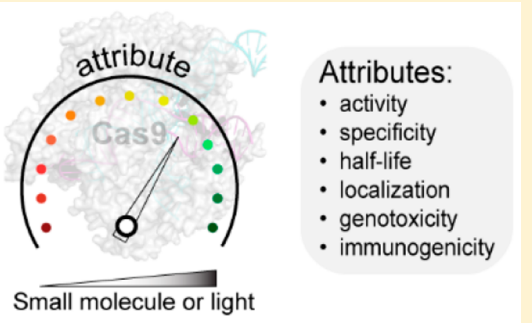
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Precision Control of CRISPR-Cas9 Using Small Molecules and Light

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ABSTRACT: The CRISPR (clustered regularly interspaced short palindromic repeat)-Cas system is an adaptive immune system of bacteria that has furnished several RNA-guided DNA endonucleases (e.g., Cas9) that are revolutionizing the field of genome engineering. Cas9 is being used to effect genomic alterations as well as in gene drives, where a particular trait may be propagated through a targeted species population over several generations. The ease of targeting catalytically impaired Cas9 to any genomic loci has led to development of technologies for base editing, chromatin imaging and modeling, epigenetic editing, and gene regulation. Unsurprisingly, Cas9 is being developed for numerous applications in biotechnology and biomedical research and as a gene therapy agent for multiple pathologies. There is a need for precise control of Cas9 activity over several dimensions, including those of dose, time, and space in these applications. Such precision controls, which are required of therapeutic agents, are particularly important for Cas9 as off-target effects, chromosomal translocations, immunogenic response, genotoxicity, and embryonic mosaicism are observed at elevated levels and with prolonged activity of Cas9. Here, we provide a perspective on advances in the precision control of Cas9 over aforementioned dimensions using external stimuli (e.g., small molecules or light) for controlled activation, inhibition, or degradation of Cas9.



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The CRISPR-Cas system is composed of clustered regularly interspaced short palindromic DNA repeats (CRISPR) and CRISPR-associated (Cas) genes that protect bacteria against invading phages and mobile genetic elements.[1−7] These Cas genes have furnished DNA endonucleases that are transforming biomedical research and gene therapies.[8−11] The most studied CRISPR-associated nuclease is SpCas9 (henceforth called Cas9) from *Streptococcus pyogenes*, but several next-generation nucleases are rapidly emerging.[8] The CRISPR-associated nucleases recognize their substrate via a protospacer adjacent motif (PAM) sequence and base pairing of the target DNA by a guide RNA (gRNA) borne by the nuclease. Upon target recognition, the two DNA strands are cleaved by distinct nuclease domains of Cas9 (Figure 1A): the HNH domain cleaves the target strand, while the RuvC domain cleaves the nontarget strand.[8] The resulting double-stranded break in the DNA can be repaired by error-prone nonhomologous end joining (NHEJ) or the more precise homology-directed repair (HDR) pathways.[12,13] NHEJ leads to insertion/deletion (indel) mutations, causing frame-shift mutations or premature stop codons and gene knockout. HDR, on the other hand, can be used to introduce specific edits in the genome using an exogenously supplied repair template.[14]

In addition to introducing genomic alterations, CRISPR-Cas9-based gene drives have ushered in an era of "active genetics" where an engineered gene/trait can potentially self-propagate in the species population.[15] In a normal Mendelian inheritance, the probability of passing on an engineered gene to the progeny is only 50%, while the same probability for gene drives approaches 100% (Figure 1B).[16−18] Central to the molecular mechanism of super-Mendelian inheritance in the gene drive is the Cas9-induced double-stranded break on the wild type allele that is repaired by copying the allele from the engineered parent, essentially causing replacement of the wild type allele with the engineered one (Figure 1C). The super-Mendelian inheritance by gene drives has allowed ∼100% transmission of antimalarial or infertility genes in mosquitoes, enabling efficient population modification or suppression, respectively.[19−22] Unsurprisingly, gene drives are under investigation for the elimination of diseases (e.g., malaria and Dengue fever) and could potentially be used to reverse pesticide resistance.[16,18,19,21,22]

The ease of targeting catalytically inactive Cas9 (called dCas9) to any genomic loci has led to the generation of transcriptional activators, repressors, epigenetic modifiers, and chromatin imaging systems. For instance, dCas9 has been fused to a variety of transcriptional activators to initiate transcription at a specific site (Figure 1D).[23−30] Similarly, fusing dCas9 to transcriptional repression domains and

