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ADVANCES OF CRISPR CAS9 TECHNOLOGY FOR GENOME AND EPIGENOME EDITING.

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Abstract: The advent of CRISPR/Cas9 technology has revolutionized gene editing. Through diverse delivery methods, it has enabled effective gene editing across in vitro, ex vivo, and in vivo applications, thanks to ongoing advancements in the field. Viral vectors, despite their potential to trigger immune responses, limited cloning capacity, and risk of insertional mutagenesis, remain widely used in these systems. While chemical delivery methods still require extensive optimization to boost their efficiency for in vivo applications, physical delivery techniques are mostly confined to in vitro and ex vivo contexts. One of the most challenging aspects of gene editing remains developing a safe and effective in vivo delivery method for CRISPR/Cas9. CRISPR-Cas technology has also paved the way for a diverse range of molecular systems now widely used in research and increasingly in medical treatment. For instance, Cas proteins without nucleolytic activity—referred to as dead Cas proteins or dCas—can deliver functional cargo to specific, preselected genome locations. This review starts by examining the advantages and limitations of various physical methods for delivering Cas9. Next, it highlights key applications of CRISPR systems in epigenetic modifications. Finally, it explores the use of CRISPR-Cas9 technology in genome editing, with a particular focus on base editing and prime editing, along with its future prospects.

Keywords: CRISPR/Cas9 technology, dCas-system, gene editing, epigenetics, chromatin

1. Introduction:

CRISPR, or **Clustered Regularly Interspaced Short Palindromic Repeats**, was first identified in prokaryotes as part of their adaptive immune system (Lander, 2016). It consists of short DNA sequences integrated into prokaryotic genomes during past bacteriophage infections, providing a defense mechanism against subsequent infections by related viruses (Lander, 2016). This discovery eventually led to the development of CRISPR as a gene-editing tool for eukaryotic cells, enabling its application to treat human diseases (Mali et al., 2013); (Doudna & Charpentier, 2014).

The CRISPR/Cas9 system consists of a Cas9 endonuclease and a target-specific single guide RNA (sgRNA), which directs the Cas9 protein to a specific DNA sequence. The sgRNA is created by fusing CRISPR RNA (crRNA) with trans-activating CRISPR RNA (tracrRNA). Upon binding to the target

site, the Cas9 protein induces a double-stranded DNA break (DSB), facilitating genomic modifications.

Among CRISPR systems, the **Type II CRISPR-Cas** is the most commonly used, although other types, such as Types V and VI, are also employed for genomic and epigenomic editing. In the Type II system, the Cas9 protein contains two nucleolytic domains—RuvC and HNH—that cleave DNA strands to create DSBs (Jinek et al., 2012). Point mutations in these domains (D10A and H840A) can inactivate their nucleolytic function while preserving DNA-binding capabilities, resulting in "dead Cas9" (dCas9). This modified protein has expanded CRISPR's utility beyond cutting DNA, such as in gene regulation (Qi et al., 2013).

The development of CRISPR/Cas9 earned the 2020 Nobel Prize in Chemistry, highlighting its transformative impact on biological and clinical research. Despite its versatility and efficiency, further refinement is necessary to optimize DNA editing and minimize risks. A critical consideration is the method of Cas9 delivery, which significantly influences editing efficiency and safety.

Cas9 can be delivered as DNA, mRNA, or protein, each with distinct advantages and drawbacks. Plasmid DNA delivery is cost-effective and ensures prolonged Cas9 expression, which can benefit long-term editing needs. However, this approach requires transcription and translation, delaying the onset of editing. Prolonged Cas9 expression also increases the likelihood of off-target effects and carries risks of insertional mutagenesis (Wu et al., 2014); (Chen et al., 2020). mRNA delivery accelerates editing by bypassing the transcription step, but its instability and susceptibility to RNase degradation limit its duration. Chemical modifications can enhance mRNA stability, reducing off-target effects while maintaining moderate efficiency. Protein delivery allows immediate editing by introducing Cas9 directly into the nucleus, offering high efficiency and minimal off-target effects. However, this method is costly and poses safety concerns, such as potential contamination with bacterial endotoxins, particularly in clinical trials (Yin et al., 2017); (You et al., 2019).Delivery strategies are broadly classified into viral and nonviral vector-based approaches, with nonviral methods including physical and chemical techniques. Nanomaterials are emerging as promising nonviral vectors, including lipid nanoparticles (LNPs), cationic liposomes, gold nanoparticles, and exosomes (Chen et al., 2020). Exosomes, in particular, have demonstrated great potential for effective CRISPR/Cas9 delivery in both in vitro and in vivo settings, making them innovative tools for enhancing clinical applications. Selecting the appropriate delivery method requires a careful balance between safety, efficiency, and application-specific needs, particularly in clinical settings where stringent safety requirements must be met. As CRISPR technology advances, improving delivery systems will remain critical for its success as a therapeutic tool.

In this review, we will first explore various non-viral physical methods for delivering the CRISPR/Cas9 system. We will also examine different approaches to genome editing using CRISPR/Cas9, with a particular focus on base editing and modifications of noncoding genomic regions. Additionally, we will summarize the applications of CRISPR/Cas9 in introducing epigenetic modifications, with special emphasis on 3D genome organization, chromatin remodeling, and the associated molecular techniques. Finally, we will discuss anticipated future advancements in these areas.

Typical Approaches for Delivering CRISPR/Cas9 system for genome and epigenome editing.

Effectively delivering the CRISPR/Cas9 system for gene editing poses a challenge. The Cas9 protein, with a molecular weight of around 160 kDa (Jinek et al., 2014), and the sgRNA's long phosphate backbone collectively give the formed RNP complex a net negative charge (Sun, Ji, et al., 2015). These characteristics make it challenging for the Cas9 RNP to traverse the cell membrane. Once inside cells, both the Cas9 protein and sgRNA must withstand cellular degradation processes and migrate into the nucleus to facilitate gene editing. Therefore, the selection of an appropriate delivery strategy for the CRISPR/Cas9 system is crucial for achieving efficient and precise gene editing. In clinical applications, consideration of the safety profile is essential to prevent or minimize insertional mutagenesis. Currently, delivery strategies for CRISPR/Cas9 can be broadly categorized into viral or nonviral approaches, depending on viral transduction. Nonviral approaches encompass various

physical and chemical delivery methods. Each of these methods has its advantages and disadvantages. (Table 1). We will discuss in detail the non-viral modes of CRISPR/Cas9 delivery.

1 various types of Physical Methods of CRISPR/Cas9 delivery system.

