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A Decade of Genome Editing: Comparative Review of ZFN, TALEN, and CRISPR/Cas9

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Abstract: Recent advances in genome editing technologies, including ZFNs, TALENs, and CRISPR/Cas9 systems, have redefined our ability to probe and precisely modify the genome and epigenome in vivo and in vitro. ZFNs and TALENs pioneered targeted editing through engineered nucleases, offering high specificity and accuracy, while the RNA-guided CRISPR/Cas9 system has revolutionized the field with its simplicity, efficiency, and adaptability across diverse biological systems. Emerging innovations enhance precision. Broader applicability and enable gene editing even in traditionally intractable models. This collection highlights the progress, comparative strengths, and expanding applications of these genome editing tools in research, therapeutic, and agricultural fields

Keywords: *Genome Editing, ZFNS, Talens, CRISPR/Cas9, Nucleases.*

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I. INTRODUCTION

➢ *Zinc Finger Nucleases (ZFNs)*

Zinc finger nucleases (ZFNs) were the first generation of programmable nucleases that laid the foundation for the current revolution in genome editing. These chimeric proteins function by fusing a sequence-specific DNA-binding domain derived from zinc finger proteins (ZFPs) to the catalytic domain of the FokI endonucleases [1]. Originally adapted from eukaryotic transcription factors, ZFPs consist of approximately 30 amino acid motifs stabilized by zinc ions, each recognizing a 3-bp DNA sequence through specific α-helix contacts within the major groove of DNA [2]. By modularly linking multiple zine fingers in tandem (typically 3 to 6 per array), ZFNs can be designed to recognize extended DNA sequences of 9 to 18, allowing customizable targeting of genomic loci with considerable specificity [3].

The FokI endonuclease domain in ZFNs must dimerization to cleave DNA effectively. Hence, ZFNs function as obligate heterodimers; two individual ZFN monomers bind to adjacent DNA sequences separated by a 5–7 bp spacer, bringing the FokI domains into proximity to induce a targeted double-strand break (DSB) [4]. This DNA break activates the cell's natural repair mechanisms, primarily non-homologous end joining (NHEJ) or homology-directed repair (HDR). These pathways enable facilitation of site-specific gene knockouts or precise gene insertions,

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respectively [5]. ZFNs exhibit a modular architecture, and dimerization-dependent cleavage mechanism helps minimize off-target effects when properly designed, However, early versions of ZFNs faced challenges with significant cytotoxicity due to off-target site double-strand breaks (DSBs) [6].

ZFNs were the first genome-editing tool platform for highly precise modifications in human cells. Early pioneering work demonstrated the ability to correct disease-causing mutations, such as the IL2RG gene in X-linked severe combined immunodeficiency (X-SCID) in patient-derived cells [7]. ZFNs rose to prominence with the successful knockout of the CCR5 gene in human CD4+ T cells, conferring resistance to HIV-1 infection, a landmark achievement that entered clinical trials [8]. Further, studies validated therapeutic potential, including the correction of F9 mutations in haemophilia B models and the Glu6Val mutation in sickle cell disease models [7], [9]. Despite their precision and early success, ZFNs encountered technical difficulties during modular assembly because of their intricate, context-dependent binding. Individual zinc finger modules often influence adjacent fingers, unpredictably affecting binding affinity and specificity [10]. To overcome these limitations, techniques like the Context-Dependent Assembly (CoDA) and OPEN (Oligomerized Pool Engineering) platforms were developed [11], [12]. These tools democratized ZFNs design, enabling more accurate targeting and extending their use across specific. Subsequently, high-throughput zinc finger selection platforms, such as ZiFiT and modular assembly kits, provided standardized protocols to generate custom ZFNs for virtually any genomic target [13], [14].

