Gene activation by a CRISPR-assisted trans enhancer

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Abstract

The deactivated CRISPR/Cas9 (dCas9) is now the most widely-used gene activators. However, the current dCas9-based gene activators are still limited by their unsatisfactory activity. In this study, we developed a new strategy, CRISPR-assisted trans enhancer, for activating gene expression in high efficiency by combining dCas9-VP64/sgRNA with the widely used strong CMV enhancer. In this strategy, a CMV enhancer DNA was recruited to target gene in trans by two systems, dCas9-VP64/csgRNA-sCMV and dCas9-VP64-GLA4/sgRNA-UAS-CMV. The former recruited trans enhancer by the annealing between two short complementary oligonucleotides at the ends of sgRNA and trans enhancer. The latter recruited trans enhancer by the binding between GLA4 fused to dCas9 and UAS sequence of trans enhancer. The trans enhancer activated gene transcription as the natural looped cis enhancer. The trans enhancer could activate both exogenous reporter gene and variant endogenous genes in various cells, with much higher activation efficiency than the current dCas9 activators.

Keywords: CRISPR/dCas9, CMV enhancer, trans enhancer, gene activation
Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) is originally an immune system of bacteria to destroy the invaded microphage DNAs by enzymatically digestion. The system was developed into a high efficient gene editing tool (Doudna and Charpentier 2014; Mali et al. 2013c). Additionally, the system was also developed into new gene activators. For example, the dead Cas9 (dCas9) and its associated single guide RNA (sgRNA) were widely used to regulate gene expression in recent years (Dominguez et al. 2015; Hilton et al. 2015; Jinek et al. 2012; Kiani et al. 2015; Mali et al. 2013b; Radzisheuskaya et al. 2016; Wang et al. 2016). For this end, both dCas9 and sgRNA have been widely engineered for activating or repressing gene expression. For instance, the dCas9 protein was fused with various gene activation or repression domains, such as VP48 (Cheng et al. 2013), VP160 (Perrin et al. 2017), VP64 (Maeder et al. 2013; Perez-Pinera et al. 2013), VPR (VP64-p65-Rta) (Chavez et al. 2015), and KRAB (Zheng et al. 2018). Additionally, the dCas9 protein was also fused with other functional domains with transcriptional regulatory functions, such as p300 (Hilton et al., 2015), LSD1 (Kears et al. 2015), Dnmt3a (Liu et al. 2016a; Saunderson et al. 2017), and Tet1 (Choudhury et al. 2016; Liu et al., 2016a). Based on these domains, more elaborate activators have been developed for more potent activation of target genes in mammalian cells, such as SunTag (dcas9-GCN4/sgRNA plus scFV-VP64) (Tanenbaum et al. 2014), and SPH (dCas9-GCN4/sgRNA plus scFV-p65-HSF1) (Zhou et al. 2018). Furthermore, some inducible dCas9 systems were also developed to control activity of dCas9 activators in cells, such as light-activated CRISPR/Cas9 effector (Nihongaki et al. 2015; Polstein and Gersbach 2015), hybrid drug inducible CRISPR/Cas9 technology (HIT) (Lu et al. 2018), and CRISPR activator gated by human antibody-based chemically induced dimers (AbCIDs) (Liu et al. 2018). However, it is difficult to package most of these dCas9 fusion proteins into adeno-associated virus (AAV) for their in vivo application.
Except dCas9 engineering, sgRNA was also widely engineered to develop new dCas9-based activators. Compared with dCas9 engineering, sgRNA is more simple, flexible and efficient to redesign. Moreover, the engineered sgRNA is more helpful for the in vivo application of dCas9-based activators due to its limited length for virus packaging. The most widely used engineered sgRNA-based gene activator is the synergistic activation mediator (SAM) system, in which MS2 loops were fused to the 3’ end of sgRNA (Konermann et al. 2015; Liao et al. 2017). Similarly, Pumilio/FBF (PUF), modular scaffold RNAs (MS2, PP7, and com), and riboswitches were fused to sgRNA (Cheng et al. 2016; Liu et al. 2016b; Zalatan et al. 2015). However, these chimeric sgRNA-based strategies were limited by their complicated RNA aptamers and the cognate RNA-binding fusion proteins.

Although variant dCas9-based activators have been developed (Chen and Qi 2017), the current dCas9-based transcriptional activators are still at low inefficiency in endogenous gene activation and cell reprogramming (Gao et al. 2014). By a systematic comparing of their relative potency and effectiveness across various cell types and species (human, mouse, and fly) (Chavez et al. 2016), it was found that majority of second-generation activators had higher activity than dCas9-VP64, in which three most potent activators were VPR, SAM, and Suntag. The three activators were consistently better than VP64 across a range of target genes and cellular environments. Moreover, the three activators generally had same magnitude of activity and more potent activators could not be further built by fusing their elements (Chavez et al., 2016). Therefore, new potent dCas9-based activators may be built by creating other architectures.

