A Single-Chain Photoswitchable CRISPR-Cas9 Architecture for Light-Inducible Gene Editing and Transcription

Xin X. Zhou,†‡ Xinzhi Zou,†‡ Hokyung K. Chung,†‡§ Yuchen Gao,¶ Yanxia Liu,† Lei S. Qi,†‡ and Michael Z. Lin*†‡§¶

†Department of Bioengineering, Stanford University, Stanford, California, United States
‡Department of Neurobiology, Stanford University, Stanford, California, United States
§Department of Biology, Stanford University, Stanford, California, United States
¶Program in Cancer Biology, Stanford University, Stanford, California, United States

Supporting Information

ABSTRACT: Optical control of CRISPR-Cas9-derived proteins would be useful for restricting gene editing or transcriptional regulation to desired times and places. Optical control of Cas9 functions has been achieved with photo-uncageable unnatural amino acids or by using light-induced protein interactions to reconstitute Cas9-mediated functions from two polypeptides. However, these methods have only been applied to one Cas9 species and have not been used for optical control of different perturbations at two genes. Here, we use photodissociable dimeric fluorescent protein domains to engineer single-chain photoswitchable Cas9 (ps-Cas9) proteins in which the DNA-binding cleft is occluded at baseline and opened upon illumination. This design successfully controlled different species and functional variants of Cas9, mediated transcriptional activation more robustly than previous optogenetic methods, and enabled light-induced transcription of one gene and editing of another in the same cells. Thus, a single-chain photoswitchable architecture provides a general method to control a variety of Cas9-mediated functions.

CRISPR-Cas9 proteins from bacteria serve as versatile platforms for genome editing1−12 and transcriptional modulation13 due to their RNA-guided DNA-binding activity. Directed by a single-guide RNA (sgRNA), Cas9 proteins can be targeted to any endogenous genomic sequence that contains the requisite paralogue-specific protospacer-associated motif (PAM). Mutation of the native Cas9 endonuclease domains and attachment of functional domains enables outputs other than DNA cleavage, including transcriptional repression,3,4 transcriptional activation,4−7 histone modification,7 and somatic hypermutation.8,9

Temporal regulation of transcriptional repression or activation by Cas9-based systems using drugs has enabled the study or control of gene function at specific times in cells or in organisms.10,11 Optical regulation of Cas9 or various Cas9-derived proteins would be highly desirable for confining gene editing or transcriptional regulation to specific places as well as specific times. For this purpose, several approaches for optical control of Cas9-mediated functions have recently been introduced, including light-induced complementation of Cas9 fragments,12 light-induced two-hybrid systems,10,13 and photolysis of a caged unnatural amino acid.14

The Cas9 proteins from Streptococcus pyogenes and Staphylococcus aureus recognize guide RNAs orthogonally, a feature that has been used to direct different functional outputs to two different genes.10,11 Optically regulated Cas9 systems, however, have only been developed from S. pyogenes Cas 9 (SpCas9) and thus cannot currently mediate optical induction of different effects at different genes in the same cell. While it might be possible to develop analogous systems for other Cas9 species, existing methods have significant drawbacks. Systems for light-mediated recruitment of functional domains to Cas9 have demonstrated poor inducibility,10,11 while systems based on complementation of Cas9 fragments or incorporation of unnatural amino acids have required extensive screening and engineering to develop.12,14 Furthermore, all existing systems require expression of multiple genes, e.g., fusions of photoswitching domains with Cas9 fragments, fusions of photoswitching domains with a Cas9 DNA-binding domain, and a transcriptional regulatory domain, or a mutated Cas9 and a tRNA synthetase.8−13 This introduces complexity compared to a single-component system. Thus, a method for optical control

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of Cas9 variants in a single polypeptide format that could be easily applied to different Cas9 species and functional outputs would be useful, but such a method does not currently exist.

We explored using the photodissociable dimeric green fluorescent protein pdDronpa to create single-chain Cas9-derived proteins with photoswitchable function. pdDronpa dimerizes in the dark but dissociates to monomers upon exposure to cyan light and redimerizes in response to violet light.15 We hypothesized that we could genetically attach pdDronpa domains at two locations flanking the DNA-binding cleft, so that the intramolecular dimer would block DNA access in the dark. Illumination would convert the polypeptide from a circular caged conformation into a linear open conformation with two flexibly linked domains, allowing for DNA binding (Figure 1a).

We applied this strategy first to SpCas9. We examined the SpCas9-RNA crystal structure to identify locations where insertion of pdDronpa domains would prevent DNA binding. SpCas9 adopts a bilobed structure consisting of a nuclease lobe and a recognition lobe.16−18 In the bound state, the heteroduplex of the target DNA strand and the guide RNA is positioned in the cleft between the two lobes. We first identified a loop in the protospacer-associated motif domain of the nuclease lobe (aa 1242−1247)16,18 which is absent in the electron density maps of SpCas9 crystals, suggesting flexibility.

