Engineering CRISPR–Cpf1 crRNAs and mRNAs to maximize genome editing efficiency

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Cpf1, a type-V CRISPR–Cas effector endonuclease, exhibits gene-editing activity in human cells through a single RNA-guided approach. Here, we report the design and assessment of an array of 42 types of engineered Acidaminococcus sp. Cpf1 (AsCpf1) CRISPR RNA (crRNA) and 5 types of AsCpf1 mRNA in human cell lines. We show that the top-performing modified crRNA (cr3’-SF, containing five 2′-fluoro ribose at the 3′ terminus) and AsCpf1 mRNA (full ψ-modification) improved gene-cutting efficiency by, respectively, 127% and 177%, with respect to unmodified crRNA and plasmid-encoding AsCpf1. We also show that the combination of cr3’-SF and ψ-modified AsCpf1 or Lachnospiraceae bacterium Cpf1 (LbCpf1) mRNA augmented gene-cutting efficiency by over 300% with respect to the same control, and discovered that 11 out of 16 crRNAs from Cpf1 orthologues enabled genome editing in the presence of AsCpf1. Engineered CRISPR–Cpf1 systems should facilitate a broad range of genome editing applications.

CRISPR (clustered regularly interspersed short palindromic repeats) and CRISPR-associated proteins are part of the adaptive immune system of bacteria and archaea.12 CRISPR-associated protein 9 (Cas9) induces double-stranded DNA breaks through complexation with two RNA molecules: crRNA and trans-activating crRNA (tracrRNA).3 Recently, two Cpf1 (CRISPR from Prevotella and Francisella)1 proteins from Acidaminococcus sp. (AsCpf1) and Lachnospiraceae bacterium (LbCpf1) have been shown to display comparable genome-editing capability to Cas9 (refs 4–11). Genome-wide analysis has suggested that Cpf1 may cause fewer off-target cleavages in comparison to Cas9 (refs 12,13). To exert sequence-specific endonuclease activity, Cpf1 is functional through a single crRNA without an additional tracrRNA.6,14 This crRNA (43 nucleotides) consists of a 5′-handle (20 nucleotides) and a guide segment15 (23 nucleotides; Fig. 1a). The Cpf1 protein interacts with the pseudoknot structure formed by the 5′-handle of crRNA15,16. A guide segment, composed of a seed region and the 3′ terminus, possesses complementary binding sequences with the target DNA sequences. This protein–RNA complex recognizes a T-rich protospacer–adjacent motif and leads to a staggered DNA double-stranded break.6

To increase the genome editing efficiency of CRISPR–Cas systems, previous studies have explored a variety of approaches16–19. For example, chemical modifications of CRISPR–Cas9 led to enhanced activity in a number of human cells.19,21 Also, a chimaeric single-guide RNA with three chemically modified nucleotides at both the 5′ and 3′ ends strongly improved Cas9-mediated genome editing in human primary T cells.19 The incorporation of chemically modified nucleotides in guide RNAs has been shown to retain insertion and deletion (indel) percentages in the CRISPR–Cas9 nucleic system.19,20 Furthermore, the structure of guide RNAs also plays a notable role in gene cutting for the CRISPR–Cas9 system20,21. Yet, to the best of our knowledge, the genome editing efficiency and off-target effects of engineered crRNAs and Cpf1 mRNAs have not been explored. Here, we report the systematic investigation of 42 chemically or structurally engineered crRNAs, and establish comprehensive structure–activity (genome editing efficiency) relationships. We also show that a ψ-modification is a suitable chemical alteration for AsCpf1 mRNA. Notably, we demonstrate that the combination of lead crRNA and ψ-mRNA substantially increased the gene-cutting efficiency by over 300% compared to the control group. This combination induced a more pronounced improvement in the gene-cutting efficiency when using LbCpf1 than when using AsCpf1. We also show that the applicability of crRNAs from Cpf1 orthologues is different for AsCpf1 and LbCpf1, as LbCpf1 is more conservative in the recognition of the loop structure at the 5′ handle. Our findings could facilitate a wide range of genome editing applications.