Almost three decades ago, the human cytomegalovirus (CMV) enhancer/promoter (referred to as CMV enhancer hereafter) was found as a natural mammalian promoter with high transcriptional activity (Boshart et al. 1985). The late studies gradually found that the CMV enhancer is a known strongest enhancer in various mammalian cells (Boshart et al., 1985; Foecking and Hofstetter 1986; Ho et al. 2015; Kim et al.
Therefore, this enhancer has been widely used to drive the ectopic expression of various genes in wide range of mammalian cells. The CMV enhancer is therefore used to drive the ectopic expressions of exogenous genes in broad tissues in transgenic animals (*Furth et al. 1991; Schmidt et al. 1990*), protein production by gene engineering, and gene therapy. We have recently further improved the transcriptional activity of the CMV enhancer by changing the natural NF-κB binding sites in this enhancer into artificially selected NF-κB binding sequences with high binding affinity (*Wang et al. 2018*). Therefore, we conceived that a unique architecture may be constructed to further improve dCas9-based activators by using the CMV enhancer.

In this study, mimicking the natural enhancer activating gene expression by a loop structure (*Carter et al. 2002; Tolhuis et al. 2002*), we developed a new dCas9-based activator by combining dCas9/sgRNA with CMV enhancer. The 3′ end of sgRNA was redesigned to contain a short capture sequence in complementary with a stick-end of a double-stranded CMV enhancer. The CMV enhancer was anchored to the promoter region of target gene by dCas9/sgRNA. The dCas9/sgRNA-recruited CMV enhancer thus functioned like a natural looped *cis* enhancer in a *trans* form. We found that the new activator could efficiently activate exogenous and endogenous genes in various cells. More importantly, the CMV enhancer could be also recruited to target gene *in trans* by using another system consisting of dCas9-VP64-GAL4/sgRNA and UAS-CMV.

**Results**

**Principle of gene activation by a CRISPR-assisted trans enhancer**

The principle of activating gene expression by a CRISPR-assisted *trans* enhancer is schematically illustrated in *Figure 1a*. A capture sgRNA (csgRNA) was produced by adding a capture sequence to the 3′ end of normal sgRNA sequence. A linear stick-end CMV (sCMV) enhancer was produced by adding a 3′ end single-
strand overhang. The overhang can anneal with the csgRNA capture sequence. When dCas9 protein was guided to the promoter of target gene by csgRNA, sCMV could be recruited by csgRNA. The recruited sCMV may activate the transcription of target gene like natural looped cis enhancer. Because the dCas9/csgRNA-anchored sCMV functions as transcription factors in trans, we named it as trans enhancer in order to distinguish it from the natural cis enhancer.

**Effect of capture sequence on the function of sgRNA**

To find whether the capture sequence affects the function of sgRNA, we prepared a normal sgRNA and three csgRNAs targeting a same site of HNF4α promoter. The three csgRNAs had different capture sequences. We used these sgRNAs to associate with the Cas9 endonuclease to cut a 732-bp HNF4α promoter fragment. The results indicated that the target DNA could be digested by all sgRNAs (Figure 1b), indicating that the capture sequence didn’t affect the sgRNA function.

**Activation of exogenous reporter gene by trans enhancer**

To find whether the CRISPR-assisted trans enhancer can activate gene expression, we constructed a reporter construct of HNF4α promoter (pEZX-HP-ZsGreen). 293T cells were then transfected with various vectors (Figure 2a-figure supplement 1). The transfection indicated that the ZsGreen expression could be successfully activated by dCas9/csgRNA2-sCMV but not activated by dCas9/csgRNA2-blunt CMV (bCMV). Although the dCas9/csgRNA2-sCMV showed a similar activation level of Cas9-VP64/sgRNA, it was far inferior to cis CMV enhancer. To improve the performance of trans CMV, we tried transfecting 293T cells with dCas9-VP64/csgRNA2-sCMV. The results indicated that the ZsGreen expression was highly activated by the transfection. In contrast, the dCas9-VP64/csgRNA2-bCMV showed the similar activation level of dCas9-VP64/sgRNA. These data revealed that the trans CMV not only truly functioned in trans via dCas9/csgRNA but also synergistically interacted with dCas9-fused VP64.
transfections indicated that the ZsGreen expression could be also highly activated by the combination of dCas9-VP64, sCMV and other two csgRNAs, csgRNA1 and csgRNA3.

To further verify the function of CRISPR-assisted trans enhancer, we transfected 6 different cell lines with reporter construct and dCas9-VP64/csgRNA2-sCMV, dCas9/csgRNA2-sCMV or dCas9-VP64/sgRNA (Figure 2b-figure supplements 2–4). The results revealed that dCas9-VP64/csgRNA2-sCMV always showed the highest gene activation efficiency in all cell lines. Additionally, dCas9/csgRNA2-sCMV always showed higher activity than dCas9-VP64/sgRNA. These results indicated that gene could be activated by the CRISPR-assisted trans enhancer. Importantly, the trans sCMV could synergistically function with dCas9-fused VP64 in gene activation.

