Gene Regulation

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# Conditional Control of CRISPR/Cas9 Function

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*The recently discovered CRISPR/Cas9 endonuclease system, comprised of a guide RNA for the recognition of a DNA target and the Cas9 nuclease protein for binding and processing the target, has been extensively studied and has been widely applied in genome editing, synthetic biology, and transcriptional modulation in cells and animals. Toward more precise genomic modification and further expansion of the CRISPR/Cas9 system as a spatiotemporally controlled gene regulatory system, several approaches of conditional activation of Cas9 function using small molecules and light have recently been developed. These methods have led to improvements in the genome editing specificity of the CRISPR/Cas9 system and enabled its activation with temporal and spatial precision.*

## 1. Introduction to Conditionally Controlled CRISPR/Cas9

The clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) endonuclease system is an adaptive immune defense system present in many bacteria and archaea for the elimination of invading mobile genetic elements.[1] The type II CRISPR system recognizes and cleaves target DNA[2] through the RNA-guided endonuclease Cas9 directed by two non-coding RNAs (the CRISPR RNA, crRNA, and the *trans*-activating crRNA, tracrRNA),[3] which can be combined into a single guide RNA (gRNA).[4] The gRNA recruits the Cas9 nuclease to the target DNA by complementary base pairing of a 20 nucleotide region at the 5′ end, followed by the rearrangement of the nuclease lobe of Cas9 (containing the HNH and RuvC domains), leading to double-strand break (DSB) of the target DNA (Figure 1 a).[5] Following the site-specific DNA cleavage, non-homologous end-joining (NHEJ) and homology-directed repair (HDR) can result in gene insertion, gene deletion, and gene mutation in eukaryotes.[6] The introduction of insertions or deletions (indels) through NHEJ, and the resulting frameshift, lead to inhibition of expression of the targeted gene. Through introduction of two DSBs, the intervening genomic DNA can be deleted, enabling deactivation or activation (if a disruptive sequence is removed) of gene function. HDR allows for highly specific insertion of small or large DNA sequences from a corresponding repair template at a predetermined locus after generation of a DSB (Figure 1 b). Further engineering of the Cas9 nuclease to a nickase variant has shown reduced off-target effects by employing a paired nicking strategy, where a successful gene excision requires the recognition of two different gRNAs on each strand of the

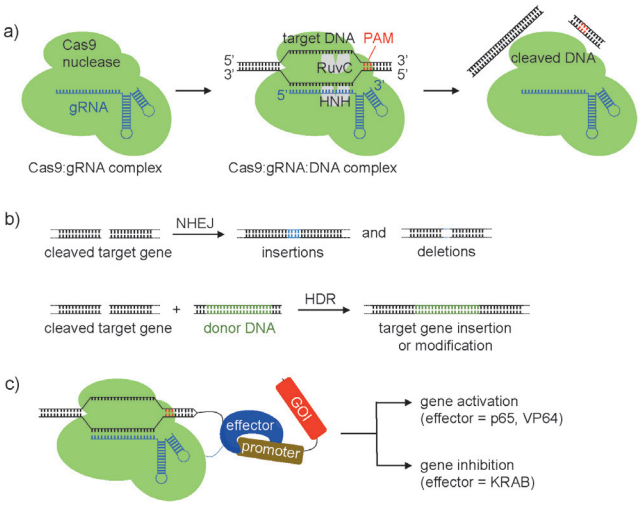


***Figure 1.*** a) gRNA-directed double-strand DNA cleavage by Cas9 nuclease. PAM: protospacer adjacent motif, a Cas9 recognition motif (5′-NGG-3′) required at the 3′ end of the target DNA sequence; HNH and RuvC: nuclease domains. b) Gene mutation by non-homologous end joining (NHEJ) and gene insertion by homology-directed repair (HDR). c) Activation of transcription using a dCas9-effector fusion protein. GOI: gene of interest; p65: p65 transcription activator domain; VP64: tetrameric VP16 transcription activator domain; KRAB: Krüppel-associated box domain.

