A more efficient CRISPR-Cas12a variant derived from Lachnospiraceae bacterium MA2020

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CRISPR effector proteins introduce double-stranded breaks into the mammalian genome, facilitating gene editing by non-homologous end-joining or homology-directed repair. Unlike the more commonly studied Cas9, the CRISPR effector protein Cas12a/Cpf1 recognizes a T-rich protospacer adjacent motif (PAM) and can process its own CRISPR RNA (crRNA) array, simplifying the use of multiple guide RNAs. We observed that the Cas12a ortholog of Lachnospiraceae bacterium MA2020 (Lb2Cas12a) edited mammalian genes with efficiencies comparable to those of AsCas12a and LbCas12a. Compared to these well-characterized Cas12a orthologs, Lb2Cas12a is smaller and recognizes a narrow set of PAM TTTV. We introduced two mutations into Lb2Cas12a, Q571K and C1003Y, that increased its cleavage efficiency for a range of target sequences beyond those of the commonly used Cas12a orthologs AsCas12a and LbCas12a. In addition to the canonical TTTV PAM, this variant, Lb2-KY, also efficiently cleaved target regions with CTTN PAMs. Finally, we demonstrated that Lb2-KY ribonucleoprotein (RNP) complexes edited two hemoglobin target regions useful for correcting common forms of sickle-cell anemia more efficiently than those of AsCas12a and LbCas12a. Thus, Lb2-KY has distinctive properties useful for modifying a range of clinically relevant targets in the human genome.

INTRODUCTION
CRISPR-Cas systems evolved as a component of bacterial and archaeal adaptive immune systems, protecting them against viruses and other mobile genetic elements. Cas effector proteins are guided by small CRISPR RNAs (crRNAs) that recognize complementary DNA or RNA sequences next to a protospacer adjacent motif (PAM). After recognition, the effector protein mediates a double-stranded break in the target nucleotide sequence. With their ease of programmability and high efficiency, CRISPR-Cas proteins have been used in a range of gene engineering applications in various organisms. Given the range of potential applications, there is a need for additional high-efficiency effector proteins with diverse PAM preferences, low off-target activities, and low immunogenicity. Two subtypes of CRISPR-Cas effector proteins are commonly used to engineer mammalian genomes. Subtype II-A includes Cas9 proteins, and subtype V-A includes Cas12a proteins, previously known as Cpf1. Although less studied than Cas9, Cas12a has useful properties for many genome-editing applications. Cas12a recognizes T-rich PAMs, enabling targeting of genomic regions that lack the G-rich PAM sequences recognized by Cas9. Cas12a mediates more efficient homology-directed repair (HDR), in part because it generates staggered DNA ends and because it rapidly releases the PAM-distal end after cleavage. Unlike Cas9, Cas12a has an RNase domain that can excise its crRNA from a larger CRISPR array, simplifying editing or gene regulation with multiple crRNAs. Cas12a may also be safer than Cas9 because it is less permissive to mismatches between crRNA and target sequences, limiting off-target activities. In addition, commonly used Cas12a proteins derive from non-pathogenic bacteria, and therefore they are less likely to be recognized by pre-existing host immune responses. In contrast, major Cas9 variants derive from Streptococcus pyogenes (SpCas9) and Staphylococcus aureus (SaCas9), two major human pathogens.

To date, the most efficient and widely used Cas12a effector proteins derive from Acidaminococcus sp. (AsCas12a) and Lachnospiraceae sp. (LbCas12a). In addition to its canonical TTTV PAM motif, AsCas12a has been engineered to recognize TACV, TYCV, VTTV, and TTTN while maintaining its target specificity. However, the AsCas12a gene is relatively large, approximately 3.9 kb, precluding its delivery by adeno-associated virus (AAV). LbCas12a is smaller and has been reported to have higher editing efficiency than that of AsCas12, but it also has been shown to have higher off-target activity.

Received 19 January 2021; accepted 14 February 2021; https://doi.org/10.1016/j.omtn.2021.02.012.
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Here we characterize another Cas12a ortholog, that of *Lachnospiraceae bacterium* MA2020 (Lb2Cas12a). Despite the similarity of its name and origin to LbCas12a, it is more closely related to *Butyrivibrio sp*. NC3005 Cas12a (Figures 1A and 1B; Figure S1). We show that Lb2Cas12a edits mammalian genomes with efficiencies comparable to AsCas12a and LbCas12a at lentivirally integrated targets. We further engineered Lb2Cas12a, markedly enhancing its editing activity and broadening its PAM recognition to include CTG as well as its cognate TTTV PAM. We show that the improved Lb2Cas12a, namely Lb2-KY, outperformed AsCas12a and LbCas12a at targets with both TTTN and CTTV PAMs when delivered as plasmids or as ribonucleoproteins (RNPAs). In addition, Lb2-KY RNP efficiently edited a key target site of the hemoglobin subunit beta gene, as well as the promoter region of hemoglobin subunit gamma 1 and 2 genes, and the Fc region of human IgG1 gene. Thus, this Lb2Cas12a variant, Lb2-KY, increases the potency and expands the range of Cas12a-mediated gene editing.