Comparison of trans CMV enhancer with VPR

VPR was a more potent transcriptional activation domain than VP64. We next compared the trans enhancer with VPR. 293T and HepG2 cells were respectively transfected with reporter construct and dCas9-VP64/csgRNA, dCas9-VPR/csgRNA, or dCas9-VP64/csgRNA-sCMV (Figure 2c). The results reveal that dCas9-VPR/csgRNA had better activity than dCas9-VP64/csgRNA as previously reported. However, the dCas9-VP64/csgRNA-sCMV always showed significantly higher activity than dCas9-VPR/csgRNA.

Activation of endogenous genes by trans enhancer

To further evaluate the activity of CRISPR-assisted trans enhancer, we activated endogenous genes with trans sCMV. The csgRNAs targeting ten different genes was designed and their linear expression vectors were produced. Seven different cell lines were transfected with dCas9-VP64/csgRNA2-sCMV, dCas9-VP64/sgRNA and dCas9/csgRNA2-sCMV (Figure 3-figure supplement 1). The quantitative PCR (qPCR) detection of gene expression revealed that almost all genes were most significantly activated by dCas9-VP64/csgRNA2-sCMV in all cells. Moreover, most of genes were more significantly activated by
dCas9/csgRNA-sCMV than dCas9-VP64/sgRNA in all cells. These results demonstrated that the CRISPR-assisted \textit{trans} enhancer could be used to activate variant endogenous genes in various cells. In addition, by activating the HNF4α gene in 293T cells, we found that dCas9-VP64/csgRNA-sCMV had better activity than dCas9-VPR/csgRNA-sCMV in activating endogenous gene (\textit{Figure 3-figure supplement 2}).

It was reported that the cancer cells HepG2 and PANC1 could be differentiated into normal liver- and pancreas-like cells by exogenously expressing transcription factor HNF4α and E47. In above assays, we found that the endogenous HNF4α and E47 genes were highly activated by the CRISPR-assisted \textit{trans} enhancer in HepG2 and PANC1 cells (\textit{Figure 3}). To further confirm the cellular effects of HNF4α and E47 activation, we detected the expressions of other genes related to the differentiation of the two cancer cells (\textit{Figure 4}). The results indicated that the genes related to stemness (CD133 and CD90) and pluripotency (Oct3/4, Sox2, Nanog, c-Myc, LIN28, and Klf4) were down-regulated, but those related to normal liver (GS, BR, ALDOB, CYP1a2, PEPCK, APOCIII, G-6-P, and HPD) and pancreas (MIST1, PRSS2, CELA3A, and CPA2) functions were highly up-regulated in HepG2 and PANC1 cells. Additionally, the cell cycle arrest-related gene p21 (HepG2 and PANC1) and TP53INP1 (PANC1) was highly up-regulated.

**Activation of genes by other \textit{trans} enhancers**

To explore if other enhancers could be also used as \textit{trans} enhancer, we fabricated the blunt- and stick-end versions of two other widely used promoters EF1a and PGK (bEF1a, bPGK, sEF1a, and sPGK). 293T cells were co-transfected with these \textit{trans} enhancers and dCas9-VP64/csgRNA and reporter construct. The results indicated that the ZsGreen expression was also activated by the two \textit{trans} enhancers; however, the activation levels were lower than that of sCMV (\textit{Figure 5a-figure supplement 1}). All other transfections as controls didn’t activate ZsGreen expression (\textit{Figure 5a-figure supplement 1}). The qPCR detection of HNF4α expression in the same transfected 293T cells revealed that the endogenous HNF4α gene expression was
also significantly activated by three stick-end trans enhancers, but not activated by all blunt-ended trans enhancers (Figure 5b). The subsequent HepG2 cell transfections with the same trans enhancers and dCas9-VP64/csgRNA indicated that the endogenous HNF4α gene expression could be also significantly activated by all stick-ended trans enhancers, but not activated by all blunt-ended trans enhancers (Figure 5b). These results indicated that the variant enhancers could be used as the CRISPR-assisted trans enhancer.

Activation of genes by the GAL4/UAS-based trans enhancer

To further improve the in vivo application of CRISPR-assisted trans enhancer, we finally tried realizing trans enhancer with the GAL4-UAS system. A dCas9-VP64-GAL4 expression vector and a UAS-CMV trans enhancer was thus constructed. Two forms of trans UAS-CMV enhancers, linear UAS-CMV (LUAS-CMV) and circular UAS-CMV (CUAS-CMV), were expected to be recruited to the target gene by the dCas9-VP64-fused GAL4 (Figure 6a). By transfecting 293T cells with dCas9-VP64-GAL4/sgRNA-LUAS-CMV/CUAS-CMV and reporter construct, the ZsGreen expression of the exogenous reporter gene was significantly activated by both LUAS-CMV and CUAS-CMV, but not activated by all transfections as controls (Figure 6b-figure supplement 1). By transfecting 293T and HepG2 cells with dCas9-VP64-GAL4/sgRNA-LUAS-CMV/CUAS-CMV, the expression of endogenous HNF4α gene was highly activated in the two cells (Figure 6c). More importantly, both trans LUAS-CMV and CUAS-CMV enhancers showed significant higher activity than the trans sCMV (Figure 6c). In contrast, all transfections as controls didn’t activate the expression of endogenous HNF4α gene in the two cells (Figure 6c). These results revealed that the CRISPR-assisted trans enhancer could be better realized with the GAL4-UAS system.

