Synergistic engineering of CRISPR-Cas nucleases enables robust mammalian genome editing

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GRAPHICAL ABSTRACT

1. Cas nucleases with low efficiency
   - Cas12i
   - Cas12b
   - CasX

2. Robustly enhanced editing activity
   - MIDAS
   - Cas12i
   - Cas12b
   - CasX

a. High efficiency
   - Efficiency (%)
   - 0 1 2 3 4
   - 0 40 60 80

b. Broad targeting range
   - FRT
   - Cas12iMax → Cas12iHiFi

c. Increased specificity
   - Cas12iMax
   - Cas12iHiFi

PUBLIC SUMMARY
- Improving Editing Activity by Synergistic Engineering (MIDAS) of Cas nucleases
- MIDAS can improve the activity of Cas12i, Cas12b, and CasX
- Engineering high-efficiency Cas12iMax and high-specificity Cas12iHiFi
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RESULTS

Rationale for MIDAS

CRISPR-Cas nuclelease-based DNA editing generally includes two processes: recognition and then hydrolysis of DNA. The former ensures the binding of a Cas nuclease to the correct PAM duplexes and the formation of a cleavage-ready complex, while the latter allows cleavage of DNA substrates encased in the catalytic pocket. The ability to accomplish these two intrinsic steps determines the catalytic activity of a given Cas nuclease. However, relative to microbial cells, the more complex cellular environments of mammalian cells may make it harder to cleave DNA.

To that end, we developed MIDAS to concurrently enhance the interactions between Cas nucleases and the PAM duplex (EIP), and the interactions between the catalytic pocket and ssDNA substrate (EIS), aiming for facilitating both DNA recognition and hydrolysis.

MIDAS enhances the nuclease activity of Cas12i in human cells

Cas12i is a recently discovered CRISPR-Cas system from metagenomes with a promising potential due to its small protein size, simple CRISPR RNA (crRNA), and PAM requirements as well as its pre-crRNA processing capacity. We first evaluated the efficiency of the wild-type Cas12i nuclease to cleave DNA in human cells. The Cas12i protein flanked by two SV40 nuclear localization signals (NLSs) was expressed from a CAG promoter, while its cognate mature crRNA was with a 20 nucleotide (nt) spacer was expressed by a U6 polymerase III promoter. The two vectors were transfected into human HEK293T cells. Subsequently, 72 h post-transfection, EGFP-positive cells were collected by fluorescence-activated cell sorting (FACS), and high-throughput sequencing was used to measure the gene-editing efficiency.

We then used MIDAS to engineer CRISPR-Cas12i variants. At the EIP step, two amino acids of Cas12i, Q163 and N164, whose side chains stack on the last base pair of the PAM duplex, were first engineered to facilitate the opening of the downstream double-stranded DNA (dsDNA). While mutating both amino acids into an alanine to disrupt the stacking interactions impairs the catalytic activity of Cas12i, here, we substituted them with residues carrying bigger, flat aromatic rings (e.g., phenylalanine [F], tyrosine [Y], or tryptophan [W]) to reinforce the stacking interaction with the last base pair of the PAM duplex. The editing efficiencies of the engineered Cas12i variants and the wild-type enzyme at two gene loci (CCR5 and RNF2) were evaluated in the human HEK293T cells. Notably, variants Q163W, N164Y, and N164F showed robustly increased gene-editing efficiency at both sites. Next, we substituted the amino acids surrounding the PAM duplex with positively charged arginine to increase their electrostatic attraction to the negatively charged phosphated backbone. A similar strategy was used for energy compensation in previous studies during the engineering of Cas nucleases with a broadened PAM recognition profile. In our study, 10 variants were tested at two human genomic sites, and four of these variants (E176R, K238R, T447R, and E563R) exhibited increased indel frequency. New variants harboring combinations of these four mutations had higher gene-editing activities.
Subsequently, we combined amino-acid alterations of variant E176R/K238R/T447R/E563R and variant N164Y/E176R/K238R/E176R/K238R/T447R/E563R as the final candidate from the EIP step optimization.

For the EIS step efficiency optimization, we mutated the non-catalytic residues in the catalytic pocket into arginine to increase their binding energy to the ssDNA substrate, aiming to substantially enhance enzymatic catalysis.7,15 First, residues around the ssDNA substrate in the catalytic pocket of Cas12i were candidates for mutagenesis (Figure 3A). Following screening of 14 variants, I926R showed the highest gene-editing activity (Figure 3B). To search for more gain-of-function amino-acid mutations, we superimposed a 9 nt ssDNA substrate from Cas12i into the catalytic pocket of Cas12i (Figures 3C and 3D). Of note, centered around the ssDNA substrate in the catalytic pocket of Cas12i were candidates from the EIP step optimization.