RESULTS

**Lb2Cas12a efficiently edits mammalian genomes and has a TTTN PAM preference.** We investigated the nuclease activity and PAM usage of a range of Cas12a orthologs identified by Zetsche et al. In contrast to Zetsche et al., who used the enzyme-mismatch cleavage Surveyor assay, we observed that Lb2Cas12a had significant nuclease activity using a double-stranded-break gain-of-expression assay (Figure 1C). This assay uses five 23-nt-target sequences from the EGFP, DNMT, FANC, or EMX1 genes, each inserted upstream of an out-of-frame luciferase gene (Figure 1D). Cleavage of the target sequence and subsequent non-homologous end-joining (NHEJ) repair puts approximately one-third of the luciferase gene in frame, allowing its correct translation. We observed that Lb2Cas12a-mediated editing efficiency was similar to or better than those of AsCas12a and LbCas12a when targeting these five sequences (Figure 1E). To determine whether the difference in Lb2Cas12a activity between this observation and Zetsche et al. was due to use of different assays, we repeated these studies using the T7E1 nuclease assay and the DNMT1-3 target used in Zetsche et al. (Figures 2C and 2D). Lb2-KY is more efficient than wild-type Lb2Cas12a.

**Rational engineering of Lb2Cas12a to increase editing efficiency.** We reasoned that Lb2Cas12a may contain divergent residues that impair its expression or editing efficiency. Therefore, we aligned Lb2Cas12a peptide sequence with the eight nearest available Cas12a orthologs and converted residues unique to Lb2Cas12a to the consensus residues among these orthologs (Figure S2). These Lb2Cas12a variants, each containing one consensus mutation, were characterized using the gain-of-expression assay with EGFP-1 as a target sequence. Most of these variants were approximately as efficient as wild-type Lb2Cas12a. However, replacement of glutamine 571 with a lysine (Q571K) resulted in a 50% increase in nuclease activity (Figure 2A). We also characterized a subset of these Lb2Cas12a variants and observed that the C1003Y variant consistently expressed more efficiently than wild-type Lb2Cas12a by approximately 40% (Figure 2B). Perhaps due to this higher expression, the C1003Y variant edited more efficiently than wild-type Lb2Cas12a, although this difference was not statistically significant in this initial screening assay (Figure 2A). Alternative mutations at C1003 position did not improve Lb2 editing efficiencies (Figure S3), suggesting that the gain of tyrosine rather than the loss of cysteine contributed to the improved expression of C1003Y mutant. A model of Lb2Cas12a based on the structure of LbCas12a (PDB: 5XUS) localizes Q571 to the loop-lysine-loop complex that interacts with PAM and promotes melting of the target DNA duplex (Figures 2C and 2D).

**Lb2-KY utilizes a wider range of PAMs than Lb2Cas12a and has higher editing efficiencies than AsCas12a and LbCas12a.** To determine if Lb2-KY utilizes different PAMs than Lb2Cas12a, we established a novel screen, represented in Figure 4A, that assesses PAM usage in mammalian cells. As shown, the EMX1 target precedes by six randomized nucleotides was retrovirally integrated along with a puromycin selection marker into HEK293T cells. Cells were selected with puromycin and transfected with plasmids encoding mCherry and the EMX1-targeting crRNA together with plasmids encoding
Figure 1. Lb2Cas12a efficiently cleaved mammalian genomes at both integrated and endogenous targets

(A) A phylogenetic tree generated by Phylo.io based on an alignment of Cas12a orthologs of the indicated species.21,22 (B) A representation of the indicated Cas12a orthologs with domains indicated. WED: crRNA binding and processing domains, split by REC and PI domains into WED-I, II, and III (green). REC: DNA binding domains REC1 and REC2 (gray). PI: PAM-interacting domain (orange). RuvC: DNA cleavage domains, split by BH and Nuc domains into RuvC-I, II, and III (blue). BH: bridge helix (light green). Nuc: DNA processing domain (red). Number at right indicates the number of amino acids of each ortholog. (C) A diagram illustrating the double-stranded-break-induced gain-of-expression assay used in subsequent figures. A construct with an in-frame (+1) crRNA target sequence (orange) preceding an out-of-frame (+3) luciferase gene (light green) is stably integrated into the genomes of HEK293T cells. Upon cleavage by a Cas12a/crRNA complex, non-homologous end-joining (NHEJ) repair places approximately one-third of the downstream luciferase genes in frame (dark green), enabling their expression.11 Luciferase activity reflects the efficiency of Cas12a-mediated cleavage. (D) A list of target sequences used in subsequent panels with their preceding PAMs (blue). (E) The editing efficiency of AsCas12a, LbCas12a, and Lb2Cas12a, measured with the assay shown in panel (C), were compared using the six indicated lentivirally integrated targets. The means of three independent replicates are shown, with error bars indicating ± standard error of the mean (SEM). The significance of differences with Lb2Cas12a are indicated above bars representing AsCas12a and LbCas12a (*p < 0.05; **p < 0.01; ***p < 0.001; values of p > 0.05 are indicated in the graph), as determined by two-way ANOVA, followed by Tukey’s multiple comparison tests. (F) Lb2Cas12a edited an endogenous locus of the DNMT1 gene (DNMT1-3) with high efficiency. A T7E1 assay was used to compare the editing efficiencies of the Cas12a orthologs. Percent edited, indicated beneath the figure, is calculated as in Guschin et al.23 and indicates the average of three independent biological replicates. Neg: negative-control cells transfected with vector alone. (G) Expression of AsCas12a, LbCas12a, and Lb2Cas12a in HEK293T cells used in (F). A western blot, representative of three independent biological replicates with similar results, is shown.
active Cas12a variants, or with an empty vector. mCherry-positive cells were selected by fluorescence-activated cell sorting, and the sites of integration were analyzed by next-generation sequencing. As shown in Figure 4B, in which preferences for the first three nucleotides of PAM sequence (i.e., positions 1 to 3) were described, wild-type Lb2Cas12a efficiently modified TTTN PAM sequences, but not most other PAMs. In contrast, Lb2-KY more efficiently modified a wider range of PAM sequences including all NTTN PAMs. LbCas12a also showed a broad PAM preference, corroborating previous research, while control mCherry-positive cells transfected with an empty vector had a low level of background variation. Of note, Lb2-KY showed consistent preference for all four CTTN PAMs, as shown in Figure 4C, where the fourth PAM nucleotide was included. We did not observe any significant contribution of the nucleotides at position 5 and 6 to the PAM preferences of the studied Cas12a nucleases.

