REVIEW ARTICLE OPEN

Check for updates

Therapeutic in vivo genome editing: innovations and challenges in rAAV vector-based CRISPR delivery

Jin-Seok Gil[1,4], Soyeon Lee[1,4] and Taeyoung Koo[1,2,3]✉

The integration of CRISPR systems with recombinant adeno-associated virus (rAAV) vectors has opened new possibilities for therapeutic genome editing, offering potential treatments for both genetic and non-genetic disorders. rAAV vectors have emerged as promising vehicles for in vivo gene therapy due to their favorable safety profile, high tissue specificity, and ability to induce sustained transgene expression. However, their limited packaging capacity has been a significant challenge for delivering large CRISPR molecules. To overcome this limitation, innovative strategies have been developed, including the use of compact Cas orthologs, dual rAAV vector systems, and trans-splicing rAAV vectors. These approaches have significantly improved the efficiency of genome editing for therapeutic applications. This review presents recent advancements in rAAV-CRISPR-mediated in vivo gene therapy, highlighting key technological innovations, current challenges, and the therapeutic potential of these strategies in the development of next-generation gene therapies.

Gene Therapy; https://doi.org/10.1038/s41434-025-00573-2

INTRODUCTION

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system represents a groundbreaking genome editing technology. Therapeutic applications of CRISPR-based genome editing are increasingly being explored for the treatment of a wide range of genetic disorders. A significant milestone in the therapeutic use of the CRISPR-Cas system was achieved with the FDA approval of Casgevy, the first ex vivo CRISPR-based therapeutic. Casgevy is designed to treat severe sickle cell disease and transfusion-dependent beta-thalassemia by editing the erythroid-specific enhancer of the *BCL11A* gene in a patient's hematopoietic stem cells to reactivate fetal hemoglobin expression, thereby compensating for defective adult hemoglobin [1]. In addition, recent clinical advances using LNP-mediated base editing to correct severe carbamoyl-phosphate synthetase 1 deficiency further underscore the therapeutic potential of CRISPR technologies, exemplified by the first patient-specific in vivo gene editing study for a rare genetic disease [2]. These clinical trials have demonstrated significant reductions in disease-related symptoms and transfusion requirements, underscoring the therapeutic potential of CRISPR technology. In this review, we summarize recent advances in recombinant AAV (rAAV) vector-based in vivo CRISPR applications, focusing on therapeutic applications. We also discuss emerging strategies, vector innovations, clinical progress, and challenges that must be addressed to translate these approaches into safe and effective treatments for genetic diseases.

CRISPR-BASED GENOME EDITING MECHANISMS

The CRISPR-Cas system, originally derived from the adaptive immune mechanism of microbes, functions through the sequence-specific guidance of small RNAs known as guide RNAs (gRNAs). In CRISPR-mediated genome editing, two key RNAs, the CRISPR RNA (crRNA) and the trans-activating CRISPR RNA (tracrRNA), are transcribed to direct target recognition. To enhance genome editing efficiency, a chimeric single-guide RNA (sgRNA) was developed by linking crRNA and tracrRNA. The Cas9: gRNA complex identifies specific protospacer adjacent motif (PAM) sequences, inducing double-strand breaks (DSBs) in the genome [3, 4]. Following the induction of DSBs, DNA repair pathways such as non-homologous end joining (NHEJ) and homology-directed repair (HDR) are activated. NHEJ often results in small insertions or deletions (indels) at the cleavage site, while HDR enables precise genetic modifications using a homologous DNA template. These pathways are harnessed to introduce targeted genetic modifications.

CRISPR-BASED PRECISION GENOME EDITING TOOLS

The ability to reprogram Cas proteins for site-specific DNA recognition has spurred the development of advanced genome editing platforms that bypass the reliance on DSBs. Base editors (BE), such as the cytidine base editors (CBEs), facilitate the conversion of cytosine (C) to thymine (T) (or guanine (G) to adenine (A)) by integrating cytidine deaminase enzymes [5]. Similarly, adenine base editors (ABEs) enable the conversion of A to G (or T to C) via adenosine deaminase [6]. These advancements enable precise nucleotide substitutions without inducing DSBs, thereby minimizing potential off-target effects and enhancing genome editing specificity.

Prime editors (PEs) have subsequently been developed, enabling more versatile genome modifications. PEs consist of a

[1]Department of Regulatory Science, Graduate School, Kyung Hee University, Seoul, Republic of Korea. [2]Department of Pharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Seoul, Republic of Korea. [3]Institute of Integrated Pharmaceutical Sciences, Kyung Hee University, Seoul, Republic of Korea. [4]These authors contributed equally: Jin-Seok Gil, Soyeon Lee. ✉email: taeyoungkoo@khu.ac.kr

Received: 17 June 2025 Revised: 22 September 2025 Accepted: 13 October 2025
Published online: 12 November 2025