The first demonstration of ZFNs in plant biotechnology was in Arabidopsis thaliana, where the ADH1 locus was targeted for gene mutagenesis [15]. ZFNs made it possible to precisely introduce the PAT gene for herbicide resistance in major crops such as maize (Zea mays), a milestone in precision agriculture [16], [17]. ZFN-mediated gene editing in soybean, canola, and rice has allowed the development of non-transgenic edited crops with commercial value [18]. ZFNs have revolutionized the development of genetically engineered animal models, particularly in species previously resistant to such modifications. In rats, which were traditionally recalcitrant to genetic manipulation, ZFNs enabled precise knockout of key genes such as Lepr (leptin receptor) and p53, generating valuable models for metabolic disorders and cancer research [7]. Beyond laboratory animals' biosafety, ZFNs have been successfully applied in livestock engineering. Notably, cattle with ZFN-edited calves that specifically disrupt the PRNP gene to prevent bovine spongiform encephalopathy (BSE), These applications highlight ZFNs' versatility in advancing both biomedical research and agricultural biotechnology [19].

To enhance the specificity concerns, ZFNs have been optimized by incorporating engineered FokI variants such as the Sharkey and ELD/KKR mutations, which enhance catalytic activity while reducing homodimerization, thereby limiting off-target cleavage [20]. These modifications parallel similar approach developments in TALEN technology and demonstrate convergent evolution toward high-fidelity editing systems [21]. Notably, the implementation of obligate heterodimeric FokI domains in ZFNs led to substantial reductions in off-target mutagenesis, with studies in human K562 cells demonstrating a greater than >75% reduction in unintended indels compared to earlier versions [22]. Additionally, ZFNs have also emerged as powerful tools for epigenome editing by fusing zinc finger proteins (ZFPs) to various regulatory domains, when coupled with effector domains like KRAB (for repression), VP64 (for activation), or p300 (for chromatin remodeling), ZFNs enable precise transcriptional control without modifying the DNA sequences [23]. This approach has yielded important mechanistic insights, for instance, fusion of ZFPs with DNA methyltransferases (DNMT3A) has allowed site-specific DNA methylation at the CDKN2A promoter in cancer cells, revealing how epigenetic silencing affects tumor suppressor genes[24]. To further highlight ZFNs' versatility beyond genome editing, ZFP-HDAC fusions were employed to investigate the impact of histone deacetylation on gene expression [25].

In terms of delivery, ZFNs have been effectively incorporated into cells using lentiviral vectors, adenoviral, mRNA microinjection, and electroporation, contingent on the application and target organism [26]. The predominant technique for ex vivo treatments is still electroporation, particularly for hematopoietic stem cells and T cells [27]. However, due to size constraints and toxicity associated with persistent expression, transient delivery of ZFN mRNA or protein is often employed to minimize adverse effects while maintaining high editing efficiencies. Similarly, direct protein delivery offers an even more precise temporal control of nuclease activity [28]. Their ability to access GC-rich, repetitive, or structurally complex regions of the genome makes ZFNs a superior context where gRNA mispairing or off-target effects plague CRISPR tools [29]. Emerging technologies such as base editing, prime editing, and deaminase fusions have inspired similar adaptations in ZFNs, with early-stage reports exploring ZFN-deaminase chimeras for precise base conversions [30], [31]. Although the modularity of ZFNs poses engineering challenges compared to gRNA-directed systems, their compatibility with synthetic biology and protein engineering keeps them relevant [32]. Next-generation sequencing (NGS) integration for real-time off-target detection and AI-based zinc finger design is set to enhance ZFN usability and safety, particularly in therapeutic gene correction [33], [34].

II. LIMITATIONS OF ZFN TECHNOLOGY

ZFNs are limited by their complex structure design, as each zinc finger must be customized to recognize specific DNA triplets, making specificity difficult to achieve and labor-consuming process. Off-target effects are a concern due to imperfect DNA binding, which can lead to unintended mutations [5]. Additionally, ZFNs have relatively low targeting flexibility, and their effectiveness varies depending on chromatin accessibility. Like other nucleases, ZFNs can induce cytotoxicity due to double-strand breaks, which restricts their potential for therapeutic applications [35].

