Improving FnCas12a genome editing by exonuclease fusion

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Abstract

Among current reported Cas12a orthologs, Francisella novicida Cas12a (FnCas12a) is less restricted by protospacer adjacent motif (PAM), which would help to target previously inaccessible genomic sites. However, the activity of FnCas12a nuclease is relatively low or unobservable in human cells, limiting its application as desirable genome engineering tools. Here, we describe TEXT (Tethering EXonuclease T5 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 shows higher indels (insertions and deletions) efficiency than FnCas12a using different spacer lengths from 18nt to 23nt, in which 18nt results in highest fold increase, with up to 11 folds higher efficiency than FnCas12a. Deep sequencing shows that TEXT substantially increased the deletion frequency and size at target locus. Collectively, TEXT enhances the activity of FnCas12a nuclease and expand its application in human cell genome engineering.

Introduction

CRISPR (clustered regularly interspaced short palindromic repeats)/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 et al (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). CRISPR-Cas12a system has been developed for genome editing applications with distinct features, enabling recognizing of T-rich PAM sequences, cleaving DNA with staggered cut distal to 5' T-rich PAM, and processing of its own crRNA arrays by Cas12a nuclease (9-12). In addition, Cas12a only requires a single short (about 40 nucleotide) CRISPR RNA (crRNA) for its targeting, which also simplifies the preparation of its constructs (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 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, TRTV, there are still a large number of genomic sites that cannot be accessed by Cas12a (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 would also expand its targeting scope in genome editing applications.

To date, two types of engineering strategies have been used to increase the knockout efficiency of CRISPR–Cas12a, either enhancing the nuclease activity of Cas12a by rational protein mutagenesis or improving the stability of crRNA by optimizing its secondary structures (18-21). However, none of those approaches can fundamentally leverage the decisive step of gene knockout, the endogenous NHEJ (non-homologous end-joining) DNA repair pathway. Once the DNA double strand break (DSB) is created at target locus, 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-stranded overhangs at the DSB ends (25). When the overhangs are perfectly compatible, NHEJ usually repairs the break accurately (22-24). Imprecise NHEJ repair, which contribute 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, 25-28). 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 DSB ends, the NEHJ pathway can be biased to use the error-prone repair, thereby improving the overall knockout efficiency.

Here, to direct the cellular NHEJ repair more frequently into error-prone pathway, we fused a series of 5’ to 3’ ssDNA exonuclease to different ends of FnCas12a for testing, and demonstrate TEXT system, in which T5 exonuclease is tethered to N terminal of FnCas12a, enabling higher indel efficiency than FnCas12a alone and all other fusions. Compared to FnCas12a, TEXT exhibits 2 to 3 folds higher genome editing activity at all tested sites, also increases the deletion size under different spacer lengths from 18nt to 23nt, in which 18nt results in highest fold increase. Notably, when using 18nt spacer crRNA, the deletion size of TEXT is mainly 8nt or 9nt. In conclusion, we developed an improved FnCas12a system that has wide range of genome editing applications.

Results

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

Since Cas12a generates a staggered DSB, which can be combined based on complementary base pairs and easy to be accurately repaired (Figure 1a). We assume that fusion a ssDNA exonuclease to FnCas12a might degrade the sticky end in time and increase the editing efficiency (Figure 1a). To test our hypothesis, six ssDNA exonuclease , including Artemis from human, Recj and polA-exo from Escherichia coli, exonuclease from phage T5, phage T7 and phage λ, were cloned or synthesized and fused to the N or C terminus of FnCas12a (Figure 1b). T7E1 assay demonstrated that two exonuclease –RecJ and T5 EXO can enhance the gene editing efficiency of FnCas12a (Figure 1c, Supplementary Figures S1). Considering that T5 EXO is smaller, and the improved efficiency of gene editing is higher, we choose T5 EXO for subsequent test. Although the editing efficiency has been improved obviously after the fusion of FnCas12a N-terminal and C- terminal with T5 EXO (141% and 216% increase, respectively; Figure 1c), the potency of fusion in N- terminal is better. Thus, we create the TEXT

system by tethering T5-exonuclears to the N terminal of FnCas12a (Figure 1b).

