ACS Synthetic Biology
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Modularized CRISPR/dCas9 Effector Toolkit for Target-Specific Gene Regulation

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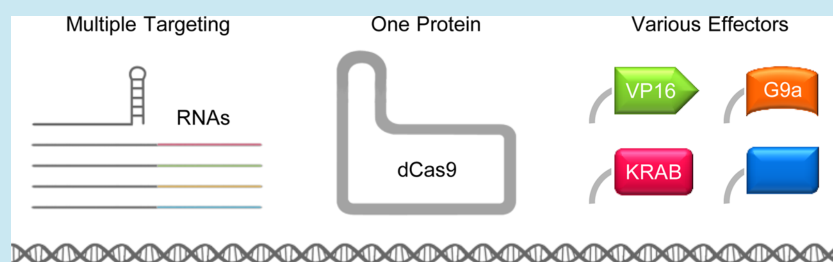
Supporting Information



ABSTRACT: The ability to control mammalian genes in a synergistic mode using synthetic transcription factors is highly desirable in fields of tissue engineering, stem cell reprogramming and fundamental research. In this study, we developed a standardized toolkit utilizing an engineered CRISPR/Cas9 system that enables customizable gene regulation in mammalian cells. The RNA-guided dCas9 protein was implemented as a programmable transcriptional activator or repressor device, including targeting of endogenous loci. For facile assembly of single or multiple CRISPR RNAs, our toolkit comprises a modular RNAimer plasmid, which encodes the required noncoding RNA components.

The CRISPR/Cas (Clustered regularly interspaced short palindromic repeats) system of *Streptococcus pyogenes* has recently gained increasing attention as a tool for genome editing and gene regulation.[1−3] The CRISPR associated nuclease protein Cas9 binds to a small CRISPR-RNA (crRNA) mediated by a second small trans-activating crRNA (tracrRNA). Guided through 30 basepairs within the crRNA sequence (spacer), Cas9 is able to specifically bind to a complementary DNA sequence and cleave the targeted region. We have inactivated the nuclease functions of Cas9 through inserting point mutations in the HNH and RuvC-like domains. By fusing effector domains to the catalytically inactive dCas9 protein synthetic transcription factors were constructed.[4,5] This engineered CRISPR system can be used to control transcription of multiple genes simultaneously when cotransfected with plasmids encoding the respective crRNAs.

Here, we provide a standardized dCas9 toolkit that enables flexible effector domain exchange and facile cloning of desired spacer sequences (for complete part list refer to Supporting Information Table S1). The effectors tested include the histone methyltransferase G9a-SET domain (G9a-SD),[6] the trans-activating herpes simplex protein VP16 domain and the repressive Krüppel-associated box (KRAB) domain.[7] Furthermore, we developed a modular plasmid encoding the RNA-components for user-friendly crRNA sequence exchange and assembly of multiple crRNAs, the RNAimer plasmid.

RESULTS AND DISCUSSION

We generated a nuclease-deficient *dcas9* sequence (based on ref 1). The human influenza hemagglutinin derived HA-tag and a nuclear localization sequence (NLS) derived from the adeno-associated virus serotype 2 (AAV2-NLS) were fused N-terminally to dCas9 via BioBrick standard assembly (Figure 1a). A human cytomegalovirus promoter (hCMV) was used to drive expression of the constructs in mammalian cells. We fused effector domains C-terminally to dCas9 to achieve transcriptional regulation of genes of interest. For effector fusions with