1.1 Lipid-Based Nanoparticles

Lipid nanoparticles (LNPs), the traditional method for nucleic acid delivery, have been extensively studied. Through host-guest and electrostatic interactions, negatively charged nucleic acids combine with positively charged lipids to form complexes, which are subsequently internalized via endocytosis. LNPs can effectively deliver plasmids or mRNAs while protecting them from nuclease degradation. Their ability to transport mRNA and small interfering RNA (siRNA) has been well established in both preclinical and clinical studies. However, the delivery efficiency of LNPs for packaging CRISPR/Cas9 plasmids has not yet met clinical standards, particularly in primary cells or in vivo animal models. Enhancing the efficiency of the lipid nanoparticle delivery system through modifications could significantly improve its performance and broaden its clinical applications.

Researchers have developed a universal engineering approach to preserve the integrity of ribonucleoprotein (RNP) complexes by incorporating persistent cationic additives, such as DOTAP, into ionizable lipid nanoparticle (LNP) formulations. These lipid components facilitate the encapsulation of CRISPR RNPs while maintaining their activity, enabling precise DNA editing in target tissues. Low-dose intravenous injections using this method can effectively direct therapeutic delivery to specific tissues, including the brain, liver, lungs, and sphincter muscles (Wei et al., 2020), (Guo et al., 2020) developed an antibody-conjugated, tumor-targeted nano lipo gel designed to deliver CRISPR/Cas9 plasmids specifically into triple-negative breast cancer cells. This approach achieved an 81% efficiency in knocking out the Lipocalin 2 gene, resulting in a 77% reduction in tumor growth. These findings highlight the potential of tumour-targeted nano lipo gels (tNLGs) as a safe, precise, and efficient platform for CRISPR-mediated genome editing with targeted specificity. Overall, lipid nanoparticles (LNPs) are proven to be safe, effective, and straightforward for delivering CRISPR/Cas9 DNA and mRNA. While commercial Lipofectamine falls short of meeting the delivery needs for CRISPR/Cas9, functional lipid modifications offer the potential to create a new generation of highly efficient gene-editing delivery systems.

1.2 Polymer-Based Nanoparticles

The chemical diversity and functional versatility of cationic polymer carriers enable a wide range of adaptable structural designs. Cationic polymer nanoparticles have been successfully used to deliver various nucleic acid forms, including plasmid DNA and mRNA. Among these, cationic polymers such as chitosan and polyethyleneimine are the most commonly employed for CRISPR/Cas9 delivery. Similar to lipid carriers, polymer nanoparticles can penetrate cell membranes via endocytosis while protecting the loaded cargo from nuclease degradation and immune system detection. (Yin et al., 2013)demonstrated the intracellular delivery of Cas9 mRNA or CRISPR/Cas9 plasmids to macrophages using the amphiphilic block polymer polyethylene glycol-b-poly (lactic-co-glycolic acid) (PEG-b-PLGA) in combination with cationic lipids. Intravenous injection of a macrophage-specific promoter driving Cas9 expression precise gene editing in macrophages. Additionally, a multifunctional nuclear-targeting nanostructure was developed using polypeptide RGD-R8-modified hyaluronic acid and perfluoro butanamide-modified oligo PEI. This innovative design efficiently loaded CRISPR/Cas9 plasmids, facilitated endosomal escape, and delivered the cargo to the nucleus, achieving significant target gene knockout. Chitosan has proven to be an effective polymer carrier for CRISPR/Cas9 delivery. (Qiao et al., 2019)demonstrated the use of red fluorescent protein (RFP)-tagged chitosan to form positively charged nanoparticles capable of simultaneously delivering Cas9 ribonucleoproteins (RNPs) and glutamate residues. This system also co-delivers single-stranded DNA (ssDNA) donors to the cytoplasm, where they are subsequently released and transported to the nucleus for homology-directed repair (HDR)-mediated genome editing. Additionally, (B. Y. Liu et al., 2018) developed dual-targeted inorganic/polymer hybrid nanoparticles. This design incorporated co-precipitation to enable the CRISPR knockout of cyclin-dependent kinase 11 (CDK11). The Cas9

plasmid was encapsulated in the nanoparticle core, comprising calcium carbonate, protamine sulfate, and carboxymethyl chitosan, stabilized with calcium phosphate. The system utilized S1411 aptamer ligands, leveraging electrostatic interactions to enhance delivery efficiency. This dual-targeted polymer nano efficiently delivers the CRISPR/Cas9 plasmid to the tumor cell nucleus, enabling precise knockout of the CDK11 gene. The CRISPR/Cas9 system holds significant potential for designing polymeric vectors with adaptable structural features, optimizing therapeutic outcomes.

1.3 DNA Nanostructures

DNA has been utilized to design nanostructures for targeted drug delivery and imaging, leveraging its ability to self-assemble into complex structures due to its predictable interactions and controllable sequences. These DNA nanostructures offer advantages such as biodegradability, biocompatibility, and high cargo-loading capacity. Traditionally, DNA nanostructures are formed through Watson-Crick base pairing of short DNA strands, but this process often requires large quantities of DNA and intricate assembly methods. To simplify this process, (Sun, Ji, et al., 2015) employed rolling circle amplification (RCA) to streamline the production of DNA nanostructures. Using RCA, (Sun, Lu, et al., 2015) developed a novel yarn-like self-assembled DNA nanomaterial capable of delivering CRISPR/Cas9 ribonucleoproteins (RNPs) both in vitro and in vivo. DNA nanoparticles can be employed to deliver a miRNA-responsive system and encode DNA aptamers that specifically target tumor cells. Research has demonstrated that genome editing efficiency can be significantly enhanced by integrating nanoflowers with a stimulus-responsive approach for Cas9/sgRNA release. This strategy allows for cell type-specific targeting through the controlled release of miR-21-responsive Cas9/sgRNA (Shi et al., 2020).

