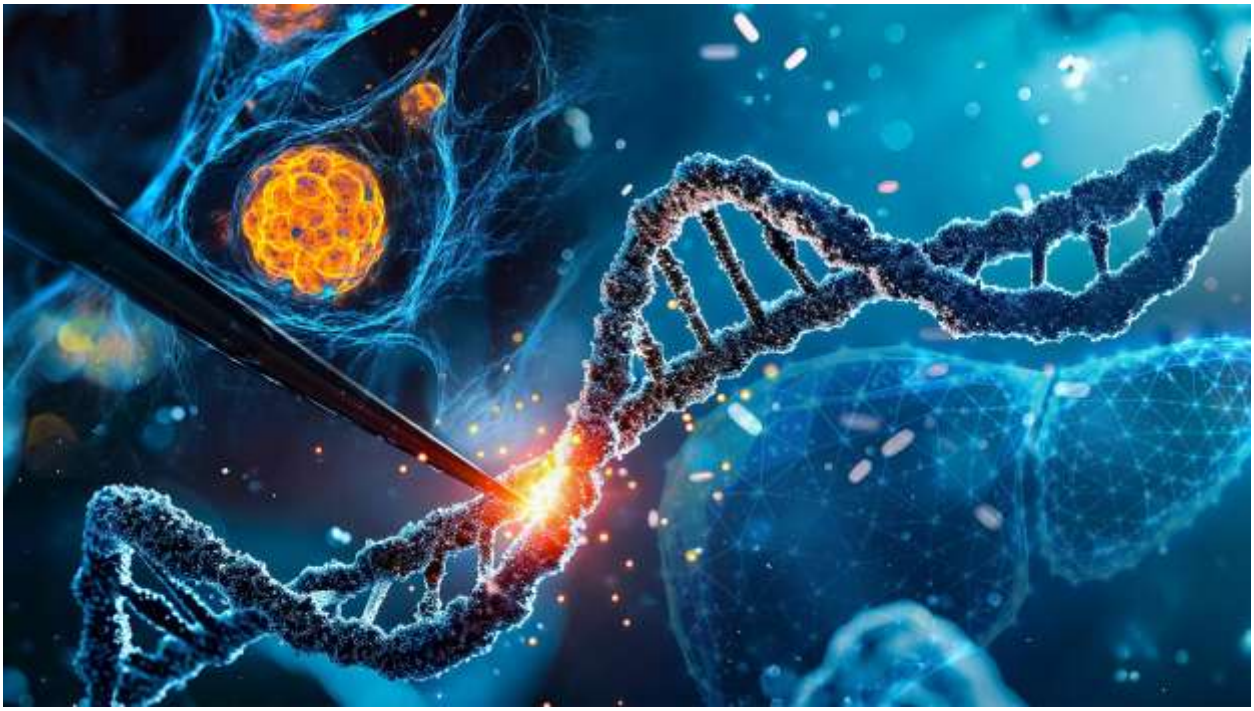
NanoCas: A Newly Identified Compact Nuclease May Expand CRISPR's Reach



The revolutionary CRISPR-Cas system has proven to be a precise method for genome editing, showing great promise in tackling genetic disorders. However, clinical trials for CRISPR-based treatments have faced delivery challenges due to the large size of the editing system. In the quest to overcome these limitations, researchers recently discovered a smaller CRISPR system that demonstrated efficient in vivo editing of the dystrophin gene in multiple types of muscles in mice and non-human primates.

CRISPR: how it works and its size limitation

CRISPR-Cas9 is a gene editing tool that acts like a pair of molecular scissors, cutting DNA at a specific location so bits of DNA can be added, disabled, modified, or removed. Engineered CRISPR systems use an RNA molecule called guide RNA (gRNA) to both find and cut a specific DNA sequence. Currently, Cas9 and Cas12 are the most frequently used cutting proteins, but at 1,000-1500 amino acids, their large sizes limit the editing modalities they can be paired with (1).

In December 2023, the first CRISPR-based therapy — Casgevy — was approved in the U.S. to treat sickle cell disease. The treatment requires extracting a patient's blood stem cells and using CRISPR to edit them before reinfusing the cells back into the body. The ex vivo therapy is a promising treatment for the thousands of people living with the disease in the U.S., but its method is time-intensive and costly.

