CRISPR-Cas Systems: Origins and Their Impact on Functional Genomic

Daniela Rodriguez-Carrascal

ABSTRACT

The CRISPR-Cas mechanism was first discovered as an intricate adaptive defense system in prokaryotic organisms, and it has profoundly influenced the evolution of biology and biotechnology today. This review article explores key aspects of these systems, beginning with their evolutionary origin and their categorization into two main classes according to the structure of their effector modules. Their roles in functional genomics are examined, with a focus on technologies such as Cas9 and dCas9 in transformational genomic editing and gene regulation. Moreover, the significance of CRISPR-Cas across various domains, including biomedicine, agriculture, and diagnostics, is examined, illustrating its advancements in rapid genomic screenings and the swift depiction of nucleic acids. Nevertheless, the potential trajectories that CRISPR-Cas technologies might explore are scrutinized, considering that many of its applications, such as CRISPR-associated transposons, are still in their nascent stages. At the same time, the prospective roles in synthetic biology, gene therapy, and enhancements in agriculture are thoughtfully evaluated. Even with the remarkable strides achieved in gene manipulation through the ingenuity of CRISPR-Cas, numerous obstacles persist, such as unintended off-target impacts and fluctuations in effectiveness. This paper emphasizes the adaptive capacity of CRISPR-Cas and its essential contribution to functional genomics, thus revealing new avenues for genetic research and biotechnological innovations.

Keywords: CRISPR-Cas systems, Genome editing, Functional genomics, Gene therapy, Synthetic biology

Departamento de Química Biológica, Cátedra de Química Biológica Patológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

Correspondence to: Daniela Rodriguez-Carrascal, danielarcarrascal@gmail.com

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Background

Cas protein systems, intertwined with clustered regularly interspaced short palindromic repeats (CRISPR-Cas), have dramatically reshaped our understanding of biology and opened new possibilities in biotechnology. To start with, these elaborate systems were recognized as defense structures within the bacterial and archaeal domains, as they emerged to shield against genetic intruders like mobile genetic elements (MGEs), plasmids, and viruses. Constant interaction with these invaders has allowed them to shape their evolution and develop sophisticated functions, making them essential tools.

CRISPR-Cas systems are characterized by modular architectures. They possess an adaptation module that serves as the system's core and includes proteins such as Cas1 and Cas2, which are responsible for incorporating new spacers into CRISPR arrays.[1–3] Cas proteins evolved from transposons known as casposons. This evolutionary process allowed microorganisms to record fragments of invading DNA in their genomes, creating an "immunological memory" that protects them from future infections.[4,5] In addition, CRISPR-Cas systems have an effector module that meticulously handles the CRISPR RNA (crRNA) and uses it to neutralize unwanted DNA or RNA.[6] This module includes well-known proteins such as Cas9 and Cas12, which also have a particular origin, as they evolved from nucleases related to mobile genetic elements, adapting to perform specific functions in host defense.[7] This flexibility and modular design have played an important role in the transformation and variety of CRISPR-Cas systems, allowing them to meet the diverse requirements of various organisms.[8]

Although initially designed as a shield against invaders, certain CRISPR-Cas systems have lost that function. They are now involved in diverse biological activities, such as signaling and carefully integrating genetic components.[9,10] This evolutionary versatility makes us question how to define these systems; understanding their adaptive capacity and the variety of tasks they can perform is necessary to use them as innovative tools for genome manipulation.[11]

This captivating review delves into the intricate features of CRISPR-Cas systems, weaving together the diverse classifications of their forms, their impact on functional genomics, potential applications, and visions for the future. It aims to provide an extensive synthesis of how these systems have revolutionized our understanding of biological processes and their use in genetic research.

Classification of CRISPR-Cas Systems: Organizing Diversity

The classification of CRISPR-Cas mechanisms presents a formidable puzzle, owing to the vast array of types and subtypes that abound. At present, they are segmented into two main classes according to the architecture of their effector module:[12]

Class 1 CRISPR-Cas Systems

This class includes types I, III, and IV, which rely on protein complexes composed of multiple subunits. These systems, commonly found in bacteria and archaea, are characterized by structures such as the Cascade complex, a CRISPR-associated complex for antiviral defense.[12]