Special Issue: iGEM 2013

Received: January 17, 2014
Published: December 19, 2014

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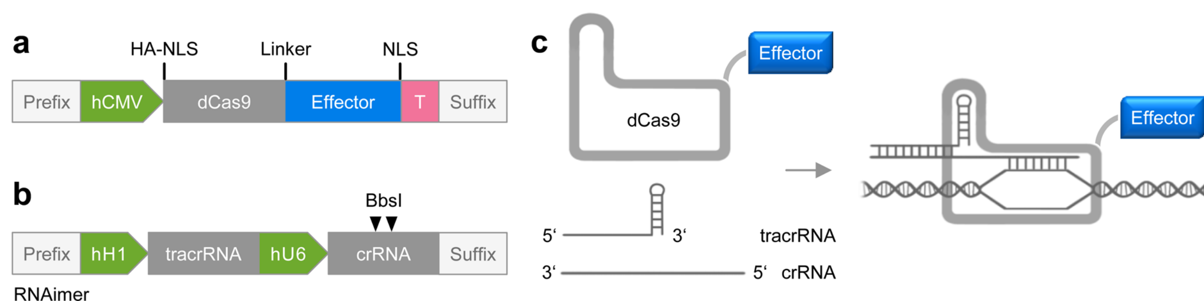

Figure 1. CRISPR/dCas9 effector toolkit components for target-specific gene regulation. (a) The standardized and nuclease-deficient dCas9 is combined with an effector domain for transcriptional regulation. The protein sequence is flanked by nuclear localization sequences (NLS) and an HA-tag is added N-terminally, P: hCMV promoter, T: BgH transcription terminator. (b) The noncoding trans-activating crRNA (tracrRNA) and CRISPR RNA (crRNA) are driven by RNA polymerase III dependent promoters (human H1 and human U6) and located on the RNAimer plasmid. The crRNA guides the protein to a specific DNA target and must therefore in part be complementary to this sequence. The appropriate DNA sequence can easily be inserted by annealed oligonucleotide ligation into the *Bbs*I digested RNAimer plasmid. (c) Expression products of plasmids (a) and (b); both RNAs are needed to form an RNA-dCas9-complex which can specifically bind to the DNA sequence of interest. Prefix contains *Eco*RI, *Not*I, and *Xba*I restriction sites. Suffix contains *Spe*I, *Not*I, and *Pst*I restriction sites.