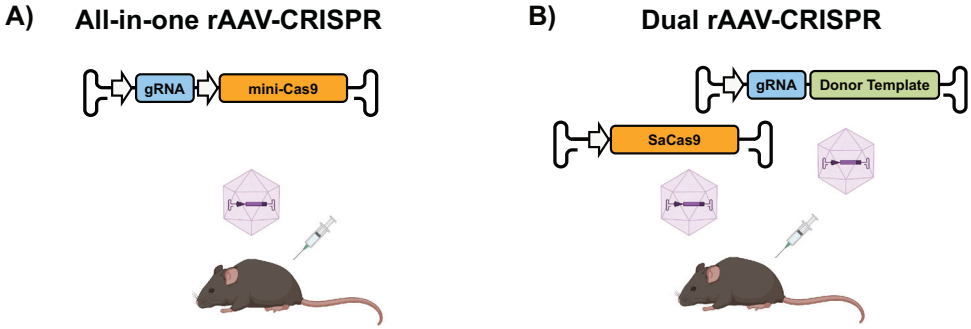


Fig. 1 Schematic diagram of single- and dual-rAAV vector strategies for in vivo delivery of complete CRISPR components. A All-in-one rAAV vector encoding a hypercompact mini-Cas9 along with its gRNA. **B** Dual-rAAV vector system encoding SaCas9 with its gRNA and a donor template, delivered separately by two vectors.

Cas9 nickase fused to a reverse transcriptase enzyme, allowing targeted incorporation of precise edits without inducing DSBs [7]. The prime editing guide RNA (pegRNA) guides the PE protein to the target site and provides a template for the desired genome editing. However, the efficiency of PEs remains suboptimal, and ongoing research aims to improve their efficiency and broaden their applicability.

RECOMBINANT AAV VECTORS FOR IN VIVO CRISPR DELIVERY

rAAV vectors are attractive vehicles for efficient and safe in vivo gene delivery. Numerous independent clinical trials and approved gene therapy products have demonstrated their strong potential for treating various disorders [8–12]. rAAV vectors offer several advantages over other viral vectors, primarily due to their non-pathogenic nature, which makes them safer for clinical use [13]. Secondly, rAAV vectors largely persist in an episomal form within cells, ensuring sustained transgene expression in the targeted organ. Animal studies and emerging human data suggest that transgene expression can persist for over a decade [14]. This durability of therapeutic response is critical for long-term treatment success, particularly since rAAV vector-based therapies cannot be re-administered with the same vector serotype [15]. Furthermore, rAAV vectors elicit a low immune response, with limited activation of both innate inflammatory pathways and adaptive immunity, including neutralizing antibody production and cytotoxic T-cell responses, thereby allowing them to persist in cells for extended periods [13]. Lastly, rAAV vectors exhibit high tissue tropism, facilitating efficient delivery of transgenes to targeted organs [16].

Despite these advantages, the limited packaging capacity of rAAV vectors (<4.7 kb) remains a significant challenge for in vivo delivery of CRISPR components. To address this, several strategies have been developed, including the utilization of compact Cas orthologs and rational engineering of rAAV vectors. These approaches have improved delivery efficiency and broadened therapeutic applicability. We review recent advancements in rAAV-mediated CRISPR genome editing that help circumvent size limitations, enabling more efficient delivery of CRISPR tools and expanding the scope of treatable genetic conditions.

RAAV VECTOR-MEDIATED CRISPR CLINICAL TRIALS

Among the various delivery platforms under investigation, rAAV vectors have emerged as one of the most widely used systems for in vivo therapeutic applications, owing to their favorable safety profile, broad tissue tropism, and capacity for long-term transgene expression. These features make rAAV vectors particularly suitable for delivering CRISPR tools directly into target tissues, enabling precise genome editing via systemic or localized administration. Building upon the success of ex vivo strategies, in vivo CRISPR therapeutics are now advancing toward clinical realization. EDIT-101, the first in vivo CRISPR-based therapy to enter human trials, targets the IVS26 mutation in the *CEP290* gene, which causes Leber Congenital Amaurosis type 10 (LCA10), a severe inherited retinal disorder. Delivered via rAAV serotype 5 (AAV5) vectors through subretinal injection, EDIT-101 delivers SpCas9 and two gRNAs targeting intronic regions flanking the IVS26 mutation directly into the retinal cells, excising the aberrant splice donor site and thereby restoring normal splicing and functional CEP290 expression. Early findings from the phase 1/2 BRILLIANCE trial (NCT03872479) report favorable safety outcomes and improved photoreceptor function in eleven of fourteen treated participants, supporting the feasibility of rAAV vector-mediated in vivo gene editing in humans [17]. Nevertheless, the limited efficacy observed, combined with the small target patient population, led to the discontinuation of further enrollment and a shift toward seeking a development partner. Notably, these clinical advances underscore the growing potential of CRISPR-based therapeutics for the treatment of previously intractable genetic disorders. The demonstrated feasibility of rAAV vector-mediated in vivo genome editing further supports its clinical relevance, particularly for targeting tissues that are inaccessible to ex vivo approaches. Moving forward, systematic evaluation and optimization of rAAV vector-based delivery platforms will be critical to achieving safe, efficient, and tissue-specific CRISPR delivery across a broad spectrum of genetic diseases.

