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# Optical Control of CRISPR-Cas9 system for gene editing using photolabile crRNA

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**Abstract:** Currently CRISPR-Cas9 is a widely used efficient tool for gene editing. Precise control over CRISPR-Cas9 system in temporal and spatial resolution is essentially important for studying gene regulation and editing. Here, we presented a novel light-controlled crRNA via coupling vitamin E and a photolabile linker at 5' terminus to inactivate CRISPR-Cas9 system. The vitamin E modification did not affect ribonucleoprotein (RNP) formation of Cas9/crRNA /tracrRNA complexes, but did inhibit the association of RNP with the target DNA. Upon light irradiation, vitamin E caged crRNA was successfully activated to achieve light-induced genome editing of Vascular Endothelial Cell Growth Factor A (VEGFA) in human cells through T7E1 assay and Sanger sequencing as well as gene knock-down of EGFP expression in EGFP stably expressing cells. This new caging strategy for crRNA is readily achievable and will possibly provide new methods for spatiotemporal photoregulation of CRISPR-Cas9 mediated gene editing.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) is a type of immune system widely existing in bacteria[1]. Among different Cas proteins in the subtypes of CRISPR systems, Cas9 protein is currently the most commonly used system for gene editing[2]. In the process of CRISPR-Cas9 system, chimeric single-stranded guide RNA (sgRNA) or CRISPR RNA/trans-activating CRISPR RNA (crRNA/tracrRNA) duplex directs Cas9 to recognize the target locus containing Protospacer Adjacent Motif (PAM)[3] and the complementary spacer sequence of guide RNA. The target dsDNA is unwound to form the complex with Cas9/guide RNA, which further induces the conformational changes of Cas9 and dsDNA cleavage[1c, 4]. Finally, DNA repair is performed through non-homologous end joining (NHEJ) or homologous recombination (HR)[5].

Although CRISPR-Cas9 system is now widely applied as a gene editing tool in recent years[6], unwanted off-target effects are often observed[7]. Precise control of CRISPR-Cas9 function with spatiotemporal resolution is advantageous to reduce the off-target effects and increase its specificity[8], which enables more precise gene editing and raises its potential as a powerful tool for both basic research and therapeutic applications. Various methods had been developed to control CRISPR-Cas9 functions with small molecules and endogenous substances[9]. One commonly used method to control the activity of guide RNA was to add a blocking sequence that was hybridized to the spacer region of the guide RNA, thereby blocking the complex formation of Cas9/guide RNA/the target DNA. And then the blocking sequence/spacer sequence duplex portion was unwound in different ways. The linker between the blocking sequence and the spacer could be designed as theophylline-responsive aptazyme sequence or RNase H-responsive sequence for triggered release of sgRNA[10]. Therefore, the cleavage reaction was used to separate the blocking sequence. The spacer was released through the branch migration of the blocking sequence to obtain the guide RNA in response to endogenous RNA[11]. mRNA-responsive sgRNA, microRNA-inducible sgRNA and reversible ligand-activated sgRNA were developed to trigger the unfolding of the guide RNA for activation of CRISPR system[12]. In addition, masked guide RNA obtained by covalent attachment of the azidomethylnicotinyl (AMN) groups was able to trigger CRISPR systems efficiently under the action of Staudinger reduction[13].

Light is one of the essential means of regulation in CRISPR-Cas9 system. Most of light control methods were currently used to control Cas9 protein[8d, 8e, 14]. Light-activated Cas9 was obtained via site-specific incorporation of photosensitive lysine[8e]. A pair of light-controllable proteins was developed to achieve reversible regulation of the CRISPR-Cas9 system via the dimerization of two Cas9 fragments under light irradiation[8d]. Cas9 nuclease activity was also controlled through photoinduced separation of dimer fluorescent protein on the Cas9 domain[14c]. Few cases of light-induced guide RNA had been reported before, which required the optimization of different inhibitory oligodeoxynucleotides (ODNs) with multiple photolabile linkers for blocking gRNAs[15], or multiple photolabile nucleotides on guide RNAs for inhibiting their binding to complementary the target DNA sequences[8a, 8b, 16]. And spatiotemporal regulation of guide RNA activity was also achieved[8a, 8b].

