Two chemical-controlled switchable Cas9s for tunable gene editing

Meng Liang^{1,2}, Yang Cui^{1,2}, Jie Lan^{1,2}, Guangtao Song^{1,2}✉, Jizhong Lou^{1,2}✉

¹ Key Laboratory of RNA Biology, CAS Center for Excellence in Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

² University of Chinese Academy of Sciences, Beijing 100049, China

Received: 21 January 2019 / Accepted: 24 February 2019 / Published online: 4 July 2019

INTRODUCTION

The clustered regularly interspaced short palindromic repeats (CRISPR) loci and their associated (Cas) genes are found in many bacterial and archaeal genomes as adaptive defense systems against phage infection and plasmid transfer (Mohanraju *et al.* 2016). In the CRISPR-Cas system, single or multiple Cas proteins were complexed with a small CRISPR RNA (crRNA), forming an RNA protein complex (RNP), which uses the crRNA as the guide to destruct the invading nucleic acids (Mohanraju *et al.* 2016). Based on CRISPR locus organization and the Cas gene content, CRISPR-Cas systems are divided into two classes. Class 1 systems use a complex of several Cas proteins called Cascade, while class 2 systems use a single Cas protein (such as Cas9), as interference module to recognize and cleave the target nucleic acids (Mohanraju *et al.* 2016). In 2013, a class 2 CRISPR-Cas system, CRISPR-Cas9, from *Streptococcus pyogenes*, was repurposed as a revolutionary tool for precise gene editing and gene expression control (Cong *et al.* 2013). In this system, the *Streptococcus pyogenes* Cas9 (SpyCas9) first binds to an artificial chimeric single-guide RNA (sgRNA) comprising crRNA and a transactivating crRNA (tracrRNA) module to form an effector complex (Cas9–sgRNA complex) (Jinek *et al.* 2012). After the recognition of a short protospacer-adjacent motif (PAM) sequence in target DNA, Cas9–sgRNA complex binds and induces an R-loop in target

DNA through base-pairing between the guide sequence in crRNA and the DNA target. SpyCas9 finally uses its HNH and RuvC nuclease domains, to cleave the target and nontarget strands, respectively (Fig. S1) (Jiang *et al.* 2016).

Despite much progress in CRISPR-Cas9 system in genome editing, numerous studies have assessed off-target DNA binding and cleavage by the Cas9 complex, both *in vitro* and *in vivo* (Fu *et al.* 2013; Hsu *et al.* 2013). Efforts to improve the Cas9 specificity have accordingly been of great interest in the field (Tycko *et al.* 2016). Two different strategies have been developed: engineering the high-fidelity Cas9 protein or guide RNA (Fu *et al.* 2014; Kleinstiver *et al.* 2016), spatiotemporal control of Cas9 or guide RNA via external trigger signals (Gao *et al.* 2016; Nihongaki *et al.* 2018; Richter *et al.* 2017). For the latter strategy, the Cas9 activity is controlled at the post-translational stage either by modifying Cas9 protein or sgRNA using an external stimulus such as chemicals or light. This conditional control of Cas9 function has the potential to provide spatiotemporal gene manipulation in user-defined cell population at a specific time and improve the accuracy of Cas9-mediated genome editing. To date, diverse chemical-inducible Cas9s have been developed, including controlling assembly of split Cas9, fusing Cas9 with chemical-inducible protein domains. Another approach for controlling Cas9 activity is using the photo-switchable protein domains to construct the light-inducible Cas9s (Nihongaki *et al.* 2018). These inducible Cas9 systems have significantly expanded the CRISPR toolkits. It is known that during the target binding and cleavage by Cas9–sgRNA complex, the HNH domain undergoes a big structural transition from an inactivated state, in which the catalysis is hampered, to an activated state prone for

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s41048-019-0093-3>) contains supplementary material, which is available to authorized users.

✉ Correspondence: gsong@moon.ibp.ac.cn (G. Song), jlou@ibp.ac.cn (J. Lou)

the cleavage of the target strand (Fig. 1A). DNA cleavage of Cas9 is controlled by the conformational state of the HNH nuclease domain. Here, we designed two types of switchable Cas9 based on controlling the conformational switch of HNH domain by redox state or zinc ion of the surrounding buffers.