Applicability of TEXT at multiple loci and in many cell lines

In order to study the general applicability of TEXT system, we test DNMT1, CCR5 and GAPDH loci in HEK293T cells, Hela cells and human lens epithelial B3 (HLEB3) cells, respectively. For the DNMT1 locus, the TEXT system can improve gene-editing efficiency up to 172% in Hela cells and up to 330% in HLEB3 cells compared to FnCas12a (Figure 2a). For the CCR5 locus, the trend is consistent with that for the DNMT1 locus (Figure 2b). Notably, for the GAPDH locus which could be hard to edit, the TEXT system improved gene editing efficiency by about 10-fold in HEK293T cells (Figure 2c). And for FnCas12a, GAPDH locus cannot be detected after editing in Hela and HLEB3 cells. While TEXT system can edit GAPDH locus in Hela and HLEB3 cells obviously (Figure 2c). These results suggest that for general target sites, the TEXT system can improve gene editing efficiency about twice as much as FnCas12a. And the TEXT system has more significant advantages for the low editing efficiency site either.

Maximizing efficiency by regulate the spacer length of crRNA

crRNA is another important part of the CRISPR/Cas12a genome-editing system or TEXT system. It has been reported that optimizing the spacer length of crRNA could improve CRISPR–Cas12a cutting efficiency and found that crRNA with a 21nt, but not 23nt spacer sequence which is commonly used as spacer sequence for Cas12a, achieved the highest efficiency (16). Following a similar fashion, we try to investigate the optimal spacer length of crRNA for TEXT-mediated gene editing in human cells based on the DNMT1 locus. We constructed a series of crRNAs with different spacer sequence lengths (18nt-23nt) and tested its gene editing efficiency. Similarity, we found that crRNA with 20-21nt achieved a higher gene editing efficiency for FnCas12a system (Figure 3b). While we show that TEXT system with spacer length of 21-23nt could achieve maximum cutting efficiency (Figure 3b).

TEXT system increases FnCas12 editing efficiency at different crRNA spacer lengths

At first, it was generally believed that Cas12a cleave after 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 FnCas12a cleavage sites were 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). It showed that the FnCas12a has a different cleavage pattern with different length of protospacer (29, Figure 3a). When the spacer length of crRNA is 18nt, which typically direct Cas12a to generate 8-9nt sticky end, the gene editing efficiency of TEXT system is 11 times higher than FnCas12a (Figure 3a, 3c). When the spacer length is 19nt, which direct Cas12a to generate a 5-9nt sticky end, TEXT increases the editing efficiency of FnCas12a by 5 times. (Figure 3a, 3c). When the spacer length is 20-23bp, with 3-5nt sticky end, the gene editing efficiency of TEXT is around two-fold higher than FnCas12a (Figure 3a, 3c).

TEXT system has different indel patterns with different spacer length of crRNA

Furthermore, we analyzed the indel pattern of TEXT and FnCas12a by deep sequencing. In general, T5 EXO-FnCas12a induced a higher frequency of deletions than FnCas12a (Figure 3d). By using the 18nt spacer crRNA, the ratio of deletion to insertion increased the most, from 20.17 to 92.86, while when using the 23nt spacer crRNA, the increase was the smallest, from 56.78 to 80.84. (Figure 3d). Then we measured the lengths of deletions generated by T5 EXO-FnCas12a and FnCas12a with different spacer length of crRNA. When using FnCas12a, under different crRNA spacer length from 18nt to 23nt, the proportion of deletion size less than 3bp

and less than 6bp are about 20% and 30% - 40%, respectively; while using T5 EXO-FnCas12a, the deletions size that less than 3bp and less than 6bp account for 10% and 20%, respectively (Figure 3e). It is worth noting that when the spacer length is 18nt, the deletions size that less than 6 bp is 42.6% by FnCas12a and 14.3% by T5 EXO-FnCas12a (Figure 3e). In addition, we analyzed the deletion pattern and found that the proportion of 8-9nt deletion of EXO-FnCas12a is up to 41.2% with 18nt spacer length crRNA, while that of FnCas12a is 9.8%. (Figure 3f)

Discussion

FnCas12a has less PAM limitation and is a flexible gene editing tool. However, AsCas12a and LbCas12a but not FnCas12a are more widely used. Although a later research reported that FnCas12a possesses DNA cleavage activity for genome editing in human cells and explain the inconsistency of FnCas12a (16). The adoption rate of fnCas12a is still very low compared with AsCas12a and LbCas12a. This is probably caused by the low editing efficiency of FnCas12a. This is the first study to report a method that can significantly improve the editing efficiency of FnCas12a based on the theory of endogenous DNA repair pathway. In addition, we also explored whether this method is suitable for LbCas12a and AsCas12a, and unexpectedly, this system does not work well for others Cas12a (data not shown). A recent report shows that AsCas12a does not create the conventional viscous end, which will degrade several bases on non-target strand (30). As the structure of LbCas12a and AsCas12a is very similar, which means that LbCas12a may also exist this phenomenon. This may explain why the T5 EXO is not work for LbCas12a and AsCas12a.

