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Research Progress of CRISPR-Cas3

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Abstract. The CRISPR-Cas system has revolutionized genome editing. Among its nucleases, Cas9 and Cas12 have been studied most extensively. However, CRISPR-Cas3 stands out as a distinct nuclease, possessing both helicase and exonuclease activities. Despite this unique feature, it remains under-explored—especially considering its potential for large-scale genome manipulation. This review emphasizes the challenge of multidrug-resistant bacteria in current antimicrobial therapy, where Cas3 may provide a breakthrough solution by degrading the entire pathogen genome, rather than targeting single genes. To compile this review, a comprehensive literature search was conducted, covering publications from 2018 to 2025 across databases such as PubMed, Web of Science, and Nature. A comparative analysis of Cas3 and other CRISPR systems was also carried out to identify key research gaps. Key findings highlight Cas3's ability to degrade long DNA segments, making it valuable for deleting large genomic regions, such as integrated viral DNA, and addressing challenges in antimicrobial therapy. Nevertheless, low targeting precision and complex delivery processes hinder its clinical translation. The review concludes that to unlock Cas3's full potential, two steps are essential: optimizing its specificity through protein engineering and developing targeted delivery systems.

Keywords: CRISPR-Cas3, genome editing, antimicrobial therapy, large-scale deletion, bacterial adaptive immunity

1. Introduction

The CRISPR-Cas system, a prokaryotic adaptive immune mechanism, protects microbes from foreign nucleic acids by integrating viral DNA segments into the CRISPR locus. These segments are then transcribed into crRNAs, which guide Cas nucleases to degrade invading nucleic acids. In 2013, multiple groups reported the first use of CRISPR-Cas9 to induce targeted double-strand breaks (DSBs) in mammalian cells, enabling efficient gene knockout and insertion [1]. Among various CRISPR-Cas subtypes, two have become research focuses: Cas9 (Class 2, Type II) and Cas12 (Class 2, Type V). In contrast, CRISPR-Cas3 (Class 1, Type I) has unique functions. It exhibits dual helicase-exonuclease activities. These activities allow it to gradually degrade lengthy DNA fragments. This feature is not present in Cas9 or Cas12. Yet, it is still not widely applied in genome engineering [2].

Key gaps in Cas3 research include variable molecular mechanisms across bacterial species; unclear regulation of its activities in heterologous systems (e.g., human cells); lack of

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comprehensive comparisons with Cas9/Cas12 for practical applications; and unresolved technical challenges in clinical translation, with few reviews summarizing solutions. This review addresses these gaps by analyzing Cas3's mechanism, applications (large-scale editing, antimicrobial therapy, and diagnostics), and optimization strategies. A literature search (2018–2025) across PubMed, Web of Science, and Nature using keywords like "CRISPR-Cas3," "large-scale genome editing," and "Cas3 delivery" prioritized recent studies and foundational work. By consolidating existing knowledge, it deepens the comprehension of Cas3's distinct biological roles. These roles are fundamentally different from those of Cas9 and Cas12. In contrast to the CRISPR-Cas9 system, this distinctive feature of CRISPR-Cas3-mediated genome editing may expand the scope of genome editing applications by enabling efficient gene knockouts/knock-ins and supporting future therapeutic applications [2]. Ultimately, the review highlights pathways to overcoming technical challenges. This can speed up the transition of Cas3 from laboratory research to clinical practice.

2. Method

2.1. Literature search strategy

A comprehensive literature review was performed to ascertain that most of the research on CRISPR-Cas3 occurred between January 2018 and April 2025. This time frame was selected to ensure coverage of the latest advancements. This is especially important for studies on Cas3 variants or innovative applications. Four primary databases were utilized: PubMed, Web of Science, ScienceDirect, and Nature/Science journals.

The most relevant keywords were combined for the search. The combinations were: ("CRISPR-Cas3" / "Cas3") AND ("mechanism" / "structure" / "genome editing" / "large-scale deletion" / "antimicrobial therapy" / "diagnostics" / "delivery" / "optimization").

2.2. Inclusion and exclusion criteria and sorting of literature

Studies were included only if they met three criteria: (1) published in English; (2) addressed at least one of the review's core objectives (mechanism, applications, challenges, or optimization); (3) provided empirical data/original research or systematic syntheses to support claims. Several types of studies were excluded: (1) non-English publications; (2) duplicate studies (e.g., preprints later published in peer-reviewed journals); (3) abstracts or conference proceedings lacking full-text information.

