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Genome Engineering Tools for Building Cellular Models of Disease

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Abstract

With the recent development of methods for genome editing of human pluripotent stem cells, study of the genetic basis of human diseases has been rapidly advancing. Genome-edited differentiated stem cells have provided new and more accurate insights into genomic underpinnings of diseases for which there have not been adequate treatments, and moving toward clinical application of genome editing holds great promise for acceleration of therapeutic translation. Here we review recent advances in genome-editing technologies and their application to human biology in disease modeling and beyond.

Graphical abstract

Genome-editing methods involve the formation of a double-stranded DNA break followed by a repair strategy resulting in either a knockout or site-specific mutagenesis. This approach canfacilitate the study of gene function in induced pluripotent stem cells (iPSCs) from healthy individuals or individuals with disease mutations. iPSCs can then be differentiated into the celltype of interest for further study.

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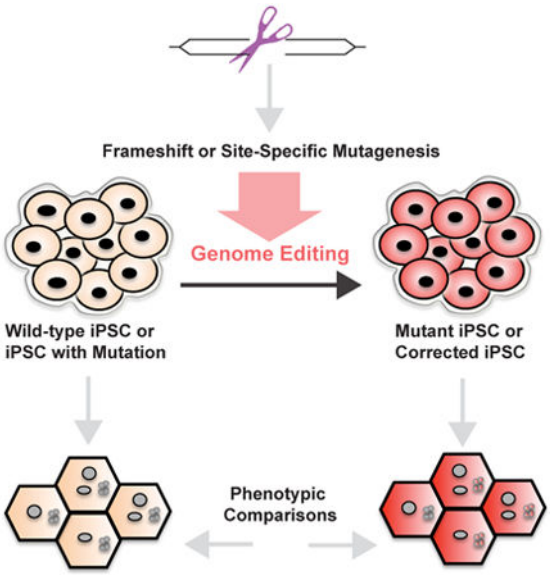

Keywords

ZFNs; TALENs; CRISPR/Cas9; genome editing; iPSC

Introduction

With increasing availability of human genome-sequencing data from individuals with diseases or disease-related traits, the role of genetics in human disease has become a major focus in translational biomedical research.[1] From genome-wide association studies,[2-4] to functional laboratory follow-up,[1,5-7] and ultimately to development of targeted therapeutics,[2-4,8] the genetic basis of disease has been a hotbed of research geared towards precision medicine—clinical strategies that account for individual variability, e.g., genomics.[9,10] Mechanistic study of potential causal genes for diseases or traits requires genetic manipulation in cellular models, which have advanced in their utility for variant-specific and cell-specific studies with the advent of genome-editing technologies.

Prior to genome editing, viral transgene expression and RNA interference (RNAi) were commonly used for functional studies of specific genes. However, viral vectors and RNAi are falling out of favor due to (1) dysregulated transgene expression from insertion-site mutagenesis and (2) incomplete time-limited ablation of gene function with poor specificity, respectively.[11-13] One approach to circumvent these issues in studying disease mechanisms has been the use of human pluripotent stem cells derived from patients with the disease of interest. With recent advances in technology, induced pluripotent stem cells (iPSCs) can be generated from a skin biopsy or blood sample from any patient, facilitating the derivation, expansion, and differentiation of somatic cells genetically matched to the patient.[14-17] This can offer advantages to studying disease-associated genetic variants in cells that do not contain the severely abnormal karyotypes of immortalized cell lines, cells that are difficult to obtain for primary culture (e.g., neurons), and cells that are difficult to

transfect or transduce (e.g., T-cells and macrophages).[15,16,18,19] However, iPSC-based studies also have their own limitations. For example, differences in genetic background between disease cases and healthy controls can confound studies, even those using healthy siblings as controls. Because siblings share only 50% of the genome on average, phenotypic differences observed in studies using disease versus healthy siblings could arise from variants in the unshared portion of the genome rather than the disease-associated mutations being interrogated. In addition, iPSCs from different individuals can vary in terms of genomic methylation patterns where some may retain epigenetic "memory" from the somatic cell of origin from which they were reprogrammed, thus allowing for an iPSC line to differentiate preferentially into some cell types over others.[20-23]

All these limitations of vector-based transgene expression, RNAi, and iPSC studies could potentially be overcome through the use of isogenic (derived from the same parental cell line) genome-edited iPSCs in which wild-type and mutant cell lines differ only in terms of disease mutations. Genome editing allows investigators to introduce a variety of genetic alterations with a high degree of target specificity within a controlled genetic background; these alterations range from single nucleotide modifications to whole-gene addition or deletion. Here we review the different nuclease-based genome-editing technologies based on programmable nucleases, discuss considerations for their use in cellular models, and present their potential and challenges for clinical translation.