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target DNA sequence.[6c] A catalytically dead variant, termed dCas9, has been developed as a sequence-specific transcriptional repressor[7] and a genomic anchor for site-specific transcriptional regulation when coupled with various kinds of genomic effectors, or for more precise DNA cleavage when coupled with other nucleases (Figure 1c).[8]

The CRISPR/Cas9 system has several advantages over previous gene editing systems, such as zinc finger nucleases, transcription activator-like effector nucleases, meganucleases, and recombinases (including the Cre/*loxP* system).[9] Owing to its customizable gRNA design (alleviating the need for protein engineering to target different DNA sequences and for pre-installed sequences, such as *loxP*), its genomic specificity, its efficiency, and its ability for multiplexing, Cas9 and its variants have shown great potential in the generation of genetically modified cell lines and organisms, the interrogation of dynamic gene function, improved gene therapies, and other applications.[7,10] As a next step, the utility of these applications will be further expanded through the development of conditionally controllable CRISPR/Cas9 systems that show rapid response, spatiotemporal specificity, and reversibility. Conditional activation and reversible control may direct Cas9 activity to a certain time window, providing safer gene therapies through a reduction of off-target effects caused by prolonged Cas9 activity; a rapid response to exogenous signals may enable synchronization of CRISPR/Cas9 activity with precisely orchestrated genetic networks, such as during circadian rhythms or in different developmental stages of an embryo; and an array of orthogonally inducible Cas9 molecules may be used as building blocks for synthetic transcriptional networks. Very recently, numerous efforts have resulted in the conditional control over CRISPR/Cas9 function through different strategies, including small-molecule-triggered Cas9 and light-triggered Cas9 proteins.

## 2. Small Molecule Control of Cas9 Function

The regulation of the RNA-guided endonuclease Cas9 in response to small molecules has been achieved by different approaches, based on previous developments of fully characterized and conveniently modularized small-molecule-binding domains.[11] Small-molecule-triggered self-splicing inteins have been developed,[12] and when a 412 amino acid intein was inserted at either Ser219 or Cys574 of the Cas9 protein, the enzyme was found inactive until the intein was activated by 4-hydroxytamoxifen (4-HT) and removed through a self-splicing reaction (Figure 2a).[13] Activation efficiencies varied between 3- to 10-fold, depending on the intein insertion site and the target gene, with little leakiness, ranging only between

