The CRISPR Journal
Volume 3, Number 6, 2020
© Mary Ann Liebert, Inc.
DOI: 10.1089/crispr.2020.0073

The CRISPR Journal

RESEARCH ARTICLE

Improving FnCas12a Genome Editing by Exonuclease Fusion

Yongqiang Wu,[1,*] Qichen Yuan,[2,*] Yufeng Zhu,[3] Xiang Gao,[4] Jiabao Song,[5] and Ziru Yin[6]

Abstract

Among current reported Cas12a orthologs, *Francisella novicida* Cas12a (FnCas12a) is less restricted by protospacer adjacent motif (PAM). However, the activity of FnCas12a nuclease is relatively low or undetectable in human cells, limiting its application as desirable genome engineering tools. Here, we describe TEXT (*T*ethering *EX*onuclease *T*5 with FnCas12a)—a fusion strategy that significantly increased the knockout efficiency of FnCas12a in human cells at multiple genomic loci in three different cell lines. TEXT results in higher insertion and deletion efficiency than FnCas12a under different spacer lengths from 18 nt to 23 nt. Deep sequencing shows that TEXT substantially increased the deletion frequency and deletion size at the targeted locus. Compared to other Cas12a orthologs, including AsCas12a and LbCas12a, TEXT achieves the highest on-targeting efficiency and shows minimal off-targeting effects at all tested sites. TEXT enhances the activity of FnCas12a nuclease and expands its targeting scope and efficiency in human cell genome engineering.

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.

Introduction

CRISPR-Cas adaptive immune systems protect bacteria or archaea from viral invasion.[1] Leveraging CRISPR-Cas systems, a variety of genome engineering technologies have been developed, greatly accelerating the study of synthetic biology, gene therapy, diagnostics, plant engineering, and so on.[2–6] The development of alternative CRISPR nucleases other than commonly used *Streptococcus pyogenes* Cas9 (spCas9) would expand the genome engineering toolbox with new and potentially advantageous properties.[7–9] The CRISPR-Cas12a system has been developed for genome editing applications with distinct features, such as recognizing T-rich protospacer adjacent motif (PAM) sequences, cleaving DNA with staggered cut distal to 5′ T-rich PAM, and the ability to process its own crRNA arrays by Cas12a.[9–12] In addition, in contrast to Cas9 that uses crRNA/tracrRNA hybrid for DNA targeting, Cas12a only requires a single short (about 40 nt) CRISPR RNA (crRNA), which is easier to prepare.[9] Cas12a orthologs from *Acidaminococcus* sp. BV3L6 (As), *Lachnospiraceae* bacterium (Lb) have been used for genome engineering in many organisms, including human cells.[9–13] However, the targeting range of AsCas12a and LbCas12a has been hindered by the strict requirement of a TTTV PAM sequence. Although several protein engineering strategies have generated new AsCas12a variants that can target non-canonical PAMs, including TATV, TYCV, TTYN, VTTV, and TRTV, there are still a large number of genomic sites that cannot be accessed by those variants.[14,15] FnCas12a has been reported to use shorter and more frequently occurring PAM sequences (TTN or TTV) *in vitro* and in human cells,[9,16,17] which is less restricted than AsCas12a and LbCas12a, and holds promise for targeting more currently inaccessible genomic regions. However, the nuclease activity of FnCas12a is relatively lower or unobservable in human cells.[16,17] Therefore, strategies that can improve the editing efficiency of FnCas12a will give full play to its advantages of wide targeting ability.

To date, two types of engineering strategies have been used to increase the knockout efficiency of CRISPR-Cas12a, either enhancing the nuclease activity of

[1]*Gene Editing Research Center, Hebei University of Science and Technology, Shijiazhuang, PR China;* [2]*Department of Chemical and Biomolecular Engineering, Rice University, Houston, Texas, USA;* [3]*Institute for Science and Technology Development, Hebei University of Science and Technology, Shijiazhuang, PR China;* [4]*School of Environmental Science and Engineering, Hebei University of Science and Technology, Shijiazhuang, PR China;* [5]*Department of Bioscience and Bioengineering, Hebei University of Science and Technology, Shijiazhuang, China; and* [6]*Periodical Press, Hebei University of Science and Technology, Shijiazhuang, PR China.*

**Address correspondence to: Yongqiang Wu, Gene Editing Research Center, Hebei University of Science and Technology, Shijiazhuang, Hebei 050018, China,* Email: wuyongqiang@hebust.edu.cn; *Qichen Yuan, Department of Chemical and Biomolecular Engineering, Rice University, Houston, TX 77005, USA,* Email: yqc@rice.edu

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.

Cas12a by rational protein mutagenesis or improving the stability of crRNA by optimizing its secondary structures.[18–21] However, neither of those approaches can fundamentally leverage the decisive step of gene knockout, the endogenous non-homologous end joining (NHEJ) DNA repair pathway. Once the DNA double-strand break (DSB) is created at the target locus, the NHEJ pathway will be responsible for ligating the break ends back together. Typically, NHEJ will use short homologous DNA sequences referred to as microhomologies to guide the repair. The microhomologies usually exist in the form of single-strand overhangs at the DSB ends.[22] When the overhangs are perfectly compatible, NHEJ usually repairs the break accurately, without causing insertions and deletions (indels).[23–25] However, the imprecise NHEJ repair, which contributes to the knockout efficiency, can disrupt genes by randomly introducing unwanted indels at the ligation joint, occurring much more commonly when the overhangs are not compatible.[22,23,26–28] FnCas12a has been characterized to generate staggered, perfectly compatible 5′ single-strand overhangs at DSB ends,[9] which is a desired substrate for error-free rather than indel-involved NHEJ repair. Therefore, we reason that by resecting the 5′ single-strand overhang at the DSB ends, the NEHJ pathway can be biased to use the error-prone repair more frequently, thereby improving the overall knockout efficiency of FnCas12a.

