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Architectural Determinants of Genome-Editing Outcomes: A Comparative Analysis of DNA Recognition and Cleavage Mechanisms in ZFNs, TALENs, and CRISPR-Cas9

Sana Khan [1], Muhammad Uzair [2,*], Ejlal Haider [3], Sohaib Usman [4] and Saba Samar [5]

[1] *University Institute of Biochemistry and Biotechnology, Arid Agriculture University, Rawalpindi, Pakistan.*
[2] *Department of Biological Sciences, Virtual University of Pakistan.*
[3] *School of Natural Sciences, University of Chester, England.*
[4] *Department of Biosciences, Comsats University Islamabad Sahiwal campus, Pakistan.*
[5] *Department of Biological Sciences, Superior university Lahore, Pakistan.*

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Abstract

Genome editing has been well recognized as a genome engineering tool that allows scientists to permanently modify the DNA contented at a particular genomic location. Earlier, it was carried out by delivering a DNA template with a long homologous arm to the targeted genomic site. This process was time-consuming and required synthesis and the delivery of a long DNA template. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 are three extensively used genome editing technologies that were developed in response to these limitations. This paper provides a comparative analysis of the origin, structure, function, and working advantages over each other, limitations, and their application in different organisms for disease treatment and genetic modifications of these three technologies. ZFNs are earliest genome editing technology. ZFN is a protein having Zn finger domains that recognize the DNA and the Fok domain that cuts the DNA sequences. TALENs are similar to ZFNs but use transcription activator-like effectors instead of zinc fingers to recognize DNA. CRISPR-Cas9 is more recent technology that uses RNA guides to target specific DNA sequences. One advantage of ZFNs and TALENs is their high specificity but they are time-consuming and expensive processes to design and synthesize each nuclease for a specific target sequence. Whereas CRISPR-Cas9 is faster and more cost-effective, as it requires only simple RNA guide design to target specific DNA sequence. In conclusion, ZFNs, TALENs, and CRISPR-Cas9 are all useful genome editing technologies. Each of these technologies have advantages and limitations that will be discussed in the article.

Key words: Bioengineering; Technologies; CRISPR-Cas9; Genome Editing; TALEN; ZFN

1. Introduction

The field of genome exploration has undergone a significant transformation with the introduction of programmable nucleases in nuclease-dependent genome editing procedures. Three types of engineered nucleases that have played a pivotal role in this advancement: Zinc Finger Nucleases, commonly known as ZFNs, Clustered Regularly Interspaced Short Palindromic Repeats associated with Cas9 Protein (CRISPR-Cas9), and Transcription Activator-Like Effector Nucleases (TAL effectors or TALENs) [1]. These nucleases allow efficient and precise modifications to DNA sequences, creating it potential to introduce targeted mutation, deletions, insertions, or replacements in antargeted genome. TALEN and ZFNs has mechanism for DNA recognition and cleavage, but Cas9 has different has different mechanism for DNA recognition. Which leads to in their specificity, versatility, and efficiency? ZFNs and TALENs use DNA-binding domains, while CRISPR-Cas9 uses guide RNAs (gRNAs) of recognize specific DNA sequences. ZFNs and TALENs have

* Corresponding author: Muhammad Uzair

previously been used effectively in a range of species and cellular structures, but CRISPRs-Cas9 has gained appeal because to its simplicity, convenience of use, and superior effectiveness [2]. In response to concerns regarding the off-target effects of CRISPR-Cas9, efforts have been made to develop Cas9 variants with reduced off-target effects. Before selecting a nuclease for a specific experiment or application, it is essential to thoroughly grasp the advantages and disadvantages associated with each option [3].INTRODUCTION OF ZFN:

A widely used programmable DNA-restricting enzyme called Zinc Finger Nucleases (ZFNs) was discovered in the African torn frog Xenopus laevis. ZFNs create DSBs (Double strand breaks) using unique DNA endonucleases. ZFNs fit in with the group of naturally occurring transcription factors and endonucleases FokI. Zinc Finger (ZF) domains and restriction nuclease domains (FokI) are found in ZFN subunits as a heterodimer (regular severing movement). On specific DNA sequences, the FokI regions dimerize for DNA cleavage and produce DSBs. The DSBs render it feasible for the editing tools in the genome to function [4].

ZFNs offer a complete method for conveying double-strand break (DSB) inside the genome which is site-specific. [5]. The ZFNs have two domains i.e., DNA-cleaving and DNA-Binding Domains, which were studied first by Chandrasegaran[6].

Until now, significant protein domains have been encoded inside the genome. Cys2His2 is usually prevalent in 700 proteins, and 4000 of these domains are detected. According to the frequency of encoding Cys2-His2 ZF domains in eukaryotes, the human genome has the second most utilized protein domain. [7].

1.1. Structure of ZFN

ZFNs have separate DNA- recognitionand DNA-cleavage domains. These modified proteins first appeared in Chandrasegaran's paper.. Chandrasekaran showed that the cleavage domain is generic, allowing for the adoption of several recognition domains in addition to the natural one [8]. Each finger creates contact with 3bp of the DNA [9].

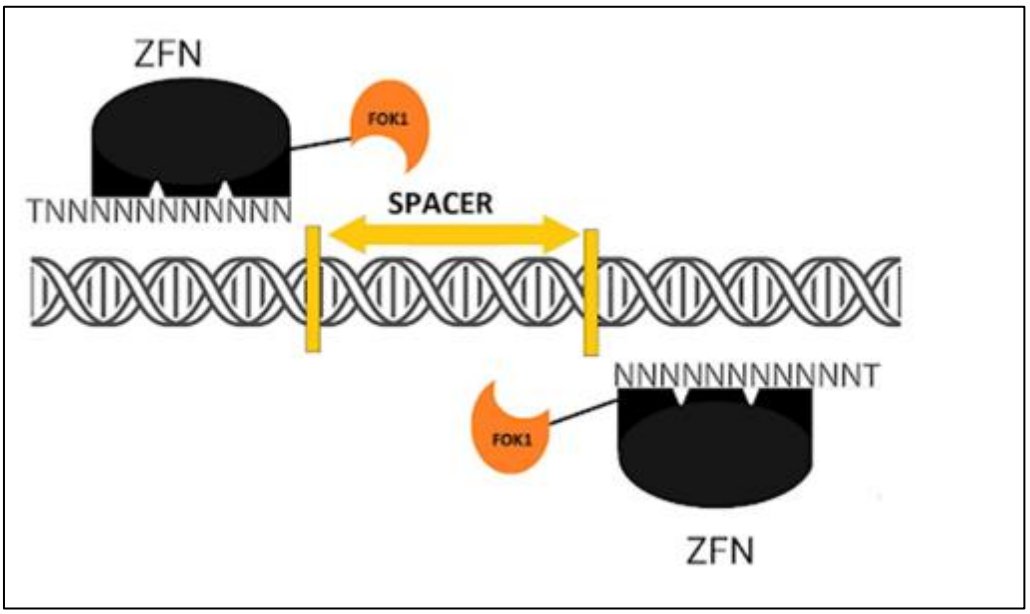

Figure 1 Structure of ZFNs; two finger modules are part of the DNA binding domain, each finger paired with nucleotides. The DNA-cleavage domain is made up of restriction endonucleases of type II i.e., Fok1