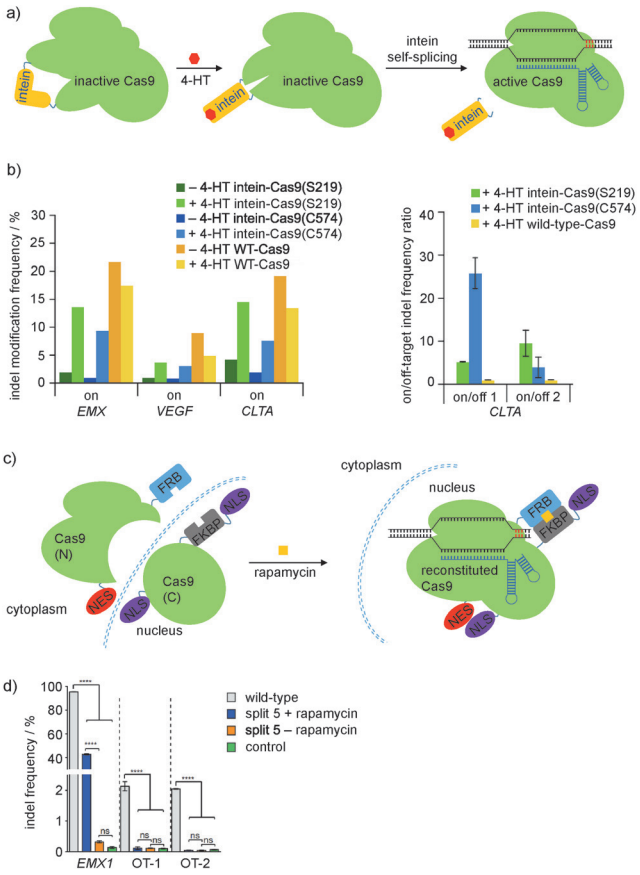


***Figure 2.*** a) Upon binding to 4-HT, the inserted intein undergoes self-splicing and restores active Cas9 structure and function. b) Indel frequency determined by DNA sequencing of amplified genomic on-target sites (*EMX*, *VEGF*, and *CLTA*) and their on/off target ratio (two *CLTA* sites). Adapted from Ref. [13] with permission from Macmillan Publishers Ltd: Nature Chemical Biology, copyright 2015. c) Upon rapamycin-induced dimerization of FKBP (rapamycin FK506-binding protein 12) and FRB (FKBP rapamycin-binding protein) domains, NLS-mediated nuclear import allows for target binding of Cas9. d) Indel frequency measured by sequencing at the *EMX* locus and its two off-target (OT) sites. Adapted from Ref. [15] with permission from Macmillan Publishers Ltd: Nature Biotechnology, copyright 2015.

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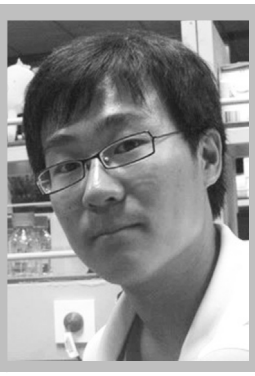

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2–5% in indel formation frequency (Figure 2b).[13] In addition to the innovative temporal control over gene editing, an up to 25-fold improvement in the on/off-target ratio of the gene editing event was observed in comparison to wild-type Cas9, indicating enhanced genomic specificity and reduced off-target effects with the small-molecule-controlled Cas9 (Figure 2b).

A second approach to the generation of a small-molecule-triggered Cas9 cleverly takes advantage of a split-protein system.[14] Split-Cas9 proteins were generated at two different sites, Arg 535 and Glu 573,[14] and for each pair the C- and N-terminal Cas9 fragments were fused to FKBP12 and FRB domains,[15] respectively. Rapamycin-induced heterodimerization (Figure 2c) enabled the conditional reconstitution and activation of split-Cas9, resulting in indel formation at targeted loci with low mutation frequencies at off-target sites, OT-1 and OT-2 (Figure 2d). Adding to its versatility, the split-protein strategy was also applied to the generation of a small-molecule-induced transcriptional activator by combining split-dCas9 (catalytically dead Cas9) with a VP64 domain.[14]

Screening of several mutants was employed in both approaches in order to identify disrupted Cas9 enzymes followed by restoration of their function. Observed challenges in first-generation systems included: 1) Background activities in the absence of rapamycin (approx. 15–30% of wild-type Cas9 activity) due to spontaneous self-assembly of the two split-Cas9 fragments.[14] 2) Cas9 reconstitution by 4-HT led to lower activity than wild-type Cas9 due to incomplete splicing and relatively slow splicing kinetics:[13] self-splicing of this intein has a maximum yield of 80% in mammalian cells that is reached after 24 h.[12b] To address the first issue, split-Cas9 fragments were cleverly sequestered by attachment to an NES (nuclear export sequence) and an NLS (nuclear localization sequence) in order to spatially separate them in different cellular compartments (Figure 2c).[14] This enabled an improvement in switching behavior as background levels were reduced to below the detection limit. Furthermore, it increased genome editing specificity compared to wild-type Cas9 (Figure 2d). Overall, small-molecule-control of Cas9 represents an important advance in the field. However, some challenges remain: not only does small molecule induction lack spatial specificity, it is also dependent on the kinetic and biochemical properties of the inducers and functionalizing domains (for example, the rates and efficiencies of intein splicing, as well as the irreversibility of protein splicing and of the rapamycin-induced protein dimerization).