DEVELOPMENT OF RAAV VECTOR PLATFORMS FOR IN VIVO CRISPR DELIVERY

All-in-one rAAV vectors employing compact nucleases

The size limitation of rAAV vectors remains a significant challenge for in vivo CRISPR delivery. However, the use of compact Cas variants offers a promising strategy to overcome this obstacle. The discovery of hypercompact CRISPR systems, such as *Campilobacter jejuni* Cas9 (CjCas9) [18, 19], *Staphylococcus aureus* Cas9 (SaCas9) [20], and Cas12f [21], has enabled efficient in vivo delivery using a single rAAV vector for therapeutic applications (Fig. 1A and Table 1). As one example, subretinal delivery of rAAV8 vectors encoding CasMINI_v3.1/ge4.1 targeting the *Nr2e3* gene achieved transduction efficiencies of over 70% in GFP+ retinal cells of $Rho^{P23H/+}$ mice, a disease model of retinitis pigmentosa (RP) [22]. One month post-injection, a significant improvement in cone photoreceptor function was shown, as evidenced by increased photopic b-wave values compared to control groups [22]. As another example, rAAV9 vectors were utilized to deliver the compact Nme2-ABE8e, derived from *Neisseria meningitidis*, to correct the *Fah* mutation responsible for hereditary tyrosinemia type 1 (HT1) in the $Fah^{PM/PM}$ mice. Although the overall editing efficiency was relatively low (0.34%), the treatment successfully restored 6.5% FAH+ hepatocytes, exceeding the therapeutic

threshold of repairing 1 in 100,000 hepatocytes, demonstrating the therapeutic potential of all-in-one rAAV-mediated in vivo CRISPR delivery [23].

Recent advances have identified effectors such as IscB [24, 25] and TnpB [26], putative ancestors of modern Cas proteins, as promising tools for ultra-compact genome editing. Due to their small molecular size, they offer enhanced compatibility with the packaging constraints of rAAV vectors and may present a reduced immunogenicity profile compared to conventional CRISPR systems. In preclinical studies, systemic delivery of the rAAV8 vectors encoding EnIscB–ωRNA-based ABE effectively corrected a pathogenic mutation in the *Fah* gene in a tyrosinemia mouse model. This treatment resulted in 15% editing efficiency and restoration of *Fah* expression [24]. In a separate study, intramuscular injection of an rAAV9 vector encoding IscB.m16*-CBE led to 30% exon skipping and recovery of dystrophin expression in a humanized mouse model of DMD (hDMD) [25]. Furthermore, a scAAV9 vector encoding TnpB targeting *Pcsk9* delivered via systemic injection achieved up to 56% editing in the liver and significantly reduced blood cholesterol levels [26]. These findings highlight the therapeutic potential of AAV-mediated small CRISPR-derived platforms for in vivo genome editing.

Dual-rAAV vectors for full-length CRISPR delivery

Dual rAAV vectors encoding CRISPR components, in which the Cas9 nuclease and its corresponding gRNA are delivered on separate vectors, have been developed to overcome the packaging capacity limitation of rAAV. This strategy enables delivery of full-length Cas nuclease together with multiple gRNAs and/or large HDR donor templates, thereby broadening the therapeutic scope of genome editing (Fig. 1B). As a representative application, two rAAV9 were employed, one encoding SaCas9, and the other encoding a gRNA with a donor spanning exon 52–79 of the *DMD* gene. This approach successfully restored full-length dystrophin expression in treated muscles through precise homology-independent targeted integration in *hDMD*-KO mice lacking *DMD* exon 52 [27]. Notably, systemic delivery via facial vein injection at postnatal day 2 achieved 3% genomic correction and over 25% restoration of dystrophin transcripts in treated tissues, underscoring the therapeutic potential of dual rAAV vector-based editing for large-fragment insertion in vivo [27]. Despite these advances, efficient co-transduction of both vectors into the same target cell remains a major challenge, as insufficient co-delivery can markedly reduce editing efficiency. Moreover, large CRISPR platforms such as BEs and PEs still exceed the packaging capacity of rAAV vectors, precluding their delivery as full-length molecules in this system.