Genome Editing Technologies

Zinc Finger Nucleases (ZFNs)

ZFNs comprise a nuclease class used for genome editing through binding of a target DNA sequence by a chimeric enzyme consisting of target site-specific binding domains—adapted from zinc finger transcription factors—fused to the sequence-agnostic nuclease domain of bacterial restriction enzyme *Fok*I. ZFNs are typically designed in pairs to recognize DNA sequences flanking the genomic target site of interest. Each zinc finger domain recognizes a 3-4 basepair (bp) DNA sequence, and multiple domains can be engineered to exist in tandem for each ZFN monomer to bind an extended unique 9-18 bp sequence adjacent to the target site. Upon binding the sequences flanking the excision target, the *Fok*I nuclease domains of the ZFN pair dimerize and generate a double-stranded break (DSB).[24,25] DSBs are then repaired by the cell through one of two methods: non-homologous end joining (NHEJ) or homology-directed repair (HDR) (see Figure). NHEJ repairs the lesion by directly rejoining the DSB ends in an error-prone process that does not require the presence of a repair template. This strategy introduces insertion or deletion mutations (indels) that bridge the break site, and when introduced into a coding portion of the target gene these indels can cause frameshifts that lead to nonsense mediated mRNA decay and/or production of non-functional truncated proteins, thus effectively knocking out the target gene.[24] Unlike NHEJ, HDR involves the use of an exogenous DNA repair template to incorporate desired changes in the DSB repair, i.e., introduction of a specific mutation of interest. The repair template can be a double-stranded DNA vector or a single-stranded DNA oligonucleotide; the single-stranded oligonucleotide can consist of the desired mutation flanked by homology

arms of as little as 20 nucleotides in length, offering reasonable repair efficiency that does not require antibiotic selection for screening of correctly targeted clones.[26]

Although ZFN technology offers advantages over RNAi, its use is still accompanied by challenges worth consideration by investigators new to genome editing. For example, the first steps of engineering ZFNs to target a specific site may prove to be difficult as assembly of desired zinc finger domains to bind an extended nucleotide sequence requires substantial protein engineering expertise.[27] These first steps are still technically challenging and potentially expensive despite publicly available protocols for ZFN screen optimization and alternative ZFN engineering platforms.[28-31] In addition, target-site selection is limited: ZFNs do not facilitate targeting of sequences that are guanine-poor, and ZFN components can only be used for binding sites located every few hundred bp throughout the genome.[11]

Transcription activator-like effector nucleases (TALENs)

Like ZFNs, TALENs are chimeric enzymes with a DNA-binding domain fused to a *Fok*I nuclease domain that generates a DSB within a target region and facilitates repair either through NHEJ or HDR. However, the TALEN's DNA-binding strategy differs in the use of modified TALEs, which consist of DNA binding modules called TAL repeats originally discovered in plant-pathogenic bacteria.[32] Each TAL repeat contains 33-35 amino acids that include a repeat-variable di-residue (RVD), a unit consisting of two adjacent amino acids that specifically recognize and bind to one of four DNA bases.[32,33] With the affinity of specific RVD amino acid combinations for each type of nucleotide, a DNA sequence can be targeted by engineering a tandem array of TAL repeats that contain RVDs in the order of their corresponding target nucleotides, in a 1-RVD to 1-bp ratio (see Figure). TALENs are easier to design than ZFNs through use of the RVD “code” to create *de novo* extended TAL repeat arrays that bind with high affinity to target genomic sequences, allowing for the construction of hundreds of TALENs at a time[34,35] with robust gene-targeting in human embryonic stem cells and iPSCs. TALENs also do not have the same target-site limitations that ZFNs do, with more potential binding sites as the main requirement is that the 5′ targeted base should be a thymine.[11]