## 3. Optical Control of Cas9 Function

Light has been widely adopted as an inducer of protein function, because it is non-invasive and conveys spatial and temporal specificity as well as reversibility to the system under study. One approach to the optical regulation of gene function is the engineering of a CRISPR/Cas9-based transcriptional activation system. Here, Cas9 function was not directly regulated with light; however, a catalytically dead Cas9 (dCas9) was used as a genomic anchor (with a corresponding gRNA) and a transcriptional activator (VP64 or p65) was recruited. The two proteins were each fused to light-controlled protein dimerizers, specifically the CRY2/CIB pair, which has been extensively characterized and used to trigger protein dimerization in response to blue light,[16] enabling light-induced genomic targeting of the transcriptional activators (Figure 3a). Applying this innovative approach, two groups independently reported very similar

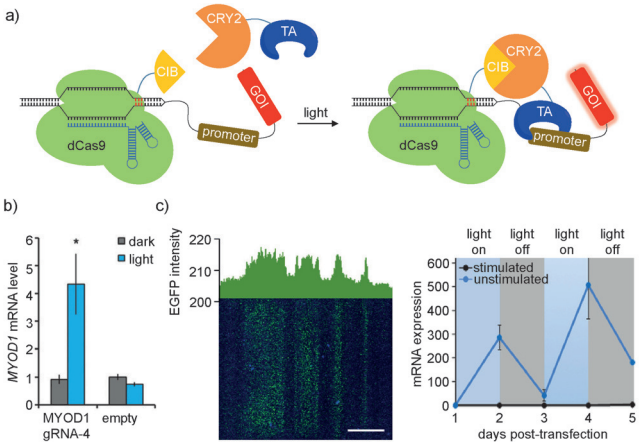


***Figure 3.*** a) Overview of the dCas9-based photoactivatable system: one or two transcriptional activators (TAs) are recruited to dCas9 through CRY2 (cryptochrome circadian clock 2) and CIB (cryptochrome-interacting basic-helix-loop-helix) protein dimerization. b) Light-activated expression of the *MYOD1* gene measured by qPCR. Adapted from Chemistry & Biology, Ref. [17], copyright 2015, with permission from Elsevier. c) An EGFP reporter revealed spatial specificity and reversibility of the dCas9-based photoactivation system. Scale bar: 2 mm. Adapted from Ref. [18] with permission from Macmillan Publishers Ltd: Nature Chemical Biology, copyright 2015.

photoactivatable transcriptional control systems: a p65-CRY2/dCas9-CIB construct[17] and a VP64-CRY2/CIB-dCas9-CIB construct,[18] and both of them successfully demonstrated light-induced activation of target gene expression with minimal background activity,[17] spatially localized activation with submillimeter resolution, and reversibility. In the first approach,[17] p65 (activator domain of NF-κB) was recruited to the C-terminus of dCas9 following light irradiation. When combined with MYOD1 gRNA-4, the p65 domain activated the transcription of the *MYOD1* gene, while in the absence of light only basal gene expression was detected (Figure 3b). Using an EGFP reporter, localized activation and reversibility of the system were demonstrated, with a spatial resolution of at least 1.5 mm, and induction times of 18 h for deactivation and 9 h for reactivation. The second approach,[18] in which the VP64 activator was recruited to both the N- and C-terminus of dCas9, also showed spatial control, reversibility (Figure 3c), and only basal levels of background activity (comparable to the absence of the VP64 activator). When transfected cells were subjected to light irradiation through a photomask of slits ranging from 0.2–3.0 mm in width, patterned expression of EGFP was observed owing to localized activation of the target gene. Also, using one-day intervals with or without light exposure, the photoactivatable dCas9 transcriptional activation system was able

  

to switch between the stimulated and the non-stimulated form.

While light-induced recruitment of a transcriptional effector domain (activator or repressor) to a catalytically dead Cas9 enables control of gene expression, it does not provide optical triggering of gene editing. Two approaches have been developed to achieve that goal: 1) site-specific incorporation of a photocaged lysine into Cas9, and 2) generation of a split-Cas9 that can be reconstituted through irradiation.