The Cascade complex, a hallmark of type I systems, is most frequently found in archaea and has a helical structure, which assembles multiple Cas proteins, such as Cas5e, Cas6e, Cse1, Cse2, and Cas7, which bind crRNA to recognize and bind invading DNA.[12] The Cascade complex has been extensively analyzed using crystallography and electron microscopy, providing insights into its helical structure and interaction with crRNA.[13–15] In particular, Zhao et al.[14] and van der Oost et al.[15] demonstrated how crRNA binding induces conformational changes that enhance DNA target recognition. This assembly undergoes conformational changes upon binding to the invading DNA,

positioning Cascade to recruit Cas3. Cas3 is an enzyme with nuclease and helicase activity, which efficiently and processively degrades the invading DNA.[16–20]

On the other hand, Type III systems, also part of Class 1, have a more advanced functionality as they can bind to DNA and also to invading RNA. In addition, these systems generate molecular signals that activate other defensive proteins, demonstrating a sophistication that goes beyond just destroying invading genetic material.[21,22]

Class 2 CRISPR-Cas Systems

After learning about Class 1 systems, we can discuss Class 2 CRISPR systems, which include Types II, V, and VI. These systems are characterized by effector modules with unique proteins, such as Cas9 and Cas12, capable of performing multiple functions. These proteins have a denser configuration and are easier to manipulate, which makes them particularly ideal for innovative biotechnological uses.[23]

Within the realm of intricate frameworks, Type II systems emerge as a straightforward and efficient paradigm, famously exemplified by the renowned Cas9 system, which hails from the bacterium *Streptococcus pyogenes*.

The complex has a guide RNA that functions as a specific RNA sequence that recognizes the DNA region of interest, directing the Cas nuclease to the DNA for editing. In some cases, the single guide RNA (sgRNA) is observed, which is a version of the naturally occurring two-piece guide RNA complex designed in a single continuous sequence.

The Cas9 enzyme wields a sgRNA to slice DNA into twin-stranded shattered pieces. The parts can then be restored using strategies such as homology-directed repair or non-homologous end joining. These processes allow the introduction of specific mutations or insertions.[24,25] This approach has revolutionized genome editing by allowing precise DNA modifications.[23,26] Furthermore, the inactive variant of Cas9 (dCas9) has created a field of opportunities for CRISPR that goes beyond simple sequence changes. When combined with active domains, dCas9 allows the activation (CRISPRa) or inhibition (CRISPRi) of gene expression without causing irreversible changes in the genetic blueprint.[27] These tools have opened new possibilities for gene regulation, targeted epigenetic modifications, and base editing, providing innovative approaches for genetic and epigenetic research.[28]

Structure investigations have revealed that Cas9 assumes a self-controlled form when it lacks its guide RNA. Upon binding to the guide RNA and target DNA, Cas9 undergoes a significant structural reorganization, forming a central channel where DNA cleavage occurs. This intricate mechanism relies on the presence of a Protospacer adjacent motif (PAM) sequence, a brief yet crucial string of nucleotides that needs to be adjacent to a DNA sequence for Cas9 to latch onto and snip. Here, a harmonious Watson-Crick-type base pairing occurs that connects the guide RNA to the desired DNA.[29]

Comparison Between Class 1 and Class 2 CRISPR Systems

Although Class 1 and Class 2 CRISPR systems share the goal of recognizing and degrading invasive genetic material, their structural and functional mechanisms exhibit key differences. Class 1 systems rely on multi-subunit complexes such as Cascade and the helicase Cas3. In contrast, Class 2 systems, like those of Type II, use multifunctional proteins such as Cas9, which integrate recognition and cleavage into a single molecule. These structural and functional distinctions make Class 2 systems more suitable for biotechnological applications.[6,17] The following table elucidates the distinctions outlined herein (Table 1).

Table 1 | Feature comparison between class 1 and class 2 crispr systems

Feature	Class 1	Class 2
Structural Complexity	Dependence on multi-subunit complexes, such as Cascade.	Use of a single multifunctional protein, such as Cas9.
Key Proteins	Cascade and Cas3.	Cas9.
Main Function	DNA recognition and degradation through joint action.	DNA recognition and cleavage in a single molecule.
Functional Efficiency	Processive, requiring multiple proteins and steps.	Direct, combining functions in a single protein.
Applications	Less common in biotechnology due to its complexity.	Widely used in genome editing and biotechnology.
Guide Structure	crRNA assembled in the complex.	The dual complex of crRNA and tracrRNA.
Structural Reorganization	Complex conformational changes during function.	Auto-inhibited reorganization until guide RNA binding.
References	13,29	23,28

Classification of CRISPR-Cas Subtypes

The structural composition of CRISPR-Cas systems has allowed the differential evolution of adaptation and effector modules, promoting the exchange of components between different variants. This evolutionary mechanism is reflected in the wide diversity of forms and functions these systems present, specifically the coexistence of multiple subtypes within type I and type III systems.