Despite this improvement over ZFNs, widespread use of TALENs has been limited by the suboptimal performance of a significant portion of designed TALEN pairs, thus requiring the screening of a large number of candidate pairs in order to find one with a high level of activity.[35] A potential contributor to variable performance of engineered TALEN pairs is the methylation state of the target region, as the standard TAL repeats cannot bind well to methylated cytosines, often found in CpG islands.[36,37]

CRISPR/Cas9

More recently, the genome editing field has undergone a “revolution” with the rise of clustered regularly interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9) systems, which have been adapted from bacterial immune systems that use CRISPR RNA in conjunction with Cas proteins to direct the cleavage and degradation of invading viral and plasmid DNAs. In response to these invading genomes, bacteria incorporate fragments of the foreign DNA into the CRISPR locus as a nucleic acid “vaccination record”; these DNA

sequences are “protospacers” transcribed into “guide RNAs” that can be bound by Cas proteins and that can then recognize other foreign DNAs with the protospacer sequence via complementary binding. Protospacer sequences in DNA must be flanked by protospacer-adjacent motifs (PAMs) that are recognized by Cas proteins directed to target sites by guide RNAs binding to the DNA protospacers; only then will Cas proteins cleave the targeted foreign DNAs.[38,39] For genome editing, this bacterial adaptive immune strategy was re-engineered to use heterologous expression of the *Streptococcus pyogenes* Cas9 (SpCas9) protein alongside short, investigator-designed guide RNA complexes in mammalian cells. Leveraging the combination of guide RNA-mediated target recognition and Cas9 endonuclease activity, investigators can create a DSB within a genomic target site that has a 20-bp DNA sequence matching the protospacer of the guide RNA and a flanking PAM, which for SpCas9 is a NGG nucleotide sequence (see Figure).[39-41] With this design, sequences can be targeted by changing the 20-bp guide RNA sequence without re-engineering the Cas9 portion, thus allowing for greater ease in engineering CRISPRs. In addition, multiple guide RNAs can be introduced in series within a single vector to allow for multiplexed targeting of multiple sites within the same cell.[39]

Despite its ease of engineering, CRISPR/Cas9 still has its own limitations. For example, SpCas9 genome editing requires the presence of an NGG PAM, which occurs roughly once every 8 bp, and this requirement may lead to less target sequence density than TALENs, which may on average have a dimeric target site every 3 bp.[35,42,43] The PAM requirement may limit the use of CRISPR/Cas9 when target specificity is required for introducing a DSB at a precise location for HDR-mediated repair to introduce a specific mutation. To address this, non-canonical PAM sequences and Cas9-like proteins derived from alternative bacteria have recently been explored to increase the potential for target specificity,[44-46] although their relative efficacy and ease of use in iPSCs compared to established methods remain to be determined. For example, a novel method based on the general concept of CRISPR uses *Natronobacterium gregoryi* Argonaute, a eukaryotic DNA-guided endonuclease that does not require the presence of a PAM and thus may offer more flexibility in choosing target sequences; more studies are needed to assess precision and efficiency of this recently developed method.[47] In addition, CRISPR/Cas9 efficiency has much room for improvement, as investigators currently need to derive clonal cell lines to study genome-edited cells, a process that can take several weeks to complete. Other issues, including off-target effects, are discussed in more detail below.

Other Genome Editing Tools

In addition to ZFNs, TALENs, and CRISPR/Cas9, strategies for genome editing include use of meganucleases,[48] adeno-associated viruses,[49] and adenoviruses.[50,51] These tools have not been as widely used as ZFNs, TALENs, and CRISPR/Cas9 due to lower adaptability. However, given how rapidly all of these genome-editing strategies have been developed, additional modifications to current approaches and the creation of additional novel genome-editing technologies, such as the DNA-guided endonuclease approach described above,[47] are likely to be on the horizon.

Application in Cellular Models

With the feasibility of using genome-editing tools to study gene function, increasing numbers of studies are demonstrating the power of genome editing in disease modeling and potential future therapeutic translation. Here we present examples of recent seminal studies that leverage genome editing for functional studies.

