

CRISPR and gene editing technologies for bleeding disorders

Thierry VandenDriessche^{ID}, Mathias Janssens^{ID} and Marinee K. Chuah^{ID}

Ther Adv Hematol

2025, Vol. 16: 1–22

DOI: 10.1177/
20406207251387402

© The Author(s), 2025.
Article reuse guidelines:
[sagepub.com/journals-](https://sagepub.com/journals-permissions)
permissions

Abstract: The first gene therapy products for hemophilia A and B have recently been approved by the regulatory authorities. Although this is an important milestone for people with hemophilia, there is still a need to further improve on the efficacy, safety, and stability of expression and to ultimately include pediatric patients before the onset of arthropathy and other complications caused by uncontrolled bleeding. To overcome some of the limitations of conventional gene therapy strategies, gene editing is currently being explored in preclinical studies. Gene editing allows for targeted modifications of the human genome with unprecedented specificity based on zinc finger nuclease, meganuclease, transcription activator-like endonuclease, or clustered regularly interspaced short palindromic repeats (CRISPR) technologies that induce double-strand DNA breaks (DSB). Next-generation gene editing strategies, such as those dependent on CRISPR-derived base or prime editors, allow targeted genetic modification independent of the induction of DSBs, offering a potential safer alternative. Sustained efficacy and production of factor VIII or factor IX can be achieved after gene editing in patient-derived cells or in adult or newborn hemophilia A or B mouse models. These preclinical studies pave the way toward phase I/II clinical trials in patients with severe hemophilia. The potential risk of undesired off-target modifications of the human genome and adverse immune reactions, and the need for efficient delivery of the gene editing components, need to be rigorously addressed before the promise of gene editing for hemophilia can ultimately be fulfilled.

Plain language summary

CRISPR and gene editing technologies for bleeding disorders

Gene therapy has recently made big steps forward, with the first approved treatments now available for people with hemophilia A and B. These treatments are promising, but there is still room to improve how well they work, how long they last, and how safe they are. Eventually, we want to be able to treat children early, before they start having joint damage and other bleeding-related problems. To make gene therapy even better, gene editing is being developed, a more precise way to fix faulty genes. This includes advanced tools like CRISPR, TALENs, ZFNs, and meganucleases, which can make small cuts in the DNA to help correct genetic mistakes. Newer methods, such as base editing and prime editing, can fix genes without cutting the DNA, which might be even safer. So far, gene editing has worked well in lab-grown cells from patients and in mouse models of hemophilia, showing it can restore the production of clotting factors (Factor VIII or IX). These early successes are helping to prepare for future human trials. However, there are still important challenges. We need to make sure the edits do not accidentally change the wrong parts of the DNA (called off-target effects), avoid immune system reactions, and find the best ways to deliver the gene editing tools to the right cells. Solving these problems is key before gene editing can become a routine treatment for hemophilia.

Correspondence to:
Marinee K. Chuah
Department of Gene
Therapy and Regenerative
Medicine, Faculty of
Medicine and Pharmacy,
Vrije Universiteit Brussel,
Building D, Room
JD.3.56, Laarbeeklaan
103, Brussels B-1090,
Belgium Center for
Molecular and Vascular
Biology, Department of
Cardiovascular Sciences,
University of Leuven,
Leuven, Belgium
marinee.chuah@vub.be
Thierry VandenDriessche
Department of Gene
Therapy and Regenerative
Medicine, Faculty of
Medicine and Pharmacy,
Vrije Universiteit Brussel,
Building D, room JD.3.56,
Laarbeeklaan 103,
Brussels B-1090, Belgium
thierry.vandendriessche@vub.be
Mathias Janssens
Department of Gene
Therapy and Regenerative
Medicine, Vrije Universiteit
Brussel, Brussels,
Belgium

Keywords: adeno-associated, base editing, CRISPR, factor VIII, factor IX, hemophilia, nanoparticles, TALEN, ZFN

Received: 24 April 2025; revised manuscript accepted: 16 September 2025.

Introduction

Hemophilia is a rare, inherited bleeding disorder where the blood does not clot properly due to a deficiency in clotting factors, such as Factor VIII (FVIII; for Hemophilia A) or Factor IX (FIX; for Hemophilia B). People with hemophilia are at risk of prolonged bleeding, internal bleeding, and joint damage, requiring frequent and lifelong treatment through factor replacement therapies. However, these treatments often require regular infusions and can be costly, with a significant impact on the quality of life for those affected. In recent years, gene therapy has emerged as a groundbreaking approach to treating hemophilia. Unlike traditional treatments that manage symptoms, gene therapy aims to address the root cause of the disorder by delivering a functional copy of the defective gene responsible for the condition into hepatocytes. This innovative therapy offers the potential for long-term or ideally even permanent relief, reducing the need for frequent infusions and improving overall quality of life for patients.

Conventional gene therapy for hemophilia A and B

To achieve efficient gene delivery, adeno-associated virus (AAV) gene therapy has emerged as a promising and innovative approach for treating various genetic disorders, with a particular focus on liver-targeted therapies. The liver, being a central organ for metabolic processes and protein synthesis, is an ideal target for gene therapy, as it can produce therapeutic proteins directly into the bloodstream, such as the clotting factors, once a functional copy of the defective gene is delivered. AAV vectors, which are non-pathogenic and have a proven ability to transduce liver cells efficiently, have become the preferred delivery system for this purpose. AAV-based gene therapy works by utilizing these viral vectors to deliver a corrected copy of the FVIII or FIX gene into the liver, along with its regulatory elements and promoter to drive expression. The goal is for the liver to

produce the missing or defective clotting factors responsible for these bleeding disorders.^{1,2}

The regulatory approval of the first gene therapies for hemophilia A (BioMarin's Roctavian®—*valoctocogene roxaparvovec*) and hemophilia B (CSL Behring/uniQure's Hemgenix®—*etranacogene dezaparvovec* and Pfizer's BEQVEZ™—*fidanacogene elaparvovec-dzkt*) has transitioned from experimental to clinical reality, offering hope to those living with severe forms of hemophilia. Therapeutic levels of FVIII or FIX could be achieved with these approved AAV-based products, which are sustained for several years and result in reduced factor usage and fewer bleeding episodes.^{3–5} This achievement not only marks a significant milestone in the care of hemophilia but also serves as a significant step forward in our broader efforts to combat disease and alleviate human suffering, potentially impacting the wider medical field.

Despite the long-term benefits of gene therapy, a major hurdle is the significant variability in patient outcomes, which lead to differences in both FVIII or FIX levels and the duration of expression. Some patients may experience a gradual decline in factor production over time, eventually even requiring additional factor replacement therapy. The exact causes for this variation remain unclear but are believed to involve factors such as the efficiency of vector entry, intracellular transport, gene expression regulation, and immune responses. A persistent challenge in gene therapy for hemophilia is the contrasting long-term outcomes observed between hemophilia A and B treatments. Notably, expression of FVIII in hemophilia A tends to be less sustained over time compared to the more stable expression of FIX seen in hemophilia B. Although the underlying causes are not yet fully understood, several plausible mechanisms have been proposed to explain this decline in FVIII activity. These include (i) transcriptional silencing of the F8 transgene, (ii) cellular stress caused by FVIII accumulation

leading to an unfolded protein response, (iii) sub-clinical immune responses targeting the transduced cells—potentially directed at the AAV capsid or the FVIII protein itself, and (iv) differences in vector properties linked to the method of vector production.^{6–9} A potential solution to improve FVIII durability may involve targeting expression to endothelial cells, which are the native source of FVIII, rather than hepatocytes, where ectopic overexpression may trigger cellular dysfunction.¹⁰

Immune responses directed against either the AAV vector or the expressed therapeutic protein poses a major barrier to the success of gene therapy. These responses can compromise the therapeutic outcome and, in some cases, trigger adverse events such as liver toxicity that requires careful monitoring. Managing such immunogenicity remains a key obstacle in translating gene therapy into widespread clinical practice. In addition, a substantial portion of the population harbors preexisting neutralizing antibodies against AAV, acquired through natural infection. This preexisting immunity can impair vector transduction efficiency, reduce therapeutic benefit, and may render certain patients ineligible for AAV-based treatments.^{11,12}

As a result, gene therapy is not appropriate for all individuals with hemophilia. Eligibility also depends on a range of other factors, including liver function, history of prior treatments, the presence of factor inhibitors, and the patient's age. These variables can significantly influence both the safety and therapeutic efficacy of the intervention, making careful patient selection essential for successful outcomes.

Gene therapy is ideally administered to pediatric patients prior to the onset of hemophilic arthropathy and other disease-related complications. However, the use of AAV vectors presents challenges in this age group. These vectors predominantly remain episomal within transduced hepatocytes, with only rare integration into the host genome. In infants and young children, the liver undergoes rapid growth and frequent hepatocyte proliferation, which leads to the progressive dilution and eventual loss of nonintegrated AAV genomes during cell division.¹³ As a result, transgene expression diminishes over time, rendering AAV-based gene therapy less effective in

younger patients. A potential strategy to address this issue is to postpone gene therapy until hepatic growth slows, typically in adolescence, thereby enhancing the durability of transgene expression. However, delaying treatment may not be suitable for patients with severe forms of hemophilia, who would benefit from early intervention to prevent irreversible joint damage and bleeding complications. In contrast, the adult liver is largely quiescent, supporting more sustained and stable expression of the transgene. Nevertheless, low-level hepatocyte turnover still occurs and may be exacerbated by hepatotoxic factors such as medications, alcohol, or viral hepatitis, which can compromise long-term expression of FVIII or FIX.¹⁴

Despite these limitations, ongoing preclinical research and clinical trials are focused on overcoming these challenges and optimizing gene therapy for hemophilia A and B. This includes exploring different vector designs, capsids, immunological interventions, and vector manufacturing methods to improve efficacy and reduce the risk of adverse events.