Quality evaluation was conducted for original research. Quality evaluation was conducted in a sequential manner, first focusing on original research and then on review articles, to ensure the rigor and reliability of included studies. Two factors were analyzed: study design (e.g., sample size, control groups, reproducibility) and journal impact factors. The latter was used to prioritize rigorous, peer-reviewed publications. The final reference list included 12 high-impact studies that best represent key advances in Cas3 research.

3. Mechanism of CRISPR-Cas3

CRISPR-Cas3 differs from well-characterized nucleases like Cas9 in its structure and editing mode, enabling its unique capability for large-scale DNA degradation. This section will detail three key aspects of CRISPR-Cas3.

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3.1. Structural features

Cas3 contains an HD-nuclease domain and a Superfamily 2 (SF2) helicase domain, which cooperate to degrade DNA. The HD-nuclease domain cleaves single-stranded DNA and requires metal ions like iron, manganese, and calcium. The helicase domain, powered by ATP hydrolysis, unwinds the DNA duplex in a 3′-to-5′ direction [3]. Unlike CRISPR/ Cas9, which induces large-fragment deletion by generating DSBs, CRISPR-Cas3 systems progressively shred target DNA through a multistep process [4]. Cas3's DNA degradation begins with the Cascade complex, guided by crRNA, identifying the target DNA. As shown in Figure 1, Cascade identifies a Protospacer Adjacent Motif (PAM) sequence next to the target DNA. This recognition forms an "R-loop," where the crRNA binds to one DNA strand, displacing the other. Once the R-loop forms on the target DNA, Cas3 is recruited [5]. In contrast, Cas9 acts as a single protein and does not require an accessory subunit—a feature that simplifies its use but limits its ability to degrade extensive DNA fragments.

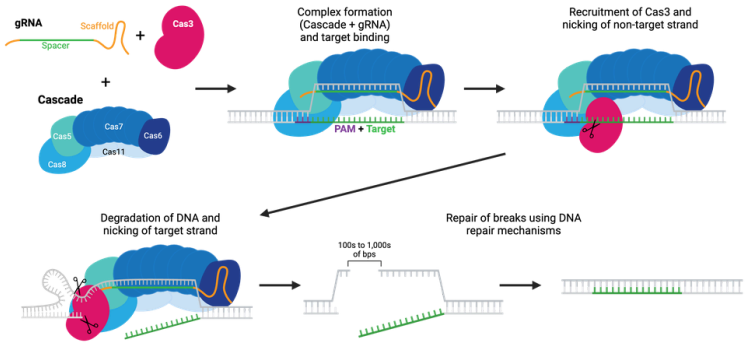


Figure 1. Overview of Cascade-Cas3 mechanism. Created with BioRender.com

Table 1. The structural differences between Cas3 and Cas9

Component	CRISPR-Cas3	CRISPR-Cas9
Core Domains	1. Helicase domain (SF2 family)	1.RuvC-like endonuclease domain
	2. HD nuclease domain	2. HNH endonuclease domain
Accessory Subunits	Requires Cascade complex (CasA-CasE)	No accessory subunits needed
DNA Interaction Mode	Binds ssDNA after unwinding by Cascade	Binds dsDNA directly via sgRNA-PAM interaction
Degradation/Cleavage Mode	Progressive 3'-5'exonuclease degradation	Site-specific DSBs (3 bp upstream of PAM)

Note: This diagram summarizes core structural differences. It is adapted from key findings in [2-4].

3.2. Editing process

The CRISPR-Cas3 editing process involves three sequential steps, distinct from Cas9's DSB-mediated mechanism (shown in Table 1). First, targeting recognition occurs as the Cascade-crRNA complex scans dsDNA for a PAM (e.g., 5'-AAG-3' in Type I-E), located upstream of the target protospacer. PAM binding triggers dsDNA unwinding, enabling crRNA to hybridize with the target

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strand and form an R-loop. For example, Yoshimi et al. successfully used CRISPR-Cas3 to edit the Il2rg gene in rat embryos, achieving deletion mutations in 38.5% of offspring [6]. Next, Cas3 recruitment and activation take place when the R-loop exposes an ssDNA segment that binds to Cas3's helicase domain, activating Cas3 to initiate DNA degradation. Finally, progressive degradation occurs as Cas3 degrades DNA unidirectionally from the PAM-proximal to the PAM-distal site, resulting in deletions ranging from 100 bp to over 100 kb. Unlike Cas9, which cleaves dsDNA at a fixed site, this process can produce large deletions but requires caution in gene-dense regions or near regulatory elements [4]. Notably, the spacer and PAM sequences remain intact in the absence of small indel mutations [2].