1.4 Gold Nanoparticles

old nanoparticles (AuNPs) represent a novel carrier for CRISPR/Cas9 RNP delivery. The ability to form Au-S bonds allows for easy cross-linking of AuNPs with sulfhydryl (-SH) compounds, enabling control over their hydrophilicity and surface charge (Lévy et al., 2010). Once AuNPs are surface-modified with cationic peptides, electrostatic interactions can facilitate the adsorption of pCas9. (Wang et al., 2018) modified the TAT peptide (C-terminal cysteine) on the surface of AuNPs containing pCas9 protein. After intravenous injection, a laser directed at the AuNPs generates a thermal effect, triggering the release of Cas9. The cationic TAT peptide then directs pCas9/sgPLK-1 (Polo-like kinase 1) into the nucleus, where it disrupts the PLK-1 gene, inhibiting tumor growth.Cationic arginine-functionalized gold nanoparticles (ArgNPs) were developed by (Mout et al., 2017) for the delivery of sgRNA and chemically modified Cas9 protein. A glutamate peptide tag was added to the N-terminus of Cas9, which neutralizes the protein's positive charge by introducing negatively charged amino acids. This allows the Cas9 protein to bind to the positively charged arginine residues on the ArgNPs, forming self-assembled nanocomponents. The cytoplasmic and nuclear transport efficiency of this Cas9 delivery method was approximately 90%, with genome editing efficiency ranging from 23% to 30% (Yang et al., 2011); (Mout et al., 2017).

The use of gold nanoparticles (AuNPs) in gene editing has advanced with the development of CRISPR-Gold technology. This innovative approach helps mitigate some of the negative effects associated with CRISPR, such as off-target mutations, and allows for better control over the amount needed for each injection. By using AuNP surfaces combined with thiol-modified oligonucleotides (DNA-thiol), donor ssDNA can hybridize with DNA-thiol, facilitating the loading of Cas9 ribonucleoproteins (RNPs) through their affinity for ssDNA and the Cas9 nuclease. CRISPR-Gold is produced by encapsulating the system with the cationic polymer PAsp (DET). After intramuscular injection, CRISPR-Gold has been shown to reduce muscle fibrosis in X-linked muscular dystrophy (MDX) mice, effectively correcting the mutant dystrophin gene with a 5.4% correction rate (Lee et al., 2017). CRISPR-Gold technology's ability to precisely modify specific brain cells opens up promising possibilities for treating various neurogenetic disorders and targeted therapies for social behavior disorders. By enabling the targeted knockout of the metabotropic glutamate receptor 5 (mGluR5) gene, CRISPR-Gold facilitates the intracranial delivery of RNA-guided endonucleases,

such as Cas9 and Cpf1, into adult mouse brains. This approach has been shown to effectively reduce mGluR5 levels and reverse the repetitive behaviors associated with autism caused by fragile X syndrome (Lee et al., 2018). CRISPR-Gold technology, utilizing dual sgRNA, extends its application beyond single-gene disorders to address polygenic diseases such as Huntington's disease. Gold nanoclusters (AuNCs), with their exceptional fluorescence emission and customizable surface functionalization, enable real-time monitoring of biological effects during gene editing (Tao et al., 2021). The development of additional AuNC-based nanocomposites will further enhance the therapeutic and diagnostic potential of CRISPR technologies.

1.5 Microinjection

It refers to the direct injection of Cas9 and sgRNAs into cells using a microscope and needle. The needle penetrates the cell membrane, facilitating the direct delivery of payloads into the nucleus, alleviating concerns about the molecular weight of Cas9 typically encountered in viral infections. The limited cloning capacity associated with vector-mediated delivery does not pose a hindrance in microinjection. Moreover, manual injection enables accurate dosing of payloads into cells. Nevertheless, microinjection is a process that demands considerable time and technical expertise, leading to a restricted throughput. Additionally, the requirement for a microscope in the injection process makes it unsuitable for application on live patients. Indeed, the primary use of microinjection is in animal zygotes for the creation of transgenic animals. (Horii et al., 2014), (Long et al., 2014). Electroporation is a widely adopted method of physical delivery that employs electrical current pulses to temporarily open pores in cell membranes, facilitating the entry of cargo into cells. Its effectiveness in transporting payloads across various cell types makes electroporation a common choice for in vitro and ex vivo gene editing applications. This is advantageous compared to conventional transfection methods, which often face challenges in transfecting cell types that are typically resistant, such as primary cells. Certainly, the application of electroporation for ex vivo gene editing has played a pivotal role in advancing stem cell therapies, specifically in the context of treating hematologic malignancies (Dever et al., 2016), (Romero et al., 2018). Following ex vivo modification, patient-derived hematopoietic stem/progenitor cells are reintroduced into patients as part of the treatment process (Romero et al., 2018)

	VIRAL VECTOR DELIVERY			
APPROACH	LV	AAV	AV	EV
Cas9 Delivery Method	DNA	DNA	DNA	DNA
Delivery Efficiency	High	Medium	Medium	Medium
Key Advantages	- High efficiency - Supports larger gene insert	Non-integrating	Non-integrating	-Non-integrating -Short term delivery -Supports multiplexing -All-in-one design
Application	in vitro and ex vivo	in vivo	in vivo	in vitro, ex vivo and in vivo

	NON-VIRAL VECTOR DELIVERY			
APPROACH	Microinjection	Electroporation	Cell Penetrating Peptide	Lipid-based Nanoparticle
Cas9 Delivery Method	DNA, mRNA or Protein	DNA, mRNA or Protein	Protein	DNA, mRNA or Protein
Delivery Efficiency	Low	High	Low	Low
Key Advantages	-Direct delivery -Dosage more controllable	-Efficient delivery -Easy to operate	No risk of virus	-FDA approved -Low cell stress
Application	in vitro and ex vivo	in vitro and ex vivo	in vitro and in vivo	in vitro and in vivo

Table 1. Comparison of Common Strategies for Delivery CRISPR-Cas9 System

1.6 Electroporation

Electroporation is a widely used technique for delivering proteins and nucleic acids into mammalian cells (Tebas et al., 2014), (Mali et al., 2013) This method temporarily increases the permeability of the cell membrane, allowing these molecules to enter the cell. It is applicable to various CRISPR-Cas9 systems, including plasmid-based setups, Cas9/sgRNA ribonucleoprotein complexes (RNPs), and combinations of Cas9 mRNA and sgRNA , However, a significant limitation of electroporation is its low efficiency, with only about 0.01% of target cells successfully incorporating plasmid DNA. Additionally, the process often results in substantial cell death. Electroporation of plasmid-based CRISPR-Cas9 systems has been extensively utilized in gene-editing studies of vertebrate