We then implemented the last SE step of MIDAS by combining the optimized mutations from the EIP and EIS steps. Robust and significant synergistic effects were observed when we combined E176R/K238R/T447R/E563R, and/or E176R (Figures 3G and 3H). Based on the above data, we chose variants I926R, E176R/K238R/T447R/E563R, and E176R/K238R/I926R as candidates from the EIS step optimization.

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Next, we investigated the expandability of MIDAS for engineering other classes of the Cas nucleases.16 Considering the generally similar catalytic mechanism of different single-effect Cas nucleases,16 we next investigated the expandability of MIDAS in engineering other classes of the Cas nucleases. Specifically, in the EIP step, amino acids stacking with the last PAM base pair were mutated into W, Y, or F and/or amino acids surrounding the PAM duplex were mutated into R. In the EIS step, amino acids surrounding ssDNA substrate were mutated into R. In the last SE step, the modification from EIP and EIS were combined into one protein. R, W, Y, and F represent arginine, tryptophan, tyrosine, and phenylalanine, respectively.

Efficacy of MIDAS in engineering Cas nucleases

Next, we investigated in depth the efficacy of MIDAS in engineering Cas nucleases. MIDAS-engineered Cas12i variant N164Y/E176R/K238R/T447R/E563R, which was named Cas12iMax, was chosen to test (Figure 4A). First, we assessed the gene-editing activity of Cas12iMax at 62 randomly chosen sites from five different genes, covering all of the possible canonical NTTN PAMs. Notably, Cas12iMax displayed highly robust genome-editing activity at all of the sites tested, with the average editing efficiency reaching up to 73% (Figure 5A). Importantly, the indel frequency of Cas12iMax at 56 sites surpassed 60%, indicating that the high gene-editing activity of Cas12iMax would be less target-focus dependent (Figure 5A). Next, we explored the activity of Cas12iMax at targeted sites carrying non-NTTN PAMs, which are not amenable for cleavage by wild-type Cas12i. Here, 60 targeted sites bearing NTVN, NTNN, and NVTN PAMs (V = C, G, or A) were randomly chosen for testing. Impressively, Cas12iMax potently edits sites bearing NTVN, NTNN, NVTN, and NVTN PAMs (V = C, G, or A), with an average editing efficiency over 40% (Figure 5B). To confirm the robustness of Cas12iMax in recognizing a broad range of PAMs, wild-type Cas12i and Cas12iMax proteins were purified (Figure S4A), and their cleavage activities were compared at targets carrying the same protospacer and different NNNN PAMs in vitro. Remarkably, Cas12iMax nearly fully digested dsDNA with NTVN, NTAN, NTNN, NAAN, and NCAN PAMs and was efficient at NTGN, NACN, and NGAN, while wild-type Cas12i only partially cleaved dsDNA with an NTTN PAM (Figures 5C and 5B). Encouraged by these results, we systematically characterized the PAM recognition profile of Cas12iMax using a previously reported assay called PAM-DOSE19 with some modifications. Briefly, DNA fragments containing recognizable PAMs of Cas12iMax were collected by gel purification for high-throughput-sequencing analysis (Figure S4B). Of note, only subtle base preferences at -3, -2, and -1 positions of 5’-NNNN PAM were observed (Figure 5E). Further analyzing the frequency of each NNNN demonstrated that NGGN, NCGN, and NGCN were the only ones with minor proportions, indicating that Cas12iMax exhibits very simple PAM requirements (Figure 5F). This result is consistent with our observation that Cas12iMax enables dsDNA cleavage at all of the NNNN PAMs except at NGGN, NCGN, and NGCN (Figures 5A–5D). Collectively, these data demonstrate that the MIDAS enables the Cas nuclease with highly potent gene-editing activity both at canonical and non-canonical PAMs.

To further evaluate the efficacy of MIDAS, we compared the gene-editing activity of Cas12iMax with currently widely used Cas nucleases, while the PAMs of the
The Innovation

The advantageous features of Cas12iMax, including smaller protein size, high editing efficiency, and capability of pre-crRNA processing, make it highly suitable for multiplexed gene editing in vivo. To explore this potential, we tested the editing efficiency of AAV expression plasmids containing Cas12iMax and crRNA targeting single genes (Pcsk9, Angptl3, and Apcoc3) or one pre-crRNA array simultaneously targeting the three genes in mouse-derived Hepa1-6 cells (Figure 5H). Of note, Cas12iMax robustly edited the three genes with a single pre-crRNA array at a comparable or higher indel level as a single crRNA (Figure 5H).