The screen shown in Figure 4 uses a single integrated EMX1 target sequence. To confirm and extend these observations, we used TIDE analysis to compare AsCas12a, LbCas12a, Lb2Cas12a, Lb2-Y, and Lb2-KY for their ability to modify 31 distinct endogenous target sites in four genes. The complete target list is provided in Table S1. As shown in Figure 5A, the editing efficiencies of Lb2-KY markedly
exceeded those of AsCas12a and LbCas12a, whereas Lb2-Y activity was similar to those of LbCas12a and AsCas12a for most target regions. Both Lb2-KY and Lb2-Y showed improvements over the wild-type Lb2Cas12a. In accordance with the PAM screening assay, Lb2-KY showed enhanced activity at non-canonical TTTT and CTNT PAMs (Figures S5B and S5C; Figure S4). We conclude that Lb2-KY is more efficient than AsCas12a and LbCas12a for modifying targets with TTTN PAMs and consistently performed better than these latter Cas12a orthologs with CTNTPAMs. Finally, we compared Lb2 and Lb2-KY with additional Cas12a orthologs reported recently to be efficient in mammalian cells\(^{32}\) (Figure 5D).

Lb2-KY, but not Lb2Cas12a, edited CTNT PAMs similarly to, or more efficiently than, Mb2Cas12a, Mb3Cas12a, and BsCas12a.

**Lb2-KY RNP complexes can precisely edit therapeutically relevant targets bearing CTNT PAMs**

RNP complexes composed of a CRISPR effector protein and a crRNA more efficiently edit their targets than plasmid-expressed protein and RNA.\(^{33,34}\) Accordingly, we produced Lb2-KY RNP complexes with an optimized nuclear localization signal (NLS7; Figures S3A, S5B, and S6A) and assessed the editing efficiency of these RNP complexes using physiologically relevant target sites with CTNT PAMs.
regions. Using the HDR-dependent editing strategy previously described for Cas12a proteins,7,35 we delivered Lb2-KY RNP with single-stranded oligodeoxynucleotide (ssODN) repair templates complementary to the target strand, with either 77/37- or 37/77-nucleotide homology arms. Lb2-KY RNP complexes efficiently used a CTTC PAM to introduce two mutations, M428L and N434S, into the human IgG1 Fc region (IGHG1 gene), which lacks TTTN PAM sequences (Figures 6A and 6B). These mutations have been shown to markedly increase the half-life of antibodies in the serum.37 Note that ssODN enhanced NHEJ-mediated indel

Figure 4. PAM usage of Lb, Lb2, and Lb2-KY
(A) A diagram outlining the mammalian-cell PAM identification assay used in subsequent panels. A library of randomized 5’-NNNNNN-3’ PAMs preceding a crRNA target was stably expressed in HEK293T cells. Plasmids encoding Cas12a proteins, crRNAs, and the fluorescent marker mCherry were transfected to these cells. Cells were sorted for mCherry expression, and a region containing the randomized PAM and the target sequence was deep-sequenced with paired-end reads of 300 base pairs. Indel frequencies were then calculated for each four-nucleotide PAM. Note that the first two nucleotides of the 6-mer randomized PAM sequence had no detectable impact on the activities of any Cas12a variant. (B) Heatmaps showing the result of mammalian-cell PAM screen for Lb2Cas12a, Lb2-KY, LbCas12a, and cells lacking a Cas12a ortholog (control). PAMs and the related editing efficiencies were pooled by the first three nucleotide positions. Each heatmap indicates an average of two or three independent replicates. The range of replicates for each 3-nucleotide PAM sequence is less than 10% of the signal at each position. (C) An expansion of the data generated in (B), except that all 64 four-nucleotide PAMs are plotted. The range of replicates for each 4-nucleotide sequence is less than 25% of the average signal for each four-nucleotide PAM.
formation as well as precise HDR-mediated editing, as previously reported.38

We further directed Lb2-KY RNPs to two chromosomal targets relevant to sickle-cell and beta-thalassemia diseases, using a cell line, K562, that resembles early-stage erythrocytes. We compared Lb2-KY RNP with a commercially produced Cas12a RNP, namely Alt-R AsCas12a V3 (denoted here as As-V3; Integrated DNA Technologies [IDT]). We first targeted a region of the hemoglobin subunit beta (HBB) gene proximal to the codon for glutamic acid 6. This glutamic acid is a valine in a common form of sickle cell disease. Although this region lacks a canonical TTTV PAM site, Lb2-KY RNP was still able to mutate this codon to encode a valine by targeting an adjacent CTTG PAM (Figures 6C and 6D), again consistent with the efficiency with which Lb2-KY recognizes CTTV PAM sites. Lb2-KY RNPs were even more efficient at introducing an engineered deletion into the

