Dean&Francis
ISSN 2959-409X

Classification and Functional Diversity of CRISPR-Cas Systems with Emerging Editing and Delivery Tools

Runqi He

College of Agricultural and Environmental Sciences, University of California, Davis, CA 95616, USA
rqihe@ucdavis.edu

Abstract:

The CRISPR-Cas system originates from the adaptive immune mechanism of microorganisms and has now evolved into a revolutionary gene-editing tool, driving rapid development in the field of molecular biology. This system is systematically classified into two major categories: Class 1 (multi-protein complexes) and Class 2 (single-protein effectors), covering six main types, including DNA-targeting Cas9, Cas12 and RNA-targeting Cas13. These types possess diverse editing and regulatory functions. In recent years, this technology has not only achieved precise gene knockout and repair, but also derived new tools such as base editing and prime editing, which have significantly improved editing accuracy and expanded application scope. In addition, CRISPR technology has gone beyond gene editing itself and been applied in transcriptional regulation (CRISPRa/i) and molecular diagnostics (e.g., high-sensitivity nucleic acid detection). However, this technology still faces challenges in terms of delivery efficiency, safety and specificity. In particular, the limitations of viral vectors in packaging capacity and immunogenicity have promoted research on non-viral vectors (such as lipid nanoparticles, LNP) and physical delivery methods. This article reviews three aspects of the CRISPR system: classification and functions, evolution and optimization of editing tools, and delivery technologies, and looks forward to its broad prospects in the fields of genetic disease treatment, infectious disease detection and cell programming.

Keywords: CRISPR-CAS; gene editing; delivery systems.

ISSN 2959-409X

1. Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the partner proteins, Cas, originally evolved as a microbial immune system. It was found in various bacteria and archaea, and its main intention was to help defend from invading genetic material such as viruses and plasmids by utilizing RNA-guided enzymes to seek out and snip foreign DNA [1]. In 2012, scientists were able to re-engineer the CRISPR-Cas9 system to edit genes in eukaryotic cells, which led to a new era in molecular biology. In 2020, Emanuelle Charpentier and Jennifer Doudna were awarded the Nobel Prize in Chemistry for developing a way to edit genomes through CRISPR-Cas9. Since then, CRISPR-based technologies have evolved to allow DNA and RNA modifications across various organisms. This has allowed CRISPR-based systems to extend beyond their original role and be used for: therapeutic genome editing, transcriptional regulation, epigenetic modifications, functional genomics, and nucleic acid diagnostics [2]. During this time, CRISPR-Cas systems have been classified into various types and classes.

A systematic classification framework is essential for navigating this complexity. The current categorization has two major classes and six principal types (Types I-VI), which provide a foundational structure for selecting the appropriate CRISPR-Cas system based on the clinical or experimental requirements [1]. Additionally, parallel innovations such as base editors, prime editors, RNA-targeting Cas13 variants, and engineered delivery platforms have further expanded the scope and precision of CRISPR [3].

This research review provides a comprehensive view of the biological roles of CRISPR-Cas families, their development into versatile tools for genome editing, and the delivery technologies that shape the next generation of CRISPR applications.

2. CAS Classification and Function

CRISPR is often thought of as a single technology, but in reality, it is more complicated. CRISPR started as a microbial immune system, but it has since evolved into a diverse toolset that has different advantages and applications depending on the type. To better organize the family of CRISPR systems, scientists developed a classification framework based on the structure and composition of the Cas (CRISPR-associated) proteins. Today, they're grouped into two major classes: Class 1 and Class 2, which can be further divided into six main types, I through VI. Each offers its own editing capabilities and biological roles [1].

Class I systems, which include Types I, III, and IV, are essentially multi-protein task forces. They rely on large complexes made of several Cas proteins working together to find and destroy invading genetic material. These work like an immune system, with specific proteins having specific roles; some will be used to identify targets, and others do the cutting.

