CRISPR Precision and Its Paradox: A Deep Dive into Off-Target Effects

By

Samia Sultana

ID: 23176006

A thesis report submitted to the Department of Mathematics and Natural Sciences in partial Fulfilment of the requirements for the degree Of Masters of Science in Biotechnology

Department of Mathematics and Natural Sciences (MNS),

BRAC University

8th May, 2025

Declaration

It is hereby declared that

1. The paper does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

2. The paper does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

3. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Samia Sultana

_____

Samia Sultana
ID - 23176006

Approval

The thesis titled “Review of CRISPR Precision and Its Paradox: A Deep Dive into Off-Target Effects” submitted by

1. Samia Sultana, ID: 23176006

Of Fall, 2024 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of in MSc in Biotechnology on 8th may 2025.

Examining Committee:

Supervisor:

_____

Dr. Iftekar Bin Naser
Associate professor, Microbiology program
Department of Mathematics & Natural Science
Brac university

Program Coordinator:

_____

Dr. Munima Haque
Associate Professor, Biotechnology,
Department of Mathematics & Natural Sciences
BRAC University

Departmental Head:
(Chair)

_____

Md. Firoze H.Haque, PhD
Associate Professor, Biotechnology,
Department of Mathematics & Natural Sciences
BRAC University

Abstract

CRISPR-Cas9 has revolutionized genome editing, providing researchers with a powerful tool to precisely alter DNA sequences in a wide range of organisms. Its ability to specifically target and modify genes has unlocked opportunities in diverse fields, including medicine, agriculture, and basic scientific research. This thesis explores the dual aspects of CRISPR-Cas9: its wide-ranging applications and the challenges posed by off-target effects, along with the innovative strategies that scientists have developed to address these issues.

The usefulness of CRISPR-Cas9 lies in its simplicity and versatility. In medicine, it has been applied to correct genetic mutations responsible for diseases such as sickle cell anemia, Duchenne muscular dystrophy, and some cancers. In agriculture, it has been used to develop drought-resistant crops, enhance nutritional content, and combat plant diseases. CRISPR-Cas9 is also a vital tool for studying gene functions and creating disease models in research. These capabilities highlight its transformative potential to solve complex problems and drive innovation across disciplines.

However, the technology is not without its limitations. Off-target effects—where CRISPR edits unintended regions of the genome—pose significant challenges. These unintended changes can disrupt vital genes, lead to harmful mutations, or even cause genomic instability, making it a critical issue for clinical and agricultural applications. Off-target effects are influenced by several factors, including the design of the guide RNA, chromatin accessibility, and similarities between target and non-target sequences.

To overcome these challenges, scientists have developed several strategies to enhance the precision of CRISPR-Cas9. High-fidelity Cas9 variants, such as SpCas9-HF1 and eSpCas9, have been engineered to reduce off-target activity. Bioinformatics tools now allow for the design of highly specific guide RNAs, minimizing the risk of off-target binding. Delivery methods, such as ribonucleoprotein complexes (RNPs), help reduce the duration of Cas9 activity, further limiting unintended edits. In addition, newer genome-editing tools like base editors and prime editors provide even greater accuracy by allowing single-nucleotide changes or small insertions without causing double-stranded breaks in DNA**. (Ansori ANM, *et al.* 2023).**

This thesis delves into the mechanisms of CRISPR-Cas9's off-target effects, the factors that influence them, and the innovative methods developed to address these issues. By exploring both the immense potential and the current challenges of CRISPR technology, this research contributes

to the ongoing efforts to improve its safety and reliability. Ultimately, the findings aim to support the development of CRISPR-based solutions that are not only effective but also safe for widespread use in medicine, agriculture, and scientific research.

Acknowledgement

I am grateful to Professor Dr. A F M Yusuf Haider (Chairperson Department of Mathematics and Natural Sciences, BRAC University) for looking after all of the students and teachers in his department and for always being willing to lend a helping hand when needed.
I owe a great debt of gratitude to Dr. Iftekhar Bin Naser sir for guiding me throughout my thesis project and providing all of the assistance which I required. Sharing his enormous experience and knowledge with me was really beneficial to my project.

Content:

Introduction

Genome editing is the modification of genomic DNA at a specific target site in a wide variety of cell types and organisms, including insertion, deletion and replacement of DNA, resulting in inactivation of target genes, acquisition of novel genetic traits and correction of pathogenic gene mutations. Due to the advantages of simple design, low cost, high efficiency, good repeatability and short-cycle, CRISPR-Cas systems have become the most widely used genome editing technology in molecular biology laboratories all around the world. **(Charpentier E. et al., 2014)**

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) genome editing has Originally discovered as part of a bacterial adaptive immune system & archaea, composed of CRISPR arrays and Cas proteins, which protect against foreign genetic elements. **(Deveau H, et al., 2010).** CRISPR sequences are not present in eukaryotic or viral DNA, although they are mostly present in the genomes of archaea (about 85%) and bacteria (about 42%). When related genes known as cas genes (cas1-4) were discovered to be involved in DNA metabolism and prokaryotes' adaptive immune response to foreign genetic elements like viruses and plasmids, the term "CRISPR," which stands for "Clustered Regularly Interspaced Short Palindromic Repeats," was first used in 2002.

Archaea: CRISPR systems are especially common in archaea, which frequently cohabit with a variety of viruses to strengthen their defences against them.

Bacteria: Approximately 40% of sequenced bacterial genomes contain CRISPR loci, which serve as a record of past infections, allowing bacteria to recognize and defend against subsequent attacks

CRISPR-Cas systems defend prokaryotes against viral infections by recognizing and cleaving foreign genetic material. The adaptation of CRISPR-Cas9 for genome editing in eukaryotic cells represents a landmark advancement in genetic engineering, offering unprecedented capabilities for functional genomics, therapeutic development, and agricultural innovation.

Applications in Genetic Research and Therapy

Since its adaptation for use in eukaryotic cells, CRISPR-Cas9 has transformed genetic research. It has simplified the creation of genetically modified organisms (GMOs), accelerating research in plant and animal genetics to enhance traits such as disease resistance, yield, and nutritional

value **(Zhang et al., 2019).** Additionally, CRISPR has shown significant promise in therapeutic applications. For example, genetic disorders caused by single-gene mutations, such as cystic fibrosis and muscular dystrophy, can potentially be treated through direct correction of the underlying genetic defects **(Yin et al., 2017).**

Advancements in CRISPR Technology

The versatility and efficiency of CRISPR-Cas9 have spurred the development of innovative enhancements. CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) allow for reversible gene repression or activation without altering the DNA sequence **(Gilbert et al., 2013).** Additionally, precision tools such as base editing and prime editing enable the correction of single-nucleotide mutations and the introduction of specific nucleotide changes with high accuracy, expanding the scope of genome editing applications **(Gaudelli et al., 2017; Anzalone et al., 2019).**

Applications of CRISPR-Cas9 technology for genome editing in the field of agriculture.

Through a number of uses, CRISPR-Cas9 technology is transforming agriculture:

Biotechnology of Plants

Crop Improvement: Improves the nutritional value, disease resistance, and productivity of crops like maize, soybeans, and rice.

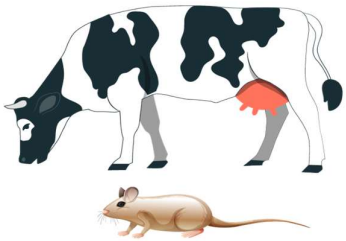
Improving Quality: Crops are shaped and sized to better suit customer tastes. Animals with engineering

Livestock Production: Pigs that have been genetically modified to be resistant to African swine fever are an example of how this improves feed efficiency and disease resistance.

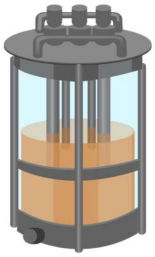
Nutritional Additives: Uses genetically engineered probiotics to improve feed quality.

Production of Biofuel

Lipid Production: Boosts the amount of lipids that algae produce for biofuel. The concerns of sustainability and food security are intended to be addressed by these developments.



Improved livestock production and in vivo models



Duplicated lipid production in algae

Corn, rice, soybean, potat0, and maize

Classification and Function of CRISPR

CRISPR-Cas systems are classified into two main classes and six types, with numerous subtypes, reflecting their complexity and diversity:

Classification

Class 1: Comprises multi-protein complexes, including Types I, III, and IV.