Engineering crRNAs to increase gene-cutting efficiency

To study the effects of engineered crRNAs on genome editing efficiency, we first used three types of chemically modified nucleotides, which were modified by phosphorothioate (PS), 2′-O-Me, and 2′-F-modifications (Supplementary Fig. 1). Modified crRNAs targeting the DNMT1 locus were purified on denaturing polyacrylamide gels and validated by mass-spectrometry analysis (sequence information and mass-spectrometry data are included in Supplementary Table 1). On the basis of previous findings for CRISPR–Cas9 (refs 18,19), we first treated human HEK293T cells with newly synthesized full-backbone phosphorothioate-modified cr42PS (all 42 phosphodiester linkages were substituted with phosphorothioate linkages) or cr5′/3′-3F2PS (three 2′-F modifications with two phosphorothioate linkages at both 5′ and 3′ ends) and plasmid-encoding AsCpf1 complexed with Lipofectamine 3000 (Fig. 1b). Genome editing efficiency was quantified using the T7E1 assay, and normalized to the treatment of unmodified (wild-type) crRNA (crWT) and plasmid-encoding AsCpf1. Both crRNAs showed a marked reduction in gene-cutting capability (Fig. 2a), which suggests that chemical modifications of AsCpf1 crRNA do not have the same pattern as Cas9 guide RNAs18,19. Subsequently, we systematically examined...
the effects of chemical modifications at the 5'-handle (U(−20) to U(−1)), seed region (C(+1) to U(+8)), and 3'-terminus (C(+9) to C(+23))(Fig. 1a). We introduced five, ten or twenty nucleotides with 2'-O-Me- or 2'-F-modifications at the 5'-handle of the crRNAs, which led to complete loss of activity. These data indicate that the 5'-handle is not suitable for current chemical modifications. Then we installed different numbers of modifications at the seed region and synthesized crS3M3PS, crS3F, crS2F and crS1F (Fig. 1b). Except for crS1F, all crRNAs lowered the indel percentage (Fig. 2a), indicating that the seed region may tolerate slight modifications. Furthermore, at the 3'-terminus, we incorporated five or ten nucleotides with 2'-O-Me-or 2'-F-modifications(Fig. 1b). Notably, 2'-F-modifications (crS3'F and crS3'10F) were more suitable compared to their corresponding 2'-O-Me-modifications (crS3'M and crS3'10M). crS3'5F improved the efficiency by 127% compared to the crWT (P < 0.01, Fig. 2a). We subsequently incorporated four additional phosphorothioate linkages along with five 2'-F-modifications (crS3'5F4PS), or replaced five 2'-F-modifications with five unlocked (crS3'5U) or locked (crS3'5L) nucleotides (Supplementary Fig. 1). crS3'5F4PS and crS3'5L reduced the potency, whereas crS3'5U completely lost the function of gene cutting (Fig. 2a). These results imply that modifications at the 3'-terminus are critical to gene editing activity. We next engineered the stem duplex (U(−15) to C(−11) and G(−6) to A(−2)) of the 5'-handle by split of the crRNA (crSplit), by deletion of base pairs (crDel2–crDel8) or by insertion of nucleotides (crIns4–crIns12) (Fig. 1c and Supplementary Table 1). Current crRNAs with split sequences and deletion of nucleotides exhibited no gene-cutting function. Insertion of four (crIns4') or six nucleotides (crIns5') retained the activity to some extent, which was dependent on the inserted base pairs (Fig. 2b).

AsCpf1 mRNA improved gene-cutting efficiency

We also investigated the effects of chemically modified AsCpf1 mRNAs on their gene-cutting efficiency. On the basis of previous results, we designed pseudouridine-modified (ψ), N6-methylpseudouridine-modified (me6ψ) and 5-methoxyuridine-modified (5moU) AsCpf1 mRNAs with split sequences and deletion of nucleotides (Supplementary Fig. 2) and the same chemically modified crRNAs (crIns5'). AsCpf1 mRNAs with split sequences and deletion of nucleotides exhibited no gene-cutting function. Insertion of four (me6ψ') or six nucleotides (5moU') retained the activity to some extent, which was dependent on the inserted base pairs (Fig. 2b).
mRNA, which were produced through in vitro transcription (Fig. 3a and Supplementary Fig. 2). We then treated HEK293T cells with crWT and modified AsCpf1 mRNA. Unmodified AsCpf1 (wild-type AsCpf1 mRNA), ψ- and meψ-modified AsCpf1 mRNA showed higher activity (154%, 177% and 168%, respectively) than plasmid-encoding AsCpf1, whereas the activity of the 5moU-modified AsCpf1 mRNA resembled that of the AsCpf1 plasmid (Fig. 3b). In addition, it has recently been reported that plasmid-encoding AsCpf1 with a S1228A mutation may improve genome editing efficiency. We therefore constructed AsCpf1 mRNA with both a S1228A mutation and ψ-modification (S1228A/ψ mRNA), which displayed comparable activity to the original ψ-modified mRNA (Fig. 3b).