RESULTS AND DISCUSSION

The intracellular oxidation–reduction reactions play central roles in cell metabolism and are integral components of cellular signaling and cell fate decisions. Changes in the redox equilibrium of cells are accompanied with transitions of many cell functions, such as proliferation, differentiation, immune responses, senescence, and death (Banaszynski *et al.* 2006; Beal 1995). The ability to respond and control the intracellular redox equilibrium is thus of great interest in biomedical research. Redox condition is one of the most effective local stimuli that can be exploited. Alterations in the redox equilibrium are precipitated by changing either the glutathione/glutathione-disulfide (GSH/GSSG) ratio and/or the reduced/oxidized thioredoxin ratio. By placing artificial disulfides onto fluorescent proteins, several redox-sensitive GFPs were developed to probe the redox states of the cell (Belousov *et al.* 2006). In these designs, pairs of cysteines were introduced in different positions of the GFP such that the two residues

are capable of forming an intramolecular disulfide bridge, resulting in a significant decrease of fluorescence intensity. These redox-sensitive GFPs (roGFPs) have been widely used to probe the intracellular redox state. Inspired by these designs, it is possible to construct a redox-sensitive Cas9 by controlling the conformation change of its active domain. Previous structural studies revealed that the distance between S867 and N1054 is less than 0.5 nm in spyCas9–sgRNA complex, whereas more than 5 nm in R-loop complex (Jiang *et al.* 2016). We thus incorporate a disulfide bound between HNH and RuvC domain of Cas9 (S867C–N1054C), constructing a disulfide-controlled Cas9 which is sensitive to the redox state of its surroundings.

We next isolate and purified the Cas9 proteins with single or dual sites mutations, S867C, N1054C, and S867C/N1054C. The mobility shift analysis of the purified Cas9 and its mutants under nonreducing conditions revealed that wtCas9 protein and its single-site mutants consist of a major band around 150 kDa, whereas the dual mutant consists of a major band around 190 kDa. This indicates that the isolated Cas9 formed an intra- or inter-molecular disulfide bond. Boiling of Cas9 in an SDS-PAGE buffer containing 5 mmol/L DTT converted slower component to the faster component similar to that of wtCas9 (Fig. 1B). We then asked whether the Cas9 activity could respond to the redox state of the surrounding conditions. The *in vitro* plasmid cleavage assay was next used to probe the redox-dependent Cas9

Fig. 1 **A** Schematic of the conformational change of SpyCas9 during R-loop formation. **B** SDS-PAGE analysis of the Cas9 mutants. **C** Effect of Cu²⁺ on the target cleavage activity of Cas9 and its mutants; **D** Effect of DTT on the target cleavage activity of Cas9 and its mutants. SC: supercoiled DNA, L: linear DNA, N: nicked DNA

activity. It is known that the Cu^{2+} could increase the degree of oxidation and induce disulfide bond. As shown in Fig. 2B, it could be found that the addition of Cu^{2+} decreases the redox-Cas9 activity, not the wtCas9, demonstrating that the target cleavage activity was switched off when increasing the oxidation state of the buffer. We next used DTT as the reducing agent to test the Cas9 activity. As shown in Fig. 1C, upon increase of the DTT concentration, the percentage of linearized plasmid increases. Generally, the intracellular redox changes are precipitated by changing either the GSH/GSSG ratio and/or the reduced/oxidized thioredoxin ratio (Simonian and Coyle 1996). We thus used GSH/GSSG ratio to probe the redox effect on Cas9 activity. It could be seen that after the increase of GSH/GSSG ratio, the target DNA could be totally linearized by redox-Cas9 (Fig. S2). All these data showed that we have successfully constructed a switchable Cas9 which could be responsible to the redox state of buffer *in vitro*.

We also used a bacterial-based negative selection system to prove the function of the redox-Cas9 *in vivo*. As shown in Fig. 2A, a pET41a plasmid encodes a redox-Cas9 and sgRNA was first transformed into the *E. coli* Rosetta (DE3) strain. Another pUC19-based plasmid containing the target sequence of the sgRNA was then transformed to the Cas9-expressed *E. coli* cells. The inactive Cas9 cells which could not target and digest the plasmids enable cell survival due to the presence of an

antibiotic-resistance gene, whereas cells with active Cas9 are depleted from the library. As expected, the dual-mutated redox Cas9 showed much less activity than that of wtCas9 or single-site mutants (Fig. 2B). These data indicate that the redox state in *E. coli* cell is moderate, which enable a high percent of disulfide bond. Unfortunately, we were not able to modulate the redox-Cas9 activity by simply changing the concentration of DTT or ratio of GSH/GSSG (data not shown). Further work will focus on maximizing the design to screening new mutant which could effectively respond to the redox state *in vivo*.