organogenesis. This approach has been applied to study the development of chicken embryos, mouse brains, zebrafish fins, and axolotl tissues (Thummel et al., 2006),(Fei et al., 2014) More recently, plasmid-based CRISPR-Cas9 systems have been successfully delivered via electroporation to various cell types, including cancer cells, CD4+ T cells, CD34+ stem cells, and embryonic stem cells. (Yang et al., 2013) Additionally, electroporation has been employed to deliver Cas9 mRNA and sgRNA into cells. For example, to generate a mouse model with specific genetic modifications, Cas9 mRNA, sgRNA, and donor DNA were introduced into mouse zygotes using this method. Electroporation has also been used to deliver ribonucleoprotein (RNP) complexes to various cell types, such as CD4+ human T cells, fibroblasts, and embryonic stem cells (Kim et al., 2014) In axolotl spinal cord cells, electroporation of CRISPR-Cas9 ribonucleoproteins (RNPs) has demonstrated higher gene-editing efficiency compared to plasmid-based CRISPR-Cas9 systems (Fei et al., 2016). Liang and colleagues conducted a comparative study of RNPs, Cas9 mRNA/sgRNA, and plasmid-based CRISPR-Cas9 systems, showing that RNP electroporation achieved superior gene-editing outcomes in specific target cells. For instance, RNP electroporation yielded editing efficiencies of 87% in Jurkat T cells and 94% in induced pluripotent stem cells (iPSCs). In contrast, electroporation with plasmid-based CRISPR-Cas9 and Cas9 mRNA/sgRNA resulted in much lower efficiencies: 63% and 42% in Jurkat T cells, and 20% and 32% in iPSCs, respectively (Liang et al., 2015)

1.7 Hydrodynamic injection

Hydrodynamic injection involves the rapid infusion of a nucleic acid solution into rats via the tail vein, typically in volumes equal to 8–10% of their body weight (M. S. Al-Dosari, J. E. Knapp and D. Liu Adv Genet 2005). Since its development, this method has become one of the simplest and most efficient techniques for delivering nucleic acids to the liver (Liu et al., 1999). The rapid administration of a large volume generates hydrodynamic pressure, creating temporary pores in endothelial cell membranes that facilitate nucleic acid entry into cells. In a study addressing hereditary tyrosinemia, researchers corrected the Fah mutation in a mouse model by delivering a plasmid-based CRISPR-Cas9 system to hepatocytes via hydrodynamic injection (Zhen et al., 2015). The plasmid utilized the pX330 backbone, containing cassettes for the Cas9 nuclease and sgRNA, with a Fah-targeting sequence incorporated. Along with the plasmid, a modified Fah DNA template was co-injected, leading to the production of Fah protein in approximately 1 out of 250 liver cells. Building on this work, the same group later used hydrodynamic injection to deliver a pX330 plasmid system co-expressing a sgRNA targeting the PTEN gene. This approach resulted in genomic alterations in approximately 2.6% of the liver's genome sequences. (Yin et al., 2014)

Hydrodynamic injection is highly effective in small animals but presents significant challenges for use in larger animals (Suda & Liu, 2007). It can cause transient heart dysfunction, liver enlargement, elevated blood pressure, and, in severe cases, death (Khorsandi et al., 2008). Its clinical application remains difficult, and its effects on large animals are not fully understood. For example, a clinical trial using hydrodynamic gene therapy to treat cirrhosis patients was ultimately unsuccessful due to hepatotoxicity.

2 CRISPR-Cas9 and Epigenetics

Epigenetics has emerged as a critical field of research in recent decades, providing deep insights into the regulation of gene expression and genetic stability without altering the underlying DNA sequence. Epigenetic mechanisms, such as DNA methylation, histone modifications, and chromatin remodeling, influence cellular phenotypes and behavior, often transmitting these traits across generations (Bhattacharjee et al., 2023). These regulatory systems are highly sensitive to environmental signals, shaping adult phenotypes and playing significant roles in the onset and progression of various diseases, including cancer and neurological disorders ((Bhattacharjee et al., 2023); (Vojta et al., 2016). At the core of epigenetics are molecular tools known as **epigenetic effectors**, which modify chromatin states, DNA, or histones to regulate gene expression without changing the genetic code. These modifications, such as DNA methylation and histone acetylation, result in stable yet reversible changes in gene function, demonstrating the intricate interplay between the genome and its regulatory

environment. Despite these advances, the causal relationship between specific histone modifications and gene expression remains unresolved (fig,1). Addressing this requires innovative methods to directly study the effects of targeted epigenetic changes on gene regulation at defined genomic loci (Vojta et al., 2016).

The advent of CRISPR-Cas9 technology has transformed our ability to interrogate and manipulate the epigenome. By coupling **CRISPR systems**, especially nuclease-deactivated Cas9 (dCas9) variants, with epigenetic effectors such as DNA methyltransferases, histone modifiers, and chromatin remodelers, researchers can achieve precise, localized epigenetic modifications (Nakamura et al., 2021) . This approach allows for the modulation of chromatin states, transcriptional activity, and higher-order chromatin structures, bridging the gap between genome organization and functional outcomes (Li et al., 2021).

Key techniques within this framework include:

1. **CRISPRi and CRISPRa**: Tools for targeted transcriptional repression or activation.
2. **Chromatin Editing**: Precise modifications of histone marks and DNA methylation.
3. **3D Genome Engineering**: Manipulating chromatin loops, enhancer-promoter interactions, and topologically associating domains (TADs) (Nakamura et al., 2021).

The integration of dCas9 with epigenetic effectors has also enabled the development of synthetic transcriptional regulators, which provide a powerful means to dissect the relationships between genomic structure and gene expression. For instance, dCas9 fused to heterodimerization domains can tether genomic regions to control enhancer-promoter interactions, resulting in persistent gene activation. Additional approaches, such as light-activated looping and CRISPR-GO, facilitate the study of spatial genome organization by directing genomic loci to specific nuclear compartments, such as Cajal bodies or the nuclear envelope (Nakamura et al., 2021); (Li et al., 2021).

Furthermore, CRISPR-based strategies have elucidated the role of CTCF, a protein critical for TAD formation, in chromatin topology and gene regulation. Targeted deletion, methylation editing, or binding site occlusion using CRISPR tools has revealed how CTCF-driven interactions shape genomic architecture and transcriptional outcomes (Nakamura et al., 2021). These advancements highlight the immense potential of CRISPR-Cas9 in unraveling the complex interplay between genome structure and function.

Overall, the utilization of CRISPR technology with epigenetics provides an unprecedented window into gene regulatory networks. This fusion not only enhances our understanding of development and disease mechanisms but also paves the way for novel therapeutic approaches that harness the power of precise epigenetic editing (Bhattacharjee et al., 2023); (Vojta et al., 2016); (Nakamura et al., 2021).