1.2. Mechanism of ZFN

The DNA cleavage process occurs after DNA recognition by the ZF domain and requires the FokI dimerization. In the event of the existence of both the particular DNA and the metal divalent ion, two FokI molecules cut double-stranded DNA. The dimerization occurs at the interface of two FokI catalytic domains [10]. The interface of the dimerization is weak; hence two arrangements of fingers are coordinated. High concentration is achieved when both sets achieve their target sequence and hence the dimerization and cleavage are achieved [11]. The dimerization is requiredfor specificity as the monomer is not active and doesn't allow the cleavage.

1.3. Hurdles in ZFN

In living cells, the use of ZFN is not always a success. ZFNs created in vitro usually don't succeed to reproduce genome editing at endogenous loci in vivo.

Fingers were streamlined against target triplet DNA sequences but they had errors. Off-target breaks cause cytotoxicity in the DNA of target cells. Multiple copies of a target-related sequence can serve as extra target sites, as a result of which off-target breaks occur. So, optimizing ZFN design must take into account both specificity and cytotoxicity [13].

Three fingers are required for suitable affinity although all the fingers do not provide equal contribution [14].Compact chromatin and DNA modification structure may make the gene inaccessible [14].Adding fingers may not always increase activity [15].Excessive nuclease levels may trigger target cell death [16]. Lethality is achieved by excessive cleavage expression of ZFNs[17].

1.4. Developments in ZFN

The specificity of ZFNs can be enhanced through three general approaches: (i) enhancing the binding specificities of the ZF domains, (ii) optimizing the linker sequence that connects the ZF domain to the FokI cleavage domain, and (iii) controlling the DNA-cleavage activity of the FokI nuclease domain [18].

Off-target cleavages are reduced by using heterodimeric ZFNs, which restrict the undesired cleavage caused by ZFN homodimerization. Heterodimeric ZFNs consist of two different monomers, each recognizing a distinct DNA sequence. This dual recognition system makes it less likely for ZFNs to bind and cleave off-target sites compared to homodimeric ZFNs, which are made up of two identical monomers and may exhibit broader binding specificity and are less likely to cause unintended DNA damage, reducing the risk of cellular toxicity. . That is why some researchers used adeno-associated virus (AAV) vectors to deliver zinc finger nucleases (ZFNs) and a corrective DNA template to the livers of adult mice with hemophilia. This approach successfully led to the production of high levels of human factor IX, a protein deficient in hemophilia B. The researchers has found a way to reduce off-target DNA cleavage by using obligate heterodimeric ZFNs, which minimized unwanted cleavage associated with homodimeric ZFNs by using AAV/ZFN-mediated genome editing in the liver cellsas a promising therapeutic strategy and might be applicable to other non-replicating cell types.The use of Zinc Finger Nucleases (ZFNs) for genome editingof liver cells may potentially extend this approach to other non-replicating cell types [19].

Other researchers found that by linking three two-finger domains instead of two three-finger units, they can achieve greater target specificity, making it more selective against mutations or closely related DNA sequences. These new peptides can also span short gaps of unbound DNA while maintaining strong binding to their target sites. The authors believe that this new construction method for zinc finger arrays can improve the efficacy of gene therapy and the creation of transgenic organisms compared to previous methods.In conclusion using short linker allowed improved specificity [20].

Several methods for identifying Zinc Finger Proteins (ZFPs) with appropriate affinity and specificity for use in genome engineering have been developed.Also, various methods have been created to find zinc finger proteins (ZFPs) suitable for genome engineering without using the modular assembly approach. One of these methods is the 'OPEN' system, which uses bacterial selections to discover combinations of zinc finger modules that function well together.Oligomerized Pool Engineering (OPEN) is a technique used in genetic engineering to create customized zinc finger nucleases (ZFNs), which are molecular tools used for gene editing.OPEN is a method for efficiently generating a diverse pool of ZFNs with different target specificities. The OPEN system involves two key steps i.e. Low-Stringency Selection and High-Stringency Selection. Stringency selections refer to the conditions or criteria used in a laboratory experiment or process to determine how closely a molecule, such as DNA or proteins, matches its target. The low-Stringency Selection is the first step, multiple low-stringency selections are conducted in parallel. These selections involve testing randomized zinc finger modules to identify those that can bind to each part of the targeted DNA sequence. The use of mild conditions ensures that the resulting pools of zinc finger modules maintain a considerable diversity. Subsequently, in High-Stringency Selection, the selected zinc finger modules from these diverse pools are connected in a combinatorial manner. The products of this combination are then subjected to high-stringency selections to assess their binding capability to the final target DNA sequence. The 'OPEN' framework, for example, make customized ZFNs to bind with bacterial targeted sequences.. Following that, fingers from various pools are combined, as the resultant products are subjected to high stringent selection to ensure that they bind exactly to the desired target.

An alternative method for creating zinc finger proteins (ZFPs) with new DNA sequence specificities is introduced. This method is called “bacterial one-hybrid” which is similar to OPEN but involves a different strategy for building the library

of potential ZFPs. In this approach, for each target DNA sequence (triplet), a library of ZFPs is generated. However, instead of randomizing all the amino acid residues at the zinc finger-DNA interaction interface, only a subset of them is randomized. The remaining positions are selected deliberately, choosing specific amino acid residues known to establish well-understood interactions with the DNA bases. Essentially, this approach combines randomization with carefully selected amino acids to create ZFPs that can interact with the target DNA sequence while maintaining specificity. This method aims to balance the generation of diverse ZFPs with the preservation of critical DNA-binding properties.