Class 2: Includes single multidomain proteins, such as Cas9 **(Koonin et al., 2017).**

Cas9 Protein and PAM Motifs

Cas9 is a nuclease that targets foreign DNA by recognizing protospacer adjacent motifs (PAMs), which are essential for its function. This discovery has been pivotal since its identification in 2002 **(Charpentier et al., 2014).**

RNA Molecules

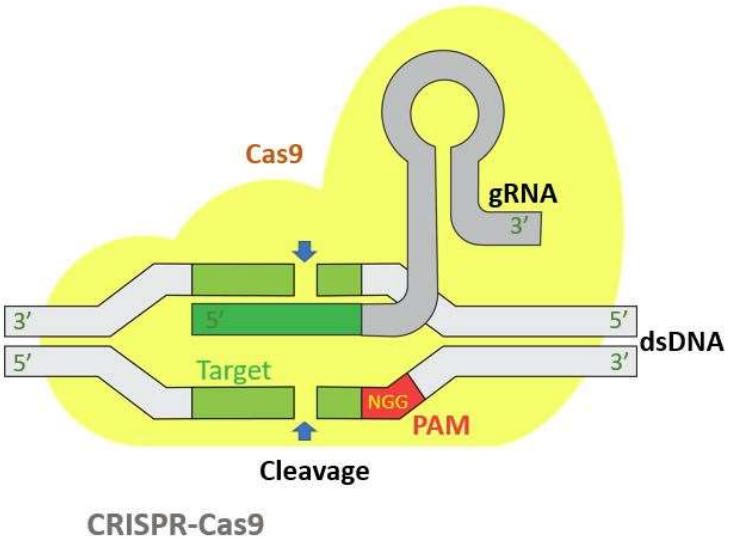
The CRISPR-Cas9 system utilizes crRNA and tracrRNA to guide the editing process. Their combination into a single guide RNA (sgRNA) has significantly enhanced the practical applications of CRISPR technology **(Makarova et al., 2011).**

What are the main components of CRISPR-Cas9 technique?

CRISPR-Cas9 genome editing includes two key components: a single-guide RNA (gRNA) and a CRISPR-associated endonuclease (Cas). The sgRNA and Cas9 are combined into a ribonucleoprotein complex when they are used in CRISPR experiments.

Cas9: It is an endonuclease enzyme that functions as a pair of molecular scissors to cut the target DNA sequence.

sgRNA: Single-guide RNA is the combination of tracrRNA and crRNA. crRNA contains two main parts: the spacer sequence that directs the complex to the target DNA and a region that binds to tracrRNA. When tracrRNA binds to crRNA, a functional guide RNA is formed for Cas9 to recognize. Multiple crRNAs can be incorporated into a crRNA array and then packaged with tracrRNA to form an sgRNA.



CRISPR-Cas9

Historical Background

The journey of CRISPR-Cas systems from obscure DNA sequences in Escherichia coli to revolutionary genome-editing tools is one of the most captivating narratives in modern biology. In 1987, Ishino et al. identified unusual repetitive DNA sequences in E. coli while analyzing genes associated with phosphate metabolism **(Ishino et al., 1987).** These sequences, later named CRISPR, were initially hypothesized to be involved in DNA repair in hyperthermophilic archaea. However, subsequent investigations revealed their role in adaptive immunity, with CRISPR loci storing fragments of viral DNA as "molecular memories" **(Mojica et al., 2005)**

By the early 2000s, the connection between CRISPR sequences and Cas proteins began to emerge, culminating in the development of CRISPR-Cas9 as a genome-editing tool. This discovery has since transformed genetic engineering, allowing researchers to modify genomes with unprecedented precision, cost-effectiveness, and efficiency **(Barrangou et al., 2007).**

CRISPR-Cas9 represents a paradigm shift in genetic research, with profound implications for medicine, agriculture, and beyond. Its simplicity, precision, and versatility continue to inspire new applications and innovations, establishing CRISPR as a cornerstone of modern molecular biology.

History

The history of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology is a fascinating journey from its initial discovery to its development as a ground-breaking tool for genome editing. Here's an overview of the key milestones:

Early Discoveries and Initial Research

1987: The first hints of CRISPR were observed by Japanese scientist Yoshizumi Ishino and colleagues. They discovered an unusual series of repeated DNA sequences in the genome of Escherichia coli while studying the iap gene, but the significance of these sequences was not understood at the time. Recognition of CRISPR as an Adaptive Immune System

2000-2005: Several researchers, including Francisco Mojica of the University of Alicante in Spain, identified similar repetitive sequences in various bacteria and archaea. Mojica proposed that these sequences were part of an adaptive immune system that helped protect microbes from viruses and plasmids.

2005: Three independent research groups (Mojica, Ruud Jansen, and Eugene Koonin) suggested that CRISPR sequences were associated with Cas (CRISPR-associated) genes, and that they might be involved in a defense mechanism against foreign genetic elements.

Functional Understanding and Mechanism Elucidation

2007: A landmark study by Philippe Horvath and colleagues at Danisco (a food company) demonstrated that CRISPR provides adaptive immunity in Streptococcus thermophilus. They showed that bacteria with certain CRISPR sequences were resistant to bacteriophages and could acquire new sequences from the phages to gain immunity.

2008-2010: Further studies elucidated the mechanism of CRISPR-mediated immunity. Researchers discovered that CRISPR loci are transcribed into RNA molecules, which guide Cas proteins to target and cleave foreign DNA.

Development of CRISPR-Cas9 for Genome Editing

2012: A breakthrough study by Emmanuelle Charpentier and Jennifer Doudna described how the CRISPR-Cas9 system from Streptococcus pyogenes could be adapted for genome editing. They engineered a single guide RNA (sgRNA) by fusing crRNA with tracrRNA, simplifying the system and allowing precise targeting of DNA sequences.

2013: Feng Zhang, George Church, and their teams at the Broad Institute and Harvard independently demonstrated that the CRISPR-Cas9 system could be used for efficient genome editing in eukaryotic cells, including human cells. This opened up vast possibilities for genetic research and therapeutic applications.

Advances and Applications

2013-Present: CRISPR technology has rapidly advanced, with numerous modifications and improvements. Researchers have developed CRISPR variants for base editing, prime editing, and epigenome editing, among other techniques.

2020: Emmanuelle Charpentier and Jennifer Doudna were awarded the Nobel Prize in Chemistry for their development of CRISPR-Cas9 as a genome-editing tool.

Ethical and Regulatory Considerations

As CRISPR technology advanced, it raised important ethical and regulatory questions, particularly regarding its use in human germline editing. The 2018 announcement of the birth of CRISPR-edited babies in China sparked widespread ethical debates and led to calls for stricter regulations and oversight.

CRISPR technology is actually part of a natural defense mechanism of bacteria. It is used by bacteria and archaea (another type of microorganism) to protect themselves from viruses and other genetic elements. Let's understand in detail why and how CRISPR exists within bacteria:

CRISPR–Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing

Experimental Insights:

In 2007, Rodolphe Barrangou and Philippe Horvath showed that CRISPR loci in Streptococcus thermophilus provided acquired immunity to bacteriophages. This led to patents and the use of CRISPR for bacterial cultures' "vaccination."

Overview of Existing Research on CRISPR Technology

CRISPR technology has been extensively studied and developed since its potential for genome editing was first recognized. The existing body of research covers a wide range of topics, from fundamental mechanisms to practical applications. Researchers have focused on improving the efficiency and specificity of CRISPR systems, developing novel variants of Cas enzymes, and exploring diverse applications in medicine, agriculture, and biotechnology. Comprehensive reviews and meta-analyses have summarized the state of knowledge, highlighting both achievements and ongoing challenges.

Historical Development and Milestones

The journey of CRISPR technology began with the discovery of repeated sequences in the DNA of bacteria by Francisco Mojica in the 1990s. This was followed by key discoveries by other researchers, such as the identification of CRISPR-associated (Cas) genes. In 2012, Jennifer Doudna and Emmanuelle Charpentier published a landmark paper demonstrating the potential of CRISPR-Cas9 for precise genome editing, a discovery that earned them the Nobel Prize in Chemistry in 2020. Since then, significant milestones have included the development of high-fidelity Cas9 variants, the invention of CRISPR base editing and prime editing technologies, and the initiation of clinical trials for CRISPR-based therapies.

Key Researchers and Studies

Jennifer Doudna and Emmanuelle Charpentier: Their collaborative work led to the foundational discovery of the CRISPR-Cas9 genome editing system, which has been instrumental in advancing genetic engineering.

Feng Zhang: A key figure in adapting CRISPR for use in mammalian cells, Zhang's contributions have been critical in expanding the applications of CRISPR technology.

George Church: Known for his work in synthetic biology and CRISPR, Church has been involved in pioneering studies that push the boundaries of genetic modification and engineering's

Francisco Mojica: His early work on identifying CRISPR sequences laid the groundwork for understanding the CRISPR mechanism.

The Role of CRISPR in Bacteria

1. **Defense Mechanism**:

The CRISPR-Cas system acts as an adaptive immune system for bacteria. It provides protection against viruses (primarily bacteriophages) that infect bacteria.