The classification of the subtypes is complex, since it depends on the presence of specific genes, such as csn2 or dinG, and requires the detailed analysis of their genetic sequences. In addition, derived variants that have lost their original immune functions have been identified but have evolved to fulfill other functions in other biological processes. This phenomenon highlights the remarkable evolutionary versatility of CRISPR systems, particularly those of Class 2, which have been shown to adapt to a wide range of functions beyond their initial defensive role.[1,9,10]

CRISPR-Cas in Functional Genomics

Since its unearthing in 2012, the CRISPR-Cas mechanism has transformed the landscape of genomics by presenting an accurate, versatile, and notably straightforward approach to genome manipulation. Born as a clever defense mechanism in prokaryotic life forms, its remarkable capacity to precisely hone in on particular DNA sequences has transformed it into a vital instrument for a myriad of uses, spanning from gene therapy to agricultural enhancements and breakthroughs in biotechnology.[30]

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Before the advent of CRISPR, genome editing primarily relied on zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN). While these technologies enabled significant advancements, they posed challenges in terms of complexity and cost. ZFN, as the first developed approach, required highly specific designs, making it less accessible. TALEN improved precision and flexibility, but its large size hindered its application in certain systems. The arrival of CRISPR-Cas transformed the realm of genome editing, unveiling a method that is not only simpler and more efficient but also brimming with versatility. Unlike its predecessors, CRISPR uses an RNA guide to direct the editing process, significantly reducing design time and cost, and making it the preferred tool in research and biotechnology.[31]

Overall, CRISPR-Cas has blossomed into a groundbreaking instrument that clears the path for extensive functional exploration. In functional genomics, researchers examine the detailed interactions of genes and the outcomes of those interactions, elucidating their contributions to biological systems and weaving together the rich network of genetic variety and phenotypic expressions by investigating numerous biological domains, such as the transcriptome, epigenome, and proteome. The synergy of this method alongside innovative tools like CRISPR-Cas-driven functional screening has propelled advancements in vital research domains such as oncology, drug resistance, infectious ailments, and metabolic imbalances. These advances have been instrumental in identifying target genes involved in these diseases, thus contributing to the understanding of their underlying biological mechanisms.[22,32–35]

Moreover, the advent of modified variants like dCas9 and dCas13, which possess no catalytic prowess yet maintain their exceptional affinity for attaching to DNA or RNA, has remarkably broadened the horizons of CRISPR-Cas. These tools enable the regulation of gene expression, pathogen detection, and genomic imaging, opening new frontiers in research and applications within functional genomics.[30,36–38]

Functional Screenings and Genomic Visualization with CRISPR-Cas

CRISPR-Cas-based functional screenings have transformed the study of genetic functions by enabling large-scale investigations in diverse biological contexts. There are two main formats for these analyses: arrayed screenings and pooled screenings. Arrayed screenings, conducted in multi-well plates, allow detailed study of complex phenotypes in individual cells. Conversely, pooled screenings are a more cost-effective, high-throughput option, ideal for simultaneously analyzing thousands of genes using technologies like oligonucleotide synthesis and high-throughput sequencing.[33,39,40]

The success of these screenings depends on careful design and meticulous execution. Choosing a suitable guide RNA (sgRNA) library is essential and demands the latest genomic insights customized to the experimental framework and cellular variety being investigated. Maintaining library representation throughout the process is essential to ensure accurate quantification of results. Additionally, optimizing assays and ensuring the relevance of the experimental model are crucial steps before conducting the analysis. Secondary screenings using customized libraries are particularly useful for validating initial findings and deepening the understanding of results.[33]

Lentiviruses are the premier choice for delivering CRISPR-Cas elements into cells, owing to their extraordinary efficiency and capacity to transport large payloads. Nevertheless, other innovative methods like lipid nanoparticles and piggyBac transposons are rising in favor, especially in research focused on primary cells and applications in living organisms.[32,41,42] The workflow diagram below summarizes the general steps involved in performing genomic functional screenings using CRISPR-Cas technology (Figure 1).

