sgRNA level determines CRISPRi knockdown efficiency in K562 cells

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Abstract

To determine whether nuclease deactivated Cas9 (dCas9) or sgRNA expression level or both determine the knockdown efficiency of CRISPRi, we carried out the following experiments: Cell clones expressing KRAB-dCas9 either under the control of the inducible Tet-on system or SFFV promotor were created by lentiviral transduction, and several single clones were selected by fluorescence-activated cell sorting (FACS) for further study. Six genes with various expression levels were targeted using lentiviral sgRNA from two libraries, and the only clone with the high expression level of KRAB-dCas9 effectively knocked down target gene expression. In the high KRAB-dCas9 expressing cell clone, the knockdown efficiency was neither affected by the target gene expression level nor does it correlate with the KRAB-dCas9 expression level, which remained relatively constant (CV=2.2%) across knockdown experiments. 74.72%, 72.28%, 39.08% knockdown of MMADHC, RPIA, ZNF148 genes were

achieved, and the knockdown efficiency correlated well with the sgRNA expressing level, which is controlled by a different multiplicity of infection (MOI). Cell clones with low KRAB-dCas9 expression levels did not achieve high knockdown efficiency.

Keywords: CRISPR interference, knockdown efficiency, inducible Tet-on system, the multiplicity of infection, sgRNA expression level

1. Introduction

CRISPRi is gradually replacing the siRNA technique for mechanistic investigations in various fields [1]. However, there is a lack of mechanistic study on the efficiency of the CRISPRi system. How the two major players of the system, the dCas9 fusion protein, and the guide RNA affect the knockdown efficiency has not been systematically studied.

Three protein domains were commonly used for repressing target gene transcription: KRAB, MeCP2, dCas9 and its synthetic derivatives [2-3]. KRAB recruits KAP1, which serves as a scaffold for various heterochromatin-inducing factors [4]; MeCP2 binds to the methylated DNA and interacts with histone deacetylase and the co-repressor SIN3A [5]. A combination of dCas9 with KRAB and MeCP2 has been shown to improve the knockdown efficiency [2-3], and dCas9, KRAB-dCas9, dCas9-KRAB-MeCP2 are three commonly used combinations.

Effective knockdown of target gene expression started from the sgRNA being expressed from the transgene, formed a complex with KRAB-dCas9 protein, and roaming in the nucleus until the sgRNA hybrid to target DNA [6]. Previously inducible KRAB-dCas9 has been reported [2], but how knockdown efficiency was affected by dCas9 fusion protein or sgRNA expression level has not been studied systematically. In this study, we found that the dCas9 protein level has to be highly expressed for an efficient knockdown, and the knockdown efficiency correlated well with the sgRNA expressing level.

2. Materials and methods

2.1.Cell culture

HEK293T and K562 cells were obtained from the National Infrastructure of Cell Line Resource. HEK293T cells were cultured at 37℃ in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose supplemented with 10% (v/v) Fetal Bovine Serum (FBS). K562 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS. Cells were tested every 3 months for mycoplasma contamination and consistently tested negative.

2.2.Construction of plasmids

The pTRE3G promoter was PCR-amplified with the primers using the pTRE3G plasmid as a template. The PCR product was digested with EcoRI and ligated into EcoRI digested pHR-SFFV-KRAB-dCas9 vector (Addgene, 60954), to generate a

pHR-pTRE3G-KRAB-dCas9 vector. Two complementary oligonucleotides were annealed and inserted into the BstXI and BlpI sites of the linearized pCRISPRia-v2 vector (Addgene, 84832) using T4 DNA ligase. Oligonucleotides are listed in Supplementary Table 1. Primers are listed in Supplementary Table 2.

2.3.Transfection and transduction

For transfection, 2.1×10^7 HEK293T cells were seeded into T175 flasks 1d before transfection. Cells were transfected with pSPAX2, pMD2.G, Tet3G/dCas9/sgRNA plasmid (4:1:4, mole ratio) using PEI (1 mg/mL) with N/P ratio was 20 [2, 7]. Viral supernatants were collected at 48 h and 72 h after transfection. For lentiviral concentration, the cell culture supernatant was filtered using 0.45 um filters. 4 mL 20% sucrose solution was added to the bottom of the ultracentrifuge tube with 32 mL filtered cell culture supernatant, centrifuge for 2 h at 82,700 g, 4℃ [8], and the supernatant was disinfected and discarded. 100 uL of pre-cooled PBS without Ca^{2+}/Mg^{2+} was added to resuspend the pellet. The titer of the lentiviral vector was determined by fluorescence titering assay. For transduction, 1×10^5 K562 cells were infected with a mixture of lentivirus and 10 ug/mL polybrene.

