial challenge to overcome. Humanizing Cas9 has been proposed,[133] but not extensively tested, to address this immune response to Cas9 editors. Furthermore, small molecules such as rapamycin can be used simultaneously as inducers and immunosuppressants.

Prolonged editor activity *in vivo* can be genotoxic or cytotoxic in addition to immunogenic. Hence, research has focused on methods to limit the life of Cas9 once editing is completed. Delivery itself can be engineered to control various parts of the CRISPR-Cas9 system, either through transient delivery or sequential delivery. Future studies that tackle these challenges could use combinations of strategies that include inducible activators, inhibitors, and degrons. Split variations of Cas9 can have little to no background editing but present a major challenge for clinical translation, as researchers have to ensure that all split vector systems are produced with the same purity, infectivity, and potency. While Acr protein inhibitors for Cas9 exist, none have been tested without light being the inducer. Using small molecules such as rapamycin or doxycycline to induce Acr protein activity is likely to be effective. Degrons have been relatively understudied in conjunction with CRISPR-Cas systems, and the different degraders suggested in this review may be further combined with other genome editing systems.

As the CRISPR-Cas editors continue to increase in precision, accuracy, and diversity, new editors may have different kinetics of editing and molecular targets for off-targets (e.g., base editors not utilizing DNA repair and off-targeting of RNA). These strategies would likely build upon control strategies established with Cas9. Tight control over both established and next-generation CRISPR genome editors will likely remain an important goal in the field in order to broaden therapeutic applications *in vivo*.

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AUTHOR CONTRIBUTIONS

N.K. and K.S. were involved in drafting and revising the manuscript. N.K. conducted the literature review, drafted the manuscript, and designed the tables and figures.

DECLARATION OF INTERESTS

K.S. receives sponsored research support from Spotlight Therapeutics and Synthego. N.K. declares no competing interests.

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