We next examined the structure and sequence of the recognition lobe for a second insertion site. We identified a loop (aa 258−269) in the REC2 domain that lacks sequence conservation across species and is situated across the DNA-binding cleft from the loop at aa 1242−1247. We hypothesized that pdDronpa domains inserted at these two loops should be able to dimerize, as the distance in between them in the SpCas9/RNA complex structure is ∼3.0 nm, comparable to the 3.4 nm separation between the N- and C-termini of the pdDronpa dimer. Importantly, because the two loops are situated on opposite sides of the DNA-binding cleft, dimerization of pdDronpa domains attached to them should create a bridge that would make the binding of a continuous DNA strand to the cleft topologically impossible (Figure 1a).

To test the above design, we inserted pdDronpa1 domains after Ala-259 and Lys-1246 (Figure 1b) with linkers of two amino acids (aa) on each side of each domain. For an unblocked control, we also inserted monomeric Dronpa145K domains in these two locations (Figure 1b, construct a). Active Cas9 cleaves a targeted endogenous genomic locus to induce indel mutations by nonhomologous end-joining.19 Using a nuclease assay to measure indel frequency,20 we observed that SpCas9 fused to Dronpa145K domains was as active as wild-type SpCas9 in HEK293T cells, indicating that the monomeric fusions did not affect the enzymatic activities...
(Figure 1c). In contrast, cells expressing SpCas9 fused with pdDronpa1 domains exhibited very low indel frequencies comparable to untransfected cells, indicating effective arrestment of protein activity (Figure 1c). Exposure of cells to 10 mW/cm² cyan light for 40 h took place, chosen to allow sufficient time for then induced indel formation at both gene loci tested, human GRIN2B and human MOV10 (Figure 1d,e). We thus named this construct photoswitchable SpCas9, or ps-SpCas9. Quantification of indel frequency showed that photoinduction of ps-SpCas9 introduced indels at 16.0 ± 2.5% of MOV10 genes (mean ± standard error of the mean in three independent experiments), compared to 46.8 ± 1.7% for wild-type SpCas9 (Figure 1f). This activity level is similar to that of a photoinduced two-component Cas9 system.22 Uniquely, the bright green fluorescence of the pdDronpa domains fused to ps-dSpCas9 can serve as protein expression and localization markers, and the off-switching of its fluorescence can be used to monitor and quantify protein activation (Supporting Figure s1).

We investigated whether the ps-SpCas9 design can also be used to regulate gene transcription. To create a ps-SpCas9-based transcriptional activator, we introduced mutations D10A and H841A to abolish DNA cleavage activity, creating photoswitchable enzymatically dead SpCas9 (ps-dSpCas9), and fused a VP64-p65-Rta (VPR) transactivation module to its N-terminus (Figure 2a).11 We tested this construct in HEK293T cells with a stably integrated TRE3G-mCherry reporter gene11 by coexpressing a sgRNA directed to the tetO sequences in the TRE3G promoter (sgTRE3G). We observed a low but detectable level of mCherry expression with our initial construct containing two pdDronpa1 domains (Supporting Figure s2). This problem was resolved by replacing pdDronpa1 with pdDronpa1.2 (Supporting Figure s2), which we previously showed improved protein caging.18 We compared the resulting construct, named VPR-ps-dSpCas9, to the optimized light-activated Cas9 effector (LACE) system10,13, which uses photoassociation between full-length cryptochrome2 (CRY2) and CIb1 to recruit transactivation domains to dSpCas9 (Figure 2b). dSpCas9-mediated transactivation activation takes ~24 h to reach the maximum,10 but both pdDronpa photodissociation and CRY2-CIB1 photoassociation reverse after a few minutes,15,21 so we illuminated with cyan light (10 mW/cm²) for 24 h to ensure robust transcriptional activation. VPR-ps-dSpCas9 mediated 58-fold induction of mCherry by light (Figure 2c,d), achieving levels 28.7 ± 2.2% (mean ± standard error of the mean in three independent experiments) as high as a chemically inducible system previously shown to have comparable activity to wild-type dSpCas9 (Supporting Figure s3).11 By comparison, LACE mediated 22-fold induction by light, to levels ~60% of VPR-ps-dSpCas9 (Figure 2c,d). While LACE uses VP64 rather than VPR for transactivation, substitution of VPR in place of VP64 in a recent published study yielded insignificant light-induced activity.11 When different light doses were tested, the VPR-ps-dSpCas9-induced mCherry response plateaued at 100–1000 μW/cm² (Figure 2e), demonstrating that this low intensity of light is sufficient for long-term photoactivation.