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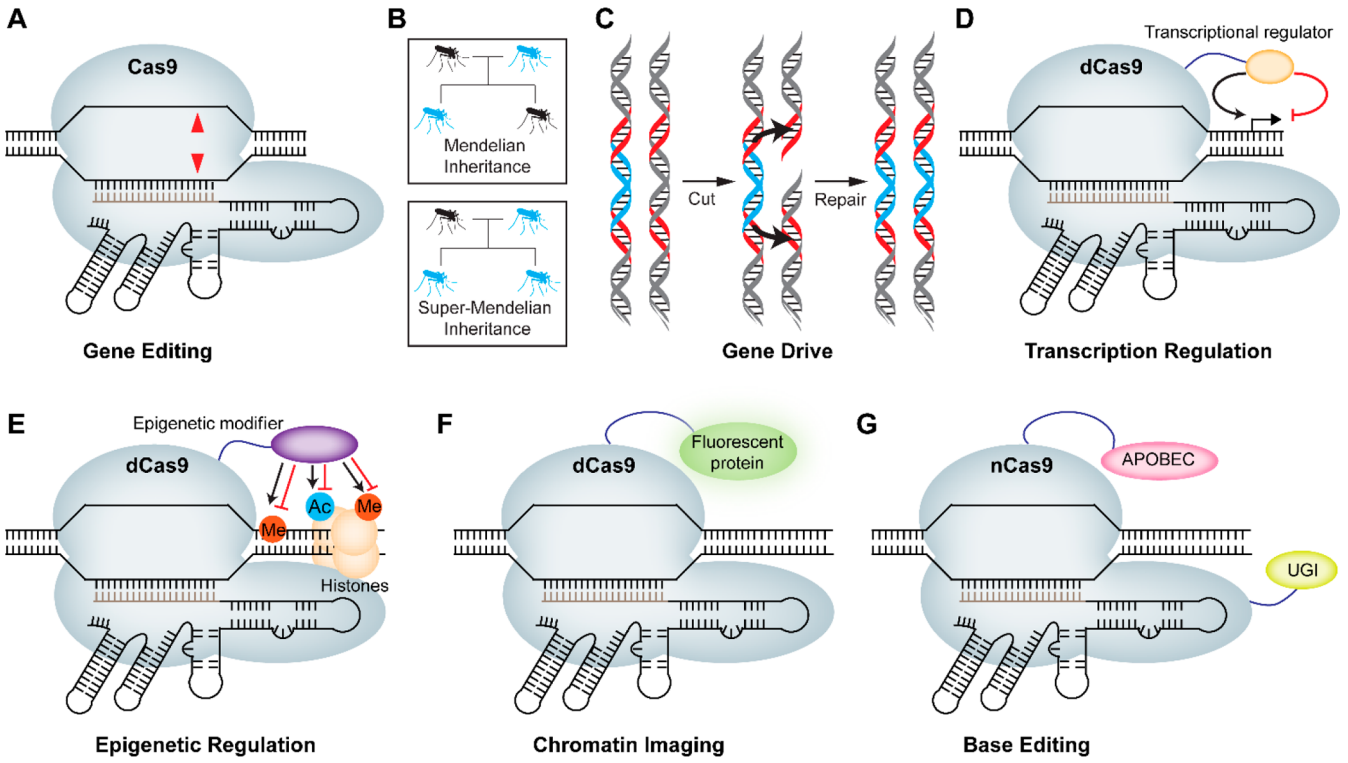


Figure 1. Major applications of CRISPR-Cas9. (A) Cas9 enables gene editing through its RNA-guided DNA endonuclease activity leading to double-stranded breaks (red triangles). (B) CRISPR-Cas9-based gene drive allows self-propagation of an engineered gene and/or trait in a species population. The probability of passing on an engineered gene (blue) to the progeny in Mendelian inheritance is 50%, while the same probability for gene drives approaches 100%. (C) The molecular mechanism of super-Mendelian inheritance in the gene drive involves a Cas9-induced double-stranded break on the wild type allele (gray) that is repaired by copying the drive allele (blue) from the engineered parent via HDR, causing replacement of the wild type allele with the drive element. Red regions indicate homology arms. (D) Nuclease dead Cas9 (dCas9) can be fused to an activator or repressor domain to regulate gene expression. (E) dCas9 can be fused to DNA-demethylating enzymes/methyltransferase as well as histone demethylase/methyltransferase or deacetylase/acetyltransferase domains to regulate epigenetic modifications in a sequence-specific manner. (F) dCas9 can be fused to a fluorescent protein such as GFP to enable imaging of a specific locus. (G) Nickase Cas9 (nCas9) can be fused to cytosine deaminase (APOBEC1) and uracil glycosylase inhibitor (UGI) to enable conversion of cytosine (C) to thymine (T) without double-stranded breaks.

targeting to gene promoters or exonic sequences has allowed for sequence-specific gene repression (Figure 1D).[24,28,31−34] dCas9 has also been used to design programmable epigenetic modifiers targeting both the DNA locus of interest and the surrounding histones (Figure 1E). The alteration of DNA methylation status at a specific target locus was attained by fusing the catalytic domain of DNA methyltransferase (DNMT3A), while dCas9-TET1 fusions were used for DNA demethylation.[35−42] Additionally, dCas9-LSD1 and dCas9-PRMD9 methyltransferase fusion complexes have been applied to demethylate and methylate histones, respectively, while dCas9-p300 and dCas9-HDAC3 fusions have been used to acetylate and deacetylate histones, respectively.[43−46] Fluorescent protein fusions to dCas9 have allowed genomic imaging in live cells as well as probing molecular interactions at specific genomic loci (Figure 1F).[32,33,47−49] Finally, a catalytically impaired "nickase" variant of Cas9 has been instrumental in base editing technologies (Figure 1F). Nickase Cas9 fusions to both APOBEC1 deaminase and uracil glycosylase inhibitor (UGI) afford a base editor that can convert C to T at the target site. Similarly, fusion of an evolved adenosine deaminase to nickase Cas9 has led to the development of a base editor capable of converting A to G.[50,51]

Precision control of Cas9 across multiple dimensions, including those of dose, time, and space, is critical for its several applications. Toxicology's adage "The dose makes the poison" is particularly relevant for Cas9, as the substrate DNA is present at a concentration much lower than those of the nuclease.[52−59] Unsurprisingly, significant off-target effects, chromosomal translocations, and genotoxicity are observed with elevated Cas9 activity.[52−59] In addition, as off-target activity often occurs at a rate slower than that of on-target activity,[60] restriction of the nuclease activity to a narrow temporal window is highly desired. Rapid degradation of Cas9 upon target editing may be required to evade the host's immune surveillance of this bacterial protein. Following germline editing in embryos, the lingering Cas9 activity causes undesirable mosaicism at both genotypic and phenotypic levels.[61−64] In the context of human germline editing, molecular safeguards are even more important for multiple reasons, including the potential to alter the human gene pool. Here, we discuss various approaches for precision control of Cas9 function over the dimensions of dose, time, and space. We describe methods for conditional activation of Cas9 using small molecules or light as well as molecules that can mediate deactivation through inhibition or degradation of Cas9.