In a study of *NOTCH1* nonsense mutations leading to calcific aortic valve disease (CAVD), Theodoris *et al.* demonstrated that genome-edited iPSCs differentiated into endothelial cells (ECs) can be used to probe the transcriptomic and epigenomic effects of disease-mutation heterozygosity.[52] The investigators used TALENs to correct the disease mutation in iPSCs derived from *NOTCH1*$^{+/-}$ individuals diagnosed with CAVD to create isogenic control lines. *NOTCH1*$^{+/-}$ iPSCs, isogenic corrected control iPSCs, and unrelated wild-type control iPSCs were then differentiated into ECs and subjected to shear stress to interrogate whether the disease mutations affected transcriptional responses to this stimulation. They found that shear stress induced aberrant upregulation of pro-osteogenic and inflammatory signaling pathways in *NOTCH1*$^{+/-}$ iPSC-derived ECs compared to isogenic and unrelated controls. In both static and shear-stress states, *NOTCH1*$^{+/-}$ iPSC-derived ECs had pro-inflammatory STAT and IRF motifs enriched for H3K27 acetylation, an epigenetic mark of active transcription. Additionally, bisulfite sequencing revealed that *NOTCH1*$^{+/-}$ iPSC-derived ECs had many differentially methylated regions compared to controls. The investigators showed that genome-edited iPSC-derived cells can reveal important information about epigenetic and transcriptional events in Mendelian diseases.

Genome editing can be used not only to study disease-related intracellular pathways and regulation but also to rescue disease phenotypes in cellular models. This process was first demonstrated in the study of Huntington's disease[53] and Parkinson's disease,[18,54] and more recently the approach was extended to the study of cystic fibrosis (CF). In the CF study, the investigators generated iPSCs from a CF patient homozygous for the disease-associated ΔF508 *CFTR* mutation, which prevents the proper trafficking of the chloride channel to the cell membrane, leading to aberrant secretions in the lungs that ultimately result in premature respiratory failure and death.[55] They corrected the mutation using CRISPR/Cas9 to create a clonal isogenic control. When stimulated with a cocktail that induces chloride channel currents in wild-type lung epithelial cells, differentiated iPSCs with the ΔF508 *CFTR* mutation did not exhibit any chloride current response, whereas their CRISPR/Cas9-corrected isogenic controls did respond partially in terms of chloride current and appropriate glycosylation of the CFTR protein. This phenotypic rescue through genome editing holds promise that gene therapy for the ΔF508 *CFTR* mutation may be a viable approach to improving the health of CF patients.

Current Challenges in Genome-Edited Cellular Models

The potential for achieving a high level of specificity in genetic manipulation is one of the main draws of using genome-editing technologies. Successful design of genome-editing tools can facilitate the precise study of a genetic variant on an isogenic background to limit potential confounders, but genome editing does not guarantee absolute precision given that it

involves DSBs, which can induce unanticipated perturbations in the genome if cleavage occurs at off-target sites. Introduction of off-target mutations could have major impact on functional studies in terms of evaluating cellular phenotypes and mechanisms; as genome editing moves toward therapeutic translation, these off-target mutations could be problematic in introducing oncogenic potential and reduced cellular fitness.

Only a limited number of studies have attempted to evaluate and report the targeting specificities of ZFNs, TALENs, and CRISPR/Cas9 systems. Studies addressing specificity of ZFNs and TALENs have demonstrated the challenges of detecting off-target activity for these nuclease-based tools.[56-58] Recently, studies reporting the use of whole-genome sequencing of CRISPR/Cas9-edited stem cell lines revealed a low incidence of off-target mutation, with higher cleavage efficiency for on-target sites.[59-61] Although off-target effects were found to be low, continued improvements are being made to CRISPR/Cas9 technology to improve nuclease specificity. For example, increasing numbers of *in silico* tools are becoming available to improve target specificity in nuclease engineering.[62] Also, alteration of the Cas9 protein into a single-strand DNA nickase to facilitate the use of two separate guide RNAs to create single-strand breaks on opposite DNA strands have led to reduction of indel formation at computationally predicted off-target sites.[63,64] Furthermore, use of truncated guide RNAs, the development of pairs of RNA-guided *Fok*I nucleases fused to catalytically inactive Cas9, and novel variant Cas9 proteins are new modifications to the CRISPR/Cas9 system that also have been demonstrated to improve target specificity.[65-68]

Even if off-target effects occur with low frequency when using genome-editing tools, sound study design and use of multiple pairs of wild-type and mutated clones will still be needed to address the possibility of off-target effects that may confound an experiment if only one mutated clone with off-target indels were compared to one wild-type clone. In addition, for eventual therapeutic translation, the rapid development of improvements to genome-editing strategies mentioned above will also enhance specificity that will be needed for safe application of these tools for gene therapy.