Gene editing

The application of gene editing technologies has the potential to overcome several limitations associated with conventional gene therapy approaches. Unlike traditional gene therapy strategies, gene editing is an advanced molecular technology that enables precise and targeted modifications to the human genome.¹⁵ It holds transformative potential to correct disease-causing mutations at their source, offering the possibility of long-lasting or even permanent cures for a wide range of genetic disorders, including hemophilia.¹⁶

Among the various gene editing platforms, zinc finger nucleases (ZFNs), meganucleases (MNs; synonymous with homing endonucleases), and transcription activator-like effector nucleases (TALENs) were among the first to be developed (Figure 1).¹⁷ While these protein-based systems have been successfully applied in therapeutic contexts, they are often limited by complex and time-consuming design requirements for targeting specific DNA sequences. In contrast, the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9) system, developed more recently, has

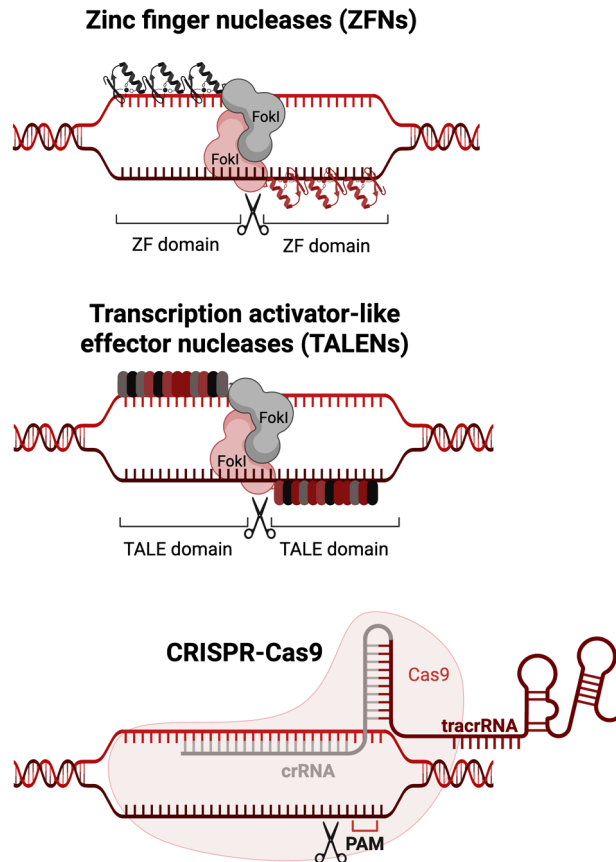


Figure 1. Overview of genome editing nucleases. ZFNs are engineered nucleases composed of zinc finger protein domains linked to the FokI endonuclease cleavage domain. For effective double-stranded DNA cleavage, ZFNs must form a dimer, with each monomer binding to adjacent DNA sequences. TALENs function similarly, utilizing DNA-binding motifs derived from TALE, each consisting of 34 amino acids. The specificity of TALENs is primarily determined by two amino acids at positions 12 and 13, termed RVDs. Like ZFNs, TALENs require dimerization of left and right arms to induce targeted DNA breaks. CRISPR-Cas9 nucleases use a guide RNA complex, composed of a crRNA and a tracrRNA, to direct the Cas9 protein to a specific genomic sequence. Binding occurs adjacent to a PAM, and the Cas9 nuclease introduces a double-stranded break at the targeted site. CRISPR-Cas9, clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9; crRNA, CRISPR RNA; PAM, protospacer adjacent motif; RVDs, repeat variable di-residues; TALE, transcription activator-like effector; TALENs, transcription activator-like effector nucleases; tracrRNA, trans-activating CRISPR RNA; ZFNs, zinc finger nucleases.

rapidly become the most widely used gene editing tool due to its high efficiency, ease of design, and programmability¹⁸ (Figure 1). ZFNs, MNs, TALENs, and CRISPR/Cas9 differ in the mechanism of target sequence recognition, but all of these technologies function by inducing targeted double-strand breaks (DSBs) in DNA at specific

genomic loci.¹⁹ Site-specific mutations in engineered nucleases have led to the development of synthetic nickases, which introduce single-stranded breaks rather than DSBs in DNA.²⁰ DSBs are repaired by the cell's natural DNA repair mechanisms—primarily nonhomologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ; Figure 2).²¹ NHEJ and MMEJ are imperfect repair mechanisms that result in insertions and deletions (indels). When indels occur within a gene's coding region, they can disrupt the reading frame or remove critical amino acids. This may result in the loss of functional protein synthesis or alterations to essential regions of the encoded protein, potentially impairing its biological activity.²²

Homology-directed repair (HDR) requires the presence of nucleases or nickases and enables precise gene correction or insertion when a suitable repair template is available (Figure 2).²³ However, HDR is significantly less efficient than NHEJ and is dependent on the cell's progression through the S and G2 phases of the cell cycle. As a result, it is generally ineffective in nondividing or slowly dividing cells, limiting its applicability in certain therapeutic contexts.²⁴

DNA repair templates used in HDR can consist of either double-stranded DNA (dsDNA)—linear or circular—or single-stranded DNA (ssDNA). These donor molecules contain regions of sequence homology flanking the genomic cut site, which guides precise integration. The required length of these homology arms varies depending on the template type: long dsDNA templates typically require several hundred base pairs of homology, whereas short ssDNA templates can function effectively with much shorter homology arms.²⁵ Though the efficiency of HDR is limited, several strategies have been explored to enhance HDR efficiency. These include inhibition of components of the NHEJ pathway, such as DNA Ligase I, or enhancing the effect of HDR-promoting factors like RAD51.²⁶ In the CRISPR-Cas9 system, introducing blocking mutations in the donor sequence—for example, modifying the protospacer adjacent motif (PAM) site—can prevent repeated cutting of the edited locus and thereby improve editing efficiency.²⁷ In addition to HDR and NHEJ, MMEJ can also serve as a repair mechanism. The activation of MMEJ depends on the cellular context and DNA repair environment.

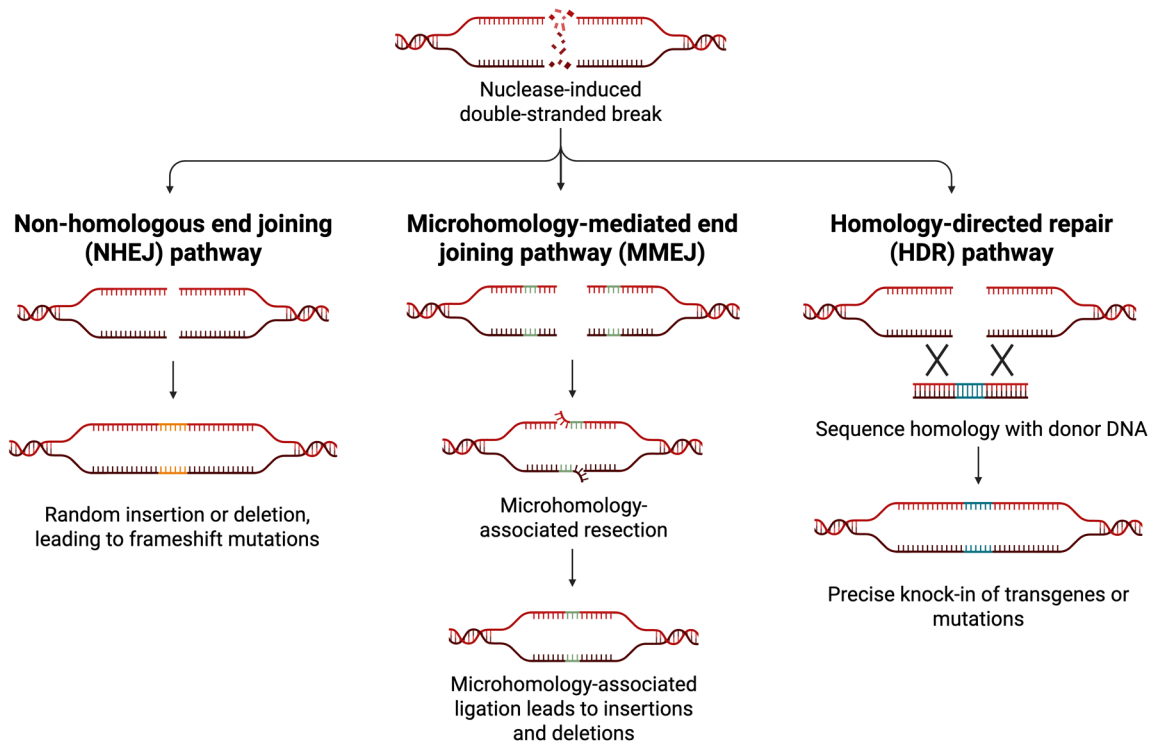


Figure 2. DNA repair mechanisms following genome editing-induced breaks. Once a DSB is introduced by any of the above nucleases, the cell's intrinsic repair pathways are activated. These include NHEJ, MMEJ, and HDR. The pathway choice can influence the outcome of genome editing and may be used strategically depending on the desired genetic modification.

DSB, double-strand break; HDR, homology-directed repair; MMEJ, microhomology-mediated end joining; NHEJ, nonhomologous end joining.

For instance, loss of Ku proteins, which are essential for classical NHEJ, increases the likelihood of MMEJ activity.²⁸ Notably, MMEJ can occur concurrently with HDR, and in some contexts, may contribute significantly to repair outcomes.²⁹

FokI-based nucleases: ZFNs and TALENs

ZFNs are synthetic enzymes that combine multiple zinc finger DNA-binding motifs—typically three to five—with the catalytic domain of the FokI restriction endonuclease, a type IIS enzyme. These zinc finger motifs, originally derived from Cys2His2-type transcription factors, each recognize a triplet of DNA bases. Structurally, a zinc finger is about 30 amino acids in length and consists of an α -helix paired with two β -strands. The key amino acid residues responsible for DNA recognition are generally located at the first, third, and sixth positions of the α -helix. Effective target recognition generally requires at least three zinc

fingers. ZFNs operate as dimers: two ZFN monomers bind to adjacent DNA sequences, allowing the FokI nuclease domains to dimerize and induce a DSB at the target site. These nucleases have been used to successfully edit genes across a range of species, including in human cells.^{30,31}

TALENs follow a similar mechanism of action but use a different DNA recognition strategy. TALENs are constructed from TALE proteins, originally found in *Xanthomonas* bacteria, which assist in modulating host gene expression during plant infection.³² TALEs contain multiple 34-amino-acid repeat units, each recognizing a single nucleotide based on a two-residue sequence—called a repeat variable di-residue (RVD)—located at positions 12 and 13. Common RVDs (e.g., NI, HD, NG, NN) specify binding to particular nucleotides (A, C, T, G).^{33,34} By customizing the sequence of these repeats, TALENs can be designed to target virtually any

DNA site. Like ZFNs, TALENs are fused to the FokI cleavage domain and must bind as dimers to induce targeted DSBs. Before the widespread adoption of CRISPR-Cas systems, TALENs were among the most widely used gene editing tools. However, their highly repetitive DNA-binding domains complicate assembly and cloning and increase the likelihood of recombination during viral packaging. Additionally, the relatively large size of TALEN constructs poses delivery challenges, especially when using vectors such as AAV, which has limited cargo capacity.³⁵

Meganucleases

Meganucleases, also referred to as homing endonucleases, are naturally occurring enzymes derived from self-propagating genetic elements, such as inteins and introns. These endonucleases function by introducing highly specific DSBs at extended DNA target sites, typically ranging from 14 to 40 base pairs, thereby facilitating the mobility of their own encoding genes through homologous recombination mechanisms.^{36,37} Structurally and phylogenetically, meganucleases are categorized into five major families based on their conserved motifs and catalytic domains: LAGLIDADG, GIY-YIG, HNH, His-Cys box, and PD-(D/E)XK (which includes Vsr-like endonucleases).^{38,39} Among these, the LAGLIDADG family, especially those found in archaeal and eukaryotic systems, has been the most extensively investigated due to its high target specificity and well-characterized structural features. LAGLIDADG homing endonucleases typically recognize asymmetric DNA sequences and engage their substrates through antiparallel β -sheets that create a saddle-shaped structure, fitting into the major groove of the DNA double helix. Despite their favorable specificity, engineered meganucleases have not been widely adopted for genome editing due to the complex and labor-intensive nature of their protein design. Unlike CRISPR-Cas9 or TALENs, meganucleases lack a modular code, making it difficult to establish direct correspondence between amino acid residues in the DNA-binding domain and specific DNA bases.

