Al3Cas12f RKK: A New, Enhanced Compact Editor that is Highly Efficient

CRISPR-based gene-editing tools have transformed the biomedical field, but delivering these systems directly into human tissues — known as in vivo gene editing — remains a major challenge. Many therapeutic CRISPR approaches still rely on ex vivo editing, where the patient's cells are removed, edited outside the body, and reinfused. This method is used partly because commonly used CRISPR proteins are relatively large, making it difficult to package them efficiently into delivery vehicles such as adeno-associated viruses (AAVs), which have strict cargo limits.

To overcome these packaging restrictions, researchers have increasingly focused on smaller CRISPR-Cas systems that could be delivered directly into the body. One promising family is the Cas12f enzymes — compact bacterial "molecular scissors" that are significantly smaller than traditional CRISPR editors. Their unusually small size may allow the entire editing system, including the CRISPR enzyme and guide RNA (gRNA), to fit within a single AAV vector for in vivo delivery. This could make compact editors particularly useful for tissues that are difficult to treat, including muscle, eye, liver, brain, and heart tissue.

In addition to standard DNA cutting, compact Cas12f systems are being adapted for a growing range of genome-engineering applications, including base editing, gene activation or repression, epigenetic modulation, and exon skipping or deletion. However, many naturally occurring Cas12f enzymes have shown inconsistent or target-dependent activity in human cells, limiting their therapeutic potential. A newly identified Cas12 enzyme may help overcome these limitations.

Al3Cas12f: A Newly Identified Cas12f Variant

Researchers from the University of Texas at Austin, funded by the National Institutes of Health (NIH) and in partnership with the biotech company Metagenomi, have identified and characterized a naturally occurring Cas12f variant, Al3Cas12f, that demonstrates robust genome editing across multiple human cell lines. Their findings, published in Nature Structural & Molecular Biology, suggest that the compact editor could help advance more practical in vivo gene-editing therapies.

The newly identified enzyme consists of roughly 400–600 amino acids, making it significantly smaller than widely used CRISPR editors like Cas9. In experiments, the system demonstrated high editing rates across multiple genomic sites, with multiple targets achieving editing efficiencies exceeding 90% in human cell line screens (e.g., AAVS1 and APOA1 loci), though results varied across genomic sites (1).

Running the Analysis: Why Al3Cas12f May Perform Better

The researchers found that Al3Cas12f outperformed the two other Cas12f orthologs — OsCas12f and RhCas12f — across several tested genomic targets. Previous work had already established OsCas12f1 and RhCas12f1 as among the most promising miniature CRISPR systems for therapeutic editing, making them important benchmarks for evaluating the newly identified Al3Cas12f enzyme (2). Earlier studies also showed that miniature Cas12f nucleases exhibit diverse PAM preferences — the short DNA sequences CRISPR systems must recognize before cutting — expanding the range of genomic sites accessible to compact CRISPR editors and highlighting the substantial functional diversity within the Cas12f family (3).

To understand why the enzyme performed so well, the team compared the three systems using cryo-electron microscopy, biochemical assays, and kinetic experiments designed to measure how quickly the enzymes bind DNA, form editing complexes, and cleave their targets (1).

Their analyses revealed several structural advantages unique to Al3Cas12f. The enzyme forms a highly stable "dimer," meaning two protein units fit together in an unusually secure configuration. David Taylor, a UT molecular biosciences professor and study co-author, explained that compared to the other orthologs, Al3Cas12f "basically comes preassembled," allowing it to form an active editing complex more readily. The authors found that this stable interface helps the enzyme bind DNA more efficiently and remain active during editing (1).

The researchers also discovered that Al3Cas12f appears naturally optimized to interact with its guide RNA (gRNA), the molecule that directs the enzyme to the correct DNA sequence. These optimized interactions stabilize the editing complex and improve DNA targeting and cleavage efficiency. In addition, the study identified differences among the Cas12f systems in how they recognize PAM sequences, bind guide RNA, and regulate DNA cleavage.