CRISPR-Cas-based genomic screenings have been fundamental tools for identifying critical genes in various cellular processes. For instance, knockout (KO) screenings enable the identification of essential genes through targeted genome editing. Additionally, combining CRISPR with single-cell RNA sequencing has facilitated detailed analyses of transcriptomic and phenotypic changes following editing events, providing key insights into mutations and their functional impact.[43–44]

CRISPR-Cas technology has also enabled significant advances in genomic visualization. Tools such as Live-FISH, which combines dCas9 and dCas13 labeled with fluorophores, allow simultaneous observation of DNA and RNA in live cells, offering valuable insights into genomic dynamics and gene regulation. In plants, techniques like CASFISH and RGEN-ISL have been used to visualize telomeres and centromeric repeats with high specificity, enhancing our ability to study genome organization and function.[45–49]

Moreover, tools like CRISPR-view have transformed genomic screenings by centralizing data from CRISPR-Cas9, CRISPRa, CRISPRi, and RNAi, standardizing the analysis of over 11,000 studies in humans and mice. Utilizing technologies such as MAGeCK-VISPR, this platform enables the exploration of complex phenotypes related to cellular proliferation, viral infections, and immune responses, democratizing access to genomic data and facilitating the discovery of novel genotype-phenotype relationships.[50]

Despite CRISPR technology's exceptional versatility, its widespread adoption has posed challenges. The abundance of available methodologies necessitates strategic planning that considers both the system's strengths and inherent limitations, including off-target effects and variability in efficiency.[33] These advancements highlight the importance of a well-designed and executed approach to maximizing CRISPR's potential in functional research.

From Research to Application

The CRISPR-Cas system has fundamentally transformed numerous domains within biotechnology and

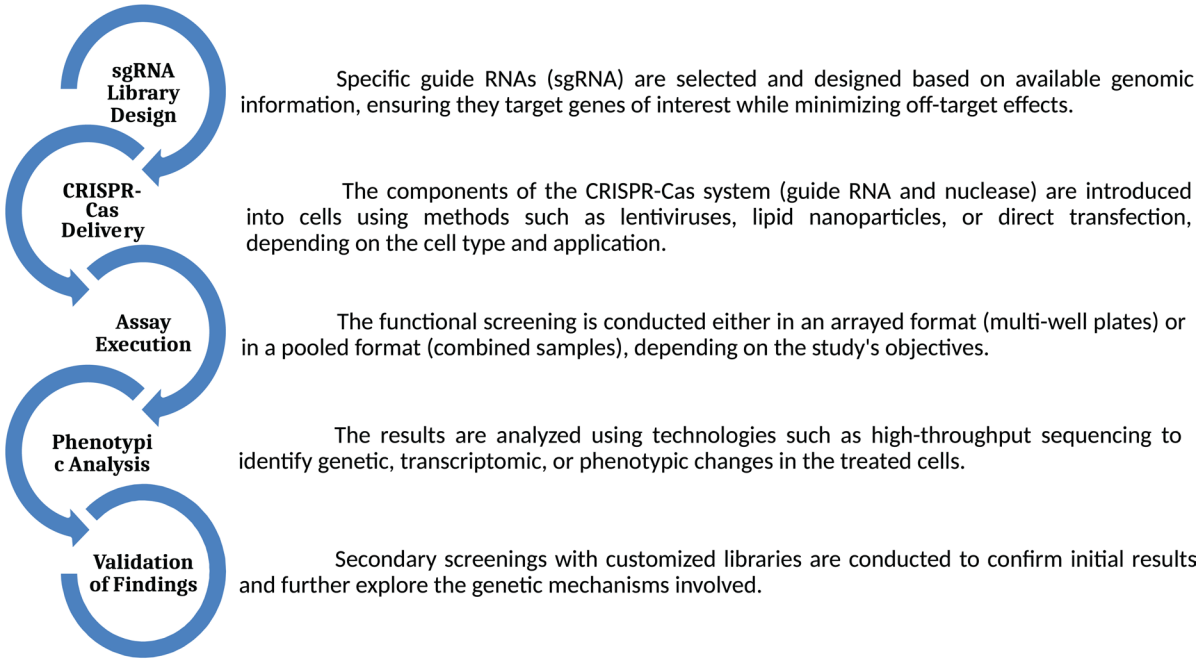

Fig 1 | Workflow for functional screenings using CRISPR-Cas. Includes sgRNA design, delivery into cells, assay execution, phenotypic analysis, and validation of findings to explore gene functions and genetic mechanisms