**Figure 5. Efficiencies of Cas12a variants at TTTN and CTTN PAMs**

(A) As, Lb, Lb2, Lb2-Y, and Lb2-KY Cas12a variants were characterized for their ability to modify 31 endogenous target sequences preceded by TTTN or CTTN PAMs within four genes—DNMT1, EMX1, FANCF, and GRIN2B—as determined by TIDE analysis.30 Indel levels were ranked and compared with Lb2-KY using nonparametric tests with Steel’s corrections for multiple comparisons. p values are indicated in the legends. (B and C) Heatmaps summarizing data from (A) for target regions with CTTN (B) and TTTN (C) PAMs. (D) Editing efficiency of additional Cas12a orthologs at endogenous CTTN loci in the DNMT1 gene. Lb2-KY was compared with Mb2Cas12a (Mb2), Mb3Cas12a (Mb3), and BsCas12a (Bs). The means of three independent replicates are shown, with error bars indicating ±SEM. Significance of comparisons with Lb2-KY was determined by two-way ANOVA, followed by Dunnnett’s multiple comparisons tests (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; values of p > 0.05 are shown in the graph).
distal BCL11A binding motif preceding the hemoglobin subunit gamma 1 and 2 (HBG1/2) genes (Figures 6E and 6F). This deletion was previously reported to augment expression of fetal hemoglobin in adult reticulocytes. Thus, Lb2-KY RNP can be used to efficiently edit CTTV PAM sites in physiologically relevant genomic regions.

During the preparation of our manuscript, a more enhanced commercial version of AsCas12a, namely Alt-R AsCas12a Ultra, was introduced (denoted here as As-Ultra; IDT). As-Ultra was engineered through directed evolution to achieve higher, more robust editing efficiencies compared to the earlier version As-V3. In order to thoroughly compare the efficiencies of available Cas12a RNPs, we produced wild-type Cas12a orthologs, As and Lb2 RNPs (Figures S6B–S6E) and compared the efficiencies of Lb2-KY RNP with As and Lb2 RNPs and the commercially available As-Ultra and EnGen Lba Cas12a (New England BioLabs; denoted here as Lb-NEB; note that neither commercial product is marketed to cleave CTTN PAM motifs). All homemade RNP variants include the optimized nuclear-localization signal NLS7. Lb2-KY RNP outperformed all the tested variants at all but one target from different genes with the canonical TTTN PAMs and the non-canonical CTTN PAMs (Figures 6G and 6H). At the DNMT1-3 target, Lb2-KY activity was slightly less than that of As-Ultra.

The trans-cleavage and off-target effects of Lb2-KY

Cas12a nucleases have been shown to indiscriminately cleave ssDNA in vitro after being activated by a bona fide double-stranded DNA target.8,9,41 We used ssDNA substrates with fluorophore-quencher to study the trans-cleavage activity of Lb2-KY, as previously described.10,42 Lb2-KY showed comparable trans-cleavage activity to LbCas12a (i.e., EnGen Lba Cas12a, NEB); both were activated by targets containing TTTT and CTTT PAMs, but not AGCG or ACTG targets (Figures S7A–S7D). Note that it is not clear that this trans-cleavage activity is deleterious in physiological settings, because ssDNA in replication bubbles and homologous recombination complexes are protected by DNA-binding proteins.43–45 and because LbCas12a-mediated HDR with ssDNA repair templates is as efficient as that mediated by AsCas12a.7

We also characterized the target specificity of Lb2-KY RNP compared to those of AsCas12a and As-Ultra RNPs using iGUIDE assay.46 When directed at the DNMT1-3 target, Lb2-KY showed a moderate off-target profile as compared to the other nucleases, with one prominent off-target (Figures S7E–S7H; Table S2). The on-target frequency of Lb2-KY RNP was slightly less than that of AsCas12a RNP but higher than As-Ultra. No novel off-targets containing CTTN PAM were detected with this target. When delivered as plasmids, Lb2-KY on-target frequency was somewhat reduced as expected (Figure S7I; Table S2). Finally, we detected no significant loss of cell viability when Lb2-KY, as well as other Cas12a variants, were expressed from plasmids in HEK293T cells (Figure S7J).

DISCUSSION

In this study, we show that Lb2Cas12a can edit mammalian genomes with efficiencies similar to other, better characterized Cas12a orthologs. By converting unique residues in Lb2Cas12a to residues common among its closely related Cas12a orthologs, we created an Lb2Cas12a variant, Lb2-KY, with enhanced activity and expression in mammalian cells and a broadened PAM preference. Lb2-KY has markedly greater cleavage activity than AsCas12a and LbCas12a at both the canonical PAM TTTT and the non-canonical PAM CTTV. The difference is especially pronounced with sequences bearing CTTV PAM, expanding the range of possible PAMs available for gene editing. In addition to its greater efficiency, Lb2-KY has an additional advantage: at 1,206 residues, it is the smallest of the Cas12a variants that function efficiently in mammalian cells. It thus can be readily introduced into AAV vector along with a crRNA array, perhaps encoded as a single transcript.

We have also developed a mammalian PAM assay that tracked the PAM preference of Cas12a proteins in the mammalian genomes (Figure 4). This assay may better reflect conditions in the eukaryotic nucleus than in vitro assay or those in E. coli.15,47 However, this assay is limited to only a single target sequence per library, and therefore its results should be confirmed with multiple endogenous targets, as shown in Figure 5. Nonetheless, it may be useful as a rapid screen for evaluating Cas12a variants engineered for novel PAM preferences in mammalian cells.