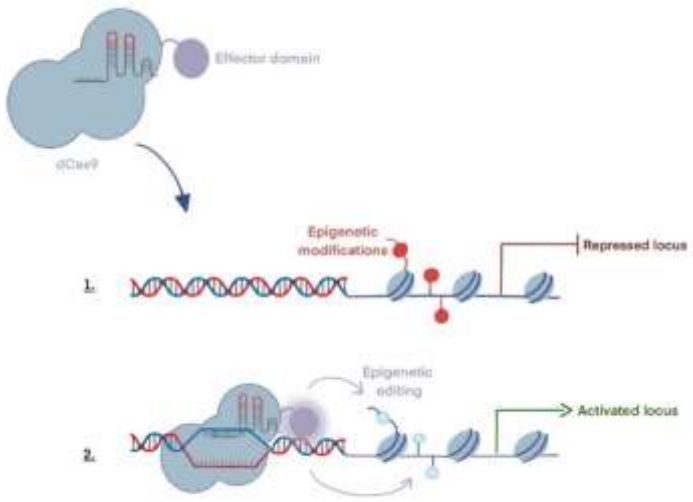

Figure 1: Mechanism of CRISPR-Cas9 mediated epigenetic editing. A catalytically inactive Cas protein (dCas9) is fused to a variety of epigenetic modifiers, including methyltransferases, demethylases, acetyltransferases, and histone deacetylases, allowing for the precise targeting of

particular epigenetic marks at desired genomic locations. There are two primary steps involved in epigenome editing: directing the editing complex to a particular genomic region via gRNA. The modified Cas protein is precisely guided toward DNA of interest by the gRNA. Once the fused epigenetic effector domain has been directed to the intended genomic region, it modifies the epigenetic landscape, a process known as epigenetic mark modulation.

2.1 Applications of CRISPR-Cas9 Technology in Epigenetics

CRISPR-Cas9 technology has enabled precise manipulation of epigenetic markers such as DNA methylation, histone modifications, as well as chromatin remodelers. These advancements provide us with valuable information about role of epigenetics in gene regulation, genome organization and also, potential therapeutic applications, allowing researchers to target specific epigenetic factors with accuracy.

2.1.1DNA Methylation and Demethylation

The DNA is epigenetically marked either by methylation or demethylation. These modifications are added by epigenetic effectors known as DNA methyltransferases that add 1-3 methyl residues at the 5^{th} carbon of cytosine leading to the formation of 5-methylcytosine. DNA methylation acts as an inactive mark due to which transcriptional repression takes place. However, not all cytosine residues can be methylated, cytosines must immediately be followed by guanine for the pyrimidine ring to be acted upon by epigenetic effectors, these are called CpG sites. CpG dinucleotides are not abundant in the genome, with 70% methylation and concentrated in intra- and intergenic areas, but unmethylated CpGs cluster, called "CpG islands", are located in transcription start sites and within genes (Sweatt et al., 2013) DNMT enzymes regulate DNA methylation, with DNMT1 maintaining existing methylation patterns and DNMT3a/3b creating new ones during cell fate determination (Willyard, 2017). Methylation normally reduces gene transcription, particularly in promoters, although methylation within gene bodies can boost transcription, demonstrating the epigenetic mark's location-dependent effects. By fusing deactivated Cas9 (dCas9) with DNA methyltransferases, researchers have developed a system to introduce methylation at specific CpG sites to study its effect. For example, targeted methylation of the IL6ST and CDKN2A promoters silenced their expression, demonstrating potential therapeutic applications in autoimmune disorders and cancer (McDonald et al., 2016). Similarly, methylation of the APP promoter reduced amyloid-beta production in Alzheimer's models, suggesting a strategy to combat neurodegenerative diseases (Park et al., 2022). Conversely, dCas9-TET1 and dCas9-ROS1 facilitate active DNA demethylation by removing methyl groups, thereby reactivating silenced tumor suppressor genes and addressing hypermethylation-associated conditions such as cancer and neurodevelopmental disorders (Xu et al., 2016), (Devesa-Guerra et al., 2020).

2.1.2 Histone Modifications

The nucleosome is the fundamental unit of chromatin, consisting of an octomer of histone proteins (two copies of histones 2A, 2B, 3, and 4). The DNA double helix is wrapped around it, in a bead on a string manner. Chemical changes to the N-terminal tails of histone proteins have a role in determining the condensed or packed state of nucleosomes, which affects the transcriptional activity of the DNA linked to it. Structural studies show that N-terminal tails extend from nucleosomes and undergo substantial post-translational modifications (Luger et al., 1997). Currently, four post-translational modifications of histone tails are well understood: acetylation, methylation, ubiquitination, and phosphorylation. These alterations function as epigenetic tags or markers (Strahl & Allis, 2000) , (Strahl & Allis, 2000).

In histone methylation, methyl groups are added to lysine or arginine residues by histone methyltransferases (HMTs), its function varying according to its location and degree of methylation. For example, H3K4 methylation is linked to transcriptional activation, whereas H3K9 and H3K27 methylation are associated with gene silence. In contrast, histone acetylation performed by histone acetyltransferases (HATs), which adds acetyl groups to lysine residues, is commonly associated with

gene activation because it promotes open chromatin state, making DNA accessible for transcriptional activity. Both changes are reversible and controlled by histone demethylases (HDMs) and histones deacetylases (HDACs), respectively. CRISPR-dCas9 coupled with HMTs or HDMs allows for precise alteration of histone methylation patterns, providing insights into its functional functions and therapeutic potential. Similarly, CRISPR-dCas9 coupled with HATs or HDACs allows for precise modulation of histone acetylation patterns.

In *Aspergillus niger*, dCas9-p300 was used to acetylate histones and activate genes such as *breF* and *fuml*, enhancing metabolite production, while dCas9-HosA repressed gene expression through histone deacetylation. The study highlighted the importance of sgRNA positioning relative to transcription start sites in determining the efficiency of these modifications, illustrating the role of histone acetylation in regulating fungal metabolism.

In another study, dCas9-p300 was used to install H3K27 acetylation at specific promoters, which enriched H3K4 trimethylation, activating gene expression. However, introducing H3K4me3 alone with a dCas9-SET fusion did not induce H3K27ac or transcription, revealing H3K27ac as an upstream regulator of transcriptional activation. The role of BRD2 as a mediator of H3K27ac-driven effects was validated by inhibiting BRD2-H3K27ac interactions with JQ1, which impaired downstream transcriptional events. These findings emphasize the utility of CRISPR-dCas9 in validating the causal relationships between histone modifications and transcription (Zhao et al., 2021).