Type I systems are particularly common and specialize in recognizing foreign DNA. Once they identify their target, they will utilize Cas3, a molecular shredder that unwinds and degrades the invader's genetic code [1]. This allows foreign genetic material to be dismantled and destroyed. Type III systems are more versatile because they can target both DNA and RNA. Their versatility has potential benefits in immunity defense and could be used in future research; however, current research on Type III systems is limited due to their complexity compared to other systems. Type IV is also less understood compared to other systems, but current research suggests it focuses on DNA targeting, especially in plasmids. Generally, Class I systems are great for natural immunity, but they require a multi-protein architecture and can be complicated to package for human use due to their larger delivery systems.

In contrast, Class II systems, which include Types II, V, and VII, are streamlined and efficient. Instead of utilizing a team of proteins, each system only relies on a single, multifunctional effector. The simplicity of Class II systems makes it easier for researchers to program, engineer, and deliver into cells, thus making them the go-to tools for genome editing. The most common protein of the type II systems is Cas9, which is used to cut double-stranded DNA at specific sites guided by a short strand of RNA. Cas9 is used to make blunt-ended cuts and has well-understood targeting rules, which makes it the first CRISPR system to be widely used in labs and clinics across the world [2].

Cas9 is not the only widely used system. Cas12a, the effector in Type V, offers some advantages. It recognizes a different PAM sequence, which gives researchers more flexibility in targeting. It also creates sticky-ended cuts, which can be more useful for certain genetic modifications, and it can process multiple guided RNAs from a single array, to allow multiplexed editing [2]. This allows Cas12a to be more precise and handle multiple splices at once compared to Cas9.

Type VI systems also have Cas13, which is unlike Cas9 and Cas12a. Unlike the previous two, Cas13 targets RNA, which opens a new layer of gene regulation. Instead of making permanent changes, Cas13 can temporarily silence or modify gene expression by cutting RNA transcripts. Cas13 also has a unique "collateral cleavage" behavior, which causes it to shred nearby RNA indiscriminately after finding its target. Cas13 has been used in diagnostic tools like SHERLOCK, which can detect various genetic

material from viral RNA to cancer biomarkers with high sensitivity [4].

3. Transformation and Expansion Editing Tools

One of the earliest challenges with Cas9-based editing was its off-target activity. The enzyme could cleave DNA with sequences similar to the intended target, which raised the question of unintended mutations. To address this, researchers engineered more accurate Cas9 variants such as SpCas9-HF1, which introduced mutations in the protein's DNA-binding interface to reduce unintended interactions without compromising the on-target efficiency [2]. As Tao et al. explain, these variants are able to reduce off-target editing significantly, thus dropping indel formation at mismatched sites while retaining efficient cleavage at perfectly matched targets. Analysis demonstrated that engineered Cas9 variants such as SpCas9-HF1 or eSpCas9 displayed up to ten times the reduction in off-target activity across genomic sites, therefore demonstrating protein engineering could be used to alter and tune DNA-binding specificity [2].

Beyond accuracy, Cas12a has also become a complementary tool to Cas9 by offering distinct structural and functional advantages. Cas12a creates staggered cuts with 5' overhangs, which are more conducive to certain forms of DNA repair and gene insertion. It also recognizes T-rich PAM sequences to expand the targeting range to genomic regions that would otherwise be inaccessible to Cas9 [2]. Moreover, Cas12a can process a single crRNA array into multiple individual guides, thus enabling simultaneous editing for multiple genes. In comparative experiments, Cas12a achieved higher efficiency for multiplex editing than Cas9, with Tao et al. reporting successful editing of several genomic sites using only one crRNA array. Additionally, Cas12a's use of staggered cuts further away from PAM sites reduced unwanted indels compared to Cas9, which demonstrates its advantage for applications requiring precise knock-ins [2]. Overall, these optimizations have improved safety and expanded its genome editing capabilities.