Unlike transcriptional preparation, chemical synthesis of guide RNA with modified nucleotides (2'-OMe, 2'-F et al.) or back-

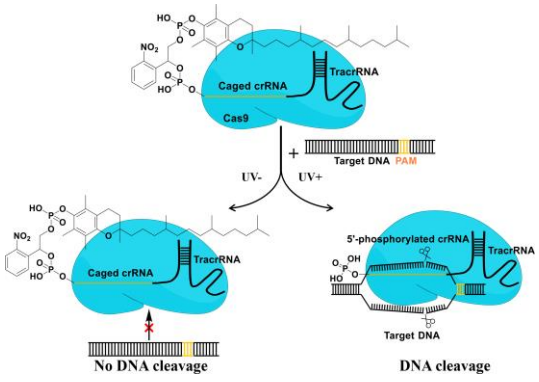



**Figure 1.** Schematic representation of light-regulation of CRISPR/Cas9 induced DNA editing.


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bones (phosphorothioate et al) greatly improves their stability and specificity for gene editing[17]. In this study, we designed and constructed a new caged CRISPR-Cas9 system (Figure 1) through a novel photolabile crRNA with simple terminus vitamin E modification. Although 5' terminus of crRNA was previously demonstrated to be tolerant to the modification with rhodamine or dibenzocyclooctyne[18], we here demonstrated that the gene editing function of CRISPR-Cas9 was efficiently blocked when vitamin E was directly linked to the 5' terminus of crRNA via a photolabile linker for point modification. Under the irradiation of UV at 365 nm, vitamin E together with photolabile linker was removed and guide RNA was recovered to restore the functional CRISPR-Cas9 system effectively.

To photomodulate the gene editing activity of CRISPR-Cas9 system, we synthesized vitamin E modified crRNA with photolabile linker at 5' terminus (Figure 2A). The phosphoramidites of nitrophenyl ethyldiol and vitamin E were first synthesized and then coupled to the 5' terminus of crRNA. The caged crRNA (Vitamin E-PL-crRNA) was then obtained after the cleavage from solid resin and further purification and characterization. With caged crRNA in hand, we first evaluated the photosensitivity of caged crRNA under different light irradiation for 1 min, 2 min, 3 min, 4 min and 5 min, respectively. As shown in Figure 2B and Figure S1, vitamin E and photolinker were completely detached from caged crRNA at 4 min light irradiation. To further confirm the ability of caged crRNA for controlling CRISPR-Cas9 function, we performed an *in vitro* DNA cleavage assay. With the crRNA/tracrRNA and Cas9 protein, the DNA cleavage efficiency was around 58% in 1 h (Figure 2C and 2D). However, with vitamin E modified caged crRNA together with tracrRNA and Cas9 protein, only less than 6.6% DNA cleavage was observed, which indicated that vitamin E modification did significantly inhibit CRISPR-Cas9 activity. Upon exposure to light irradiation for 4 min, up to 7.6-fold enhancement of Cas9-induced DNA cleavage was observed with the efficiency of 50.2%, similar to the positive control crRNA. This result clearly indicated that caged crRNA with vitamin E modification at 5' terminus was effective in photocontrolling CRISPR-Cas9 activity *in vitro*.

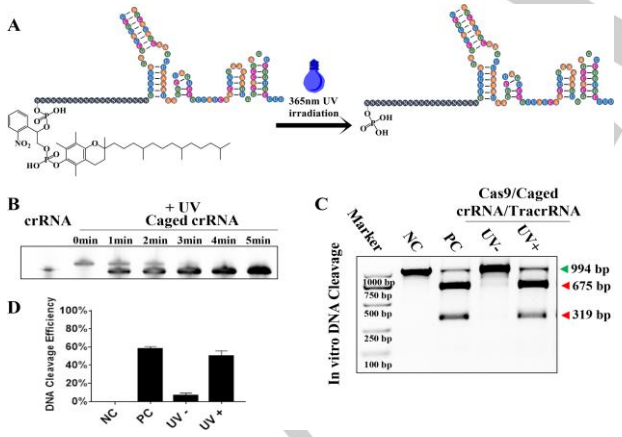



**Figure 2.** Caged crRNA (vitamin E-PL-crRNA) with vitamin E modification at 5' terminus blocked Cas9-mediated DNA cleavage *in vitro*. A) Light activation of CRISPR-Cas9 system for gene editing process upon light exposure; B) Gel running assay for validation of photocleavage of caged crRNA$_{EGFP}$ before or after light irradiation (λ = 365nm, 7mW/cm$^2$). C) Representative 2% agarose gel electrophoresis data from the DNA cleavage assay *in vitro*. NC for untreated target DNA. PC for target DNA treated with Cas9/crRNA$_{EGFP}$/tracrRNA. The uncleaved DNA band (green triangle) and the cleaved DNA bands (red triangle). D) Quantification of replicate DNA cleavage assays *in vitro* from (C). The cleavage efficiency was calculated according to eq (1) in supporting information. Error bars are the standard errors of three independent experiments.