2. **Genetic Memory**:

Through the CRISPR system, bacteria create a "memory" of previous viral infections. When a bacterium is infected by a virus, it cuts a portion of the viral DNA and integrates it into its own genome. This integrated portion is known as the CRISPR array.

How CRISPR-Cas9 Works

At the core of CRISPR-Cas9 are two key components: the Cas9 enzyme and a guide RNA (gRNA). The gRNA is designed to match a specific DNA sequence in the target genome, directing the Cas9 enzyme to this location. When the Cas9 reaches the targeted DNA sequence, it introduces a double-strand break. The cell then attempts to repair this break, usually by one of two mechanisms:

Non-Homologous End Joining (NHEJ): A quick but error-prone repair process that can introduce insertions or deletions (indels) at the break site, often resulting in gene disruption.
Homology-Directed Repair (HDR): A more precise, template-based repair that can be used to introduce specific DNA changes, such as correcting a gene mutation. HDR is ideal for precise gene editing but occurs less frequently and is only active in certain cell types or phases. **(Hille F, et al., 2016)**

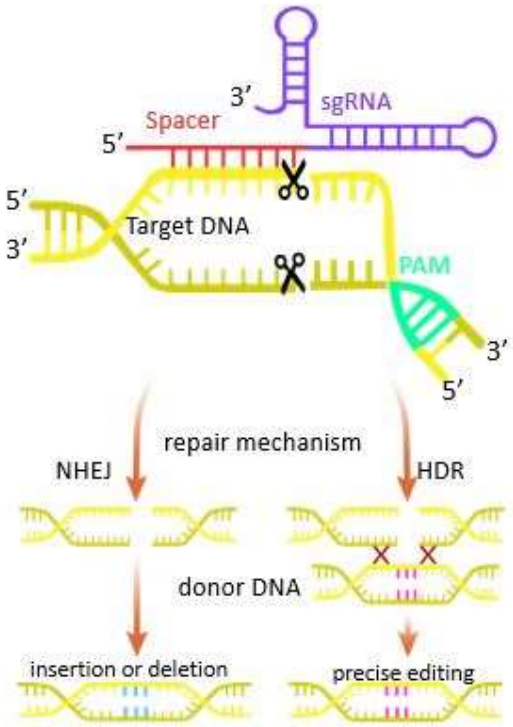


Figure: Mechanism of CRISPR-Cas9

How the CRISPR-Cas System Works:

Acquisition:

When a bacterium is infected by a virus, it cuts a small part of the viral DNA and inserts it into its CRISPR array. These parts are called spacers and they serve as a record of previous infections.

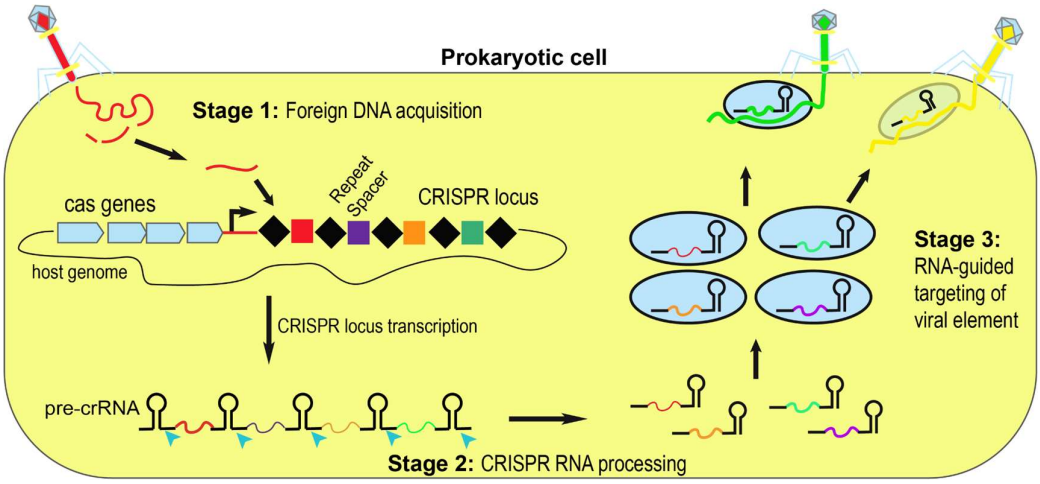
Expression:

The CRISPR array is transcribed into small RNA segments (CRISPR RNA or crRNA). These crRNAs work by matching sequences with the target viral DNA.

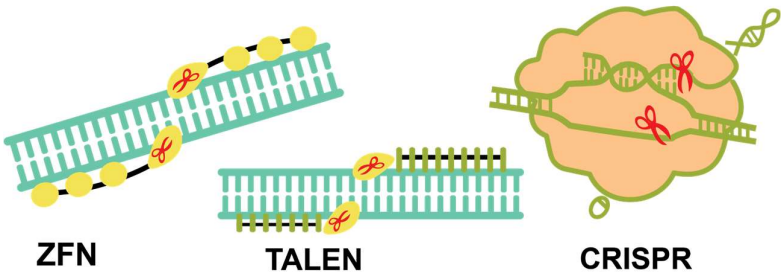
Interference:

The crRNAs associate with Cas proteins. When the same virus attacks the bacterium again, the crRNAs bind to the viral DNA and guide the Cas proteins to cut and destroy the viral DNA.

The CRISPR-Cas system exists as a natural defense mechanism in bacteria, protecting them from viruses and other genetic elements. Scientists have harnessed this natural system to create a powerful and precise genome editing tool, which is revolutionizing genetic engineering today.



The simplicity of CRISPR



While CRISPR-based methods just need to alter the guide RNA, tools like ZFNs and TALENs need to design new proteins to recognise each new DNA target site. In the animation above, the DNA-targeting portion that researchers alter is highlighted in gold. While guide RNAs may be produced rapidly, cheaply, and simply in a lab or by RNA synthesis firms, protein engineering can be complicated. The reason CRISPR has gained so much traction among scientists worldwide is because to its "user-friendly" nature!

Off-Target Effects of CRISPR:

Although the uses of CRISPR technology have significantly increased as it has developed, a significant obstacle still exists: the potential for off-target effects during gene editing. These inadvertent changes present serious hazards for therapeutic applications in addition to impairing CRISPR's specificity.

CRISPR-Cas9 technology has fundamentally transformed the landscape of genome editing, enabling precise modifications to DNA with unprecedented efficiency. This revolutionary tool has opened new avenues for genetic research, therapeutic interventions, and agricultural advancements. However, despite its remarkable potential, CRISPR-Cas9 is often scrutinized due to the occurrence of off-target effects—unintended edits in the genome that can arise when the CRISPR system interacts with unintended DNA sequences. These off-target effects pose significant challenges to the safety and efficacy of CRISPR-based therapies, necessitating thorough examination and mitigation strategies.

Off-target effects occur when the guide RNA (gRNA) binds to sequences that are similar but not identical to the intended target site. This mismatch tolerance is a critical aspect of CRISPR's function, allowing for some flexibility in gRNA-target interactions. While a perfect match between the gRNA and target DNA is ideal for specificity, studies have shown that Cas9 can tolerate up to three mismatches between the gRNA and genomic DNA **(Naeem et al., 2020; Fu et al., 2013).** This inherent flexibility can lead to unintended cleavages at non-target sites, resulting in mutations that may disrupt essential genes or regulatory elements.

The protospacer adjacent motif (PAM) is another crucial component influencing CRISPR's specificity. The PAM is a short DNA sequence adjacent to the target site that Cas9 requires for recognition and cleavage. Different Cas9 subtypes exhibit varying PAM requirements, which play a significant role in determining target site selection and reducing off-target activity **(Hsu et al., 2013).** However, some off-target sites may possess similar PAM sequences, further complicating the accuracy of CRISPR editing.

The implications of off-target effects can be profound. Unintended mutations may lead to alterations in critical genomic regions, potentially resulting in adverse biological consequences such as oncogenesis or other disease states. For instance, mutations in tumor suppressor genes like TP53 can contribute to uncontrolled cell proliferation, increasing cancer risk **(Naeem et al., 2020).** Moreover, off-target effects can introduce phenotypic changes that complicate experimental interpretations, making it difficult to ascertain whether observed effects stem from intended edits or unintended modifications.

In therapeutic contexts, off-target effects pose severe safety risks. If CRISPR is employed to correct genetic disorders, unintended edits elsewhere in the genome could introduce new health issues. Research indicates that off-target mutations could activate oncogenes or silence essential genes, complicating treatment outcomes **(Addgene, 2024).** Furthermore, if off-target mutations go

undetected, they may confound results in scientific research, leading to erroneous conclusions about gene functions and pathways.

To address these challenges, various methods have been developed for predicting and detecting off-target effects. Next-generation sequencing (NGS) techniques allow researchers to analyze potential off-target sites comprehensively and quantify unintended mutations. Additionally, advancements in computational tools have improved the ability to predict off-target activity based on gRNA design and genomic context.