CONTROL BY SMALL-MOLECULE ACTIVATORS

A relatively straightforward approach to control Cas9 activity is to regulate its transcription through an inducible promoter, as

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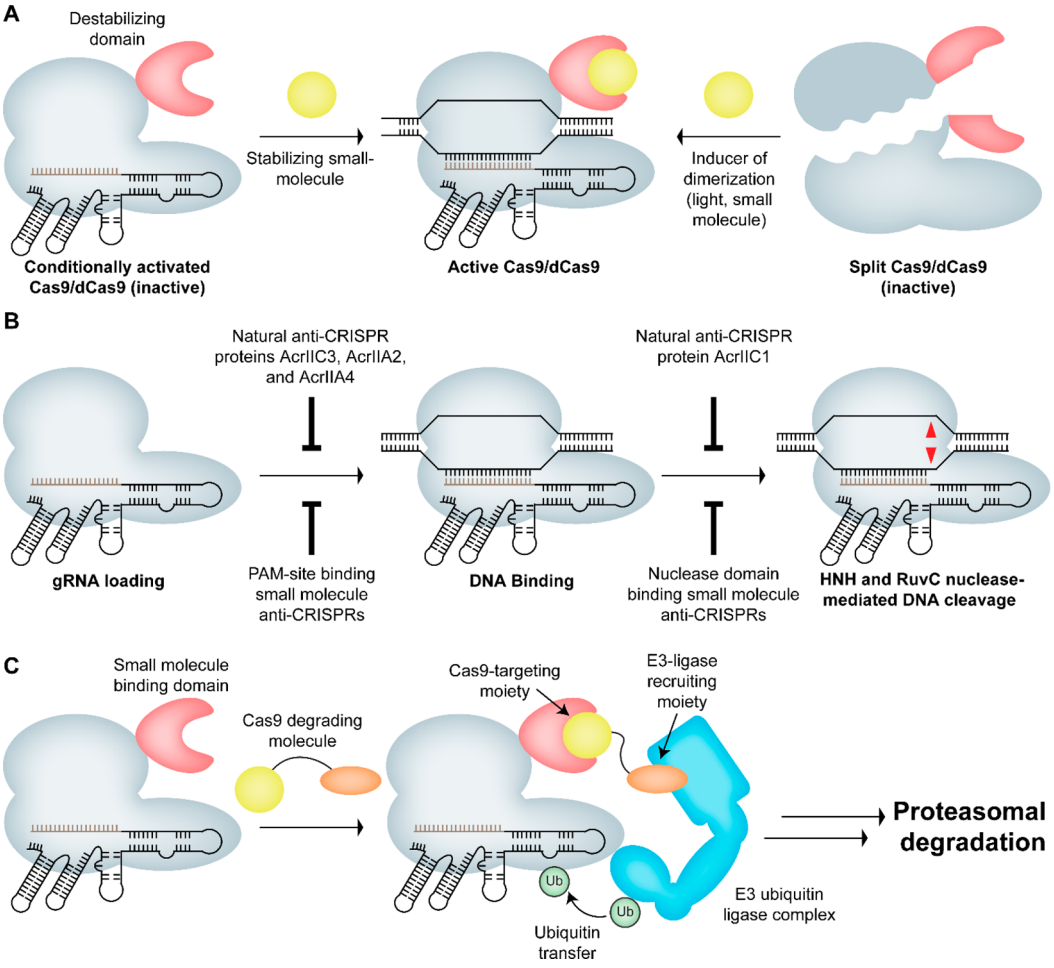


Figure 2. Strategies for conditional control of Cas9 activity. (A) Cas9 can be inactivated by fusing a small molecule or light-responsive domain, or it can be split into N- and C-terminal fragments that can be reconstituted in response to a small molecule or light, leading to the formation of active Cas9. (B) Steps involved in Cas9-mediated strand DNA cleavage. Any of the steps shown above can be inhibited by a protein or small-molecule inhibitor leading to loss of Cas9 activity. (C) Strategy for Cas9 degradation using a heterobifunctional small molecule such that one end of the molecule binds to the small-molecule binding domain fused to Cas9 and the other end binds to CRBN, resulting in ubiquitination and proteasomal degradation of Cas9.

demonstrated in the work by Dow et al.[66] and Gonzalez et al.[65] in mammalian cells and mice, respectively. In addition, Kiani et al. and Aubry et al. have developed doxycycline-inducible gRNA systems for controlling Cas9-mediated genome regulation.[67,68] Although robust gene editing was observed upon induction of Cas9 or gRNA in response to doxycycline treatment, these approaches have slow response times because of the lengthy time scale of transcription and translation[65,66] and require additional factors (e.g., reverse tetracycline transactivator). In addition, transcriptional control is incapable of limiting Cas9 activity to the short temporal window that is necessary to maximize genome editing specificity. In contrast, strategies that post-translationally control protein activity offer a much higher temporal resolution.[69−71]