Dual-rAAV vectors for split delivery of CRISPR components

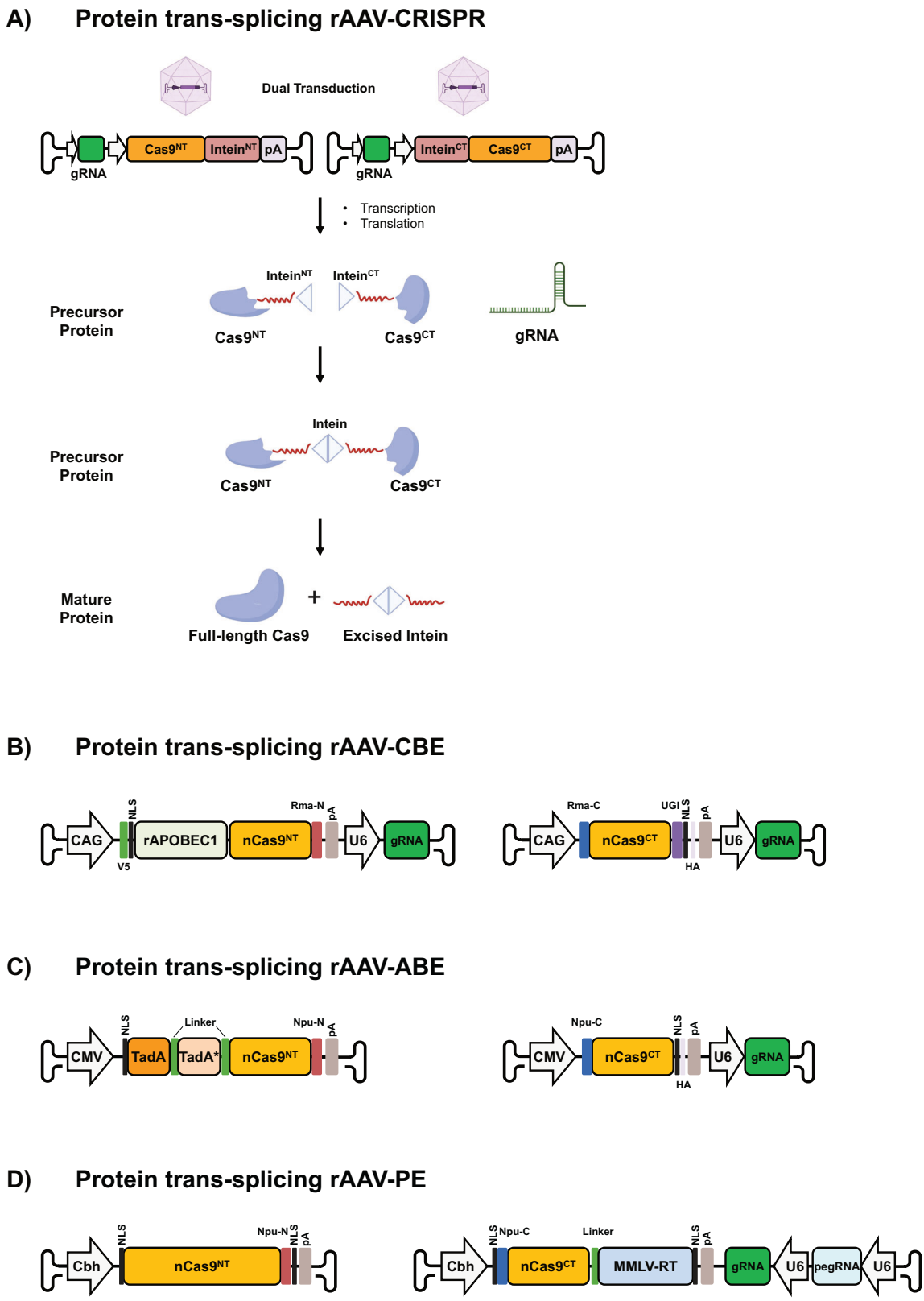
Protein trans-splicing rAAV vectors encoding split-CRISPR molecules. Protein trans-splicing rAAV vector platforms represent a promising strategy to overcome the packaging limitation of rAAV vector-mediated delivery of large CRISPR components, such as BEs or PEs, which include additional functional domains, by employing an intein-mediated protein reconstitution strategy [28–33]. In this system, the Cas9 transgene is split into N-terminal and C-terminal fragments, each packaged into a separate rAAV vector. Upon co-delivery and expression in the same target cell, the two protein fragments are reconstituted into a functional full-length protein via intein-mediated splicing (Fig. 2A) [34]. Inteins are self-catalyzing protein elements that are capable of self-splicing, ligating flanking exteins to form a full-length protein [35]. During the splicing process, the intein excises itself, joining the N- and C-terminal fragments of Cas9 via a peptide bond.

This strategy has demonstrated therapeutic efficacy in several preclinical studies (Table 2). For example, a split CBE was employed to treat amyotrophic lateral sclerosis (ALS) caused by mutations in the *SOD1* gene. Delivered in two parts via rAAV9, one

Table 1. All-in-one rAAV vector-based CRISPR delivery.