Discussion

In this study, we developed a new dCas9-based gene activation strategy, CRISPR-assisted trans enhancer,
in which a *trans* enhancer could be recruited to target promoters by dCas9-VP64/csgRNA or dCas9-VP64-GAL4/sgRNA. The results revealed that the expression of variant exogenous and endogenous genes could be highly activated by the CRISPR-assisted *trans* enhancers in various mammalian cells, more efficiently than the current widely-used dCas9-VP64 and dCas9-VPR. This strategy has its unique advantages over the current dCas9-based gene activation systems.

First, only one csgRNA was used in activating all target genes in various cells with the CRISPR-assisted *trans* enhancer. However, in the current dCas9-based gene activations, multiple sgRNAs were used. In general, three or more sgRNAs were often used to activate an interested gene (*Cheng et al., 2013; Maeder et al., 2013; Mali et al. 2013a; Perez-Pinera et al., 2013*). In many assays with various numbers of sgRNAs, one sgRNA often produced very low or undetectable expression. Using multiple sgRNAs, each sgRNA has to be independently transcribed by a long U6 promoter. Second, csgRNA is the simplest sgRNA used in dCas9-based gene activators, which only extended a 24-bp short sequence at the 3′ end of normal sgRNA. However, the current dCas9/sgRNA activators often used long complex chimeric sgRNAs that harbor multiple tandem aptamers of various RNA-binding proteins, such as SAM sgRNA (MS2) (*Konermann et al., 2015; Liao et al., 2017*), Casilio sgRNA (Pumilio/FBF) (*Cheng et al., 2016*), and scaffold RNAs (MCP, PCP, and Com) (*Zalatan et al., 2015*).

The capture sequences of csgRNA can be easily designed. We originally designed three different capture sequences. All of them functioned in the CRISPR-assisted *trans* enhancer. However, csgRNA2 showed the best performance. The capture sequences were artificially designed short sequences, they have no complementary sequences in human cells, which is important for their specific annealing with sCMV in high efficiency. This study demonstrated that sCMV could be efficiently captured by csgRNA in the nuclear of human cells. To our knowledge, this is the first time to report that gene could be activated by an artificial
In this study, we realized the CRISPR-assisted trans enhancer with two forms. One was csgRNA-sCMV, the other was GAL4-UAS. Two forms can be easily used to activate genes in the in vitro cultivated cells. As to the in vivo applications, the csgRNA-sCMV-based trans enhancer can be used via nanoparticle gene carriers, while the GAL4-UAS-based trans enhancer can be easily transferred by virus vectors such as AAV. Especially, AAV has already been approved to be used as gene vector in clinics. We found that the GAL4-UAS-based trans enhancer had better performance than the csgRNA-sCMV-based trans enhancer, especially the linear UAS-CMV. In the in vivo applications, the linear UAS-CMV can be easily transferred by AAV vector.

As a typical application, the dCas9-based transcriptional activators were used to reprogram cells in vitro and in vivo for biomedical applications by activating endogenous genes. For example, the fibroblasts were reprogrammed into the induced pluripotent stem (iPS) cells by activating endogenous the Oct4 and Sox2 genes with dCas9-SunTag-VP64 (Liu et al., 2018). The mouse embryonic fibroblasts were converted into neuronal cells by activating endogenous the Brn2, Ascl1, and Myt1l genes with $^{VP64}dCas9^{VP64}$ (Black et al. 2016). The in vivo target genes were activated by MPH to ameliorate disease phenotypes in mouse models of type I diabetes, acute kidney injury, and muscular dystrophy (Liao et al., 2017). The brain astrocytes were converted into functional neurons in vivo by activating the Ascl1, Neurog2 and Neurod1 genes with SPH (Zhou et al., 2018). These studies makes CRISPR therapies the grade not the cut (Burgess 2018).

In this study, we selected 10 endogenous genes to be activated by the CRISPR-assisted trans enhancer. Most of these genes coding transcription factors, including HNF4α, E47, Ascl1, Ngn2, Sox2, Oct4, and Nanog. The Ascl1, Ngn2 and Sox2 were used to directly reprogram fibroblasts into nerve cells (Zhao et al. 2015). The Oct4, Sox2 and Nanog were widely used to reprogram fibroblasts into iPS cells (Takahashi et
al. 2007; Takahashi and Yamanaka 2016; Yu et al. 2007). The HNF4α and E47 were used to differentiate the liver and pancreas cancer cells into normal cells (Kim et al. 2015; Yin et al. 2008). TNFAIP3 is a well-known natural NF-κB inhibitor (Cooper et al. 1996), having the potential to treat NF-κB-overactivated diseases such as inflammation and cancers. Caspase9 is a key gene making cell apoptosis (Li et al. 2017). CSF3 codes granulocyte-colony stimulating factor (G-CSF), a glycoprotein that stimulates the bone marrow to produce granulocytes and stem cells and release them into bloodstream (Cetean et al. 2015), which is widely used in chemotherapy to enhance the immunity of cancer patients. We selected these genes for pre-exploring the future in vitro and in vivo applications of the CRISPR-assisted trans enhancer.