To generate Lb2-KY, we introduce two mutations into wild-type Lb2Cas12a. Noting that wild-type Lb2Cas12a expressed less efficiently than either LbCas12a or AsCas12a (Figure 1G), we initially
sought mutations that increased expression in mammalian cells. We observed that replacement of cysteine 1003 with a tyrosine markedly enhanced Lb2Cas12a expression. Although the effect of this mutation on editing was modest, we retained it for two reasons. First, the greater expression may suggest that it is better folded or more stable in mammalian cells. Second, this expression may be more relevant in contexts where Cas12a levels are limiting, for example, in AAV-transduced cells in vivo.

The utility of the Q571K mutation is more obvious. This mutation restores a critical PAM binding lysine present in every Cas12a ortholog that functions efficiently in mammalian cells, including AsCas12a, LbCas12a, FnCas12a, and MbCas12a (Figure S1). Analysis ofFnCas12a crystal structure (PDB: 5NFV) has shown that FnCas12a K667, homologous to Lb2Cas12 Q571K, participates in a cation–π interaction with final PAM nucleotide, disrupting base-pairing in the seed region of the target DNA. 25,26 Presumably a glutamine at this position does not interact as strongly with this nucleotide.

Finally, we showed that Lb2-KY RNP complexes efficiently edited therapeutically important targets using CTTV PAMs in mammalian cells. At two loci critical to sickle cell and β-thalassemia diseases, namely glutamic acid 6 in HBB gene and BCL11A-binding motif near HBG1/2 genes, Lb2-KY RNPs outperformed the commercially produced As-V3 RNPs. In addition, Lb2-KY RNP showed moderate off-target activity compared other AsCas12a and As-Ultra RNPs. Although SpCas9 RNPs have been shown to efficiently edit these therapeutically relevant targets as well, 48–50 this CRISPR effector protein in general has greater off-target activities, and it presents epitopes from a common human pathogen. Thus, cells expressing SpCas9 might be more efficiently cleared by the adaptive immune system. 12,14,21,22 During the preparation of our manuscript, a few novel Cas12a nucleases have been described to utilize non-canonical PAMs 53–56 or utilize more stringent T-rich PAMs with reduced off-target effects compared to AsCas12a and LbCas12a. 57 Although the cleavage activities of these nucleases have not been extensively compared with the commonly used Cas12a orthologs, it will be interesting to directly test Lb2-KY activity with these novel variants at both TTTN and CTTN PAMs. In summary, the inherent editing efficiency of Lb2-KY, its smaller size, the ease with which efficient Lb2-KY RNP can be produced and used, and its markedly greater ability to edit regions with CTTV PAM sites suggest that Lb2-KY will be a useful contribution to the expanding toolbox of CRISPR effector proteins.

 MATERIALS AND METHODS

 Plasmids

 Wild-type AsCas12a (pcDNA3.1-hAsCpf1), LbCas12a (pcDNA3.1-hLbCpf1), and Lb2Cas12a (pcDNA3.1-hLb2Cpf1) and pX330 (pX330-U6-chimeric_BB-CBh-hSpCas9) plasmids were gifts from Dr. Feng Zhang (Addgene plasmid numbers 69982, 69988, 69983, and 42230, respectively). pMAL-his-LbCpf1-EC was a gift from Dr. Jin-Soo Kim (Addgene plasmid number 79008) and was used to express Cas12a variants in E. coli for protein production. Lb2Cas12a point mutations were introduced by PCR using the Phusion site-directed mutagenesis kit (Thermo Fisher Scientific). Changes to the N and C termini of Cas12a plasmids were made by ligation of gBlock gene fragments (IDT) into the original plasmids linearized by appropriate restriction enzymes. pcDNA-3.1 plasmids expressing Cas12a direct repeats and double BsmBI recognition sites under U6 promoters (pcDNA3.1-U6-promoter) were created by PCR amplification of the U6 promoter region from pX330 with a U6-promoter forward primer and a reverse primer carrying the Cas12a direct-repeats-BsmBI sequence in the 5’ end. crRNA plasmids were generated by cloning annealed spacer-sequence between BsmBI-double-digested restriction sites in pcDNA3.1-U6-promoter plasmids. For Cas12a protein production, each Cas12a gene was codon-optimized for E. coli using OptimumGene Codon Optimization (GenScript), synthesized, and cloned into pMAL-his-LbCpf1-EC between EcoRI and NotI restriction sites.

 Cell culture

 Human embryonic kidney 293T (HEK293T) cells (ATCC) and HEK293T-derived reporter cell lines for gain-of-expression experiments 11 were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM Glutamax-I (Life Technologies), and 100 μM nonessential amino acids (Life Technologies). JeKo-1 cells (ATCC) were maintained in RPMI-1640 Medium (Life Technologies) supplemented with 20% FBS. K-562 cells (ATCC) were maintained in Iscove’s modified Dulbecco’s medium (IMDM, Life Technologies) supplied with 10% FBS. All growth media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies), except during transfection and electroporation. Cells were confirmed mycoplasma-free by the provider.