Nuclease	Size (bp)	Gene	Administration route	Editing efficiency	rAAV serotype	Animal model	References
CasMINI_3.1	1587	*Nr2e3*	Subretinal	>70% in GFP^{+} cells	8	*Rho*$^{P23H/+}$ mouse model of RP	Cui et al. [22]
Nme2-ABE8e	3840	*Fah*	Intravenous	0.34%	9	Mouse model of HT1	Zhang et al. [23]
IscB-ABE	2793	*Fah*	Intravenous	15%	8	Mouse model of HT1	Guo et al. [24]
IscB-CBE	2136	*hDMD*	Intramuscular	7%	9	Humanized DMD^{E51del} mice	Xiao et al. [25]
TnpB	1224	*Pcsk9*	Intravenous	56%	9	C57BL/6	Marquart et al. [26]

rAAV Recombinant adeno-associated virus, *CBE* Cytosine base editor, *ABE* Adenine base editor, *RP* Retinitis pigmentosa, *DMD* Duchenne muscular dystrophy, *HT1* Hereditary tyrosinemia type 1.



encoding rAPOEC1 fused to nCas9-NT and the other encoding nCas9-CT, the CBE was expressed as two half-length proteins that reassembled into a full-length protein through intein-mediated protein splicing (Fig. 2B). This approach achieved 1.2% editing at the *SOD1*, improving neuromuscular function and reducing SOD1 immunoreactive inclusions by 40% in the mouse model of ALS [28]. In another application, intein-mediated trans-splicing rAAV9-CBE was used to correct the *Npc1* c.3182T>C mutation associated

Fig. 2 Protein trans-splicing rAAV vectors for in vivo delivery of split-CRISPR components. A Schematic diagram of the protein trans-splicing rAAV-CRISPR system, in which split-inteins mediate the reconstitution of full-length proteins from N-terminal (NT) and C-terminal (CT) precursors of the Cas effector. **B** Trans-splicing rAAV-CBE vector system utilizing split nickase Cas9 (nCas9), rAPOBEC1 deaminase, and split inteins for cytidine base editing. $nCas9^{NT}$ is fused to rAPOBEC1 and the N-terminal fragment of the DnaB split-intein (Rma-N) derived from *Rhodothermus marinus*, whereas $nCas9^{CT}$ is fused to uracil-DNA glycosylase inhibitor (UGI), and the C-terminal fragment of the Intein (Rma-C). **C** Trans-splicing rAAV-ABE vectors encoding split nCas9, TadA deaminase, and N- and C-terminal inteins for reconstitution of the full-length ABE. $nCas9^{NT}$ is fused to the TadA/TadA* heterodimer and the N-terminal fragment of DnaE intein derived from *Nostoc punctiforme* (Npu-N), while $nCas9^{CT}$ is fused to the corresponding C-terminal fragment (Npu-C). **D** Trans-splicing rAAV-PE vector system encoding split nCas9, Moloney murine leukemia virus reverse transcriptase (MMLV-RT), pegRNA, and gRNA for efficient prime editing. All vector schematics indicate the positions of inverted terminal repeats (ITRs), promoters (CAG, CMV, Cbh, and U6), polyadenylation signals (pA), nuclear localization signals (NLS), epitope tags (V5 and HA), and other regulatory elements.

Table 2. Protein trans-splicing rAAV vectors encoding split-CRISPR molecules.

Nuclease	Gene	Administration route	Editing efficiency	rAAV serotype	Animal model	References
CBE4	*SOD1* G93A	Intrathecal	1.2%	9	mouse model of ALS	Lim et al. [28]
CBE3.9max	*Npc1* I1061T	Retro-orbital	0.3% (unsorted), 42% (sorted)	9	*Npc1*^{I1061T} homozygous mice	Levy et al. [29]
ABEmax	*Rpe65* R44X	Subretinal	13.5%	9	rd12 mouse model of LCA	Jo et al. [30]
v3emPE	*Pcsk9* Q155H	Retro-orbital	39%	9	C57BL/6	Davis et al. [31]
PEmax	*Pde6a* D670G	Subretinal	9.4%	2.NN	*Pde6a*$^{nmf363/nmf363}$ mice	Liu et al. [32]
PE^{SpRY}	*Pde6b* R560C	Subretinal	40.9% (unsorted), 76.3% (sorted)	8	*Pde6b*^{rd10} mice	Qin et al. [33]

Npc1 Niemann-Pick disease type C1, *LCA* Leber congenital amaurosis, *ALS* Amyotrophic lateral sclerosis.

Table 3. RNA trans-splicing rAAV vectors encoding split-CRISPR molecules.

Nuclease	Gene	Administration route	Editing efficiency	rAAV serotype	Animal model	References
ABE	*Dmd*	Intramuscular	3.3%	9	*Dmd*-KO mice	Ryu et al. [37]
NG-ABE8e	*MAPT*	Intracranial	5.7%	9	PS19 mice	Gee et al. [38]
PE	*Rpe65*	Subretinal	6.4%	8	rd12 mouse model of LCA	Jang et al. [39]

KO Knockout.

with Niemann-Pick disease type C [29]. In the mouse cerebellum, editing efficiencies were 0.3% in unsorted nuclei and 42% in sorted nuclei. This led to 10% increase in lifespan, improved Purkinje cell survival, and reduced inflammation, demonstrating the therapeutic potential of this approach for CNS disorders. Similarly, trans-splicing rAAV9 vectors encoding ABEmax targeting the *Rpe65* R44X mutation achieved 13.5% editing efficiency in a mouse model of LCA (*rd12*), restoring light-induced electrical responses in the retina (Fig. 2C) [30].