2.4.FACS and antibiotics selection

To generate KRAB-dCas9-mCherry-expressing stable cell lines, cells were analyzed based on the expression of mCherry by BD FACS Aria II and sorted into 96-well plates. After cell clones expanded, positive cells expressing mCherry were confirmed by flow cytometry using CytoFLEX S. For the selection of cells expressing pLVX-Tet3G, 100

ug/mL G418 was applied for 10 days. For cells expressing sgRNA, 5 ug/mL puromycin was applied 72 h after infection, for an additional 72 h.

2.5.Western blot analysis and ELISA

Total protein was extracted using RIPA lysis and extraction buffer with 1 mM PMSF. Total protein concentration was measured by BCA protein assay kit. For Western blot analysis, equal amounts of total protein were loaded in 8% SDS-PAGE and transferred onto a 0.22 um nitrocellulose membrane at 150 V, 90 min. The primary antibody rabbit anti-human Cas9 Polyclonal Antibody (Clontech) was diluted 1: 2500 and the secondary antibodies IRDye^{TM}-800CW goat anti-rabbit and goat anti-mouse were diluted 1:12,000. Specific protein was detected by the Odyssey^{TM}CLx Imaging system (Li-COR Bioscience). α-Tubulin was used as an internal control. For ELISA, the dCas9 protein was quantified using the CRISPR/Cas9 assay ELISA kit (Epigentek).

2.6.RNA isolation and qPCR

Total RNA was extracted using the Universal RNA Extraction Kit, and 1ug RNA was reverse-transcribed using the PrimeScript RT master mix. The reaction system included 10 uL of SYBR Premix Ex Taq II, 6.4 uL of ddH_2O, 0.8 uL each of forward and reverse primers at 10 uM and 2 uL of cDNA, with the following cycling conditions: 95 ℃ for 30 s, and 45 cycles of 95 ℃ for 5 s, annealing temperature for 30 s, melting: 95 ℃ for 10 s, 65 ℃ for 60 s, 97 ℃ for 1 s, cooling: 37 ℃ for 30 s (LightCycler® 96, Roche). For relative quantification, RNA expression was normalized to actin, and the PCR

amplification efficiency of primers was estimated by the slope of a standard curve. qPCR primers are listed in Supplementary Table 2.

3. **Results**

Weissman lab lentiviral plasmid expressing KRAB-dCas9 under SFFV promoter were obtained and packaged in HEK293T cells with packaging plasmids, for obtaining cell clones that constitutively expressing KRAB-dCas9. To establish cell clones that express KRAB-dCas9 in doxycycline-inducible fashion, we replaced the SFFV promoter with doxycycline-inducible pTRE3G promoter (Fig. 1A). The Tet-on trans-activator rtetR-VP16 (Tet3G plasmid) was introduced into K562 cells by lentiviral infection, and the cells were selected using antibiotic G418. The inducible KRAB-dCas9 lentiviral plasmid was then packaged and used for infection into the G418 selected cells. The clonal K562 cells that either constitutively or inducible express KRAB-dCas9 were then selected by FACS utilizing the mCherry marker. To compare the cells with high or low KRAB-dCas9 expression level, we choose two clones, K562-idCas9 #49 and K562-idCas9 #60 from the 60 clones obtained from the inducible expressing cell selection. Similarly, K562-dCas9 #2 and K562-dCas9 #3 were chosen from the constitutive expressing clones for further study (Fig. 1B). Six different sgRNAs from two libraries were tested to determine CRISPRi knockdown efficiency by qPCR (Fig. 1C).

