sgRNA level is a major factor affecting CRISPRi knockdown efficiency in K562 cells

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Abstract—To determine how nuclease deactivated Cas9 (dCas9) or sgRNA expression level affects the knockdown efficiency of CRISPRi, K562 cell clones expressing KRAB-dCas9 protein either with the inducible Tet-on system or with the constitutive SFFV promotor were created by lentiviral transduction, and 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 in four cell clones with various KRAB-dCas9 expression levels. We determined the knockdown efficiency and the expression level of the dCas9 protein /sgRNA level by flow cytometry. The cell clone with the highest KRAB-dCas9 expression level achieved effective CRISPRi knockdown, and is statistically different from other clones, indicating enough KRAB-dCas9 expression might be a prerequisite for CRISPRi. Utilizing this clone, we modified the expression level of sgRNA by adopting different multiplicity of infection (MOI) in lentiviral transduction and found that the knockdown efficiency was neither affected by the target gene expression level nor does it correlate with KRAB-dCas9 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 expression level. Linear regression modeling of the data revealed that the knockdown efficiency is significantly affected by both KRAB-dCas9 and sgRNA level,

31 and the sgRNA level has a greater impact, based on the standardized coefficient (0.525
32 for KRAB-dCas9, 0.981 for sgRNA) , indicating that sgRNA level is a major factor
33 affecting CRISPRi efficiency.

34

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

37

38 INTRODUCTION

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

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

51 Although the dynamics of KRAB-dCas9 and sgRNA mediated CRISPRi
52 knockdown has not been studied thoroughly, the mechanistic study of the CRISPR
53 system could provide some clues due to the similarity: effective knockdown of target
54 gene started from the sgRNA being expressed from the transgene, formed a complex
55 with Cas9 protein, and roaming in the nucleus until the sgRNA hybrid to target DNA,
56 then the Cas9 protein cut the target gene [6]. Cas9 is essential for guide RNA stability
57 and that the nuclear Cas9-guide RNA complex levels limit the targeting
58 efficiency. Similar dynamics could exist in the CRISPRi system, and the very first step

59 is to determine how dCas9 fusion protein and the sgRNA expression level affect
60 CRISPRi efficiency.

61 In this study, different cell clones with various KRAB-dCas9 protein level and
62 subclones with different sgRNA expression level was evaluated for CRISPRi efficiency.
63 Linear regression was applied to determine the major factor that determines CRISPRi
64 efficiency.

65

66 EXPERIMENTAL

67 **Cell culture and construction of plasmids.** HEK293T and K562 cells were
68 obtained from the National Infrastructure of Cell Line Resource. HEK293T cells were
69 cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L
70 glucose (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco). K562
71 cells were maintained in Iscove's modified Dulbecco's medium (IMDM, Gibco)
72 supplemented with 10% FBS. Cells were tested every 3 months for mycoplasma
73 contamination and consistently tested negative. To induce the expression of
74 KRAB-dCas9 protein using doxycycline, we replaced the promotor of
75 SFFV-KRAB-dCas9 plasmid (plasmid Addgene, #60954 provided by Jonathan S.
76 Weissman) with the pTRE3G promoter (plasmid Addgene # 96964 provided by Elena
77 Cattaneo) using classic cloning method, primers are listed in Supplementary Table 1.

78 **Lentiviral Packaging.** 2.1×10^7 HEK293T cells were seeded into T175 flasks 1d
79 before transfection. Cells were transfected with the packaging plasmids of pSPAX2
80 (11.6 ug) and pMD2.G (2.8 ug) (plasmid Addgene #12260 and #12259 provided by
81 Didier Trono), and the target plasmids (11.6 ug) using Polyetherimide (PEI) (1 mg/mL),
82 with the nitrogen to phosphorus (N/P) ratio 20 [2, 7]. Viral supernatants were collected
83 at 48 h and 72 h after transfection and filtered using 0.45 um filters. Subsequently, 4 mL
84 20% sucrose solution was added to the bottom of the 32 mL viral supernatants, and
85 centrifuge for 2 h at 82,700 g, 4°C [8], and the pellet was resuspended by 100 uL
86 pre-cooled PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$. The titer of the lentivirus was determined using
87 flow cytometry method as described [8].