In cancer research, CRISPR-dCas9 tools have been instrumental in targeting the cancer epigenome. Using fusions with epigenetic effectors like DNA methyltransferases or histone acetyltransferases, researchers have reactivated tumor suppressor genes like *PTEN* and *KLF4*, reducing cancer proliferation and metastasis. Conversely, silencing oncogenes like *Myc* has shown promising therapeutic results. This dual ability to activate and repress genes highlights CRISPR-dCas9's potential in reversing cancer-related epigenetic changes and advancing personalized cancer therapy (Zhao et al., 2021).

2.1.3 Chromatin Remodeling

Chromatin remodelers are protein complexes that modify the location, composition, or density of nucleosomes, which are chromatin's structural components. These remodelers play an important function in regulating DNA access for activities including transcription, replication, and repair. They perform this by modulating chromatin in a variety of ways, including sliding or removing nucleosomes, swapping histone variations, and changing histone modifications.

By fusing dCas9 with chromatin remodeling factors such as PRDM9, researchers enhanced homology-directed repair efficiency through targeted histone methylation (Chen et al., 2022). Additionally, studies have shown that CRISPR can improve access to compacted chromatin regions by inhibiting histone deacetylases like HDAC1 and HDAC2, facilitating efficient gene editing and knockout in otherwise inaccessible genomic regions (Liu et al., 2020). This approach has significant implications for therapeutic gene editing, particularly in diseases involving chromatin abnormalities. Furthermore, CRISPR systems such as dCas9-SunTag have been used to remodel chromatin at key pluripotency loci, such as *Oct4* and *Sox2*, enabling the reprogramming of fibroblasts into induced pluripotent stem cells (P. Liu et al., 2018).

Studies have further revealed the SWI/SNF chromatin remodeler's role in maintaining chromatin accessibility at developmental loci, highlighting MLF2 and RBM15 as stabilizers of SWI/SNF function and m6A RNA methylation, which regulate subunit stoichiometry and chromatin dynamics (Schwaemmle et al., 2024). Similarly, CRISPR-based analyses showed that the INO80 remodeler promotes H2A.Z occupancy at bivalent promoters, coordinating histone modification and chromatin remodeling to regulate developmental gene expression during pluripotency transitions (Yu et al., 2021). Additionally, CRISPR-Cas9 constructs targeting Trithorax (TrxG) and Polycomb (PcG) proteins, including ISWI, provide insights into their roles in chromatin remodeling, gene regulation, and genome organization (Wani et al., 2019).

In *Trypanosoma cruzi*, CRISPR-Cas9 technology enabled the functional characterization of ISWI (TcISWI), revealing its involvement in transcriptional silencing, mRNA export, and chromatin

compaction. This study identified novel TcISWI interactors, such as DRBD2, DHH1, and SMC domain proteins, underscoring the diverse functions of chromatin remodeling in nuclear processes (Díaz-Olmos et al., 2020).

2.1.4 Applications in Disease and Therapeutics

Modified CRISPR-based editing has also been applied to study diseases linked to epigenetic dysregulation. For instance, by targeting H3K9me3-associated proteins, CRISPR has been used to enhance tumor immunogenicity and improve responses to checkpoint blockade therapy in cancer (Willyard, 2017). Similarly, chromatin remodeling factors such as the cBAF and INO80 complexes have been studied to improve T-cell functionality in cancer immunotherapy (Belk et al., 2022). In fungal systems, the ability to activate or repress specific genes has broadened applications in industrial biotechnology and disease models (Li et al., 2021). Moreover, CRISPR has been employed to target specific chromatin states using modifiers like HP1α, demonstrating precise control over higher-order chromatin structures and their transcriptional outcomes (Kim et al., 2014). These applications underscore CRISPR's potential in addressing complex diseases, including rare imprinting disorders such as Angelman syndrome and Prader-Willi syndrome, through targeted epigenetic modifications (Syding et al., 2020).

3 CRISPR cas9 technology in genome editing:

CRISPR-mediated genome editing creates double-strand breaks (DSBs) in DNA by employing the Cas enzyme that is further repaired either by Homology Directed Repair (HDR) or Non-Homologous End Joining (NHEJ). The former introduces precise genomic snips by employing a template donor DNA and the latter introduces insertions or deletions (indels), that can disrupt gene function (Sander & Joung, 2014). However, the HDR-mediated pathway is characterized by limited efficiency. Due to reliance on homologous recombination, it is restricted to dividing cells only, limiting the range of diseases that can be targeted (Bollen et al., 2018). To address these challenges, base editing and prime editing techniques have been devised which generates efficient and precise point mutations in the genome (DNA or RNA) of living organisms (Porto et al., 2020).

3.1 Base editing:

Base editing bypasses the limitations of HDR, enabling precise changes at a single base pair which significantly reduces the risk of off-targets. It is unique in its approach as it avoids DNA cleavage and directly introduces point mutations, thus improving both efficiency and accuracy (Komor et al., 2016). Base editors (BEs) bypass the need for DSBs and repair pathways. They convert one

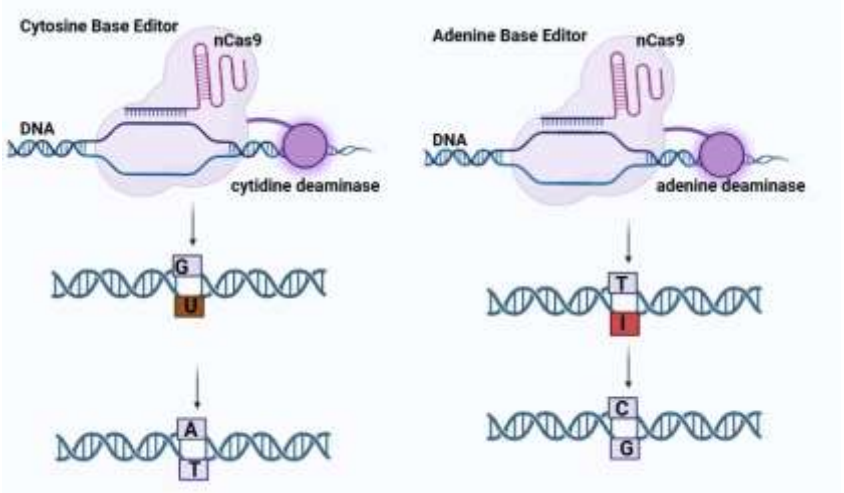

Figure 2: Two classes of DNA base editors have been outlined based on the type of base they target: cytosine base editor (CBE) and adenine base editor (ABE), enabling precise single-base conversions without introducing double-strand breaks (DSBs). CBEs convert cytosine (C) to thymine (T) while ABEs convert Adenine (A) to Guanine (G).