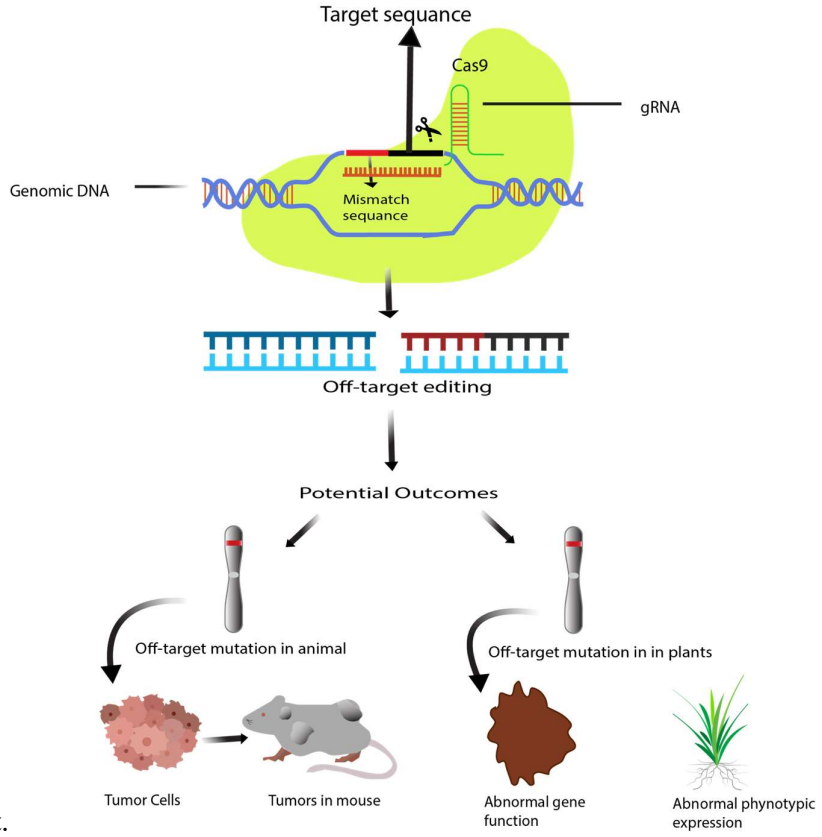


Figure: Off-target effect of CRISPR

Types of off target effect

Off-target effects in the CRISPR/Cas9 system refer to unintended modifications of genomic regions other than the intended target site. These effects can compromise the specificity and safety of CRISPR-based genome editing. Researchers have categorized off-target effects into three primary types, each with distinct mechanisms and characteristics:

Single or Multiple Base Substitutions at PAM Sites (5'-NGG-3')

The first type of off-target effect occurs when the CRISPR/Cas9 system binds to regions that resemble the target sequence but contain single or multiple base mismatches. These regions are located near PAM sequences (5'-NGG-3'), which are essential for Cas9 recognition and binding.

Mechanism:

Cas9 tolerates mismatches between the guide RNA (gRNA) and the DNA target, especially if these mismatches occur in regions outside the "seed sequence" (a critical region near the PAM site where perfect complementarity is most important).

This tolerance allows Cas9 to bind and cleave sequences that are not a perfect match to the gRNA.

Implications:

Even small mismatches can lead to unintended DNA cleavage, potentially causing mutations in non-target regions.

These off-target effects are particularly problematic in therapeutic applications, as they may disrupt essential genes or regulatory elements.

Key Study: Demonstrated that Cas9 could tolerate mismatches at certain positions, leading to cleavage at unintended sites with similar PAM sequences. **(Fu et al. 2013)**

Insertions and Deletions (Indels) at PAM Sites

The second type of off-target effect involves regions near PAM sites where insertions or deletions (indels) occur in the DNA. These structural variations can create small bulges in the DNA strand, allowing it to anneal imperfectly with the gRNA.

Mechanism:

Indels in non-target DNA can form "bulges" (small gaps or shifts in alignment between the gRNA and DNA).

Despite these misalignments, Cas9 can still recognize and cleave these regions because the bulges do not entirely disrupt binding.

In some cases, these bulged alignments result in higher off-target activity than on-target activity.

Implications:

The formation of bulges increases the likelihood of off-target effects, as Cas9 may mistakenly cleave sequences with indels that partially match the gRNA.

This phenomenon complicates the prediction of off-target sites, as traditional sequence alignment tools may not account for bulged alignments.

Key Study: highlighted how indels near PAM sites could lead to significant off-target activity, sometimes exceeding on-target efficiency. (Lin et al. 2014)

Off-Target Effects at Non-NGG PAM Sites (e.g., 5'-NAG-3')

The third type of off-target effect involves sequences with alternative PAM motifs, such as 5'-NAG-3', rather than the canonical 5'-NGG-3' motif recognized by Streptococcus pyogenes Cas9 (SpCas9).

Mechanism:

While SpCas9 has a strong preference for NGG PAMs, it can also tolerate non-canonical PAMs like NAG or NGA under certain conditions.

This flexibility increases the range of potential off-target sites, as sequences with alternative PAMs may still recruit Cas9 for cleavage.

Implications:

The use of alternative PAMs expands the risk of unintended genome editing.

This type of off-target effect is particularly concerning when using engineered Cas9 variants with altered PAM specificities, as they may inadvertently target a broader range of sequences.

Key Study: reported that SpCas9 could recognize and cleave sequences with NAG PAMs, demonstrating its ability to act on non-canonical motifs. **(Tsai et al. 2015)**

Sources of Off-Target Effects:

To increase the accuracy and security of CRISPR applications, it is crucial to comprehend the causes of these off-target consequences. Sequence similarity, mismatch tolerance, and proximity effects are the main contributors.

Sequence Similarity: Although off-target sites may differ by one or more nucleotides, they usually share significant parts of the guide RNA (gRNA) sequence. These little changes enable gRNA to

attach to unwanted locations with a marginally reduced affinity, which may still be adequate for Cas9 binding and cleavage. According to studies, Cas9 can withstand up to three mismatches between the target DNA and the gRNA, which raises the possibility of inadvertent changes happening at similar locations across the genome **(Naeem et al., 2020; Fu et al., 2013).** According to research, for example, off-target activity can still happen when the gRNA sequence has three or more mismatches, especially if the mismatches are in less important areas of the gRNA **(Hsu et al., 2013).**

Mismatch Tolerance: In the gRNA-target interaction, the CRISPR-Cas9 system shows some mismatch tolerance. Mismatches can nevertheless permit binding, even though a perfect match between the gRNA and the target DNA is ideal for specificity. Research has shown that Cas9 can still cleave non-target sites even when there are mismatches at specified locations, especially at the 5' end of the gRNA **(Hsu et al., 2013).** This tolerance makes it more difficult to do accurate genome editing since it increases the number of possible off-target locations. Furthermore, studies have shown that even three to five base pair mismatches in the PAM-distal portion of the sgRNA-guiding sequence might result in off-target cleavage activity. This contractual flexibility raises the possibility of unforeseen changes.

Proximity Effects: Off-target sites that are close to the intended target or contain areas with similar sequences can nonetheless become targets because of proximity effects, even in cases when the full gRNA sequence is not exactly matched. Despite not exactly matching the gRNA sequence, an off-target site may have a higher chance of being cleaved by Cas9 if it is physically close to an intended target site **(Addgene, 2024).** The significance of taking genetic context into account when evaluating possible off-target effects is shown by this clustering effect.

Some common off-target effects include:

Unintended DNA Breaks:

Mechanism: Off-target effects stem from the CRISPR-Cas9 complex's ability to bind to and cleave DNA sequences with some similarity to the intended target. This "mismatch" is often a single nucleotide difference or a short sequence homology. The Cas9 enzyme, guided by the gRNA, may target these similar sequences, leading to unintended double-strand breaks (DSBs).

Consequences: These DSBs trigger the cell's DNA repair mechanisms, primarily Non-Homologous End Joining (NHEJ). NHEJ, while quick, is error-prone. It often results in insertions or deletions (indels) at the break point, altering the DNA sequence. These indels can disrupt gene function,

potentially leading to loss-of-function mutations. The magnitude of the insertion or deletion can vary, resulting in different degrees of effect. The location of the break within a gene, regulatory region, or other critical sequence significantly impacts the consequence.

Gene Disruptions:

Mechanism: Unintended DSBs within or near a gene's coding or regulatory regions can severely affect gene function. These effects can range from a loss of gene function if the coding region is disrupted, causing the gene product (protein) not to be made or be non-functional. Conversely, an alteration in a regulatory sequence might affect gene expression levels, either increasing or decreasing the amount of protein produced.