88 **Generating cell clones by lentiviral transduction.** To generate single clones with
 89 constitutive KRAB-dCas9 protein expression, 1×10^5 K562 cells were transduced with
 90 the lentiviral particles for SFFV-KRAB-dCas9 using polybrene (final concentration 10
 91 $\mu\text{g/mL}$) at MOI 20 in six-well plates. 72 h after transduction, cells were analyzed and
 92 sorted into 96-well plates using a cell sorter(BD FACS Aria II). Single clones emerged
 93 after 14 days and were expanded and cultured for further analysis.

94 For inducible KRAB-dCas9 expressing cells, 1×10^5 K562 cells were infected with
 95 lentiviral particles for expressing the trans-activator of the pTRE3G promotor
 96 (pLVX-Tet3G) and selected for 10 days using G418 (Final concentration 100 $\mu\text{g/mL}$).
 97 Subsequently, the cell clones were transduced with the lentiviral particles for
 98 pTRE3G-KRAB-dCas9 using polybrene, the following step with the same procedure
 99 described in the above section.

100 **Flow cytometry analysis.** The flow cytometry analysis was performed on
 101 Beckman CytoFLEX S. The data were analyzed using Flowjo 10 and CytoExpert 2.0
 102 software.

103 **Western blot analysis and ELISA.** To detect the expression level of
 104 KRAB-dCas9 protein, K562-idCas9 #49 were induced by the doxycycline of 200 nM
 105 and 2000 nM for 48 h. Then, total protein was extracted using RIPA lysis buffer with 1
 106 mM PMSF, and concentration was determined by the BCA protein assay kit (Solarbio,
 107 PC0020). For Western blot analysis, total protein (20 μg) were separated by 8%
 108 SDS-PAGE and transferred onto a 0.22 μm nitrocellulose membrane at 150 V, 90 min.
 109 The membrane was incubated with the Cas9 polyclonal antibody (Clontech, 632607)
 110 and α -Tubulin (ABclonal, AC012), and the secondary antibodies IRDyeTM-800CW(BD,
 111 Lot No. C50113-06). The membrane was scanned by the OdysseyTMCLx Imaging
 112 system (Li-COR Bioscience). For ELISA, the KRAB-dCas9 protein was quantified
 113 using the CRISPR/Cas9 assay ELISA kit (Epigentek, P-4060-48) according to the
 114 manufacturer's protocol.

115 **The evaluation of the CRISPRi knockdown efficiency.** Single clones were
 116 infected with the lentivirus of sgRNA (MOI 20 if not explicitly stated otherwise) with
 117 polybrene (Final concentration 10 $\mu\text{g/mL}$). After 72 h transduction, clones were selected

118 by puromycin (Final concentration 5 ug/mL) for 72 h. The expression level of target
119 genes was detected by qPCR using the relative quantification method. qPCR primers are
120 listed in Supplementary Table 1.

121 **Statistical analysis.** ANOVA and multiple comparisons were performed to
122 compare the four-cell clones. Linear regression modeling was performed to analyze the
123 relationship between the knockdown efficiency and the expression level of either
124 KRAB-dCas9 or sgRNA. All statistical analysis was performed using the SPSS
125 package.

126

127 RESULTS

128 To analyze how KRAB-dCas9 protein and/or the sgRNA expression level affects
129 the CRISPRi knockdown efficiency, we adopted a hierarchical design that firstly
130 compared cell clones with various KRAB-dCas9 expression level, then compared cell
131 subclones with various sgRNA expression level (Fig. 1a). For the first comparison, we
132 established K562 cell clones that have either constitutive or inducible expression of the
133 KRAB-dCas9-mCherry fusion protein.