**Applicability of crRNAs from Cpf1 family orthologues**

Inspired by crRNAs from 15 other members of the Cpf1 family* (see Methods for a list of the Cpf1 family orthologues), we engineered the loop (U(−10)−C(−9)−U(−8)−U(−7)) of the 5′-handle by substituting the loop of wild-type AsCpf1 crRNA (crWT here is defined as AscRNA to distinguish it from other crRNAs from the Cpf1-protein family) with those from crRNAs of other Cpf1 family orthologues to investigate the effects of the loop on gene-cutting activity (Fig. 3c and Supplementary Table 1). crRNAs were termed by combining the initials of the Cpf1 protein family member and crRNA. crRNAs of PbcPfp1, PecPfp1 and LicPfp1 share the same loop (UUUU) and crRNAs of LbcPfp1, PmpPfp1 and PmpPfp1 share the same loop (UAUU). Notably, co-delivery of ψ-modified AsCpf1 mRNA and a panel of loop-engineered crRNAs revealed that crRNAs with a four-membered loop including FncPfp1, PecPfp1 and LicPfp1 share the same loop (UUUU). In addition, when the U at position −10 was replaced by G (LbcCcrRNA and BpCcrRNA) or A (ScCcrRNA) on the five-membered loop, the function of the crRNAs was completely lost (Fig. 3d). This result suggests that U (−10) is a critical position for gene editing, consistent with the findings from the crystal structure of Cpf1.
Figure 3 | Gene-cutting efficiency of chemically modified AsCpf1 mRNAs and loop-engineered crRNAs. a, Schematic depicting chemical modifications applied to CRISPR–Cpf1 mRNAs. Modified Cpf1 mRNAs were generated by fully substituting natural uridines (blue box) of unmodified mRNA (wild-type mRNA) with pseudouridine (ψ), N1-methylpseudouridine (me1ψ) or 5-methoxyuridine (5moU). b, Gene-cutting efficiency of chemically modified AsCpf1 mRNAs. Indel percentage was determined by the T7E1 cleavage assay and normalized to the treatment with crWT plus plasmid-encoding AsCpf1. ND, not detectable. (***P < 0.001, ψ-modified AsCpf1 mRNA versus AsCpf1 plasmid; two-tailed t-test). c, Loop engineering of crRNAs. Nucleotides of the loop were altered according to crRNAs from other 15 Cpf1 family orthologues (see Methods). crRNAs were termed by combining the initials of the species of origin followed by crRNA. crRNAs of PbCpf1, PeCpf1 and LiCpf1 share the same loop (UUUU); crRNAs of Lb2Cpf1, PcCpf1 and PmCpf1 share the same loop (UAUU). Differences in nucleotides between AscrRNA and other crRNAs in the loop are highlighted in red. d, Gene-cutting efficiency of loop-engineered crRNAs in the presence of ψ-modified AsCpf1 mRNA. Red nucleotides denote sequence differences in the loop between AscrRNA and other crRNAs. Gene-cutting efficiency was determined by the T7E1 cleavage assay and normalized to the treatment with AscrRNA plus ψ-modified AsCpf1 mRNA. ND, not detectable. Data are expressed as the mean ± s.d. from three biological replicates (*P < 0.05, Lb2crRNA versus AscrRNA; two-tailed t-test).
Maximizing efficiency through crRNA and AsCpf1 mRNA