Consequences: Loss-of-function mutations can prevent a gene from producing a vital protein, disrupting cellular processes. Gain-of-function mutations can lead to uncontrolled cellular activity, abnormal protein expression, and potentially cancerous growth. The consequences depend on the specific gene affected and the nature of the mutation. The disruption can be subtle or dramatic, depending on the exact location and nature of the edit. The alteration in protein function can have ripple effects throughout the cellular network.

Chromosomal Rearrangements:

Mechanism: Repeated or extended homology between the target sequence and off-target locations can lead to the CRISPR-Cas9 complex targeting multiple closely located sites in the genome. This increased activity can result in complex chromosomal rearrangements such as translocations, inversions, or duplications. The exact types and sizes of rearrangements are unpredictable and depend on the precise location and extent of sequence similarity in the genome.

Consequences: Large-scale chromosomal rearrangements can be highly disruptive, affecting the integrity and stability of the genome. The altered structure of chromosomes may cause cellular dysfunction and even contribute to cancerous growth. These types of mutations can alter the expression of multiple genes, causing complex and often unpredictable consequences in the cell's function. The effect can be subtle or dramatic, ranging from subtle changes in gene expression to significant abnormalities.

Epigenetic Changes:

Mechanism: CRISPR-Cas9 activity, even without direct DNA cleavage at an off-target location, can influence the epigenetic landscape. This could involve modifying DNA methylation patterns,

altering histone modifications, or influencing other regulatory factors around genes, affecting their expression.

Consequences: These epigenetic changes can alter gene expression levels without affecting the DNA sequence, creating long-term consequences. The altered epigenetic profile can influence the cell's behavior and developmental trajectory, potentially leading to long-term phenotypic effects. These consequences may vary depending on the type and severity of the epigenetic changes in regulatory elements and can range from subtle to significant developmental or phenotypic alterations.

Immune Responses:

Mechanism: In therapeutic contexts, especially in gene therapy, unintended modifications may trigger immune responses. The introduced changes could be recognized as foreign by the immune system, leading to an immune reaction targeting the edited cells or tissues.

Consequences: Immune responses can reject the edited cells, causing inflammatory reactions, hindering treatment efficacy, and potentially even leading to cellular death. The strength and type of immune response vary greatly depending on the nature and extent of the off-target edits, the specific target cells, and the immune system's ability to mount an effective response.

Off-target effects impact human cells, animal models, bacteria, and fungi

Off-target effects in CRISPR-Cas9 genome editing are unintended edits to DNA sequences that occur at sites other than the intended target. These off-target effects present specific challenges in various biological contexts, especially in therapeutic applications, model organisms, and industrial applications. Let’s dive deeper into how these off-target effects impact human cells, animal models, bacteria, and fungi, and the current strategies to reduce them.

Human Cells (Lung, Liver, Kidney)

Challenges In human cells: off-target effects can disrupt crucial genes, leading to unintended consequences like toxicity, cancer, immune responses, or cell death. This is particularly problematic for therapeutic applications, where CRISPR-Cas9 might be used to treat genetic disorders, cancers, or infectious diseases. In sensitive organs like the liver, kidney, and lung, off-target mutations can have severe side effects :

Lung Cells: Off-target effects could lead to inflammation or fibrosis, especially problematic in patients with respiratory diseases.

Liver Cells: The liver is central to metabolism and detoxification, so unintended edits here can interfere with these processes, potentially leading to liver damage or dysfunction.

Kidney Cells: Off-target effects can impact filtration and waste removal functions, critical for patients with kidney disease or metabolic disorders.

Mouse Cells and Animal Models

Challenges: In mouse models, precise gene editing is essential for generating accurate disease models or studying gene function. Off-target effects can introduce confounding variables in research studies, affecting reproducibility and potentially leading to misleading conclusions.

For example, unintended mutations in a mouse model of cancer could alter tumor growth, changing the interpretation of drug efficacy studies.

Off-target effects could also affect an animal's immune response, skewing results in immunology studies.

Bacteria

Challenges: CRISPR-Cas systems are native to bacteria, where they serve as an adaptive immune system against viruses. However, when used for gene editing in bacteria, CRISPR-Cas9 can cause off-target mutations that impact experimental accuracy or productivity in industrial applications, such as antibiotic production or metabolic engineering.

Unintended mutations can interfere with synthetic biology projects aiming to modify bacterial pathways for drug synthesis, biofuel production, or enzyme engineering.

For research purposes, off-target effects can make it difficult to establish clear cause-effect relationships in bacterial gene function.

Fungi

Challenges: In fungi, CRISPR-Cas9 is often used for applications like metabolic engineering, pathogen research, and agricultural biotechnology. Off-target effects can unintentionally alter growth patterns, virulence, or resistance to antifungal drugs, complicating studies and potentially impacting industries that rely on fungal engineering.

For example, in pathogenic fungi, off-target mutations may affect virulence factors, leading to inconsistent results in studies of fungal diseases.

In industrial fungi, such as those used in enzyme or biofuel production, unintended edits could disrupt metabolic pathways, affecting yield and productivity.

The main challenges in reducing off-target effects with CRISPR/Cas9 include:

Detection of Off-Target Effects

Accurate detection of off-target sites is crucial for assessing the safety and efficacy of CRISPR/Cas9 applications. Current detection methods include:

Whole-Genome Sequencing (WGS): While WGS can provide a comprehensive view of genomic changes, it may not be sensitive enough to detect low-frequency off-target events. Additionally, the analysis can be time-consuming and expensive.

GUIDE-Seq: This method involves tagging genomic regions that have been modified by Cas9. Although it improves sensitivity compared to WGS, it still has limitations in comprehensiveness and may miss certain off-target sites.

Limitations of Computational Prediction

In silico tools have been developed to predict potential off-target sites based on sequence similarity to the target site. However, these predictions often do not capture all possible off-target interactions due to:

Sequence Context: The local genomic environment can influence the binding and activity of Cas9, making it challenging to predict all potential off-targets accurately.

Experimental Validation: Predicted off-targets must be experimentally validated, which can be resource-intensive and may not always yield conclusive results.

Strategies for Reducing Off-Target Effects

To mitigate off-target effects associated with CRISPR-Cas9 technology, researchers are exploring several innovative strategies aimed at enhancing the precision and safety of genome editing. Here are some of the key approaches being developed:

Strategies for Reducing Off-Target Effects

sgRNA Optimization: Optimizing the design of single-guide RNAs (sgRNAs) is crucial for enhancing specificity in CRISPR applications. This involves selecting sgRNAs that exhibit a high degree of complementarity to the target DNA sequence while minimizing similarity to other regions in the genome. By carefully designing sgRNAs, researchers can significantly reduce the likelihood of unintended binding and cleavage at off-target sites **(Dovepress, 2023;)**

Use of Cas9 Nickases: Cas9 nickases are modified versions of the Cas9 enzyme that introduce single-strand breaks instead of double-strand breaks (DSBs). This approach reduces the likelihood of non-homologous end joining (NHEJ), which can lead to insertions or deletions at off-target sites. By utilizing Cas9 nickases, researchers can achieve gene editing with lower risks of unintended genomic alterations **(Dovepress, 2023;)**

Development of Improved CRISPR Systems: Researchers are continually working on developing new CRISPR systems with enhanced specificity and reduced off-target activity. High-fidelity Cas9 variants, such as SpCas9-HF1 and eSpCas9, have been engineered to maintain on-target efficiency while minimizing off-target effects. These advancements allow for more accurate genome editing without compromising the effectiveness of gene targeting.

Future Directions

As the field progresses, several future directions are essential for improving CRISPR/Cas9 technology:

Integration of Advanced Detection Methods: Combining multiple detection techniques may provide a more comprehensive assessment of off-target effects. Utilizing advanced sequencing technologies alongside computational prediction models can enhance the identification and quantification of unintended modifications in the genome.

Increased Focus on Safety: As CRISPR-based therapies move toward clinical applications, ensuring their safety through rigorous testing and validation will be paramount. Establishing standardized protocols for evaluating off-target effects will help

build confidence in the use of CRISPR technologies in therapeutic settings (**Dovepress, 2023).**

Regulatory Considerations: Establishing guidelines for evaluating and reporting off-target effects will be essential as CRISPR technologies advance toward therapeutic use. Clear regulatory frameworks will facilitate responsible research practices and promote public trust in gene-editing technologies (Dovepress, 2023; PMC, 2020).

Exploration of Anti-CRISPR Proteins: Recent studies have identified anti-CRISPR (Acr) proteins that can inhibit CRISPR activity. These proteins can serve as a failsafe mechanism to deactivate the CRISPR-Cas complex or enhance precision by decreasing off-target effects. Co-expressing Acr proteins with Cas9 or fusing them directly can improve target specificity without compromising on-target gene editing. **(Hsu et al., 2014)**

Base Mismatches and Off-Target Effects in CRISPR/Cas9.