A different method being discussed is referred to as "two-finger modules." In this method, instead of using individual zinc fingers, two-finger modules are used as the primary units for DNA recognition. This approach offers several advantages. By using two-finger modules, it becomes easier to optimize the connections or junctions between the zinc fingers within each module. This optimization enhances the cooperation and specificity of base recognition. Using two-finger modules reduces the number of untested junctions between zinc fingers in new ZFP designs. For example, a four-finger ZFP assembled from two-finger modules has only one new junction, whereas assembling it from one-finger units would result in three new junctions. This reduces the risk of a weak interaction between the newly combined fingers. This approach has been used to create zinc finger nucleases (ZFNs) with four, five, or six zinc fingers, allowing for a wide range of applications. A five-finger ZFN, for instance, is constructed using one one-finger and two two-finger units. However, it's important to note that this approach comes with a limitation. Developing and characterizing the extensive panel of two-finger units can require a substantial initial investment, as there are potentially up to 4,096 different two-finger units needed to recognize all possible 6-base-pair DNA sequences[21].

1.5. Application of ZFN in animals:

ZFN has been used successfully in different organisms in both in germ line and somatic cells:

- ZFN is successfully allowed mutagenesis in Zebra Fish (Danio rerio) with golden and no tail/Brachyury genes. It shown that ZFN may be applied to species that allow mRNA transport into the eggs that are fertilized [22].
- ZFN is used in Frog (Xenopus tropicalis) by seeing the high frequency loss of the fluorescence phenotype, here the off-target effects are not found and the germline and somatic cell gene editing both are found effective [23].
- Knockouts are done in embryo of rats by ZFN. The modification which was done in these mice were in two types of genes i.e., Immunoglobulin M (IgM) and Rab38. Both of the knockouts done were properly inherited in the germline of the subjects[24].
- Using ZFN, successful genome editing in mice has been demonstrated. In mice of the types FVB/N and C57BL/6, the genes for jagged 1 (Jag1), notch homolog 3 (Notch3), and multidrug resistant 1a (Mdr1a) were targeted proceeded with a range of specific gene deletions. [25].
- zinc finger DNA recognition domain, were assessed for their ability to locate and cut specific DNA sites in living *Xenopus laevis* oocyte nuclei. The cleaved DNA was activated for homologous recombination, achieving nearly 100% recombination efficiency under optimal conditions, even when the DNA was in chromatin form.[26].
- The sea urchin embryo enables ZFN-driven gene disruption. The presence of populated mesenchyme cells in the embryos is a direct outcome of mRNA insertion. The sequencing analysis also demonstrated that the HesC gene's chosen HpHesC (Hemicentrotuspulcherrimus) homologous locations experienced deletions and insertions. [27].
- ZFN gene editing in Drosophila is performed. The yeast LYS2 gene's cleavage sites might be inserted thanks to the rose locus. [28].

1.6. Applications of ZFN in plants:

- The rice SSIVa gene is subjected to site-directed mutagenesis using ZFN [29]
- The ZFN-edited maize crop was successful in disrupting *ZmIPK1* by insertion of the PAT gene, leading to the development of the inositol phosphate pattern in maize growing seeds and herbicide tolerance [30].
- Herbicide-resistance mutations were successfully added to the Sur loci by ZFN gene targeting in endogenous genes of Tobacco where 40% of recombinant plants were mutated successfully [31].
- ZFN performs targeted mutagenesis in Arabidopsis. The QQR ZFN (a particular type of ZFN) can produce up to 0.2 mutations at each target site in the rabidopsis genome. This suggests that native loci may be targeted for mutation at rates as large as 0.2 alterations per gene or 0.4 alterations per cell. Prior to this, there were 10-7 GT events (Gene Targeting events) each cell or 10-6 to 10-4 GT events each integration

when using homologous recombination-based approaches. Therefore, QQR ZFN is effective for application in plant mutagenesis [32].

1.7. Application of ZFN in humans

ZFN is used in disruption of CCR5 cell receptors (responsible for regulating immune responses and is a co-receptor for the entry of certain strains of HIV into human cells) of humans, which successfully blocked the HIV entry into cells. [33]

1.8. Decline in the use of ZFN

In spite of widespread applications of ZFN from 2002 to 2016[34], some limitations like less stability, complexity, low efficiency[35]high toxicity[36], tremendous expertise and time during manufacturing[37] slow down their effective use [34].

2. Introduction of TALEN

To modify gene transcription in host plant cells, Xanthomonas bacteria employ proteins known as transcription activator-like effectors (TALEs). These bacterial TALENs served as a model for the development of engineered nucleases used in gene editing. They may be created efficiently and swiftly by scientists using a straightforward "protein-DNA code" that links specific DNA-binding.TALEs use specific binding sites to target individual DNA bases within repeat domains. [38].

The ability of these proteins to bind to DNA was first demonstrated in 2007, and in 2009, two research teams deciphered the mechanism allowing the TALE proteins to recognize the target DNA. When the effector protein mimics eukaryotic transcription factors, it binds to DNA and activates the expression of the target genes. Further investigation into the mechanism of the effector protein's action has revealed this process. [39].

2.1. Composition and working of TALEN:

A TALEN is formed by combining a TALE repeat sequence with the FokI nuclease domain. . The repeating unit contains 33–35 amino acids, two of which are hypervariable residues “repeat variable Di residues” (RVD) at positions 12 and 13. These (RVD) direct protein-DNA collaboration which is done in order for the repetitive variable Di residues NN, NI, HD, and NG to be able to distinguish between the four DNA bases of guanine, adenine, cytosine, and thymine. Each RVD detects only one base in this manner making the paired cluster of repeats a high-explicitness determinant for the DNA restricting of the molecule in general. The "code-like" connection between the amino acid arrangement of RVD and perceived/bounded nucleotides takes into account the design of certain DNA-binding domains in a manner similar to the group of Lego blocks. By selecting a mixture of repetitions with particular RVDs, it is possible to collect DNA-binding domains with the desired specificity. Curiously, the RVD code is quite distinct between the species of Xanthomonas and Ralstonia{Nesme, 1995 #1,Heuer, 2007 #2}{Doyle, 2013 #3}.

We looked into the possibility of employing specially created TALEN effector nucleases for precise genome editing. TALEN causes site-specific double-stranded DNA breaks, leading to homology-directed repair with an external donor template. Every 35 base pairs of DNA have an average number of TALEN binding sites, and TALENs can be swiftly built from freely available modules. [40].

2.2. Single-Molecule Analysis of TALE Protein DNA Search Mechanism:

Genetic engineering typically employs a class of programmable proteins that bind to DNA known as TALE proteins. Despite the late advancement, there is little open knowledge of their sequence search methodology. Here, described the TALE search along DNA using single-molecule analyses. Despite staying connected to DNA templates throughout the seeking mechanism, TALEs use a rotationally decoupled system for nonspecific search. That TALEs can adopt a loosely folded conformity across DNA sequences during the nonspecific search, operating with fast one-dimensional diffusion under the scope of solving conditions. Besides, this model is reliable with a formerly detailed model is two-state TALE search mechanism that enables these proteins to overcome the search speed-stability issue. Among a vast class of sequence-specific proteins that bind to DNA, the TALE search is distinctive and promotes effective 1D diffusion along DNA. [41].