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III. TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES (TALENS)

Transcription Activator-like effector nucleases constitute a robust and highly specific genome editing platform that predates the advent of CRISPR/Cas9, offering unparalleled modularity for targeted DNA modifications [36] TALENs are chimeric proteins engineered as endonucleases by fusing a customizable DNA-binding domain derived from Xanthomonas spp. bacterial effector proteins to the non-specific nuclease domain of FokI restriction enzyme [37]. The DNA binding domain comprises tandem repeats of 33-35 amino acids, each containing hypervariable residues at positions 12 and 13, termed Repeat Variable Di-residues (RVDs), which confer nucleotide binding specificity [38]. Structural and biochemical studies have elucidated that the RVDs NI, NG, HD, and NN exhibit preferential binding to adenine (A), thymine (T), cytosine (C), and guanine (G) or adenine (A), respectively, enabling programmable DNA recognition with single-base precision [39]. The FokI nuclease domain functions as an obligate dimer, necessitating the pairing of two TALEN monomers flanking a spacer region 12-20 base pairs (bp) to induce a site-specific double-strand break (DSB). This break activates endogenous repair pathways, facilitating gene knockouts via non-homologous end joining (NHEJ) or precision knock-ins via Homology-directed repair (HDR) [40], [41].

The modular architecture of TALENs allows for flexible target site selection, though their design complexity and labor-intensive cloning process initially limited widespread adoption [42]. The advent of Golden Gate assembly, high-throughput solid phase synthesis, and different TALENs assemblies has revolutionized TALEN construction, enabling large, rapid generation of designer nucleases for large-scale functional genomics projects [43], [44]). Empirical studies have validated TALENs' efficacy across phylogenetically diverse organisms, including zebrafish *(Danio rerio),* murine models *(Mus musculus),* and agriculturally relevant plant species, with demonstrably reduced off-target effects compared to early CRISPR/Cas9 systems [45], [46]. For instance, TALEN-mediated knockout of the golden gene in zebrafish achieved 90% mutagenesis efficiency, recapitulating hypopigmentation phenotypes with Mendelian inheritance patterns [47]. TALENs have been deployed to engineer herbicide resistance, glyphosate resistance in crops via targeted mutagenesis of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, underscoring their utility in precision agriculture [48]. Additionally, therapeutically, TALENs have been harnessed to disrupt the C-C chemokine receptor type 5 (CCR5) in human CD34+ hematopoietic stem cells, conferring resistance to HIV-1 infection in ex vivo gene therapy paradigms [49].

To improve TALEN efficiency and specificity, researchers have developed engineered FokI variants with enhanced cleavage activity and reduced toxicity [41]. Heterodimeric FokI domains, such as ELD/KKR and Sharkey mutations, minimize off-target cleavage by preventing homodimerization, thereby increasing target specificity [50]. Incorporating obligate heterodimer FokI variants has reduced unintended genomic rearrangements in human K562 leukaemia cell lines, where ELD/KKR TALENs reduced indel frequencies at off-target loci >90% [50]. Furthermore, modifications to the TALEN scaffold, including truncations of N and C-terminal domains (+63 truncation variant), have optimized DNA binding affinity and nuclease activity, as demonstrated in Drosophila melanogaster target gene editing studies where truncated TALENs achieved 95% germline transmission rates [51], [52]. TALENs have also been adapted for epigenome editing through fusion with transcriptional regulators or chromatin modifiers. Fusions with transcriptional activators (VP64, p65) or repressors (KRAB, SID) enable targeted gene regulation without altering DNA sequences [53]. In induced pluripotent stem cells (iPSCs), TALEN-KRAB fusion has been used to silence the employed oncogenic MYC amplification [54]. At the same time, TALENs-YP64 constructs have activated endogenous OCT4 expression to enhance somatic cell reprogramming efficiency by 3.5-fold [55]. Additionally, Fusion of TALENs to histone deacetylases (HDACs) or DNA methyltransferases (DNMT3A) has enabled locus-specific epigenetic modification, facilitating mechanistic studies of CpG island hypermethylation in glioblastoma multiforme (GBM), where promoter hypermethylation of tumor suppressor genes is a hallmark [56].