Utilizing an expanded genetic code in mammalian cells, numerous light-activated proteins have been generated through the site-specific incorporation of caged amino acids.[19] We applied this approach to the first optical control of Cas9 enzymatic function. An essential lysine residue (K866) was identified and mutated to a photocaged lysine (PCK), rendering the enzyme inactive through minimal modification of the protein: the molecular weight of the caging group (238 Da) is dramatically lower than that of light-responsive protein domains. UV-induced photolysis of the caging group generated wild-type Cas9, which was fully active and allowed for gRNA-targeted DNA cleavage (Figure 4a).[20] High OFF to ON switching ratios were observed, with complete inactivity before light-exposure and enzymatic activity comparable to wild-type Cas9 after decaging. Patterned UV irradiation of cells enabled spatially controlled switching of gene expression from DsRed to EGFP in a dual-reporter system (Figure 4b). The perturbation of Cas9 through introduction of a caged amino acid may provide kinetic advantages over other methods that require the recruitment of complimentary domains/split fragments, by limiting reestablishment of an active conformation to a confined region of the protein, and by providing complete activation through photochemical generation of the wild-type enzyme. However, it should be noted that this strategy requires the expression of the engineered tRNA synthetase and cognate tRNA for caged lysine incorporation, which may be a limiting factor. Also, while UV irradiation could have phototoxic effects, recently encoded lysine analogues have introduced visible light for decaging reactions.[19d]

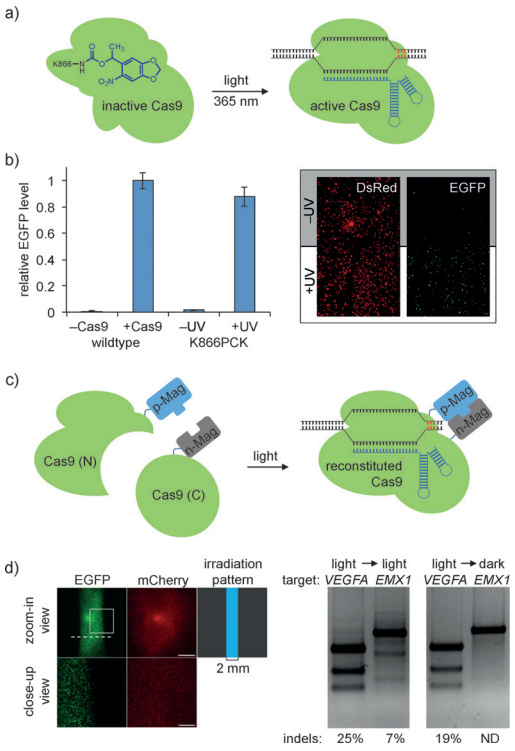


***Figure 4.*** a) A site-specifically incorporated photocaged lysine (the caging group is shown in blue) blocks Cas9 function until it is photochemically cleaved, generating active wild-type Cas9 protein. b) UV-induced decaging of photocaged Cas9 leads to nearly full restoration of nuclease activity in a fluorescent reporter assay and enables spatial control of gene editing. Adapted with permission from Ref. [20], copyright 2015 American Chemical Society. c) Magnet-enabled split-Cas9 activation through light-induced reconstitution of Cas9 through dimerization of its N- and C-terminal domains. d) Spatial specificity and reversibility of Magnet-enabled split-Cas9 gene editing. Adapted from Ref. [21] with permission from Macmillan Publishers Ltd: Nature Biotechnology, copyright 2015.