2.3. Delivery of TALENS into Targeted Cells

The development of TALENs has made it possible to modify the genome specifically in novel ways. In any case, the huge size of TALEN proteins and the highly repetitive structure of the TALE DNA-recognition domain provide a considerable

barrier to their delivery into target cells.When delivered via lentiviral vectors, the TALE sequences with a lot of repetitions are favourable for broad improvement.

Given the constraints of space in delivery vectors, such as adenoviruses, the transfection of plasmid DNA or mRNA encoding TALENs offers an alternative to viral-based methods. These viral-based methods are limited to a small number of cell types and can be highly toxic.

There has recent TALENs delivery via a novel cell-penetrating peptide approach. To confer cell-penetrating functionality to TALEN proteins, cell-penetrating peptides can be chemically attached to Cys residues located on the outer surface of TALE domains.They demonstrated that, in HeLa and HEK293 cells, separately, R9-conjugated TALENs induced the deletion of BMPR1A genes in the human CCR without causing any apparent damage.[42].

2.4. Toolbox For Construction of TALE

A toolbox uses the hierarchic binding method to quickly create unique TALE transcription factors (TALE-TFs) and nucleases (TALENs). This toolbox may easily be expanded to create TALEs for numerous targets simultaneously and works with the reasonable and quick construction of custom TALE-TFs and TALENs in one week [43].

2.5. TALEN Effectors

Major virulence factors known as TAL effectors are present in the bacterial plant pathogen Xanthomonas, which affects numerous species of plants, including important crops like citrus, rice, and pepper. By restricting the activity of target gene promoters, TAL proteins are translocated into host cells by bacteria through the type III secretion system. The TAL-DNA binding domain permits the proteins to bind with any given DNA sequence specifically [44].

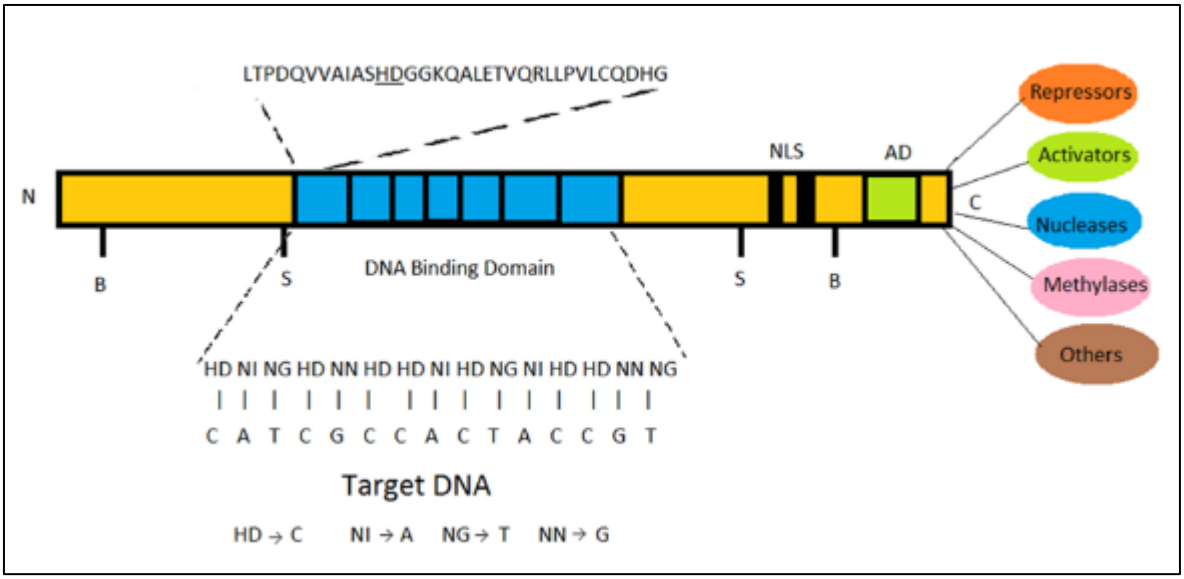

Figure 2 Structure of TALEN effectors: it consists of N-terminal and C-terminal. N-terminal is for nuclear localization signals, RVD to target nucleotide sequence and bacterial type III secretion. while the C-terminal is for transcriptional activation. It contains repressors, activators, nucleases, and methylases for transcriptional activity for the target sequence editing

2.6. Genome Editing in Eukaryotes by TALEN

Scientists may now edit genes of interest to research their function and investigate possible uses in biotechnology for genetic modification in plants and other species thanks to the development of targeted genome modifications. An essential step in achieving the necessary site-specific DNA double-strand break for effective genetic modification is the subsequent DNA repair process. This repair can occur through non-homologous end joining (NHEJ) when variable insertions or deletions (Indels) are introduced, or through homology-directed repair (HDR) when a donor sequence is available for recombination.Through the use of its flanking homologous sequences, HR (homologous recombination) is often a perfect DSB repair mechanism and in order to facilitate the genetic exchange of host and foreign DNA strands. It is possible to use the NHEJ (non-homologous end joining) and HR repair mechanisms for nuclease-based genome editing. Two complementary DNA recognition sites (TALEN-F and TALEN-R) are required by standard TALENs, which

are flanked by an unspecific spacer region that is centered. For DSB, the dimeric FokI nuclease domain, coupled with a single polypeptide. Necessary genome alterations can be achieved by restoring the genomic DSBs caused by TALENs via HR or NHEJ. Little INDELs (insertion and deletions) are created via the NHEJ-mediated pathway. Double-stranded HR using a repair template DNA triggers precise gene replacement and genetic editing [45].

Genetic engineering typically employs a class of programmable proteins that bind to DNA known as TALE proteins. Despite the late advancement, there is little open knowledge of their sequence search methodology. Here, described the TALE search along DNA using single-molecule analyses. Despite staying connected to DNA templates throughout the seeking mechanism, TALEs use a rotationally decoupled system for nonspecific search. That TALEs can adopt a loosely folded conformity across DNA sequences during the nonspecific search, operating with fast one-dimensional diffusion under the scope of solving conditions. Besides, this model is reliable with a formerly detailed model is two-state TALE search mechanism that enables these proteins to overcome the search speed-stability issue. Among a vast class of sequence-specific proteins that bind to DNA, the TALE search is distinctive and promotes effective 1D diffusion along DNA. [41].