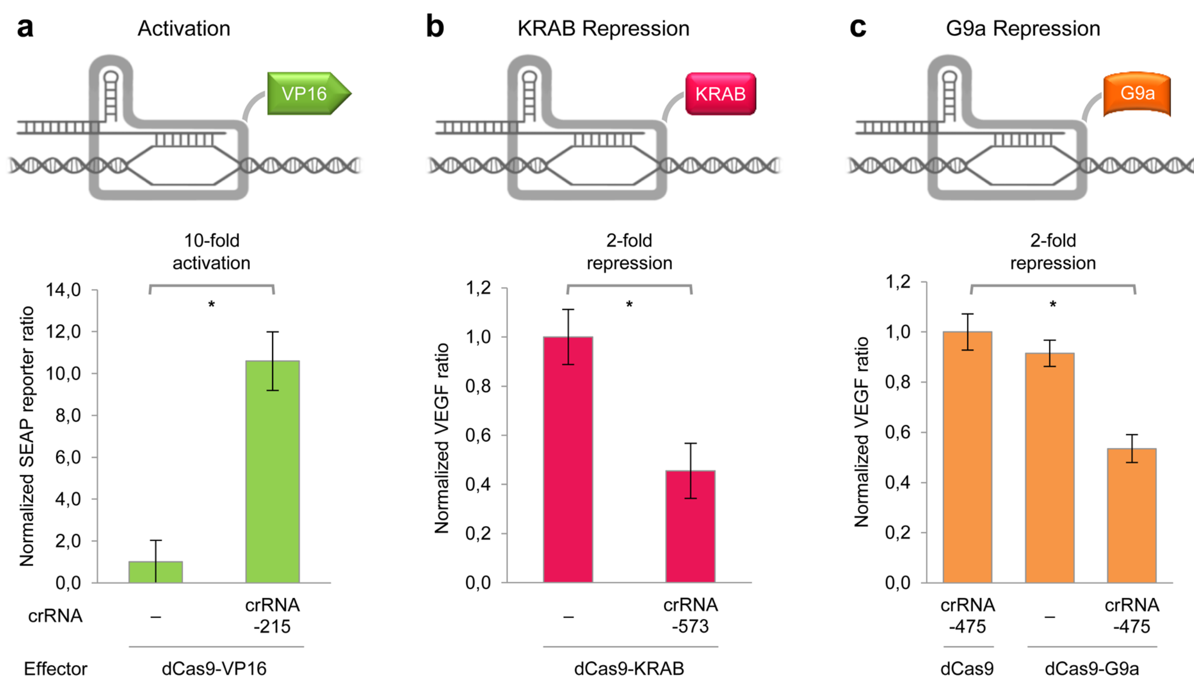

Figure 2. Gene up- and downregulation achieved with different effectors fused to dCas9. (a) dCas9-VP16 activates SEAP reporter expression. HEK-293T cells were cotransfected with the dCas9-VP16 construct, a SEAP reporter plasmid, a construct for constitutive Renilla luciferase expression, as well as an RNAimer vector comprising a crRNA sequence for targeting the SEAP reporter 215 bp upstream of the translational start site. In control wells, no RNAimer plasmid was transfected. Twenty-four hours after transfection, SEAP activity in the cell supernatant was analyzed and normalized to a Renilla luciferase reporter assay. Control was set to 1. (b) dCas9-KRAB represses endogenous VEGF-A expression. HEK-293T cells were cotransfected with constructs for dCas9-KRAB, constitutive SEAP expression and an RNAimer plasmid comprising a crRNA sequence for targeting 573 bp upstream of the transcriptional start site of the VEGF-A locus. In control wells, no RNAimer plasmid was transfected. Forty-eight hours after transfection, VEGF-A levels in cell supernatant were assessed and normalized to SEAP activity. Control was set to 1. (c) dCas9-G9a represses endogenous VEGF-A expression through H3K9 dimethylation. HEK-293T cells were cotransfected with the dCas9-G9a construct, without or with an RNAimer plasmid targeting the VEGF-A promoter 475 bp upstream of the transcriptional start site, and a constitutive SEAP expression plasmid. As additional control cells were transfected with a dCas9 construct without an effector domain and directed to the same sequence. Forty-eight hours after transfection, VEGF-A levels in cell supernatant were measured and normalized to SEAP activity. One scale unit corresponds to 0.966 ng/mL VEGF-A. Results are means ± standard deviation (SD) ($n = 3$). For each effector statistically significant difference to the control is indicated with an asterisk (student's t test, two-sided with unequal variance, $p < 0.05$). Relative expression of all controls was set to one. SEAP: secreted alkaline phosphatase, VEGF-A: vascular endothelial growth factor-A.

dCas9, a short flexible linker of seven amino acids (TGAGSTG) was introduced, as well as a second NLS and the bovine growth hormone (BgH) transcription terminator (Figure 1a). On a second vector, the crRNA and tracrRNA sequences[1] were assembled adjacently under control of RNA polymerase III dependent promoters (Figure 1b). This so-called RNAimer plasmid completes the set of components necessary for transcriptional regulation using the dCas9 system (Figure 1c).

We tested three effector domains in fusion with dCas9 to demonstrate the functionality of our dCas9 toolbox in mammalian cells. VP16 is a widely used transcriptional

transactivation domain.[7] A plasmid coding for a dCas9-VP16 fusion protein and an RNAimer plasmid were cotransfected into HEK-293T cells to achieve activation of expression of a secreted alkaline phosphatase (SEAP) reporter. Figure 2a shows relative SEAP reporter levels for dCas9-VP16 targeting a sequence upstream of the minimal promoter hCMVV_{min} driving SEAP gene expression. As an internal standard a plasmid encoding Renilla luciferase was cotransfected. SEAP activity was normalized to internal standard. Compared to the negative control (no RNAimer plasmid transfected, normalized value set to 1), SEAP expression could be raised by 10-fold (Figure 2a).

Second, the transcriptional repressor domain KRAB was fused to dCas9. dCas9-KRAB was directed toward the endogenous Vascular Endothelial Growth Factor (VEGF) promoter region in order to decrease VEGF production. The amount of secreted VEGF-A was analyzed by ELISA (see Supporting Information for methods). A construct for constitutive SEAP expression was cotransfected and used as an internal standard. VEGF expression was normalized to internal standard. Figure 2b shows a dCas9-KRAB mediated repression of 50% of VEGF-A expression compared to the negative control (no RNAimer plasmid transfected, normalized value set to 1).

