

Engineered Circular Guide RNAs Enhance Miniature CRISPR/Cas12f-based Gene Activation and Adenine Base Editing

Corresponding Author: Professor Zhili Rong

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this research, the sgRNA of Un1Cas12f1 was engineered from a linear RNA to a circular one, which increase the efficiency of the Cas12f-VPR and Cas12f-ABE. In addition, the authors also fused the Cas12f-VPR with FUS-IDR to make it more efficient. This study is of great potential, but some issues need to be addressed before publication.

Major points:

- 1.Although the delivery advantage was mentioned in the introduction, Cas12f-ABE was not used for gene editing in animals. These results are necessary to prove the delivery advantage of Cas12f-ABE.
- 2.Page 27, figure 3a: Only 4 targeting sites were selected for the test of dCas12f-VPR in HEK293T, we recommend to test more targeting sites (about 6-8).
- 3.Page 29, figure 5d: Only 2 targeting sites were selected for the test of dCas12f-ABE in HEK293T, we recommend to test more targeting sites (about 6-8).
- 4.It would be interesting to test whether the linker-gRNA-linker-gRNA-linker construct can be used to simultaneously active and edit different genes.
- 5.It would be helpful to compare the activating and editing efficiency between Cas12f RNA or protein with gRNA and circular gRNA.
- 6.In figure 5, to draw the narrowing editing window conclusion, two better controls of U6gRNAs with AC5 and AC10 linker are needed.

Minor points:

- 1.Page 4: "Next, to directly assess the effect of cgRNA on gene editing, we construed two reporter cell lines", there is a spelling error here, "construed" should be "constructed".
- 2.Page 4: "the downstream gene mNeonGreen was activated", we suggest to explain as "the downstream mNeonGreen gene expression was activated".
- 3.Page 25, figure 1d: The G3/G5 gRNA binding sites are depicted with 7 same squares and Tet Response Element (TRE) is depicted with one square. This diagram can be misunderstanding as the regular TRE is composed with 7 TetO, also there are no obvious description about the tandem G3/G5 gRNA binding sites (also in page 31, figure S1a).
- 4.Page 25, figure 1e: The histogram shows the percentage of mNeonGreen positive cells, we recommend to change this with mNeonGreen/BFP ratio, as the cgRNA was co-transfected with BFP, which might provide a more calibrated result.
- 5.Page 29, figure 5b and 5d: The results were redundantly displayed in these two figures. Figure 5d results were obtained through sanger sequencing, this sanger sequencing results does not need to be shown in this figure, as the NGS results have been shown.

Reviewer #2

(Remarks to the Author)

Overall Comments:

In this manuscript, Zhang et al. report the utility of cgRNA in the application of CRISPR-Cas12f system for gene expression regulation and base editing. A sophisticated design of cgRNA allows the significant improvements in gene expression and base editing in terms of efficiency and specificity. They attempted to engineer a linker sequence to further improve the utility of cgRNA. This appears to be novel because the authors convince that cgRNA is functional for Cas12f system, but the utility is limited because they failed to show that cgRNA is working for the indel formation of Cas12f1. Besides these significant limitations, the manuscript needs to be considered for improvements with respect to experimental designs and consistency. The major questions related to these concerns are as follows:

Major concerns:

Q1) The activity of Cas depends on the level of intracellular level of gRNA, apart from whether it is circular or linear. However, the authors did not provide the quantitative data for the intracellular expression for the gRNAs under comparison. Because it is not assumed that cgRNA is formed at 100% circularization efficiency, it is important to address that what percentage of gRNA is linearized or remained as linear form. However, no relevant data was not provided. What's the level of cgRNA expressed in HEK293T cells in comparison of canonical sgRNA? The cgRNA is homogeneous or only a certain fraction of gRNA is circularized?

Q2) Throughout the manuscript, the authors selected the HBG and HBB sites to investigate the alterations in gene expression in HEK293T cells and to test if the cgRNA further improves the gene activation by dCas12f-VPR. However, the HBB or HBG activation is not biologically relevant because the HEK293T cell does not express those gene per se. Plus, there are other regulators for the gene expression of HBG. The CRISPR-activation test should be tested in HBB or HBG-expressing cells, such as several myeloid cells, and the authors need to show that the cgRNA increases HBB or HBG expression in the HBB or HBG-positive cells. To my knowledge, the Ct value for HBG and HBB mRNA is quite high, so there can be significant fluctuations in the Ct value between experimental sets, rather than improvements by cgRNA. Can you provide western blot data where cgRNA boosted the HBB or HBG expression levels in HEK293T cells?

Q3) There is a significant discrepancy between the experimental sets. In Fig. 1d-e using a 0-nt linker, the % mNeonGreen-positive cells were ~20%. However, the level was significantly decreased to 1-2% even in the same G3 reporter cell line as shown in Fig.2c. Which is true? If it's a matter of expression level, the authors need to control the expression level so that the cgRNA with a 0-nt linker shows ~20% positive cells identically in both experimental sets. And then, the authors should convince that cgRNA further increase the number of positive cells higher than 30 or 40%.

Q4) In Fig. 3, how many non-specific genes are upregulated for cgRNA? Plus, what's the reason for the higher specificity of cgRNA, compared to other gRNA types?

Q5) In Fig. 5, the Cas12f-ABE system has already been reported to show a narrow base-editing window at the positions 3-4 from the PAM sequence. Nonetheless, the authors tested the base editing at the A3/A6 and A3/A8, which is not useful for the comparison of base editing windows for cgRNA. There are many sites that have a consecutive multiple A's in the positions 3-6, and thus I recommend that the authors test any alterations in the editing windows in those sites.

Q6) The authors only tested gRNA-4.0 for cgRNA-mediated improvements in gene expression and base editing. What's the cgRNA effect for gRNA-4.1? The relevant study would generalize the cgRNA effects on the improvements in gene editing.