This protein trans-splicing rAAV vector system has also been successfully applied for in vivo delivery of PEs. In *C57BL/6* mice, systemic injection of trans-splicing rAAV9 vectors encoding v3emPE achieved 39% editing efficiency of *Pcsk9* Q155H in the liver at eight weeks post-injection (Fig. 2D) [31]. This treatment led to a 20% reduction in plasma cholesterol levels compared to untreated controls. Similarly, subretinal delivery of trans-splicing rAAV2.NN vectors encoding PE targeting the *Pde6a* D670G mutation achieved 9.4% gene editing in the mouse retina, preserving visual functions and photoreceptor integrity, along with improving retinal structure and supporting functional recovery [32]. In a separate study, subretinal administration of trans-splicing rAAV8 vectors encoding PE achieved 40.9% editing in total retinal cells of *rd10* mice carrying the *Pde6b* R560C mutation [33]. This editing preserved photoreceptor cells, improved retinal structure and function, and restored PDE6 activity, which is essential for phototransduction and vision.

Collectively, these outcomes highlight the potential of trans-splicing rAAV-mediated CRISPR delivery to slow disease progression. However, intein splicing efficiency may vary across tissue types, or disease context approaches present several limitations [36]. In addition, efficient co-transduction and proper stoichiometric expression of both vector components potentially result in incomplete protein reconstitution. Immune responses against inteins or misfolded intermediates may also compromise in vivo expression and stability. Nonetheless, the protein trans-splicing rAAV vector-mediated CRISPR delivery system is a versatile and powerful platform for expanding the therapeutic applicability of CRISPR-based genome editing.

RNA trans-splicing rAAV vectors encoding split-CRISPR molecules. Unlike protein trans-splicing rAAV vectors, which rely on reconstitution at the protein level, RNA trans-splicing rAAV vectors exclusively produce full-length mRNA, without expression of nonfunctional or deleterious intermediates and provide greater flexibility in selecting split sites, independent of protein-folding constraints. The RNA trans-splicing approach has demonstrated compatibility with both BE and PE systems and has been successfully applied across various tissues, underscoring its versatility for therapeutic genome editing (Table 3) [37–39]. As a proof-of-concept, RNA trans-splicing rAAV vectors encoding an

ABE were developed. The 5'-half vector carries the N-terminal Cas nuclease fused with TadA together with a splicing donor (SD), while the 3'-half vector carries the C-terminal Cas nuclease along with a splicing donor (SA) and poly(A) signal. Upon co-transduction, homologous recombination between the ITRs of the two rAAV vectors mediates concatemer formation, generating a fused vector genome. Transcription across these concatemers produces pre-mRNA transcripts that contain both SD and SA sequences. The endogenous splicesome recognizes these splice sites and mediates precise RNA trans-splicing, resulting in a full-length mature ABE mRNA that is subsequently translated into a complete ABE protein (Fig. 3A).

Subsequently, intramuscular delivery of trans-splicing rAAV-ABE vectors in *Dmd* knockout mice achieved 3.3% editing, correcting a premature stop codon in the *Dmd* gene to the wild-type at eight weeks post-injection (Fig. 3B). This targeted editing restored dystrophin expression in 17% of myofibers, demonstrating the functional efficacy of the system [37]. Building on this approach, a separate study utilized RNA trans-splicing rAAV9 vectors to deliver NG-ABE8e in PS19 mice, a tauopathy model harboring the *MAPT*-P301S mutation [38]. Intracranial administration achieved 5.7% editing efficiency in hippocampal cells, leading to a significant reduction in insoluble tau aggregates and phosphorylated tau levels. Notably, treated mice exhibited marked improvements in cognitive performance compared to mock control groups. Furthermore, the utility of RNA trans-splicing rAAV vectors has been extended to PE systems for delivery in the *rd12* mice (Fig. 3C) [39]. Subretinal administration of rAAV8 vectors encoding PE components resulted in a 6.4% editing efficiency in retinal pigment epithelial cells, restoring *Rpe65* expression and significantly improving visual function. The use of RNA trans-splicing rAAV-CRISPR systems highlights their therapeutic potential. However, precise vector design, including optimization of splicing signals and ITR homology regions, is critical to ensure consistent and efficient genome editing performance.

Building upon these findings, the mRNA trans-splicing-based rAAV vector approach, reconstitution via mRNA trans-splicing (REVeRT), has been developed to deliver the CRISPR components [40]. In this system, Dual rAAV vectors each transcribe a separate pre-mRNA. Vector carries a promoter, the NT coding sequence of Cas nuclease, SD, and binding domain (BD). The 3'-half vector carries a promoter, a SA, the C-terminal Cas nuclease, and BD. After co-transduction of rAAV vectors, the pre-mRNAs hybridize via their complementary BDs, which juxtapose the engineered SD and SA. This interaction facilitates the physical proximity required for a trans-splicing event. This process results in the reconstitution of a mature full-length mRNA and its subsequent translation into the complete CRISPR protein (Fig. 4). In wild-type mice, REVeRT-mediated rAAV8 vector delivery of split dCas9-VPR achieved robust reconstitution across multiple tissues, including retina, hippocampus, liver, lung, heart, and skeletal muscle, leading to efficient transactivation of the *Myo7b* gene (Fig. 4).