Several methods exist for post-translational control of protein function using small molecules (Figure 2A).[70,71] Davis et al. inserted an evolved 4-hydroxytamoxifen (4-HT)-responsive intein at specific sites within Cas9 to disrupt enzymatic activity.[69] Addition of 4-HT causes splicing of the intein to release active Cas9. Although the overall activity of this engineered Cas9 was slightly lower than that of Cas9, the ratio of on-target to off-target edits was approximately 6-fold higher. In another study, Liu et al. developed iCas9 by fusing the hormone binding domain of the estrogen receptor (ERT2) directly to Cas9, enabling tight temporal control of Cas9 using 4-HT.[72] In the absence of 4-HT, the ERT2 domains sequester Cas9 in the cytoplasm, but upon addition of 4-HT, the fusion protein rapidly translocates to the nucleus where it can engage with genomic DNA. The cleavage activity of iCas9 was observed at multiple endogenous genomic loci as early as 4 h after 4-HT treatment. A comparison of iCas9 with the 4-HT intein-Cas9 system revealed that in the presence of inducer, iCas consistently showed a DNA cleavage efficiency higher than that of the 4-HT intein-Cas9 at all genomic loci and time points tested, although both systems had comparable background activity in the absence of an inducer. In addition, the iCas9 system was reversible and toggled between on and off states. The activity of iCas9 could be reversed in 72 h by removing the inducer 4-HT. Additionally, this system displayed improved specificity over wild type Cas9 at most endogenous loci and sgRNAs tested when the duration of the 4-HT treatment was restricted to 4−8 h. However, iCas9 still had residual activity in the absence of 4-HT, and the most optimal construct retained only 60% of wild type Cas9 activity.[72] In another study, Oakes et al. used randomized insertional mutagenesis to insert a small domain into the Cas9

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sequence and screened for active variants, thus identifiying structural "hot spots" within Cas9 that could tolerate additional protein domains.[73] Insertion of the ligand binding domain of human estrogen receptor-α into position 231 of Cas9 or dCas9 afforded a 4-HT-responsive Cas9 (arC9) or dCas9 (darC9), respectively. The authors demonstrated 4-HT dose-dependent repression by darC9 (EC_{50} = 440 ± 70 nM) in CRISPRi studies as well as dose-dependent control of Cas9 (arC9) with an EC_{50} of 1 nM. With arC9, almost no background was observed in the absence of 4-HT, but the maximum activity attained was still only 30% of that of wild type Cas9. Reversibility studies indicated that the activation of arC9 could be turned off by removing 4-HT from the media after 6 days. However, a small amount of residual arC9 activity remained even after ligand removal, possibly due to the high binding affinity of arC9 for 4-HT and slow dissociation of the complex.[73]

Several groups have developed various small-molecule-controlled Cas9 systems based on the chemically induced dimerization of split protein fragments (Figure 2A). A well-studied example of this type of system uses the rapamycin-mediated dimerization of FK506 binding protein 12 (FKBP) and FKBP rapamycin binding domain (FRB) of the mammalian target of rapamycin (mTOR).[74,75] Zetsche et al. designed a split Cas9 system in which the C-terminal fragment of Cas9 was fused to FKBP and the N-terminal fragment was fused to the FRB domain.[76] Additionally, they appended a nuclear export signal (NES) to the N-terminal fragment and a nuclear localization signal (NLS) to the C-terminal fragment to prevent spontaneous reconstitution of the two fragments, reducing basal activity in the absence of rapamycin. This design produced low levels of Cas9 activity in the absence of the molecule but irreversible activation upon rapamycin addition. Furthermore, the authors demonstrated substantial indels at the intended genomic loci with no significant off-target effects upon induction of this split-Cas9 system with rapamycin.[76] The availability of orthogonal small-molecule regulators that utilize multiple chemically induced dimerization systems has led to the development of orthogonal gene regulation systems. Gao et al. demonstrated dose-dependent and reversible transcriptional activation/repression using abscisic acid-inducible ABI-PYL1 and gibberellin-inducible GID1-GAI heterodimerization domains.[77−80] In this case, dCas9 was fused to either ABI or GAI while the effector (transcription activator or repressor) domains were fused to PYL1 or GID1, respectively, allowing orthogonal and multiplexed transcription regulation without significant background. Detectable levels of transcriptional activation were observed within 24 h. Furthermore, these systems were reversible upon removal of the inducer, with the activity reaching baseline levels in 4−5 days.[77,78] In another study, Bao et al. utilized the gibberellin- and rapamycin-mediated dimerization systems to demonstrate orthogonal and temporal regulation of multiple endogenous genes.[77,81]

A complementary approach to inducing dimerization of protein domains using small molecules is to disrupt the dimerization process using small molecules. Rose et al. used this method to develop a rapidly inducible Cas9 (ciCas9) system using the interaction between BCL-xL and BH3 peptide as an intramolecular inhibitory switch.[82,83] In this case, the REC2 domain of Cas9 was replaced with BCL-xL to yield Cas9.BCL, and a BH3 peptide was attached to both N- and C-termini of Cas9.BCL.[83] The intramolecular interaction between BCL-xL and the BH3 peptides keeps Cas9 in an inactive state. Addition of the small-molecule inhibitor of BCL-xL, A-385358 (A3),[84] disrupted the interaction between BH3 and BCL-xL, activating Cas9. The ciCas9 system showed dose-dependent control of nuclease activity with the degree of activation being higher than that of the 4-HT-induced intein-Cas9 and iCas9 systems.[69,72,83] Furthermore, ciCas9 showed lower background activity and off-target effects compared to those of Cas9 and could induce indel formation in as little as 2 h. However, the maximal activity reached by this system is still lower than that of Cas9.[83]