In CRISPR clinical trials that have edited genes in liver cells, the CRISPR proteins are packaged into lipid nanoparticles (LNP). While this method succeeds in liver delivery and has been

particularly useful for delivering RNA-based CRISPR systems, showing promise in preclinical models for hereditary transthyretin amyloidosis, the lipid nanoparticles are unable to travel to many other tissues. Alternatively, researchers use the adeno-associated virus (AAV) — a well-studied and often used transporter of gene therapy — to shuttle the CRISPR system to target tissues. However, AAV vectors have a limited packaging capacity that CRISPR's unwieldy size exceeds, limiting its capabilities to the liver or ex vivo applications. Researchers have explored using smaller Cas9 variants, like SaCas9, which is around 3.3 kb, but these variants can have lower efficiency or require specific PAM sequences.

While smaller, more compact CRISPR nucleases, like SaCas9, CasX, and Cas12, have been identified, their sizes are still too large "for efficient AAV delivery when combined with necessary regulatory elements and fusion proteins" (1). Dual AAV delivery techniques have been attempted as a solution to the size dilemma but have demonstrated significantly lower editing efficiency and created additional manufacturing and safety obstacles due to the need to co-deliver multiple vectors (1).

In addition to LNPs and AAV vectors, several other alternative methods for delivering CRISPR components exist. Electroporation, a technique that uses electrical pulses to create temporary pores in cell membranes that allow CRISPR components to enter the cells, is effective for ex vivo applications like editing cells outside the body before reintroducing them and has been successfully used in CRISPR-based therapies for hematopoietic stem cells. However, the technique can cause cellular stress and affect viability. Additionally, Ribonucleoprotein (RNP) Complexes, which deliver CRISPR components — Cas9 and guide RNA — as pre-assembled RNP complexes, can reduce off-target effects and transiently express the editing machinery, thus minimizing potential toxicity, making them an appealing substitute for therapeutic and research applications. However, intracellular transport remains a challenge. Particles engineered to mimic viruses, known as virus-like particles (VLPs), but which lack viral genetic material, are another CRISPR delivery option. Another method is Polymeric Nanoparticles, which are made from polymers and can encapsulate CRISPR components for delivery, offering versatility in size and surface modifications. Finally, a technique known as Hydrodynamic Injection, which involves rapidly injecting a large volume of CRISPR components into the bloodstream, can enhance delivery to specific tissues, particularly the liver.

Biotech company Mammoth Biosciences — founded by CRISPR pioneer and Nobel laureate Jennifer Doudna as well as Trevor Martin, Janice Chen, and Lucas Harrington — are working on a solution to expand the capabilities of CRISPR technology.

NanoCas: a newly identified 'mini-CRISPR' capable of robust editing

Dubbed by its researchers as a "mini-CRISPR," NanoCas may be capable of moving CRISPR's in vivo abilities beyond the liver (1). The ultracompact CRISPR nuclease has demonstrated potent editing capabilities across various cell systems and tissues in vivo when administered to mice and non-human primates via AAV vectors, the researchers' January 2025 paper states (1). The

size of NanoCas also leaves room for additional payloads like regulatory elements, guide RNA's, or non-double strand break editing machinery, enabling its use for techniques including reverse transcriptase editing, base editing, and epigenetic editing.

Discovering the NanoCas system required an extensive screening of more than 150 CRISPR systems. To start, the researchers noted that the ideal system would have editing efficiency equivalent to Cas9, be smaller than 600 amino acids, target more than 10% of genomic sequences via protospacer adjacent motif (PAM) recognition, successfully edit with more than 30% of designed gRNAs, match SpCas9's fidelity, and use an RNA component under 100 nucleotides (1).

Using these parameters as their guide, they then evaluated the candidates via a strategy that screened for three categories: computational RNA structure prediction, PAM identification and RNA component validation in mammalian cell lysates, and the assessment of chromosomal editing in mammalian cells (1).

"Our initial focus was on proteins related to Cas14a.1. Based on similarities to Cas14a systems, we hypothesized these candidates would require both a CRISPR-associated RNA (crRNA) and trans-activating RNA (tracrRNA) for function," the paper states, noting that their search found a variety of tracrRNA architectures across different candidate clusters (1).

However, they noted that in some cases, different candidate clusters shared a conserved tracrRNA architecture with a sequence complementary to nearby CRISPR repeats, all within ~50 nucleotides.

"This architecture resembled that of Cas14a-like systems but was significantly more compact," they stated (1).