Collectively, the REVeRT system demonstrates the feasibility of RNA-level reconstitution without relying on ITR-mediated recombination. Its efficiency depends largely on the rational design of binding domains and splice site architecture, which are essential to ensure precise splicing and to minimize aberrant or nonfunctional transcripts.

ENGINEERING AAV CAPSIDS FOR TARGETED IN VIVO CRISPR DELIVERY

rAAV vectors exhibit tissue-specific tropism that has been exploited to target organs such as muscle, liver, and the central nervous system (CNS) for therapeutic gene delivery. However, this tropism is not absolute, and most AAV serotypes display a strong preference for the liver. This hepatic tropism, while useful for liver-targeted therapies, poses a significant challenge for systemic delivery aimed at extrahepatic tissues, as it can reduce the proportion of vectors reaching the intended target. In addition, it raises concerns regarding a reduction in therapeutic efficacy and the risk of off-target toxicity and immune responses. To overcome these limitations, a range of rAAV capsid engineering strategies has been developed to enhance tissue specificity [41–44]. For example, insertion of a 7-mer PHP.B peptide into the AAV1 capsid, identified through library-based directed evolution, enabled efficient blood-brain barrier penetration and enhanced CNS transduction in the mouse brain following intravenous delivery of AAV1-PHP.B carrying CRISPRa [41]. This approach resulted in robust transgene activation of endogenous genes such as *Camk2a*, *Nrf2*, and *Keap1* in mouse brain tissue. Similarly, the muscle-tropic variant AAVMYO, also derived through library-based directed evolution, demonstrated superior skeletal muscle targeting compared with conventional serotype AAV9, while markedly reducing off-target expression in the liver [42]. In parallel, rational design approaches have yielded variants such as myoAAV, which exhibit enhanced muscle specificity and reduced off-target biodistribution [43].

More recently, machine learning-guided approaches such as Fit4Function have enabled the design of multi-trait rAAV capsids with enhanced liver tropism and cross-species predictive performance [44], achieving up to 1000-fold greater transduction efficiency in human hepatocytes relative to rAAV9 vectors. These advances underscore the potential of library-based approaches, rational design, and AI-driven engineering to generate next-generation rAAVs tailored for precise and safe in vivo CRISPR delivery.

ENGINEERING HIGH-FIDELITY CRISPR PLATFORMS FOR RAAV VECTOR DELIVERY

Enhancing the fidelity of CRISPR systems is essential to improving the safety of rAAV vector-delivered therapeutics. Extensive protein engineering has yielded high-fidelity (HF) variants including eSpCas9 [45], SpCas9-HF1 [46], and efSaCas9 [47]. Similarly, next-generation BEs such as ABE8e-V106W [48] and eCBE [49] incorporate point mutations that reduce off-target editing at unintended genomic sites without substantially reducing efficiency. PEs, although generally associated with fewer off-target activity compared with nucleases or BEs, continue to benefit from fidelity improvements, particularly through optimization of pegRNA scaffolds. Recent refinements, such as the development of mpegRNAs, have further enhanced editing precision by reducing undesired indels while maintaining editing efficiency [50]. Collectively, these advances demonstrated that the development of high-fidelity nucleases, BEs, and PEs, together with optimized gRNA structures, represents a critical strategy for ensuring the specificity of rAAV vector-delivered CRISPR therapeutics, particularly as these systems progress toward clinical applications.

OVERCOMING IMMUNOLOGICAL CHALLENGES OF RAAV VECTORS FOR CRISPR-BASED THERAPIES

Immune responses to both rAAV vectors and CRISPR components pose significant obstacles to the long-term success of in vivo genome editing therapies. rAAV vectors, though generally considered to have low immunogenicity, can still elicit robust immune responses, particularly in individuals with pre-existing neutralizing antibodies (NAbs) against AAV capsid proteins. Moreover, even in the absence of pre-existing immunity, the host immune system can mount adaptive responses following rAAV vector administration. Cellular immune responses directed against transduced cells expressing foreign proteins, such as bacterial-derived CRISPR enzymes, may lead to cytotoxic T lymphocyte (CTL)-mediated clearance of the edited cells [51, 52]. This not only limits the duration of therapeutic benefit but also raises safety concerns due to inflammation or tissue damage.