Here, to improve the editing efficiency of FnCas12a in human cells, we fused T5 ssDNA exonuclease to N terminal of FnCas12a, namely TEXT (*T*ethering *EX*onuclease *T*5 with FnCas12a), which can direct the cellular NHEJ repair more frequently into the error-prone pathway, enabling higher indel efficiency than when using FnCas12a alone. TEXT exhibits two- to threefold higher genome editing activity than FnCas12a at all tested sites, and also increases the deletion size under different spacer lengths from 18 to 23 nt, in which 18 nt results in the highest fold increase. Notably, when using 18 nt spacer crRNA, the deletion size of TEXT is mainly 8 or 9 nt. In addition, compared to FnCas12a, LbCas12a, and AsCas12a, TEXT showed the highest editing efficiency among all test sites with minimal off-target effects. In conclusion, we developed an improved FnCas12a system that has a wide range of genome editing applications, which will help target previously inaccessible genomic sites.

Methods

Plasmid construction

Polymerase chain reaction (PCR) was performed using Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA), and specific primers were synthesized by Genewiz (Suzhou, PR China). Human codon-optimized exonuclease from phage T5, phage T7, and phage λ were synthesized by Genewiz. The Artemis gene was amplified from complementary DNA (cDNA) of HEK293T cells. Recj and PolA-exo were amplified from *Escherichia coli* genomic DNA. Briefly, the FnCas12a/EXO-FnCas12a driven by the cytomegalovirus promoter and crRNA expression cassette driven by the U6 promoter were constructed into one plasmid base on the backbone of pcDNA3.1(+) plasmid and separated by a spacer sequence. The amino acid sequence of TEXT is shown in Supplementary File S1, and the sequences of crRNA used in this study are listed in Supplementary File S2.

Human cell culture and transfection

HEK293T cells and Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin and streptomycin (Solarbio, Beijing, PR China). HLEB3 cells were cultured in DMEM supplemented with 15% FBS and 1% penicillin and streptomycin. Cells were maintained at 37°C with 5% CO_2. Cells were seeded onto 96-well plates (Corning, Corning, NY) and transfected at approximately 60% confluency. Plasmids (200 ng) were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) following the manufacturer's recommended protocol.

Genomic DNA preparation and PCR amplification

Sixty hours after treatment, cells were washed with PBS genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Epicentre Technologies, Madison, WI) following the manufacturer's protocol. Concentrations of gDNA were determined on a Nanodrop 2000. Genomic regions flanking the target sites were amplified using 200 ng purified gDNA template, Q5 high-fidelity DNA polymerase, and specific primers (Supplementary File S2 and S4) on a T100 thermal cycler (Bio-Rad, Hercules, CA).

T7E1 cleavage assay

PCR fragments were generated using Q5 high-fidelity DNA polymerase, and then hybridized in NEB Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, pH 7.9; New England Biolabs) by heating to 98°C for 3 min, followed by a 2°C/s ramp down to 85°C, 1 min at 85°C, and a 0.1°C/s ramp down to 25°C on a T100 thermal cycler. Subsequently, the annealed samples were digested by T7 Endonuclease I (New England Biolabs) for 30 min and separated by a 2% agarose gel, and quantification was based on relative band intensities. Digitalized images were analyzed to calculate indel

efficiency using the Image J software. Indel percentage was determined by the formula:

$$100 \times [1 - \sqrt{1 - (b + c)/(a + b + c)}]$$

where a represents the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product.

Deep sequencing of Cas12a gene editing results in HEK293T cells

HEK293T cells were transfected and harvested as described for assessing activity of Cas12a cleavage. The genomic-region-flanking DNMT1 targets were amplified using a pair-specific primer with sample-specific barcodes added to the ends of the target amplicons (Supplementary File S3). PCR products were run on 1.6% agarose gel and purified using Spin Column (Sangon Biotech, Shanghai, PR China) as per the manufacturer's recommended protocol. Equal amounts of the PCR products were pooled, and samples were sequenced commercially (Genewiz) by paired-end read sequencing using the Illumina HiSeq X Ten platform.

Off-target analysis for FnCas12a, TEXT, LbCas12a, and AsCas12a

To profile the off-targeting effect of FnCas12a, TEXT, LbCas12a, and AsCas12a, the off-target sites were predicted by CRISPR RGEN Tools (Cas-OFFinder). The fragments containing potential off-target sites were amplified and tested by T7E1 cleavage assay. The sequences of crRNA and off-target sites are listed in Supplementary File S4.

Statistics

Statistical significance was calculated using Mann–Whitney tests using GraphPad Prism v8.4.0 (GraphPad Software, Inc., La Jolla, CA). The error bars in all figures show the standard error of the mean ($n = 3$). p-Values are reported using GraphPad style, where ns indicates not significant, $*p > 0.05$, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

Fusion of FnCas12a and 5′-3′ ssDNA exonuclease can enhance editing efficiency in HEK293T cells