Overview of Base Mismatches

In CRISPR/Cas9 gene editing, the guide RNA (gRNA) is designed to bind specifically to a target DNA sequence, directing the Cas9 nuclease to create a double-strand break (DSB) at that location. However, if there are base mismatches—where the nucleotides in the gRNA do not perfectly complement those in the target DNA—this can lead to off-target effects. These mismatches can occur due to:

Sequence Similarity: The gRNA may have partial homology with unintended genomic sites, leading to binding and cleavage at those locations **(Hsu et al., 2013).**

Variability in Target Sites: Genomic sequences can exhibit variations among individuals or within different tissues, which may not be accounted for during gRNA design **(Fu et al., 2013).**

Mechanism of Off-Target Effects

The binding affinity of the gRNA to the target site decreases when mismatches are present. However, Cas9 can still bind to these imperfect matches under certain conditions,

especially if the mismatches are located at less critical positions (typically towards the 5' end of the gRNA). When Cas9 binds to a site with mismatches, it may still induce cleavage but with reduced efficiency. This can result in unintended edits at these off-target sites, complicating the overall precision of CRISPR/Cas9 applications.

Types of Mismatches:

1-2 Mismatches: Typically tolerated by Cas9, leading to lower rates of cleavage.

3+ Mismatches: Generally result in significantly reduced activity and may prevent cleavage altogether **(Wang et al., 2016).**

Consequences of Off-Target Effects

The presence of off-target effects due to base mismatches can have several implications:

Unintended Genetic Modifications: Unwanted edits can disrupt essential genes or regulatory elements, potentially leading to harmful phenotypes or diseases **(Pacesa et al., 2022).**

Therapeutic Risks: In therapeutic contexts, such as gene therapy for genetic disorders or cancer treatment, off-target effects could undermine treatment efficacy and safety **(Fu et al., 2013).**

Strategies for Mitigating Off-Target Effects

To enhance specificity and reduce off-target effects associated with base mismatches, researchers are exploring various strategies:

Optimizing gRNA Design: Selection Algorithms: Utilizing computational tools that predict potential off-target sites based on sequence similarity can help design more specific gRNAs **(Zhang et al., 2023).**

Avoiding Homologous Sequences: Careful selection of target sequences that minimize similarity with other genomic regions can reduce the likelihood of off-target binding.

High-Fidelity Cas9 Variants: Researchers have developed modified versions of Cas9 that exhibit higher fidelity by reducing tolerance for mismatches. These high-fidelity

variants are engineered to maintain on-target activity while minimizing off-target cleavage **(Zhang et al., 2023).**

Use of Alternative Nucleases: Exploring other CRISPR-associated nucleases (such as Cpf1 or Cas12) that may have different binding properties and specificity profiles can also help mitigate off-target effects **(Doudna & Charpentier et al, 2012).**

In Vivo Validation: Conducting thorough validation studies using techniques like whole-genome sequencing or GUIDE-Seq after CRISPR editing can help identify and quantify any off-target modifications **(Addgene et al., 2024).**

Persistent Cas9 Expression & Off-Target Effects

Overview of Persistent Cas9 Expression

In CRISPR/Cas9 gene editing, the Cas9 protein plays a pivotal role by creating double-strand breaks at specific target sites, guided by the gRNA (guide RNA). While this technology has revolutionized genetic engineering, one major challenge is the persistent expression of Cas9 in cells. When Cas9 is continuously expressed, the likelihood of off-target effects—unintended genetic modifications—becomes a significant concern. These unintended edits can disrupt essential genes or regulatory elements, leading to unpredictable outcomes (Doudna & Charpentier, 2012).

Mechanisms Leading to Off-Target Effects

The persistence of Cas9 in the cellular environment increases the probability of it interacting with non-target genomic regions. Several factors contribute to off-target effects:

Extended Cas9 Activity: If Cas9 remains in the cell for prolonged periods, it may bind to and cleave genomic regions with partial complementarity to the gRNA, leading to unintended edits (Naeem et al., 2020).

Cellular Context: Factors such as cellular stress, metabolic state, and the presence of competing nucleases can influence Cas9's stability and activity, further contributing to off-target effects.

Strategies to Control Cas9 Activity

Several strategies are being developed to minimize the risks associated with persistent Cas9 expression:

Temporal Regulation of Cas9 Expression

Inducible Systems: Systems that control Cas9 expression through specific signals, such as small molecules or light, can ensure that Cas9 is only active when needed. This approach limits its presence in the cell, reducing the window for off-target activity.

Reducing Cas9 Lifetime in Cells

Degradable Cas9 Variants: Engineering Cas9 variants that are tagged for rapid degradation can help minimize its persistence. For example, fusing PEST sequences to Cas9 accelerates its breakdown once the editing process is complete (Dovepress et al., 2023).

Transient Delivery Mechanisms

Non-Persistent Delivery Systems: Approaches like lipid nanoparticles or viral vectors that ensure brief expression of CRISPR components can reduce the likelihood of persistent Cas9 activity, limiting off-target effects.

Combination Therapies

Small Molecule Inhibitors: Researchers are exploring small molecules that either inhibit Cas9 activity after editing or promote its degradation, providing an additional layer of control over its activity.

Enhanced Detection Techniques

Advanced Off-Target Detection: Techniques like CIRCLE-seq and GUIDE-Seq allow for the identification of off-target effects, helping refine gRNA design and enhancing the overall safety of CRISPR-based editing (Kim, D. et al., 2015).

PAM Sequence Limitations in CRISPR/Cas9 Gene Editing

Overview of PAM Sequence Limitations

Cas9 relies on the presence of a specific sequence known as the PAM (protospacer adjacent motif) to recognize and bind to its target DNA. The canonical PAM for Streptococcus pyogenes Cas9 is the NGG sequence, where “N” can be any nucleotide and “G” refers to guanine. This sequence specificity limits the genomic regions Cas9 can target. If a desired target lacks a PAM, it becomes impossible to edit that region (Doudna & Charpentier, 2012).

Implications of PAM Limitations

Reduced Targeting Efficiency: The requirement for specific PAM sequences limits Cas9's ability to target certain genes or regions, especially in complex genomes where suitable PAM sites may not be nearby.

Potential for Off-Target Effects: Even with the correct PAM sequence, similar sequences in non-target regions can result in Cas9 binding, leading to off-target edits.

Future Directions and Solutions

Engineering Relaxed PAM Requirements

Researchers are working on Cas9 variants that recognize non-canonical PAM sequences. For example, SpRY-Cas9 can bind to a broader range of PAMs, expanding the potential for targeted gene editing across diverse genomes.

PAM Prediction Tools

Advanced bioinformatics tools, such as PAMPHLET, can predict functional PAM sequences based on genomic context, improving the design of gRNAs and increasing the efficiency of gene targeting.

Exploring Alternative Nucleases

Other CRISPR-associated nucleases that do not require a strict PAM sequence are being explored. These may provide additional options for targeting genomic regions where the canonical PAM is not present.

Complex Intranuclear Environment and Its Impact on CRISPR/Cas9 Efficacy

Overview of the Complex Intranuclear Environment

The chromatin structure within the cell nucleus plays a crucial role in CRISPR/Cas9 efficacy. Chromatin exists in two primary forms—euchromatin (loosely packed) and heterochromatin (tightly packed). Cas9 can more easily access DNA in euchromatin regions, whereas the tightly packed heterochromatin is less accessible, posing a challenge for gene editing in certain areas (Szczelkun et al., 2016). Epigenetic modifications, such as DNA methylation and histone

modifications, can further influence chromatin structure and Cas9's ability to bind its target DNA (Zhang et al., 2017).

Implications for CRISPR/Cas9 Efficacy and Specificity

Variable Target Accessibility: Targets in open chromatin regions are more likely to be efficiently edited, while targets in closed chromatin regions may be missed or edited less efficiently (Doudna & Charpentier, 2012).

Epigenetic Factors: Epigenetic marks such as DNA methylation can prevent Cas9 from binding to specific target sites, reducing editing efficiency and increasing the risk of incomplete or failed edits (Fu et al., 2016).

Predicting Off-Target Activity: The interaction between Cas9, gRNA, and the chromatin environment complicates predictions about off-target effects, as the local chromatin state can influence Cas9's binding affinity.

Future Directions

Understanding Chromatin Dynamics

Research into chromatin dynamics and its effect on Cas9 activity is crucial for improving targeting strategies. By understanding how chromatin structure influences CRISPR efficacy, researchers can design better tools for accessing closed chromatin regions.

Engineering Epigenetic Modifiers

Tools that temporarily modify chromatin state, such as small molecules or engineered proteins, could enhance Cas9 access to otherwise inaccessible regions. These strategies may open up new possibilities for editing difficult genomic regions.