 Cas12a on-target DNA cleavage assays

HEK293T cells or HEK293T-derived reporter cells were seeded in 48-well plates with antibiotic-free medium 16 h prior to transfection. Cells were transfected with 100 ng of Cas12a plasmids and 100 ng of crRNA plasmids using 0.75 μL of Lipofectamine 2000 (Thermo Fisher Scientific). Eight hours later, transfection medium was completely removed and replaced with regular growth medium containing 2% or 10% FBS. About 72 h post-transfection, cells and culture medium were harvested for reporter gene expression in luminescence assays, T7E1 assay, Sanger sequencing and subsequent TIDE analysis, or western blotting. Luminescence assays measuring Gaussia luciferase, firefly luciferase, and Cypridina luciferase and T7E1 mismatch cleavage assay were conducted as previously described.11

 TIDE and TIDER analysis

Genomic DNA from transfected cells was isolated using Nucleospin Tissue kits (MACHEREY-NAGEL). About 100 ng of the purified DNA was used as templates for PCR amplification with primer pairs flanking a 1.5–2 kbp region inclusive of the target site. PCR products were purified using Nucleospin Gel and PCR Clean Up kit (MACHEREY-NAGEL), then sent to Genewiz for Sanger sequencing using primers binding about 200–400 bp up- or downstream of the Cas12a cleavage site. Table S3 contains a list of primers used. The
chromatograms were decomposed and indel levels or precise-editing efficiencies predicted using TIDE package available at http://shinyapps.datacurators.nl/tide/ or TIDER package at http://shinyapps.datacurators.nl/tider/. For TIDER analysis, reference sequences were generated by sequencing gBlocks resembling the target region and carrying the designed mutations.

**Western blots**

Transfected cells were washed with cold PBS and incubated in PBS on ice for 5 min to detach from the plate, then collected and spun down at 1,600 x g, 4°C, for 5 min. The cell pellet was resuspended in RIPA (Sigma Aldrich) and incubated on a tube rotator at 4°C for 15 min, then spun down at 13,000 x g, 4°C, for 15 min. Supernatant was collected and total protein was quantified with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). About 10 μg of total protein was mixed with 2 x Laemmli buffer (Sigma Aldrich), boiled at 95°C for 5 min, and then resolved on a Novex 8% Tris-glycine mini gel (Invitrogen) in Tris-glycine SDS running buffer. Proteins on the gel were transferred to a 0.45 μm PVDF membrane (Invitrogen) in 20% methanol-tris-glycine buffer using a semi-dry transfer module. The membrane was blotted with anti-HA and anti-β-actin antibodies (Sigma Aldrich) in 5% milk blocking buffer overnight at 4°C in a shaking incubator, and all the colonies on the plate were collected. The cell suspension was cultured for 6 h at 37°C to the EMX1 sequence and a reverse primer (Table S3). The PCR products were assembled into pQCXIP vector between NotI and PmeI (GE Healthcare Life Sciences).

**PAM screening assay**

A randomized PAM library was constructed by amplifying the EMX1 target sequence TCATCTGTGCCCCCTCCCTCCCTG with forward primers that contained a randomized 5’ region and carrying the designed mutations. Heatmaps predicted using TIDE package available at http://shinyapps.datacurators.nl/tide/ or TIDER package at http://shinyapps.datacurators.nl/tider/. For TIDER analysis, reference sequences were generated by sequencing gBlocks resembling the target region and carrying the designed mutations.

Western blots

Transfected cells were washed with cold PBS and incubated in PBS on ice for 5 min to detach from the plate, then collected and spun down at 1,600 x g, 4°C, for 5 min. The cell pellet was resuspended in RIPA (Sigma Aldrich) and incubated on a tube rotator at 4°C for 15 min, then spun down at 13,000 x g, 4°C, for 15 min. Supernatant was collected and total protein was quantified with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). About 10 μg of total protein was mixed with 2 x Laemmli buffer (Sigma Aldrich), boiled at 95°C for 5 min, and then resolved on a Novex 8% Tris-glycine mini gel (Invitrogen) in Tris-glycine SDS running buffer. Proteins on the gel were transferred to a 0.45 μm PVDF membrane (Invitrogen) in 20% methanol-tris-glycine buffer using a semi-dry transfer module. The membrane was blotted with anti-HA and anti-β-actin antibodies (Sigma Aldrich) in 5% milk blocking buffer overnight at 4°C in a shaking incubator, and all the colonies on the plate were collected. The cell suspension was cultured for 6 h at 37°C to the EMX1 sequence and a reverse primer (Table S3). The PCR products were assembled into pQCXIP vector between NotI and PmeI (GE Healthcare Life Sciences).

**PAM screening assay**

A randomized PAM library was constructed by amplifying the EMX1 target sequence TCATCTGTGCCCCCTCCCTCCCTG with forward primers that contained a randomized 5’ region and carrying the designed mutations. Heatmaps predicted using TIDE package available at http://shinyapps.datacurators.nl/tide/ or TIDER package at http://shinyapps.datacurators.nl/tider/. For TIDER analysis, reference sequences were generated by sequencing gBlocks resembling the target region and carrying the designed mutations.
an Akta explorer fast protein liquid chromatography (FPLC) system (GE Healthcare). In brief, cells were harvested by centrifugation and resuspended in sonication buffer (50 mM NaH2PO4, 500 mM NaCl, 10 mM imidazole [pH 8.0], and 10% glycerol), sonicated on an icer-water bath for 20 min at 18 W output, and centrifuged for 25 min at 50,000 × g. Cas12a was isolated from the sonicated supernatant by adsorption to a 16 mL HisTrap FF column (GE Healthcare) and eluted with linear gradient from 10 mM to 300 mM imidazole in sonication buffer. The elution fraction containing the protein was pooled and concentrated to 20 mL using a 50 kDa cutoff ultrafiltration unit (Millipore). A ratio of 1 mg of TEV protease to 50 mg of Cas12a was added to the concentrate and dialyzed against TEV protease buffer (250 mM NaCl, 20 mM HEPES pH 7.4, 0.5 mM EDTA, and 1 mM DTT) for 48 h at 4°C.