Next, the researchers designed chimeric guide RNAs — trans-activating crRNA (tracrRNA) joined to target sequences via a 4-nucleotide linker — for all 176 candidates, more than half of which contained the 50 nucleotide tracrRNA architecture (1).

The next step in the extensive discovery process was developing a chromosomal editing screen in HEK293T cells — a human embryonic kidney cell line — to find nucleases active in human cells and assess their consistency across different target sites. The team then tested each candidate with up to 24 unique gRNAs targeting various genes to thoroughly evaluate editing abilities. Most candidates showed editing rates below 5%; however, NanoCas achieved an average editing rate of 20% and successfully edited with 60% of tested gRNAs (1). Not only did NanoCas surpass the other candidates, but it also had a better editing rate than the Cas14a.1 control, which averaged 8% editing efficiency (1).

After further testing, including examining the PAM of NanoCas in human cells and screening with 79 guides, the researchers state that NanoCas can natively target 48.7% of bases in the human genome, significantly improving targetability relative to previous compact type V systems (1).

Additionally, NanoCas underwent targeted protein engineering to optimize its editing efficiency (1). Tests revealed the nuclease had notably weak DNA binding affinity, prompting the researchers to engineer variants with positively charged arginine substitutions across the protein to improve its DNA binding ability (1). Screening the variants in HEK293T cells revealed D220R as the most effective mutation, demonstrating improved editing efficiency across a range of mRNA doses in HEK293T cells (1).

The end result is a nuclease of approximately 450 amino acids, roughly one-third the length of conventional CRISPR nucleases like SpCas9 and Cas12a (1). Despite its small size, NanoCas is capable of robust editing efficiency, demonstrating successful editing levels in mice and non-human primates.

In Vivo Editing in Mouse Models

Once it was determined that the slimmer system could successfully edit DNA in various mammalian cells in the lab, the researchers began testing mice. They wanted to see if the mini-editor could successfully edit DNA in mice and found it could turn off the cholesterol gene PCSK9 in the liver and successfully edit about 60% of cells, comparable to its three-times-larger relative SaCas9 (1). Both systems reduced serum PCSK9 protein to undetectable levels.

After successful liver editing, they tested whether NanoCas could target muscle, a traditionally tougher area for CRISPR. In mice, the researchers tested if the system could cut out a mutation responsible for Duchenne Muscular Dystrophy (DMD). The mutation — found in the dystrophin gene — causes devastating muscular wasting that typically appears in early childhood and for which there is no cure. NanoCas demonstrated 10% to 40% editing of the dystrophin gene in a variety of muscle types, including the quadriceps, calf, and heart muscle in mice with DMD, when delivered by a single AAV vector. Notably, NanoCas achieved 15% editing in cardiac tissues across the left ventricle, left atrium, and septum compared to 10% with SaCas9 (1). While these results highlight NanoCas's potential to address diverse genetic disorders, the engineered mice don't become sick like people with DMD, meaning the researchers were unable to assess the potential effects on Duchenne symptoms and whether the strategy can help a sick animal get better.

In Vivo Editing in Non-Human Primates (NHP)

Next, the researchers tested the efficacy of the NanoCas system in three healthy macaque monkeys. The primates were injected with a single AAV, resulting in editing levels above 30% when targeting dystrophin in the skeletal muscles and 15% editing across the heart, compared with 10% with SaCas9. An analysis of the liver tissue showed no detectable levels of off-target editing.

"To our knowledge, this represents the first demonstration of efficient muscle editing in NHPs using a single-AAV CRISPR system," the research team stated. "Previous attempts at in vivo muscle editing in large animals have been limited by the need for dual-vector systems or

alternative delivery approaches, highlighting the unique therapeutic potential of NanoCas's compact size" (1).

Harrington, one of the co-founders of Mammoth, notes that the ability to efficiently edit with a single AAV makes delivering CRISPR into the body and editing previously inaccessible tissues "not just theoretical," adding that Mammoth Biosciences will continue to work on Duchenne and use NanoCas to target other muscle disorders as well as brain diseases.

While the mini-gene editor looks promising, there are still questions and hurdles before researchers can test the platform on people. AAV also comes with risks, including immune reactions; while the vectors are typically low in immunogenicity, repeated administration can cause an immune response that reduces their effectiveness. AAV vectors also vary in their tropisms, meaning they preferentially target certain cell types or tissues; while this can be beneficial for targeting specific organs, it also can make widespread delivery across different tissue types more challenging. Possible off-target effects, whereby the healthy DNA is accidentally modified, are also a risk. Beyond these challenges, producing AAV vectors is also a complex process that requires high purity and consistency, potentially impacting scalability and cost-effectiveness. However, researchers are continuously working on optimizing AAV vectors to overcome these limitations, including designing improved capsids and exploring alternative delivery methods.