Q7) As the authors acknowledged, cgRNA is not functional for indel formation of Cas12f1. I am wondering how hard the authors were attempting to overcome those limitations. Even a longer linker sequence is not effective? A longer and more flexible linker would provide a cleavage opportunity for Cas12f. The failure of indel formation is the very reason that undermines the utility of cgRNA for Cas12f.

Minor concerns:

1) In Fig. 1d, I don't understand what 0-nt linker 1 and linker 2 refers to. Is a 0-nt linker indeed a linker?

2) Reverse transcription PCR (RT-PCR) assay with two outward-facing primers confirmed that cgRNA (Circular), not normal gRNA (U6) or linear gRNA (Linear), was circularized in cells (Fig.1b) -> Reverse transcription PCR (RT-PCR) assay with two outward-facing primers confirmed the circularization of cgRNA (Circular), which was not observed for normal gRNA (U6) or linear gRNA (Fig.1b)

3) Because cgRNA is expressed under U6 promoter, it is confusing to note the normal gRNA as (U6) throughout the manuscript. The authors need to consider the way of clearing the possibility of this confusion.

4) What's Ploy A? is it a PolyA signal?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The author have addressed all my concerns, I do not have other questions.

Reviewer #2

(Remarks to the Author)

In the revised manuscript, the authors attempted to address the claims raised in the previous one. I evaluate most of the concerns were addressed, but some of the questions remain elusive.

Given the difficulty in analyzing HBB / HBG protein levels in HEK293 cells, why didn't you measure the protein levels in K562 cells? You said that HBB mRNA levels were increased in K562 cells. Without quantifying protein levels, you cannot convince that cgRNA effectively activates the gene transcription.

I don't understand if cgRNA is more specific or not, considering the Fig 3 and the rebuttals. cgRNA is responsible for the non-specific activation of non-target genes, thus evoking the issue of non-specificity.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Most of concerns were addressed and I recommend this revised manuscript for publication without further experimental revision.

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Responses to reviewers' comments:

(Line numbers mentioned in a report may not coincide with the original line numbers.)

Referee: 1

Comments for the Authors

In this research, the sgRNA of Un1Cas12f1 was engineered from a linear RNA to a circular one, which increase the efficiency of the Cas12f-VPR and Cas12f-ABE. In addition, the authors also fused the Cas12f-VPR with FUS-IDR to make it more efficient. This study is of great potential, but some issues need to be addressed before publication.

Major points

1. Although the delivery advantage was mentioned in the introduction, Cas12f-ABE was not used for gene editing in animals. These results are necessary to prove the delivery advantage of Cas12f-ABE.

We sincerely appreciate the valuable suggestion and we fully agree with you that it is better to perform gene editing in animals. As we know, AAV vector is currently the most valuable delivery tool for *in vivo* tissue targeted delivery^{1, 2}. However, the size of the most widely used *SpCas9* (~4.1 kb) plus a gRNA sequence and base editor functional module (TadA-TadA* dimer) exceeds the ~4.5 kb AAV capacity limit³. To achieve *in vivo* base editing, two split AAV vectors are generally used, and only when the two vectors enter the same cells the base editor could be reconstituted via an intern protein splicing system⁴. Fortunately, with the discovery of more compact Cas (such as *SaCas9* and *CjCas9*) and Cas-like (such as *IscB* and *TnpB*) proteins, a spectrum of groups have reported the delivery advantage of these proteins than the big-sized *Cas9* proteins^{5, 6, 7, 8}. In particular, single-AAV-delivered Cas12f-based ABE has been reported to edit the *Ttr* gene in heart, liver, and muscle, demonstrating the delivery advantage of this system *in vivo*⁹. Therefore, based on theory and practice, we believe that Cas12f-ABE exhibits delivery advantage.

To make this point clear, we have modified the introduction and cited the delivery related articles. Please refer to the revised manuscript.

2. Page 27, figure 3a: Only 4 targeting sites were selected for the test of dCas12f-VPR in HEK293T, we recommend to test more targeting sites (about 6-8).

Thanks for your helpful suggestion, we have added 2 more targeting sites in Fig. 3a. Please refer to the revised manuscript.

3. Page 29, figure 5d: Only 2 targeting sites were selected for the test of dCas12f-ABE in HEK293T, we recommend to test more targeting sites (about 6-8).

Thanks for your helpful suggestion, we have added seven more targeting sites in Fig. 5. Please refer to the revised manuscript.

4. It would be interesting to test whether the linker-gRNA-linker-gRNA-linker construct can be used to simultaneously active and edit different genes.

Thanks for your helpful suggestion, we have tested whether the linker- *gHBB*- linker-*gHBB*- linker construct can be used to simultaneously activate different genes. The results showed that the tandem arrayed crRNAs hardly activated the expression of the target genes, while single gRNA efficiently activated gene expression (Fig. R1). These observations indicate that Cas12f might not exhibit gRNA self-processing ability.

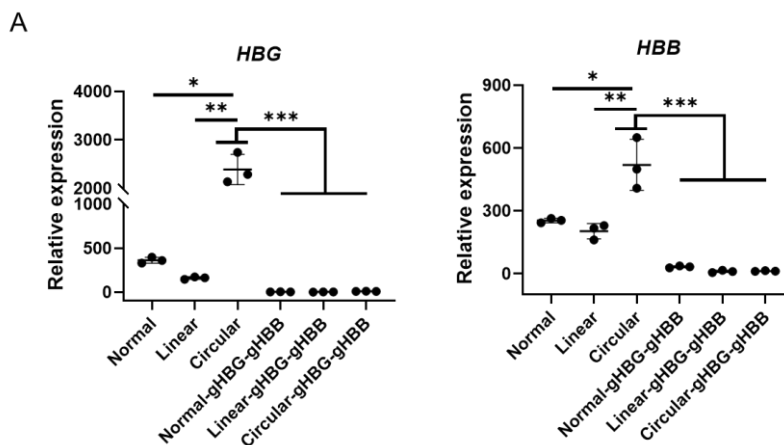


Figure R1: The linker-gHBB-linker-gHBB-linker construct hardly activated gene expression. A, Targeted gene activation guided by single gRNA or the linker-gHBB-linker-gHBB-linker construct in the dCas12f-VPR knock-in (KI) HEK293T cell line transfected with 16 ng gRNAs. n = 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA test.