The digested protein was diluted 2-fold with 20 mM HEPES (pH 7.0) and loaded on a 5 mL Hitrap SP HP column (GE Healthcare) equilibrated with 100 mM NaCl, 20 mM HEPES (pH 7.0). Adsorbed protein was eluted with a linear gradient from 100 mM to 2 M NaCl. Cas12a was purified further by gel filtration through a Superdex 200 26/60 column (GE Healthcare) equilibrated with gel filtration buffer (500 mM NaCl, 20 mM HEPES pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol). Fractions containing Cas12a were pooled and concentrated to 4 mL with a 50 kDa cutoff ultrafiltration unit (Millipore). Finally, the concentrate was divided and loaded to 1 mL endotoxin removal columns (Pierce) for overnight incubation. The next morning, each column was washed with 3 mL of sample buffer (400 mM NaCl, 20 mM HEPES, pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol). The eluted protein was pooled and concentrated to 40 mg/mL using a 50 kDa cutoff ultrafiltration unit (Millipore). Purity of Cas12a protein was confirmed by SDS-PAGE. A 260/280 ratio of ~0.5 was measured using NanoDrop (Thermo Scientific), indicating no nucleic acid contamination.

RNP formation and electroporation
crRNAs for Cas12a variants were ordered from IDT. crRNAs were resuspended in IDTE (10 mM Tris, 0.1 mM EDTA; IDT) and annealed by incubation at 95°C for 5 min and cooling down on bench top for 1 h. For each electroporation sample, RNP complexes were formed by mixing 240 pmol of each Cas12a variant with 480 pmol of crRNA and PBS. The RNP mixture was incubated at room temperature for 30 min to 1 h, then added with 400 pmol of single-stranded DNA repair templates and immediately mixed with cell suspension in electroporation solution. HEK293T, K562, and JeKo-1 cells (ATCC) were electroporated using Lonza 2B or 4D modules according to Lonza electroporation protocol. HEK293T cells were transfected with varying amounts of Cas12a and crRNA plasmids. 36 h after transfection, glycyl-phenylalanyl-amino-fluorocoumarin (GF-AFC) substrate was added to each well as indicated by manufacturer’s protocol (CellTiter-Fluor Cell Viability Assay, Promega) and incubated for 30 min. Digitonin was added to positive control wells minutes before substrate addition. Fluorescence was detected in a SpectraMax M5 plate reader with excitation at 380 nm and emission at 505 nm.

Protein sequence and structure analyses
Alignments of Lb2Cas12a with its orthologs were performed using MUSCLE, available at https://www.ebi.ac.uk/Tools/maa/muscle/ and were visualized using ESPript 3.0, available at http://espript.ibcp.fr/ESPript/ESPript/.

A model of Lb2Cas12a was generated from the LbCas12a structure (42% sequence similarity; PDB: 5XUS) using Chimera Modeler. The model score GA341 equals 1.00, indicating a probability of having the correct fold that is >95%, and the normalized discrete optimized protein energy (zDOPE) score is −0.47.

Statistics
Student’s t test, one-way ANOVA, and two-way ANOVA with Tukey’s, Dunnett’s, or Sidak’s multiple comparison tests were performed in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Rank ordered indel % values (Figure 5A) were compared using nonparametric comparisons with control using the Steel method, where control was Lb2-KY. The analysis was done in JMP Pro 15.1.0 (SAS Institute, Cary, NC, USA). In all tests, differences were considered significant at p <0.05.
REFERENCES

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

M.H.T., G.Z., H.M., and M.F. conceived of this study. C.L.N. and M.F. wrote the manuscript.

ACKNOWLEDGMENTS

This work is supported by NIH grants R37 AI091476 and DP1 DA043912 (PI: M.F.). The authors would like to thank Brian Quinlan, PhD, Shruti Choudhary, PhD, and Matthew Costales, PhD, for experimental advice, and Matthew Gardner, PhD, and Meredith Davis Gardner, PhD, for experimental advice and careful critique of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Barrangou, R., Fremeaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709–1712.

2. Marraffini, L.A. (2015). CRISPR-Cas immunity in prokaryotes. Nature 526, 55–61.

3. Mohanraj, P., Makarova, K.S., Zetsche, B., Zhang, F., Koonin, E.V., and van der Oost, J. (2016). Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. Science 353, aad5147.

4. Pickar-Oliver, A., and Gersbach, C.A. (2019). The next generation of CRISPR-Cas technologies and applications. Nat. Rev. Mol. Cell Biol. 20, 490–507.

5. Makarova, K.S., Wolf, Y.I., Iranzo, J., Shmakov, S.A., Alkhinbashi, O.S., Brouns, S.J.J., Charpentier, E., Cheng, D., Haft, D.H., Horvath, P., et al. (2020). Evolutionary classification of CRISPR-Cas systems: a bust of class 2 and derived variants. Nat. Rev. Microbiol. 18, 67–83.

6. Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Vols, S.E., Joung, J., van der Oost, J., Regev, A., et al. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163, 759–771.