Cas12a generates staggered DSB ends, which can be easily combined by Watson–Crick base pairing and thus will favor the accurate NHEJ repair pathway that does not contribute to the overall editing efficiency (Fig. 1A). To increase the gene editing efficiency of FnCas12a, we assume to bias the DSB repair pathway into imprecise NHEJ by fusing an ssDNA exonuclease with FnCas12a that will degrade the perfectly compatible sticky ends (Fig. 1A). To test our hypothesis, we selected six ssDNA exonuclease candidates, including Artemis from human, RecJ and polA-exo from *Escherichia coli*, and exonuclease from phage T5, phage T7, and phage λ. Each of them was fused to either the N- or C-terminus of FnCas12a as candidate fusions for comparison tests of the editing efficiency (Fig. 1B). T7E1 assay showed that T5 EXO had the most significant effect on improving the indel efficiency of FnCas12a, increasing by 216% at the N-terminus of FnCas12a and 141% at the C-terminus of FnCas12a (Fig. 1C). Although FnCas12a-Artemis and ecRecJ-FnCas12a showed slightly higher indel efficiency than FnCas12a, the rest of the candidates did not improve the editing efficiency of FnCas12a. Considering that N-terminus-fused T5 EXO-FnCas12a exhibited higher indel efficiency and a smaller size than all other candidate fusions, we chose it for subsequent genome editing tests, and hereafter we refer to it as TEXT.

TEXT genome editing at multiple loci of different cell lines

To profile the TEXT system further, we designed FnCas12a crRNAs for targeting three loci—DNMT1, CCR5, and GAPDH—in different cell lines, including HEK293T cells, Hela cells, and human lens epithelial B3 (HLEB3) cells. Compared to FnCas12a at DNMT1 locus, the TEXT system improved gene editing efficiency by up to 172% in Hela cells and 330% in HLEB3 cells (Fig. 2A). At CCR5 locus, TEXT showed two- to three-fold higher editing efficiency than FnCas12a in all three cell lines (Fig. 2B). Notably, at GAPDH locus, FnCas12a only showed around 0.3% editing efficiency in HEK293T cells, and undetectable levels of editing in both Hela and HLEB3 cells, while TEXT dramatically improved gene editing efficiency, with a 10-fold increase in HEK293T cells and with significant editing efficiency in Hela and HLEB3 cells (Fig. 2C). These results indicate that the TEXT system can generally increase gene editing efficiency of FnCas12a in human cells, ranging from 2- to 10-fold higher efficiency in our tests, and can significantly edit locus that previously could not be edited by FnCas12a.

Varying spacer length of crRNA to optimize editing efficiency of TEXT

The spacer region of crRNA uses Watson–Crick base paring to locate the Cas12a–crRNA complex or TEXT system at specific genomic loci. It has been reported that by optimizing the spacer length of crRNA ranging from 18 to 23 nt, the cutting efficiency of CRISPR-Cas12a can be increased. Typically, 21 nt can achieve

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.