5. It would be helpful to compare the activating and editing efficiency between Cas12f RNA or protein with gRNA and circular gRNA.

Thanks for your helpful suggestion. The formation of circular gRNA undergoes the Twister ribozymes spontaneous autocatalytic cleavage, which leaves a hydroxyl group at the 5' end and a 2',3'-cyclic phosphate at the 3' end¹⁰. These unique termini are subsequently ligated by the ubiquitously expressed endogenous RNA ligase RtcB in cells¹⁰. Regrettably, our lab has no technique for generating circular RNA *in vitro*, which poses a challenge in constructing the RNA or the RNP system of Cas12f/cgRNA to assess gene activation and gene editing. Actually, it has been reported that highly stable circular RNA vaccines against SARS-Cov-2 and Zika virus enable effective protection against corresponding virus in mice and monkeys^{11, 12}. Therefore, we believe that cgRNA could boost gene activation and ABE base editing efficiency via an RNP complex delivery form. We have a plan to find a cooperator to test the RNA or RNP system in the future.

6. In figure 5, to draw the narrowing editing window conclusion, two better controls of U6 gRNAs with AC₅ and AC₁₀ linker are needed.

Thanks for your helpful suggestion. We have added the controls of U6-driven gRNAs with AC₅ and AC₁₀ linkers (namely, L-AC₅ and L-AC₁₀) in Fig. 5. In addition, we also tested seven more sites for the A-to-G base editing assay. With these controls at the tested nine sites, we could draw the conclusion that cgRNA narrows the ABE editing window and achieve more precise A-to-G base editing.

Minor points:

1. Page 4: "Next, to directly assess the effect of cgRNA on gene editing, we construed two reporter cell lines", there is a spelling error here, "construed" should be "constructed".

Thank you for pointing out the typo. And we have corrected it in the revised manuscript.

2. Page 4: "the downstream gene mNeonGreen was activated", we suggest to explain as "the downstream mNeonGreen gene expression was activated".

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Thanks for your helpful suggestion. The TRE is indeed composed of 7 TetO, so we have changed the diagram of the TRE to 7 squares, as shown in Fig.1d and Fig. S1a. Meanwhile, we also added a description about the tandem G3/G5 gRNA binding sites in the figure legend of Fig. S1a

4. Page 25, figure 1e: The histogram shows the percentage of mNeonGreen positive cells, we recommend to change this with mNeonGreen/BFP ratio, as the cgRNA was co-transfected with BFP, which might provide a more calibrated result.

Thanks for your helpful suggestion. We agree that the mNeonGreen/BFP ratio provides a more calibrated result. However, the fluorescence signal of BFP was too weak to distinguish BFP-positive cells from BFP-negative cells clearly. Additionally, in many cells transfected with low amounts of gRNA, mNeonGreen expression was significantly activated, while the BFP signal remained too weak to distinguish from the negative population. Therefore, we chose to present the percentage of mNeonGreen-positive cells in the main figures, while also including the mNeonGreen/BFP ratio (mNeonGreen⁺ BFP⁺ / BFP⁺) in Supplementary Fig. S1–S9 for reference.

5. Page 29, figure 5b and 5d: The results were redundantly displayed in these two figures. Figure 5d results were obtained through sanger sequencing, this sanger sequencing results does not need to be shown in this figure, as the NGS results have been shown.

Thanks for your helpful suggestion. We have removed the Sanger sequencing data to Fig. S14.

Referee: 2

Comments for the Authors

In this manuscript, Zhang et al. report the utility of cgRNA in the application of CRISPR-Cas12f system for gene expression regulation and base editing. A sophisticated design of cgRNA allows the significant improvements in gene expression and base editing in terms of efficiency and specificity. They attempted to engineer a linker sequence to further improve the utility of cgRNA. This appears to be novel because the authors convince that cgRNA is functional for Cas12f system, but the utility is limited because they failed to show that cgRNA is working for the indel formation of Cas12f1. Besides these significant limitations, the manuscript needs to be considered for improvements with respect to experimental designs and consistency. The major questions related to these concerns are as follows:

Major concerns:

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Thanks for your helpful suggestion. We agree that it is essential to quantify or semi-quantify the expression levels of canonical (normal) sgRNA, linear gRNA and cgRNA in cells. To this end, we utilized quantitative real-time PCR to measure the gRNA expression levels when 500ng normal, linear or circular gRNA plasmids were transfected into HEK293T cells. The results showed that the expression level of circular gRNA increased approximately 392.9-fold and 194.6-fold compared to normal gRNA and linear gRNA, respectively (Fig. 1c and Fig. R2B).

The formation of circular gRNA undergoes several distinct stages¹⁰. First, the U6 promoter drives the transcription of linear pre-circular RNA. Second, as the pre-circular

gRNA properly folds, the Twister ribozymes undergo spontaneous autocatalytic cleavage, producing a hydroxyl group at the 5' end and a 2',3'-cyclic phosphate at the 3' end. Third, the endogenous RNA ligase RtcB, which is widely expressed in cells, ligates these unique termini to form mature circular gRNA. As gRNA continues to be expressed, folded, processed, and matured, the three forms of gRNA (pre-circular, linear, and circular) co-exist in a state of dynamic balance, maintaining specific proportions (Fig. R2A). As the expression of all the three types of gRNAs (Normal, linear, and circular) are driven by the same U6 promoter, the gRNA producing rates should be comparable. And as cgRNA is much more stable (Fig. 1d), we believe that the about 400- and 200-fold increase of cgRNA is resulted from slow degradation rate (high gRNA stability). Therefore, we believe that the cgRNA is heterogeneous and most of gRNA is circularized.