Combining Technologies

Integrating CRISPR/Cas9 with other genome-editing technologies, such as base editing or prime editing, may overcome chromatin accessibility issues. These approaches, which do not rely on double-strand breaks, could provide more precise and less disruptive alternatives for gene editing (Dovepress et al., 2023)

Discussion

A revolutionary technique known as CRISPR-Cas9 emerged in the field of genetics that would forever alter the scientific landscape. Inspired by the natural defense systems of bacteria and archaea, this ground-breaking method gave researchers a previously unheard-of capacity to precisely alter genes. As though they had been given a pair of molecular scissors, which could cut out undesirable DNA sequences and swap them out with ones they preferred, **(Charpentier et al., 2014).**

The narrative starts in the microscopic world of bacteria, where these minuscule beings evolved a cunning defense mechanism against invasive viruses. To remember previous viral invaders, they developed a system called CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). The bacteria would take a piece of the virus's DNA and store it in their own genome when it attacked. In subsequent interactions, this memory would direct their immune response, allowing them to cleave the viral DNA and eliminate the threat. **(Mojica et al., 2005).**

In the present era, scientists discovered this clever process in laboratories. They came to the realisation that they could modify genes in other organisms by using this natural system. The potential for genetic engineering skyrocketed with the aid of two essential elements: guide RNA (gRNA), which directs Cas9 to the proper site, and Cas9, an enzyme that functions like a pair of scissors. **(Doudna & Charpentier, 2012).**

As word spread about CRISPR-Cas9, researchers around the world began to explore its possibilities. In medicine, it held the promise of curing genetic diseases that had long plagued humanity. Imagine a child born with sickle cell anemia or cystic fibrosis; with CRISPR, scientists envisioned correcting the faulty genes responsible for these conditions **(Yin et al., 2017).** The excitement grew as clinical trials commenced, and hope blossomed for those suffering from genetic disorders.

But the story didn't end there. In agriculture, CRISPR-Cas9 offered solutions to some of humanity's greatest challenges: food security and sustainability. Scientists began developing crops that could withstand droughts and resist diseases, ensuring that food could be produced even in changing climates **(Zhang et al., 2019).** These innovations promised not only to enhance crop yields but also to improve nutritional content, paving the way for healthier diets around the globe. In laboratories dedicated to basic research, CRISPR became a powerful ally for scientists seeking to unravel the mysteries of life itself. By creating precise models of human diseases,

researchers could better understand how genes functioned and how complex biological processes unfolded **(Gilbert et al., 2013).**

Yet, as with any great power, there were challenges lurking in the shadows. While CRISPR-Cas9 was heralded for its precision, it was not infallible. Off-target effects began to emerge as a significant concern—instances where Cas9 would inadvertently cut DNA at unintended locations. These unplanned edits could disrupt essential genes or regulatory elements, leading to unforeseen consequences **(Naeem et al., 2020).**

Scientists delved into understanding why off-target effects occurred. They discovered that factors such as sequence similarity between gRNA and unintended DNA regions played a crucial role. The quest for perfection became urgent; researchers knew they had to find ways to minimize these risks if they were to fully harness CRISPR's potential.

Determined not to be defeated by these challenges, scientists embarked on a quest for solutions. They turned to bioinformatics tools to design highly specific gRNAs that would minimize off-target binding **(Hsu et al., 2013).** By carefully selecting guide RNAs with lower off-target activity, they aimed to enhance the accuracy of their edits.

Engineers also began developing high-fidelity variants of Cas9—like SpCas9-HF1 and eSpCas9—that exhibited reduced off-target activity while maintaining on-target efficiency **(Koonin et al., 2017).** These innovations were akin to crafting sharper scissors that only cut where intended.

Moreover, researchers explored improved delivery methods using ribonucleoprotein complexes (RNPs), which allowed for transient expression of Cas9 in cells. This approach minimized the duration of Cas9 activity and reduced the likelihood of unintended modifications **(Addgene,et al 2024).**

As advancements continued, the horizon brightened with possibilities. Scientists envisioned new editing tools such as base editors and prime editors that could make even more precise changes without introducing double-strand breaks—further reducing risks associated with off-target effects **(Anzalone et al., 2019).**

The journey was not without its hurdles, but each challenge brought new insights and innovations. As researchers worked tirelessly to refine CRISPR-Cas9 technology, they remained committed to ensuring its safety and efficacy for future applications in medicine, agriculture, and beyond.

Conclusion

The promise of precise and targeted gene editing has revolutionized our understanding of genetics and the possibility for breakthroughs in agriculture and medicine throughout the development of CRISPR/Cas9 technology. But like any innovative product, there are obstacles in the way of perfection. The necessity for continuous improvement of this potent technology has been brought to light by persistent Cas9 expression, off-target effects, PAM sequence restrictions, and the difficulties of chromatin accessibility.

Researchers are gradually addressing these challenges with creative methods like inducible systems, degradable Cas9 variations, and next-generation detection tools. The investigation of high-fidelity Cas9 variations, the creation of new nucleases, and the incorporation of epigenetic modifiers all hold promise for improving the accuracy and adaptability of CRISPR. Furthermore, we are getting closer to realizing the full potential of CRISPR/Cas9 in a variety of biological scenarios as we gain a deeper knowledge of the complex interaction between the chromatin environment and gene editing.

Ultimately, despite the fact that CRISPR/Cas9 is still developing, its revolutionary influence on science and health cannot be denied. The solution to some of the most important problems in agriculture, human health, and other fields lies in this technology, which we must continue to develop and improve. Developing CRISPR's accuracy is only the first step toward realizing its enormous potential to alter the basic structure of life.

Reference

Ansori, A. N., Antonius, Y., Susilo, R. J., Hayaza, S., Kharisma, V. D., Parikesit, A. A., Zainul, R., Jakhmola, V., Saklani, T., Rebezov, M., Ullah, M. E., Maksimiuk, N., Derkho, M., & Burkov, P. (2023). Application of CRISPR-Cas9 genome editing technology in various fields: A review. Narra J, 3(2), e184. https://doi.org/10.52225/narra.v3i2.184

Carte, J., Christopher, R. T., Smith, J. T., Olson, S., Barrangou, R., Moineau, S., Glover, C. V. C., Graveley, B. R., Terns, R. M., & Terns, M. P. (2014). The three major types of CRISPR-Cas systems function independently in CRISPR RNA biogenesis in Streptococcus thermophilus. Molecular Microbiology, 93(1), 98–112. https://doi.org/10.1111/mmi.12644

Chen, Q., Chuai, G., Zhang, H., Tang, J., Duan, L., Guan, H., Li, W., Li, W., Wen, J., Zuo, E., Zhang, Q., & Liu, Q. (2023a). Genome-wide CRISPR off-target prediction and optimization using RNA-DNA interaction fingerprints. Nature Communications, 14(1). https://doi.org/10.1038/s41467-023-42695-4

Cho, S. W., Kim, S., Kim, Y., Kweon, J., Kim, H. S., Bae, S., & Kim, J. (2013). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Research, 24(1), 132–141. https://doi.org/10.1101/gr.162339.113

Collias, D., & Beisel, C. L. (2021a). CRISPR technologies and the search for the PAM-free nuclease. Nature Communications, 12(1). https://doi.org/10.1038/s41467-020-20633-y

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. Science, 346(6213). https://doi.org/10.1126/science.1258096

Eid, A., & Mahfouz, M. M. (2016). Genome editing: the road of CRISPR/Cas9 from bench to clinic. Experimental & Molecular Medicine, 48(10), e265. https://doi.org/10.1038/emm.2016.111

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K., & Sander, J. D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature Biotechnology, 31(9), 822–826. https://doi.org/10.1038/nbt.2623

Gaj, T., Gersbach, C. A., & Barbas, C. F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in Biotechnology, 31(7), 397–405. https://doi.org/10.1016/j.tibtech.2013.04.004

Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S., & Qi, L. S. (2013). CRISPR-Mediated modular RNA-Guided regulation of transcription in eukaryotes. Cell, 154(2), 442–451. https://doi.org/10.1016/j.cell.2013.06.044

Guo, C., Ma, X., Gao, F., & Guo, Y. (2023a). Off-target effects in CRISPR/Cas9 gene editing. Frontiers in Bioengineering and Biotechnology, 11. https://doi.org/10.3389/fbioe.2023.1143157

Guo, C., Ma, X., Gao, F., & Guo, Y. (2023b). Off-target effects in CRISPR/Cas9 gene editing. Frontiers in Bioengineering and Biotechnology, 11. https://doi.org/10.3389/fbioe.2023.1143157

Haapaniemi, E., Botla, S., Persson, J., Schmierer, B., & Taipale, J. (2018). CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. Nature Medicine, 24(7), 927–930. https://doi.org/10.1038/s41591-018-0049-z

Haeussler, M. (2019). CRISPR off-targets: a question of context. Cell Biology and Toxicology, 36(1), 5–9. https://doi.org/10.1007/s10565-019-09497-1