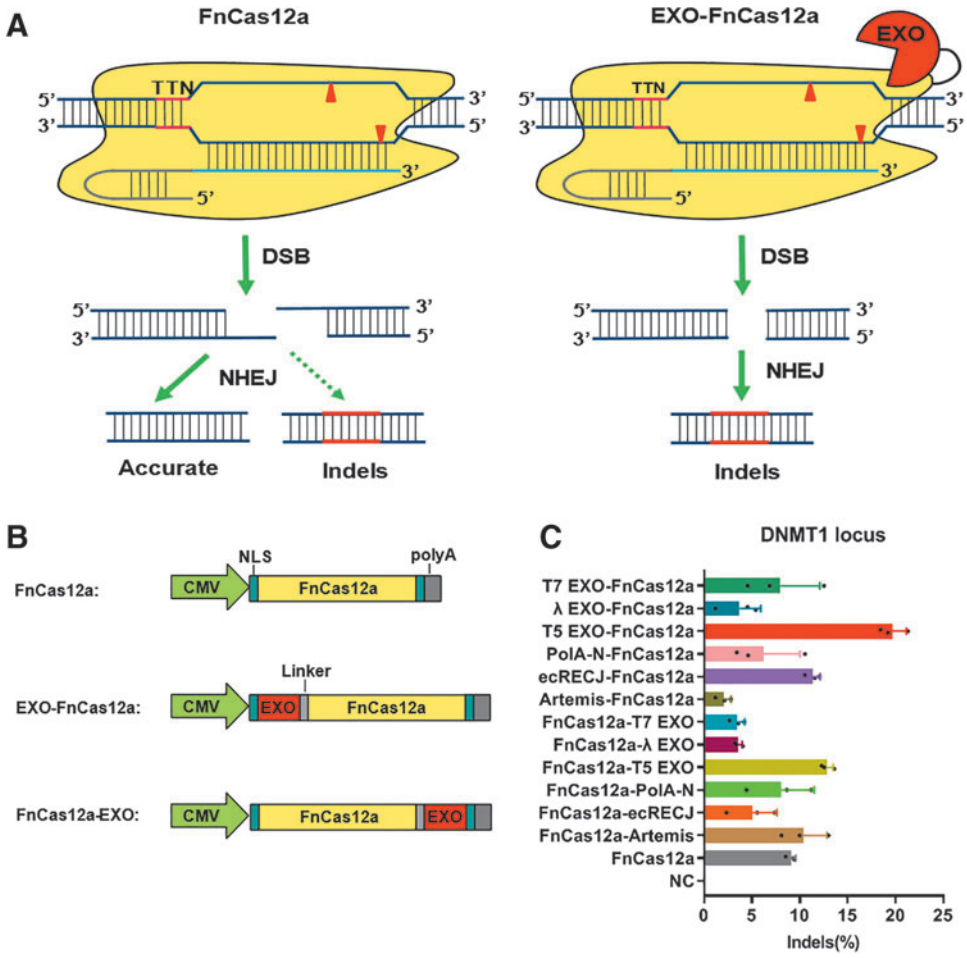


FIG. 1. FnCas12a, EXO-FnCas12a, and FnCas12a-EXO mediated gene editing in HEK293T cells. **(A)** Illustration of the FnCas12a and EXO-FnCas12a/FnCas12a-EXO system and corresponding DNA repair pathways. FnCas12a generates fully compatible sticky ends, which are mainly repaired by the precise non-homologous end joining (NHEJ) pathway, and thus will not contribute to insertion and deletion (indel) efficiency. Exonuclease fused FnCas12a can degrade the single-strand DNA overhang after cleavage, and could bias the cellular repair pathway mainly into imprecise NHEJ, which will result in the enhancement of indel efficiency. **(B)** Schematic illustration of the FnCas12a/EXO-FnCas12a/FnCas12a-EXO expression cassette. **(C)** Relative indel frequencies induced by FnCas12a, EXO-FnCas12a, and FnCas12a-EXO. Gene editing efficiency was analyzed using T7E1 assay. (Gel images are shown in Supplementary Figure S1.) Color images are available online.

higher editing efficiency than other lengths when using FnCas12a.[16] In a similar fashion, in order to investigate the optimal spacer length of crRNA for TEXT-mediated gene editing in human cells, we constructed a series of crRNAs with different spacer lengths ranging from 18 to 23 nt, and we tested the gene editing efficiency of TEXT at DNMT1 locus. Consistent with a previous report,[16] we found that crRNA with a 20–21 nt spacer length can enable higher gene editing efficiency when using FnCas12a (Fig. 3B), while the TEXT system requires a 21–23 nt spacer length to achieve maximum cutting efficiency (Fig. 3B). In addition, it is worth noting that when the spacer length of crRNA was 18 nt, the editing efficiency of FnCas12a sharply decreased to 1.2%. However, the TEXT system retained more than 10% editing efficiency. Overall, these results confirmed that the TEXT system is compatible with a wide range of guide lengths.

TEXT improves gene editing under different lengths of crRNA

Previously, it was commonly believed that Cas12a would cut DNA at the 18th base downstream of the PAM site on the non-target strand and the 23rd base on the target strand.[9] However, recently, it has been reported that

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.

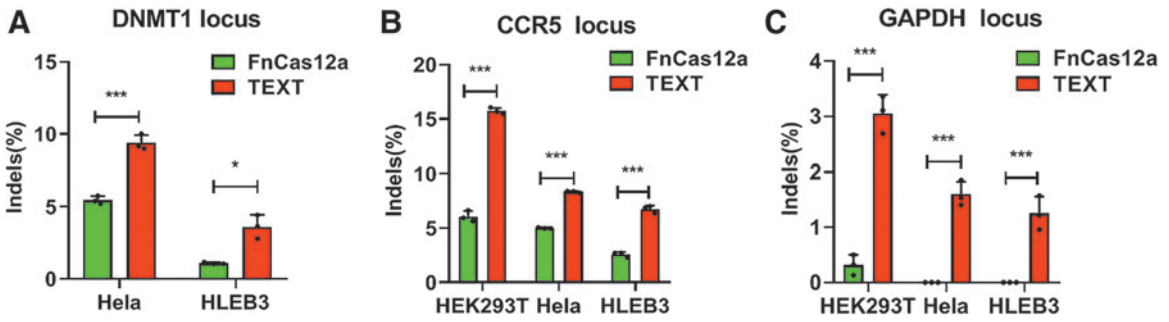


FIG. 2. Comparison of gene editing efficiency of the FnCas12a system and the TEXT system in HEK293T, Hela, and HLEB3 cell lines. **(A)** Gene editing efficiency of the FnCas12a system and the TEXT system for the human *DNMT1* gene in Hela and HLEB3 cells. **(B)** Gene editing efficiency of the FnCas12a system and the TEXT system for the human *CCR5* gene in HEK293T, Hela, and HLEB3 cells. **(C)** Gene editing efficiency of the FnCas12a system and the TEXT system for the human *GAPDH* gene in HEK293T, Hela, and HLEB3 cells. Indel percentage at each locus was determined using the T7E1 assay and is expressed as the mean ± standard deviation from three biological replicates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; two-tailed *t*-test). (Gel images are shown in Supplementary Figures S2–S4). Color images are available online.