In addition to the use of small-molecule-inducible protein domains, another strategy that adds variety to the repertoire of regulated CRISPR-Cas9 systems is the fusion of conditional degrons to Cas9 (Figure 2A). Conditional degrons can be activated or deactivated by one of several external factors such as a small-molecule ligand, light, temperature, or another protein.[85] We and others have adopted this approach to design destabilized Cas9 systems whose activity can be tightly regulated by a small-molecule stabilizer. Work from our laboratory demonstrated the application of destabilized Cas9 systems for both gene editing and transcription activation by fusing the destabilized domains from *Escherichia coli* dihydrofolate reductase (DHFR) and estrogen receptor (ER50) to Cas9 or the transcription activation domains. Transcriptional activation by these destabilized Cas9 systems could be reversed within 48 h by removing the stabilizing small molecule (TMP or 4OHT), with a rapid decrease in mRNA transcript levels seen in as little as 8 h. Furthermore, these systems showed improved specificity over that of wild type Cas9 upon administration of an optimal dose of the stabilizing small molecules.[56] Senturk et al. developed a destabilized Cas9 system by fusing the FKBP-derived destabilized domain to Cas9, which rapidly degrades the protein upon its expression.[86] However, the stabilizing small molecule shield-1 affords rapid, reversible, and temporal control of FKBP-Cas9 gene editing activity. Analysis of the protein induction kinetics revealed that destabilized Cas9 was expressed within 2 h of treatment with shield-1. This effect could be reversed within 2 h of withdrawal of the ligand from the media, with protein levels becoming undetectable after 12 h. Furthermore, treatment with shield-1 led to the formation of indels at the target locus within 24 h to an extent comparable to that seen for wild type Cas9. The authors also demonstrated the application of this destabilized Cas9 system in three-dimensional organoid models, indicating the utility of this system as a screening tool in primary tumor cells. They also demonstrated temporal and independent control of gene editing and Cre-mediated recombination. Therefore, this method could potentially enable interrogation of genetic interactions in various preexisting mouse models of human diseases based on the Cre-lox system.[86] Kleinjan et al. utilized the auxin-inducible degron (AID) and the *E. coli* DHFR degron to develop conditionally degradable dCas9-based effector proteins. The AID-dCas9 system exhibited rapid degradation kinetics upon addition of auxin (1−2 h). Similarly, this effect was rapidly reversed when auxin was removed from the media, leading to the reappearance of detectable levels of the fusion protein in 30 min and maximum expression in the next 3−7 h. The AID approach was also extended to specific orthogonal Cas9 and Cpf1 proteins, affording orthogonal, auxin-degradable synthetic transcription factors.[87] Jacobs et al. inserted stabilizable polypeptide linkages (StaPLs) based on hepatitis C virus NS3 protease domain into internal loops of

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dCas9 to enable conditional control of Cas9 activity. StaPLs undergo autoproteolysis to cleave the proteins to which they are attached, though the presence of a protease inhibitor prevents their cleavage to preserve protein function. Using this system, they demonstrated dose-dependent transcription activation, but the system was irreversible.[88] In a similar approach, Tague et al. used the NS3 protease domain and its inhibitor BILN-2061 as a ligand-inducible connection (LInC) to control the association of DNA binding and transcription activation domains. In their design, the viral protease was incorporated into dCas9-VPR such that the protease was positioned between the DNA binding scaffold and the C-terminal region that contained a nuclear localization sequence (NLS) and the VPR transcription activation domain (dCas9-NS3-NLS-VPR). In the absence of the protease inhibitor, NS3 autocleavage separated the transcription activation domain from dCas9, preventing transcriptional upregulation. Using the LInC-containing dCas9-VP64 system, the authors demonstrated transcription activation of the target gene in a dose-dependent manner, with activity comparable to that of the dCas9-VP64 system without LInC. They also demonstrated the application of LInC in recruiting a transcription activation domain to hairpin-modified sgRNA.[89]

Control of Cas9 activity has also been exercised at the level of the gRNA. Post-transcriptional control of gRNA can potentially be used to independently regulate multiple targets, because each target has its unique guide.[90] Toward this end, aptamers and aptazymes have been fused with gRNA to attain ligand-dependent control. Liu et al. engineered the gRNA by incorporating tetracycline- and theophylline-responsive riboswitches within the gRNA.[91] In the absence of a small-molecule trigger, the spacer portion of the gRNA is base-paired with a designed antisense strand, preventing gRNA:DNA base pairing. Upon addition of the small molecule, the aptamer undergoes a conformational change that allows gRNA to bind to the target DNA. This allowed transcriptional regulation of endogenous genes in a dose-dependent manner upon stimulation by riboswitch-responsive signals.[91] Similarly, Tang et al. incorporated theophylline- and guanine-responsive aptazymes into gRNA sequences, which afforded dose control of transcriptional and nuclease activity but were irreversible and exhibited significant background activity in the absence of small-molecule triggers.[92] To overcome some of these shortcomings in the first-generation systems, Kundert et al. developed a ligand-responsive gRNA that can be activated or deactivated upon ligand addition.[90] The ligand-activated gRNA (ligRNA$^+$) was obtained by inserting the theophylline aptamer into the hairpin region while the ligand-deactivated gRNA (ligRNA$^-$) had the aptamer inserted into the nexus. Both ligRNA$^+$ and ligRNA$^-$ showed robust and titratable activity over a large range of ligand concentrations. Using two different ligRNA$^-$ variants allowed simultaneous and reversible regulation of two genes.[90] Additionally, Pu et al. utilized evolved, split RNA polymerase-based biosensors that drive the production of target gRNAs when activated using small molecules or protein−protein interacting domains. Using this system, the authors demonstrated multidimensional control of SaCas9 function.[93]