CRISPR-Cas nucleases

In prokaryotic organisms such as bacteria and archaea, the CRISPR-Cas system provides a form of adaptive immunity against invading nucleic acids including phages and plasmids. This defense

mechanism functions by incorporating short sequences of foreign DNA into host genomic loci known as CRISPR arrays, creating a molecular memory of past infections. These incorporated sequences, termed spacers, are transcribed into CRISPR RNAs (crRNAs) that guide the Cas proteins to recognize and cleave the corresponding nucleic acids during reinfection episodes.^{40,41} CRISPR-Cas systems are categorized into three primary types—Type I, II, and III—based on their cas gene content, operon arrangement, and repeat sequence architecture. Type II systems, characterized by the cas9 gene, are the most commonly utilized in genome engineering.⁴² Within type II, three subtypes (II-A, II-B, and II-C) have been defined, with II-A distinguished by the presence of the *csn2* gene.⁴³ Universal to nearly all CRISPR-Cas systems are *cas1* and *cas2* genes, which encode proteins required for spacer acquisition and integration into the CRISPR array.⁴⁴ The Cas9 protein contains dual nuclease domains: a RuvC-like domain and an HNH domain, enabling it to generate site-specific DSBs when complexed with a crRNA and trans-activating crRNA. For genome editing applications, these RNAs are typically fused into a single guide RNA (gRNA), which directs Cas9 to the complementary genomic target.¹⁸ Among the orthologs, SpCas9 (from “*Streptococcus pyogenes*”) is the most extensively characterized. Its activity requires a PAM, specifically the NGG sequence, adjacent to the 3' end of the target DNA.^{45,46} A notable advantage of CRISPR-Cas9 is its simplicity and programmability, which has driven its widespread adoption as a versatile RNA-guided endonuclease (RGEN). Additionally, the system supports multiplex genome editing, allowing simultaneous modification of multiple loci, a critical feature for investigating polygenic traits and diseases.⁴⁷ To improve its specificity and utility, numerous modifications have been implemented, including truncated gRNAs,^{46,48} nickase variants (e.g., paired Cas9 nickases),⁴⁹ catalytically inactive Cas9 (dCas9) fused to FokI nuclease,⁵⁰ compact Cas9 variants for better delivery,⁵¹ and recently, CRISPR-associated transposase systems that enable RNA-guided DNA insertion without DSB formation.⁵²

Base and prime editors

The CRISPR-Cas system led to the creation of new precision tools like base editors, which

facilitate targeted single-nucleotide alterations without generating DSBs. These editors are created by fusing catalytically inactive or nickase forms of Cas9 (dCas9 or nCas9) to nucleobase deaminases, enabling the permanent conversion of one DNA base into another. Cytosine base editors (CBEs) consist of a cytidine deaminase fused to either nCas9 or dCas9 and catalyze the conversion of cytosine to uracil, which is ultimately recognized as thymine by DNA replication or repair machinery, resulting in C·G to T·A conversions (Figure 3).⁵³ The use of nCas9, which introduces a single-strand nick on the nonedited strand, enhances the efficiency and specificity of editing compared to dCas9, which binds DNA but lacks any cleavage activity.⁵⁴

Adenine base editors (ABEs) similarly utilize nCas9 or dCas9, but are instead fused to a tRNA adenosine deaminase (TadA) enzyme, often in both wild-type and evolved forms (Figure 4). ABEs convert adenine to inosine, which is read as guanine during DNA synthesis, effectively enabling A·T to G·C substitutions.⁵⁵ Further extending base-editing capabilities, C-to-G base editors (CGBEs) have been developed to achieve specific transversion changes, such as C → G, expanding the available base conversion portfolio.⁵⁶ Collectively, CBEs, ABEs, and CGBEs enable C → T, A → G, C → G, and limited G → C changes.

However, several transversions (e.g., A → T or G → T) remain inaccessible using current base-editing platforms. Additionally, base-editing efficiency is dependent on the location of the editable nucleotide within a constrained editing window, limiting broader applicability. To overcome these limitations, prime editing (PE) was developed to support all 12 base substitutions, as well as insertions and deletions, without requiring donor DNA or DSBs (Figure 5). Prime editors are engineered by tethering a reverse transcriptase to a Cas9 H840A nickase and guiding it with a prime editing guide RNA (pegRNA), which contains both a primer binding site (PBS) and a reverse transcription template encoding the desired genetic change.⁵⁷ The pegRNA initiates reverse transcription at the target genomic site, integrating the edit through endogenous repair processes. PBS regions of ≥8 nucleotides have demonstrated optimal activity in human cell lines like HEK293T. Importantly, prime editors can be applied in contexts where base editors are

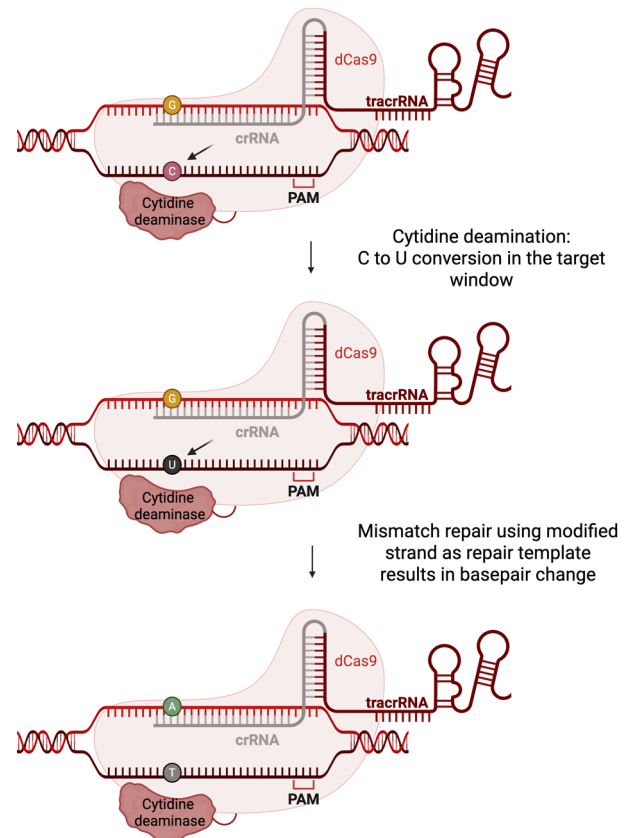


Figure 3. Cytosine base editing for C-to-T transition. CBEs facilitate the conversion of cytosine (C) to thymine (T) without introducing a double-strand break. The guide RNA targets the editing complex to a specific genomic region. Within the editing window, cytidine deaminase catalyzes the deamination of cytosine to uracil (U). During DNA replication or repair, uracil is read as thymine, resulting in a permanent C·G to T·A substitution. Editing can be performed using a Cas9 nickase (nCas9) or catalytically inactive Cas9 [dead or dCas9]. crRNA, tracrRNA, and PAM are indicated. CBEs, Cytosine base editors; crRNA, CRISPR RNA; PAM, protospacer adjacent motif; tracrRNA, trans-activating CRISPR RNA.

inapplicable due to PAM sequence or spatial constraints.

CRISPR-based gene activation and repression

In addition to genome editing, CRISPR technologies have expanded to regulate gene expression epigenetically and transcriptionally using methods that have also been developed for other gene editing platforms based on ZF or TALE. By fusing dCas9 to transcriptional activation domains such as VP64, synthetic activators like CRISPRa (CRISPR activation) can upregulate the expression of endogenous genes at specific loci.⁵⁸ These

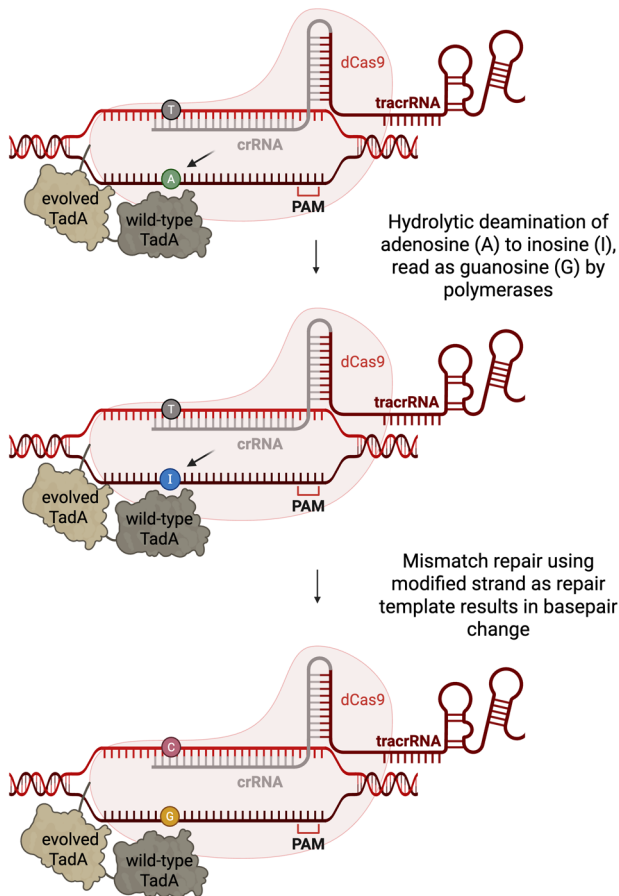


Figure 4. Adenine base editing for A-to-G transition. ABEs mediate A·T to G·C conversions by enzymatically converting adenosine to inosine (I), which is interpreted as guanosine during DNA synthesis. This process is initiated by a guide RNA that directs the base editor to the desired DNA sequence. The editing is achieved through deamination by an engineered TadA fused to a Cas9 variant, typically a Cas9 nickase (nCas9) or a catalytically dead Cas9 (dCas9). crRNA, tracrRNA, and PAM are indicated. ABEs, adenine base editors; crRNA, CRISPR RNA; PAM, protospacer adjacent motif; TadA, tRNA adenosine deaminase; tracrRNA, trans-activating CRISPR RNA.

systems are effective in transiently activating silent or lowly expressed promoters, though their impact may wane over time and require repeated application.