7. Wang, Y., Liu, K.I., Srinivasan, H., Zhang, J., Li, J., Zhang, F., Laibh, C.R.I., Xing, H., Shanmugam, R., et al. (2018). Systematic evaluation of CRISPR-Cas systems reveals design principles for genome engineering in human cells. Genome Biol. 19, 62.

8. Singh, D., Mallon, J., Poddar, A., Wang, Y., Tippapa, R., Yang, O., Bailey, S., and Ha, T. (2018). Real-time observation of DNA target interrogation and product release by the RNA-guided endonuclease CRISPR Cpf1 (Cas12a). Proc. Natl. Acad. Sci. USA 115, 5444–5449.

9. Hewes, A.M., Sansbury, B.M., and Kmiec, E.B. (2020). The Diversity of Genetic Outcomes from CRISPR/Cas Gene Editing is Regulated by the Length of the Symmetrical Donor DNA Template. Genes (Basel) 11, 1160.

10. Zetsche, B., Heidenreich, M., Mohanraj, P., Fedorova, I., Kneppers, J., DeGennaro, E.M., Winblad, N., Choudhury, S.R., Abudayyeh, O.O., Gootenberg, J.S., et al. (2017). Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. Nat. Biotechnol. 35, 31–34.

11. Zhong, C., Wang, H., Li, Y., Tran, M.H., and Farzann, M. (2017). Cpf1 proteins excise CRISPR RNAs from mRNA transcripts in mammalian cells. Nat. Chem. Biol. 13, 839–841.

12. Yan, W.X., Mirrazazadeh, R., Garnerone, S., Scott, D., Schneider, M.W., Kallas, T., Custodio, J., Wernersson, E., Li, Y., Gao, L., et al. (2017). BLISS is a versatile and quantitative method for genome-wide profiling of DNA double-strand breaks. Nat. Commun. 8, 15058.

13. Strehlendl, L., Suifuldin, F.A., Rybarski, J.R., Finkelstein, I.J., and Russell, R. (2018). Kinetic Basis for DNA Target Specificity of CRISPR-Cas12a. Mol. Cell 71, 816–824.e3.

14. Charlesworth, C.T., Deshpande, P.S., Dever, D.P., Camarena, J., Lempart, V.T., Comer, M.K., Vakulskas, C.A., Collingwood, M.A., Zhang, L., Bode, N.M., et al. (2019). Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nat. Med. 25, 249–254.

15. Gao, L., Cox, D.B.T., Yan, W.X., Manteiga, I.B., Schneider, M.W., Yamano, T., Nishimaru, H., Nureosi, C., Cot illego, N., and Zhang, F. (2017). Engineered Cpf1 variants with altered PAM specificities. Nat. Biotechnol. 35, 789–792.

16. Kleinstiver, B.P., Sousa, A.A., Walton, R.T., Tak, Y.E., Hsu, J.Y., Clement, K., Welch, M.M., Horng, J.E., Malagon-Lopez, J., Scarfo, I., et al. (2019). Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. Nat. Biotechnol. 37, 276–282.

17. Grieger, I.C., and Samulski, R.J. (2005). Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. J. Virol. 79, 9933–9944.

18. Kim, H.K., Song, M., Lee, J., Menon, A.V., Jung, S., Kang, Y.M., Choi, J.W., Woo, E., Koh, H.C., Nam, J.W., and Kim, H. (2017). In vivo high-throughput profiling of CRISPR-Cpf1 activity. Nat. Methods 14, 153–159.

19. Töth, E., Weinhardt, N., Bencsura, P., Huszár, K., Kuleszir, P.I., Tálas, A., Fodor, E., and Welker, E. (2016). Cpf1 nuclease demonstrates robust activity to induce DNA modification by exploiting homologous directed repair pathways in mammalian cells. Biol. Direct 11, 46.

20. Kleinstiver, B.P., Tsai, S.Q., Prew, M.S., Nguyen, N.T., Welch, M.M., Lopez, J.M., McCaw, Z.R., Aryee, M.J., and Joung, J.K. (2016). Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. Nat. Biotechnol. 34, 869–874.

21. Robinson, O., Deylus, D., and Deslimoz, C. (2016). Phylo.io: Interactive Viewing and Comparison of Large Phylogenetic Trees on the Web. Mol. Biol. Evol. 33, 2163–2166.

22. Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 770–780.

23. Guschin, D.Y., Waite, A.J., Katiabah, G.E., Miller, J.C., Holmes, M.C., and Rebar, E.J. (2010). A rapid and general assay for monitoring endogenous gene modification. Methods Mol. Biol. 649, 247–256.

24. Vuillot, L., Thelie, A., and Pellet, N. (2015). Comparison of T7EI and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3 (Bethesda) 5, 407–415.

25. Stella, S., Alcón, P., and Montoya, G. (2017). Structure of the Cpf1 endonuclease R-loop complex after target DNA cleavage. Nature 546, 559–563.

26. Swarts, D.C., van der Oost, J., and Jinek, M. (2017). Structural Basis for Guide RNA Processing and Seed-Dependent DNA Targeting by CRISPR-Cas12a. Mol. Cell 66, 221–233.e4.

27. Cofsky, J.C., Karandur, D., Huang, C.J., Witte, I.P., Kuriyan, J., and Doudna, J.A. (2020). CRISPR-Cas12a exploits R-loop asymmetry to form double-strand breaks. eLife 9, e55143.

28. Wilson, K.A., Kellie, J.L., and Wetmore, S.D. (2014). DNA-protein π-interactions in nature: abundance, structure, composition and strength of contacts between aromatic