We confirmed the inducible expression of KRAB-dCas9 in K562-idCas9 #49 by western blot and ELISA (Fig. 2A-B) and found that KRAB-dCas9 protein mainly localized in the nucleus, meeting expectation. mCherry median fluorescent intensity (MFI), the tag that co-expressed with KRAB-dCas9, exhibits a similar pattern as the ELISA signal, indicating that mCherry reflected the KRAB-dCas9 expression level. Both inducible clones have a dose-responsive increase in mCherry MFI upon doxycycline induction (Fig. 2C). The inducible KRAB-dCas9 expressing clones have lower expression levels compared to the constitutive clone K562-dCas9 #2, suggesting that inducible expression may not be able to reach the expression level of constitutive expression (Fig. 2D).

We assessed the knockdown efficiency of the four KRAB-dCas9 expressing clones using six different sgRNAs from two published CRISPRi libraries, the hCRISPRi-v2 library and the Dolcetto library [9-10] (Supplementary Table 1). The relative position of the sgRNA to the transcription start site of the target genes varies (Fig. 3A), probably for meeting the specificity requirement. sgRNA was constructed and packaged into lentivirus and used for infection, and the cells expressing sgRNA were selected using puromycin. The knockdown efficiency was assessed by qPCR. The relative expression level of the target genes in four cell clones differed from each other, probably due to the single cloning procedure [11] (Fig. 3B). K562-dCas9 #2, the clone with the highest KRAB-dCas9 expression level knocked down most genes effectively (Fig. 3C); yet all other clones have relatively low or no knockdown efficiency or the target gene expression level increased (Fig. 3C). MMADHC, RPIA, ZNF148, LSM4, and LIMA1

145 genes have been knocked down in clone K562-dCas9 #2. The NPEPPS gene expression
146 level increased in 3 cell clones, which could be due to the following reasons: NPEPPS
147 is a puromycin responsive gene, and the puromycin selection after sgRNA transduction
148 activated its expression; and/or the sgRNA is too far from the TSS. Similarly, the RPIA
149 gene expression increased in the inducible clones, probably due to doxycycline
150 treatment, as RPIA expression increased upon doxycycline treatment [12]. The
151 KRAB-dCas9 protein expression level in K562-dCas9 #2 is at least 24-fold more than
152 all other clones, indicating that high expression of KRAB-dCas9 is probably a
153 prerequisite for effective CRISPRi knockdown. We analyzed whether knockdown
154 efficiency is affected by the target gene expression level, and found no correlation (Fig.
155 3D).

156 Utilizing K562-dCas9 #2, we assessed the impact of sgRNA expression level on
157 knockdown efficiency. We infected K562-dCas9 #2 with sgRNA lentivirus at different
158 MOI and determined the gene transfer rate using the BFP MFI, which is co-expressed
159 with the sgRNA. We found an increase in the gene transfer rate, correlated with MOI,
160 fitting a polynomial curve (Fig. 4A). Based on this curve, the gene transfer rate was
161 34.1% at MOI 0.5, the usually used MOI for CRISRPi library, which is close to the
162 previously reported gene transfer rate 28.6% at MOI 0.5 in K562 cells [13], confirming
163 an effective lentiviral packaging and transduction. BFP MFI in positive sgRNA
164 expression cells linearly correlated with MOI, indicating that the sgRNA level increased
165 with increasing MOI (Fig. 4B). Correspondingly, a linear relationship was observed
166 between the MOI and the knockdown efficiency, as expected (Fig. 4C). In our research,

based on the linear relationship between the MOI and the knockdown efficiency, around 45% knockdown could be achieved by MOI 0.5 for the MMADHC gene (Fig. 4C). Moreover, we studied the relationship between the KRAB-dCas9 expression level and the knockdown efficiency and found that the KRAB-dCas9 level remained relatively constant across different MOI of sgRNA infection (CV=2.2%), which does not correlate with the knockdown efficiency (Fig. 4D).