On the flip side, gene silencing can be achieved by combining dCas9 with repressor elements such as the KRAB (Kruppel-associated box) domain. When directed to promoter or enhancer regions, KRAB-dCas9 complexes recruit chromatin-modifying enzymes that establish repressive histone marks, compacting chromatin and repressing transcription.^{58,59} This strategy enables targeted, programmable transcriptional repression. Furthermore, CRISPR-based epigenome editors

have been developed by fusing dCas9 with catalytic domains of enzymes that alter epigenetic marks. For instance, dCas9 fused to DNA methyltransferases (e.g., DNMT3A) enables site-specific methylation of CpG dinucleotides, which can lead to heritable gene silencing.^{60,61} Similarly, histone acetyltransferases or deacetylases can be tethered to dCas9 to modify chromatin structure by altering histone tail acetylation states, influencing transcriptional accessibility and gene activity.^{62,63}

Safety considerations: Off-target effects

A primary concern in the clinical application of gene editing is the risk of off-target activity, which can result in unintended genomic modifications and potentially deleterious phenotypic consequences, including tumorigenesis. Off-target activity has been observed with any type of gene editing technology (ZFN, TALEN, MN, CRISPR) but in the context of this review, we will focus specifically on CRISPR. Although multiple high-sensitivity detection platforms have demonstrated that such events are generally infrequent, their occurrence is highly dependent on the sequence specificity of the gRNA and the genomic context of the targeted site.^{63,64} Notably, techniques such as CHANGE-seq have shown that off-target effects are more likely to occur near actively transcribed genomic regions, including promoters and enhancers.⁶⁵ Therefore, careful optimization of gRNA design and the use of high-fidelity Cas nucleases are critical for improving safety without sacrificing editing efficiency. Different strategies have been developed to increase the fidelity and safety of gene editing.

The identification and engineering of diverse Cas orthologs and optimized variants have significantly broadened the targeting range and improved the fidelity of CRISPR-Cas systems. The choice of Cas protein is largely dictated by the PAM requirements of the target site. The most widely utilized nuclease, *S. pyogenes* Cas9 (SpCas9), has been modified for enhanced specificity.⁶⁶ Strategies to mitigate off-target activity include truncated gRNAs, chemically modified ribonucleotides, paired nickases, and catalytically inactive dCas9 fused with effector domains.^{26,49,63,67,68} Innovations in promoter design have enabled spatial control of CRISPR-Cas9 expression, facilitating tissue- and cell-type-specific gene editing. This specificity is especially important when delivering editing

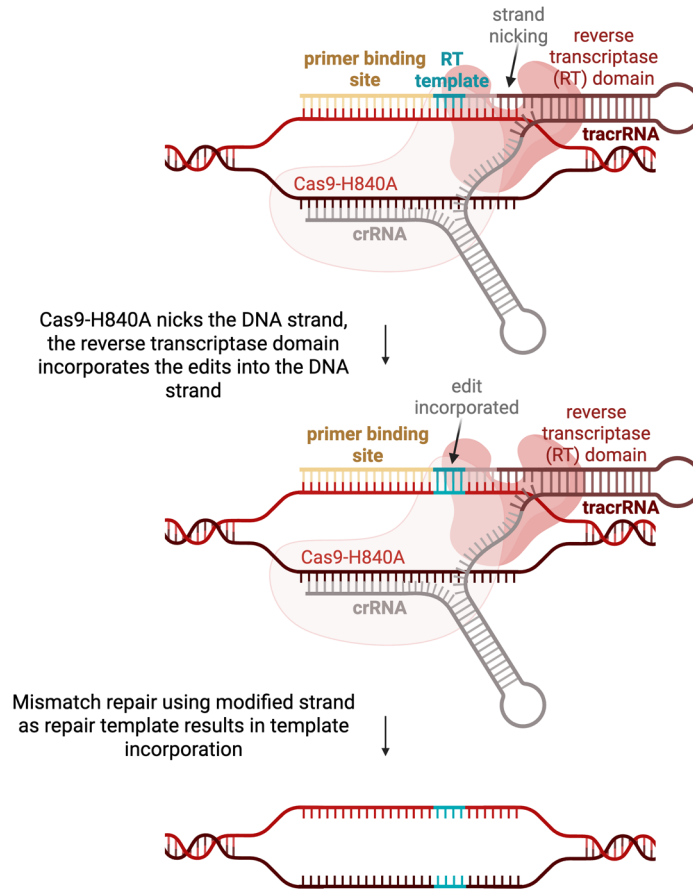


Figure 5. Prime editing for precise DNA insertions. Prime editing enables the introduction of targeted insertions, deletions, or point mutations without requiring donor DNA or creating double-strand breaks. The prime editor protein is a fusion of a RT (derived from M-MLV) and a Cas9 H840A nickase. The pegRNA not only directs the complex to the target locus but also provides the sequence template for reverse transcription. The prime editor introduces a nick in the DNA and incorporates the desired edit encoded in the pegRNA. crRNA and tracrRNA are indicated.
crRNA, CRISPR RNA; M-MLV, mouse Moloney leukemia virus; pegRNA, prime editing guide RNA; RT, reverse transcriptase; tracrRNA, trans-activating CRISPR RNA.

machinery via AAV vectors, which are constrained by packaging limits (e.g., SpCas9 ~4.2 kb). Following gRNA-guided DSB induction, repair is mediated by NHEJ, MMEJ, or HDR pathways. However, these repair processes can introduce undesired insertions or deletions. In vivo studies have reported AAV integration at CRISPR target loci, raising concerns over insertional mutagenesis and long-term safety.⁶⁹ Thus, vector selection, promoter design, and gRNA specificity must be tailored to minimize systemic exposure and genotoxicity.

The development of advanced gene editing systems has paralleled progress in molecular tools to

detect off-target effect. Early assays like the T7 endonuclease I (T7E1) assay⁷⁰ and the surveyor nuclease assay⁷¹ provided basic but limited sensitivity for identifying indels at on-target sites. For off-target detection, more comprehensive methods have emerged that leverage next-generation sequencing (NGS) in combination with high-resolution bioinformatics. Current approaches employ a combination of PCR-based enrichment, gel or capillary electrophoresis, and targeted amplicon sequencing to detect low-frequency off-target mutations.^{50,72,73} Deep sequencing can uncover rare variants, although its sensitivity is affected by read depth and coverage uniformity. Techniques that incorporate tagmentation chemistry enhance

detection by capturing DSBs with high precision. A summary of prominent off-target detection platforms is provided in Table 1. Even at intended loci, CRISPR-mediated editing can result in unanticipated alterations due to the stochastic nature of DNA repair. Such on-target events may include large deletions, inversions, translocations, and base substitutions.⁷⁴ These outcomes can arise from off-pathway repair activity or structural instability during DSB resolution. While many of the methods used for off-target detection are also suitable for identifying on-target aberrations, the detection of complex rearrangements remains challenging and often requires high-throughput or long-read sequencing technologies.

Gene editing for hemophilia

Gene editing strategies for hemophilia aim to restore functional coagulation factor expression through either the insertion of a corrected copy of the *F8* or *F9* gene or the precise repair of causative mutations. These interventions often rely on creating DSBs followed by insertion or correction at genomic “safe harbor” loci, such as the albumin or *F9* loci (Table 2). ZFNs and CRISPR-Cas systems remain the primary tools utilized in these editing approaches.

Preclinical studies have demonstrated the feasibility of using ZFNs for targeted gene insertion in hemophilia models. For instance, ZFN-mediated targeting of the *F9* or albumin locus in mice has resulted in stable expression of human *F9* cDNA, achieving therapeutic levels of FIX in hemophilia B models. In a landmark study, Li et al. demonstrated sustained FIX activity following intravenous delivery of AAV vectors encoding ZFNs and an AAV vector containing a *F9* donor template in neonatal and adult mice, achieving correction without detectable off-target toxicity.⁹⁹ However, more comprehensive and sensitive off-target analysis would be required to rule this out. Similarly, ZFNs have also been explored in hemophilia A models, where targeted integration of B-domain deleted *FVIII* cDNA led to partial restoration of clotting function.¹⁰¹ These preclinical successes provided foundational proof-of-concept that helped justify the transition of ZFN therapies into early-phase clinical trials for hemophilia.⁹⁸ A notable ZFN-based clinical trial for hemophilia B employed two AAV-delivered ZFNs designed to target the albumin locus, enabling integration of a

functional *F9* cDNA under the albumin promoter.⁹⁸ However, this trial was halted due to unchanged recombinant FIX usage. Gene editing in nonhemophilia patients is typically assessed by determining base pair insertions and/or deletions (indels) via NGS in DNA extracted from liver biopsies. However, in this clinical trial, liver biopsies were not taken from the treated hemophilia B subject due to increased risk of bleeding associated with taking liver biopsies in the context of hemophilia.⁹⁸ Consequently, the subject had declined to undergo this relatively invasive procedure.

Building upon the experience with ZFNs, CRISPR was subsequently explored for targeted genomic integration based in the induction of sequence-specific DSBs. Different types of vectors (viral, nonviral), vector designs, transgenes, and CRISPR/Cas systems were employed. Some of the highlights and concepts are summarized below and in Table 2. Though some of the vector systems that were employed to deliver the various gene editing components (such as plasmid transfection and adenoviral vectors)^{106,114} were useful to establish initial proof-of-concept, they cannot readily be translated to clinical trials. Instead, most studies relied on either AAV or lipid nanoparticles (LNPs), which are also most attractive from a translational perspective. Initial studies were based on targeted CRISPR-dependent integration of a normal *F9* cDNA sequence, into the *F9* locus itself allowing high, liver-specific expression of the clotting factor in hemophilia B mice.^{113,115} However, to further improve the outcomes, a codon-optimized hyperactive human *F9*-Padua variant was integrated into the native *F9* locus, resulting in sustained FIX levels that matched or even exceeded the physiological range.¹⁰⁷

Alternatively, the *F9* cDNAs were targeted to safe harbour loci distinct from the *F9* locus (like albumin or ApoC3). This typically allowed for higher levels of FIX expression since these promoters are also more potent than the endogenous *F9* promoter. In hemophilia B models, this resulted in stable therapeutic or sometimes even physiologic *F9* expression levels in hemophilia B mice, restoring coagulation capacity in treated animals.^{105,108,109,112} Similarly, targeted insertion of a B-domain-deleted (BDD) *F8* cDNA into the albumin (*Alb*) gene resulted in dose-dependent

Table 1. Methods of detecting off-target gene editing.