Another significant innovation for CRISPR's capabilities was the addition of base editors, which are tools that enable single-nucleotide changes without adding double-stranded breaks (DSBs). The two main classes of base editors are cytosine base editors (CBEs), which convert C/G base pairs to T/A, and adenine base editors (ABEs), which help with A/T to G/C transitions. These editors typically fuse a catalytically impaired Cas9 (dCas9 or nCas9) with a deaminase enzyme, which then allows targeted, irreversible base conversions within a defined editing window [2]. Because they avoid DSBs and donor templates, base editors offer a lower-risk alternative for treating point mutation diseases. Tao et al., outline how CBEs and ABEs demonstrated efficient correction of single-base mutations in disease-relevant loci, with transition editing efficiencies often going above 50% in mammalian cells while producing fewer byproducts compared to HDR-based editing [2]. Base editors are useful in medical and research fields, but their usage is limited to transition mutations. To overcome this, prime editing was introduced as a search-and-replace system to edit genomes. It was developed by Anzalone et al. and uses a Cas9 nickase fused to a reverse transcriptase, and guided by a prime editing guide RNA (pegRNA) that codes the target site and intended edit [3]. This method can induce all 12 types of point mutations as well as insertions and deletions, without relying on DSBs or external donor DNA. Anzalone et al. reported that prime editing corrected pathogenic mutations in human cells with fewer unintended edits than Cas9 HDR methods. This included corrections of sickle-cell-causing mutations in HBB. Newer versions, including PE3 and PE5, helped further improve efficiency across loci, which demonstrates that prime editors can perform complex edits for therapeutic purposes [3]. Further innovations, such as PE3 and PE5, have improved efficiency and reduced unintended edits, to be used in clinical applications [2].

CRISPR's versatility goes beyond sequence editing. Catalytically inactive variants of Cas proteins, such as dCas9 and dCas12a, have been used as programmable gene regulators. When they are fused to transcriptional activation or repression domains, these systems become CRISPRa (activation) and CRISPRi (inference) platforms. By targeting promoter or enhancer regions, CRISPRa/i systems can modulate gene expression without altering the DNA sequence, thus providing control over gene networks [5]. Bendixen et al. outline that CRISPRa/i systems are able to be used therapeutically, such as upregulating genes that compensate for loss-of-function mutations, or downregulating oncogenes. In one case, CRISPRi was able to effectively repress MYC transcription in human cancer cells and reduce proliferation without having permanent genomic changes [5].

Meanwhile, Cas13 helps open new pathways by targeting DNA instead of RNA. Cas13's RNA-guided ribonuclease activity allows it to degrade specific RNA molecules. Using Cas13's collateral cleavage, researchers created SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing), which is a molecular diagnostic platform that can detect RNA from viruses with high sensitivity [4]. Gootenberg et al. demonstrate that SHERLOCK could detect attomolar levels of Zika virus RNA and still

ISSN 2959-409X

distinguish between different strains with a single-base specificity. This is more sensitive than qPCR-based detection and demonstrates that Cas13 could be used as a diagnostic tool in clinical applications, and allows Cas13 to be an important non-editing form of CRISPR [4].

4. Delivery Tools

Delivery is an integral part of CRISPR editing because it determines which tissues receive the editor. It also impacts how long the nuclease/guide is expressed (minutes for RNPs vs. days for mRNA and longer DNA). This can also have an impact on safety. In practice, CRISPR cargos can be delivered through plasmid DNA, mRNA, or ribonucleoprotein (RNP) complexes, with each method benefiting from different delivery vehicles, including: viral, nonviral, and physical [6,7].

Viral vectors have been the most commonly used delivery systems for CRISPR because of their natural ability to transfer genetic material to host cells. Two of the most common systems are adeno-associated viruses (AAVs) and lentiviruses. AAVs are important for in vivo gene therapy due to their relatively low immunogenicity and strong tropism; however, they have a limited packaging capacity at around 4.7kb, which creates a challenge for delivering large Cas proteins such as SpCas9, which is about 4.1kb before accounting for the promoter, guide RNAs, or other regulatory elements. To address the size constraint, groups often swap to compact nucleases such as SpCas9, or use split-Cas9 strategies with split inteins so the nuclease can reassemble inside cells [6]. Conversely, lentiviral vectors can package larger genetic elements and integrate into the host genome to ensure long-term expression. While this can be beneficial for stability in the cell line generation, it can also raise safety concerns for therapeutic use due to the risk of insertional mutagenesis. Thus, lentivirus is typically favored for in vivo applications. In practice, lentiviral vectors are often used ex vivo in applications such as T cells and HSCs, whereas AAV is predominantly used for in vivo delivery because it supports efficient tissue transduction with lower genotoxicity risk [6].