CONTROL BY LIGHT

Over the past decade, light has emerged as an attractive tool to control protein function. The high spatiotemporal resolution and non-invasiveness of light induction provides several advantages over other external stimuli. As such, several optically controlled Cas9 systems have been designed to enable precise genomic modifications. Nihongaki et al. developed a system based on cryptochrome 2 (CRY2) and calcium and integrin binding protein 1 (CIB1), which can heterodimerize in the presence of blue light.[94] By fusing dCas9 with CIB1, and a transcriptional activator domain (VP64 or p65) with the CRY2 domain, the authors demonstrated spatiotemporal and multiplexed regulation of endogenous genes with light. The duration of blue light irradiation required to produce detectable levels of upregulation ranged from 1 h for a reporter gene to 3 h for an endogenous gene. This effect could be reversed by incubating the cells in the dark for 18 h.[94] Polstein et al. pursued a similar strategy using multiple copies of the N-terminal fragment of CIB1 (CIBN) fused to dCas9's N- and C-termini and CRY2 fused to VP64. CIBN-dCas9-CIBN showed robust and time-dependent upregulation of the target gene upon exposure to light, which was reversed by simply switching off the light source, with time scales similar to those of the system of Nihongaki et al.[95]

Another study by Nihongaki et al. describes a photoactivatable split Cas9 (paCas9) strategy (Figure 2A).[96] Here, the N- and C-terminal fragments of Cas9 were fused to engineered, light-responsive domains called positive magnet (pMag) and negative magnet (nMag). Upon exposure to blue light, pMag and nMag dimerize, affording a new split system with a lower background and higher fold induction of Cas9 activity. Additionally, paCas9 exhibited similar nuclease activity and targeting specificity to the wild type Cas9 and could be used to induce random indel mutations as well as more precise genomic modifications through HDR in response to light. As with the other protein-based light-inducible systems, paCas9 activity could also be reversed by switching off the light source.[96] In contrast to paCas9, the psCas9 system developed by Zhou et al. employs a single-polypeptide architecture designed by inserting the photodissociable dimeric fluorescent protein pdDronpa1 into the REC2 and PI domains of Cas9.[97,98] In the dark, the dimerization of the inserted pdDronpa1 domains sterically inhibits Cas9 activity, but upon illumination with 500 nm light, pdDronpa1 dissociates and enables Cas9 to carry out gene editing functions as well as transcriptional upregulation.[98] Hemphill et al. developed a genetically encoded light-activated Cas9 system by incorporation of a photocaged lysine to a residue that is critical for gRNA binding (e.g., K866). The activity of this photocaged Cas9 was restored upon a brief (120 s) exposure to 365 nm light. However, unlike some of the other light-activated systems, this system is irreversible due to the irreversible nature of the lysine chemical modification.[99]

As with small-molecule-inducible systems, the bulk of light-controlled Cas9 systems involve engineering of the Cas9 protein. In the only study involving photocaged gRNA, Jain et al. used photocleavable protector oligonucleotides that hybridize with the gRNA, preventing gRNA:DNA base pairing. Upon exposure to light, the protector oligo undergoes photolysis, producing short fragments with reduced binding affinity for the gRNA because of their lower melting temperature. However, once activated, the gRNA cannot be deactivated by removal of illumination, making this system irreversible.[100]

While several methods for precision activation of Cas9 in mammalian cell culture exist, genome editing is being used in several nonmammalian organisms (e.g., plants and insects) for which methods for precision activation are underdeveloped. In

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addition, methods for precision activation of Cas9 in organismal settings are still a challenge even for mammalian systems. Such controls will be useful in several therapeutic settings and within the context of gene drives.[101] For example, currently switching on gene drive involves a split system in which the *Cas9* gene is delivered from one parent and gRNA from another; however, this genetic method is slow and inefficient, and the output is binary (i.e., 0 or 100% super-Mendelian inheritance). Much interest exists to develop gene drives whose output can be fine-tuned to any values from 0 to 100% super-Mendelian inheritance rapidly. Such control, which can be accomplished using conditional activators of Cas9, will enable facile animal husbandry and population expansion of insects with gene drives propagating lethal traits. In addition, numerous CRISPR systems that target RNA have been discovered and have been used to develop highly sensitive diagnostic tools for diseases caused by Zika and Dengue virus.[102−104] The RNA targeting Cas13a has also been used to mediate RNA base editing.[105] Development of inducible RNA targeting CRISPR systems and exploration of their application for conditional control of various types of RNAs in cells will be interesting. Such programmable and inducible RNA-targeting modules could find applications in cancer diagnosis and therapy without affecting wild type transcripts or making any genomic alterations.[106]