134 Tet-on system was adopted to establish cell clones that express
135 KRAB-dCas9-mCherry fusion protein in doxycycline-inducible fashion: trans-activator
136 (Tet3G plasmid) of the pTRE3G promotor was first introduced into K562 cells by
137 lentiviral transduction, followed by G418 selection, and subsequently undergone
138 lentiviral transduction of the KRAB-dCas9-mCherry expressing vector with the
139 inducible pTRE3G promotor (Fig. 1b). After FACS selection based on mCherry
140 expression, sixty inducible clones (K562-idCas9 #1 to #60) were randomly chosen from
141 twelve 96-well plates for further expansion, and their transgene expression pattern was
142 analyzed with flow cytometry upon doxycycline induction (Supplemental Fig. 1-2).
143 Based on the fold change of transgene expression level, represented by the medium
144 fluorescent intensity (MFI), with or without doxycycline induction (Induced
145 MFI/Uninduced MFI) , the 60 clones were ranked (Fig. 1c) and five clones

146 (K562-idCas9 #9, #25, #49, #54, #60) with various fold changes were chosen for further
147 analysis.

148 We first confirmed the inducible expression of KRAB-dCas9-mCherry in
149 K562-idCas9 #49 and #60 by detecting mCherry, the tag that co-expressed with
150 KRAB-dCas9, using flow cytometry (Fig. 2a), then by detecting dCas9 with western
151 blot and ELISA (Fig. 2b-c). KRAB-dCas9 protein mainly localized in the nucleus, as
152 expected. MFI of mCherry exhibits a similar pattern as the ELISA signal, confirming
153 that mCherry represented the KRAB-dCas9 expression level. For sgRNA, two sgRNA
154 libraries, the hCRISPRi-v2 library [9] and the Dolcetto library [10], were obtained from
155 Addgene, both contain multiple sgRNAs targeting one gene and ranked them (Table 1).
156 Pilot CRISPRi knockdown experiments were carried out using the lentiviral particle of
157 highest-ranking sgRNA against UBE4A and DPH2, from the hCRISPRi-v2 library.
158 K562-idCas9 #9, #25, #54 were excluded from further analysis based on their
159 performance in the pilot experiments (Supplementary Fig. 3a-d). K562-idCas9 #49
160 displayed 24% knockdown efficiency in the DPH2 gene with 100 nM doxycycline
161 (Supplementary Fig. 3e), has relatively higher KRAB-dCas9-mCherry expression level,
162 and were chosen for further analysis, together with K562-idCas9 #60, which has
163 relatively low KRAB-dCas9-mCherry expression (Fig. 2d).

164 Similarly, we established constitutive KRAB-dCas9-mCherry expressing cell
165 clones, and chose three clones, K562-dCas9 #A, K562-dCas9 #B, and K562-dCas9 #C.
166 Pilot knockdown experiments revealed that K562-dCas9 #A failed to knockdown
167 UBE4A or DPH2 genes (Supplemental Fig. 3f), and was excluded from further analysis.

168 To compare the knockdown efficiency of cell clones with various KRAB-dCas9
169 expression levels, the constitutive K562-dCas9 #B, #C and the inducible K562-idCas9
170 #49, #60 were evaluated for their CRISPRi efficiency. The inducible KRAB-dCas9
171 expressing clones have lower expression levels compared to the constitutive clones,
172 indicating that inducible expression may not be able to reach the expression level of
173 constitutive expression (Fig. 2d). For sgRNA, we randomly choose three sgRNAs per
174 CRISPRi library of high rankings (Table 1). After lentiviral transduction of sgRNA into
175 the cell clones, puromycin selection was applied, and the knockdown efficiency was

assessed by qPCR. *mmadh*, *rpia*, *znf148*, *lsm4*, and *lim1* genes have been knocked down in clone K562-dCas9 #B, the clone with the highest KRAB-dCas9 expression level (Fig. 3a); yet all other clones have relatively low or no knockdown efficiency or the target gene expression level increased (Fig. 3a), indicating sufficient KRAB-dCas9 expression level is a prerequisite for efficient CRISPRi.