Methods	Acronyms/description	General techniques involved	Features	Refs
T7E1	T7 endonuclease I	Gel electrophoresis	Low sensitivity and no data about exact sequence	Kim et al. ⁷⁵
Surveyor	Surveyor nuclease	Gel electrophoresis	Low sensitivity and no data about exact sequence	Guschin et al. ⁷⁶
HTGTS	High-throughput genome-wide translocation sequencing	DNA capture in DSBs	High-throughput method	Chiarle et al. ⁷⁷
TC-Seq	Translocation-capture sequencing	Linker ligation + Solexa seq	Quantifies chromosomal rearrangements	Klein et al. ⁷⁸
BLESS	Direct in situ breaks labeling, enrichment on streptavidin and NGS	Tracks DSBs in fixed cells	Unbiased labeling of DSBs. Can detect telomere ends	Crosetto et al. ⁷⁹
TIDE	Tracking of indels by decomposition	Sanger sequencing-based method	Low sensitivity	Brinkman et al. ⁸⁰
IDAA	Indel detection by amplicon analysis	Capillary electrophoresis	Medium sensitivity and no data about exact sequence	Yang et al. ⁸¹
Digenome-seq	Nuclease-digested genome sequencing	Off-target prediction and WGS-based	In vitro approach	Kim et al. ⁸²
GUIDE-seq	Genome-wide unbiased identification of DSBs enabled by sequencing	Tagging of DSBs + NGS	High sensitivity	Tsai et al. ⁵⁰
Multiplex Digenome-seq	Multiplexed digenome sequencing	Digenome-seq and classification by edit distance	In vitro approach	Kim et al. ⁷³
IDLV assay	Integrase-defective lentiviral vector integration	LAM-PCR-based approach	In vivo translatable; high sensitivity	Martin et al. ⁸³
LAM-PCR HTGTS	Linear amplification-mediated high-throughput genome-wide sequencing	LAM-PCR-based cloning + HTGTS	Scalable	Hu et al. ⁸⁴
SITE-Seq	Selective enrichment and identification of tagged genomic DNA ends by sequencing	Biotin-streptavidin affinity-based NGS	In vitro biochemical mapping of Cas9 cleavage sites	Cameron et al. ⁷²
CIRCLE-seq	Circularization for in vitro reporting of cleavage effects by sequencing	Circularization of fragmented DNA + NGS	In vitro	Tsai et al. ⁸⁵
BLISS	Breaks labeling in situ and sequencing	Tracks DSBs in fixed cells	High sensitivity	Yan et al. ⁸⁶
TIDER	Tracking of insertions, deletions, and recombination events	Sanger sequencing-based method	Low sensitivity	Brinkman et al. ⁸⁰
qEva-CRISPR	Quantitative evaluation of CRISPR-Cas9-mediated gene editing	Off-target prediction and capillary electrophoresis-based	Requires knowledge of predicted cut sites	Dabrowska et al. ⁸⁷

(Continued)

Table 1. (Continued)

Methods	Acronyms/description	General techniques involved	Features	Refs
DIG-Seq	Digenome sequencing to decipher chromatin states	Digenome-seq-based approach	In vitro approach	Kim and Kim ⁸⁸
VIVO	Verification of in vivo off-targets	CIRCLE-seq + in vivo off-target validation	In vivo	Akcakaya et al. ⁸⁹
UDiTAS	Unidirectional targeted sequencing	Tn5 transposon-based approach	Can quantify translocations	Giannoukos et al. ⁹⁰
iBLESS	Direct genome-wide DNA DSB labeling in cells immobilized in agarose beads	Immobilization on agarose beads (modified BLESS)	Ultrasensitive	Biernacka et al. ⁹¹
WGS	Whole genome sequencing	Unbiased approach	High cost for detecting low-frequency off-targets	Dong et al. ⁹²
iGUIDE	Improvement of the GUIDE-seq method	Guide-seq-based approach	High sensitivity	Nobles et al. ⁹³
iHTGTS	Improved HTGTS	HTGTS with size-selection beads	High-throughput method	Yin et al. ⁹⁴
GOTI	Genome-wide off-target analysis by two-cell embryo injection	FACS + WGS based	—	Zuo et al. ⁹⁵
CHANGE-seq	Circularization for high-throughput analysis of nuclease genome-wide effects by sequencing	Circularization of fragmented DNA + NGS	In vitro	Lazzarotto et al. ⁶⁵
TTISS	Tagmentation-based tag integration site sequencing	Tagging of DSBs + NGS	High-throughput method	Schmid-Burgk et al. ⁹⁶
DISCOVER-seq	Discovery of in situ Cas off-targets and verification by sequencing	Chromatin immunoprecipitation followed by NGS	Track the precise recruitment of MRE11 to DSBs	Wienert et al. ⁹⁷

DSB, double-strand break; FACS, fluorescence activated cell sorting; NGS, next-generation sequencing.

and durable expression of therapeutic FVIII levels in hemophilia A models.^{102–104}

An important target for CRISPR-mediated correction in hemophilia A is the intron 22 inversion within the F8 gene, a mutation responsible for approximately 45% of severe hemophilia A cases. This inversion disrupts the gene by separating the promoter and early exons from the remaining coding sequence. Recent studies have demonstrated that CRISPR-Cas9 technology can correct this inversion by generating dual cuts flanking the inverted segments, facilitating homologous recombination or inversion reversion. For

example, Park et al. derived induced pluripotent stem cells (iPSCs) from patients with intron 22 inversions and used CRISPR-Cas9 nucleases to revert these chromosomal segments back to the wild-type configuration.¹²⁰ Endothelial cells differentiated from these corrected iPSCs expressed the F8 gene and functionally rescued FVIII deficiency in a mouse model of hemophilia.¹²⁰ Similarly, Wu et al. achieved in situ genetic correction of the F8 intron 22 inversion in patient-specific iPSCs using TALENs, resulting in the rescue of both F8 transcription and FVIII secretion in endothelial cells and mesenchymal stem cells derived from the gene-corrected iPSCs.¹²¹

Table 2. Gene editing strategies to treat hemophilia.

Gene editing method	Mechanism	Vector	Study	References
ZFN	ZFN-mediated insertion of <i>F9</i> gene at albumin locus	AAV-ZFN and <i>F9</i>	Human clinical phase I trial NCT02695160 (terminated)	Harmatz et al. ⁹⁸
	ZFN-mediated insertion of partial cDNA cassette containing exons 2–8 of the wild-type human <i>F9</i> gene	AAV8-ZFN and <i>F9</i>	Preclinical HB mouse	Li et al., ⁹⁹ Anguela et al. ¹⁰⁰
	ZFN-mediated insertion of FVIII cDNA cassette	AAV8-ZFN and <i>F8</i>	Preclinical HA/CD4KO mouse	Sharma et al. ¹⁰¹
CRISPR-Cas nuclease	CRISPR-Cas9-mediated BDD- <i>F8</i> insertion at albumin locus	AAV8-SaCas9 and AAV8-BDD- <i>F8</i>	Preclinical HA mouse	Chen et al. ¹⁰²
	CRISPR-Cas9-mediated integration of promoter region and exon1 of the <i>F8</i> coding sequence	AAV8	Preclinical HA mouse	Luo et al. ¹⁰³
	Knock-in of BDD- <i>F8</i> at Alb locus	AAV8-Cas9, guide RNA and BDD- <i>F8</i>	Preclinical HA mouse	Zhang et al. ¹⁰⁴
	CRISPR-Cas9-mediated site-specific integration of therapeutic transgene within the albumin gene	AAV8-SpCas9 and AAV8-gRNA-donor	Preclinical HB mouse	Wang et al. ¹⁰⁵
	CRISPR-Cas9 plasmid and donor DNA for correcting <i>F9</i> mutation	Plasmid DNA	Preclinical HB mouse	Huai et al. ¹⁰⁶
	CRISPR-Cas9-mediated promoterless partial <i>F9</i> insertion at <i>F9</i> locus	AAV8-SaCas9 and codon-optimized h <i>F9</i> -Padua	Preclinical HB mouse	Wang et al. ¹⁰⁷
	CRISPR-Cas9-mediated insertion of <i>F9</i> CDS at APOC3 locus	AAV8-CjCas9 and <i>F9</i> donor	Preclinical APOC3-humanized HB mouse	Lee et al. ¹⁰⁸
	Liver-specific knock-in of hyperactive h <i>F9</i> R338L (Padua) variant	AAV8	Preclinical HB mouse	He et al. ¹⁰⁹
	Homology-independent targeted integration h <i>F9</i> -Padua variant	AAV8	Preclinical HB rat	Chen et al. ¹¹⁰
CRISPR-Cas9 nuclease (continued)	Homology-independent targeted integration of BDD-FVIII into Alb	AAV8	Preclinical HA mouse	Esposito et al. ¹¹¹
	Promoterless donor vector containing h <i>F9</i> cDNA, carrying the V86A, E277A, and R338L-Padua mutations with CRISPR-Cas9 targeted at the albumin locus	AAV8	Preclinical HB mouse	Lisjak et al. ¹¹²
	Insertion of exons 2–8 mouse <i>F9</i> cDNA into intron 1 of <i>F9</i> and HDR	AAV8	Preclinical HB mouse	Ohmori et al. ¹¹³
	Knock-in of murine <i>F9</i> at Rosa26 locus	Adenoviral vector expressing Cas9 and gRNA	Preclinical HB mouse	Stephens et al. ¹¹⁴

(Continued)

Table 2. (Continued)

Gene editing method	Mechanism	Vector	Study	References
	Antithrombin disruption	AAV8	Preclinical HB mouse	Ohmori et al. ¹¹³
	Antithrombin disruption	Cas9-mRNA LNP	Preclinical HA and HB mouse	Han et al. ¹¹⁵
	Antithrombin disruption and human factor 9 gene knock-in	LNPs and AAV8	Preclinical HB mouse	Lee et al. ¹¹⁶
Nuclease-free	Promoterless nuclease-free gene addition at albumin locus	AAV8- <i>F9</i>	Preclinical HB mouse	Barzel et al. ¹¹⁷
Base editing	Correction of <i>F9</i> G20519A mutation by adenine base editing	Poly-L-lysine-delivered ABE8e system	Preclinical in vitro HEK293-FIXmut and Huh7-FIXmut stem cells	Rong et al. ¹¹⁸
	Correction of <i>F8</i> p.R2166* and p.R2228Q mutations by adenine base editing	Lentiviral vector expressing ABE8e-NG and gRNA	Preclinical In vitro Human BOEC	Tonetto et al. ¹¹⁹
AAV, adeno-associated virus; BDD, B-domain-deleted; BOEC, blood outgrowth endothelial cells; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9; gRNA, guide RNA; HA, hemophilia A; HB, hemophilia B; LNPs, lipid nanoparticles; ZFN, zinc finger nuclease.				

These approaches hold promise for developing autologous cell-based therapies and could eventually be adapted for in vivo correction with optimized delivery methods.