Materials and methods

Vector construction

A lac operon fragment was amplified from pEASY-Blunt-simple (Transgen) using primers Lac-px-F and Lac-px-R. The product was ligated into px458 (Addgene) to construct px458-lac using BbsI (NEB) and BsaI (NEB). The U6-sgRNA-lac fragment was amplified from px458-lac using primers U6-F and U6-R/U6-1-R/U6-2-R/U6-3-R. The products were cloned into the pEASY-Blunt-simple to produce pEASY-sgRNA and pEASY-csgRNA (Supplementary File 2), which were used to construct particular sgRNA/csgRNA expressing plasmid. The sgRNAs targeting interested genes were designed by CHOPCHOP. The chemically synthesized complementary oligonucleotides containing sgRNA/csgRNA targets were annealed and ligated into pEASY-sgRNA/pEASY-csgRNA. The ligation reaction consisted of 10 U BbsI (NEB), 120 U T4 DNA ligase (NEB), 1× T4 DNA ligase buffer, 0.1 mg/mL Bovine Serum Albumen, and 50 ng plasmid pEASY-csgRNA. The reaction was run as fellow: 10 rounds of 37°C 5 min and 16°C 10 min, 37°C 30 min, and 80°C 5 min. The reaction was then used to transfect DH5α competent cells. The white clones were screened by blue-white screening on LB agar plates with 100 μg/mL Ampicillin, 40 μL of 20 mg/mL X-gal and 40
µL of 0.1 M IPTG. The vectors were validated by sequencing. Then the linear sgRNA/csgRNA expression vectors were amplified from the validated pEASY-sgRNA/pEASY-csgRNAs using primers U6-F and U6-R/U6-1-R/U6-2-R/U6-3-R. The primer U6-R was used to amplify the normal sgRNA expression template (named as U6-sgRNA) from pEASY-sgRNA. The primer U6-1/2/3-R was used to amplify the csgRNA expression template (named as U6-csgRNA) from pEASY-csgRNA. The PCR products were purified with PCR clean kit (Axygen) and used to transfect cells as sgRNA/csgRNA expression vector.

The CMV enhancer fragment was amplified from pEGFP-N1 using primers CMV-F and CMV-1-R/CMV-2-R/CMV-3-R. The PCR products were purified with PCR clean kit and used as linear blunt-end CMV (bCMV). To prepare stick-end CMV (sCMV), the PCR products were firstly digested with Nt.BbvCI and then added with complementary oligonucleotide CS-1-R/CS-2-R/CS-3-R. The PCR products were denatured at 85°C for 10 min and then naturally cooled to room temperature. The PCR products was purified with PCR clean kit and used as linear sCMV. The blunt-end EF1α/PGK promoters were all amplified from pEF1a-FB-dCas9-puro (Addgene) by using primers EF1-a-F/R and PGK-F/R. The stick-end EF1α/PGK promoters were similarly prepared by treating blunt-end EF1α/PGK promoters.

A 1000-bp HNF4α promoter sequence was amplified from the genomic DNA of HepG2 cells using primers HNF4α-P-F and HNF4α-P-R. The amplified promoter fragment was ligated into pEZX-ZsGreen, producing an HNF4α promoter reporter construct pEZX-HP-ZsGreen. The VP64 sequence was deleted from pcDNA-dCas9-VP64 (Addgene) to construct pcDNA-dCas9. The VPR sequence was cloned into pcDNA-dCas9 to construct pcDNA-dCas9-VPR.

The GAL4 fragment was amplified from pGBKT7 (MiaoLing Plasmid Sharing Platform) using primers GAL4-BsiEW-F and GAL4-BspE-R, which was then ligated into pcDNA-dCas9-VP64 using BsiWI and BspEI to prepare pcDNA-dCas9-VP64-GAL4. A chemically synthesized 5xUAS fragment was ligated into
pEASY-Blunt using BglII and HindIII to obtain pEASY-Blunt-UAS. A CMV fragment was amplified from pEGFP-C1 using primers UAS-CMV-Bgl-F and UAS-CMV-Hind-R. The CMV fragment was then ligated into pEASY-Blunt-UAS to obtain pEASY-Blunt-UAS-CMV, which was used as circular UAS-CMV (CUAS-CMV). The linear UAS-CMV (LUAS-CMV) fragment was amplified from pEASY-Blunt-UAS-CMV using primers CMV-UAS-Bgl-F and UAS-CMV-R.