To understand the blockage mechanism of caged crRNA for CRISPR-Cas9 function, we investigated the interaction of Cas9 protein with crRNA/tracrRNA duplex and the target DNA (Figure S2 and S3). The affinity of Cas9 protein and crRNA/tracrRNA duplex was measured via biolayer interferometry assay (Table S2). There was no obvious difference in terms of affinities between Cas9 protein and caged crRNA/tracrRNA duplex before and after exposure to light irradiation ($K_d$ = 0.68 ± 0.02 x $10^{-9}$ M and $K_d$ = 1.09 ± 0.04 x $10^{-9}$ M), which was also virtually indistinguishable from that of Cas9 and unmodified crRNA/tracrRNA duplex ($K_d$ = 1.08 ± 0.02 x $10^{-9}$ M). This result indicated that vitamin E modification of crRNA at 5' terminus didn't inhibit the formation of Cas9/crRNA/tracrRNA ribonucleoprotein (RNP) complexes (Figure S2A-C), which was further confirmed by gel analysis of RNP (Figure S2D). The RNPs were formed for both unmodified crRNA and caged crRNA, and vitamin E modification slightly retarded the mobility of RNP complex, which was recovered after light activation. The gel retardation assay was also performed to investigate the interaction between different RNP complexes and the target DNA. When both dCas9 and crRNA/tracrRNA were mixed with the target DNA, the electrophoretic mobility of the target DNA was retarded, indicating that RNP already bound the target DNA and form the complex as expected (Figure S3). While in the presence of dCas9 and caged crRNA/tracrRNA, the target DNA showed the same electrophoretic mobility as the mixture of only the target DNA and crRNA/tracrRNA without dCas9, which suggested that the target DNA didn't bind to RNP with caged crRNA. After light irradiation of above mixture of RNP and the target DNA, the recovery of RNP/DNA complex formation resulted in slow electrophoretic mobility of the target DNA, similar to that of positive control with dCas9/crRNA/tracrRNA. This observation demonstrated that vitamin E modification of caged crRNA at 5' terminus efficiently inhibited the interaction between RNP and the target DNA for the formation of RNP/DNA complex.

To further investigate the ability of caged crRNA to control gene editing in human cells, photomodulation of gene editing of endogenous VEGFA was then examined using caged crRNA$_{VEGFA}$ in HEK293T cells. The effect of ratios between Cas9 and caged crRNA$_{VEGFA}$/tracrRNA duplex on gene editing efficiency was first optimized before and after light activation. For VEGFA locus, caged crRNA$_{VEGFA}$-mediated gene editing efficiency was the highest when Cas9: RNA duplex was 1: 1.5 (Figure S4). Under this optimized ratio, the insertion-deletion (indel) efficiency of VEGFA caused by crRNA$_{VEGFA}$/tracrRNA was 61.5% without UV irradiation, and light irradiation induced a little decrease of indel efficiency to 53.4% probably due to the light induced partial loss of Cas9 activity (Figure 3A and Figure S5). However, for caged crRNA$_{VEGFA}$, less than 3.1% indel background efficiency of VEGFA was observed when Cas9 and caged crRNA$_{VEGFA}$/tracrRNA were electroporated without UV irradiation, and further Sanger sequencing indicated that no indel mutation was detected, similar to the negative control (Figure 3B). Upon light irradiation for 4 min, caged crRNA$_{VEGFA}$/tracrRNA duplex-mediated indel efficiency of VEGFA reached to 45.2% with more than 14.6-fold enhancement of gene editing efficiency, and indel mutations in Sanger sequencing were clearly observed with similar observation as that of the



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positive control. The difference in indel efficiency between crRNA$_{VEGFA}$/tracrRNA duplex-mediated and caged crRNA$_{VEGFA}$ /tracrRNA duplex-mediated CRISPR-Cas9 gene editing was consistent with that *in vitro* DNA cleavage assay. These results demonstrated the possibility of photomodulation of CRISPR-Cas9 system using single vitamin E modified caged crRNA in human cells. In addition, although electroporation and light irradiation induced a certain degree of cell toxicity (Figure S6), but no gene editing was observed (Figure 3B).