4. Discussion

We discovered that only cell clone with high KRAB-dCas9 expression can CRISPRi effectively knockdown target gene, suggesting that care shall be taken when choosing cells/cell clones for CRISPRi applications. The requirement of a high KRAB-dCas9 level mandated selection of the cells after lentiviral infection. However, it is known that single cloning affected the transcription profile of the KRAB-dCas9 expressing cells, suggesting that poly-clonal cell populations are better in CRISPRi applications [11]. To meet the requirement of high KRAB-dCas9 expression, cell sorting shall be carried out, and more than one selection may be required, as the lentiviral transgene promoter could be methylated and transgene expression shut down in the continuous culture of the cells. For CRISPRi library screening, it can be challenging to obtain the cells with universal high KRAB-dCas9 expression level due to a large amount of cell required: the number of the cell in each group shall exceed 1000× the sgRNA number in the library. A high-speed cell sorter is required, which is unfortunately not available in most

188 laboratories. CRISPRi library screening without such an instrument might have to rely
189 on obtaining and expanding single-cell clone that stably expressing a high level of
190 KRAB-dCas9. Effects on transcriptional profile brought by the single cloning procedure
191 can be canceled out, though, as comparisons were made between groups using the same
192 cell clone. Alternatively, with a more potent CRISPRi system, the high expression level
193 of dCas9 fusion protein might not be necessary, and drug selection based on antibiotic
194 resistance could be applied. The dCas9-KRAB-MeCP2 fusion protein is more effective
195 in knockdown [14] and may offer such an option. Alternatively, iterative transfection
196 could be applied to inprove the expression of both dCas9 and sgRNA level [15].

197 We observed that once KRAB-dCas9 expression is high enough, CRISPRi efficiency
198 depended on the sgRNA expression level. This observation corroborated the previous
199 findings in the CRISPR system, that the nuclear concentration of the guide RNA is the
200 limiting factor for efficient DNA targeting [6, 16], indicating that the dynamics of
201 ribonucleoprotein formation and DNA targeting is probably similar between CRISPR
202 and CRISPRi procedure. To improve the knockdown efficiency, it is critical to
203 increasing the expression level of sgRNA, and a change of promotor could achieve this
204 goal. Alternatively, since sgRNA is very unstable when it was not bound with Cas9
205 protein [6], improvement of the sgRNA stability could potentially increase efficiency.
206 The correlation between the sgRNA expression level and the knockdown efficiency of
207 the CRISPRi system has indications for the CRISPRi library screening: inducible
208 sgRNA will provide better control of the knockdown. It has been reported that inducible

sgRNA offered better control of knockout in the CRISPR system [17], again pointing to the similarities between the two tools.

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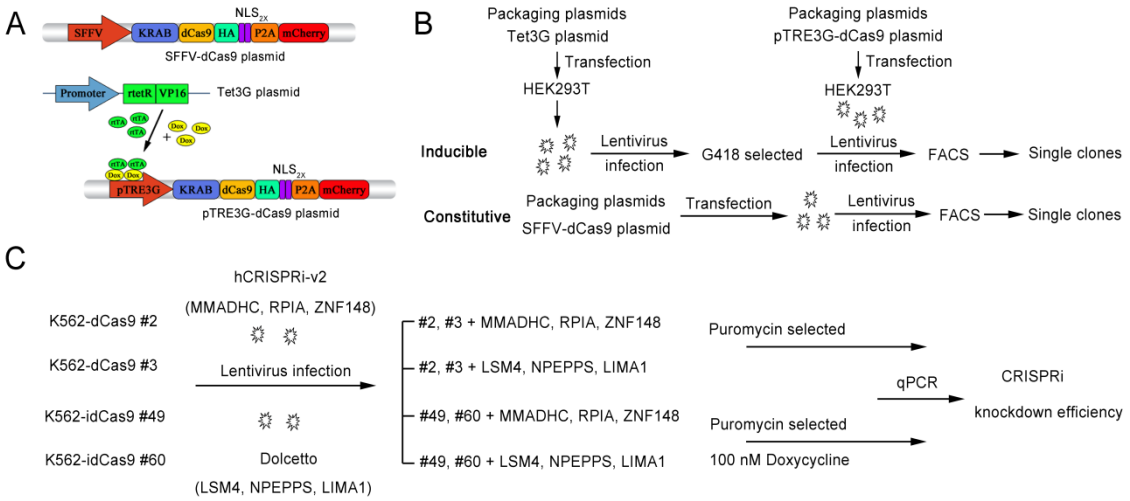
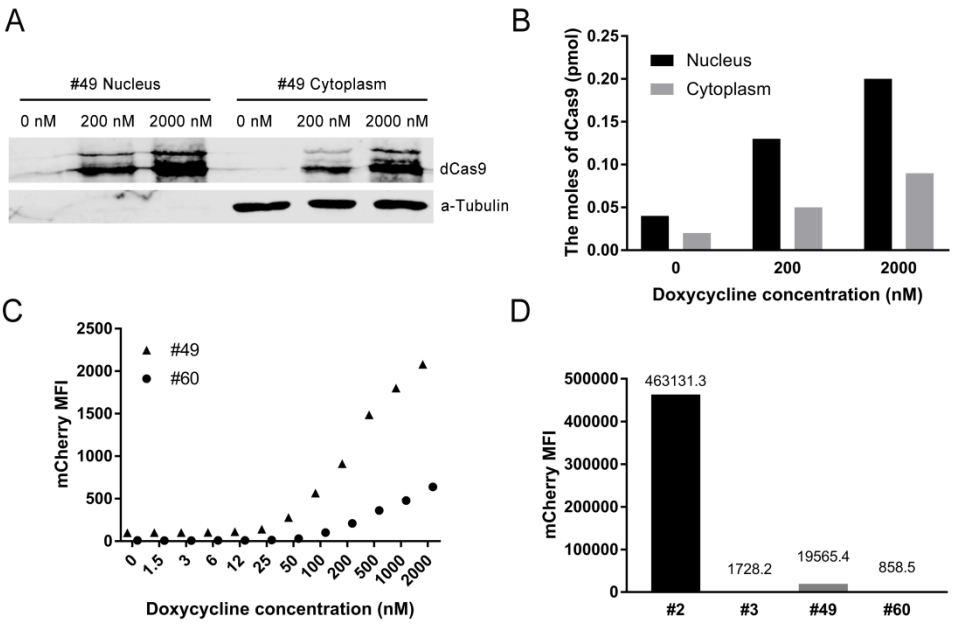