The sequences of all PCR primers used in above vector construction were shown in the Supplementary File 1–Table 1. The chemically synthesized complementary oligonucleotides used to construct pEASY-sgRNA/pEASY-csgRNA were shown in the Supplementary File 1–Table 2. The functional sequences of all linear and plasmid vectors were provided as the Supplementary File 3.

**DNA cutting with Cas9-csgRNA**

A sgRNA targeting to the HNF4α promoter sequence was selected. The sgRNAs were prepared by an *in vitro* transcription using T7 RNA polymerase (NEB). The sgRNA transcription template was amplified from pEASY-csgRNA using primers HNF4α-T7-F and U6-R/U6-1-R/U6-2-R/U6-3-R. A normal sgRNA (HNF4α-sgRNA) and three csgRNAs (HNF4α-csgRNAs) were prepared. A 732-bp HNF4α promoter fragment was amplified from pEZX-HP-ZsGreen using primers HNF4α-sP-F and HNF4α-sP-R. The sequences of PCR primers were shown in the Supplementary File 1-Table 1. The Cas9 digestion reaction (30 µL) consisted of 1×Cas9 Nuclease Reaction Buffer, 1 µM Cas9 Nuclease (NEB), and 300 nM HNF4α-sgRNA or HNF4α-csgRNA. The reaction was incubated at 25 °C for 10 min. Then 400 ng of purified 732-bp HNF4α promoter fragment was added to the reaction and incubated at 37 °C for 15 min. Finally, the Cas9 nuclease was inactivated at 65 °C for 10 min. The reaction was run with 1.5% agarose gel electrophoresis.

**Cell lines**

All cells including 293T, HepG2, PANC1, A549, HeLa, SKOV3, and HT29 were obtained from the
Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The identity was authenticated by the STR profiling. The mycoplasma contamination testing was performed and no mycoplasma contamination was ensured.

**Cell culture and transfection**

Cells were cultured in the Dulbecco’s Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells at >70% confluence in each well of 12-well plate were transfected with various combinations (see figures) of linear or plasmid vectors, including pcDNA-dCas9, pcDNA-dCas9-VP64, pcDNA-dCas9-VPR, U6-sgRNA, U6-csgRNA, sCMV, bCMV, and pEZX-HP-ZsGreen, by using Lipofectamine® 2000 (ThermoFisher Scientific) according to the manufacturer’s instruction. The transfected cells were incubated at 37 °C and 5% CO₂ for 36 h. Cells were then imaged with a fluorescence microscope (Olympus) at 200 × magnification.

**Flow cytometry**

The fluorescence intensity of cells was quantified with flow cytometry (Calibur). Ten thousand cells were measured for each transfection. Flow cytometry data analysis and figure preparation were done using BD software.

**Quantitative PCR**

The total RNA was extracted from cells using TRIzol (Invitrogen). The complementary DNA (cDNA) was synthesized with 3 µg of total RNA using the Hifair ⅢⅢ SuperMix (YeaSen). The gene transcription was detected with quantitative PCR (qPCR) using the Hieff qPCR SYBR Green Master Mix (YeaSen) according to the manufacturer’s instruction. GAPDH was used as an internal reference to analyze the relative mRNA
expression of different genes. The sequences of PCR primers were shown in the Supplementary File 1-Table 3 and 4. The qPCR programs were run on StepOne Plus (Applied Biosystems). Each qPCR detection was performed in at least three technical replicates. Melting curve analysis was performed. Data analysis was performed using the Applied Biosystems StepOne software v2.3, and C_t values were normalized with that of GAPDH. The relative expression level of target mRNAs was calculated as relative quantity (RQ) according to the equation: \[ \text{RQ} = 2^{\Delta \Delta C_t} \].

**Statistical analyses**

Each cell transfection for detecting gene expression activation by trans-enhancer was performed in three biological replicates. In each biological replicate, at least three technical replicates (three replicate wells) were performed. In qPCR detection of gene expression, the mean RQ value of technical replicates was used as the RQ value of one biological replicate. The mean RQ value of three biological replicates were used to calculate the final mean and standard deviation (SD). Data were analyzed by Student’s t test when comparing 2 groups. Data were shown as mean ± SD and differences were considered significant at \( P < 0.05 \).

**Additional information**

**Competing interests**

The authors declare no competing interests.

**Funding**

This work was supported by the grants from the National Natural Science Foundation of China (Grant 61571119). The funder had no role in study design, data collection and interpretation, or the decision to submit the work for publication.
**Additional files**

Supplementary files

Transparent reporting form


**Data availability**

All data generated or analyzed during this study are included in the manuscript and supporting files.