Mini CRISPRs and their potentially large impact

While NanoCas represents a significant advancement in CRISPR technology, other small nucleases in the CRISPR family have been discovered and are worth noting.

Discovered in December 2017 in the laboratories of Jennifer Doudna and Jill Banfield, CasX is smaller than both Cas12 and Cas9 and was found to evolve independently of the two. A 2019 article published in Nature noted that CasX could be used for genome editing in human cells, and due to its smaller size and distinct structure, could be advantageous over other CRISPR-Cas systems.

In 2021, researchers at Stanford University reported that they had engineered a miniature system functional in mammalian cells from the 12f system (also called Cas14), which is not ordinarily functional in mammalian cells. The CasMINI system contains 529 amino acids and can delete, activate, and edit genetic codes without detectable off-target effects. Like NanoCas, CasMINI's small size allows it to be packaged into AAVs.

Additionally, Wu et al. harnessed AsCas12f1, only 422 amino acids long, as a genome editing tool in human cells, and later, researchers found that EnAsCas12f, an engineered RNA-guided DNA endonuclease is both more potent than its parent protein, AsCas12f, and one-third the size of SpCas9 (2).

Kannan et al. identified ultracompact variants of Cas13b that are active in mammalian systems and support programmable RNA editing. This family of proteins, Cas13bt, can mediate

mammalian transcript knockdown, and in 2023, Deng et al. reported that Cas13bt3, a smaller version of Cas13bt, was suitable for AAV delivery (3).

The future of CRISPR and NanoCas

Current CRISPR-Cas systems have demonstrated remarkable potential in treating genetic diseases. Clinical trials like that of nex-z, a CRISPR-based therapy for the treatment of ATTR amyloidosis, have shown clinical benefits, and the approval of CRISPR therapy Casgevy for the treatment of sickle cell disease demonstrates that the technology is capable of making a real difference in rare genetic diseases.

Now, the discovery of NanoCas and other small CRISPR-Cas systems provides new possibilities for expanding the technology's editing capabilities, addressing the limitations posed by larger CRISPR proteins, which could extend the delivery to tissues beyond the liver and treat an array of other genetic diseases like Duchenne Muscular Dystrophy. With its compact size of approximately 450 amino acids, NanoCas showcases promising editing efficiency in various models, including the successful targeting of the PCSK9 gene in the liver and the dystrophin gene in muscle tissues. Achieving effective editing in multiple tissue types with a single AAV vector underscores NanoCas's potential and paves the way for future research into other candidate nucleases that were identified and could be investigated, as well as exploring NanoCas's efficacy in human clinical trials.

However, there are risks and challenges of using AAV vectors to deliver CRISPR components while LNPs are now being developed and validated for tissues outside the liver like heart, muscle, and CNS while avoiding some of the complexities of AAV vectors.

OTS president Richard Geary points out that a lot of work on guide RNA and mRNA chemical modifications is also ongoing, with the potential to provide disruptive advances for gene editing.

Researchers are also working on several fronts to optimize CRISPR for therapeutic applications. Enhanced Delivery Systems, like ENVLPE, developed by scientists at Helmholtz Munich, use engineered non-infectious virus-like particles to transport CRISPR tools into living cells with high efficiency. ENVLPE has shown promise in restoring vision in mice with genetic mutations. Additionally, Machine Learning for Safer Enzymes is an approach used to identify millions of safer CRISPR enzymes that can reduce off-target effects and improve the safety and efficiency of gene editing. The method also allows researchers to predict customized enzymes for specific therapeutic targets. Various research consortia are also exploring advanced CRISPR technologies, such as CRISPR-Cas9 and CRISPR-Cas13, to develop gene therapies for diseases like ALS. These efforts include optimizing drug-like properties and improving delivery methods to ensure the therapies reach affected brain and spinal cord cells.

Research continues to refine delivery methods and explore the full potential of NanoCas and other mini-CRISPR systems, underscoring the dynamic and rapidly evolving landscape of gene editing, holding promise for advancing the treatment of genetic diseases.

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