3.3. Specificity

Two key factors determine Cas3's specificity: PAM requirements and crRNA-target complementarity. PAM sequences vary across Cas3 subtypes. For instance, the SSA assay demonstrated a range of DNA cleavage activities for different PAM sequences, among which the 5′-AAG PAM had the highest activity in human cells. Other sequences like AGG, GAG, TAC, ATG, and TAG had lower activity. These results match previously reported biochemical results [2].

Recent studies have improved our understanding of Cas3's specificity. Li et al. (2024) conducted a key experiment. They fused a catalytically dead Cas9 (dCas9) to Cas3, creating a dCas9-controlled CRISPR/Cas3 system. It enhances specificity by restricting Cas3 activity to dCas9-targeted sites. Compared to wild-type Cas3, this system reduced off-target deletions by 60%. This result highlights the potential of chimeric Cas3 variants to improve precision [4]. Besides, Ruffolo et al. characterized an exemplar editor. They perform an extensive characterization of one exemplar editor (OpenCRISPR-1) and show that it is highly functional and specific. Their top hit, PF-CAS-182, had on-target activity comparable to SpCas9 (median indel rates of 56.4% versus 47.1%) while having a 95% reduction in editing at known SpCas9 off-target sites (median indel rates of 0.32% versus 6.1%) [7]. Another relevant development comes from Baglaenko et al.. They present a multi-omic single-cell sequencing approach that directly identifies genomic DNA edits, assays the transcriptome and measures cell-surface protein expression [8]. This offers indirect support for optimizing Cas3's specificity assessment.

4. Applications of CRISPR-Cas3 in biotechnology

4.1. Large-scale genome deletion

Cas3's progressive degradation mechanism makes it ideal for certain tasks. These tasks include removing integrated viral DNA, large genomic rearrangements, or non-essential genetic regions. Morisaka et al. attained a significant milestone. The CRISPR-Cas3 system exhibited editing efficiencies ranging from 4.4% to 38.2% for EMX1 and 8.0% to 16.6% for CCR5, with Cas3-mediated deletions averaging 3.2–6.3 kb (EMX1) and 4.0–7.6 kb (CCR5). Maximum deletion sizes reached 77.7 kb (EMX1) and 51.3 kb (CCR5) [2]. More recently, Li et al. made further progress. They developed a dCas9-controlled CRISPR/Cas3 system. This system generates precise large-fragment deletions in mammalian cells and mice. They targeted a 30 kb segment of the Dmd gene (mutated in Duchenne muscular dystrophy) and achieved deletion efficiencies of 35% in mouse myoblasts and 28% in vivo (mouse skeletal muscle) [4]. This study demonstrated that Cas3 holds potential for treating diseases caused by large genomic deletions or duplications.

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4.2. Antimicrobial therapy

In contrast to antibiotics, which indiscriminately eliminate both harmful and beneficial bacteria, Cas3 can be tailored to focus on genes that are specific to pathogenic bacteria, thereby reducing unintended impacts on the microbiome.

The antimicrobial mechanism of Cas3 focuses on two types of genes. It targets essential bacterial genes and antibiotic resistance genes. When delivered properly, the Cas3-crRNA complex degrades the target bacterial DNA. This degradation leads to bacterial cell death [3]. A striking case illustrates Cas3's potential. For example, a study showed that Cas3-encoding phages reduced MRSA load in mouse skin infections by 99% within 24 hours. Importantly, no detectable impact was found on the skin microbiome, such as Lactobacillus species. Cas3 offers several advantages over Cas9 in antimicrobial therapy. First, its ability to degrade large DNA segments ensures complete inactivation of bacterial genomes. This reduces the risk of resistance development. In contrast, Cas9 may leave partial gene fragments intact, which can contribute to resistance. Second, Cas3 relies on the Cascade complex. This complex is unique to prokaryotes, minimizing toxicity in eukaryotic cells and enhancing its safety profile.

4.3. Diagnostic tools

These tools are used to detect viral and bacterial pathogens. In a manner akin to diagnostics based on Cas12a (such as DETECTR), systems that employ Cas3 capitalize on the nuclease's collateral activity, the non-specific degradation of ssDNA reporters, to amplify detection signals. It is worth mentioning that Cas3 has superior processivity. Processivity refers to its ability to degrade multiple ssDNA molecules per activation, rendering it more sensitive than Cas12a when targeting low-abundance entities. Yoshimi et al., in their work on Type I-E CRISPR-Cas3 genome editing in mice and rats, indirectly supported Cas3's diagnostic potential. While their primary focus was on in vivo editing, they observed that Cas3's Type I-E system exhibited high affinity for target DNA sequences in rodent cells—even at low copy numbers of the target locus. This high affinity aligns with the sensitivity required for diagnostic applications, suggesting that Type I-E Cas3 (the same subtype used in their rodent studies) could be adapted for detecting low-abundance viral or bacterial DNA in clinical samples [6]. Another advantage of Cas3-based assays is their simplicity. They do not require thermal cycling, making them suitable for point-of-care testing in resource-limited settings.