A photoactivatable split-Cas9 was generated through the comprehensive screening of 18 potential split sites and several light-inducible heterodimerization domains.[21] A pair of domains called Magnets[22] proved most effective, since the Magnets approach imposes less steric hindrance and displays weaker homodimerization than the CRY2/CIB1 system.[23] With this novel, optically triggered split-Cas9 system (Figure 4c–d), the endonuclease activity of Cas9 was restored with genome editing specificities comparable to full-length Cas9, but with reduced efficiency. Importantly, low background activity was observed, comparable to empty, negative control vectors. Moreover, spatial control and light-induced reversibility of the system were demonstrated as well. Following modifications of the *VEGFA* gene (indel frequencies of 25% and 19%) through light-activation, subsequent transfection of a second gRNA targeting the *EMX* gene lead to successful modification (indel frequency of 7%) only in the presence of light exposure (Figure 4d).

The catalytically dead dCas9 was also converted into a split-protein and fused to Magnet domains following the same principle.[21] Its application as a target-specific genomic silencer displayed a repression efficiency of 60% compared to full-length dCas9, similar to the efficiency of photoactivated split-Cas9 when compared to wild-type Cas9. It is also noteworthy that the reversibility of this system provides an optical ON to OFF switch. This controlled reduction of Cas9 activity, once the targeted DNA locus was modified, may be utilized to minimize potential off-target effects.[13]

## 4. Other Approaches to Conditional Control of Cas9 Function

In addition to post-translational conditional regulation of the CRISPR/Cas9 system for spatial and temporal control, regulatory mechanisms at the transcriptional level have also been applied. A TRE$^{3G}$-IRES-Cas9 construct was introduced,[24] whose transcription was controlled by doxycycline to avoid embryonic lethality and genetic mosaicism caused by constitutively active Cas9 in mice. Temporal regulation was significantly slower compared to conditional control at the post-translational level. However, spatial regulation was

  

achieved with tissue-specific promoters in zebrafish embryos,[25] taking advantage of abundant, highly modularized, and finely tuned promoter toolboxes.[26] Heat-shock inducible Cas9 expression for temporal control in *C. elegans*[27] and *D. rerio*[28] was also reported.

## 5. Summary and Future Directions

As part of the rapidly developing CRISPR gene-editing field and through recent advances in the molecular structure, mechanism, and application of the Cas9 system, efficient and well-characterized conditional control over Cas9 function has been developed. Precise spatial and temporal regulation was achieved in tissue culture and improved genomic specificity owing to reduced off-target effects was observed. Through post-translational control, rapid responses to inducers, including small molecules and light, were achieved.

The innovative designs for post-translationally controlling CRISPR/Cas9 function discussed here follow similar general principles: perturbation of the Cas9 active conformation by either the insertion of exogenous motifs (that is, an intein or a photocaged amino acid), or the splitting of the Cas9 protein at carefully screened positions, followed by the restoration of Cas9 structure and its function upon induction by either small molecules or light. Regulation of the Cas9 protein function is rapid, orthogonal to the host system, and benefits from the development of modularized inducible and/or functional domains (for example, heterodimerization domains and activator/repressor domains). Additional opportunities may arise from the coupling of post-translational regulation with other methods, such as transient delivery of the Cas9:gRNA complex[29] or expression under the control of tissue-specific promoters.[30] Small molecule induction has an advantage in the context of penetrability of tissue and animals compared to light irradiation, while optical control enables high spatial specificity and fast reversibility. However, background activity caused by incomplete alteration of the Cas9 structure (for example, self-assembly of split-Cas9 fragments or of the heterodimerization domains) and weakened activity after induction caused by insufficient restoration (for example, incomplete self-splicing of inteins) are areas for further improvement of the conditionally controlled split Cas9 systems.[31]

New applications of small-molecule- or light-induced CRISPR/Cas9 function may include the design and regulation of complex genetic circuits, the investigation of gene regulatory dynamics during temporally and spatially orchestrated processes, such as cell differentiation and development, or the engineering of tissues and cell–cell interactions through spatiotemporal regulation of gene expression. In addition, high precision gene editing may be achieved through combining of programmable DNA-binding domains,[32] paired-nicking strategies with Cas9 D10A nickase,[6c] or dCas9-endonuclease fusions,[33] with conditional regulation for finely tuned genomic editing.

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