We next asked whether the same approach of caging the DNA-binding clefct using pdDronpa domains could be used to create a photoswitchable S. aureus Cas9 (SaCas9). SaCas9 recognizes a different PAM sequence, broadening the range of target sites for RNA-directed gene editing or transcriptional control.23 SaCas9 also recognizes guide RNAs orthogonally from SpCas9, enabling targeting of two genes for different perturbations.10,11 We designed fusion proteins (Figure 3a) based on the SaCas9-RNA-DNA triplex structure.23 We first inserted pdDronpa1, flanked by 2-aa linkers, after Thr-128 in a loop within the recognition lobe (Figure 3b). We then inserted pdDronpa1, flanked by 2-aa linkers, after Gly-614 in a loop within the HNH domain or after Ala-738 in a loop within the RuvC domain of the nuclease lobe (Figure 3b), either alone or in combination with the first insertion. We found that SaCas9 fused to a single pdDronpa1 domain in any of the identified

![Figure 2. Engineering of photoswitchable dSpCas9. (a) Schematic of optical regulation of VPR-ps-dSpCas9. Basally, pdDronpa dimerization prevents VPR-dSpCas9 from binding DNA. Upon illumination, pdDronpa dissociates, allowing DNA binding and transcriptional activation. (b) For comparison, the schematic of optical regulation by LACE, a light-inducible two-hybrid system comprising Cry2FL-VP64 and CiBNSpCas9-CiBN, is shown. Note that repression of transcription should be expected in the dark if there is no basal binding between the components. (c) 24 h of 10 mW/cm² cyan light induced transcription of mCherry. Cells expressing sgTRE3G only did not exhibit mCherry expression upon light stimulation. Light induced more mCherry expression in cells expressing ps-dSpCas9 compared to cells expressing Cry2FL-VP64 and CiBN-dSpCas9-CiBN. Scale bar, 20 μm. (d) Quantification by flow cytometry of average mCherry fluorescence before and after 24 h of 10 mW/cm² cyan light illumination. mCherry was normalized to mean level in nonilluminated cells with reporter only. (e) Increase in mCherry intensities at different illumination intensities for reporter cells expressing ps-dSpCas9, normalized as above and plotted in log scale. In c and e, error bars represent standard error of the mean (s.e.m.), n = 3.](https://doi.org/10.1021/acschembio.7b00603)
sites did not affect its cleavage activity (Figure 3c). SaCas9 fused to two pdDronpa1 domains after aa-128 and aa-738 did not block DNA cleavage (Figure 3c). In contrast, the construct consisting of SaCas9 fused to pdDronpa1 domains after aa-128 and aa-614, which we named ps-SaCas9, completely eliminated protein activity (Figure 3c). Exposure of HEK293T cells expressing ps-SaCas9 to 40 h of 10 mW/cm² cyan light robustly induced indel formation (Figure 3d). These results demonstrated the successful engineering of an optically controllable form of SaCas9.

We finally tested whether light-induced editing and transcription of different genes in the same cells could be performed by coexpressing ps-dSpCas9 and ps-SaCas9 (Figure 3e). In TRE3G-mCherry reporter cells coexpressing VPR-ps-dSpCas9, ps-SaCas9, and their guide RNAs, we found that 48 h of 10 mW/cm² cyan light induced both expression of mCherry and editing of the human GRIN2B gene (Figure 3f-g). In contrast, in cells only expressing VPR-ps-dSpCas9 and its sgRNA, light induced only transcriptional activation of mCherry (Figure 3f-g). A similar outcome using LACE and light-induced fragment complementation of SaCas9 (assuming the latter can be engineered) would require expressing four polypeptides in addition to the two sgRNAs, adding to complexity.

In summary, we reported the engineering of three new light-controllable Cas9 variants, ps-SpCas9, VPR-ps-dSpCas9, and ps-SaCas9, by fusing pdDronpa domains to rationally identified protein loops within Cas9 proteins. Our ps-Cas9 proteins differ from previous photocontrollable Cas9's in several ways. First, ps-Cas9 proteins require no cofactor because the maturation of the pdDronpa chromophore is autocatalytic. Second, the fluorescent nature of pdDronpa provides an inherent real-time monitoring system for the status of the controlled proteins. Third, the same ps-Cas9 unit can be used in both catalytically active and inactive versions without any further protein engineering. Last, ps-Cas9 proteins are single polypeptides, in contrast to light-induced two-hybrid or fragment complementation systems, offering greater experimental convenience.