TALENs have been instrumental in generating genetically modified model organisms for functional genomics. In *Xanopus tropicalis*, microinjection of TALEN mRNA elicited 80-95% somatic and germline cell mutagenesis, enabling genome-wide reverse genetic screens [57]. Similarly, TALEN-mediated gene knockouts in Lepr gene in rats, traditionally resistant to genetic manipulation, have produced models for cardiovascular and metabolic disorders [58]. In livestock engineering, TALENs have introduced loss-of-function mutations in PPARy in pigs, yielding animals with enhanced lean muscle mass and insulin sensitivity, a breakthrough in agricultural biotechnology [59]. Despite their precision, TALENs face challenges in delivery and scalability, particularly in vivo, while adeno-associated viral (AAV) vectors have been explored for TALEN delivery, their 4.7 kb constraints limit preclude intact TALEN expression, necessitating a dual-vector method that compromises efficiency [60]. Electroporation and nucleofection are preferred for ex vivo applications, as demonstrated in CAR-T cell engineering, where TALENs disrupted PD-1 to enhance antitumor cytotoxicity [61]. In plants, Agrobacterium-mediated transformation T-DNA delivery remains the primary delivery method, through protoplast transfection has achieved >60% high efficiency editing in crops like wheat *(Triticum aestivum)* and rice *(Oryza Sativa. L) [62], [63].*

While CRISPR/Cas9 dominates current genome editing, TALENs retain niche superiority in high specificity or targeting repetitive regions. Clinical trials employing TALENs correct factor IX (F9) mutations in haemophilia B have demonstrated <0.1% off-target activity, a critical metric for therapeutic safety [64], [65]. Moreover, TALENs exhibit enhanced activity in repetitive genome regions, where

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CRISPR systems suffer from gRNA mispairing [66]. Developing TALEN deaminase fusions (TALEN-APOBEC1) has enabled C to T base editing without DSBs, as applied in correcting the APOE4 gene in Alzheimer's patient-derived neurons [67]. Although prime editing and base editing have overshadowed TALENs in versatility, their proven reliability ensures continued use in niche applications [68]. The integration of TALENs with emerging technologies, such as single-molecule real-time sequencing for validation, may revive their prominence in precision medicine [69]. As genome editing evolves, TALENs remain a vital tool for researchers prioritizing fidelity over convenience, underscoring their enduring legacy in genetic engineering [37].

IV. LIMITATIONS OF TALEN TECHNOLOGY

Although TALENs exhibit high specificity and minimal off-target effects, their major drawbacks include the labor-intensive and time-consuming assembly of DNA-binding domains. This requires designing and constructing a unique TALE array, which complicates the large-scale or high-throughput applications [70]. TALENs are also relatively large, making delivery into cells, particularly using viral vectors like AAV, challenging. Additionally, their efficiency can be influenced by chromatin structure, and they often induce cytotoxicity due to double-stranded DNA breaks [71].

V. CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS/CRISPR-ASSOCIATED PROTEIN9 (CRISPR/CAS9)

CRISPR/Cas9 genome editing has transformed genetics and functional genomics by enabling precise modifications of DNA sequences. The system functions by creating a targeted DNA double-strand break, which activates the cell's repair mechanisms, leading to mutations through error-prone Non-Homologous End Joining (NHEJ) or precision knock-in via Homology Directed Repair (HDR) [72]. The versatility of CRISPR/Cas9 has been further enhanced by developing engineered variants that improve specificity and broaden targetable sequences [73]. Variants with single-strand nicking activity reduce off-target effects, while modifications to the protospacer adjacent motif (PAM) allow Cas9 to recognize a wider range of genomic sequences [74]. Additionally, catalytically inactivated Cas9 (dCas9) serves as a scaffold for fusion proteins with diverse gene editing functions. Epigenome editing via dCas9 fusion proteins enables targeted histone modification, while deaminase fusions facilitate base editing by converting cytosine to thymine or adenine to guanine without inducing double-strand breaks [75]. Prime editing, another powerful extension of the CRISPR/Cas9 toolbox, utilizes reverse transcriptase to introduce genome modifications using an RNA template, allowing for precise genetic changes without relying on HDR [76].