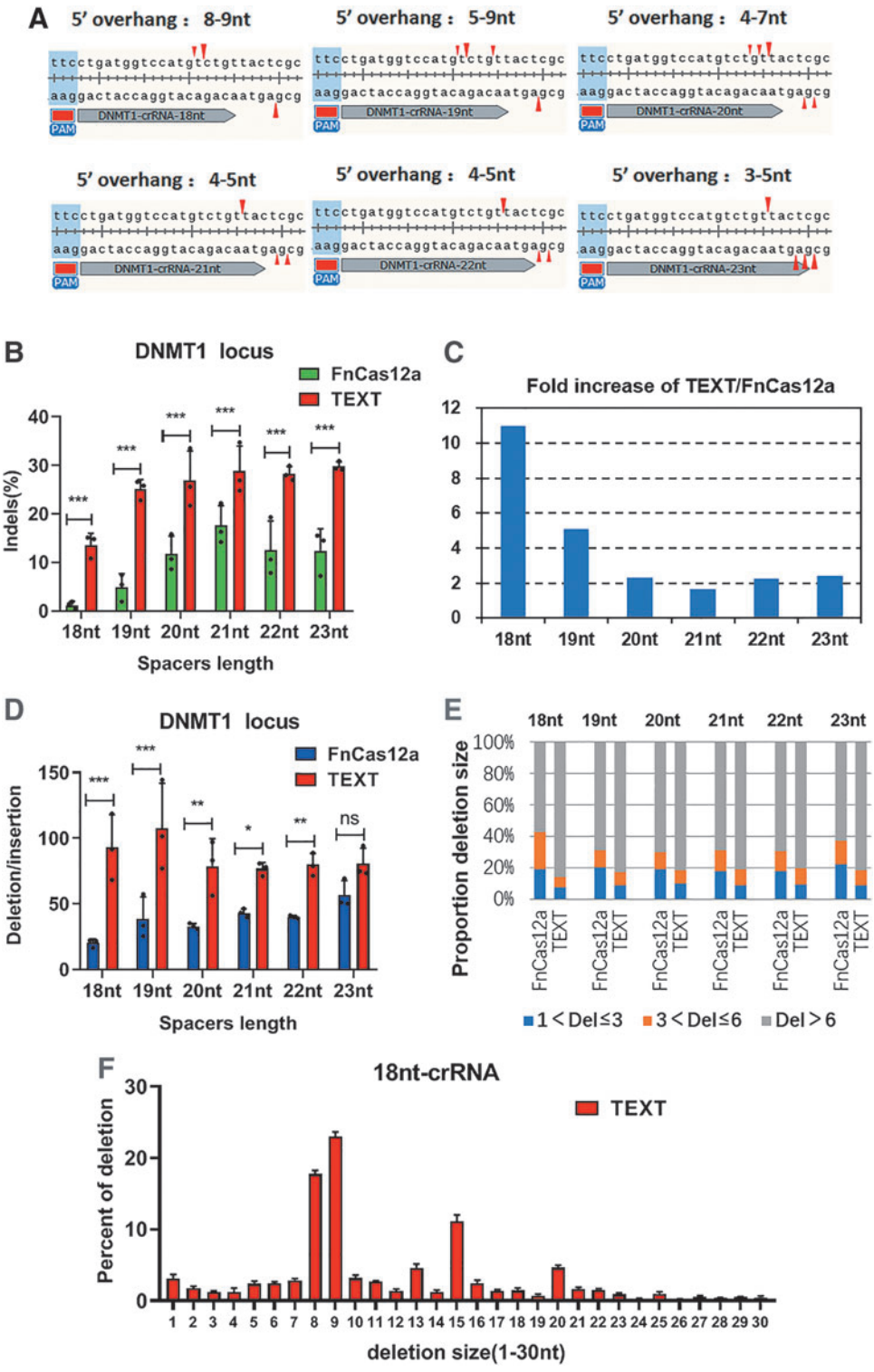
FnCas12a cleavage sites are located after the 13th, 14th, 18th, and 19th bases on the non-target strand and from the 21st to 24th bases on the target strand,[29] which supports the scheme that FnCas12a has a different cleavage pattern when using different spacer lengths of crRNA (Fig. 3A). When the spacer length of crRNA is 18 nt, which typically directs Cas12a to generate 8–9 nt sticky end, the gene editing efficiency of the TEXT system is 11 times higher than FnCas12a (Fig. 3A and C). When the spacer length is 19 nt, which directs Cas12a to generate a 5–9 nt sticky end, TEXT can increase the editing efficiency of FnCas12a by five times (Fig. 3A and C). When the spacer length is 20–23 bp, with 3–5 nt sticky end, the gene editing efficiency of TEXT is around two-fold higher than FnCas12a (Fig. 3A and C).

Deep sequencing analysis of TEXT indel patterns

Furthermore, we analyzed the indel pattern of TEXT and FnCas12a with different spacer lengths of crRNA by deep sequencing (Fig. 3D–F). In general, TEXT induced

FIG. 3. Different spacer lengths of crRNA result in distinct cleavage pattern and gene editing efficiency. **(A)** Cleavage pattern of FnCas12a under different crRNA spacer lengths (18–23 nt). With a 18 nt spacer (crRNA-18 nt), FnCas12a cleavage sites were mainly after the 13th and 14th bases on the non-target strand and from the 22nd bases on the target strand. With a 19 nt spacer, FnCas12a cleavage sites were mainly after the 14th, 13th, and 17th bases on the non-target strand and from the 22nd bases on the target strand. With a 20 nt spacer, FnCas12a cleavage sites were mainly after the 18th, 17th, and 16th bases on the non-target strand and from the 18th and 19th bases on the target strand. With a 21–22 nt spacer, FnCas12a cleavage sites were mainly after the 18th bases on the non-target strand and from the 21st and 22nd bases on the target strand. With a 23 nt spacer, FnCas12a cleavage sites were mainly after the 18th bases on the non-target strand and from the 20th, 21st, and 22nd bases on the target strand. The cleavage sites are indicated by red arrows. **(B)** Gene editing efficiency of the FnCas12a system and the TEXT system for the human DNMT1 locus with different spacer lengths of crRNA in HEK293T cells. Gel images are shown in Supplementary Figure S5. **(C)** Editing efficiency fold increase of the TEXT system compared to FnCas12a at different spacer lengths. The ratio was determined by the formula *A*/*B*, where *A* and *B* represent the gene editing efficiency of TEXT and FnCas12a, respectively. **(D)** The relative ratio of deletions to insertions induced by FnCas12a or the TEXT system with 18–23 nt spacer lengths of crRNA. **(E)** The relative proportions of different size deletions among total deletions that were induced by FnCas12a or the TEXT system with 18–23 nt spacer lengths of crRNA. **(F)** Ratio of deletions size (1–30 nt) induced by the TEXT system with a 18 nt spacer length of crRNA. (Gel images are shown in Supplementary Figure S5. Ratio of deletions size (1–30 nt) induced by FnCas12a and the TEXT system with other spacer lengths of crRNA are shown in Supplementary Figures S6 and S7). Color images are available online.

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.



Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.

a higher indel efficiency than FnCas12a (Fig. 3B and C). In addition, the ratio of deletion frequency to insertion frequency (del/in) is significantly higher in TEXT than it is in FnCas12a (Fig. 3D). When using 18 nt spacer crRNA, TEXT increased the del/in of FnCas12a from 20.17 to 92.86, while it only increased from 56.78 to 80.84 when using the 23 nt spacer crRNA (Fig. 3D). Then, we measured the lengths of the deletions generated by TEXT and FnCas12a. When using FnCas12a, under different crRNA spacer lengths from 18 to 23 nt, the proportion of deletion sizes <3 bp and <6 bp are about 20% and 30–40%, respectively, while when using TEXT, the deletion sizes that are <3 bp and <6 bp account for 10% and 20%, respectively (Fig. 3E). It is worth noting that when the spacer length is 18 nt, the deletion size that is <6 bp is 42.6% by FnCas12a and 14.3% by TEXT (Fig. 3E). In addition, we analyzed the deletion pattern and found that the proportion of 8–9 nt deletion of TEXT is up to 41.2% with a 18 nt spacer length crRNA, while that of FnCas12a is 9.8% (Fig. 3F). Next, to understand further the role of T5-EXO in TEXT editing, we analyzed next-generation sequencing data to generate detailed indels pattern of TEXT, in which most of the deletion alleles do not contain the sequence of the 5′ overhangs (Supplementary File S5). The results further confirm that the TEXT system improves the gene editing efficiency of FnCas12a by resecting the 5′ single-strand overhang at the DSB ends. Collectively, the TEXT system substantially increased the deletion frequency and deletion size at the targeted locus.

TEXT performs efficient on-target editing with minimal off-target effects

Further characterization of the TEXT system showed substantial editing activities in human cells. We compared the editing efficiency of FnCas12a, TEXT, LbCas12a,

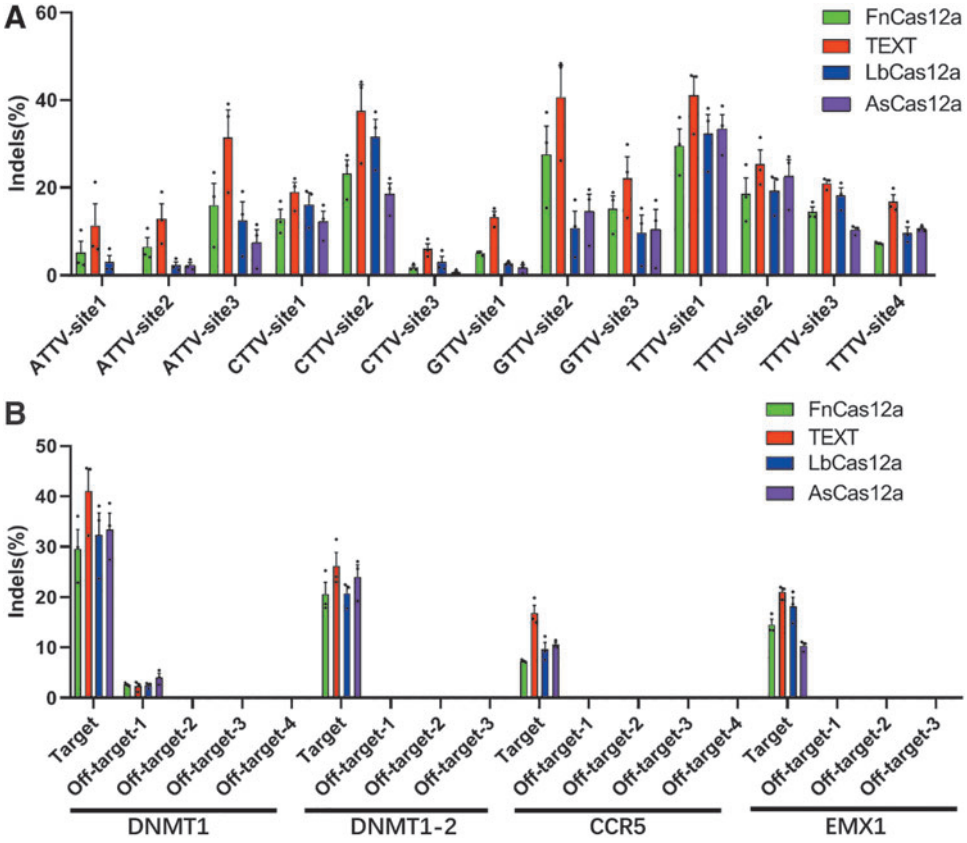


FIG. 4. Comparison of editing efficiencies and off-target effects between TEXT and Cas12a orthologs over a diverse range of endogenous targets. **(A)** Comparison of indel efficiency of TEXT, FnCas12a, LbCas12a, and AsCas12a at various protospacer adjacent motif (PAM) sites in HEK293T cells. Four types of PAM (ATTV, CTTV, GTTV, and TTTV) were analyzed, in which three to four different sites were selected to represent each type of PAM. Indel efficiency was detected by T7E1 assay. **(B)** Off-target profile of TEXT, FnCas12a, LbCas12a, and AsCas12a at selected loci in HEK293T cells. (Off-target sites and their amplification primers are listed in the Supplementary File S4. Gel images are shown in Supplementary Figures S8–S15). Color images are available online.