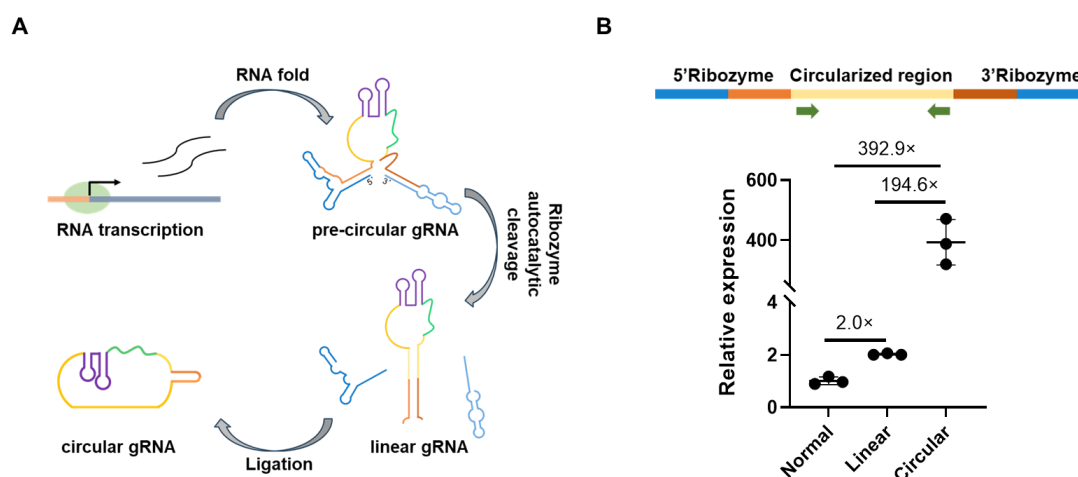


Figure R2: Circularization increases the abundance of gRNAs in human cells. A, Schematic representation of circular gRNA processing. **B,** RT-PCR analysis revealed the abundance of circular RNAs in cells. HEK293T cells were transfected with indicated plasmids encoding normal, linear or circular RNAs, and 48 hours post-transfection, total RNA was harvested for RT-PCR analysis.

2. Throughout the manuscript, the authors selected the HBG and HBB sites to investigate the alterations in gene expression in HEK293T cells and to test if the cgRNA further improves the gene activation by dCas12f-VPR. However, the HBB or HBG activation is not biologically relevant because the HEK293T cell does not express those gene perse. Plus, there are other regulators for the gene expression of HBG. The CRISPR-activation test should be tested in HBB or HBG-expressing cells, such as several myeloid cells, and the

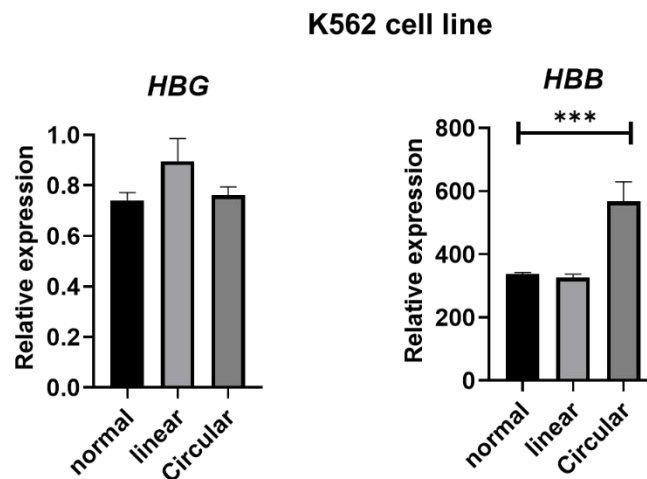
authors need to show that the cgRNA increases HBB or HBG expression in the HBB or HBG-positive cells. To my knowledge, the Ct value for HBG and HBB mRNA is quite high, so there can be significant fluctuations in the Ct value between experimental sets, rather than improvements by cgRNA. Can you provide western blot data where cgRNA boosted the HBB or HBG expression levels in HEK293T cells?

Thanks for your helpful suggestion. As suggested, we have tested the efficiency of circular RNA in the three myeloid cell lines (K562, THP1 and MV4-11). Results showed that compared to normal and linear gRNA, cgRNA significantly increased the expression of HBB and HBG in the THP1 and MV4-11 cell lines (Fig. 3b). Similarly, cgRNA markedly enhanced HBB expression in the K562 cell line, yet it did not activate HBG expression, likely due to the high basal expression level of HBG, which exceeds that of the housekeeping gene GAPDH (Fig. R3).

We fully concur with your observation: in the HEK293T and MCF cell lines, the Ct values of the non-target (NT) group for HBG and HBB mRNA are quite high, generally over 30. However, the Ct values in the cgRNA-treated group for HBG and HBB mRNA were about 21 and 28, well within the qPCR linear range, thus supporting the stability and reliability of these results. Additionally, we calculated the relative RNA expression for the normal, linear, and cgRNA groups relative to the NT control group within the same experimental run, thus eliminating variability due to high Ct values of HBG and HBB mRNA in NT controls across different experimental batches.

Following your suggestion, we also attempted to detect HBG and HBB protein expression in 293T cells. Unfortunately, Western blot results did not reveal distinct bands, possibly due to the absence of components in 293T cells required for post-transcriptional processing and transport of HBG and HBB. Nonetheless, we verified that the Ct values for the normal, linear, and cgRNA groups remained within the linear range, and agarose gel analysis confirmed the specificity and integrity of the qPCR products (Fig. R3). Thus, although we failed to detect significantly enhanced HBG or HBB protein expression in 293T cells, it did increase HBG and HBB mRNA expression.