One of the major challenges in CRISPR/Cas9-based genome editing is optimizing the efficiency of precision knock-in strategies, particularly in mammalian cells where HDR is less dominant than NHEJ [77]. Experimental parameters such as Cas9 variant selection, single versus double-stranded DNA templates, template structure, and homology arm length significantly impact knock-in success. Strategies to improve HDR efficiency include the inhibition of NHEJ repair enzymes such as DNA Polymerase θ and DNA-PK, as well as in vivo template liberation to enhance template availability [78]. The effectiveness of CRISPR/Cas9 editing is also influenced by the delivery method and expression system used for Cas9 and guide RNA (gRNA) components [73]. The replacement of gene promoters in CRISPR/Cas9 constructs with species-specific regulatory elements has been shown to improve gene expression and editing efficiency [79]. For example, the use of endogenous RPS5a promoters in Arabidopsis thaliana increased base editing efficiency by over 30% compared to the commonly used CaMV35S viral promoter [80].

Technical advancements have facilitated genome editing in previously challenging biological systems. The development of automated microinjection platforms has enabled efficient and reproducible genome modifications in mouse embryos, leading to improved survival rates of genetically modified mice [81]. In human induced pluripotent stem cells (iPSCs), where transfection efficiency is often low, the piggyBac transposon system provides a stable means of expressing prime editors, thereby increasing editing success [82]. Fluorescent reporter systems have also been utilized to monitor CRISPR/Cas9 knockout efficiency in leukemia cells, providing a visual indicator of successful gene edits [83]. The Cas9 targetable genome has been further expanded through the creation of near-PAM-less Cas9 variants, which recognize a broader range of PAM sequences, facilitating editing in a wider array of model organisms [84]. In Dictyostelium discoideum, a model for studying multicellularity, these variants have been used to generate precise knock-ins, allowing researchers to investigate the genetic basis of cellular communication and coordination [85].

Alternative approaches to double-strand break-induced HDR have also been explored. Tandem paired nicking, which employs Cas9 D10A nickases in combination with long homology templates and optimized gRNA lengths, has been shown to drive efficient knock-ins in mammalian cell lines [86]. This strategy reduces genomic instability while maintaining high editing accuracy. CRISPR/Cas9-mediated knock-in and knock-out strategies have been widely adopted for creating novel genetic models in various organisms [87]. The GeneWeld short homology arm knock-in approach has been successfully applied in zebrafish, where it enables precise Cre recombinase integration under the control of endogenous regulatory elements, enhancing lineage tracing and conditional gene studies [88]. Similarly, the PITCh (Precise Integration into Target Chromosome) method has been used to insert fluorescent calcium sensors into insect cells, advancing the study of calcium signaling and anhydrobiosis in midge larvae [89]. HDR-based knock-ins have also been employed to introduce stop codons into oncogenes, revealing key regulatory functions in glioblastoma proliferation and immune evasion [90].

The efficiency of CRISPR knockout strategies has allowed for the development of new models to study gene

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function. Dual-gRNA-mediated exon deletion has been applied to investigate exon skipping and frameshift mutations in mice, while CRISPR knockout of HOL methyltransferases in rice has provided insights into methyl iodide emissions and plant metabolism [91], [92]. Patient-derived Duchenne muscular dystrophy myoblast models have been established using CRISPR/Cas9 to facilitate drug screening efforts [93]. Promoter deletion using CRISPR/Cas9 has emerged as another effective gene knockdown strategy, as demonstrated in H19 lncRNA knockout studies that revealed its role in cell proliferation and genome stability [94]. Beyond direct sequence modifications, CRISPR-based epigenome editing enables gene regulation without altering DNA sequences [95]. Targeting gene promoters with epigenetic modifiers such as histone acetyltransferases and methyltransferases has provided insights into transcriptional regulation and chromatin dynamics, allowing researchers to dissect the complex interplay of histone modifications in gene activation [96].