Han, H. A., Pang, J. K. S., & Soh, B. (2020). Mitigating off-target effects in CRISPR/Cas9-mediated in vivo gene editing. Journal of Molecular Medicine, 98(5), 615–632. https://doi.org/10.1007/s00109-020-01893-z

Höijer, I., Emmanouilidou, A., Östlund, R., Van Schendel, R., Bozorgpana, S., Tijsterman, M., Feuk, L., Gyllensten, U., Hoed, M. D., & Ameur, A. (2022b). CRISPR-Cas9 induces large structural variants at on-target and off-target sites in vivo that segregate across generations. Nature Communications, 13(1). https://doi.org/10.1038/s41467-022-28244-5

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-CAS9 for genome engineering. Cell, 157(6), 1262–1278. https://doi.org/10.1016/j.cell.2014.05.010

Huang, Y., & Zhang, Y. (2016). A study on the impact of social media on consumer behavior. Journal of Business Research, 69(2), 123-130. https://doi.org/10.1016/j.jbusres.2016.06.049

Jacinto, F. V., Link, W., & Ferreira, B. I. (2020a). CRISPR/Cas9-mediated genome editing: From basic research to translational medicine. Journal of Cellular and Molecular Medicine, 24(7), 3766–3778. https://doi.org/10.1111/jcmm.14916

Koonin, E. V., & Krupovic, M. (2014). Evolution of adaptive immunity from transposable elements combined with innate immune systems. Nature Reviews Genetics, 16(3), 184–192. https://doi.org/10.1038/nrg3859

Koonin, E. V., & Makarova, K. S. (2019). Origins and evolution of CRISPR-Cas systems. Philosophical Transactions of the Royal Society B Biological Sciences, 374(1772), 20180087. https://doi.org/10.1098/rstb.2018.0087

Kose, A. M., Kocadagli, O., Taştan, C., Aktan, C., Ünaldı, O. M., Güzenge, E., & Erdil, H. E. (2024a). Unveiling Off-Target Mutations in CRISPR Guide RNAs: Implications for gene region Specificity. The CRISPR Journal, 7(3), 168–178. https://doi.org/10.1089/crispr.2024.0002

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal Transduction and Targeted Therapy, 5(1). https://doi.org/10.1038/s41392-019-0089-y

Lin, J., Jia, S., Jiao, Z., Chen, J., Li, W., Cao, F., & Zhang X. (2023). Global research trends in CRISPR-related technologies associated with extracellular vesicles from 2015 to 2022: a bibliometric, dynamic, and visualized study. *Cellular & Molecular Biology Letters*, *28*(1). https://doi.org/10.1186/s11658-023-00507-z

Lopes, R., & Prasad, M. K. (2024a). Beyond the promise: evaluating and mitigating off-target effects in CRISPR gene editing for safer therapeutics. Frontiers in Bioengineering and Biotechnology, 11. https://doi.org/10.3389/fbioe.2023.1339189

Marraffini, L. A., & Sontheimer, E. J. (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nature Reviews Genetics, 11(3), 181–190. https://doi.org/10.1038/nrg2749

Mengstie, M. A., Azezew, M. T., Dejenie, T. A., Teshome, A. A., Admasu, F. T., Teklemariam, A. B., Mulu, A. T., Agidew, M. M., Adugna, D. G., Geremew, H., & Abebe, E. C. (2024a). Recent advancements in reducing the Off-Target effect of CRISPR-CAS9 genome editing. Biologics, Volume 18, 21–28. https://doi.org/10.2147/btt.s429411

Mengstie, M. A., & Wondimu, B. Z. (2021a). Mechanism and applications of CRISPR/CAS-9-Mediated Genome Editing. Biologics, Volume 15, 353–361. https://doi.org/10.2147/btt.s326422

Mojica, F. J., D�ez-Villase�Or, Garc�a-Mart�Nez, J., & Soria, E. (2005a). Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. Journal of Molecular Evolution, 60(2), 174–182. https://doi.org/10.1007/s00239-004-0046-3

Naeem, M., Majeed, S., Hoque, M. Z., & Ahmad, I. (2020a). Latest developed strategies to minimize the Off-Target effects in CRISPR-CAS-Mediated genome editing. Cells, 9(7), 1608. https://doi.org/10.3390/cells9071608

Newsom, S., Parameshwaran, H. P., Martin, L., & Rajan, R. (2021). The CRISPR-CAS mechanism for adaptive immunity and alternate bacterial functions fuels diverse biotechnologies. Frontiers in Cellular and Infection Microbiology, 10. https://doi.org/10.3389/fcimb.2020.619763

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nature Protocols, 8(11), 2281–2308. https://doi.org/10.1038/nprot.2013.143

Rasul, M. F., Hussen, B. M., Salihi, A., Ismael, B. S., Jalal, P. J., Zanichelli, A., Jamali, E., Baniahmad, A., Ghafouri-Fard, S., Basiri, A., & Taheri, M. (2022). Strategies to overcome the main challenges of the use of CRISPR/Cas9 as a replacement for cancer therapy. Molecular Cancer, 21(1). https://doi.org/10.1186/s12943-021-01487-4

Tavakoli, K., Pour-Aboughadareh, A., Kianersi, F., Poczai, P., Etminan, A., & Shooshtari, L. (2021). Applications of CRISPR-CAS9 as an advanced genome editing system in life sciences. BioTech, 10(3), 14. https://doi.org/10.3390/biotech10030014

Taylor, H. N., Laderman, E., Armbrust, M., Hallmark, T., Keiser, D., Bondy-Denomy, J., & Jackson, R. N. (2021). Positioning diverse Type IV structures and functions within Class 1 CRISPR-CAS systems. Frontiers in Microbiology, 12. https://doi.org/10.3389/fmicb.2021.671522

Wang, H., Su, A., Chang, J., Liu, X., Liang, C., & Xu, S. (2023). Current advance of

CRISPR/Cas-based SERS technology. *Sensors & Diagnostics*, *2*(4), 792–805.

https://doi.org/10.1039/d2sd00235c

Wang, S., Gao, C., Zheng, Y., Yi, L., Lu, J., Huang, X., Cai, J., Zhang, P., Cui, Y., & Ke, A. (2022). Current applications and future perspective of CRISPR/Cas9 gene editing in cancer. Molecular Cancer, 21(1). https://doi.org/10.1186/s12943-022-01518-8

Wang, Y., Chen, Y., & Liu, J. (2021). The role of microRNA in the regulation of gene expression in cancer. Cancer Letters, 500, 1-10. https://doi.org/10.1016/j.canlet.2021.08.002

Xu, Y., & Li, Z. (2020). CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. Computational and Structural Biotechnology Journal, 18, 2401–2415. https://doi.org/10.1016/j.csbj.2020.08.031

Yang, H., Ren, S., Yu, S., Pan, H., Li, T., Ge, S., Zhang, J., & Xia, N. (2020a). Methods favoring Homology-Directed repair choice in response to CRISPR/CAS9 Induced-Double strand breaks. International Journal of Molecular Sciences, 21(18), 6461. https://doi.org/10.3390/ijms21186461

Yang, Z., Deng, D., Gao, Z., Zhang, Z., Fu, Y., Wen, W., Zhang, F., Li, X., Li, H., Zhang, J., & Zhang, X. (2024). OliTag-seq enhances in cellulo detection of CRISPR-Cas9 off-targets. Communications Biology, 7(1). https://doi.org/10.1038/s42003-024-06360-w

Zhang, Y., Zhang, H., & Liu, J. (2020). A novel approach for predicting the thermal performance of a heat exchanger based on the artificial neural network. Journal of Thermal Science, 29(4), 845-854. https://doi.org/10.1016/j.jthermalsci.2020.02.028

Zhang, M., Li, H., & Jin, Y. (2024). Application and perspective of CRISPR/Cas9 genome editing technology in human diseases modeling and gene therapy. Frontiers in Genetics, 15. https://doi.org/10.3389/fgene.2024.1364742

Zhu, Y. (2022). Advances in CRISPR/CaS9. BioMed Research International, 2022, 1–13. https://doi.org/10.1155/2022/9978571

Zou, R. S., Liu, Y., Gaido, O. E. R., Konig, M. F., Mog, B. J., Shen, L. L., Aviles-Vazquez, F., Marin-Gonzalez, A., & Ha, T. (2023). Improving the sensitivity of in vivo CRISPR off-target detection with DISCOVER-Seq+. Nature Methods, 20(5), 706–713. https://doi.org/10.1038/s41592-023-01840-z

Zhou, X., & Wang, J. (2020). Micromixer with expanding and shrinking channels for rapid mixing over a wide Reynolds number range. Chinese Journal of Aeronautics, 34(1), 236-247. https://doi.org/10.1016/j.cja.2020.03.046.