2.7. Application of TALEN in Yeast

TALENs have been effectively applied in approximately 25 different species, ranging from fungi to human cells, to carry out a wide spectrum of genetic modifications, including both minor edits and substantial genome duplications. This discovery will almost certainly be of incomparable value to scientists who are interested in developapplications such as targeted genome modifications or the use of artificial transcription factors to regulate the activity of particular genes. To induce targeted double-strand breaks (DSBs) for gene modification and frame shift transformations through non-homologous end joining (NHEJ) at pre-determined loci, specific nucleases are typically used.As a proof-of-concept, the TALEN method has successfully knocked out several marker genes in S. cerevisiae. These marker genes either yield easily identifiable mutations or are ideal for visual inspection and screening. .The procedure for S. cerevisiae gene knockout using the URA3 gene involves the principle of using negative selection with 5-FOA, an inhibitor specific to URA3-containing strains. The URA3 gene encodes orotidine 5-phosphate decarboxylase (OD Case), which is an enzyme involved in pyrimidine ribonucleotide biosynthesis. The use of 5-FOA negatively selects against cells with an intact URA3 gene, leading to the loss of URA3 function in the target gene knockout. This provides a selective pressure for identifying yeast cells in which the URA3 gene has been successfully disrupted.[46].

2.8. Application of TALEN in Plant

Site-specific plant genome editing with TALENs is a versatile and modern tool that can have a significant impact on crop improvement. In several plant species, TALEN-mediated genetic engineering has been used. The first crop to be improved utilizing TALENs innovation was the rice crop. The blight disease causes a major yearly loss in rice production around the world. which is caused by Xanthomonas oryza. The gene for sucrose-efflux carriers OsSWEET 14's promoter sequence is bound by bacteria effector protein during infection, activating certain disease-prone genes in rice. A pair of TALENs was designed and used to target the OsSWEET 14 gene for editing in rice. The precise modification introduced by these TALENs resulted in the alteration of the gene's promoter region and subsequent gene silencing, providing protection against M. Oryza. [47].

2.9. Application of TALEN in Animals

Advancements in genome-editing tools have enabled precise and effective manipulations of genetic material. These innovations have opened up new possibilities for improving the genetic traits of domesticated animal species, including enhancing their productivity, reproductive abilities, and resistance to infections. In particular, pigs with edited genomes are currently the only species capable of introducing genomic mRNA into animal zygotes. Additionally, gene-edited dairy cattle and sheep can be produced through the infusion of TALEN mRNA into zygotes. In this context, the focus is on the myostatin (MSTN) gene in these two species. The process involves directly infusing TALEN mRNA into zygotes, followed by transferring them to synchronized recipients, resulting in the editing of specific genes. This approach has successfully replicated the double-muscle phenotype, demonstrating the practicality of TALEN-based editing in sheep and cattle. Importantly, this technique simplifies the calving process for Nellore cattle, as breeds with double muscling typically have a normal birth weight, reducing the risk of dystocia.[48].

2.10. Application of TALEN in Human Genome

The TALEN pair targeted three human loci (CCR5, AAVS1, and IL2RG) and conducted a detailed investigation of their action, toxicity, and specificity. With allelic genetic disruption the frequencies of 15–30% in human cells, the TALENs demonstrated activity comparable to that of benchmark ZFNs. Surprisingly, minimal cytotoxicity and the lack of anomalies in the cell cycle were often associated with TALEN activity [49].

2.11. Application in Development of novel universal CAR T cells

Due to the accuracy and precision of TALEN-mediated gene editing, a novel universal CAR T cells scaffold with immune evasive properties has been developed. To better cancer therapy and patient outcomes, all CAR T cell treatments have been paused [50]. Gene-edited CD19 CAR-T cells modified by TALEN technology have a significantly reduced risk of cancer.[51]

2.12. Application in diagnostic of COVID-19

In the diagnostic of COVID-19 the TALEN is an effective gene editing tools. Because of low-costly diagnostic develop and its ability for quick on site, very low off targeting and mismatches.[52]

2.13. TALENS's advantages over other genome modification techniques

Advanced genome-editing technologies like ZFNs, TALENs, and CRISPRs-Cas9 may be used to modify the gene architecture of complex genomes and have enormous implications for basic plant research. Over ZFNs and CRISPR systems, TALENs provide a few anticipated advantages. ZFNs were the first utilized programmable genetic-modification technology, however, two crucial drawbacks interfere with its broader acceptance. ZFNs are challenging to develop and need significant expenditures for novel gene targeting, and they frequently result in undesirable alterations and chromosomal abnormalities because of their potentially dangerous off-target cleavage. [53, 54]. Based on TALEs (Transcription Activator-Like Effector proteins), Zinc fingers are expected to be less efficient as DNA-binding domains. This is due to their wider targeting range, which results in lower specificity and higher off-target activity.[55]. The main drawback of the CRISPR system is that it only targets sequences preceded by the protospacer adjacent motif, or NGG-3. TALENs concentrate on up to 16–24 repetitions, whereas CRISPR is, in a sense, limited to 20 bp. This should increase the likelihood of mismatches and Cas9's off-target cleavage. Human cells' CRISPR has a high prevalence of off-target mutations might seriously hamper this technique [56-58]. One significant advantage of TALENs over CRISPR and ZFNs is their effectiveness in targeting short DNA sequences, including those encoding enhancers and microRNA, which require specific targetable sites. [59, 60].In gene editing within heterochromatin regions, TALENs show a five-fold increase in editing efficiency compared to CRISPR-Cas9. This enhanced efficiency is attributed to TALENs' precision, as both TALEN and Cas9 can be hindered by local searches in non-specific regions within heterochromatin. .[61][62]

2.14. Limitations of TALEN

Repeat TALE arrays cloning technical hurdles are one of the fundamental specialized obstacles for cloning repeats TALE due to a huge size of ambiguous repetitive sequences. To address this limitation, several techniques have been developed to work with the quick groups of custom TALE clusters, including "Golden Gate" sub-atomic cloning, high-throughput solid-phase gathering, and association autonomous cloning methodologies [63].

2.14.1. Off-target effects of TALEN

Off-target effects are a notable concern in TALEN-mediated genome editing, particularly in the context of gene therapy. Despite this, TALENs have successfully induced modifications in endogenous genes within a wide range of organisms, including microorganisms, plants, and animals such as yeast, Drosophila, rice, human somatic cells. They have also found successful application in the creation of disease cell models and animal models (TALENs have been employed to create both in vitro cellular models of diseases and living animal models with disease-related traits for research purposes). . Contrasted with ZFNs, the editing efficiency of TALENs is comparable, however, the off-target rate is lower, a huge part of the reason is that every tandem repeat sequence perceives just a single base while a zinc finger module of ZFNs perceives 3-4 bases. Shorter TALENs have higher specificity for each base recognition, while longer TALENs have higher specificity for the recognition of the whole target sequence. Shorter TALENs require less energy to bind DNA, and the corresponding energy is conveyed to each base for recognition, and the specificity is stronger. Bringing down the saturation of the target site makes it will quite often bind to the off-target site, which will likewise decrease the specificity [64].