One of the challenges of using CRISPR for gene editing pertains to the limited packaging capacity of AAV. To address packaging size constraints of the CRISPR toolkit, researchers have leveraged compact orthologs such as *Campylobacter jejuni* Cas9 (CjCas9). Due to its smaller coding sequence, CjCas9 can be encapsulated within a single AAV vector and has been successfully utilized to mediate targeted integration of clotting factor genes in hemophilic mouse models into the ApoC3 locus, allowing high expression of the clotting factor. This strategy not only restored hemostasis but also achieved liver-restricted transgene expression by virtue of co-opting the human ApoC3 promoter, enhancing safety and therapeutic precision.¹⁰⁸

Homology-independent targeted integration (HITI) has emerged as a promising gene-editing platform for the permanent correction of genetic

diseases such as hemophilia. Unlike HDR, which is inefficient in nondividing cells, HITI leverages the NHEJ pathway to achieve targeted insertion of therapeutic transgenes, making it highly suitable for in vivo applications, particularly in the liver. The feasibility of HITI was initially demonstrated using ZFNs to restore hemostasis by integrating a codon-optimized *F9* cDNA into the albumin locus of hemophilia B mice, resulting in long-term expression of functional FIX and correction of the bleeding phenotype.^{99,100} Chen et al. further confirmed these findings by achieving durable FIX expression and phenotypic correction in hemophilia B mice using CRISPR/Cas9-mediated HITI with AAV vectors.¹¹⁰ Recent works highlighted the safety and efficacy of liver-directed AAV-mediated HITI at the albumin locus in neonatal mouse models of inherited metabolic diseases and hemophilia.¹¹¹ This approach led to long-term transgene expression without evident genotoxicity, reinforcing the translational potential of HITI for pediatric gene therapy. In hemophilia A, a dual AAV system was employed to mediate HITI-based integration of a BDD *F8* transgene into the albumin locus,

resulting in sustained therapeutic FVIII levels in a mouse model.¹¹¹ These studies demonstrate that AAV-delivered HITI represents a versatile and durable strategy for correcting hemophilia A and B in preclinical models.

Gene editing strategies in hemophilia are now also investigating targets beyond the canonical F8 and F9 gene targets. One such target is antithrombin (encoded by the *SERPINC1* gene), a physiological anticoagulant that limits thrombin activity. CRISPR-mediated inactivation of antithrombin has emerged as a viable approach to restore hemostasis in both hemophilia A and B. CRISPR-Cas9 was used to induce frameshift mutations in the *Serpinc1* gene in mice, leading to reduced antithrombin levels and enhanced thrombin generation, ultimately improving hemostasis in hemophilia A and B models and reducing need for clotting factor replacement suggesting the broad applicability of antithrombin targeting across different types of hemophilia. In these models, the gene editing components were delivered using either AAV or LNPs.^{113,115,116}

These findings support the development of anticoagulant gene editing strategies as a factor-independent therapeutic modality.

Gene integration approaches that do not depend on nuclease-induced DSBs have also gained attention for their improved safety profile.¹¹⁷ Barzel *et al.* engineered a targeted gene insertion strategy without creating DSBs. By inserting a promoterless human F9 transgene upstream of the albumin stop codon, they harnessed the strong endogenous albumin promoter to drive expression of FIX in hepatocytes. This approach achieved stable integration with an efficiency of ~0.5% and therapeutic FIX levels approaching 20% of normal in a mouse model of hemophilia B, without evidence of insertional mutagenesis or off-target effects.¹¹⁷ This strategy demonstrates the feasibility of harnessing endogenous gene regulation machinery while minimizing genotoxic risks associated with DSBs.

Recent advances in base editing (BE) have enabled precise correction of point mutations associated with hemophilia without the need for DSBs or donor templates. Rong *et al.* developed a strategy using ABEs in combination with HDR to correct the pathogenic G20519A (R226Q) mutation

in the F9 gene in Huh7-derived cells.¹¹⁸ Star-shaped poly-lysine nanocarriers were used to deliver the editing components efficiently into the target cells. This combinatorial strategy restored functional FIX expression, indicating that BE could be a viable path toward autologous gene correction therapy in hemophilia B patients carrying point mutations. Recently, single point mutations leading to severe hemophilia A were corrected in cell lines in vitro and blood outgrowth endothelial cells by DSB/HR-independent BE and PE approaches resulting in dose-dependent rescue of secreted functional FVIII.¹¹⁹

Though base and prime editors may provide more precise genetic engineering than nuclease-based approaches because they are DSB-independent. However, base and prime editors were recently shown to induced detrimental transcriptional responses that reduced editing efficiency and hematopoietic repopulation in xenotransplants and also generated DSBs and genotoxic by-products, including deletions and translocations, albeit at a lower frequency than Cas9.¹²² The potential genotoxic consequences of BE and PE for hemophilia would therefore need to be thoroughly examined prior to their clinical translation. One of the challenges of BE and PE, as opposed to conventional gene therapy, is that each product needs to be typically tailored to specific causative mutations. Although such personalized “bespoke” gene-editing poses some challenges pertaining to commercial product development, the most common causative mutations could be targeted.

Liver-directed gene-editing clinical trials

The liver has emerged as an optimal target for in vivo gene editing due to its accessibility, regenerative capacity, and role in synthesizing many circulating proteins including clotting factors. Recent advances in CRISPR-Cas9 technology have enabled precise genetic modifications in hepatocytes, allowing the correction or disruption of disease-causing genes directly within the patient. This strategy has yielded early clinical success, particularly for rare monogenic disorders. A landmark phase I clinical trial (NTLA-2001) conducted by *Intellia Therapeutics* and *Regeneron Pharmaceuticals* demonstrated the feasibility of CRISPR-Cas9-based in vivo gene editing for hereditary transthyretin amyloidosis (ATTR) amyloidosis. The

trial used LNP delivery of mRNA encoding Cas9 and a gRNA targeting the *TTR* gene in hepatocytes. This single systemic administration resulted in up to a 96% reduction in serum transthyretin protein levels, with no serious adverse events reported in early participants.¹²³ This trial marked the first evidence of successful in vivo CRISPR-Cas9 gene editing in humans, highlighting its potential for treating systemic proteinopathies.

A follow-up in vivo liver-targeted gene editing approach was employed in the treatment of hereditary angioedema. Intellia's investigational therapy, NTLA-2002, uses CRISPR-Cas9 to knockout the *KLKB1* gene, which encodes plasma prekallikrein, a protein contributing to bradykinin overproduction and recurrent angioedema attacks. Interim results from an ongoing clinical trial showed up to a 90% reduction in kallikrein levels and a substantial decrease in attack frequency in patients after a single intravenous infusion.¹²⁴ These findings support the promise of CRISPR-mediated inactivation of hepatic genes in treating diseases driven by gain-of-function mutations or toxic protein accumulation.

Although no clinical trials have yet reached human testing stages for CRISPR-based gene editing in hemophilia, robust preclinical studies have laid the groundwork for future translation. Several in vivo strategies in murine models have demonstrated efficient correction or insertion of *F8* and *F9* transgenes into the albumin locus using AAV- or LNP-delivered CRISPR-Cas9 systems, achieving sustained clotting factor expression (see above). The success of NTLA-2001 and NTLA-2002 establishes a critical precedent for the development of CRISPR-based therapies for hemophilia and other liver-targeted disorders.

Critical assessment of gene editing

The main advantage of gene editing pertains to its ability to achieve targeted integration of therapeutic *F8* or *F9* transgenes in the endogenous defective *F8* or *F9* loci or into safe harbor loci, thereby minimizing the risk of insertional oncogenesis associated with random vector integration. Targeted genomic integration will increase the likelihood that lifelong FVIII or FIX expression could be achieved, since the transgenes will not be diluted upon target cell division. This opens up new perspectives to treat pediatric patients with

hemophilia before the onset of bleeding-induced arthropathy, while their livers are still growing owing to proliferating hepatocytes.

Despite the potential long-term benefits of gene editing, gene editing is no *magic bullet* and several important challenges remain to be addressed. The risk of potential off-target effects needs to be rigorously addressed, mandating the use of more precise, high-fidelity CRISPR platforms and sensitive detection and monitoring assays as described above. The long-term effects of CRISPR-mediated editing are still under investigation. Genomic instability, unintended structural variations (e.g., large deletions or rearrangements), and persistent DNA breaks can have deleterious consequences. Concerns remain about clonal expansion of edited cells or integration of AAV vectors, which could lead to insertional mutagenesis or oncogenesis in the long-term.^{69,125} CRISPR editing relies on the cell's endogenous DNA repair mechanisms: NHEJ is active in most cells but is error-prone and unpredictable, while HDR allows precise edits but is mostly restricted to dividing cells and is relatively inefficient in vivo, particularly in adult tissues.¹²⁶ The use of HITI may overcome some of these limitations.

One of the main challenges pertains to the delivery of the gene editing components. A brief pulse of Cas9 and gRNA expression suffices to achieve targeted correction or integration while long-term Cas9 or gRNA expression should be avoided to minimize the risk of off-target effects and potential immunogenicity issues. To address this, Cas9 and gRNA could potentially be delivered as mRNA using LNP technology allowing for short-term but robust liver-directed expression and efficient gene editing, as confirmed in clinical studies for hereditary amyloidosis.^{123,124} AAV vectors have been used to deliver the *F8* or *F9* donor transgenes into the hepatocytes since DNA delivery with LNPs is not as efficient as RNA delivery. Consequently, most of the limitations associated with the use of AAV that have been identified in the context of "conventional" gene therapy will also be relevant for any gene editing application that relies on AAV to deliver the donor DNA. This is compounded by the relatively high AAV vector doses which are typically needed to achieve efficient gene correction or targeted integration. As long as gene editing requires AAV to deliver the therapeutic *F8* or *F9* transgenes, the risk of

liver inflammation and hepatotoxicity will need to be rigorously addressed. In addition, preexisting immunity to AAV will hamper effective gene editing and therefore patients with high-titer anti-AAV antibodies will be excluded. Cas proteins, being of bacterial origin, can be recognized as foreign by the human immune system. This can trigger innate and adaptive immune responses, potentially neutralizing the editing machinery or damaging edited cells expressing the foreign protein. Studies have shown that many individuals have preexisting immunity to Cas9 derived from *S. pyogenes* and *S. aureus*.⁵ However, when LNPs are used to deliver Cas9 mRNA, gene-edited cells persisted long-term^{123,124} perhaps due at least in part to the short-term Cas9 expression kinetics. Finally, once FVIII or FIX protein expression is restored after gene editing, the emergence of neutralizing antibodies (clinically referred to as inhibitors) to these de novo expressed clotting factors would need to be carefully monitored.