A



B

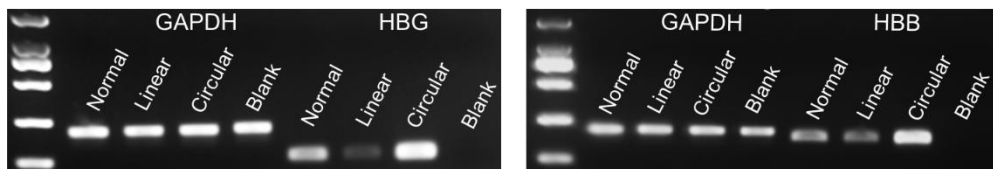


Figure R3: Cas12f/cgRNAs activates the endogenous genes expression. **A**, Targeted gene activation guided by Normal, linear and circular gRNAs in K562 cell line transiently co-transfected with dCas12f-VPR and gRNA-encoded plasmids by Lonza kit SF. **B**, Agarose gel analysis revealed the RNA levels of *HBG* and *HBB*, following RNA extraction, reverse transcription, and qPCR amplification performed for 28 and 34 cycles, respectively.

3. There is a significant discrepancy between the experimental sets. In Fig. 1d-e using a 0-nt linker, the % mNeonGreen-positive cells were ~20%. However, the level was significantly decreased to 1-2% even in the same G3 reporter cell line as shown in Fig.2c. Which is true? If it's a matter of expression level, the authors need to control the expression level so that the cgRNA with a 0-nt linker shows ~20% positive cells identically in both experimental sets. And then, the authors should convince that cgRNA further increase the number of positive cells higher than 30 or 40%.

Thanks for your helpful suggestion, and we apologize for the spelling error here. "Fluorescence-activated Cell Sorting (FACS) assay showed that cgRNA, with a 19-nt spacer and 0-nt linker1 and linker2, significantly enhanced activation efficiency compared with Normal and Linear gRNAs in both G3 and G5 reporter cell lines (Fig. 1d-e)", "0-nt linker1 and linker2" should be "10-nt linker1 and linker2". Because the structure of cgRNA

was essential to their target recognition and function, the initial circular gRNA construct used in Fig. 1e-f incorporated a 10-nt linker1 between 5' ribozyme and gRNA scaffold and a 10-nt linker2 between gRNA spacer and 3' ribozyme to ensure spatial separation of the gRNA and ribozyme (namely AC₁₀-AC₁₀ in Fig 2c). Following dosage and time-course optimization, we tested various flexible linker1 and linker2 configurations, in which 16 ng of gRNA plasmid was transfected, and cells were harvested three days post-transfection (Fig. 2c). The differences in transfection efficiency and activation levels between the data in Fig. 1e-f and the data in Fig. 2c (group AC₁₀-AC₁₀) are understandable because the different experimental conditions were used. Additionally, although differences in transfection efficiency and data variability were observed across experimental batches, the normal gRNA control group were included in each batch to standardize comparisons.

4. In Fig.3, how many non-specific genes are upregulated for cgRNA? Plus, what's the reason for the higher specificity of cgRNA, compared to other gRNA types?

Thanks for your helpful comments. 23, 15, and 10 genes other than the target HBG gene were observed up-regulated in the cgRNA group when compared to the NC, Normal, and Linear groups (Deseq2, $q < 0.05$), indicating a slightly lower specificity (Fig. 3c). The detailed list of genes is provided in the table below. Actually, the specificity of cgRNA is a little bit lower, compared to other gRNA types.

Circular vs NC	Circular vs Normal	Circular vs Linear
PARP14	PARP14	PARP14
FOSL2	LINC01013	LINC01013
DCLK1	ANKRD26P1	ANKRD26P1
ANKRD26P1	DCLK1	SHROOM2
COL9A2	ARHGAP36	ARHGAP36
ABCD2	ENSG00000286795	DCLK1
ENSG00000286795	ABCD2	ADAMTS8
KCNA2	ADAMTS8	ABCD2
ARHGAP36	COL9A2	WNT8A
SHROOM2	SHROOM2	LILRA1
LINC01013	WNT8A	
PCAT19	MARK2P9	
ADAMTS8	AZGP1	
CCN2	LILRA1	
SERPINA9	SERPINA9	
LILRA1		
PIWIL1		
GEM		
WNT8A		
MARK2P9		
MBL2		
FMR1-AS1		
CACNG7		

5. In Fig.5, the Cas12f-ABE system has already been reported to show a narrow base-editing window at the positions 3-4 from the PAM sequence. Nonetheless, the authors tested the base editing at the A3/A6 and A3/A8, which is not useful for the comparison of base editing windows for cgRNA. There are many sites that have a consecutive multiple A's in the positions 3-6, and thus I recommend that the authors test any alterations in the editing windows in those sites.

Thanks for your helpful suggestion. A recent study has revealed that the most efficient editing window of Cas12f-ABE system was at the positions 3-4 from the PAM sequence, with additional, albeit lower, editing observed at adjacent positions (Fig. R4)¹³. To comprehensively evaluate the performance of the Cas12f-ABE/cgRNA system, we introduced seven additional target loci in Fig. 5, including *FUS*, *RPH3AL*, *LOC105370393*, *DNMT1*, and *INIP*, which each feature two to three adenines within positions 3-6. Analysis of the nine sites indicates that cgRNA introduces less base editing beyond A3-A4, suggesting a more precise base editing tool (Fig. 5c and d).

Figure Redacted

Figure R4: A-T to G-C conversion base editing frequency in HEK293T cells by dCasMINI-ABE¹³. A. The schematic of the nucleotide position is shown on the top: the "R" in TTTR PAM is position "0." The highlighted yellow boxes represent the observed most efficient A-T to G-C conversion positions (positions 3 and 4). The data shown are the number of reads with A-T to G-C conversion at a specific position over the total number of reads for A-T to G-C conversion using deep sequencing. GS1, genomic site 1. Bars represent mean values and data represent three independent biological replicates.