3. Introduction of CPISPRs-Cas9

The Clustered Regularly Interspaced Short Palindromic Repeats associated Cas genes, which is present in bacteria and archaea and aids in their defense against viruses and serves as adaptive immunity, is known as the CRISPRs-Cas9 tool [65].

In *Escherichia. coli*, while researching a gene involved in the adaption of alkaline phosphatase in 1987, Japanese researchers accidently replicated a strange collection of repetitive sequences interspersed with spacer sequences. [42, 47]. This discovery directed to the introducing to CRISPRs- Cas9 tool.

CRISPRS-Cas system is categorised into two classes: (i) Class 1 has multi-subunit effector complexes and further divided into six types and thirty-three sub-types whereas (ii) Class 2 has single-protein effector modules and into three types and seventeen sub-types [48][53]. Type 2, which employs the Cas9 protein as effector, which is the widespread and greatest tacit subset of Class 2 system. [54].

The ground-breaking CRISPR-Cas9 gene-editing technique, which facilitates researchers to accurately target and modify specific genes, was developed in 2012 by Jennifer Doudna and Emmanuelle Charpentier [55, 56]. They received the Chemistry Nobel Prize in 2020 in recognition of their ground-breaking work. Numerous opportunities for scientific and medical study have been created by their discovery.

3.1. Cas9 Protein

The Cas9 enzyme family depend on the creation of a complex between the targeting crRNA and activating transRNA, to identify and cut the specific double-stranded DNA sequence. This base-paired structure allows for precise cleavage at the intended location and is a crucial aspect of the CRISPR-Cas9 genome editing tool [57].

3.2. Composition and Working of Cas9

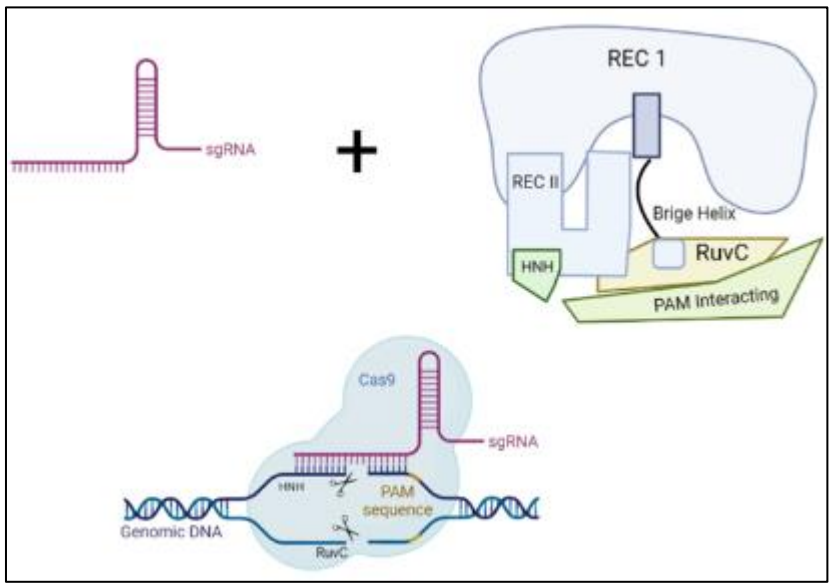

Figure 3 Cas9 nucleases complex; six domains of Cas9 protein: RECI, REC II, PAM Interacting domain, Bridge Helix, RuvC and HNH. The Binding of Cas9 and sgRNA formed the CRISPRs-Cas9 complex. The CRISPRs-Cas9 system targets DNA with the help of complementary base pairing with its bound sgRNA. A 3′ PAM sequence follows target regions. The subsequent breakage of dsDNA activates error-prone either NHEJ or HDR processes

CRISPR-Cas9 system involves three major components: (i) the Cas9 protein, (ii) Single guide RNA (sgRNA), and (iii) PAM (protospacer adjacent motif) interrelate to create a complex that can recognise target site with great accurately and selectivity. In natural and artificial CRISPRS-Cas9 systems, Cas9 nucleases is liable for tracing and cutting target DNA. Without sgRNA, Cas9 remains inactive. [60]. Cas9 protein is consisted of six domains: (i) HNH, (ii) RuvC (HNH and RuvC are nuclease domains), (iii) PAM Interacting, (iv) Bridge Helix, (v) REC I and (vi) REC II [60]. In engineered CRISPRs-Cas9 tool, gRNA comprises of a ssRNA that forms a T-shape contained two or three stem loops and one tetra-loop [57, 66, 67]. The DNA sequences that express the protospacer-containing CRISPRS-Cas9, are linked to create an artificial sgRNA, which has the target sequence (excluding PAM) for pairing to the target region in its 5′ terminal region. The original crRNA-tracrRNA duplex is faithfully mimicked by the sgRNA, which makes CRISPR system manipulation much easier. A Cas9 nuclease complex is formed by the combination of sgRNA and the Cas9 protein. Importantly, by simply adding the target sequences to the sgRNA, the sgRNA/Cas9 nuclease combination may be tailored to recognise specific target sites. This point made the CRISPR-Cas9 system dominant from ZFNs and TALENs, then rely on the DNA binding domains of proteins for target identification.

The Cas9 nucleases can explore the target DNA sequence close to PAM, which is necessary for efficient target identification, both in vitro and in vivo. The target sequence is cleavedby Cas9 that allowing the sgRNA to link with the target complementary strand, once the target region has been found. After then, three bases upstream of PAM identifies target DNA region.RucV and HNH domains cut both strands of the target DNA, anda blunt-ended DSB is formed. The DSB can be repaired either by HDR that causes gene (fragment) knocking or alteration for particular gene engineering, when homologous donor DNA is present or by NHEJ that causes mutations at the targeted spot. It's significant that several designed sgRNAs with various target sequences may lead Cas9 to the appropriate locations in the same cells. Multiple members of gene families as well as functionally linked genes that govern complex features can be altered by multiplex editing in the same cells[59].

3.2.1. Cas9 in Prokaryotes

The CRISPRs-Cas9 system originate from prokaryote, turns as an adaptive immune system to defend against viral targets (like bacteriophages) by cutting the DNA by an Cas9 nuclease in a sequence precise fashion [68, 69]. Cas9 is mostly find in Streptococcus thermophiles [70], Francisellanovicida[71] and Neisseria meningitides [71, 72]. [57]. [73].