The histone methyltransferase G9a-SD is known to dimethylate Histone 3 Lysine 9 (H3K9), thus inducing heterochromatin formation and consequently decreasing local gene expression.[6] Therefore, directing dCas9-G9a-SD to the VEGF promoter region should reduce gene expression similarly as dCas9-KRAB did. Indeed, dCas9-G9a-SD mediated a 50% repression of VEGF-A expression when cotransfected with crRNAs directed to the endogenous VEGF locus in comparison to the no-crRNA control (normalized value set to 1) (Figure 2c). A construct for constitutive SEAP expression was cotransfected and used as an internal standard. VEGF expression was normalized to internal standard.

Earlier studies proposed that binding of dCas9 within the coding region or in proximity of the transcriptional start site (TSS) can sterically hinder the recruitment of the transcriptional machinery (known as CRISPR interference (CRISPRi)).[3] This repressive effect is thought to decrease with increasing upstream distance from the TSS. To confirm that the repressive effect we observed with dCas9-G9a-SD is not caused by CRIPSRi, we included a "CRISPRi control" (dCas9 without effector) targeted to the same sequence 475 bp upstream of the VEGF TSS. In contrast to dCas9-G9a, dCas9 alone did not show any repressive effect (Figure 2c).

One major advantage of the CRISPR/Cas9 system is the ability to address several loci in parallel. As shown in Figure 3, directing dCas9-VP16 to the targeting site T1 or T2 located upstream of the hCMVV_{min} promoter led to 5- and 17-fold activation of SEAP expression, respectively, compared to the no-crRNA control. However, directing dCas9-VP16 simultaneously to T1 and T2 increased reporter expression in a synergistic manner by 45-fold.

So far, multiple targeting is associated with an increased number of plasmids to be transfected. This can lead to a decrease of transfection efficiency. Thus, we designed the RNAimer plasmid, which enables the combination of several crRNAs on one single plasmid in a simple and fast way.

We successfully demonstrated that standardizing dCas9 and fusing effector domains to it does not impair its ability to interact with specific DNA sequences. With these modularized fusion proteins, promoter activities can be altered, leading to

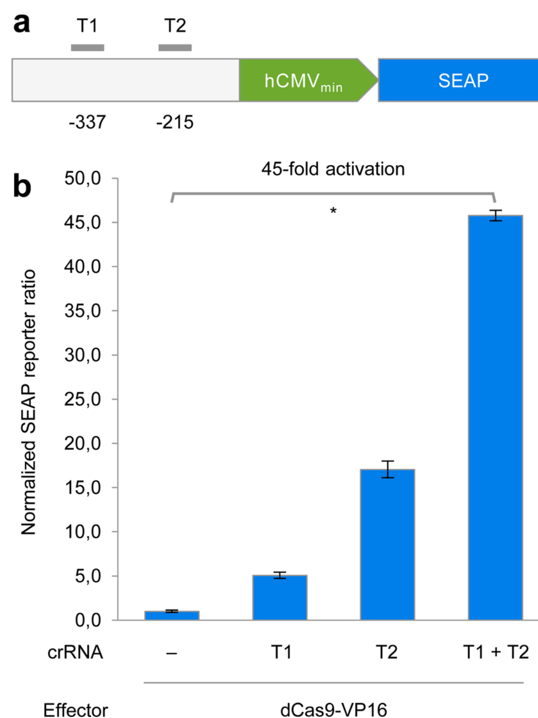

Figure 3. Combining multiple CRISPR RNA sequences to increase levels of gene expression. (a) Plasmid map of SEAP reporter under control of a CMV minimal promoter. T1 and T2 mark sequences upstream of the translational start site that were targeted with dCas9-VP16 to achieve activation of SEAP expression. (b) Relative expression of SEAP reporter levels. HEK-293T cells were cotransfected with the dCas9-VP16 construct, a SEAP reporter plasmid, a construct for constitutive Renilla luciferase expression and RNAimer plasmids coding for either crRNA T1, crRNA T2, or both. Twenty-four hours after transfection, SEAP activity in the cell supernatant was analyzed and normalized to a Renilla luciferase activity. The control (no RNAimer plasmid transfected) was set to a relative expression of 1. Results are means ± SD (n = 3). For each effector, statistically significant difference to the control is indicated with an asterisk (Student's t test, two-sided with unequal variance, $p < 0.05$). SEAP: secreted alkaline phosphatase.