To study the combined effects of chemically modified crRNA and AsCpf1 mRNA, we treated HEK293T cells with the top-performing modified crRNA and AsCpf1 mRNA (cr3′5F and ψ-modified AsCpf1 mRNA). Notably, this combination significantly enhanced the gene cutting efficiency by over 250% compared to the treatment of crWT plus the plasmid encoding AsCpf1 (Fig. 4a). We then analysed the interaction among crRNA, AsCpf1 protein and target DNA. The crystal structure of the AsCpf1 complex indicates that the 2′-OH of ribose on crRNA at the following positions has a minor role: −16, −15, −12, −11, −9, −8, −7, −5, −4, −3, −2, +1, +6, +8, +9, +10, +11, +12, +17, +19, +20, +21, +22 and +23 (ref. 4). On the basis of this analysis, we designed three additional types of crRNAs: cr121F, cr116M3F and cr116M10P85F (Supplementary Fig. 6 and Supplementary Table 1), by introducing interspersed modifications at the ribose units without interaction with AsCpf1 and target DNA and by avoiding modifications at the seed region (+1 to +8). Interestingly, cr121F was comparable to cr3′5F, whereas cr166M5F and cr166M10P5SSF markedly weakened the activity of crRNA (Supplementary Fig. 6). Because cr3′5F possesses less modifications than cr121F, we further investigated the applicability of cr3′5F in Hep3B (a human hepatoma cell line) and U87 cells (a human glioblastoma cell line). Consistent with the results in HEK293T cells, the combination of cr3′5F and ψ-modified AsCpf1 mRNA improved gene-cutting by 329% for Hep3B (P < 0.001) and by 293% for U87 cells (P < 0.001) (Fig. 4a) compared to the treatment of crWT plus AsCpf1 plasmid. In addition to DNMT1 locus, we also examined gene-cutting efficiency for another target genes, AAAS1 and FANCF (ref. 13). For the AAAS1 locus, the order of potency in HEK293T cells is (most potent to least potent): cr3′5F plus ψ-modified AsCpf1 mRNA, crWT plus ψ-modified AsCpf1 mRNA, cr3′5F plus AsCpf1 plasmid, and crWT plus AsCpf1 plasmid. The combination of cr3′5F and ψ-modified AsCpf1 mRNA is 2.77-fold more efficient than the combination of crWT and AsCpf1 plasmid (P < 0.001; Fig. 4b). The enhanced efficiency was also observed in Hep3B and U87 cells (a 257% and 394% increase, respectively, Fig. 4b). For the FANCF locus, the trend is consistent with that for the AAAS1 locus (Fig. 4c). These results demonstrate the broad applicability of the chemically modified crRNAs and Cpf1 mRNAs.

Gene-cutting efficiency and applicability of crRNAs

As with AsCpf1, LbCpf1 is another notable endonuclease in the Cpf1 family that also displayed genome editing ability in human cells14,15. To investigate whether our strategy is applicable to LbCpf1, we used similar chemical modifications for LbcrRNA, as well as its corresponding LbCpf1 mRNA. The combination of LbCpf1 crWT and LbCpf1 plasmid led to no detectable gene cutting (Fig. 5a), whereas the combination of LbCpf1 cr3′5F (Lbcr3′5F) and ψ-modified LbCpf1 mRNA induced notable gene-cutting activity in all three cell lines tested. Moreover, LbCpf1 cr3′5F was more efficient than LbCpf1 crWT (P < 0.01). These results further prove the concept of our chemical modifications to advance CRISPR–Cpf1-mediated genome editing. In addition, we investigated the applicability of crRNAs for LbCpf1. We treated cells with loop-engineered

Figure 4 | Maximizing the genome editing efficiency through the combination of chemically modified crRNA and AsCpf1 mRNA. a, AsCpf1-mediated gene-cutting efficiency for the human DNMT1 gene in HEK293T, Hep3B and U87 cells. b, AsCpf1-mediated gene-cutting efficiency for the human AAAS1 gene in HEK293T, Hep3B and U87 cells. c, AsCpf1-mediated gene-cutting efficiency for the human FANCF gene in HEK293T 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 Fig. 3.
crRNAs (Fig. 3c) in the presence of ψ-modified LbCpf1 mRNA. Except for its own LbcRNA, LbCpf1 only led to reduced indels with Lb2crRNA/PccrRNA/PmcrRNA, AscrRNA, and MbcRNA, and no activity was found for other crRNAs (Fig. 5b), indicating different crRNA applicability between AsCpf1 and LbCpf1.