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.

and AsCas12a, with NTTV PAMs in human cells. In all cases, we observed higher editing efficiency achieved by TEXT than by FnCas12, LbCas12a, and AsCas12a (Fig. 4A). In particular, TEXT showed robust editing activities on multiple endogenous target sites with ATTV and GTTV PAMs compared to AsCas12a and LbCas12a (Fig. 4A). These results confirm that TEXT recognizes TTV PAM, which will help target genomic sites that are not accessible to LbCas12a and AsCas12a. Next, we determined the off-target effects of TEXT compared to FnCas12a, LbCas12a, and AsCas12a. We used CRISPR RGEN prediction tools to generate a set of potential off-target sites and found that only one off-target site had detectable efficiency by TEXT and other Cas12a orthologs, while most predicted off-target sites showed undetectable off-target effects (Fig. 4B). The results indicated that the TEXT system does not significantly increase off-target effects when enhancing on-target editing efficiency.

Discussion

Currently, AsCas12a and LbCas12a are more widely used for genome editing than FnCas12a. Although FnCas12a has shown fewer PAM limitations, the low targeting activity of FnCas12a limits its application as a genome editing tool. We report a method that can significantly improve the editing efficiency of FnCas12a by leveraging the endogenous DNA repair pathway to favor the imprecise NHEJ repair that mainly generates indels. We also explored whether our strategy could increase the editing efficiency of LbCas12a and AsCas12a. Unexpectedly, no improvement was observed on those Cas12a variants. A recent report shows that AsCas12a not only generates sticky end, but will also degrade several bases along the non-targeting ssDNA strand,[30] and the structure of LbCas12a and AsCas12a are very similar, which indicates that the nuclease activities of AsCas12a and LbCas12a are high enough to approximate the saturation state of their indel efficiency. Therefore, it is hard to be further improved by tethering T5 exonuclease.

The crRNA of Cas12a consists of a spacer sequence and direct repeat sequence that has a huge impact on the editing efficiency of the CRISPR-Cas12a system.[9,21] *In vitro* experiments have confirmed that different spacer lengths of crRNA can result in different cutting patterns of FnCas12a.[29] We demonstrated that TEXT exclusively improved the gene editing efficiency of FnCas12a when using crRNAs of different spacer lengths (Fig. 3C).

TEXT expands the genome editing activity and scope of FnCas12a with low off-target effects and is superior to AsCas12a and LbCas12a at the tested loci. Our study provides insights into CRISPR-Cas12a systems and provides a new method for maximizing the genome editing efficiency of FnCas12a, enabling it as a promising human genome editing tool for broader research applications. It is worth noting that although no significant cytotoxicity was observed after transfection of the TEXT system, it remains unknown whether exonuclease from phage T5 has detectable off-target effect on naturally occurring DNA damage response in mammalian cells. To understand and improve the security of TEXT further, whole genome sequencing pipelines need to be developed to profile the effect of TEXT comprehensively on naturally occurring DNA lesions. Protein engineering strategies such as rational structural engineering[31] or inducible split protein[32] can be applied to enhance the precision and safety of the TEXT system.

Conclusion

In this study, to improve the editing efficiency of FnCas12a in human cells, we fused T5 ssDNA exonuclease to N terminal of FnCas12a, namely TEXT, which can direct the cellular NHEJ repair more frequently into the error-prone pathway, enabling higher indel efficiency than when using FnCas12a alone. TEXT increases the deletion size under different spacer lengths from 18 to 23 nt, with 18 nt resulting in the highest fold increase. Notably, TEXT shows higher gene editing efficiency than FnCas12a, LbCas12a, and AsCas12a at human genomic loci with NTTV PAMs. In conclusion, we developed an improved FnCas12a system with expanded targeting scope and increased editing efficiency for human cell genome engineering.

Acknowledgments

We thank the facility support of Gene Editing Research Center, Hebei University of Science and Technology. Y. W. and Q.Y. conceived the project, designed all experiments. Y.W., Y.Z., J.S., X.G. and Z.Y. performed the experiments. Y.W. and Q.Y. analyzed the data. Y.W. and Q.Y. wrote the manuscript with help from all authors. Data sets from deep sequencing experiments have been deposited with the National Center for Biotechnology Information Sequence Read Archive under BioProject ID: PRJNA642811.

Author Disclosure Statement

Y.W. and Q.Y. are the inventors on a patent application of this work with aim of ensuring this technology can be used freely and widely.

Funding Information

This work was supported by the Youth Fund of Science and Technology Research Program for Colleges and Universities in Hebei Province (QN2020262).

Supplementary Material

Supplementary File S1
Supplementary File S2
Supplementary File S3
Supplementary File S4
Supplementary File S5
Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Figure S6
Supplementary Figure S7
Supplementary Figure S8
Supplementary Figure S9
Supplementary Figure S10
Supplementary Figure S11
Supplementary Figure S12
Supplementary Figure S13
Supplementary Figure S14
Supplementary Figure S15