5. Challenges and limitations

Despite its promising potential, CRISPR-Cas3 faces three significant obstacles. These obstacles hinder its application in clinical and industrial settings: low targeting precision, delivery barriers, and limited efficacy in eukaryotic cells.

5.1. Low targeting precision and delivery barriers

The primary limitation of CRISPR-Cas3 is non-specific DNA degradation, which leads to off-target effects. Cas9 cuts DNA at a specific location. It depends on the high degree of complementarity between the crRNA and the target. Cas3 acts differently. Its continuous degradation can extend beyond the intended target site if the Cascade complex attaches to a sequence that is only partially complementary. This can result in the deletion of adjacent genes, leading to unintended phenotypic changes.

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Efficient delivery of CRISPR-Cas3 components (including the Cascade complex, Cas3, and crRNA) into target cells. This challenge is particularly acute for eukaryotic cells. Adeno-associated viruses (AAVs), with their linear double-stranded DNA genomes (8 kb capacity), are widely employed in clinical gene editing due to their non-enveloped nature, which minimizes host genome integration and off-target effects. For instance, AAV-mediated delivery has demonstrated efficacy in treating Haemophilia B by targeting the mutated Factor IX (FIX) gene [9]. However, antimicrobial applications present unique challenges: Cas3 must simultaneously breach bacterial cell walls, evade bacterial defense mechanisms, and maintain broad-spectrum activity. While bacteriophages enable bacterial penetration, their species-specificity limits clinical use [3].

5.2. Eukaryotic use limitations

CRISPR-Cas3 originates from prokaryotes, a trait that restricts its effectiveness in eukaryotic cells. Two key factors contribute to this limitation: nuclear localization and immune recognition.

Cas3 lacks a native nuclear localization signal (NLS). MSNs modified with nucleus-targeting sequences (NLS) can help carry CRISPR/Cas3 plasmids to the nucleus. This helps protect the DNA from damage [9]. While adding an NLS to Cas3 improves nuclear entry, it reduces Cas3's catalytic activity.

More notably, the prokaryotic origin of Cas3 and its accessory Cascade complex triggers robust innate immune recognition in eukaryotes via two primary molecular mechanisms. First, endosomal Toll-like receptors (TLRs) recognize pathogen-derived nucleic acids. For example, TLR9 binds unmethylated CpG DNA motifs common in bacteria, and the related receptors TLR7/8 bind single-stranded RNA. When Cas3 or Cascade genes (as DNA or RNA) enter the cell's endosomes, these TLRs engage the MyD88 adaptor and trigger NF-κB/IRF signalling. This leads to the production of type I interferons and pro-inflammatory cytokines such as IFN-α and TNF-α. Second, cytosolic nucleic acid sensors. Cytosolic receptors detect prokaryotic nucleic acids delivered into the cytoplasm. The DNA sensor cGAS binds cytosolic double-stranded DNA (such as a Cas3-encoding plasmid) and synthesizes cyclic GMP-AMP (cGAMP). cGAMP then activates STING and IRF3, driving interferon gene transcription. Similarly, the RNA sensor RIG-I recognizes uncapped 5′-triphosphate RNA (a hallmark of bacterial transcripts). RIG-I binding initiates a signalling cascade that amplifies type I IFN production.

This immune response has direct negative impacts on Cas3's editing efficiency: it can induce apoptosis (cell death) of transfected cells or trigger clearance of edited cells by immune cells (e.g., macrophages).

6. Strategies for optimizing CRISPR-Cas3

To address the aforementioned challenges, researchers have developed two primary optimization approaches: refining delivery systems and enhancing specificity via protein engineering. The goal of these strategies is to fully unlock Cas3's capabilities while ensuring safety and efficiency.

6.1. Improved delivery systems

Progress has been made in both viral and non-viral delivery systems.