CONTROL BY INHIBITORS

With the recent discovery of natural genetically encoded antagonists of CRISPR systems (called "anti-CRISPRs"), there has been a surge of interest in co-opting these proteins to achieve context-specific inhibition of Cas9. Protein-based anti-CRISPRs are small accessory proteins with fewer than ∼200 amino acids found in the genomes of bacteriophages that bind to and inhibit CRISPR-Cas machinery.[107−109] These proteins allow phage to evade the bacterial immune response, allowing phage propagation and making anti-CRISPRs crucial components in the pervasive arms race between bacteria and phage. Indeed, this arms race has resulted in a tremendous diversity of anti-CRISPR proteins, with more than 20 different anti-CRISPR families targeting type I and type II CRISPR-Cas systems currently characterized[110] (including anti-CRISPRs that target SpCas9, such as AcrIIA2 and AcrIIA4).[111] Anti-CRISPRs can interfere with the CRISPR-Cas machinery in a variety of ways as well, such as by binding to the gRNA-loaded CRISPR-Cas complex and preventing DNA binding,[112−115] binding to Cas effector proteins to block their recruitment to active Cascade complexes[112] (type I systems), or directly inhibiting nuclease activity of the Cas9 protein (Figure 2B).[115]

While anti-CRISPRs tend to show little sequence homology with other anti-CRISPR proteins, their continued discovery has been aided by their frequent association with a putative "helix−turn−helix" (HTH) regulatory protein in the phage anti-CRISPR locus (called the anti-CRISPR associated protein, or *aca*).[116] Discovery of *aca* homologues in other phage genomes has allowed putative anti-CRISPRs to be identified through "guilt by association", simplifying the characterization of these open reading frames.[117,118] Recently, expansion of bioinformatic methodologies to discover novel anti-CRISPR proteins include pipelines to identify bacteria harboring "self-targeting" CRISPR spacers, indicating that survival of the bacteria depends upon the ability to self-inhibit CRISPR proteins via bacterially encoded anti-CRISPR proteins.[111] This approach has recently led to the discovery of novel anti-CRISPRs targeting type V CRISPR systems (Cpf1 or Cas12).[119,120] Given the important role anti-CRISPRs play for phage in counteracting bacterial defenses, it is certain similarly clever bioinformatics strategies will discover anti-CRISPRs for all remaining CRISPR-Cas types, including the RNA-targeting type VI CRISPR nuclease Cas13.

Anti-CRISPRs have provided a natural solution for mitigating two of the main problems plaguing CRISPR-mediated genome editing: off-target cleavage events[110] and DNA damage-induced toxicity.[121] As off-target activity and DNA double-stranded break (DSB)-induced toxicity increase with expression time of the CRISPR-associated nuclease, handicapping or outright inhibiting Cas9 activity after the desired double-strand break would be expected to alleviate these issues. Indeed, the work by Shin et al. has shown that timed transfection of AcrIIA4 protein or plasmid can reduce the number of known off-target edits of Cas9 at the *HBB* and *VEGFA* loci in K562 human cells.[60] Li et al. have also shown that temporally staggered delivery of Cas9/gRNA and AcrIIA2 and AcrIIA4 anti-CRISPR proteins to hematopoietic stem cells via helper-dependent adenovirus improves their eventual engraftment into host mice tissue while preserving the desired edit,[122] presumably due to minimization of double-strand break genotoxicity. However, like the Cas9/gRNA complexes themselves, anti-CRISPRs suffer from poor pharmacokinetic properties and delivery issues. The efficacy of anti-CRISPRs in selectively impairing off-target editing while retaining high on-target activity is highly dependent on the timing of addition: if anti-CRISPRs are added concurrently with Cas9/gRNA, the overall editing efficiency is dramatically decreased. Ideally, the addition of anti-CRISPRs follows the introduction of active Cas9/gRNA complexes, but this timing is dependent on the method of Cas9/gRNA delivery (plasmid, a ribonucleoprotein complex, or virus) and will need to be optimized for each situation. With anti-CRISPRs, staggered addition paradigms would also require multiple cycles of nucleofection or adenovirus infection, which may introduce other sources of stress and toxicity in the cells. As such, delivery of anti-CRISPRs poses a large hurdle to the routine application as part of the genome editing toolbox. Moreover, as some anti-CRISPR proteins function as a DNA mimic, the possibility of blocking certain transcription factors by anti-CRISPR proteins cannot be ruled out, and the specificity of anti-CRISPR proteins in the context of mammalian cells should be evaluated.[123]

Small-molecule inhibitors of Cas9 can overcome some of the challenges associated with anti-CRISPR proteins without compromising functional activity. Small-molecule inhibitors can easily be delivered to cell's nuclei through passive diffusion, are stable to proteases, and are generally non-immunogenic. Small-molecule inhibitors exhibit fast kinetics, in stark contrast to genetic methods,[124] and can be synthesized with little batch-to-batch variability. Unsurprisingly, small molecules remain the preferred reagents for dose and temporal control of intracellular targets. However, developing small-molecule inhibitors of Cas9 is challenging for multiple reasons. First, Cas9 possesses novel protein folds limiting the application of rational design approaches.[125] Second, inhibitor identification requires multiple robust, orthogonal, high-throughput assays of Cas9, which are mostly unavailable. Third, Cas9 is a single-turnover enzyme that holds on to its substrate with picomolar affinity,[126] further complicating development of such high-throughput assays. Fourth, the inhibition of Cas9 activity

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requires the inactivation of two nuclease domains. Finally, Cas9 belongs to the class of DNA binding proteins that are often deemed chemically intractable.[127]