6. The authors only tested gRNA-4.0 for cgRNA-mediated improvements in gene

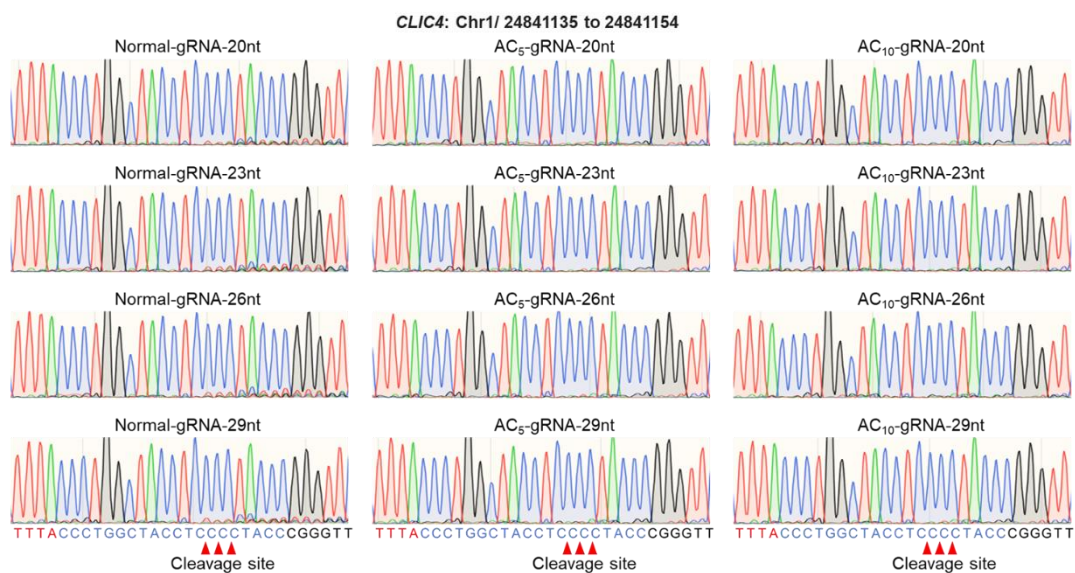
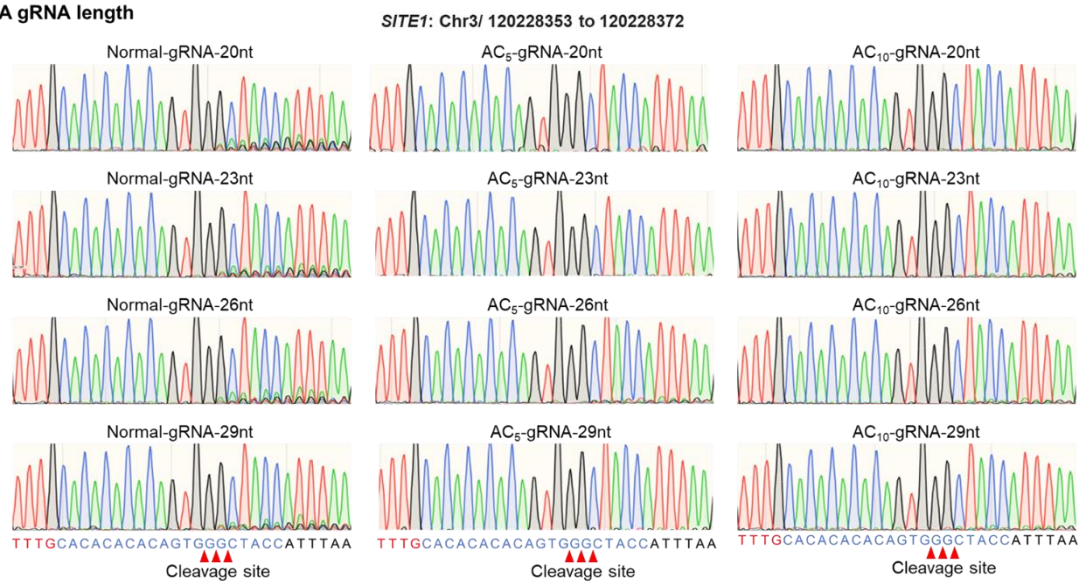
expression and base editing. What's the cgRNA effect for gRNA-4.1? The relevant study would generalize the cgRNA effects on the improvements in gene editing.

Thanks for your helpful suggestion. We have evaluated the cgRNA effect for gRNA-4.1 in gene activation. The result demonstrated that cgRNA-4.1 significantly enhanced activation efficiency compared with Normal and Linear gRNAs-4.1 in both G3 and G5 reporter cell lines (Fig. S11a-c). RT-PCR analysis also showed that cgRNAs could robustly enhance endogenous gene (*HBG* and *HBB*) activation (Fig. S11d).

7. As the authors acknowledged, cgRNA is not functional for indel formation of Cas12f1. I am wondering how hard the authors were attempting to overcome those limitations. Even a longer linker sequence is not effective? A longer and more flexible linker would provide a cleavage opportunity for Cas12f. The failure of indel formation is the very reason that undermines the utility of cgRNA for Cas12f.

Thanks for your helpful suggestion. To ameliorate the cleavage efficiency of Cas12f1/cgRNA, we explored gRNAs of varying lengths, from 20-nt to 29-nt, as well as linker sequences ranging from 0-nt to 120-nt. Despite these modifications, cgRNA was still unable to induce significant indel formation, suggesting that further optimization may be required to achieve effective target cleavage. (Please refer to Fig. S12 and Fig. R5.) We have explained this phenomenon in the maintext as followings: "It has been reported that conformation change after gRNA/Cas12f complex binding to the target site is essential for DNA cleavage ¹⁴, therefore we speculated that cgRNA/Cas12f complex retained the DNA binding capability to activate gene activation but might lose the ability for conformation change and thus failed to cleavage target DNA. " If the structure of the Cas12f1/cgRNA and Cas12f1/cgRNA/target DNA complexes are resolved, especially some dynamic binding states are revealed, it would be possible to perform structure-directed optimization and finally find a way to enhance the DNA cleavage activity via an engineered cgRNA.