Conclusion

Compared to conventional gene therapy, which primarily relies on random transgene integration or episomal expression, gene editing based on ZFNs, MNs, TALENs, or CRISPR/Cas9 offers a more precise and versatile genome-editing platform. While conventional methods are limited by vector size and promoter-driven expression, gene-editing strategies can achieve permanent correction with reduced risks of insertional mutagenesis which is typically associated with random genomic integration. These gene-editing technologies enable site-specific gene correction or insertion within endogenous *F8* or *F9* genomic loci, maintaining physiological gene regulation. Alternatively, gene editing has been used to target the integration of the therapeutic *F8* or *F9* cDNA into a safe harbor locus. In some cases, a strong endogenous promoter, like albumin, can be co-opted to drive expression of the exogenous *F8* and *F9* transgenes following their integration into the albumin locus. Alternatively, gene editing could be exploited to inactivate genes that inhibit the coagulation cascade, such as antithrombin, resulting in phenotypic correction in hemophilic models. Despite its promise, challenges remain related to the off-target effects, the immunogenicity of CRISPR/Cas components and last but not least the delivery itself. Nevertheless, the first FDA and EMA-approved CRISPR-based products have recently become available and will inevitably pave the way toward

the use of CRISPR as transformative tool in the development of next-generation therapies for monogenic disorders such as hemophilia.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors provided their consent for publication.

Author contributions

Thierry VandenDriessche: Conceptualization; Funding acquisition; Writing – original draft; Writing – review & editing.

Mathias Janssens: Conceptualization; Writing – original draft; Writing – review & editing.

Marinee K. Chuah: Conceptualization; Funding acquisition; Writing – original draft; Writing – review & editing.

Acknowledgements

Figures were created with BioRender.com.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported with a Methusalem and a VUB IOF GEAR grant.

Competing interests

VUB owns intellectual property in the field of gene therapy for hemophilia. T.V. and M.C. had obtained grant support from Pfizer, Baxter/Baxalta (Shire/Takeda), Bayer, and Catalyst Biosciences.

Availability of data and materials

Not applicable.

ORCID iDs

Thierry VandenDriessche  <https://orcid.org/0000-0003-4230-745X>

Mathias Janssens  <https://orcid.org/0009-0004-8189-4719>

Marinee K. Chuah  <https://orcid.org/0000-0001-8569-0175>

References

- High KA and Roncarolo MG. Gene therapy. *N Engl J Med* 2019; 381(5): 455–464.
- Pierce GF and Iorio A. Past, present and future of haemophilia gene therapy: From vectors and transgenes to known and unknown outcomes. *Haemophilia* 2018; 24 Suppl 6: 60–67.
- Pasi KJ, Rangarajan S, Mitchell N, et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N Engl J Med* 2020; 382(1): 29–40.
- Miesbach W, Meijer K, Coppens M, et al. Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B. *Blood* 2018; 131(9): 1022–1031.
- George LA, Sullivan SK, Giermasz A, et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *N Engl J Med* 2017; 377(23): 2215–2227.
- Handyside B, Ismail AM, Zhang L, et al. Vector genome loss and epigenetic modifications mediate decline in transgene expression of AAV5 vectors produced in mammalian and insect cells. *Mol Ther* 2022; 30(12): 3570–3586.
- Mingozzi F and High KA. Overcoming the host immune response to adeno-associated virus gene delivery vectors: the race between clearance, tolerance, neutralization, and escape. *Annu Rev Virol* 2017; 4(1): 511–534.
- De Wolf D, Singh K, Chuah MK, et al. Hemophilia gene therapy: the end of the beginning? *Hum Gene Ther* 2023; 34(17–18): 782–792.
- Fong S, Yates B, Sihn CR, et al. Interindividual variability in transgene mRNA and protein production following adeno-associated virus gene therapy for hemophilia A. *Nat Med* 2022; 28(4): 789–797.
- Chen Z, Herzog RW and Kaufman RJ. Cellular stress and coagulation factor production: when more is not necessarily better. *J Thromb Haemost* 2023; 21(12): 3329–3341.
- Ronzitti G, Gross DA and Mingozzi F. Human immune responses to adeno-associated virus (AAV) vectors. *Front Immunol* 2020; 11: 670.
- Wang D, Tai PWL and Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov* 2019; 18(5): 358–378.
- Zhang L, Yates B, Murphy R, et al. Young mice administered adult doses of AAV5-hFVIII-SQ achieve therapeutic factor VIII expression into adulthood. *Mol Ther Methods Clin Dev* 2022; 26: 519–531.
- Ragni MV, Mead H, de Jong YP, et al. Optimizing liver health before and after gene therapy for hemophilia A. *Blood Adv* 2024; 8(19): 5203–5212.
- Doudna JA and Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. *Science* 2014; 346(6213): 1258096.
- Davies K, Philippidis A and Barrangou R. Five years of progress in CRISPR clinical trials (2019–2024). *CRISPR J* 2024; 7(5): 227–230.
- Gaj T, Gersbach CA and Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 2013; 31(7): 397–405.
- Jinek M, Chylinski K, Fonfara I, et al. A programmable dual RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; 337(6096): 816–821.
- Sander JD and Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 2014; 32(4): 347–355.
- Carroll D. Progress and prospects: zinc-finger nucleases as gene therapy agents. *Gene Ther* 2008; 15(22): 1463–1468.
- Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 2010; 79: 181–211.
- Santiago Y, Chan E, Liu PQ, et al. Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. *Proc Natl Acad Sci U S A* 2008; 105(15): 5809–5814.
- Moehle EA, Rock JM, Lee YL, et al. Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proc Natl Acad Sci U S A* 2007; 104(9): 3055–3060.
- Nami F, Basiri M, Satarian L, et al. Strategies for in vivo genome editing in nondividing cells. *Trends Biotechnol* 2018; 36(8): 770–786.
- Orlando SJ, Santiago Y, DeKolver RC, et al. Zinc-finger nuclease-driven targeted integration into mammalian genomes using donors with limited chromosomal homology. *Nucleic Acids Res* 2010; 38(15): e152.
- Jin YY, Zhang P, Liu LL, et al. Enhancing homology-directed repair efficiency with HDR-boosting modular ssDNA donor. *Nat Commun* 2024; 15(1): 6843.

27. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; 339(6121): 819–823.
28. McVey M and Lee SE. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet* 2008; 24(11): 529–538.
29. Pannunzio NR, Watanabe G and Lieber MR. Nonhomologous DNA end-joining for repair of DNA double-strand breaks. *J Biol Chem* 2018; 293(27): 10512–10523.
30. Urnov FD, Rebar EJ, Holmes MC, et al. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 2010; 11(9): 636–646.
31. Carroll D. Genome engineering with zinc-finger nucleases. *Genetics* 2011; 188(4): 773–782.
32. Boch J, Scholze H, Schornack S, et al. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 2009; 326(5959): 1509–1512.
33. Moscou MJ and Bogdanove AJ. A simple cipher governs DNA recognition by TAL effectors. *Science* 2009; 326(5959): 1501.
34. Joung JK and Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* 2013; 14(1): 49–55.
35. Sanjana NE, Cong L, Zhou Y, et al. A transcription activator-like effector toolbox for genome engineering. *Nat Protoc* 2012; 7(1): 171–192.
36. Stoddard BL. Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. *Struct* 2011; 19(1): 7–15.
37. Belfort M and Roberts RJ. Homing endonucleases: keeping the house in order. *Nucleic Acids Res* 1997; 25(17): 3379–3388.
38. Chevalier BS and Stoddard BL. Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. *Nucleic Acids Res* 2001; 29(18): 3757–3774.
39. Jurica MS and Stoddard BL. Homing endonucleases: structure, function and evolution. *Cell Mol Life Sci CMLS* 1999; 55(10): 1304–1326.
40. Marraffini LA. CRISPR-Cas immunity in prokaryotes. *Nature* 2015; 526(7571): 55–61.
41. Barrangou R, Fremaux C, Deveau H, et al. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007; 315(5819): 1709–1712.
42. Makarova KS, Haft DH, Barrangou R, et al. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol* 2011; 9(6): 467–477.
43. Chylinski K, Le Rhun A and Charpentier E. The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems. *RNA Biol* 2013; 10(5): 726–737.
44. Nuñez JK, Lee ASY, Engelman A, et al. Integrase-mediated spacer acquisition during CRISPR-Cas adaptive immunity. *Nature* 2015; 519(7542): 193–198.
45. Jiang F and Doudna JA. CRISPR-Cas9 Structures and Mechanisms. *Annu Rev Biophys* 2017; 46: 505–529.
46. Fu Y, Sander JD, Reyon D, et al. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* 2014; 32(3): 279–284.
47. Kabadi AM, Ousterout DG, Hilton IB, et al. Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. *Nucleic Acids Res* 2014; 42(19): e147.
48. Chen JS, Dagdas YS, Kleinstiver BP, et al. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. *Nature* 2017; 550(7676): 407–410.
49. Ran FA, Hsu PD, Lin CY, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 2013; 154(6): 1380–1389.
50. Tsai SQ, Zheng Z, Nguyen NT, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* 2015; 33(2): 187–197.
51. Kim Y, Cheong SA, Lee JG, et al. Generation of knockout mice by Cpf1-mediated gene targeting. *Nat Biotechnol* 2016; 34(8): 808–810.
52. Strecker J, Ladha A, Gardner Z, et al. RNA-guided DNA insertion with CRISPR-associated transposases. *Science* 2019; 365(6448): 48–53.
53. Komor AC, Kim YB, Packer MS, et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 2016; 533(7603): 420–424.
54. Nishida K, Arazoe T, Yachie N, et al. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* 2016; 353(6305): aaf8729.
55. Gaudelli NM, Komor AC, Rees HA, et al. Programmable base editing of A·T to G·C in