To better understanding the difference among the four cell clones, ANOVA was performed and multiple comparisons were made. The results indicated that K562-dCas9 #B is significantly different from K562-dCas9 #C and K562-idCas9 #60, and the p-value for comparison between K562-dCas9 #B and K562-idCas9 #49 is 0.054, indicating that K562-dCas9 #B is also different from K562-idCas9 #49, corroborating our finding that only K562-dCas9 #B has effective CRISPRi (Table 2).

We also analyzed whether knockdown efficiency is affected by the target gene expression level, and found no correlation (Fig. 3b). 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. 3c). Continuous monitoring of the KRAB-dCas9 expression revealed that K562-dCas9 #C lost transgene expression over time, while other clones maintained their transgene expression (Supplementary Table 2).

Utilizing K562-dCas9 #B, we assessed the impact of sgRNA expression level on knockdown efficiency. Gene transfer rate, the rate of cells expressing a transgene in the cell population, were assessed to ensure successful transduction. BFP MFI, which is co-expressed with the sgRNA in a polycistronic transcript, was used for determining the gene transfer rate and found to correlate with MOI, fitting a polynomial curve (Fig. 4a). Based on this curve, the gene transfer rate was 34.1% at MOI 0.5, close to the previously reported gene transfer rate 28.6% at MOI 0.5 in K562 cells [12], confirming an effective lentiviral packaging and transduction.

We then assessed how MOI correlated with transgene expression and found that in positive sgRNA expression cells, based on flow cytometry detection (Fig. 4b), BFP MFI linearly correlated with MOI (Fig. 4c). Moreover, a linear relationship was observed between the MOI and the knockdown efficiency (Fig. 4d), indicating that as sgRNA expression level correlates with knockdown efficiency. In our research, based

206 on the linear relationship between the MOI and the knockdown efficiency, around 45%
207 knockdown could be achieved for the *mmadh*c gene by MOI 0.5, the common MOI
208 used for CRISPRi library screening (Fig. 4d).

209 To systematically evaluate how dCas9 and sgRNA expression level affects the
210 knockdown efficiency, a linear regression model with dCas9 MFI and sgRNA as
211 independent variable individually was applied. Results have shown that the assumption
212 of equal variance was met. When dCas9 serve as the independent variable in the linear
213 model, $R^2=0.243$, $F=8.367$, $p=0.008$, indicating that dCas9 fusion gene expression
214 affected the knockdown efficiency significantly. The standardized coefficient $\beta_1=0.525$
215 indicated that when dCas9 change for one standard deviation, the knockdown efficiency
216 changes 0.525 standard deviations. Similarly, when sgRNA serves as the independent
217 variable in the linear model, $R^2=0.949$, $F=75.84$, $p=0.003$, and standardized coefficient
218 $\beta_2=0.981$, indicating that sgRNA level affected knockdown efficiency significantly, and
219 when sgRNA level change for one standard deviation, the knockdown efficiency
220 changes for 0.981 standard deviations. The standardized coefficient value has shown
221 that sgRNA level has a greater impact on the knockdown efficiency, which corroborated
222 with the correlation analysis.

223

224 DISCUSSION

225 As the first step to dissect the dynamics of the KRAB-dCas9 mediated CRISPRi
226 system, we assessed how the expression level of dCas9 fusion protein and the sgRNA
227 affect the knockdown efficiency, which provided the basis for further optimization of
228 the CRISPRi library screening.