Figure 2. Implementing the EIP step of MIDAS to engineer Cas12i. (A) Amino-acid stacking on the last base pair of PAM of Cas12i2 is indicated in hot pink. The last base pair of PAM was annotated as -1 (when the 20 bp targeted dsDNA was counted from +1 to +20) and highlighted by a dashed box. Orange, PAM duplex. (B) Mutating Q163 and N164 into W, Y, or F for increased gene-editing efficiency. Error bars represent standard deviation (SD) of mean, n = 3 biological replicates. (C) Amino acids chosen for arginine substitution are marine blue. Orange, PAM duplex. (D) Testing the genome-editing activity of variants at two targeted sites. Error bars represent SD of mean, n = 2 or 3 biological replicates. (E) The gene-editing activity of new Cas12i2 variants with combinations of the E176R, K238R, T447R, or E563R mutations. Error bars represent SD of mean, n = 2 or 3 biological replicates. (F) The PAM duplex of Cas12i2 is indicated in orange. Variants with robustly improved genome-editing activity are listed in the table. Corresponding mutated amino acids are indicated in red. The number in each cell of the heatmap represents the mean indel frequency averaged from n = 3 biological replicates. Two-way ANOVA test, **** p < 0.0001.

An additional amino-acid mutation improves the specificity of Cas12iMax

Considering the high gene-editing activity and broad PAM recognition profile of Cas12iMax, it is particularly important to evaluate the specificity of this MIDAS-engineered system. Initially, we determined the degree of Cas12iMax to tolerate mismatch(es) between spacer and target DNA. At RNF2 endogenous locus, some degree of tolerance was observed at crRNAs with single or double mismatches (Figure S5A). These results indicated that Cas12iMax might have off-target effects when targeting genes. Indeed, GUIDE-seq analysis showed that Cas12iMax has relatively more off-target sites compared with other widely applied Cas nucleases (Figure S5C). Analysis of the off-target profiles demonstrated that these off-target effects are attributed to simple PAM requirements and some degree of mismatch tolerance of Cas12iMax (Figure S5D). In order to increase the specificity of Cas12iMax, we mutated amino acids with positive charge (arginine or lysine), which surrounds the RNA-DNA heteroduplex formed by spacer RNA and DNA, aiming to reduce the mismatch tolerance of Cas12iMax. After screening via crRNA targeting mCherry or an endogenous gene, a new variant of Cas12iMax, with only
Figure 3. Implementing the EIS step of MIDAS to engineer Cas12i

(A) Amino acids chosen for arginine substitution around the 5 nt ssDNA substrate are indicated in marine blue. Orange, PAM duplex; green, catalytic amino acids (D599, E833, D1017). (B) Testing the genome-editing activity of variants at two targeted sites. Error bars represent SD of mean, n = 2 or 3 biological replicates. (C) Protein BLAST result for Cas12i2 and Cas12i1. (D) Superimposition of the 3D structures of Cas12i2 and Cas12i1. Orange, Cas12i2; teal, Cas12i1. (E) Amino acids chosen for arginine substitution around the 9 nt ssDNA substrate aligned from Cas12i1 are indicated in red. Orange, PAM duplex; green, catalytic amino acids. (F) Testing the genome-editing activity of variants at two targeted sites. Error bars represent SD of mean, n = 2 or 3 biological replicates. (G) The gene-editing activity of new Cas12i2 variants with a combination of the E323R and D362R mutations at two targeted sites. Error bars represent SD of mean, n = 2 or 3 biological replicates. (H) The 5 nt ssDNA substrate of Cas12i2 is indicated in orange, and the 9 nt ssDNA substrate from Cas12i1 superimposed on Cas12i2 is indicated in red. Variants with robustly improved genome-editing activity are listed in the table. Corresponding mutated amino acids are highlighted in marine blue. The number in each cell of the heatmap represents the mean indel frequency from n = 3 biological replicates. Two-way ANOVA test, ****p < 0.0001.
an additional single mutation, K394A, exhibited largely decreased mismatch tolerance and maintained most on-target activity of Cas12iMax (Figure S6B, S5C, 6A, and 6B). We designated this high-fidelity version of Cas12iMax as Cas12iHiFi and further evaluated its specificity using GUIDE-seq. Impressively, no off-target sites for Cas12iMax were detected on a genome-wide scale (Figure 6C; Figure S6). Collectively, these results demonstrate that the fidelity of Cas12iMax can be further engineered to generate Cas12iHiFi, which offers minimized off-target effects for applications that require high specificity.

**DISCUSSION**

In this study, we developed a powerful Cas protein-engineering method named MIDAS, which can significantly improve CRISPR-Cas mammalian genome-editing efficiency by increasing the affinity of Cas proteins with PAM and with ssDNA substrate in the catalytic pocket. Using MIDAS, we optimized Cas nucleases from diverse CRISPR branches (Figure 1A). Of note, significant and robust synergistical effects on increasing gene-editing efficiency were observed in all cases in our study when we combined gain-of-function mutations from the EIP and EIS optimization steps into a single Cas nuclease. This is particularly obvious for Cas12a, when targeting endogenous genomic loci, which are not amenable to throughput characterization, and the variety of the powerful computational tools, MIDAS engineering has great potential to be applied to the optimization of a large numbers of Cas proteins.