**On-target and off-target effects of AsCpf1 and LbCpf1**

Potential off-target effects are one of the major concerns for CRISPR-mediated gene editing and these may limit its application. To address this issue, we examined the on-target and off-target effects of LbCpf1 with three biological samples from the HEK293 T cells used in Fig. 5a. As shown in Fig. 7a, Lbcr3’5F plus ψ-modified LbCpf1 mRNA caused 46.7% indels compared to the minimal effects for LbcWT plus the LbCpf1 plasmid. The indel pattern of LbCpf1 was consistent with that of AsCpf1, whereas LbCpf1 led to larger fragment deletion than AsCpf1 (Fig. 7a). Similarly, Lbc3’5F plus ψ-modified LbCpf1 mRNA showed comparable off-target effects to those of LbcWT plus LbCpf1 plasmid (Fig. 7a). Taken together, the combination of engineered crRNA and Cpf1 mRNA enables the effective enhancement of genome editing efficiency without increasing off-target effects.

**Discussion**

Genome editing efficiency and off-targets effects are major challenges for the broad application of CRISPR systems. To address...
these issues for the CRISPR–Cpf1 system, we designed an array of crRNA variants, including chemically modified crRNAs and structurally engineered crRNAs, and elucidated structure–activity relationships of crRNAs for improved genome editing activity. In contrast to CRISPR–Cas9 (refs 18,19), neither phosphorothioate substitutions nor dual modification of both sides of crRNA enhanced genome editing efficiency. Deletion or insertion of nucleotides at the stem duplex reduced the activity. Moreover, modifications at the stem duplex of the 5′ handle or at the seed region severely hampered cleavage activity. Notably, 2′-F modification at the 3′ terminus

Figure 6 | Targeted deep-sequencing analysis of on-target and off-target gene cutting for chemically modified crRNA and AsCpf1 mRNA. a, Indel percentage at on-target and predicted top four off-target (OT1–OT4) sites analysed by deep-sequencing data. Indel percentage was plotted as the mean ± s.d. from three biological replicates (***P < 0.001; NS, not significant; two-tailed t-test). b, Plot of representative indel size distribution (left) and position distribution (right) of all reads for crWT plus AsCpf1 plasmid. c, Representative plots of indel size distribution (left) and position distribution (right) of all reads for cr3′ 5F plus ψ-modified AsCpf1 mRNA. Dashed lines indicate the predicted cleavage position (b,c). Targeted deep-sequencing analysis for biological replicates 2 and 3 are shown in Supplementary Figs 7 and 8.
(cr3′5F) exhibited higher potency compared to unmodified crRNA (crWT). In addition, the crystal structure of the Cpf1–crRNA–target DNA complex provides useful guidance to design new crRNAs. We show for chemically modified AsCpf1 mRNA that pseudouridine (ψ) and N⁰-methyl pseudouridine (meψ) were suitable modifications compared to the mRNA with unmodified nucleotides. Notably, the combination of cr3′5F and AsCpf1 ψ-mRNA improved gene-cutting efficiency over threefold compared to crWT and plasmid-encoding LbCpf1. These results demonstrate the broad applicability of this engineering strategy for Cpf1-family-mediated genome editing. Notably, we also explored the cross complexation of AsCpf1 and LbCpf1 with crRNAs from other members of the Cpf1 family. We show that in addition to their own crRNAs, AsCpf1 was able to induce gene editing in the presence of ten other Cpf1 family crRNAs (FnCpf1, PbCpf1, LiCpf1, Lb2Cpf1, PcCpf1, PmCpf1, EcPgf1, MbPgf1, MdbPgf1 and LbPgf1), whereas LbCpf1 only effectively complexed with Lb2crRNA/PccrRNA/PmcrRNA. These results suggest that LbCpf1 is more conservative than AsCpf1 when complexing with crRNAs. Targeted deep-sequencing data confirmed that the combination of cr3′5F and ψ-modified Cpf1 mRNA significantly enhanced the genome editing efficiency without increasing the level of off-target effects. Overall, our results offer

Table 1 Representative top ten high-frequent on-target mutagenesis aligned to the target site of DNMT1 locus induced by CRISPR-Cpf1 system.