CrRNA that consists of spacer sequence and direct repeat sequence that has a great impact on the editing efficiency of CRISPR / Cas12a system (9, 21). In vitro experiments have confirmed that FnCas12a with different spacer lengths of crRNA have different cutting patterns (29). When using crRNAs of different spacer lengths, TEXT improves the gene editing efficiency of FnCas12a at different levels (Figure 3c).

In summary, we create the TEXT system which has higher gene editing efficiency than FnCas12a. Our study provides new insights into CRISPR–Cas12a systems and provide a new method for maximizing genome editing efficiency evidence of FnCas12a, which can act as an effective human genome editing tool for research applications and therapeutics.

Method

Plasmid construction.

PCR was performed using Q5 high-fidelity DNA polymerase (New England Biolabs) and specific primers that synthesized by Genewiz. Human codon-optimized exonuclease from phage T5, phage T7 and phage λ were synthesized by Genewiz. The Artemis gene were amplified from human 293T complementary DNA (cDNA). Recj and polA-exo were amplified from Escherichia coli genomic DNA. Briefly, the FnCas12a and crRNA expression cassette were constructed into one plasmid base on the backbone of PEGFPN1 plasmid and separated by a spacer sequence.

Human cell culture and transfection

HEK293T cells and Hela cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Solarbio). HLEB3 cells were cultured in DMEM supplemented with 15% FBS and 1% penicillin and streptomycin. Cells are maintained at 37 °C with 5% CO2. Cells were seeded into 96-well plates (Corning) and transfected at approximately 70% confluency. 200 ng plasmids were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer’s recommended protocol.

Genomic DNA preparation and PCR amplification.

60 hours after treatment, cells were washed with PBS genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Epicenter) 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 of purified gDNA template, Q5 high-fidelity DNA polymerase and specific primers (Supplementary table S2) on a T100 thermal cycler (Bio-Rad)

T7E1 cleavage assay.

The PCR products generated using the Q5 high-fidelity DNA polymerase were hybridized in NEBuffer 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 per s ramp down to 85 °C, 1 min at 85 °C and a 0.1 °C per 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, 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 x (1-sqrt(1-(b + c)/(a + b + c))), where a is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product.

Deep sequencing to characterize Cas12a indels 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 table S3). PCR products were run on 1.6% agarose gel and purified using Spin Column (Sangon Biotech) as per the manufacturer's recommended protocol. Equal amounts of the PCR products were pooled, and samples were sequenced commercially (GENEWIZ, Suzhou, China) by paired-end read sequencing using the Illumina HiSeq X Ten platform.

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Author contributions

Y. W. conceived the project and designed experiments. Y.W., Y.Z., J.S., X.G., Z.Y., performed the experiments. Y.W., Q.Y., analyzed data. Y.W., Q.Y., wrote the manuscript with help from all authors.

Competing interests

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.

Data Availability

Y. W. is responsible for distributing plasmids and materials of this work upon reasonable request. Data sets from high-throughput sequencing experiments have been deposited with the National Center for Biotechnology Information Sequence Read Archive under BioProject ID：PRJNA642811