To demonstrate the photomodulation EFGP gene silencing using CRISPR-Cas9 system, we synthesized vitamin E modified caged crRNA targeting EGFP locus according to the same caging strategy and developed Cas9/caged crRNA$_{EGFP}$ /tracrRNA complex for spatial regulation of EGFP expression in EGFP stably expressing HEK293T-EGFP cells. As shown in Figure 4 and Figure S8 of their quantitative analysis in supporting information, light irradiation had little effect on EGFP fluorescence in the negative control experiment. With the existence of Cas9/crRNA$_{EGFP}$/tracrRNA, EGFP expression level was dramatically down-regulated no matter whether light irradiation was applied. For Cas9/caged crRNA$_{EGFP}$/tracrRNA complex, EGFP expression was largely maintained before light irradiation, and obvious photomodulation of EGFP expression in HEK293T-EGFP cells was observed upon light-triggered activation of the corresponding Cas9/caged crRNA$_{EGFP}$/tracrRNA RNP complex.

In summary, we developed a novel photolabile crRNA modified with vitamin E via a photolabile linker at 5' terminus for the assembly of caged CRISPR-Cas9 system. With vitamin E modified caged crRNA$_{VEGFA}$, RNP complex of Cas9/caged crRNA$_{VEGFA}$/tracrRNA temporally lost its binding and cleavage ability for target VEGFA DNA in cells. Light irradiation removed the vitamin E and photolabile linker, and recovered the native crRNA, which activated the RNP complex to bind and cut the target DNA duplex for further gene editing. A 14.6-fold enhancement of gene editing efficiency (from 3.1% to 45.5%) was achieved upon light-irradiation. Further gene sequencing of VEGFA DNA indicated that the RNP complex of Cas9/caged crRNA$_{VEGFA}$ /tracrRNA did not trigger any editing pattern of VEGFA as the negative control, while gene editing pattern similar to the positive control was clearly observed upon light activation. Efficient gene knockdown was also achieved in EGFP using the same caging strategy, indicating that it could be a general approach for the spatial photomodulation of target gene expression using this new caging strategy. Different from the previously reported methods by changing the secondary structure of the longer guide RNA or designing a spacer blocking sequence[10-12, 15], this caged crRNA was easily synthesized using solid phase synthesis and was compatible with other nucleic acid modifications, leading to better application prospects. And our caged CRISPR-Cas9 system only needs to synthesize different caged crRNAs for different targets without adding other inhibitory oligonucleotides, which is more convenient than the inducible sgRNAs previously reported. In addition, it was able to readily assemble