| Indel pattern | On-target mutagenesis induced by the combination of AscrWT and AsCpf1 plasmid | Read (%) |
|---------------|--------------------------------------------------------------------------------|----------|
| WT            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 73.3     |
| D6            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 2.2      |
| D8            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 1.2      |
| D7            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 1.2      |
| D6            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 1.0      |
| D4            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 0.8      |
| D8            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 0.8      |
| D5            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 0.7      |
| D7            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 0.6      |
| D16           | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 0.6      |
| D6            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 0.6      |

On-target mutagenesis induced by the combination of Ascr3′5F and AsCpf1 mRNA (ψ)

| Indel pattern | On-target mutagenesis induced by the combination of Lbcr3′5F and LbCpf1 mRNA (ψ) | Read (%) |
|---------------|--------------------------------------------------------------------------------|----------|
| WT            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 34.0     |
| D6            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 6.7      |
| D16           | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 4.4      |
| D5            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 2.8      |
| D8            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 2.5      |
| D8            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 2.4      |
| D7            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 2.3      |
| D6            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 2.1      |
| D6            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 1.8      |
| D6            | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 1.7      |
| D25           | CAGTACGTTAATGTTTCTGGTTGTTGCTGTTAATGTTCTGTTACGTTGTTGCTGTTAATGTTCTGTTAGGG         | 1.3      |

On-target mutagenesis induced by the combination of Lbcr3′5F and LbCpf1 mRNA (ψ)
new insights into CRISPR–Cpf1 systems and provide a set of useful design criteria for maximizing genome editing efficiency.

**Methods**

List of the Cpf1 family orthologues. Acidaminococcus sp. BV316 Cpf1 (AsCpf1); Francisella tularensis subsp. Novicida U112 Cpf1 (FnCpf1); L. bacterium MC2017 Cpf1 (LbCpf1); Butyrivibrio proteoclasticus Cpf1 (BpCpf1); Porphyromonas macacae Cpf1 (PmCpf1); Candidatus Methanoplasma termnitum Cpf1 (CmCpf1); Eubacterium eigens Cpf1 (EeCpf1); Moraxella bovoculi 237 Cpf1 (MbCpf1); Prevotella disiens Cpf1 (PdCpf1); L. bacterium ND2006 Cpf1 (LbCpf1).

**Synthesis of crRNAs.** The sequence of unmodified crRNA targeting the DNMT1 site 3. AsCpf1 crRNA: 5'-UAAUUCUACUUGUAAGAUGAGUUCUGUUGUACUC-3'; LbCpf1 crRNA: 5'-AAUUCUACUUGUAAGAUGAGUUCUGUUGUACUC-3'; Other crRNA: 5'-AAUUCUACUUGUAAGAUGAGUUCUGUUGUACUC-3'. The sequence of unmodified crRNA targeting AAVS1 locus. AsCpf1 crRNA: 5'-UAAUUCUACUUGUAAGAUGAGUUCUGUUGUACUC-3'; LbCpf1 crRNA: 5'-AAUUCUACUUGUAAGAUGAGUUCUGUUGUACUC-3'; Other crRNA: 3'-UAAUUCUACUUGUAAGAUGAGUUCUGUUGUACUC-3'.

Unmodified crRNA (crWT) and all other crRNA variants including chemically modified crRNAs, stem- and loop-engineered crRNAs were synthesized using an automated solid-phase DNA/RNA synthesizer. Chemically modified crRNAs consisted of partial or total chemically modified nucleotides including phosphorothioate linkage (PS), 2'-O-Me, 2'-F modified, unlocked and locked nucleotides as well as their combinations (Fig. 1b and Supplementary Table 1). Stem-engineered crRNAs were designed by deleting a certain number of Watson–Crick base pairs from the stem duplex or inserting additional paired or unpaired bases into the stem duplex of crWT (Fig. 1c and Supplementary Table 1). crSplit was generated by incubating equimolar of relevant RNA sequences listed in Supplementary Table 1 in Tris-EDTA buffer at 95 °C for 30 s, followed by gradient cooling (95–23 °C ramping at 0.1 °C per s). Loop-engineered crRNAs were designed by using the loops from other Cpf1 orthologues (Fig. 3c and Supplementary Table 1). All crRNAs were purified on denaturing polyacrylamide gels and verified by mass spectrometry (Supplementary Table 1).