In addition to its great expandability, the efficacy of MIDAS in engineering Cas nuclease is also impressive. Exemplarily, one of the MIDAS-engineered nucleases, Cas12iMax, exhibited potent genome-editing activity at canonical NTTN PAMs with higher indel frequency than current widely used Cas genome editors like AsCas12a, BhCas12b, SpCas9, SaCas9, and SaCas9-KKH. Importantly, the editing efficiency of Cas12iMax at 56/62 sites exceeded 60%, which indicates that the potent editing activity of Cas12iMax in human cells might be less targeted-locus dependent, allowing editing of more genomic loci. A further study is required to understand the relationship between the activity profiles of Cas12iMax and the target sequence compositions in the mammalian cells. In addition, MIDAS also enables Cas12i to robustly edit at sites carrying non-NTTN PAMs. The results from high-throughput characterization, in vitro cleavage assay, and in cellular validation in multiple targeted sites demonstrated that Cas12iMax has a very broad PAM targeting range (NNNN, NNNT, NAAN, and NCAN). This expanded targeting flexibility is likely due to the amino-acid substitutions from the EIP step optimization, which created new non-specific interactions between the Cas protein and PAM duplex.

Its robust gene-editing efficiency, along with the ability to process pre-crRNA, makes Cas12iMax highly suitable for multiplexed gene editing in vivo. To test this potential, cassettes expressing Cas12iMax and pre-crRNA were cloned into a single AAV expression plasmid. The size of the entire construct was smaller than 4.7 kilobase pairs (kb), which can be efficiently packaged into one AAV. Using pre-crRNA to simultaneously edit multiple genes in the mammalian cells, we observed that Cas12iMax achieves a comparable or higher indel as using a single crRNA separately. Moreover, we systematically analyzed the targeting scope of Cas12iMax in the human genome. Remarkably, Cas12iMax can potentially target 70.1% of the entire human genome (Figure S7A), which vastly outnumbers other
more commonly used Cas nucleases that could also be effectively packaged into one AAV. To our knowledge, except the SpCas9-SpRY variant, the PAM requirement of Cas12i would be the simplest among all the Cas nucleases that have been reported. On the other hand, the ability to discriminate between NTN, NNT, NAAN, and NCAN and NRRN and NACN (R = C or G) makes Cas12i highly suitable for allele-specific gene editing, an important strategy to treat human heterozygous dominant genetic diseases. To explore this potential, we analyzed the number of human dominant point mutations in the ClinVar database to identify suitable PAMs for Cas12i to edit (Figure S7B). Notably, CassiMax could theoretically distinguish between the mutant from the wild type for 11,668 dominant alleles (52.4% of all dominant entries), which is higher than other more commonly used Cas nucleases (Figure S7C). Taken together, these results demonstrate that MIDAS-engineered Cas12iMax could be a new generation of a powerful and versatile gene-editing tool.

Previous studies have demonstrated that Cas nucleases with extremely flexible PAM recognition profiles exhibit off-target effects (e.g., SpRY).
Indeed, our GUIDE-seq experiments showed that Cas12i\textsuperscript{Max} has relatively more potential off-target sites compared with other widely applied Cas nucleases (Figure 6C). To minimize the off-target effects of Cas12i\textsuperscript{Max}, we introduced one additional amino-acid mutation to create a high-fidelity version of Cas12i\textsuperscript{Max} (Cas12i\textsuperscript{HiFi}). Notably, Cas12i\textsuperscript{HiFi} exhibited undetected off-target effects via GUIDE-seq and maintained nearly 90% on-target activity of Cas12i\textsuperscript{Max} (Figures 6B and 6C). We believe that high-fidelity Cas12i\textsuperscript{Max} offers minimized off-target effects for applications that need high specificity, especially in the biomedical field.

**MATERIALS AND METHODS**

Materials and methods related to this work are available in Supplemental information.

**Data availability**

The sequencing data have been deposited in Genome Sequence Archive for human (GSA-Human) of Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/gsa-human/). The accession number for the sequencing data reported in this paper is HRA001700.
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AUTHOR CONTRIBUTIONS

W.L., Q.Z., and Y.-C.C. conceived the project and designed the experiments; Y.-C.C., Y.-P.H., W.L., Q.Z., and Y.-C.C. wrote the manuscript with assistance from the other authors.

DECLARATION OF INTERESTS

Patients related to this work have been filed.

SUPPLEMENTAL INFORMATION

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