Emerging and Future Directions

With the genetic basis of human diseases as a major emphasis in the study of disease pathogenesis as well as in the development of precision medicine strategies, use of genome editing to generate human cell-based disease models has become an increasingly popular approach used in the laboratory setting. Genome-edited cells provide a powerful approach to interrogate cellular mechanism and phenotypes for both Mendelian and complex human diseases in an appropriate genetic background that is not well recapitulated in traditional mouse models. However, cellular models are limited to the study of phenotypes that can be evaluated in the dish, and they do not offer the means to assess complex physiological conditions. To address this issue, investigators have been developing chimeric animal models that house human cells within the *in vivo* setting of an animal. Although still very inefficient, this has been most successfully accomplished in humanized mouse models of liver disease,[69] in which human iPSC-derived hepatocytes colonize mouse livers via intrasplenic injection and demonstrate successful functional integration into mouse liver parenchyma.

Using this approach, investigators can potentially study human genome-edited iPSC-derived differentiated cells in model organisms. Indeed, methods for introducing human pluripotent stem cells into other organ systems are also being developed. For example, one group evaluated the embryonic integration of human iPSC-derived neural crest cells in murine embryonic neural crest development,[70] and another group engrafted human pluripotent stem cell-derived enteric nervous system precursor cells into the post-natal murine colon to treat mice genetically engineered to have megacolon of Hirschsprung's disease.[71] With these emerging methods, in the not-too-distant future improved disease modeling will be able to integrate the disease-appropriate genetic background of iPSCs with the *in vivo* physiology of animal models. This next step may be able to address the interspecies gap that had previously led to failures of bench-to-bedside translation.

Although chimeric animal models have not become fully established in the study of human diseases, already pre-clinical studies demonstrate that *in vivo* genome-editing has the potential to be used to correct Mendelian diseases. For example, three groups recently showed that in a mouse model of Duchenne muscular dystrophy, CRISPR/Cas9 delivered through adeno-associated virus (AAV) knocked out exon 23 of the dystrophin gene, restoring the functional form of the dystrophin protein and also improving muscle structure and function.[72-74] Two of these groups used the *Staphylococcus aureus* ortholog of Cas9 (SaCas9), as SaCas9 is smaller in size than SpCas9 and thus can be more readily packaged into AAV vectors for use in *in vivo* genome editing.[75] With careful application of AAV for gene therapy already demonstrating an acceptable toxicity profile,[76] clinical translation of genome editing to treat disease mutations in humans is on the horizon.

Conclusion

The rapid advancement of genome-editing technologies in the past decade has opened exciting new avenues in the study of the genetic basis of human disease and in the development of targeted therapeutic strategies that would not have been possible with traditional pharmacological agents. With careful study design, continued improvement in the engineering of genome-editing tools, and appropriate regulatory practices, genome editing will undoubtedly accelerate discoveries in basic science and clinical translation.

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Abbreviations

CF	cystic fibrosis
CAVD	calcific aortic valve disease
CRISPR	clustered regularly interspaced short palindromic repeat
DSB	double-stranded break
GWAS	genome-wide association studies
HDR	homology-directed repair
iPSC	induced pluripotent stem cell
NHEJ	non-homologous end joining
RNAi	RNA interference
RVD	repeat-variable di-residue
SaCas9	*Staphylococcus aureus* Cas9
SpCas9	*Streptococcus pyogenes* Cas9
TALEN	transcription activator-like effector nucleases
ZFN	zinc finger nuclease