**Reference**


doi:10.15386/cjmed-531

doi:10.1038/nmeth.3312


Figure legends

**Figure 1.** Principle of gene expression activation by the CRISPR-assisted *trans* enhancer and evaluation of designed csgRNAs. a. Schematic illustration of the principle of gene expression activation by the CRISPR-assisted *trans* enhancer. A capture sequence is added at the 3' end of sgRNA, which is used to capture a *trans* CMV enhancer with a single-stranded overhang that can anneal with the capture sequence of sgRNA. The captured *trans* CMV enhancer may function like the natural looped *cis* enhancer to activate transcription of interested gene, including exogenous and endogenous genes. b. *In vitro* target DNA cutting by the Cas9-csgRNA complex. A 732-bp DNA fragment amplified from the HNF4α promoter region were respectively cut by the Cas9/csgRNA and Cas9/sgRNA complexes. The csgRNA1, csgRNA2 and csgRNA3 had the same target sequence but different capture sequences.

**Figure 2.** Activation of an exogenous reporter gene ZsGreen under the control of a HNF4α promoter by the CRISPR-assisted *trans* enhancer in multiple cells. a. Transcriptional activation of reporter gene ZsGreen in various cells transfected by different vectors. The fluorescence intensity of cells were analyzed by flow cytometry and showed as the mean fluorescence intensity (MFI). Transfections: DVS, dCas9-VP64/sgRNA; DSC, dCas9/csgRNA-sCMV; DVSC, dCas9-VP64/csgRNA-sCMV. b. Comparison between *trans* enhancer and VPR. Cells were transfected with three different transcriptional activation systems to activate reporter gene ZsGreen. The fluorescence intensity of cells was analyzed by flow cytometry and the percentage of cells with certain fluorescence intensity was counted. Transfections: Lipo, lipofectin; DVS, dCas9-VP64/sgRNA; DVPRS; dCas9-VPR/csgRNA; DVSC, dCas9-VP64/csgRNA-sCMV.

The following figure supplements are available for figure 2:

**Figure supplement 1.** Activation of an exogenous reporter gene ZsGreen under the control of a HNF4α promoter by the CRISPR-assisted *trans* enhancer in 293T cells. Cells were transfected by various vectors.
Cells were photographed with a fluorescent microscope and their fluorescence was analyzed by flow cytometry. The reporter gene activation efficiency was indicated by the percentage of cells with green fluorescence over the threshold (cells in Q1-UR quadrant).

**Figure supplement 2.** Activation of an exogenous reporter gene ZsGreen under the control of a HNF4α promoter by the CRISPR-assisted *trans* enhancer in HepG2 and PANC1 cells.

**Figure supplement 3.** Activation of an exogenous reporter gene ZsGreen under the control of a HNF4α promoter by the CRISPR-assisted *trans* enhancer in A549 and HeLa cells.

**Figure supplement 4.** Activation of an exogenous reporter gene ZsGreen under the control of a HNF4α promoter by the CRISPR-assisted *trans* enhancer in SKOV3 and HT29 cells.

**Figure 3.** Transcriptional activation of endogenous genes by the CRISPR-assisted *trans* enhancer. 294T, HepG2 and PANC1 cells were transfected with three different transcriptional activation systems to activate the expression of 10 endogenous genes. The gene transcription was detected by qPCR and the expression level was showed as the relative RNA expression fold to house-keeping gene GAPDH. Data were shown as mean ± SD, n=3. The statistical difference was analyzed by the Student’s *t* test. *, *P* < 0.05; **, *P* < 0.01; NS, no significant statistical difference. Transfection: DVS, dCas9-VP64/sgRNA; DSC, dCas9/csgRNA2-sCMV; DVSC, dCas9-VP64/csgRNA2-sCMV.

The following figure supplements are available for figure 3:

**Figure supplement 1.** Transcriptional activation of endogenous genes by the CRISPR-assisted *trans* enhancer. A549, HeLa, SKOV, and HT29 cells were transfected with three different transcriptional activation systems to activate the expression of 10 endogenous genes.

**Figure supplement 2.** Activation of endogenous HNF4α gene in 293T cell with trans enhancers based on dCas9-VP64 and dCas9-VPR. The 293T cell was transfected with various vectors to activate the endogenous
HNF4α gene. The HNF4α and GAPDH genes were detected with qPCR.

**Figure 4.** Changes of gene expression in the HNF4α-activated HepG2 cells and E47-activated PANC-1 cells.

a and b. Change of gene expression in the HNF4α-activated HepG2 cells (a) and E47-activated PANC-1 cells (b). The gene transcription was detected by qPCR and the expression level was showed as the relative RNA expression fold to house-keeping gene GAPDH. Data were shown as mean ± SD, n=3. The statistical difference was analyzed by Student’s t test. *, P < 0.05; **, P < 0.01; NS, no significant statistical difference.

Transfection: Lipo, lipofectin; DVS, dCas9-VP64/sgRNA; DVSC, dCas9-VP64/csgRNA2-sCMV.

**Figure 5.** Activation of exogenous and endogenous genes with other trans enhancers. a. Activation of exogenous reporter gene ZsGreen. The fluorescence intensity of cells were analyzed with flow cytometry. b. Activation of endogenous HNF4α gene in 293T and HepG2 cells with two new trans enhancers, sEF1a and sPGK. The sCMV was used as a positive control for comparing. The blunt-end trans enhancers (bEF1a and bPGK) were also used as controls. Data were shown as mean ± SD, n=3. The statistical difference was analyzed by Student’s t test. *, P < 0.05; **, P < 0.01.