significant changes in gene expression. The synthetic transactivator device, based on the VP16 domain and dCas9, shows efficient upregulation of a reporter gene as also demonstrated in other studies.[4] VP16 is known to recruit general transcription factors and thereby leads to enhanced transcription.[7] Two repressor modules—based either on KRAB or the G9a-SET domain—have been shown to efficiently downregulate endogenous VEGF-A expression, which is comparable to results obtained with zinc-finger fusion proteins.[6] Compared to KRAB, which recruits a set of general epigenetic repressors,[8] the more specific dimethylation of H3K9 through dCas9-G9a-SD could be of elevated interest in fundamental research.[6] The observed repression in this study indicates successful induction of heterochromatin formation at the VEGF-A locus by dCas-G9a-SD, which might also be applied to other endogenous gene loci. However, further confirmation of histone modifications through chromatin immunoprecipitation sequencing would be of great value, as this might elucidate our finding that addressing additional VEGF sites did not lead to a stronger repression.

Our toolkit, comprising three dCas9-effectors and the RNAimer plasmid, allows multiple gene regulation. The

dx.doi.org/10.1021/sb500035y | ACS Synth. Biol. 2014, 3, 986−989

modular design enables flexible exchange and addition of custom effector domains to dCas9. The required RNA components were standardized and assembled on the RNAimer plasmid that allows simple insertion of desired target spacers as annealed oligonucleotides. With this system it should be possible to address several genes simultaneously using respective crRNAs. To facilitate this, multiple RNA cassettes may be combined into one vector, using the BioBrick cloning standard. This reduces the number of needed plasmids and can increase transfection efficiency when working in mammalian cells.

Therefore, our system can be broadly applied in research fields such as tissue engineering, gene therapy, and transcriptomic studies.

To apply dCas9 fusion constructs in biomedical research such as gene therapy, one has to consider viral vectors as tools of delivery. Noteworthy, due to the size of >170 kDa, viral packaging of dCas9-effectors is still difficult. A truncated version of Cas9 would therefore be of great benefit. Moreover, the recent development of photoinducible, synthetic transcription factors allows reversible gene regulation on light stimulus. This offers great spatiotemporal resolution.[9] Theoretically, this concept should also be applicable for dCas9-effectors and thus significantly expand our toolkit.

METHODS

Experimental procedures are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Detailed methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Matias Zurbriggen for helpful discussions on the manuscript, as well as Balder Rebmann, Hannes Beyer, Konrad Müller, Desiree Hövermann, Maximilian Hörner, and the Weber lab for experimental guidance. The authors also thank the group of Prof. Dr. Albert Jeltsch, University of Stuttgart, for supplying the G9a-SD vector. This work was generously supported by the Excellence Initiative of the German Federal and State Governments (EXC-294, BIOSS—Centre for Biological Signalling Studies), the Faculty of Medicine/University of Freiburg and the Verband der Freunde der Universität Freiburg. Additional funding was granted by A. Hartenstein, Anders + Redelfs, Biozym, Corning, Eppendorf, ERA SynBio, Genaxxon Bioscience, Geneious, GenScript, Gilson, Greiner Bio-One, HPS Labor und Bürositzmöbel, Kuhnle, Life Technologies, MathWorks, New England BioLabs, Peprotech, Promega, Roche, Roth, Scienova, Stiftungen Landesbank Baden-Württemberg, Thermo Scientific, Weso-Med and Zymo Research. The 2013 Freiburg iGEM Team wiki can be found at http://2013.igem.org/Team:Freiburg.

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