The continuous refinement of CRISPR/Cas9 technologies highlights the importance of species-specific experimental optimization in genome editing [97]. Advances in gene delivery methods, promoter selection, and repair pathway modulation have significantly improved editing efficiency across diverse biological systems [98]. These developments underscore the immense potential of CRISPR/Cas9 to revolutionize genetic research by enabling precise gene manipulation in previously intractable model organisms [99]. The widespread application of CRISPR/Cas9 is poised to drive new discoveries in functional genomics, disease modeling, and therapeutic gene editing **Fig. 1 & Table.1** [100].

VI. LIMITATIONS OF CRISPR TECHNOLOGY

Although CRISPR/Cas9 is a powerful tool, safety and precision issues severely limit its therapeutic applications. More than 50% of cases may have off-target effects, and existing approaches, such as enhanced gRNA designs and modified Cas9 variants, have limited success [101]. A system also requires a neighboring PAM sequence (5′-NGG-3′), which restricts target flexibility. Additionally, the large size of spCas9 complicates its delivery via AAV vectors, and CRISPR-induced DNA breaks can trigger apoptosis and cellular toxicity [102].

VII. CONCLUSION

Over the past decade, genome editing technologies, including ZFNs, TALENs, and CRISPR/Cas9, have significantly shaped the field. ZFNs introduced precise genetic modification but were limited by their complexity. TALENs improved specificity and modularity, while CRISPR/Cas9 revolutionized the field with its RNA-guided mechanism, ease of use, and broad applicability. CRISPR/Cas9 is expected to remain dominant, particularly with innovations like base and prime editing. However, TALENs and ZFNs are still valuable in applications requiring high precision and minimal off-target effects. The future of genome editing will rely on a tailored, application-specific use of these tools to meet the needs of medicine, agriculture, and biotechnology.

VIII. CONFLICT OF INTEREST STATEMENT

All authors declared no conflict of interest

Table 1 Comparative Analysis of Genome Editing CRISPR/Cas9, TALENs, and ZFNs

S. No	Attribute	CRISPR/Cas9	TALEN	ZFN
1.	Specificity	High with gRNA design	High, customizable repeats for specificity	High, but challenging to design
2.	Off-target effects	Moderate to high, dependent on gRNA quality	Low, less prone to off-target cleavage	Moderate, dependent on zinc-finger modules
3.	Scalability	High, simple, and scalable via gRNA design	Moderate, scalable with TALE repeat units	Low, complex protein engineering
4.	Ease of Design	Easy, only gRNA required	Moderate, repetitive unit assembly	Difficult, requires protein engineering
5.	Cost	Low	Moderate	High
6.	Delivery Mechanism	Plasmid, viral vectors, ribonucleoprotein	Viral and non-viral vectors	Viral vectors
7.	Multiplex genome editing	High-yield multiplexing is available	few models	few models
8.	RNA editing	Yes	No	No
9.	DNA catalytic domain	RuvC and HNH Cas9	FokI	FokI
10.	Spacer Length	NO spacer required	Spacer 14-16 bp	Spacer 5-8 bp
11.	Target Sequence Size	Recognizes NGG- PAM sequences +17-25bp	Recognizes 30-40bp	Recognizes 18-24
12.	Target Recognition type	DNA/RNA	DNA/Protein	DNA/Protein
13.	Target Cell Organelles	Not well-established	MLS-tagged TALENs for mtDNA editing	MLS-tagged ZFNs for mtDNA editing

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14.	Size of Protein Complex	Small (Cas9: ~160 kDa)	Large	Moderate