One of the study's most important findings was the formation of the "R-loop," a temporary opening of the DNA double helix required for gene editing. The researchers found that competing Cas12f systems often stalled during this step and formed only partial R-loops, while

Al3Cas12f formed the structure more efficiently and consistently. This appeared to allow the enzyme to proceed through the editing process more smoothly and rapidly than related systems. The team also found that once Al3Cas12f formed the R-loop, it was less likely to reverse course before cutting the DNA, which the researchers stated effectively pulls "the reaction toward the largely irreversible step of DNA cleavage" (1).

The work also revealed that closely related Cas12f enzymes can use surprisingly different structural strategies to recognize DNA and carry out editing, including differences in how they form stable dimers, interact with guide RNAs, stabilize R-loops, and rearrange catalytic domains during DNA cleavage. The authors said these mechanistic insights could eventually help researchers engineer customized CRISPR systems tailored for different tissues or diseases.

"We uncovered mechanistic features that explain why some Cas12f enzymes are more efficient than others," said Taylor in the press release. "With this understanding, we can begin to rationally design improved variants that outperform existing tools while maintaining a compact size that is ideal for delivery. Importantly, we also identified Al3Cas12f as a highly efficient nuclease across multiple genomic targets, making it a strong candidate for future therapeutic development."

RKK: Engineering a More Powerful Editor

Using these structural insights, the team engineered an enhanced version, Al3Cas12f RKK, that contains three amino acid substitutions designed to strengthen interactions with DNA. The researchers introduced the engineered system into a human leukemia-derived cell line and tested it against genomic targets associated with diseases, including cancer, atherosclerosis, and amyotrophic lateral sclerosis (ALS).

Across tested disease-relevant targets in a human leukemia cell line, the engineered Al3Cas12f RKK variant boosted editing efficiencies from 80% in several cases, with up to 26-fold improvement at individual sites (1).

While these efficiencies were measured in cultured human cells and will require validation in vivo, they highlight Al3Cas12f RKK's potential as a compact editor, consistent with improvements seen in other engineered miniature systems. "Overall, these results show that Al3Cas12f RKK is a highly efficient and compact nuclease that could be further optimized for genome editing," the authors stated (1).

This builds on prior engineering successes with other compact systems. Recent studies involving engineered systems, such as eCas12f1, have demonstrated that compact CRISPR editors can achieve editing efficiencies comparable to those of larger systems, such as Cas9, while retaining the delivery advantages of their smaller size (4). Al3Cas12f RKK joins a rapidly advancing class of compact CRISPR systems optimized for in vivo delivery. Additionally, in January 2025, researchers announced the discovery of a "mini-CRISPR" that could move the technology's in vivo abilities beyond the liver (5). Named NanoCas, in preclinical studies, the ultracompact CRISPR nuclease demonstrated potent editing capabilities across a range of cell systems and tissues in vivo when delivered to mice and in skeletal muscle of non-human primates via AAV

vectors. NanoCas's small size also leaves room for additional payloads, such as regulatory elements, guide RNAs, or non-double-strand-break editing machinery, enabling its use in techniques including reverse transcriptase editing, base editing, and epigenetic editing. Prior to NanoCas, in 2021, researchers from Stanford University engineered a small Cas system from Cas12. The system, which they call CasMINI, is 529 amino acids and has demonstrated efficient deletion, activation, and editing of the genetic code, while fitting comfortably within AAV packaging limits (6).

Additional engineering strategies — including circular guide RNAs, PAM-relaxed variants, and hypercompact fusion systems — continue to improve editing efficiency while preserving the small size advantage that makes Cas12f systems attractive for therapeutic delivery.

Smart delivery: Therapeutic Potential of Cas12f systems

Although Cas12f systems have not yet entered clinical trials, several recent preclinical studies have demonstrated their potential for in vivo gene therapy. Researchers have used engineered Cas12f editors delivered via a single AAV vector to restore dystrophin expression in mouse models of Duchenne Muscular Dystrophy (DMD), highlighting the promise of compact CRISPR systems for muscle-targeted therapies.

Other studies show that optimized Cas12f variants could successfully edit photoreceptor cells in mouse models of inherited retinal diseases, preserving retinal structure and improving visual function after AAV delivery. Additional research has targeted genes linked to cardiovascular and metabolic disorders, including PCSK9 and transthyretin, while enhanced Cas12f systems have also demonstrated cancer-related applications, such as disrupting tumor-associated genes and supporting base-editing or gene-regulation approaches.