A gRNA length



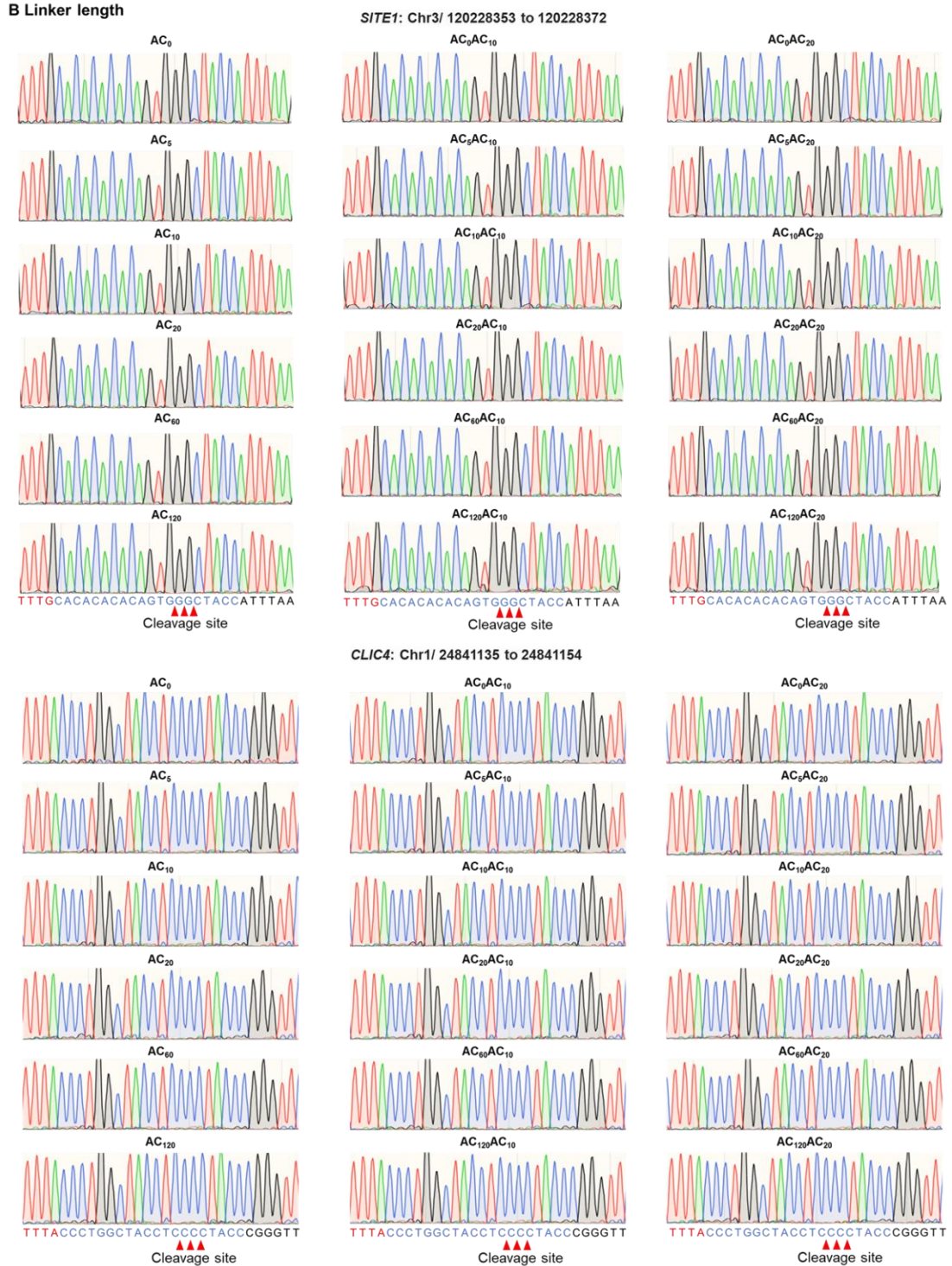


Figure R5: Circular gRNAs failed to guide Cas12f-dependent DNA cleavage. A, Sanger-seq revealed the DNA cleavage activity at the *SITE1* and *CLIC4* target sites in the HEK293T cell post-transfection with gRNA plasmids containing different lengths. **B,** Sanger-seq revealed the DNA cleavage activity in the HEK293T cell post-transfection with gRNA plasmids containing various linkers. The PAM sequences were marked in red, while the spacer sequences were marked in blue.

Minor concerns:

a

1) In Fig. 1d, I don't understand what 0-nt linker 1 and linker 2 refers to. Is a 0-nt linker indeed a linker?

Thanks for your helpful comments. I am very sorry that there is a spelling error. "Fluorescence-activated Cell Sorting (FACS) assay showed that cgRNA, with a 19-nt spacer and 0-nt linker1 and linker2, significantly enhanced activation efficiency compared with Normal and Linear gRNAs in both G3 and G5 reporter cell lines (Fig.1d-e)", "0-nt linker1 and linker2" should be "10-nt linker1 and linker2". The detailed sequences of 10-nt linker1 and linker2 are provided in the table below.

10-nt linker1	AAACAAACAA
10-nt linker2	AAACAAACAA

2) Reverse transcription PCR (RT-PCR) assay with two outward-facing primers confirmed that cgRNA (Circular), not normal gRNA (U6) or linear gRNA (Linear), was circularized in cells (Fig.1b) -> Reverse transcription PCR (RT-PCR) assay with two outward-facing primers confirmed the circularization of cgRNA (Circular), which was not observed for normal gRNA (U6) or linear gRNA (Fig.1b)

Thanks for your helpful suggestion. And we have corrected it in the revised manuscript.