DNA base pair into another, for instance, the conversion of an A to a G or a C to a T. These base editors consist of two major components: a Cas enzyme that targets the DNA precisely and a single-stranded DNA modifying enzyme meant for alteration of specific nucleotides. Two classes of DNA BEs have been outlined based on the type of base they target: cytosine base editors (CBEs) and adenine base editors (ABEs). (Figure 2). CRISPR-Cas BEs can facilitate all four transition mutations i.e. A→G,
G→A, C→T, and T→C (Anzalone et al., 2019). Current BEs are shown to induce a single type of modification thus limiting the range of possible alterations that can be done. One of the recent studies has devised a dual CRISPR BE system that can introduce both C→T and A→G transitions with minimal risk of off-targets (Grünewald et al., 2020), (Sakata et al., 2020), (Zhang et al., 2020)

3.2 Cytosine Base Editors (CBEs): Liu and co-workers engineered the first-generation BE (CBE1) by fusion of rat-derived cytosine deaminase Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 1 (APOBEC1) to the amino-terminal of dCas9 i.e. catalytically deficient or dead Cas9. CBE1 is shown to deaminate cytosine and convert it to uracil. Replication machinery then recognizes uracil as thymine, resulting in a C-G to T-A transition. Although it is shown to mediate targeted base editing in-vitro but it lacks effectiveness in human cells (Komor et al., 2016). The reduced efficacy is attributed largely to the cellular repair of the U-G intermediate via the base excision repair (BER) pathway. U-G mismatch is identified by uracil N-glycosylase and it cleaves the glycosidic bond between the uracil and the deoxyribose backbone which reverts the U-G intermediate back to its original C-G base pair. (Kunz et al., 2009) (Krokan & Bjørås, 2013).
This limitation of CBE1 is addressed by generation of a second-generation cytosine base-editor (CBE2). It is designed by the fusion of uracil DNA glycosylase inhibitor (UGI) to the C-terminus of BE1, inhibiting the activity of UDG. This inhibition of the BER pathway resulted in the increased editing efficacy in human cells. Further BE3, a third-generation BE was developed with improved editing efficiency and utility in genome editing applications. It relied upon the usage of Cas9 nickase (nCas9), introducing a nick in the G-containing strand of the U-G intermediate to bias cellular repair of the intermediate towards a U-A outcome, further converting to T-A during DNA replication (Komor et al., 2016). Further advancements in CBEs have been achieved that focus on minimizing indels, improving editing efficacy, and specificity. A range of engineered Cas9 variants with altered PAM recognition sequences and improved cleavage specificity have been generated, enabling a broader range of target sequences for CRISPR-based editing technologies (Kleinstiver et al., 2015), (Lee et al., 2016) , (Zetsche et al., 2015), (Müller et al., 2016).

Adenine Base Editors (ABEs): CBEs are limited in introducing transitions from C-G to T-A mutations, thus restricting the range of correctable disease-causing mutations. Moreover, methylated cytosines are susceptible to spontaneous deamination, further constraining the utility of CBEs. On the other hand, a new class of base editors was deployed known as adenine base editors (ABEs). This limitation was circumvented by introducing A to G conversions, which can reverse almost half of all pathogenic point mutations. ABEs are developed by fusing the deoxyadenosine deaminase domain with a dCas9 protein. The ABE-dCas9 complex binds to the target DNA sequence in a guide RNA-dependent manner. The deaminase domain catalyzes the conversion of adenine to inosine, which is interpreted as guanine during DNA replication, thus replacing the original A-T pair with a G-C pair. However, ssDNA adenosine deaminases do not occur naturally and in this context, attempts have been made to use RNA-specific deaminases with limited success. (Gaudelli et al., 2017). To circumvent this issue, David Liu and colleagues engineered an E.coli tRNA adenosine deaminase (ecTadA), which converts adenine to inosine in the single-stranded anticodon loop of $tRNA^{ARG}$. This engineered enzyme played a key role in developing of the first-generation adenine base editors. ABEs generally yield fewer indels and off-targets when compared to CBEs, hence greatly broadening the scope of genetic diseases that can be targeted using base-editing technology. (Yang et al., 2018).

3.3 Prime editing:

This editing is more versatile than base editing as it is not only limited to four transitions but encompasses a wider range of edits including insertions, deletions, transitions, transversions, and larger genetic

modifications. Similar to base editing, it also does not rely on DSBs. Prime editors (PEs) are different from base editors in the usage of a prime editing guide RNA (pegRNA) and an engineered reverse transcriptase (RT) fused to Cas9 nickase (nCas9). (Figure 2). The pegRNA comprises of two domains, a sequence complementary to the target site that directs nCas9 to its desired sequence and an additional sequence encoding the desired sequence modifications (Anzalone et al., 2019). The 5′ of the pegRNA binds to the primer binding site (PBS) on the DNA which exposes the non-complementary strand. Cas9 then nicks the unbound DNA strand containing the PAM site, generating a primer for the RT linked to nCas9. The RT uses the internal sequence of the pegRNA as a template to extend the nicked strand, thus introducing programmable modifications to the target region. Furthermore, this process

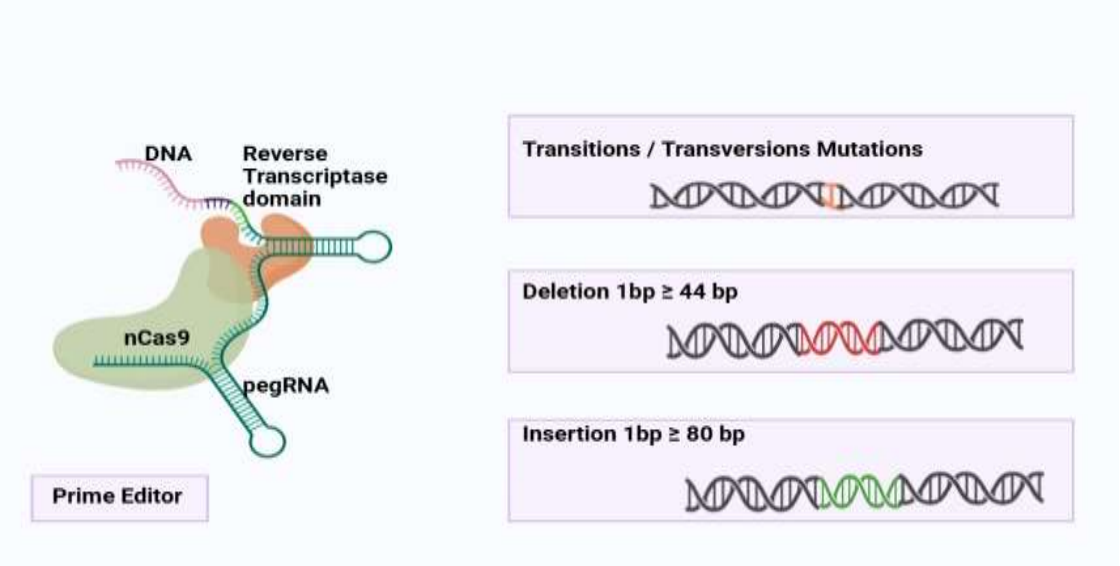

Figure 3: Prime editors (PEs) are versatile genome-editing tool that allows for precise and programmable changes to the DNA sequence without relying on double-strand breaks or donor templates. It comprises of guide RNA (pegRNA) and an engineered reverse transcriptase (RT) fused to Cas9 nickase (nCas9) and can introduce a wide range of edits, including base substitutions, insertions or deletions (indels) and combination of these edits.