Together, these studies suggest that miniature Cas12f editors could eventually support a broad range of therapies because their compact size allows them to fit within a single AAV delivery system.

"Smart delivery of gene-editing systems is a powerful notion with broad clinical implications, and this basic science finding takes us a significant step toward that future," said Erica Brown, Ph.D., acting director of NIGMS.

Limitations and Remaining Challenges

While the findings are promising, Cas12f systems are still in the preclinical stage. The Al3Cas12f study was mainly done in cultured human cells rather than in full therapeutic animal models. Additional work will be needed to evaluate off-target editing, long-term safety, immune responses, and performance in therapeutic delivery settings, including full AAV-based in vivo studies. Still, the findings provide important mechanistic insight into why some compact CRISPR systems perform better than others and offer new design principles for engineering more efficient miniature gene editors.

References:

1. Guan K, Ocampo RF, Matheus Carnevali PB, Castelle CJ, Gonzalez-Osorio L, Castanzo DT, Thomas NC, Brothers M, Dangerfield TL, Hooper MM, West MS, Appleby NM, Krudop I, Lamothe RC, Aliaga Goltsman DS, Alexander LM, Butterfield CN, Johnson KA, Brown CT, Taylor DW. Comparative characterization of Cas12f orthologs reveals mechanistic features underlying enhanced genome editing efficiency. Nat Struct Mol Biol. 2026 Apr 13. doi: 10.1038/s41594-026-01788-6. Epub ahead of print. PMID: 41975095.

2. Kong X, Zhang H, Li G, Wang Z, Kong X, Wang L, Xue M, Zhang W, Wang Y, Lin J, Zhou J, Shen X, Wei Y, Zhong N, Bai W, Yuan Y, Shi L, Zhou Y, Yang H. Engineered CRISPR-OsCas12f1 and RhCas12f1 with robust activities and expanded target range for genome editing. Nat Commun. 2023 Apr 11;14(1):2046. doi: 10.1038/s41467-023-37829-7. PMID: 37041195; PMCID: PMC10090079.

3. Sharrar A, Arake de Tacca L, Collingwood T, Meacham Z, Rabuka D, Staples-Ager J, Schelle M. Discovery and Characterization of Novel Type V Cas12f Nucleases with Diverse Protospacer Adjacent Motif Preferences. CRISPR J. 2023 Aug;6(4):350-358. doi: 10.1089/crispr.2023.0006. Epub 2023 Jun 2. PMID: 37267210.

4. Park SJ, Ju S, Jung WJ, Jeong TY, Yoon DE, Lee JH, Yang J, Lee H, Choi J, Kim HS, Kim K. Robust genome editing activity and the applications of enhanced miniature CRISPR-Cas12f1. Nat Commun. 2025 Jan 15;16(1):677. doi: 10.1038/s41467-025-56048-w. PMID: 39809780; PMCID: PMC11733285.

5. Rauch BJ, DeLoughery A, Sper R, Chen S, Yunanda S, Masnaghetti M, Chai N, Lin JC, Neckelmann A, Bjornson Y, Espino DP, Sancio A, Schmitt C, Scholes C, Shah R, Narendra PK, Ansaloni S, Tan S, Rukmini SJ, Somaiah S, Suresh S, Minami S, Tymoshenko S, Wright W, Xu S, Broughton J, Retwitzer MD, Bobbin M, Yuan D, Abe K, DeWitt M, Zhang B, Harrington LB. Single-AAV CRISPR editing of skeletal muscle in non-human primates with NanoCas, an ultracompact nuclease. bioRxiv. 2025 Jan 29. doi: 10.1101/2025.01.29.635576.

6. Xu X, Chemparathy A, Zeng L, Kempton HR, Shang S, Nakamura M, Qi LS. Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing. Mol Cell. 2021 Aug 26:S1097-2765(21)00648-1. doi: 10.1016/j.molcel.2021.08.008. Epub ahead of print. PMID: 34480847.