3.3. Cas9 in Eukaryote

CRISPRs-Cas9 is not found in eukaryotes naturally. It is used in editing, imaging, and regulating eukaryotic genomes[74]. It is used for gene engineering and editing in almost all eukaryotic organisms such as human [75, 76], plant [59], mice[77], and monkey [78] etc.

3.4. Application of Cas9

Due to simplicity, the possibility for extremely multiplexed modifications and high competence as a site precise nuclease of Cas9 targeting have introduced a wide range of applications [79].

Through the development of simple genome editing and engineering in plants and animals using RNA programmable CRISPRs-Cas9, the discipline of biology is currently going through a transformational period. The ability to accurately and effectively target, edit, regulate, modify and stain genomic loci of a widespread variety of organisms have been made possible by the simplicity of CRISPRs-Cas9 tool, in combination with a excellent DNA cutting mechanism. [80].

Most than decade years, the CRISPRs-Cas9 technique has gained attention in molecular biology research. CRISPR technology, a pioneer in genome editing, has transformed animal research as well as human gene therapy, medical research, and plant research, mostly for crop development. Making genetic knockout mutants is one of CRISPR's most widely used [81].

3.5. Editing in Plants by Cas 9

In plant species that require longer growth times, CRISPRs-Cas9 do multi-gene targeting that significantly speed up the creation and screening of higher-order mutants (organisms that carry more than one genetic modification).This is particularly important for characterisinggenes associated with cell wall formation and maintenance. Furthermore, CRISPR allows for the knockout of genes in the lack or presence of genetically connected null T-DNA mutants. These benefits make CRISPR a suitable and essential tool for conducting functional studies in research of plant cell wall. [81].

In order to attain global food security, increasing agricultural production is of great relevance. Crops has developed with increased adaptability, high production, and flexibility against a variety of biotic and abiotic stresses. The CRISPR-Cas9 technique has become an operative tool for directed mutagenesis, make qualifying for multiplex gene editing, plant transcriptional regulation, gene knockouts and single base substitution. Since CRISPR-Cas9 genome engineering has shown such enormous promise for crop improvement, genome-edited agricultural regulation is still in its infancy. [82].

3.6. Editing in Animals by Cas9

The molecular underpinnings of health and disease continue to be better understood thanks in large part to genetically engineered animals. Although other species, such as pigs, more closely match human physiology, research has primarily concentrated on genetically altered mice. Cross-species studies with phylogenetically remote species, like chickens, also offer profound perceptions into basic bio-medical methods. CRISPRs-Cas9 is one of the best adaptable genetic techniques that works with all animals such as transgenic chickens, mice and pigs. They are fertile and healthy. **[83].**

3.7. CASLFA

The CRISPRs-Cas9 tool has also been helped in the detection of pathogens with the assist of CRISPR-mediated lateral flow nucleic acid assay (CASLFA)[84].

3.8. Editing in Human by Cas9

In order to fight against human viral infections, CRISPRs-Cas9 has been usually and efficiently used. Infectious diseases like hepatitis B virus, human papilloma-virus, HIV polio and other virus's infections are still world-wide pressures with constant power to cause pandemics. CRISPR-Cas9 technology is designed to enhance the host's antiviral capabilities by directly targeting viral genetic elements, such as DNA, while avoiding modification of the host's genome. This technology aims to remove or disable the virus from the host's system. [85].

3.9. Use of Cas9 In Cancer Research and Therapy:

CRISPR assures to speed up cancer research by offering an effective mechanism to analyse the processes of tumori-genesis, recognize targets for drug design and development. CRISPR-Cas9 has the potential to advance both translational and basic cancer research, and it is still in the process of revealing its full capabilities. CRISPR-Cas9 screens are an influential well-designed genomics tool to find out new targets for treatment of cancer. Some registered clinical trials are used CRISPR-Cas9 system for treatment of neo-plasms such as for type 1 bladder cancer(tumor has spread to the connective tissue (called the lamina propria) that separates the lining of the bladder from the muscles beneath, but it does not involve the bladder wall muscle) is treated with CRISPR-Cas9. Furthermore, CRISPR-Cas9 system gives a device to control non-coding site of the DNA and will accelerate the purposeful investigation of atypical character of the genome of cancer. In the future, CRISPR-Cas9 will use in translational drug will mainly base on the skill to grow Cas9 variants with minimum or no off-target effect and new processes to develop the creative ways to create for effective engineering of accurate genomic modify by HDR, which are yet not available. Moreover, potential development of non-viral and viral transfer process will be essential to advance the in-vivo applications of CRISPR-Cas9. [86]

3.10. Advantages of CRISPR-Cas9 system over other genome editing technologies

The above-mentioned tools have a number of drawbacks, ZFNs and TALENs that are time-taking and expensive to build their constructs, which prevents them from being used widely. They can only target one spot at a time and have a poor level of efficiency. The cloning, purification, and engineering of new proteins are additional requirements in addition to the disadvantages already mentioned. [87]. Whereas Cas9 is led by short RNAs and base pairing sequence of target DNA, demonstrating that is far less expensive, simpler to develop, highly effective, specific, and compatible for multiplexed gene engineering and high-throughput for a various kind of cells and animals [88, 89].

3.11. Limitation of CRISPR-Cas9 system

In spite of their broad range of applications, Cas9 are not considerate to be precise and safe for gene therapy[90]. Gene editing has been revolutionised by CRISPR technology, although it has several technical drawbacks, such as immunogenic toxicity and off-target effect.The Cas9 mechanism requires a PAM sequence located near the target region, typically a small PAM recognition region of '5′ NGG 3′' (where 'N' can represent any nucleotide).. The use of non-viral vectors for delivering the CRISPR-Cas9 system is of interest. The system's components can be delivered in different forms, including DNA within a plasmid vector; this means that the genetic information needed for the CRISPR-Cas9 system is packaged in a plasmid that serves as a delivery vehicle for introducing the CRISPR components into the target cells. RNA using liposomes; In this case, the CRISPR components are carried within liposomes. Liposomes can encapsulate and protect the RNA, aiding in its delivery to the target cells, or as ribonucleoproteins with nanoparticles; the CRISPR components are delivered as ribonucleoproteins, which are complexes of RNA and Cas9 protein. These complexes are packaged with nanoparticles, which can enhance the stability and delivery of the ribonucleoproteins to the target cells.The most common spCas9 is hard to wrap up in AAV vectors (the utmost common vehicle in delivery of gene therapy) due to its huge mass [91].