**Production of Cpf1 mRNAs.** Anti-reverse cap analogue (ARCA) capped and polyadenylated AsCpf1 mRNA and LbCpf1 mRNA transcripts were purchased from TriLink BioTechnologies (San Diego, California, USA). For modified AsCpf1 mRNA, uridines were fully substituted with pseudouridine (ψ), 1-N-methylpseudouridine (meψ), or 5-methoxyuridine (5moU). For modified LbCpf1 mRNA, the nucleotides that encode serine (S1228) were substituted with those for alanine (A), and uridines were fully substituted with pseudouridine (ψ). These mRNAs were subjected to DNase and phosphatase treatment and silica-gel membrane spin column purification for further use. All mRNAs were verified by analytical agarose gels (Supplementary Fig. 2).
Two days after treatment, genomic DNA purification and PCR amplification. Two days after treatment, HEP3B cells were cultured in Eagle's Medium without sodium pyruvate (Corning Incorporated). Hep3B cells were cultured in Eagle's Minimum Essential Medium with sodium pyruvate (Corning Incorporated). U87 cells were cultured in Dulbecco's Modified Eagle's Medium with sodium pyruvate, high glucose (Thermo Fisher Scientific). All medium was supplemented with 10% fetal bovine serum and all cell lines used in this study are from the American Type Culture Collection (ATCC). Without further testing for mycoplasma contamination and are maintained at 37 °C with 5% CO2. After overnight incubation, cells seeded on 24-well plates at an initial density of around 100,000 per well were treated with either Cpf1 expression plasmid (provided by F. Zhang) or Cpf1 mRNA (500 ng for the DNMT1 and AAVS1 loci and 1,500 ng for the FANC locus). At the same time, crRNAs (38 pmol for the DNMT1 and AAVS1 loci and 114 pmol for the FANC locus) were added to each well. All components were formulated with Lipofectamine 3000 (Life Technologies) in Opti-MEM I reduced serum medium (Life Technologies) following the manufacturer's recommended protocol.

Genomic DNA purification and PCR amplification. The PCR products generated using the Q5 high-fidelity DNA polymerase (New England Biolabs) and specific primers (Integrated DNA Technologies, Supplementary Table 2) on a Bio-Rad T100 thermal cycler (Bio-Rad).

T7EI 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 10 min, followed by a 2 °C per s ramp down to 85 °C. 1 min at 85 °C and 0.1 °C per s ramp down to 25 °C on a T100 thermal cycler (Bio-Rad). Subsequently, the annealed samples were digested by T7 Endonuclease I (New England Biolabs) for 30 min, separated by a 2% agarose gel and quantified on ChemiDoc XRS (Bio-Rad) using Quantity One Software. The mutation frequency (indel, %) was calculated with the following formula: 100 × (1 – (1 – fraction cleaved)1/2).

MiSeq library preparation and targeted deep sequencing. To further characterize on-target and off-target effects, genomic segments (around 200–300 bp, Supplementary Note 1) spanning the sites of interest were first amplified using sequencing primers with overhang adapter sequences (Supplementary Table 3) in the first round of PCR for 25 cycles. After purification, the second limited-cycle PCR amplification (10 cycles) was performed using the Nextera Index Kit (Illumina) to attach multiplexing indices and Illumina P5/P7 sequencing adapters (Supplementary Table 4, 5) to the first round PCR products. Next, libraries were normalized and pooled, and sequenced using 2x 300 paired-end sequencing on an Illumina MiSeq system. The raw deep sequencing data from MiSeq were analysed by a bioinformatic pipeline. The raw deep sequencing data from MiSeq were analysed by a bioinformatic pipeline.

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Author contributions
B.L. and Y.D. conceived and designed the experiments; B.L., W.Z., X.L., C.Z. and X.Z. performed the experiments; B.L., W.Z., X.L., C.Z. and X.Z. and Y.D. analysed the data; B.L. and Y.D. conceived and designed the experiments; B.L., W.Z., X.L., C.Z. and X.Z. and Y.D. wrote the paper with edits and comments from all authors.

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The authors declare no competing financial interests.