3) Because cgRNA is expressed under U6 promoter, it is confusing to note the normal gRNA as (U6) throughout the manuscript. The authors need to consider the way of clearing the possibility of this confusion.

Thanks for your helpful suggestion. And we have corrected the normal gRNA as (Normal) throughout the revised manuscript.

4) What's Ploy A? is it a PolyA signal?

Thanks for your helpful comments. In this manuscript, poly-AC refers a short adenine- and cytosine-rich RNA sequence, frequently utilized as a flexible RNA linker, which has been shown to enhance RNA function^{15, 16}, such as AC₅, AC₁₀, and is distinct from the polyA

signal. The detailed sequences are shown in the supplementary Table 1.

AC ₅	AAACAA
AC ₁₀	AAACAAACAA

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16. Yi Z, Qu L. Engineered circular ADAR-recruiting RNAs increase the efficiency and fidelity of RNA editing in vitro and in vivo. *Nature biotechnology* **40**, 946-955 (2022).

Responses to reviewers' comments:

Reviewer #1 (Remarks to the Author):

The author has addressed all my concerns; I do not have other questions.

We thank the reviewer for the positive feedback on our manuscript.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, the authors attempted to address the claims raised in the previous one. I evaluate most of the concerns were addressed, but some of the questions remain elusive.

Given the difficulty in analyzing HBB / HBG protein levels in HEK293 cells, why didn't you measure the protein levels in K562 cells? You said that HBB mRNA levels were increased in K562 cells. Without quantifying protein levels, you cannot convince that cgRNA effectively activates the gene transcription.

Thanks for your helpful suggestion. As suggested, we have tried our best to check the protein expression levels of HBB in K562 cells. When we used the Lonza nucleofector kit to achieve high transfection efficiency and puromycin selection to enrich the transfected cells, we detected the protein expression of HBB with Western Blotting (Fig. R1).

Actually, in this manuscript, we have successfully used the dCas12f-VPR/cgRNA system to activate the protein expression of mNeonGreen and luciferase (Fig. 1, 2, & 6). Using flow cytometry, we measured the fluorescence intensity of the reporter gene mNeonGreen and used this reporter system to optimize the dosage, time course, and RNA linker of the dCas12f-VPR/cgRNA gene activator (Fig. 1 & 2). In addition, we employed the dCas12f-VPR/cgRNA system to activate luciferase expression in mice. The results showed that compared to the control group, cgRNA significantly increased the expression and catalytic activity of the luciferase protein (Fig. 6).

In summary, we not only assessed the mRNA expression by qPCR and RNA-seq assays, but also evaluated the corresponding protein levels of targeted endogenous HBB gene with Western Blotting, the fluorescence intensity of targeted reporter mNeonGreen gene with flow cytometry, and the catalytic activity of targeted luciferase gene with bioluminescence assay and quantification. All of the above data fully demonstrated the reliability of the dCas12f-VPR/cgRNA gene activation system. Of course, there are still room to improve the activity and specificity of the dCas12f-VPR/cgRNA system. As a miniature Cas protein to hold great potential for gene therapy, we will keep optimizing the Cas12f-based systems using various approaches, like protein engineering and directed evolution.

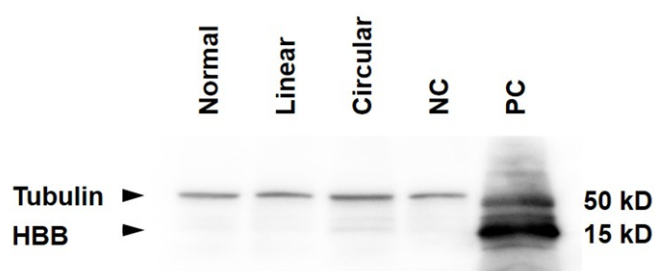


Figure R1: The dCas12f-VPR/cgRNA system activated the protein expression of HBB in K562 cells. Western blotting showed the protein expression levels of HBB gene in K562 cells transiently co-transfected with 3.8 μ g dCas12f-VPR- and indicated 0.2 μ g Normal, linear or circular gRNA-encoded plasmids. NC, a non-targeting gRNA sample transfected with a gRNA plasmid targeting no site in the human genome or transcriptome. PC, a protein sample extracted from human blood cells.

I don't understand if cgRNA is more specific or not, considering the Fig 3 and the rebuttals. cgRNA is responsible for the non-specific activation of non-target genes, thus evoking the issue of non-specificity.

Thanks for your comment. The efficiency and specificity have always been the two key factors in evaluating CRISPR systems. When improving the editing efficiency, the

specificity is generally compromised, leading to the detection of more off-target events^{1, 2,}
³. In this manuscript, we found the same phenomenon.

To more accurately assess the efficiency and specificity the Cas12f/cgRNA system, we utilized RNA-seq to detect the transcriptomic profile. The results showed that cgRNAs increased the activation efficiency of dCas12f-VPR system, resulting in an approximately 18.1-fold and 27.4-fold increase compared to Normal gRNA and Linear gRNA, respectively. However, cgRNAs also led to a slight decrease in specificity. Compared to the NC, Normal, and Linear groups, 23, 15, and 10 more off-target genes were observed to be upregulated in the cgRNA group.

We will further optimize the Cas12f/cgRNA system to improve the specificity with various approaches, like protein engineering and gRNA engineering.

Reference:

1. Kim DY, Lee JM. Efficient CRISPR editing with a hypercompact Cas12f1 and engineered guide RNAs delivered by adeno-associated virus. *Nature biotechnology* **40**, 94-102 (2022).
2. Liu J, Chen Y. CRISPR-assisted transcription activation by phase-separation proteins. *Protein & cell* **14**, 874-887 (2023).
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