Fig.1. Overview of the experimental procedure. **(A)** Vectors for inducible and constitutive KRAB-dCas9 expression. **(B)** Generate inducible and constitutive KRAB-dCas9 expressing cell clones using lentivirus. **(C)** Infect cell clones with sgRNA to determine CRISPRi knockdown efficiency.



287 **Fig.2.** The expression of KRAB-dCas9 in inducible and constitutive clonal cells. **(A-B)**
288 Western blot and ELISA detection of nuclear and cytoplasmic KRAB-dCas9 in
289 K562-idCas9 #49 induced by 200 nM and 2000 nM doxycycline. **(C)** mCherry MFI in
290 K562-idCas9 #49, K562-idCas9 #60, in response to various doses of doxycycline.
291 Triangle indicated K562-idCas9 #49, round indicated K562-idCas9 #60. **(D)** mCherry
292 MFI of two inducible cell clones induced by 100 nM doxycycline and two constitutive
293 cell clones. #2: K562-dCas9 #2; #3: K562-dCas9 #3; #49: K562-idCas9 #49; #60:
294 K562-idCas9 #60.

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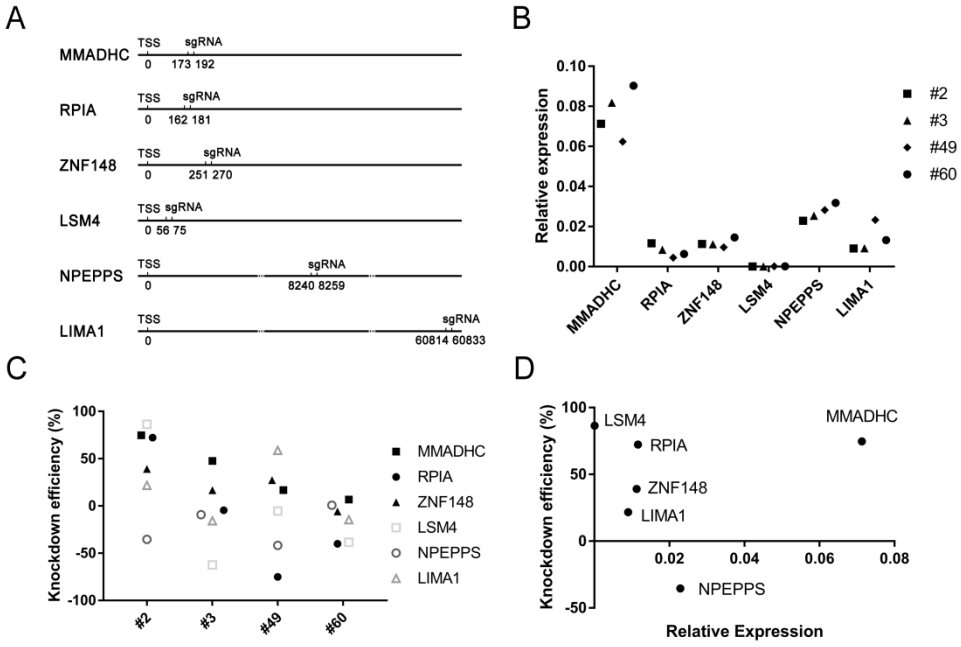