Beyond specificity enhancements, anti-CRISPR molecules will be useful for several other contexts. First, in germline editing, restricting Cas9 activity to a narrow temporal window is important, as persistent activity in dividing cells contributes to mosaicism.[61,63] Timely Cas9 degradation reduced mosaicism in non-human primate embryos.[62] Second, a Cas9 inhibitor will be useful in the context of gene drives that propagate lethal traits.[15,16,18,128] Here, temporarily switching off gene drives by inhibiting Cas9 will allow facile animal husbandry and population expansion of mosquitoes for field studies.[21] In addition, dose and temporal control of gene drives in a laboratory setting will allow precision population control and propel our understanding of the limits of this technology. Third, inhibition of Cas9-mediated genotoxicity to helper cells can enable efficient packaging of Cas9 in adeno-associated virus (AAV) for delivery.[129] Another avenue in which the synthetic inhibitors appear to be promising is organ-specific disabling of CRISPR-Cas9. Even though AAV-mediated delivery of CRISPR/Cas has been encouraging, its accumulation in the liver, heart, and kidney remains a major concern.[130] In such a scenario, small-molecule inhibitors can be administered in an organ-specific way to suppress off-target activity in tissues remote from the site of administration. Fourth, Cas9 inhibitors could help allay dual-use concerns from a biosafety perspective in the use of Cas9 in disease modeling.[131] Fifth, Cas9 inhibitors will propel the fundamental understanding of the biological functions of endogenous Cas9 and allow application of immune response-based selection pressure on bacteria for the evolution of new CRISPR-based systems. We note that the presence of CRISPR-based defense systems within bacteria has hampered efforts to develop phages as next-generation antibiotics. A cocktail of phage and the inhibitors of these defense systems may provide an approach for species-selective disruption while leaving beneficial bacteria unaffected, and such studies may lay the foundation for precision anti-infectives.

CONTROL BY DEGRADERS

In many scenarios, timely degradation of Cas9 may be preferred over outright inhibition. Several technologies have been developed for post-translational regulation of protein levels using small molecules.[132,133] Many of these methods involve the use of heterobifunctional molecules that lead to the co-localization of the target protein and specific ubiquitin ligases that are involved in the proteasomal degradation pathway.[134−136] Some of the other strategies involve fusion of a degron to the protein of interest such that degradation is induced upon addition of the small molecule or exposure to light. Examples of this include the auxin-induced degradation system, the LID domain, the B-LID domain, and the SMASh system.[137−140] Another technology that leverages the ubiquitin-independent proteasomal degradation was identified in ornithine decarboxylase (ODC) and antizyme (AZ).[141,142] Here, the protein of interest is fused to ODC that is degraded in a ubiquitin-independent manner upon co-expression of antizyme.[141] More recently, Nabet et al. have reported a small-molecule degradation system (called dTAG system) in which the target protein is fused to the $FKBP12^{F36V}$ variant.[143] Upon addition of a heterobifunctional molecule that can recruit specific E3/E2 ubiquitin ligase to the $FKBP12^{F36V}$ variant, the fusion protein is ubiquitinated and degraded. This heterobifunctional molecule (called dTAG) consists of a selective binder of $FKBP12^{F36V}$ and a CRBN binder. One can envision the application of similar strategies for the degradation of Cas9, wherein Cas9 is fused to one or more degron domains and degraded upon addition of the corresponding small molecule or light. Alternatively, the small-molecule inhibitors of Cas9 can be converted to heterobifunctional molecules bearing a ubiquitin ligase binder. These small-molecule degraders, which operate by "event-driven pharmacology", are catalytic compared to Cas9 inhibitors that operate via "occupancy-driven pharmacology" and are stoichiometric (Figure 2C).[144]

Degradation of Cas9 might be preferred over inhibition in applications in which complete removal of the Cas9 protein is desired. For example, a recent study has pointed to the existence of antibodies against Cas9 in humans,[145] and the Cas9-specific immune response is construed as a major bottleneck in the development of therapeutic applications of Cas9. Reducing the half-life of Cas9 may reduce the severity of the immune response. Cas9 degraders could also be useful for reducing Cas9-mediated genotoxicity. Cas9-induced double-strand breaks activate the p53-mediated DNA damage response leading to cell cycle arrest in the G1 phase and apoptosis.[14,146,147] Such genotoxicity not only reduces the fraction of edited cells in a treated population but also selects for apoptosis-resistant clones with potential tumorigenic properties. Cas9 is known to persist for >6 h at the site of the DNA double-strand break, perhaps preventing DNA repair and/or causing stalled replication forks that might contribute to Cas9 genotoxicity.[14,147] Cas9 degraders may also reduce the mosaicism in germline editing. While Cas9-mediated germline editing has revolutionized disease modeling, the appearance of mosaic mutations in the embryo with different mutations in different cell types in the same animal is a major problem.[61−63] Such mosaic mutations are quite extensive in zebra fish, and additional crosses are required to dilute mosaicism in small animals. In large animals (e.g., non-human primates), where sexual maturity takes multiple years, mosaic mutations can be a major obstacle to obtaining genetically modified animals. Restricting the half-life of Cas9 reduced mosaicism, but the reported method involved fusing Cas9 to a constitutively active degron without temporal control.[62] One can also envision that other strategies to degrade Cas9 in response to a specific stimulus would limit the duration of Cas9 activity to the early embryo stage. These approaches could have similar effects in reducing mosaic genotypes, thus contributing to the overall efficacy and precision of Cas9 gene editing.

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Notes

The authors declare the following competing financial interest(s): The Broad Institute has filed patent applications for many inventions related to CRISPR, including inventions by authors of this Perspective related to precision control of CRISPR.

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