It should be possible to apply ps-Cas9 to control gene editing or transcriptional regulation in vivo. We recently demonstrated that pdDronpa-based photoswitchable kinases could mediate...
developmental changes and synaptic vesicle transport in C. elegans in response to weak cyan light (0.7 mW/cm²). We thus envision that ps-Cas9 can also be easily used in transparent organisms such as C. elegans and zebrafish. While light of wavelengths >600 nm is able to propagate further into tissues outside the target area, in which case the two-photon illumination will be necessary to prevent induction of the unintended genes. We demonstrated simultaneous photoinduction and transactivation of different genes using ps-SaCas9 and VPR-ps-dSpCas9, but other possibilities exist, such as transcriptional repression of one gene and activation of another. The ps-Cas9 design thus expands the light-controllable CRISPR/Cas9 toolbox and should allow robust optical regulation of a variety of DNA-directed functions.

**METHODS**

**DNA Construction.** Plasmids were constructed by standard molecular biology methods, with complete sequences available upon request. Mutations in dsSpCas9 were introduced by overlap-extension PCR. SaCas9 plasmid was synthesized by Genescript. SpCas9, dSpCas9, and SaCas9 fragments were amplified from their parent plasmids and assembled with Droona variants using In-Fusion (Clontech). The SpCas9 and SaCas9 fusions were subcloned after the CAG promoter in the PX462 vector, a gift from F. Zhang (Addgene plasmid # 48141). Guide RNA sequences targeting human GRIN2B (CAAGCGGACCCGGATCAC) were created by oligo annealing and human GRIN2B (Addgene plasmid 48141). Guide RNA sequences targeting human GRIN2B were gel-purified on a microcentrifuge column (Zymo Research). Genomic DNA regions of interest were amplified from their parent plasmids and assembled with Droona variants using In-Fusion (Clontech). The SpCas9 and SaCas9 fusions were subcloned after the CAG promoter in the PX462 vector, a gift from F. Zhang (Addgene plasmid # 48141). Guide RNA sequences targeting human GRIN2B (CAAGCGGACCCGGATCAC) were created by oligo annealing and overlap PCR and were cloned after the U6 promoter in PX462. The VPR-dSpCas9 fusions were subcloned after the PGK promoter in a PiggyBac vector. The sgTRE3G guide plasmid encodes the guide RNA for the hMOV10 locus. PCR products were gel-purified using the DNA purification kit (Zymo Research), and 1/20 of the final products were used as a template for the secondary PCR. The secondary PCR was performed with primers 5'-TCTCATTCGT-CAGAGCAAATACCAAG-3' and 5'-ACAGTGTCCACCGGTTGAATAAGG-3' for hGRIN2B and 5'-CTCTTCCACCTCCTGGCAGGAC-3' and 5'-CAGTCTACGTCAACTTCTTCTTGG-3' for hMOV10 using the PrimeSTAR HS DNA polymerase (Takara), and PCR products were gel-purified on a microcentrifuge column (Zymo Research). A total of 400 ng of DNA was analyzed for indels using the SURVEYOR assay (Integrated DNA Technologies) following the published protocol.25

**Characterization of Photoswitchable dCas9.** HEK293T cells with a stable mCherry reporter gene were transfected with the dSpCas9 plasmids and the sgTRE3G guide RNA plasmid at a 1:1 mass ratio in 96-well plates. Cell imaging was performed using a Zeiss Axiosvert 200 M microscope with an X-Cite 120-W metal-halide lamp, a 1-m liquid light guide with a 3 mm core (Lumen Dynamics), a 560/40 nm excitation filter, a 585 nm dichroic mirror, a 630/75 nm emission filter (Chroma), and an Orca-ER camera (Hamamatsu). The microscope was operated with μManager 1.4.2 in Mac OS 10.6.8 on a 2.5-GHz Core 2 Duo MacBook Pro computer (Apple). To quantitatively characterize fluorescent protein expression, cells were dissociated using 0.05% Trypsin EDTA (Gembio) and analyzed by flow cytometry on a BD LSR II instrument at the Stanford flow cytometry facility, and data were analyzed using Flowjo. A threshold of mCherry expression was manually set in Flowjo with the standard that <0.25% of the untransfected cells would be counted as mCherry positive. The average mCherry expression was determined by analyzing the mean mCherry fluorescence of transfected cells. The TagBFP fluorescent expression in the sgTRE3G plasmid was used to determine successful cotransfection.

**ASSOCIATED CONTENT**

**Supporting Information** The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00603.

**Figures s1, s2, and s3 (PDF)**

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: mzlin@stanford.edu.

**ORCID**

Michael Z. Lin: 0000-0002-0492-1961

**Notes**

The authors declare no competing financial interest.

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