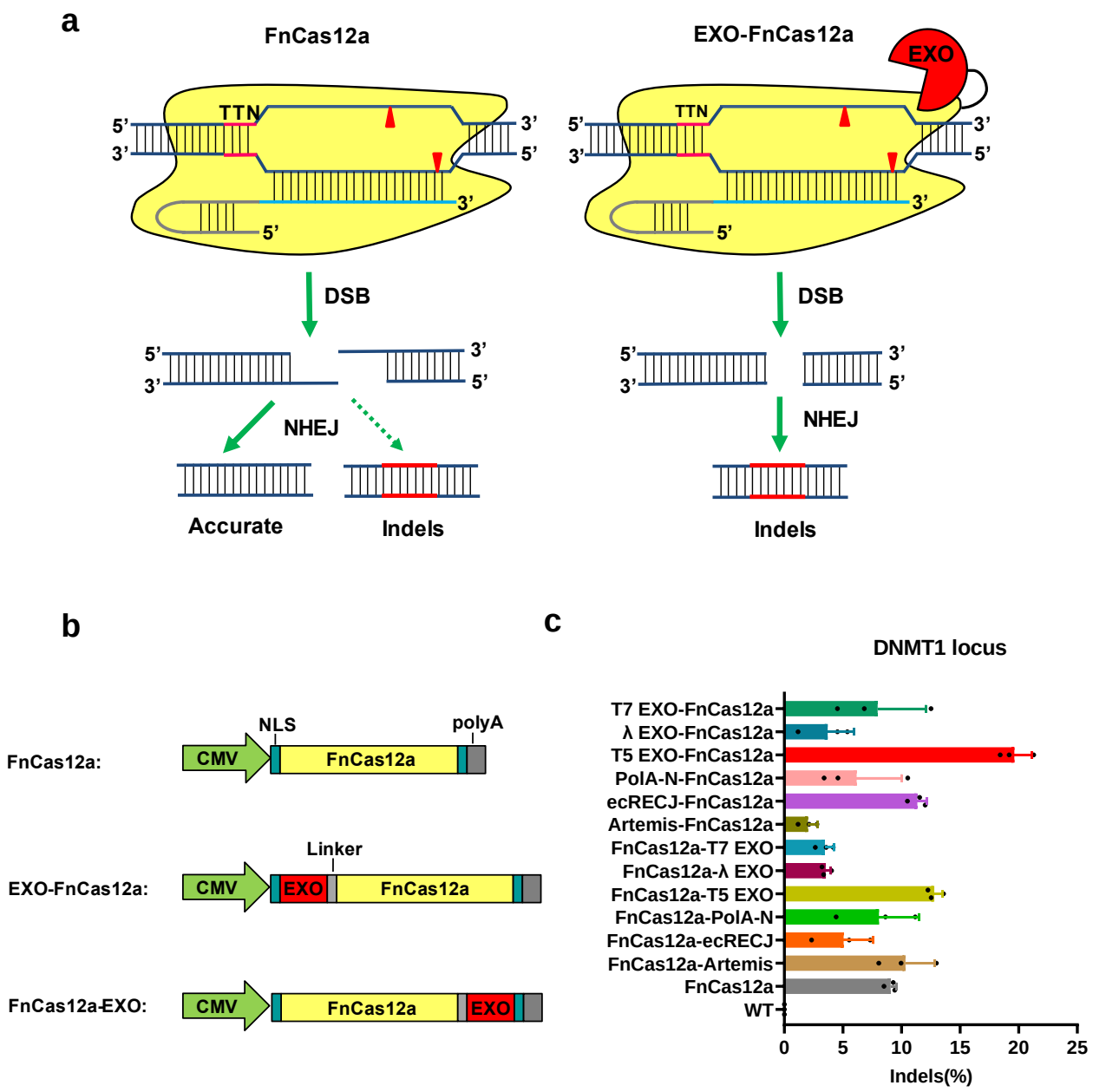


Figure 1. FnCas12a, EXO- FnCas12a and FnCas12a-EXO mediated gene editing in HEK293T cells

a. Illustration of the FnCas12a and TEXT system and the main pathway of DNA repair. For FnCas12a, simple relegation is possible with fully compatible overhangs; and also, part of cleavage target will be been excision or resynthesize in cells and only this part will be edited and detected. For TEXT, the single strand DNA exonuclease can degrade the sticky end in time, and all the target DNA that has been cut can cause indels.

b. Schematic illustration of the FnCas12a/ TEXT / 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