229 We discovered that KRAB-dCas9 expression level significantly impact CRISPRi
230 efficiency, and our data, although limited in the number of clones tested, indicated that
231 enough dCas9 fusion protein probably is a prerequisite for the efficient knockdown. A
232 study on more cell clones is mandated for clarifying whether there is a threshold of
233 KRAB-dCas9 expression above which effective knockdown is guaranteed.

234 The requirement of the KRAB-dCas9 level has implications for CRISPRi library
235 screenings: its mandated selection of the cells after lentiviral infection, which can be

236 challenging due to many cells required: the number of the cell in each group shall
237 exceed 1000× the sgRNA number in the library. A high-speed cell sorter is required,
238 which is unfortunately not available in most laboratories. CRISPRi library screening
239 without such an instrument might have to rely on obtaining and expanding a single-cell
240 clone that stably expressing a high level of KRAB-dCas9. However, the lentiviral
241 transgene promoter could be methylated and transgene expression shut down in the
242 continuous culture of the cells, as indicated in one of the cell clone K562-dCas9 #C, so
243 continuous monitoring of KRAB-dCas9 expression level is recommended, and more
244 than one selection could be required to obtain such cell clone.

245 It's known that single cloning affected the transcription profile of the
246 KRAB-dCas9 expressing cells, and poly-clonal cell populations are better in CRISPRi
247 applications [11]. Effects on transcriptional profile brought by the single cloning
248 procedure can be canceled out, though, as comparisons were made between groups
249 using the same cell clone. Alternatively, with a more potent CRISPRi system, the high
250 expression level of dCas9 fusion protein might not be necessary, and drug selection
251 based on antibiotic resistance could be applied. The dCas9-KRAB-MeCP2 fusion
252 protein is more effective in knockdown [13] and may offer such an option.

253 Among the six genes randomly chosen for CRISPRi, *npepps* and *rpia* has low
254 efficiency in most or all cell clones. Gene annotation revealed that *npepps* is a
255 puromycin responsive gene, and the puromycin selection after sgRNA transduction
256 probably activated its expression; *rpia* has been reported to be responsive to
257 doxycycline, with increased expression upon doxycycline treatment [14].

258 We observed that with enough KRAB-dCas9 expression level, CRISPRi efficiency
259 correlated very well with the MOI, which correlated equally well with the sgRNA
260 transgene expression, indicating that CRISPRi efficiency correlated well with sgRNA
261 level. Although it has not been studied in the CRISPRi system, it is known that in the
262 CRISPR system the nuclear concentration of the guide RNA is the limiting factor for
263 efficient DNA targeting [6, 15]. We found that the guide RNA level has a greater
264 impact on CRISPRi efficiency than dCas9. The dynamics of ribonucleoprotein

265 formation and DNA targeting is probably similar between CRISPR and CRISPRi
266 system.

267 To improve the knockdown efficiency, it is critical to increasing the expression
268 level of sgRNA since sgRNA is very unstable when it was not bound with Cas9 protein
269 [6], improvement of the sgRNA stability could increase efficiency [16]. Alternatively, a
270 change of promotor might serve to increase the sgRNA expression.

271 The correlation between the sgRNA expression level and the knockdown
272 efficiency of the CRISPRi system has indications for the CRISPRi library screening, too:
273 inducible sgRNA will provide better control of the knockdown. It has been reported that
274 inducible sgRNA offered better control of knockdown in the CRISPR system [17],
275 again pointing to the similarities between the two systems.

276

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281

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285

286 COMPLIANCE WITH ETHICAL STANDARDS

287 The authors declare that they have no conflict of interest. This article does not
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289 authors.