medicine, enhancing our capacity to examine, modify, and comprehend genomes. Regarding human disease scientific research, the CRISPR-Cas approach has been harnessed to expose unique genetic weaknesses in cancer cell lines, consequently enhancing the design of more significant targeted treatments. Furthermore, it has assumed an indispensable role in the exploration of infectious diseases, contributing to the identification of genes linked to resistance and vulnerability to pathogens such as HIV, malaria, and tuberculosis.[51–55]

Technological advancements in agriculture, including CRISPR-Cas9 and Cas12, have been employed in the development of virus-resistant flora, such as rice and tobacco, thereby diminishing the adverse effects of ailments induced by geminiviruses and mosaic viruses. These tools have laid the foundation for more resilient and sustainable crops.[56,57] The CRISPR-Cas9 technology has transformed the landscape of enhancing plant resilience against abiotic stresses by facilitating meticulous modifications in genes associated with drought, salinity, heat, and ion toxicity. Tools like CRISPRi and CRISPRa provide new possibilities for modulating genes without permanently altering the genome, while non-transgenic delivery systems facilitate the creation of crops without GMO labeling. This advancement is essential for developing more resilient and sustainable plants to address global food security challenges.[58]

In biomedicine, CRISPR has proven useful for generating more consistent animal models necessary for investigating gene functions and constructing precise gene therapies. Variants such as Cas13 have been used to detect viral RNA with high specificity and sensitivity, with applications in diagnosing infectious diseases and regulating key genes.[37,59] In the realm of cancer research, remarkable advancements in the art of lncRNA manipulation have been ignited by the ingenious CRISPR-Cas system, particularly through trailblazing techniques such as CRISPRi and CRISPRa. These groundbreaking technologies empower researchers to either mute or elevate specific lncRNAs, paving the way for an in-depth exploration of their contributions to cancer and their influence on the intricacies of tumor biology.

Furthermore, CRISPR-driven screenings have unveiled crucial lncRNAs linked to resistance against therapies, unlocking fresh avenues for crafting more potent and tailored treatment strategies.[60]

The Future of CRISPR-Cas in Functional Genomics

Off-target effects remain a major concern in CRISPR-Cas genetic editing. Studies have shown that Cas9 may introduce cuts in regions with sequences similar to the target due to tolerance for certain mismatches in the guide RNA. This can lead to unintended mutations with unpredictable consequences, especially in medical applications. To mitigate these risks, various strategies have been developed, including high-fidelity nucleases like Cas9-HF1, with enhanced specificity, optimized guide RNA design, and bioinformatics tools like DeepCRISPR and CRISPRoff, which predict and minimize off-target effects.[61–63]

The CRISPR-Cas9 framework is undergoing a remarkable transformation, as innovative tactics enhance its accuracy and broaden its spectrum of uses. A pivotal achievement in this journey is the advent of prime editing, which facilitates exact insertions, deletions, and

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substitutions without causing double-strand breaks, thus minimizing off-target effects. Additionally, base editors, designed to introduce single-nucleotide changes without disrupting the DNA backbone, have become invaluable tools for studying point mutations linked to genetic disorders. These innovations play a crucial role in precision medicine, allowing for more accurate modeling of human diseases and offering the potential to correct pathogenic mutations.[64]

An exciting advancement is the fusion of CRISPR-Cas elements with transcriptional and epigenetic editing technologies, showcasing dCas9-based enhancers and inhibitors. Such technologies facilitate the modulation of gene expression without the alteration of the DNA sequence, thereby presenting novel strategies for the examination of gene regulation and therapeutic interventions. Applications include the reprogramming of cellular states in regenerative medicine and the investigation of complex regulatory networks in diseases such as cancer. The scalability of CRISPR screening methodologies combined with these emerging tools further broadens the possibilities for synthetic biology, drug discovery, and functional genomics.[64]