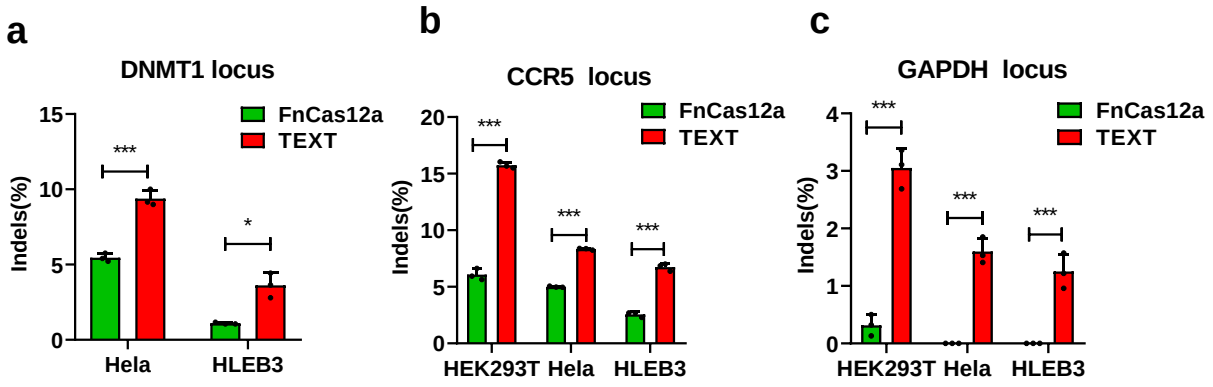


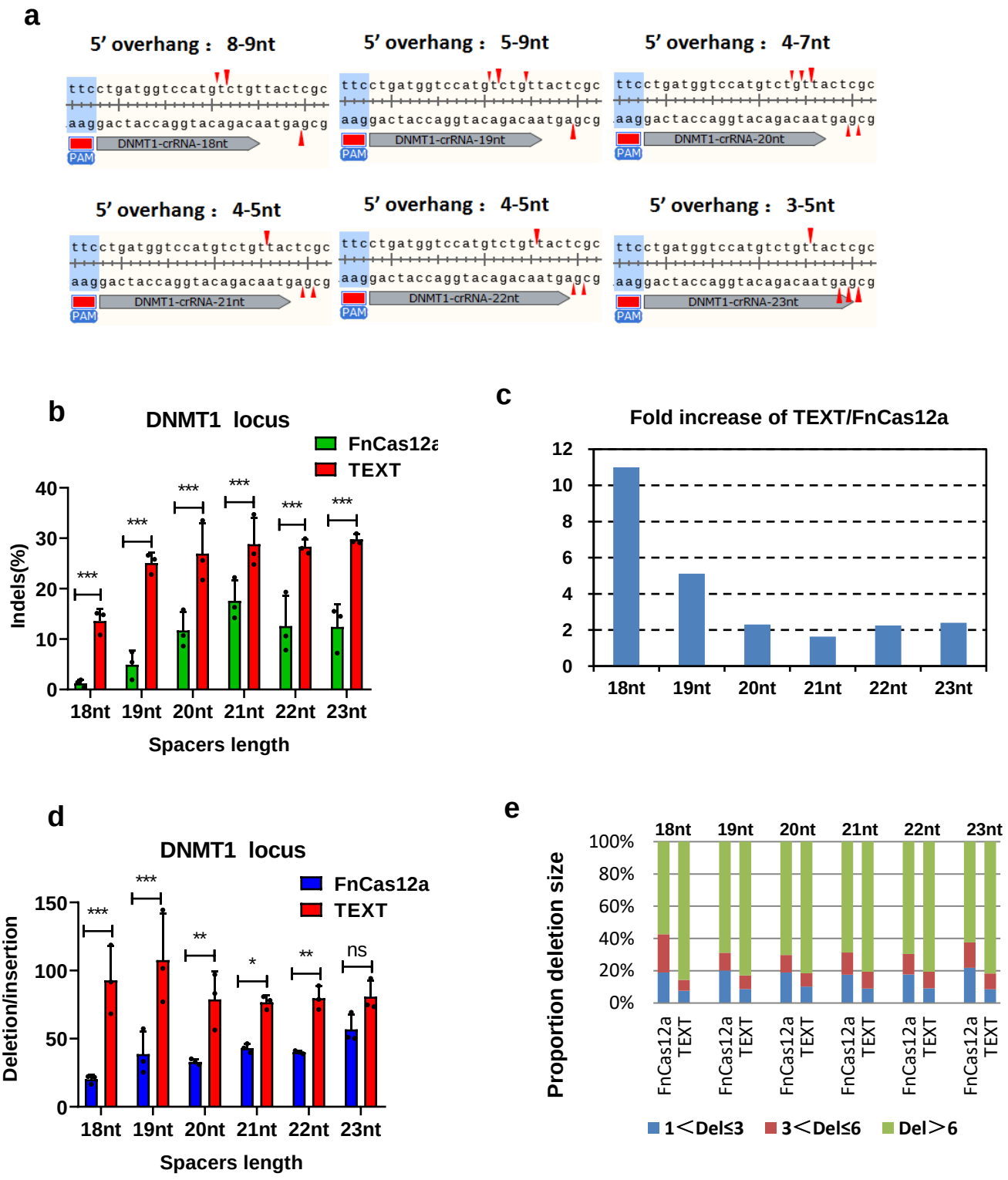
Figure 2. Comparison of gene editing efficiency of FnCas12a system and TEXT system in HEK293T, Hela and HLEB3 cell lines.