The following figure supplements are available for figure 5:

**Figure supplement 1.** Activation of exogenous and endogenous genes with other CRISPR-assisted trans enhancers. a. Activation of exogenous ZsGreen gene in 293T cells with two new trans enhancers, sEF1a and sPGK. b. Flow cytometry analysis of ZsGreen expression.

**Figure 6.** Activation of exogenous and endogenous genes with the GAL4-UAS-based trans enhancer. a. Schematic show of gene activation using the GAL4-UAS-based CRISPR-assisted trans enhancer. b. Activation of exogenous reporter gene ZsGreen. The fluorescence intensity of cells were analyzed with flow cytometry. c. Activation of endogenous HNF4α gene in 293T and HepG2 cells the GAL4-UAS-based
CRISPR-assisted *trans* enhancer. The sCMV was used as a positive control for comparing. Other three transfections were also used as controls. LUAS-CMV, linear UAS-CMV; CUAS-CMV, circular UAS-CMV. Data were shown as mean ± SD, n=3. The statistical difference was analyzed by Student’s t-test. *, *P* < 0.05; **, *P* < 0.01.

The following figure supplements are available for figure 6:

**Figure supplement 1.** Activation of exogenous and endogenous genes with CRISPR-assisted *trans* enhancer using GAL4-UAS system. a. Activation of exogenous ZsGreen gene in 293T cells with the GAL4-UAS-based CRISPR-assisted *trans* enhancer. b. Flow cytometry analysis of ZsGreen expression.

**Supplementary File 1.** Four tables showing primers and oligos.

**Supplementary File 2.** Schematic show of construction of sgRNA vectors for blue-white screening.

**Supplementary File 3.** Sequences of vectors, templates, sgRNA, csgRNA, and *trans* enhancers.
a

5' 3'

PAM

Target gene

3' 5'

Promoter

5' 3'

Transcription machine

Nt.BbCl

Stick CMV enhancer/promoter

3' 5'

Blunt CMV enhancer/promoter

Capture sequence

dCas9 or dCas9-VP64

csgRNA

5' 3'

Input DNA 732bp

Fragment 1 460bp

Fragment 2 272bp

M: 100bp ladder

1: HNF4α promoter sequence + Cas9/sgRNA
2: HNF4α promoter sequence + Cas9/csgRNA1
3: HNF4α promoter sequence + Cas9/csgRNA2
4: HNF4α promoter sequence + Cas9/csgRNA3
293T Lipofectin dCas9-VP64/sgRNA pEZX-HP-ZsGreen dCas9/csgRNA2-sCMV pEZX-HP-ZsGreen dCas9-VP64/csgRNA2-bCMV pEZX-HP-ZsGreen

Phase

ZsGreen

dCas9-VP64/csgRNA1-sCMV pEZX-HP-ZsGreen

dCas9-VP64/csgRNA2-sCMV pEZX-HP-ZsGreen

dCas9-VP64/csgRNA3-sCMV pEZX-HP-ZsGreen

dCas9/csgRNA2-bCMV pEZX-HP-ZsGreen

Phase

ZsGreen

dCas9-VP64 dCas9-VP64 +pEZX-HP-ZsGreen sgRNA pEZX-HP-ZsGreen

Phase

ZsGreen

Phase | ZsGreen | Phase | ZsGreen
--- | --- | --- | ---
dCas9-VP64 |  | dCas9-VP64/sgRNA | pEZX-HP-ZsGreen
dCas9-VP64/csgRNA-bCMV | pEZX-HP-ZsGreen | dCas9-VP64/csgRNA/sCMV | pEZX-HP-ZsGreen
dCas9-VP64/csgRNA-bEF1a | pEZX-HP-ZsGreen | dCas9-VP64/csgRNA-sEF1a | pEZX-HP-ZsGreen
dCas9-VP64/csgRNA-bPGK | pEZX-HP-ZsGreen | dCas9-VP64/csgRNA-sPGK | pEZX-HP-ZsGreen

Gate: R1

ZsGreen-A | Q1-UL 0.1% Q1-UR 5.1% | Q1-UL 1.2% Q1-UR 3.5% | Q1-UL 1.9% Q1-UR 3.7% | Q1-UL 1.2% Q1-UR 3.7%
dCas9-VP64 | dCas9-VP64/csgRNA-bCMV | pEZX-HP-ZsGreen | dCas9-VP64/csgRNA-bEF1a | pEZX-HP-ZsGreen
dCas9-VP64/sgRNA | pEZX-HP-ZsGreen | dCas9-VP64/csgRNA-sCMV | pEZX-HP-ZsGreen
dCas9-VP64/csgRNA-sEF1a | pEZX-HP-ZsGreen | dCas9-VP64/csgRNA-sPGK | pEZX-HP-ZsGreen