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6.1.1. Viral vectors

Modified viral vectors have been engineered to incorporate Cas3 and the Cascade complex. Among the various viral vectors, adeno-associated virus (AAV) emerges as the most preferred and extensively utilized vector. People like it for its low immunogenicity and its ability to exhibit serotype-specific targeting in host cells [10]. However, its ~4.7 kb packaging limit forces split "dual AAVs" for large Cas3-Cascade systems, lowering co-transduction efficiency. Lentiviruses (~8 kb capacity) carry the full Cas3-Cascade system and enable long-term expression via genomic integration but risk insertional mutagenesis. Adenoviruses (~36 kb capacity) have high transduction efficiency but induce strong immune responses and only support transient expression. AAV is top for safety/targeting, but vector engineering, like expanded AAV capacity, is needed to overcome its limitations.

6.1.2. Non-viral vectors

Non-viral vectors possess several advantages, such as a high capacity for genetic material loading, enhanced safety, ease of accessibility, and scalability for large-scale production. Numerous reviews have highlighted the progress in nanotechnology, with the development of more controllable, precise, and safe nanocarriers holding significant potential for clinical translation. Non-viral vectors are expected to have a substantial impact on cancer treatments, genetic testing, and the investigation of molecular mechanisms [9]. Building on these findings, Wang et al. employed PEG-modified nanoparticles (P-HNPs) in combination with Cas9-expressing plasmids and sgRNA for genome editing. This approach significantly improved the transfection efficiency of Cas9 and the uptake of sgRNA. Compared to existing polycation-based gene delivery methods, P-HNPs demonstrate considerable potential for non-viral delivery [11].

6.2. Protein engineering for enhanced specificity

Protein engineering efforts have focused on modifying Cas3's functional domains. The aim is to minimize off-target effects while preserving on-target activity. Two approaches have shown promise: domain mutagenesis and chimeric Cas3 variants.

Site-directed mutagenesis can be applied to Cas3's domains. Specifically, it targets the HD nuclease domain or the helicase domain. This process can reduce non-specific degradation. Morisaka et al. demonstrated that E. coli Cas3 with just a single crRNA induced large deletions more efficiently than Cas9 [2]. This mutation reduced the domain's exonucleolytic activity by 50% without affecting its ability to bind target DNA. This mutation reduced off-target deletions by 70% in human cells, with only a 10% reduction in on-target efficiency.

Fusing Cas3 with DNA-binding proteins can improve target specificity by restricting Cas3's activity to specific sites. A prominent example is the dCas9-Cas3 chimera. dCas9 is a modified Cas9 protein. It can bind to DNA but cannot cleave it. In this chimera, dCas9 directs Cas3 to the designated target site. Compared to wild-type Cas3, dCas9-controlled CRISPR/Cas3 reduced off-target effects by 60%. This is because dCas9's high specificity prevents Cas3 from binding non-target sites [4]. The performance of OpenCRISPR-1 was exceptional. In human cells, it reduced off-target deletions by 95%. It also showed lower immunogenicity than wild-type Cas3 [7].

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7. Conclusion

Key conclusions include: (1) Cas3's progressive degradation mechanism makes it suitable for two critical tasks: extensive genome deletion (e.g., excision of viral DNA) and precision antimicrobial therapy; (2) Although with these benefits, Cas3's low targeting precision, delivery barriers, and limited eukaryotic efficacy hinder its clinical translation; (3) Recent developments have made notable strides in overcoming these issues. Examples include the use of dual AAV vectors, lipid-polymer hybrid nanoparticles (LPHNs), and engineered variants like OpenCRISPR-1. This review presents multiple limitations. Firstly, it is predominantly based on in vitro and animal research, with a scarcity of clinical data regarding the safety and efficacy of Cas3 in human subjects. Secondly, comparative data on Cas3 variants from different bacterial species are insufficient, limiting our understanding of how structural differences affect performance. Lastly, the review emphasizes applications in mammals and bacteria, offering minimal insight into Cas3's potential uses in plants or industrial biotechnology. This narrow focus overlooks possible innovations in agriculture or biofuel production. Future research should prioritize three directions: (1) Developing high-throughput screening techniques to identify Cas3 variants with improved specificity and reduced immunogenicity; (2) Conducting in vivo safety studies in non-human primates to assess the long-term impacts of therapies based on Cas3. Non-human primates are closer to humans in physiology, making their data more relevant for clinical translation; (3) Expanding Cas3's application scope to include plant genome editing, such as enhancements in crop quality or disease resistance. It also includes industrial microbiology, such as the production of biofuels or industrial enzymes. By addressing these areas, CRISPR-Cas3 can fulfill its potential as a next-generation genome editing tool.

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