a gene editing efficiency of FnCas12a system and TEXT system for the human DNMT1 gene in Hela and HLEB3 cells.

b gene editing efficiency of FnCas12a system and TEXT system for the human CCR5 gene in HEK293T, Hela and HLEB3 cells.

c. gene editing efficiency of FnCas12a system and 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 ± s.d. from three biological replicates (*P < 0.05; **P < 0.01; ***P < 0.001; two-tailed t-test). Gel images are shown in Supplementary figure S2-S4.


a
5' overhang： 8-9nt
5' overhang： 5-9nt
5' overhang： 4-7nt
5' overhang： 4-5nt
5' overhang： 4-5nt
5' overhang： 3-5nt
ttcctgatggtccatgtctgttactcgc
aaggactaccaggtacagacaatgagcg
DNMT1-crRNA-18nt
DNMT1-crRNA-19nt
DNMT1-crRNA-20nt
DNMT1-crRNA-21nt
DNMT1-crRNA-22nt
DNMT1-crRNA-23nt
PAM
b
DNMT1 locus
FnCas12a
TEXT
Indels(%)
18nt 19nt 20nt 21nt 22nt 23nt
Spacers length
c
Fold increase of TEXT/FnCas12a
d
DNMT1 locus
FnCas12a
TEXT
Deletion/insertion
ns
Spacers length
e
Proportion deletion size
1＜Del≤3
3＜Del≤6
Del＞6

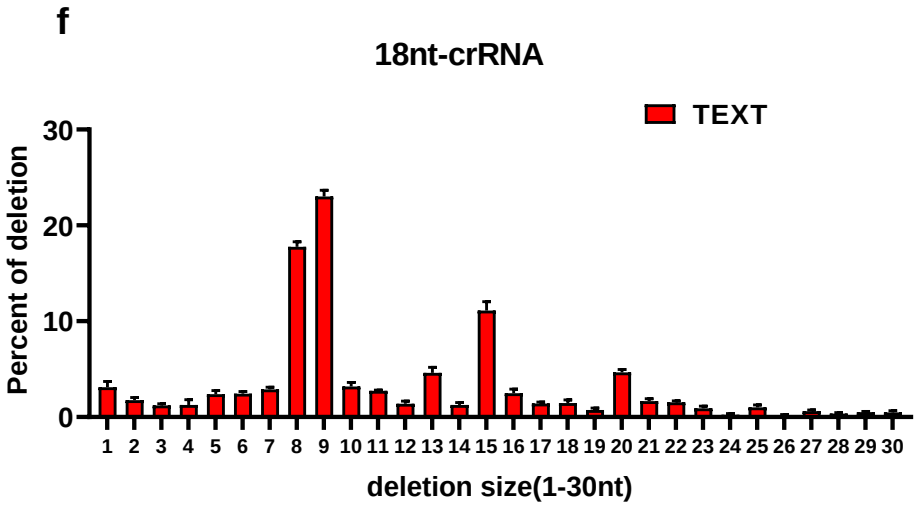


Figure 3. Different spacer lengths of crRNA result in distinct cleavage pattern and gene editing efficiency.

a. Cleavage pattern of FnCas12a under different crRNA spacer lengths (18-23nt). With 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 19-nt spacer, FnCas12a cleavage sites were mainly after the 14th,13th,17th bases on the non-target strand and from the 22nd bases on the target strand. With 20-nt spacer, FnCas12a cleavage sites were mainly after the 18th,17th,16th bases on the non-target strand and from the 18th, 19th bases on the target strand. With 21-22nt spacer, FnCas12a cleavage sites were mainly after the 18th bases on the non-target strand and from the 21st, 22nd bases on the target strand. With 23nt spacer, FnCas12a cleavage sites were mainly after the 18th bases on the non-target strand and from the 20th, 21st, 22nd bases on the target strand.

b. Gene editing efficiency of FnCas12a system and TEXT system for the human DNMT1 locus with different spacer length of crRNA in HEK293T cells. Gel images are shown in Supplementary figure S5.

c. Editing efficiency fold increase of TEXT system compare to FnCas12a at different spacer lengths. 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 TEXT system with 18-23nt spacer lengths of crRNA.

e. The relative proportions of different size deletions among total deletions that induced by FnCas12a or TEXT System with 18-23nt spacer lengths of crRNA.

f. Ratio of deletions size (1-30nt) induced by FnCas12a or TEXT System with 18nt spacer length of crRNA.

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