References

1. Carter J and Wiedenheft B. CRISPR-RNA-guided adaptive immune systems. *Cell* 2015;163:260–260. DOI: 10.1016/j.cell.2015.09.011.
2. Doudna JA. The promise and challenge of therapeutic genome editing. *Nature* 2020;578:229–236. DOI: 10.1038/s41586-020-1978-5.
3. Yin K, Gao C, Qiu JL. Progress and prospects in plant genome editing. *Nat Plants* 2017;3:17107. DOI: 10.1038/nplants.2017.107.
4. Yin H, Kauffman K J, Anderson D G. Delivery technologies for genome editing. *Nat Rev Drug Discov* 2017;16:387–399. DOI: 10.1038/nrd.2016.280.
5. Wang H, La Russa M, Qi LS. CRISPR/Cas9 in genome editing and beyond. *Annu Rev Biochem* 2016;85:227–264. DOI: 10.1146/annurev-biochem-060815-014607.
6. Jacinto FV, Link W, Ferreira BI. CRISPR/Cas9-mediated genome editing: from basic research to translational medicine. *J Cell Mol Med* 2020;24:3766–3778. DOI: 10.1111/jcmm.14916.
7. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2020;339:819–823. DOI: 10.1126/science.1231143.
8. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science* 2013;339:823–826. DOI: 10.1126/science.1232033.
9. Zetsche B, Gootenberg JS, Abudayyeh OO, et al. Cpf1 is a single RNA guided endonuclease of a class 2 CRISPR Cas system. *Cell* 2015;163:759–771. DOI: 10.1016/j.cell.2015.09.038.
10. Kim D, Kim J, Hur JK, et al. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. *Nat Biotechnol* 2016;34:863–868. DOI: 10.1038/nbt.3609.
11. Mali P, Yang L, Esvelt KM, et al. Genome-wide specificities of CRISPR–Cas Cpf1 nucleases in human cells. *Nat Biotechnol* 2016;34:869–874. DOI: 10.1038/nbt.3620.
12. Fonfara I, Richter H, Bratovič M, et al. The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature* 2016;532:517–521. DOI: 10.1038/nature17945.
13. Zetsche B, Heidenreich M, Mohanraju P, et al. Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nat Biotechnol* 2017;35:31–34. DOI: 10.1038/nbt.3737.
14. Gao L, Cox DBT, Yan WX, et al. Engineered Cpf1 variants with altered PAM specificities. *Nat Biotechnol* 2017;35:789–792. DOI: 10.1038/nbt.3900.
15. Kleinstiver BP, Sousa AA, Walton RT, et al. Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. *Nat Biotechnol* 2019;37:276–282. DOI: 10.1038/s41587-018-0011-0.
16. Tu M, Lin L, Cheng Y, et al. A "new lease of life": FnCpf1 possesses DNA cleavage activity for genome editing in human cells. *Nucleic Acids Res* 2017;45:11295–11304. DOI: 10.1093/nar/gkx783.
17. Tóth E, Czene BC, Kulcsár PI, et al. Mb- and FnCpf1 nucleases are active in mammalian cells: activities and PAM preferences of four wild-type Cpf1 nucleases and of their altered PAM specificity variants. *Nucleic Acids Res* 2018;46:10272–10285. DOI: 10.1093/nar/gky815.
18. Bin Moon S, Lee JM, Kang JG, et al. Highly efficient genome editing by CRISPR-Cpf1 using CRISPR RNA with a uridinylate-rich 3′-overhang. *Nat Commun* 2018;9:3651. DOI: 10.1038/s41467-018-06129-w.
19. Park HM, Liu H, Wu J, et al. Extension of the crRNA enhances Cpf1 gene editing *in vitro* and *in vivo*. *Nat Commun* 2018;9:3313. DOI: 10.1038/s41467-018-05641-3.
20. Li B, Zhao W, Luo X, et al. Engineering CRISPR-Cpf1 crRNAs and mRNAs to maximize genome editing efficiency. *Nat Biomed Eng* 2017;1:0066. DOI: 10.1038/s41551-017-0066.
21. Lin L, He X, Zhao T, et al. Engineering the direct repeat sequence of crRNA for optimization of FnCpf1-mediated genome editing in human cells. *Mol Ther* 2018;26:2650–2657. DOI: 10.1016/j.ymthe.2018.08.021.
22. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 2010;79:181–211. DOI: 10.1146/annurev.biochem.052308.093131.
23. Moore JK, Haber JE. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1996;16:2164–2173. DOI: 10.1128/mcb.16.5.2164.
24. Budman J, Chu G. Processing of DNA for nonhomologous end-joining by cell-free extract. *EMBO J* 2010;24:849–860. DOI: 10.1038/sj.emboj.7600563.
25. Gu J, Lu H, Tippin B, et al. XRCC4: DNA ligase IV can ligate incompatible DNA ends and can ligate across gaps. *EMBO J* 2007;26:1010–1023. DOI: 10.1038/sj.emboj.7601559.
26. Bétermier M, Bertrand P, Lopez BS. Is non-homologous end-joining really an inherently error-prone process? *PLoS Genet* 2014;10:e1004086. DOI: 10.1371/journal.pgen.1004086.
27. Löbrich M, Jeggo P. A process of resection-dependent nonhomologous end joining involving the goddess Artemis. *Trends Biochem Sci* 2017;42:690–701. DOI: 10.1016/j.tibs.2017.06.011.
28. Chang HH, Lieber MR. Structure-specific nuclease activities of Artemis and the Artemis:DNA-PKcs complex. *Nucleic Acids Res* 2016;44:4991–4997. DOI: 10.1093/nar/gkw456.
29. Lei C, Li SY, Liu JK, et al. The CCTL (Cpf1-assisted Cutting and Taq DNA Ligase-Assisted Ligation) method for efficient editing of large DNA constructs *in vitro*. *Nucleic Acids Res* 2017;45:e74. DOI: 10.1093/nar/gkx018.
30. Cofsky JC, Karandur D, Huang CJ, et al. CRISPR-Cas12a exploits R-loop asymmetry to form double-strand breaks. *Elife* 2020;9:e55143. DOI: 10.7554/eLife.55143.
31. Doman JL, Raguram A, Newby GA, et al. Evaluation and minimization of Cas9-independent off-target DNA editing by cytosine base editors. *Nat Biotechnol* 2020;38:620–628. DOI: 10.1038/s41587-020-0414-6.
32. Zetsche B, Volz S, Zhang F. A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nat Biotechnol* 2015;33:139–142. DOI: 10.1038/nbt.3149.

Downloaded by 141.105.67.194 from www.liebertpub.com at 10/06/21. For personal use only.