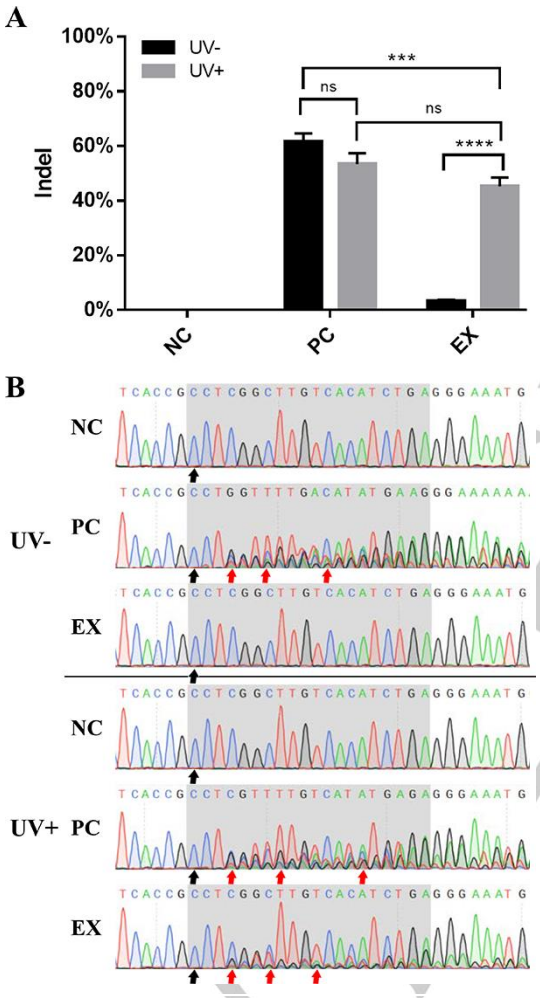



**Figure 3.** Genome editing activity of Cas9/caged crRNA$_{VEGFA}$/tracrRNA targeting VEGFA site in HEK293T cells. A) Indel mutation efficiency measured by T7E1 assay. Negative control (NC) without RNP complex; positive control (PC) electroporated with Cas9/crRNA$_{VEGFA}$/tracrRNA; experimental group (EX) electroporated with Cas9/caged crRNA$_{VEGFA}$/tracrRNA. DNA indel efficiency was calculated from the band intensities of agarose gels. Values were Mean ± SEM with n = 7. ***p<0.001. ****p<0.0001. B) Sanger sequencing of indel mutations in VEGFA locus under different conditions. The grey shaded 20 bases on the VEGFA locus that crRNA$_{VEGFA}$ targeted. Single peak (black arrow) indicated there was no mutation at this base site. Nested peak (red arrow) indicated that there were multiple bases at this site, which was due to the indel of DNA template.

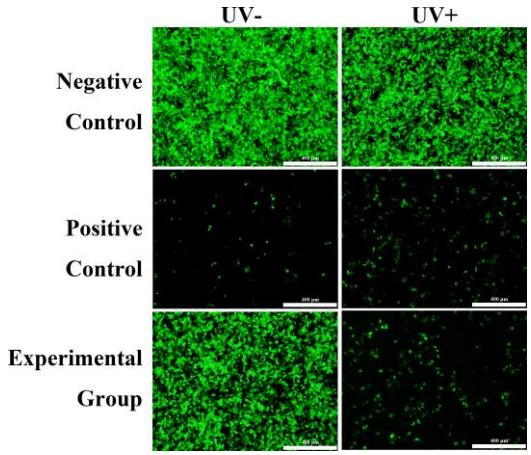



**Figure 4.** EGFP gene knockdown using Cas9/caged crRNA$_{EGFP}$/tracrRNA in HEK293T-EGFP cells. Cells were imaged with inverted fluorescence microscope (IX83, OLYMPUS). Negative control without RNP electroporation; positive control with the electroporation of Cas9/crRNA$_{EGFP}$ /tracrRNA; experimental group with the electroporation of Cas9/caged crRNA$_{EGFP}$/tracrRNA. Cells were irradiated for 4 min (λ=365nm, 7mW). Scale bar = 400 μm



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RNP complex with tracrRNA and Cas9 protein. The vitamin E modification on crRNA blocked CRISPR-Cas9 function by impacting the interaction between RNP and the target DNA with lower binding affinity until light-triggered release of caged crRNA. Substitution of vitamin E with cholesterol moiety produced the same blocking effect on CRISPR-Cas9 function (Figure S9). The results indicated that large hydrophobic molecules with possible receptor proteins and modification at the 5' terminus of crRNA via a short and rigid linker could generate a strong steric effect to block the activity of crRNA, so this terminus caging strategy might be a general caging strategy for developing caged CRISPR/Cas9 system. Currently there are few small molecule inhibitors of Cas9 discovered[19], our findings may provide new possibilities for the development of CRISPR-Cas9 inhibitors. In addition, this simple caging strategy could be compatible with chemical regulation and protein regulation, providing a new tool for possible precise spatiotemporal regulation of CRISPR-Cas9. Further researches using our terminus caging strategy for CRISPR-Cas9 system may reveal a deeper and clearer understanding of the mechanism how hydrophobic molecules like vitamin E affect the interaction between the RNP complex and the target DNA. This study may enable further optimization of the regulatory strategy and improvement of the gene editing efficiency with photolabile CRISPR-Cas9 system.

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**Keywords:** CRISPR Cas9 • caged crRNA • vitamin E terminus modification • photomodulation • gene editing

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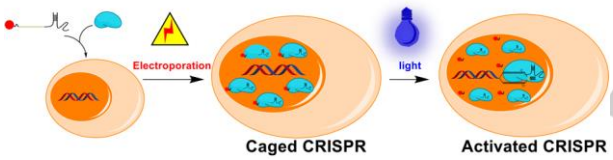


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## Entry for the Table of Contents




**Activate CRISPR with light:** Caged crRNA was readily developed with terminus modification of photolabile linker and vitamin E for construction of caged CRISPR-Cas9 system, which was successfully used for photomodulation of VEGFA gene editing and control of EGFP gene silencing through temporarily inhibiting the interaction between the target DNA and Cas9/crRNA/tracrRNA complex.

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