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- 353

Table 1. List of sgRNA sequences

sgRNA	Sequences (5'-3')	Rank	Library
DPH2	GATGTTTAGCAGCCCTGCCG	2/10	hCRISPRi-v2
UBE4A	GACAGGGTCGAAGTTCAAGA	1/10	hCRISPRi-v2
MMADHC	GAAAATACGGGCAATACCGA	1/10	hCRISPRi-v2
RPIA	GGTGACAGTCTGGGACCCG	1/10	hCRISPRi-v2
ZNF148	GCAGGCCGAGCGCTTTACCG	1/10	hCRISPRi-v2
LSM4	CAGTTGCCCTGCCCTCACCA	1/3	Dolcetto
NPEPPS	TGGCGGGCGGGCTGCCTACG	3/3	Dolcetto
LIMA1	GATGCTTTCTCCATGTGGCA	1/3	Dolcetto

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Table 2. Effects of the four cell clones on knockdown efficiency and multiple comparisons

Cell clone	The average of knockdown efficiency (%)	Standard deviation (%)
K562-dCas9 #B	43.16 ^a	45.55
K562-idCas9 #49	-3.24 ^{ab}	48.65
K562-dCas9 #C	-4.63 ^b	36.48
K562-idCas9 #60	-15.24 ^b	19.92

Different lowercase letters indicate a significant difference in knockdown efficiency under different cell clones ($p < 0.05$).

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Fig. 1. The construction and selection of inducible cell clones. (a) Experiment design. #B: K562-dCas9 #B; #C: K562-dCas9 #C; #49: K562-idCas9 #49; #60: K562-idCas9 #60. #B1, #B2, #B3, #B4, #B5 indicates K562-dCas9 #B infected with difference MOI of sgRNA. (b) Lentiviral vectors for inducible and constitutive KRAB-dCas9 expression. (c) Sixty inducible cell clones ranked based on induced MFI/uninduced MFI, the red prismatic indicated the single clones that chosen for further analysis. 2000 nM doxycycline was applied for induction for 48 h.

Fig. 2. The expression of KRAB-dCas9 in inducible and constitutive cell clones. (a) mCherry MFI in K562-idCas9 #49, K562-idCas9 #60, in response to various doses of

doxycycline. Triangle indicated K562-idCas9 #49, round indicated K562-idCas9 #60.
(b-c) Western blot and ELISA detection of nuclear and cytoplasmic KRAB-dCas9 in
K562-idCas9 #49 induced by 200 nM and 2000 nM doxycycline. (d) mCherry MFI of
two inducible cell clones induced by 100 nM doxycycline and two constitutive cell
clones. #B: K562-dCas9 #B; #C: K562-dCas9 #C; #49: K562-idCas9 #49; #60:
K562-idCas9 #60.

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Fig. 3. Efficient CRISPRi knockdown requires enough KRAB-dCas9 expression. (a)
Knockdown efficiency of six genes in four clones, #B: K562-dCas9 #B; #C:
K562-dCas9 #C; #49: K562-idCas9 #49; #60: K562-idCas9 #60. (b) The relationship
between target gene expression level and CRISPRi knockdown efficiency in
K562-dCas9 #B. (c) The relative expression level of six genes in four clones, #B:
K562-dCas9 #B; #C: K562-dCas9 #C; #49: K562-idCas9 #49; #60: K562-idCas9 #60.

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391

392 **Fig. 4.** CRISPRi knockdown efficiency correlated with sgRNA expression level. (a)
 393 Correlation between MOI of sgRNA infection of the *mmadh* gene and gene transfer
 394 rate, determined by BFP MFI, in K562-dCas9 #B. (b) The scatter plot of K562-dCas9
 395 #B with sgRNA of the *mmadh* gene. The red frame indicated positive sgRNA
 396 expression cells with BFP fluorescence. (c) Correlation between BFP MFI in positive
 397 sgRNA expression cells and the MOI of sgRNA infection of the *mmadh* gene in
 398 K562-dCas9 #B. positive sgRNA cells were defined by flow cytometry. (d) Correlation
 399 between knockdown efficiency and MOI of sgRNA infection of the *mmadh* gene in
 400 K562-dCas9 #B.