- genomic DNA without DNA cleavage. *Nature* 2017; 551(7681): 464–471.
56. Kurt IC, Zhou R, Iyer S, et al. CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. *Nat Biotechnol* 2021; 39(1): 41–46.
57. Anzalone AV, Randolph PB, Davis JR, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 2019; 576(7785): 149–157.
58. Chavez A, Scheiman J, Vora S, et al. Highly efficient Cas9-mediated transcriptional programming. *Nat Methods* 2015; 12(4): 326–328.
59. Gilbert LA, Larson MH, Morsut L, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013; 154(2): 442–451.
60. Thakore PI, D'Ippolito AM, Song L, et al. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nat Methods* 2015; 12(12): 1143–1149.
61. Liu XS, Wu H, Ji X, et al. Editing DNA methylation in the mammalian genome. *Cell* 2016; 167(1): 233–247.e17.
62. Amabile A, Migliara A, Capasso P, et al. Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. *Cell* 2016; 167(1): 219–232.e14.
63. Fu Y, Foden JA, Khayter C, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 2013; 31(9): 822–826.
64. Tsai SQ and Joung JK. Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. *Nat Rev Genet* 2016; 17(5): 300–312.
65. Lazzarotto CR, Malinin NL, Li Y, et al. CHANGE-seq reveals genetic and epigenetic effects on CRISPR-Cas9 genome-wide activity. *Nat Biotechnol* 2020; 38(11): 1317–1327.
66. Nishimasu H, Ran FA, Hsu PD, et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* 2014; 156(5): 935–949.
67. Slaymaker IM, Gao L, Zetsche B, et al. Rationally engineered Cas9 nucleases with improved specificity. *Science* 2016; 351(6268): 84–88.
68. Kim S, Kim D, Cho SW, et al. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res* 2014; 24(6): 1012–1019.
69. Hanlon KS, Kleinstiver BP, Garcia SP, et al. High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat Commun.* 2019 Sep 30;10(1):4439.
70. Vouillot L, Th  lie A and Pollet N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3 (Bethesda)* 2015; 5(3): 407–415.
71. Qiu P, Shandilya H, D'Alessio JM, et al. Mutation detection using Surveyor nuclease. *Biotechniques* 2004; 36(4): 702–707.
72. Cameron P, Fuller CK, Donohoue PD, et al. Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nat Methods* 2017; 14(6): 600–606.
73. Kim D, Kim S, Kim S, et al. Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq. *Genome Res.* 2016 Mar;26(3):406–15.
74. Kosicki M, Tomberg K and Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol* 2018; 36(8): 765–771.
75. Kim HJ, Lee HJ, Kim H, et al. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res* 2009; 19(7): 1279–1288.
76. Guschin DY, Waite AJ, Katibah GE, et al. A rapid and general assay for monitoring endogenous gene modification. *Methods Mol Biol* 2010; 649: 247–256.
77. Chiarle R, Zhang Y, Frock RL, et al. Genome-wide translocation sequencing reveals mechanisms of chromosome breaks and rearrangements in B cells. *Cell* 2011; 147(1): 107–119.
78. Klein IA, Resch W, Jankovic M, et al. Translocation-capture sequencing reveals the extent and nature of chromosomal rearrangements in B lymphocytes. *Cell* 2011; 147(1): 95–106.
79. Crosetto N, Mitra A, Silva MJ, et al. Nucleotide-resolution DNA double-strand break mapping by next-generation sequencing. *Nat Methods* 2013; 10(4): 361–365.
80. Brinkman EK, Kousholt AN, Harmsen T, et al. Easy quantification of template-directed CRISPR/Cas9 editing. *Nucleic Acids Res* 2018; 46(10): e58.
81. Yang Z, Steentoft C, Hauge C, et al. Fast and sensitive detection of indels induced by precise gene targeting. *Nucleic Acids Res* 2015; 43(9): e59.

82. Kim D, Bae S, Park J, et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 2015; 12(3): 237–243.
83. Martin F, Sánchez-Hernández S, Gutiérrez-Guerrero A, et al. Biased and Unbiased methods for the detection of off-target cleavage by CRISPR/Cas9: an overview. *Int J Mol Sci* 2016; 17(9): 1507.
84. Hu J, Meyers RM, Dong J, et al. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc* 2016; 11(5): 853–871.
85. Tsai SQ, Nguyen NT, Malagon-Lopez J, et al. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nat Methods* 2017; 14(6): 607–614.
86. Yan WX, Mirzazadeh R, Garnerone S, et al. BLISS is a versatile and quantitative method for genome-wide profiling of DNA double-strand breaks. *Nat Commun* 2017; 8(1): 15058.
87. Dabrowska M, Czubak K, Juzwa W, et al. QEva-CRISPR: a method for quantitative evaluation of CRISPR/Cas-mediated genome editing in target and off-target sites. *Nucleic Acids Res* 2018; 46(17): e101.
88. Kim D and Kim JS. DIG-seq: a genome-wide CRISPR off-target profiling method using chromatin DNA. *Genome Res* 2018; 28(12): 1894–1900.
89. Akcakaya P, Bobbin ML, Guo JA, et al. In vivo CRISPR editing with no detectable genome-wide off-target mutations. *Nature* 2018; 561(7723): 416–419.
90. Giannoukos G, Ciulla DM, Marco E, et al. UDiTaS™, a genome editing detection method for indels and genome rearrangements. *BMC Genomics* 2018; 19(1): 212.
91. Biernacka A, Zhu Y, Skrzypczak M, et al. I-BLESS is an ultra-sensitive method for detection of DNA double-strand breaks. *Commun Biol* 2018; 1(1): 1–9.
92. Dong Y, Li H, Zhao L, et al. Genome-wide off-target analysis in CRISPR-Cas9 modified mice and their offspring. *G3 (Bethesda)* 2019; 9(11): 3645–3651.
93. Nobles CL, Reddy S, Salas-McKee J, et al. IGUIDE: an improved pipeline for analyzing CRISPR cleavage specificity. *Genome Biol* 2019; 20(1): 14.
94. Yin J, Liu M, Liu Y, et al. Improved HTGTS for CRISPR/Cas9 off-target detection. *Bio Protoc* 2019; 9(9): e3229.
95. Zuo E, Sun Y, Wei W, et al. GOTI, a method to identify genome-wide off-target effects of genome editing in mouse embryos. *Nat Protoc* 2020; 15(9): 3009–3029.
96. Schmid-Burgk JL, Gao L, Li D, et al. Highly parallel profiling of Cas9 variant specificity. *Mol Cell* 2020; 78(4): 794–800.e8.
97. Wienert B, Wyman SK, Yeh CD, et al. CRISPR off-target detection with DISCOVER-seq. *Nat Protoc* 2020; 15(5): 1775–1799.
98. Harmatz P, Prada CE, Burton BK, et al. First-in-human in vivo genome editing via AAV-zinc-finger nucleases for mucopolysaccharidosis I/II and hemophilia B. *Mol Ther* 2022; 30(12): 3587–3600.
99. Li H, Haurigot V, Doyon Y, et al. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* 2011; 475(7355): 217–221.
100. Anguela XM, Sharma R, Doyon Y, et al. Robust ZFN-mediated genome editing in adult hemophilic mice. *Blood* 2013; 122(19): 3283–3287.
101. Sharma R, Anguela XM, Doyon Y, et al. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. *Blood* 2015; 126(15): 1777–1784.
102. Chen H, Shi M, Gilam A, et al. Hemophilia A ameliorated in mice by CRISPR-based in vivo genome editing of human Factor VIII. *Sci Rep* 2019; 9(1): 16838.
103. Luo S, Li Z, Dai X, et al. CRISPR/Cas9-mediated in vivo genetic correction in a mouse model of hemophilia A. *Front Cell Dev Biol* 2021; 9: 672564.
104. Zhang JP, Cheng XX, Zhao M, et al. Curing hemophilia A by NHEJ-mediated ectopic F8 insertion in the mouse. *Genome Biol* 2019; 20(1): 276.
105. Wang Q, Zhong X, Li Q, et al. CRISPR-Cas9-mediated in vivo gene integration at the albumin locus recovers hemostasis in neonatal and adult hemophilia B mice. *Mol Ther Methods Clin Dev* 2020; 18: 520–531.
106. Huai C, Jia C, Sun R, et al. CRISPR/Cas9-mediated somatic and germline gene correction to restore hemostasis in hemophilia B mice. *Hum Genet* 2017; 136(7): 875–883.

107. Wang L, Yang Y, Breton CA, et al. CRISPR/Cas9-mediated in vivo gene targeting corrects hemostasis in newborn and adult factor IX-knockout mice. *Blood* 2019; 133(26): 2745–2752.
108. Lee JH, Oh HK, Choi BS, et al. Genome editing-mediated knock-in of therapeutic genes ameliorates the disease phenotype in a model of hemophilia. *Mol Ther Nucleic Acids* 2022; 29: 551–562.
109. He X, Zhang Z, Xue J, et al. Low-dose AAV-CRISPR-mediated liver-specific knock-in restored hemostasis in neonatal hemophilia B mice with subtle antibody response. *Nat Commun* 2022; 13: 7275.
110. Chen X, Niu X, Liu Y, et al. Long-term correction of hemophilia B through CRISPR/Cas9 induced homology-independent targeted integration. *J Genet Genomics* 2022; 49(12): 1114–1126.
111. Esposito F, Dell'Aquila F, Rhie M, et al. Safe and effective liver-directed AAV-mediated homology-independent targeted integration in mouse models of inherited diseases. *Cell Rep Med* 2024; 5(7): 101619.
112. Lisjak M, De Caneva A, Marais T, et al. Promoterless gene targeting approach combined to CRISPR/Cas9 efficiently corrects hemophilia B phenotype in neonatal mice. *Front Genome Ed* 2022; 4: 785698.
113. Ohmori T, Nagao Y, Mizukami H, et al. CRISPR/Cas9-mediated genome editing via postnatal administration of AAV vector cures haemophilia B mice. *Sci Rep* 2017; 7(1): 4159.
114. Stephens CJ, Lauron EJ, Kashentseva E, et al. Long-term correction of hemophilia B using adenoviral delivery of CRISPR/Cas9. *J Control Release* 2019; 298: 128–141.
115. Han JP, Kim M, Choi BS, et al. In vivo delivery of CRISPR-Cas9 using lipid nanoparticles enables antithrombin gene editing for sustainable hemophilia A and B therapy. *Sci Adv* 2022; 8(3): eabj6901.
116. Lee JH, Han JP, Song DW, et al. In vivo genome editing for hemophilia B therapy by the combination of rebalancing and therapeutic gene knockin using a viral and non-viral vector. *Mol Ther Nucleic Acids* 2023; 32: 161–172.
117. Barzel A, Paulk NK, Shi Y, et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature* 2015; 517(7534): 360–364.
118. Rong L, Chen D, Huang X, et al. Delivery of Cas9-guided ABE8e into stem cells using poly(1-lysine) polypeptides for correction of the hemophilia-associated FIX missense mutation. *Biochem Biophys Res Commun* 2022; 628: 49–56.
119. Tonetto E, Cucci A, Follenzi A, et al. DNA base editing corrects common hemophilia A mutations and restores factor VIII expression in vitro and ex vivo models. *J Thromb Haemost* 2024; 22(8): 2171–2183.
120. Park CY, Kim DH, Son JS, et al. Functional correction of large factor VIII gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR-Cas9. *Cell Stem Cell* 2015; 17(2): 213–220.
121. Wu Y, Hu Z, Li Z, et al. In situ genetic correction of F8 intron 22 inversion in hemophilia A patient-specific iPSCs. *Sci Rep* 2016; 6: 18865.
122. Fiumara M, Ferrari S, Omer-Javed A, et al. Genotoxic effects of base and prime editing in human hematopoietic stem cells. *Nat Biotechnol* 2024; 42(6): 877–891.
123. Gillmore JD, Gane E, Taubel J, et al. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *N Engl J Med* 2021; 385(6): 493–502.
124. Cohn DM, Gurugama P, Magerl M, et al. CRISPR-based therapy for hereditary angioedema. *N Engl J Med* 2025; 392(5): 458–467.
125. Singh K, Fronza R, Evens H, et al. Comprehensive analysis of off-target and on-target effects resulting from liver-directed CRISPR-Cas9-mediated gene targeting with AAV vectors. *Mol Ther Methods Clin Dev* 2024; 32(4): 101365.
126. Davis AJ and Chen DJ. DNA double strand break repair via non-homologous end-joining. *Transl Cancer Res* 2013; 2(3): 130–143.