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297 **Fig. 3.** Efficient CRISPRi knockdown depends on highly expressed KRAB-dCas9. **(A)**
298 The location of sgRNA of six genes at the promoter region, TSS: The transcription start
299 site. **(B)** The relative expression level of six genes in four clones, #2: K562-dCas9 #2;
300 #3: K562-dCas9 #3; #49: K562-idCas9 #49; #60: K562-idCas9 #60. **(C)** Knockdown

efficiency of six genes in four clones, #2: K562-dCas9 #2; #3: K562-dCas9 #3; #49: K562-idCas9 #49; #60: K562-idCas9 #60. **(D)** The relationship between target gene expression level and CRISPRi knockdown efficiency in K562-dCas9 #2. Triangle indicated hCRISPRi-v2 libraries. Round indicated Dolcetto library.

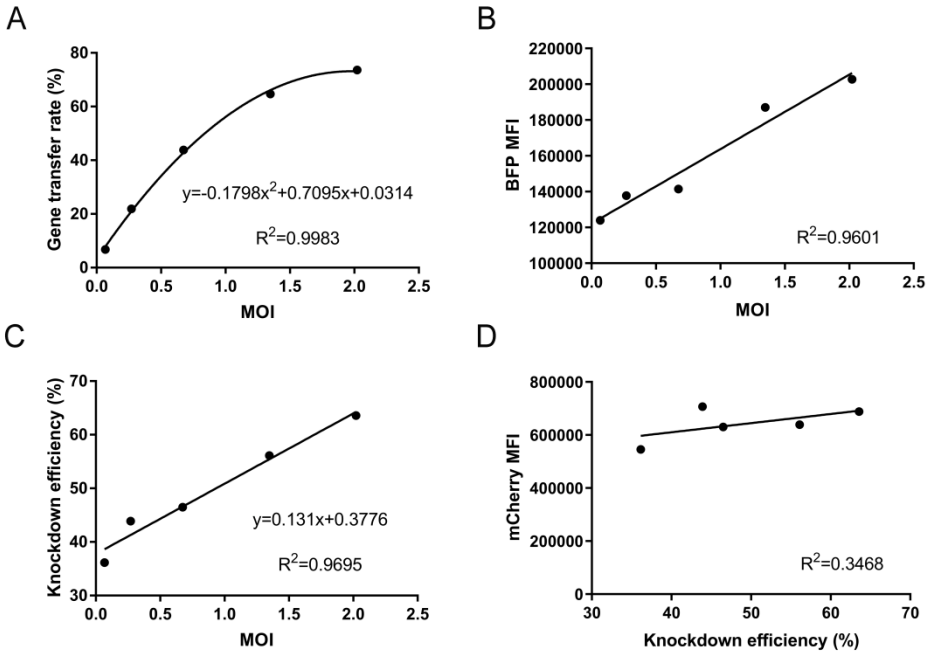

Fig. 4. CRISPRi knockdown efficiency correlated with sgRNA expression level. **(A)** Correlation between gene transfer rate and MOI of sgRNA infection of the MMADHC gene, determined by BFP MFI, in K562-dCas9 #2. **(B)** Correlation between BFP MFI in positive sgRNA expression cells and the MOI of sgRNA infection of the MMADHC gene in K562-dCas9 #2. **(C)** Correlation between knockdown efficiency and MOI of sgRNA infection of the MMADHC gene in K562-dCas9 #2. **(D)** Correlation between mCherry MFI and knockdown efficiency in K562-dCas9 #2.