While CRISPR-Cas has unequivocally demonstrated its potential as a groundbreaking instrument, its prospective developments herald even more significant progress. The expansion of genomic databases and the development of emerging tools such as CRISPR-associated transposons (CAST) are unlocking new possibilities for innovative applications. CAST systems, such as VcCAST from *Vibrio cholerae* and ShCAST from *Scytonema hofmanni*, enable DNA insertion without causing breaks in the genome, with precision rates exceeding 90%. These tools could revolutionize genetic engineering by facilitating the creation of directed libraries and the insertion of entire biosynthetic pathways.[65]

Among these emerging technologies, the ShCAST system from *Scytonema hofmanni* stands out due to its unique mechanism. It consists of transposase subunits similar to Tn7 and the Type VK CRISPR effector Cas12k, which together catalyze RNA-guided DNA transposition. This system inserts 60–66 base pair segments downstream of the protospacer, providing a high degree of precision. In *Escherichia coli*, ShCAST has successfully achieved targeted DNA integration at specific genomic sites with frequencies of up to 80%, without requiring positive selection. This not only expands our understanding of CRISPR-Cas functional diversity but also establishes a new paradigm for precise DNA insertion.[66]

A recent example of CAST use is the VcCAST system from *Vibrio cholerae*, which integrates a nuclease-deficient Type I-F CRISPR system with a Tn7-like transposase to enable guide RNA-directed transposition. The VcCAST-gRNA complex facilitates site-specific DNA insertion ~49 bp downstream of a protospacer with high precision (>99% on-target insertion). This has been applied in microbiome engineering, systematic gene disruption in *Pseudomonas aeruginosa*, and genome-scale functional genomics, demonstrating its potential for high-efficiency genetic modifications.[67]

However, these advancements come with technical challenges. Variability in guide RNA efficiency, limitations in transferring CAST systems to natural microbiomes, and off-target effects remain unresolved issues. Emerging technologies like TISCC-seq, which combines CRISPR editing with long-read sequencing, offer promising solutions by providing more precise data on endogenous mutations and their transcriptomic effects.[43,68]

In medicine, the prospective advancements of CRISPR-Cas are intrinsically associated with the progression of individualized therapeutic strategies. Integrating multi-omic data and using three-dimensional models like organoids will enable the validation and optimization of treatments, expanding CRISPR's clinical applications to detect and correct specific genetic vulnerabilities in patients.[32,69,70]

In agriculture, CRISPR is expected to continue enhancing global food security by addressing the need for more resilient and sustainable crops. The development of more specific tools for plant improvement represents a crucial step toward closing the gap between applications in animals and plants.[30]

Prospective advancements of CRISPR-Cas in functional genomics depend upon a comprehensive, interdisciplinary methodology that amalgamates innovative technologies, sophisticated experimental frameworks, and enhanced analytical precision. This trajectory promises to overcome current barriers, expanding the technology's potential in synthetic biology, personalized medicine, and agriculture.

Conclusions

The CRISPR-Cas mechanism has emerged as a groundbreaking instrument in contemporary biology, facilitating remarkable progress in genome alteration, gene modulation, and genomic representation. Its capacity to identify key genes and to study complex biological processes positions it as a cornerstone in functional genomics. Applications such as the development of gene therapies, the diagnosis of infectious diseases, and crop improvement highlight its impact on biomedicine and agriculture. However, while its simplicity and versatility have driven widespread adoption, significant challenges remain. Off-target effects, variable efficiency across systems, and limitations in complex biological models necessitate a multidisciplinary approach to overcome these barriers.

To maximize the impact of CRISPR-Cas in the realms of biotechnology and medicine, future research should focus on improving precision and safety by developing more targeted nucleases and refining techniques to minimize unintended off-target effects. Advancing epigenetic editing is another key focus, particularly through the use of variants like dCas9, which enable gene regulation without altering DNA sequences. Moreover, the triumphant clinical deployment of CRISPR-inspired treatments hinges on tackling ethical dilemmas and regulatory hurdles to guarantee their safety and effectiveness. Exploring alternative tools, such as CAST, remains essential for achieving precise genetic insertions without generating double-strand

breaks, further expanding the potential of genome editing technologies.

Innovations like CAST systems and advanced technologies such as TISCC-seq create new opportunities, further reinforcing CRISPR-Cas's potential to tackle large-scale scientific and societal challenges. Ultimately, CRISPR-Cas has revolutionized our understanding of life's intricate complexities while opening new avenues for enhanced and targeted biotechnological innovations. As research continues to refine its applications, this transformative tool solidifies its significance as an indispensable instrument in the science of tomorrow.

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