3.11.1. Immunogenic toxicity:

More over half of the subjects in a study by Charlesworth et al. had pre-existing anti-Cas9 antibodies against the two most widely utilised bacterial orthologs, SpCas9 and SaCas9. This emphasises the risk of immunogenic harm linked with the practise of CRISPR-Cas9 technology for gene engineering and gene therapy. Although they may also origin an immunological reaction, AAV vectors are routinely utilised to convey CRISPR components for gene therapy. As a result, numerous AAV serotypes and Cas9 orthologs were selected due to sequence similarity and projected obligatory power to MHC class I and MHC class II in order to find safe candidates for the recurrent delivery of AAV-CRISPR-Cas9 gene therapy and gene engineering. There were no AAV serotypes, discovered that could entirely evade immune recognition,

however three Cas9 orthologs were revealed that had strong editing effectiveness and could be administered repeatedly with less immunogenic damage in mice that had been immunised against both AAV and Cas9. This underlines the significance of taking immunogenic toxicity into account when choosing Cas9 orthologs and AAV serotypes for gene therapy[92]. To create therapy, based on CRISPR, solutions that can be used on a larger spectrum of patients, additional research is required.

3.11.2. Off Target Effects:

Mostly for therapeutic and clinical applications,Cas9 has a significant flaw called off-target activity, where the RNA-guided endonuclease frequently causes changes at undesired places (more than 50% of the time) [84, 93]. Its potential therapeutic and clinical applications are constrained as a result, and improvements are being undertaken to increase its specificity.

3.12. Modification in CRISPRs-Cas9

Off-target activity of CRISPRs-Cas9 is disrupted the competence and flexibility ofCRISPRs-Cas9 as dominant genome engineering tool. To conquer this, scientists have investigate the CRISPRs-Cas9 thoroughly (structurally and functionally) and suggest a number of strategies to improve the system elements like re-designing of Cas9 Nuclease and gRNA structure and customization of the PAM[94].

Table 1 Comparison among ZFNs, TALENs and CRISPRs- Cas9.

Characteristic	ZFNs	TALENs	CRISPRs-Cas9	References
Origin	Eukaryote/Artificial	Prokaryote/Artificial/Eukaryote	Prokaryote/Natural	(Mahfouz, Piatek, & Stewart Jr, 2014), (Mahfouz et al., 2014)
Mechanism	Protein-DNA interaction	Protein-DNA interaction	RNA-DNA interaction	(LaFountaine, Fathe, & Smyth, 2015)
DNA target recognition	Zinc finger domain	TALE domain	Guide RNA	(Mahfouz et al., 2014)
Cleavage Domain	FokI	FokI	RuvC and HNH	(LaFountaine et al., 2015)
Target specificity	Moderate	High	Variable	(LaFountaine et al., 2015)
Off-target effects	High	High	Moderate	(Wani et al., 2023)
Range	Limited	Limited	High	(Chang, 2022)
DNA binding	Modular	Modular	Specific	(LaFountaine et al., 2015)
Time	Long	Long	Short	(Chang, 2022)
Intellectual property	Patent-protected	Patent-protected	Patent-free	(LaFountaine et al., 2015) (Du et al., 2016)
Ease of design	Hard to design and assemble	Easy to design and assemble	Easy to design and assemble	(Wani et al., 2023)
Method of increasing specificity	By varying no. Of finger arrays or by using obligate	Truncated TALE domains.	Using base editors instead of nucleases, Prime editing	(Boch et al., 2009)

	heterodimeric FokI variants.			
Multiple targeting	No	No	Yes	(Wani et al., 2023)
Design flexibility	Limited	Limited	High	(Chang, 2022)
Delivery efficiency	Low to moderate	Low to moderate	High	(Hadipour et al., 2023)
Cost	High	High	Low	(Chang, 2022)
Accessibility	Limited	Limited	Widely available	(Chang, 2022)
Ease of use	Challenging	Challenging	Relatively easy	(Boch et al., 2009)
Clinical trials	Several ongoing Humans: Beta-thalassemia Animals: Mouse, Cattle, rabbit and pig	Several ongoing Zebrafish Frog Mouse Rabbit Pig Sheep Goat	Several ongoing Humans: Cancer HIV/AIDS, Huntington's disease, Glycogen storage disease type, autosomal dominant hearing loss Dogs: Duchenne muscular dystrophy Pigs Porcine endogenous retrovirus (PERV)	(Musallam, Bou-Fakhredin, Cappellini, & Taher, 2021), (Meyer, de Angelis, Wurst, & Kühn, 2010), (Verso, 1964), (Antonarakis et al., 2020), (Tuan, 2023), (Sung et al., 2013), (Morelli et al., 2023), (Song et al., 2013),(Carlson et al., 2012), (Yu et al., 2011), (Flisikowska et al., 2011), (Hauschild et al., 2011; Whyte et al., 2011), (Proudfoot et al., 2015), (Bali, El-Gharbawy, Austin, Pendyal, & Kishnani, 2021), (Gao et al., 2018), (Cui et al., 2015), (Amoasii et al., 2018), (Denner, 2021),
Application in plants (gene names are in brackets)	Soybean (FAD2-1A and FAD2-1B): produce high linoleic acid variants	Wheat (PPD-B1): Delayed flowering for improved yield Soybean (GmFAD2-1A and GmFAD2-2A):Reduced linolenic	Rice (OsSWEET13 and OsSWEET14):increased ability to tackle bacterial blight	(Pham, Lee, Shannon, & Bilyeu, 2010), (Townsend et al., 2009), (Msanne, Kim, &

	Tobacco (ALS): Herbicide resistance Potato (GBSS): Reduced acrylamide content for improved food safety	acid content for improved oil quality	Maize (ZmPDS and ZmIPK1): Improved herbicide resistance and drought tolerance	Cahoon, 2020), (Zafar et al., 2020), (Aglawe, Barbadikar, Mangrauthia, & Madhav, 2018)

4. Conclusion

This comparative analysis shows the superiority of CRISPR-Cas9 technology over ZFNs and TALENs in terms of both efficacy and precision. While these three tools exhibit potential for genome editing in prokaryotic and eukaryotic settings, they are not exempt from shared limitations that warrant further investigation. To advance the field of genetic engineering, it is imperative to prioritize the mitigation of off-target effects, refinement of precision, and the development of efficient, secure delivery methods. Genome-editing nucleases have undeniably ignited a revolution in genetic engineering and are poised to assume an increasingly central role in advancing scientific and biomedical research. The dynamic landscape of gene-editing technologies is poised to shape the future of genomics, offering more dependable, accurate, and versatile applications across diverse domains, thereby reshaping the trajectory of both scientific inquiry and biomedicine.

Compliance with ethical standards

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this review article.

Statement of ethical approval

As a comparative review article synthesizing and analysing previously published scientific literature, this study did not involve any new experiments with human participants, animal subjects, or data collected from individual persons. Therefore, formal ethical approval was not required for this work.

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