generates two overlapping PAM DNA flaps: the edited 3′ flap transcribed from the pegRNA template and the original unedited 5′ flap. The 5′ flaps are preferentially degraded by cellular endonucleases during lagging-strand DNA synthesis (Hosfield et al., 1998).The resulting heteroduplex, comprising the edited 3′ flap and the unedited complementary strand, is then resolved and integrated into the genome through cellular replication and repair mechanisms.PE1 was the first generation of PEs developed.It comprised of the Moloney murine leukemia virus reverse transcriptase (M-MLV RT), linked to the C-terminus of nCas9 and pegRNA,further expressed on a second plasmid (Anzalone et al., 2019). Prime editing is considered more advanced and versatile in approach as the risk of off-targets is minimal, reduced dependence on stringent PAM sequences due to the flexible length of the RT template, and the absence of bystander edits (Fu et al., 2023).

4 Conclusion and future perspective:

In vitro applications of CRISPR/Cas9 gene editing are highly advanced, with most delivery systems offering effective editing without significant patient safety concerns. A primary challenge in this area is the off-target effects caused by prolonged Cas9 activity. While traditional methods allow for transient Cas9 expression, lentiviral (LV) transduction induces permanent Cas9 expression, increasing the risk of unintended off-target edits. Furthermore, delivering plasmid DNA encoding Cas9 is more likely to result in off-target effects compared to direct delivery of the Cas9 protein via physical or chemical methods (Kim et al., 2014); (Ramakrishna et al., 2014). Longer exposure of Cas9 to genomes raises the risk of off-target cleavage. However, Cas9 editing specificity improves when exposure time is temporarily shortened. By minimizing off-target effects, EV-based delivery of Cas9 RNPs offers advantages over many other delivery methods due to its transient nature. (Wu et al.,

2014); (Choi et al., 2016). Electroporation often results in significant cell death, while EV (extracellular vesicle) transduction maintains high cell viability. EVs are as effective as viral systems, making them generally more efficient than chemical-based methods and requiring less optimization. This approach supports the treatment of hematopoietic diseases by enabling the collection of patient hematopoietic stem cells, which can then be modified ex vivo and autologously reintroduced into the patient (Mamcarz et al., 2019).The benefits of ex vivo cell treatment are often limited by the high cost and labor involved in extracting stem cells from each patient for autologous transplants. Consequently, iPSCs have become increasingly popular as a platform for gene editing research (Savić & Schwank, 2016). Derived from easily accessible cell types like fibroblasts and peripheral blood cells, iPSCs provide an unlimited supply of material for gene editing studies. The therapeutic potential of iPSCs has gained renewed attention due to the development of universal donor cells. By removing human leukocyte antigen classes I and II in iPSCs, differentiated cell products can serve as "off-the-shelf" treatments, making them suitable for all patients (Mattapally et al., 2018); (Zeng et al., 2017). The concept of universal donor cells has recently highlighted the therapeutic potential of iPSCs. By eliminating human leukocyte antigen classes I and II in iPSCs, differentiated cell products become "off-the-shelf" solutions suitable for any patient (Mattapally et al., 2018); (Zeng et al., 2017).

Genetic engineering has enabled directed modifications of CRISPR components, leading to highly efficient and optimized gene editing methods. This paper reviews several such tools, though some recent advances—most notably a multiplexed Cas12a-based technique that can simultaneously modulate multiple genes with unprecedented precision to control complex biological processes—are beyond its scope. In this approach, a Cas12a protein multiplexes by cleaving a single RNA transcript into multiple gRNAs, each targeting a specific gene. (Campa et al., 2019). Recently, two research teams developed an innovative method using dCas linked to transposases, enabling highly efficient on-target integration of DNA sequences. Gene engineering plays a critical role in precisely inserting desired DNA at target sites to overcome various scientific and technical challenges. (Klompe et al., 2019); (Strecker et al., 2019)

Through the use of m6A marker "writers" and "erasers," which modify the epigenetic state of RNA, dCas technologies have expanded possibilities in the field of epitranscriptomics . Additionally, combining dCas with fluorescent proteins enables the visualization of specific DNA and RNA sequences in vitro and in vivo via microscopy, a significant advancement in tracking these targets (Ma et al., 2016); (Nelles et al., 2016) Using dCas systems like CAMERA and DOMINO (Tang & Liu, 2018), researchers have developed cellular recorders capable of capturing and encoding incoming events and their parameters, enabling in-depth studies of signaling pathways and other biological processes. Innovations like CRISPR-driven evolution (Hess et al., 2016), CRISPR-based diagnostics (Zhang et al., 2017); (Qiu et al., 2018), and CRISPR-biosensors establish the groundwork for a new era of technological advancements.

The effectiveness of CRISPR-Cas-based methods and therapeutics could be compromised by off-target activity, where Cas proteins unintentionally bind or cut at undesired sites. This could lead to gene disruption, large mutations, and chromosome instability, undermining therapeutic potential. To address this, several technical advancements have been made to reduce off-target effects, including (a) improved gRNA design tools, (b) modifications to gRNAs (e.g., truncations, secondary structures), (c) engineered SpCas9 variants (e.g., eSpCas9, Sp-HF1) with reduced off-target activity, and (d) Cas proteins with altered PAM specificity. While these refinements have significantly minimized off-target risks, they have not entirely eliminated them.

In conclusion, the CRISPR field is advancing rapidly, building on years of research across biology, physics, and chemistry. This progress has enabled previously impossible biological manipulations and interventions, making CRISPR one of the most powerful molecular tools. However, significant challenges remain, and how these obstacles are addressed will determine the future scope and effectiveness of gene editing.

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