Our previous biochemical studies revealed that the conformational switch of HNH domain in Cas9 during the R-loop formation could be altered by the single-site mutation in the linkers between HNH and RuvC domain (Zeng *et al.* 2018). We anticipated that the rotation of HNH domain could be also affected by changing the flexibility of the two linkers. To prove this hypothesis, we incorporate a zinc finger domain Zif268 (Elrod-Erickson *et al.* 1996) into the C-termini of the HNH between 908 and 909 in Cas9, constructing a zfCas9 (Fig. 2C). If there are no zinc ions in the solution, the zinc finger domain is unstable, and the conformation change of HNH will not be affected. When adding zinc ions into the solution, the domain will be stable and rigid, restraining the rotation of HNH and leading to a disable Cas9 activity. Figure 2D shows the target

Fig. 2 **A** Schematic of bacterial-based target cleavage assay for the characterization of the disulfide bond-controlled switchable Cas9. **B** Transformation efficiencies of plasmids carrying target sequence to *E. coli* cells expressing sgRNA and Cas9 proteins. **C** Schematic of the conformational change of zfCas9 in the absence and presence of zinc ions. **D** Effect of zinc ion on the target cleavage activity of wtCas9 and zfCas9. SC: supercoiled DNA, L: linear DNA, N: nicked DNA

cleavage activity of the wtCas9 and zfcas9 in the absence and presence of zinc chloride (0.2 mmol/L). It could be found that the zfcas9 could keep the target cleavage activity in the absence of zinc chloride similar to that of wtCas9. In contrast, after addition of 0.2 mmol/L zinc chloride into the reaction solution, the zfcas9 loses most of its cleavage activity. We next studied the Zn^{2+} concentration dependent on zfcas9 activity. We found that, upon increase of the Zn^{2+} concentration, the percentage of linearized plasmid decreases (Fig. S3). These results clearly demonstrate that the activity of zfcas9 could be modulated by the zinc ions in the solution.

In summary, we have developed two switchable Cas9s for conditional control of gene editing. Through rational incorporation of a disulfide bond between its HNH and RuvC domain or a zinc finger domain in the linker between HNH and RuvC domain, we were able to modulate its target cleavage activity through changing the surrounding redox state or zinc ion concentration. As the redox equilibrium or zinc ion is an integral component of cellular signal processing and cell fate decision making, this redox-sensing gene editing tool may be very useful for the study of the molecular details of redox biochemistry.

MATERIALS AND METHODS

Recombinant SpyCas9 expression and purification

SpyCas9 and mutants were cloned into a custom pET-41a expression vector which contains an N-terminal GST-tag, His6-tag, and a thrombin protease cleavage site. Site-directed mutagenesis was performed to introduce point mutations into SpyCas9 and the accuracy was verified by DNA sequencing. Cas9 and its mutants were expressed in *E. coli* Rosetta 2 (DE3) (Novagen) and purified by chromatography on Ni-NTA superflow (QIAGEN). The tags were removed by thrombin at 4 °C overnight, and purified by cation exchange (SP Sepharose) chromatography. The purified Cas9 proteins were then analyzed with SDS-PAGE gel electrophoresis.

In vitro transcription and purification of RNA

sgRNAs were generated by *in vitro* transcription from DNA templates carrying a T7-promoter sequence using T7 *in vitro* transcription kit (MEGAscript T7, Invitrogen) and purified by gel as described before (Zeng *et al.* 2018).

Plasmid DNA cleavage assays

pUC19-based protospacer plasmids were generated by annealed oligonucleotides between digested EcoR I and Hind III sites in pUC19 for further *in vitro* cleavage assays. Ligated plasmids DNA were transformed into *E. coli* DH5- α cells according to a standard heat shock protocol. A custom-designed plasmid containing a 20-bp DNA target sequence and a 5'-TGG-3' PAM motif was used in plasmid cleavage assays. Purified SpyCas9 and synthesized sgRNA were pre-incubated for 15 min in cleavage buffer (20 mmol/L HEPES, pH 7.5, 100 mmol/L KCl, 2 mmol/L $MgCl_2$) to reach the final concentration of 125 and 375 nmol/L, respectively; 500 ng supercoiled plasmid was then added to the reaction mixtures and incubated at room temperature for various time points. The cleavage reactions were stopped by adding 6 $\times$ sodium dodecyl sulfate loading buffer. 1.2% agarose gels were performed to characterize cleavage products. The gels were stained by Midori Green (NIPPON Genetics Europe) and analyzed with Image J software.

Bacterial-based target cleavage assay

PET41a-based plasmid containing wtCas9 or its mutants and sgRNA expression elements were first transformed into *E. coli* Rosetta (DE3) cells. These cells were further transformed with negative selection plasmids harboring cleavable target sites. Following a 60-min recovery in LB media, transformations were plated on LB plates containing chloramphenicol, kanamycin, and ampicillin. Cleavage of the negative selection plasmid was estimated by calculating the colony forming units per mg of DNA transformed.

Acknowledgements This work was supported by the National Natural Science Foundation of China (31771015, 11672317).

Compliance with Ethical Standards

Conflict of interest Meng Liang, Yang Cui, Jie Lan, Guangtao Song, and Jizhong Lou declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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