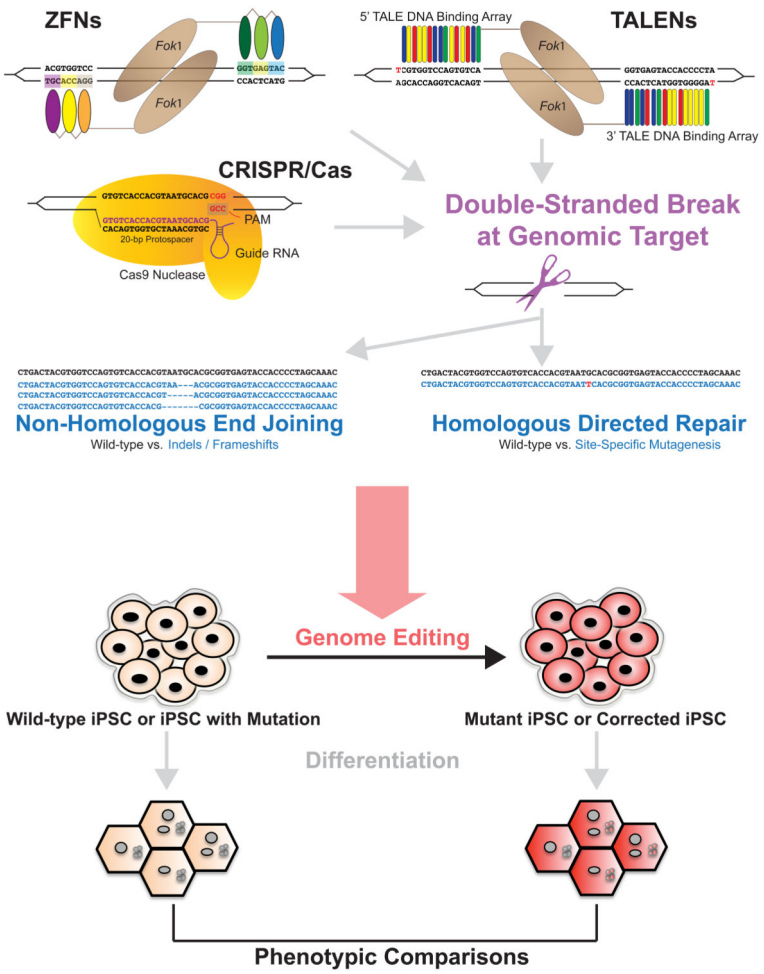

Figure. Overview of genome-editing tools and application to human iPSCs

ZFNs are a chimeric enzyme that includes a FokI nuclease (brown oval) attached to DNA-binding zinc finger domains (colored ovals) that each interact with 3-4 bp DNA sequences (each group color-coordinated with corresponding zinc finger domain), allowing for recognition of a 9-18 bp genomic sequence when engineered in tandem. The DNA binding domains flank the target genomic site, where the FokI nucleases make a DSB. TALENs are also a chimeric enzyme but with a FokI nuclease attached to a TALE DNA-binding array, which consists of RVDs (colored rectangles) that each bind to genomic DNA in a 1 RVD to 1 bp ratio. The genomic target site is cleaved by the FokI nucleases, which are flanked by the TALE DNA binding array sequences. Note that the 5′ ends of the TALE arrays begin with a thymine. The CRISPR/Cas9 system consists of a guide RNA (purple) binding to the genomic target (20 bp protospacer) and interacting with the Cas9 nuclease (yellow) that recognizes the PAM site. After a DSB is created by one of these techniques, the DNA can undergo (1) NHEJ in which the blunt ends are rejoined with introduction of additional nucleotides that induce an indel/frameshift mutation (dashes) for gene knockout or (2) HDR in which a repair template is introduced to facilitate the incorporation of a specific point mutation (red letter). This overall genome-editing strategy can then be employed to study gene function in iPSCs. iPSCs derived from humans (beige) can either be wild-type from a

healthy individual or with mutation from an individual with the disease of interest. Genome-edited iPSCs (red) can be generated through introduction of a mutation to create a mutant iPSC or corrected iPSC, providing the genetic study complement on an isogenic background. These iPSCs can then be differentiated into the cell type of interest for further study of cell-autonomous phenotypes. iPSCs = induced pluripotent stem cells, ZFNs = zinc finger nucleases, DSB = double-stranded break, TALENs = transcription activator-like effector nucleases, RVD = repeat-variable di-residue, NHEJ = non-homologous end joining, HDR = homology-directed repair.