The limitations of viral systems have promoted research into nonviral vectors, which can provide separate benefits. One of these is lipid nanoparticles (LNPs). LNPs contain CRISPR components within a protective lipid shell that promotes uptake by the cells. They can be tuned by altering the lipid composition, charge, and modifying the surface to improve targeting and stability in circulation [7]. One success of LNPs was delivering mRNA vaccines against COVID-19, which demonstrated their ability to scale and clinical usage. Other nanoparticle strategies include gold nanoparticles, which can bind nucleic acids via electrostatic interactions and deliver them into cells through endocytosis. While less developed than LNPs, gold-based systems have demonstrated high transfection efficiency and potential for combination with other therapies. According to Kazemian et al., LNP is systematically tuned through chemistry and pK_a of ionizable cationic lipids to condense the cargo and enable endosomal escape. A second way is through the ratio of helper lipids, such as cholesterol, that help with stability and fusion. A third way is through PEG-lipids to modulate circulation and aggregation, and finally through surface ligands such as sugars and peptides to bias cell-type targeting.

In addition to biological carriers, physical delivery methods provide direct and often efficient forms of introducing CRISPR machinery into target cells. One example is electroporation, which is often used in ex vivo editing. By applying short electrical pulses, cell membranes become transiently permeable, allowing Cas9 RNPs or plasmids to enter. This approach has been effective for editing T cells and hematopoietic stem cells. Microinjection is another way to deliver CRISPR components directly into a cell or embryo. This method is often demanding, but can be useful in specific situations. Electroporation offers high uptake and rapid editing kinetics for RNPs, but is constrained by cell viability and scalability. Microinjection provides single-cell precision, but comes at the cost of speed [6].

5. Conclusion

Over the past decade, CRISPR-Cas systems have evolved from their basic microbial immune systems into an integral part of molecular biology. CRISPR evolved to form different classifications to adapt to the varying medical and biological needs. Cas systems are classified into multi-protein complexes (Class I) and single-protein effectors (Class II). Together, they offer a range of tools combining systems such as Cas9, Cas12a, and Cas13 for different functions. In its evolution, CRISPR has also advanced to be able to base edit and prime edit, which has allowed it to increase accuracy in rewriting the genetic code. At the same time, non-editing applications such as CRISPRa, CRISPRi, and RNA diagnostics have evolved to alter genomes.

The promise of CRISPR still has challenges, like delivery methods. While viral vectors like AAV and lentivirus are effective, they still have limitations in packaging capacity and safety. This has led to research into non-viral alternatives such as nanoparticles and physical methods. Different delivery systems have their unique benefits and drawbacks, which demonstrates the need for further research and innovation in this field, for it to be continually

applied.

CRISPR is still evolving today to improve precision and expand its functions. Diagnostic tools such as SHERLOCK demonstrate a new direction for CRISPR, where it can expand to become a system for low-cost detection system for pathogens and genetic disorders.

References

[1] Makarova K S, Koonin E V. Annotation and classification of CRISPR-Cas systems. CRISPR: methods and protocols, 2015: 47-75.

[2] Tao J L, Bauer D E, Chiarle R. Assessing and advancing the safety of CRISPR-Cas tools: from DNA to RNA editing. Nature Communications, 2023, 14(1): 212.

[3] Anzalone A V, Randolph P B, Davis J R, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature, 2019, 576(7785): 149-157.

[4] Gootenberg J S, Abudayyeh O O, Lee J W, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science, 2017, 356(6336): 438-442.

[5] Bendixen L, Jensen T I, Bak R O. CRISPR-Cas-mediated transcriptional modulation: The therapeutic promises of CRISPRa and CRISPRi. Molecular Therapy, 2023, 31(7): 1920-1937.

[6] Huang J, Zhou Y T, Li J, et al. CRISPR/Cas systems: Delivery and application in gene therapy. Frontiers in Bioengineering and Biotechnology, 2022, 10: 942325.

[7] Kazemian P, Yu S Y, Thomson S B, et al. Lipid-Nanoparticle-Based Delivery of CRISPR Cas9 Genome-Editing Components. Molecular Pharmaceutics, 2022, 19(6): 1669-1686.