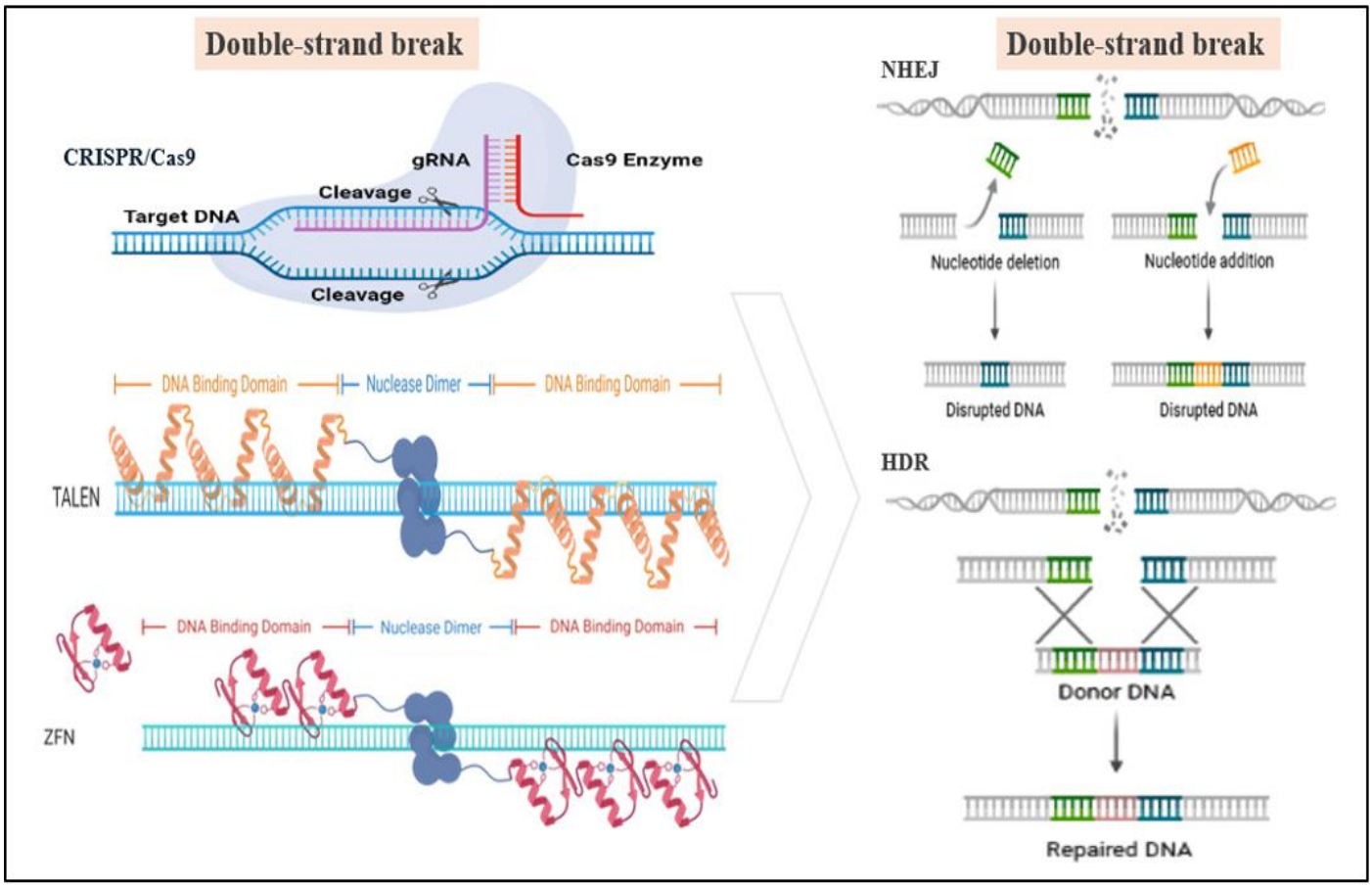

Fig 1 Schematic overview of genome editing platforms and mechanisms for DSB repair with endogenous.

Genome editing nucleases like CRISPR/Cas9, TALENs, and ZFNs induce DSBs at targeted sites. Without a repair template, the cell will repair the break via error-prone non-homologous end joining (NHEJ), leading to functional gene disruption (protein/gene knockout). Alternatively, in the presence of a repair template, repair occurs by homologous-directed repair (HDR). HDR gene correction or gene insertion involves a DSB at the desired locus.

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