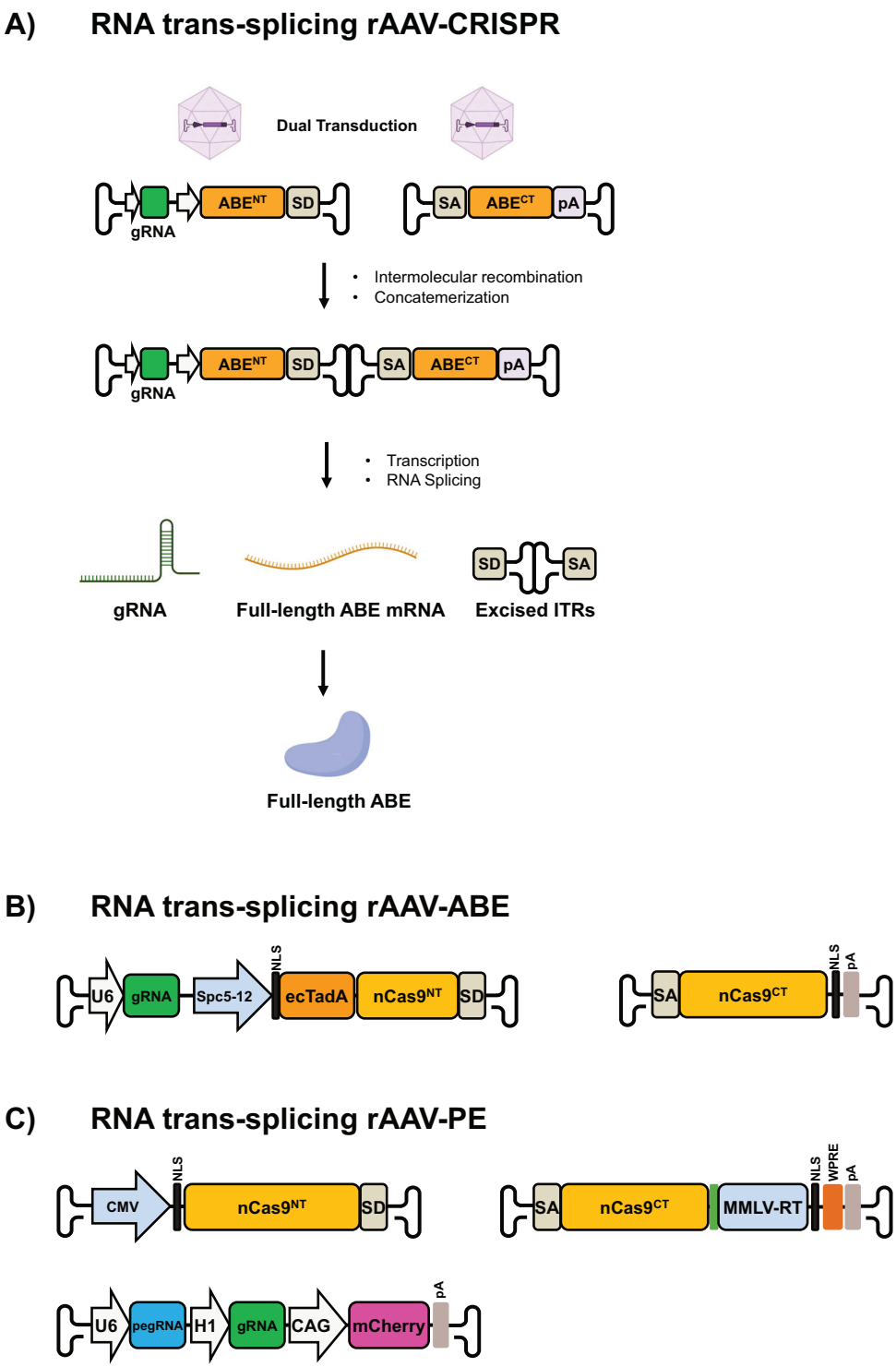


Fig. 3 RNA trans-splicing rAAV vectors for in vivo delivery of split-CRISPR components. A Schematic diagram of RNA trans-splicing rAAV-ABE vectors, which enable reconstitution of the full-length genome through intermolecular recombination and concatemerization between ITRs, followed by RNA splicing and full-length ABE mRNA expression. **B** The trans-splicing rAAV-ABE vectors contain nCas9, *E. Coli* TadA (ecTadA), a splicing donor (SD), and a splicing acceptor (SA) to mediate RNA trans-splicing. **C** The trans-splicing rAAV-PE vectors employ three separate rAAV vectors to reconstitute full-length PE. Two vectors deliver split nCas9 fragments and MMLV-RT, while the third provides pegRNA, gRNA, and mCherry. WPRE, Woodchuck hepatitis virus Posttranscriptional Regulatory Element.

To overcome these immunological barriers, several complementary strategies are being actively pursued. One approach focuses on developing less immunogenic Cas variants by protein engineering of immunogenic epitopes on the Cas nuclease, thereby reducing binding affinity to the major histocompatibility complex and attenuating the induction of cytotoxic T cell responses [53]. A second strategy employs a self-eliminating rAAV design in which Cas9 targets the vector genome, thereby restricting long-term expression and enabling self-inactivation of the vector [54]. Capsid engineering represents another major tool

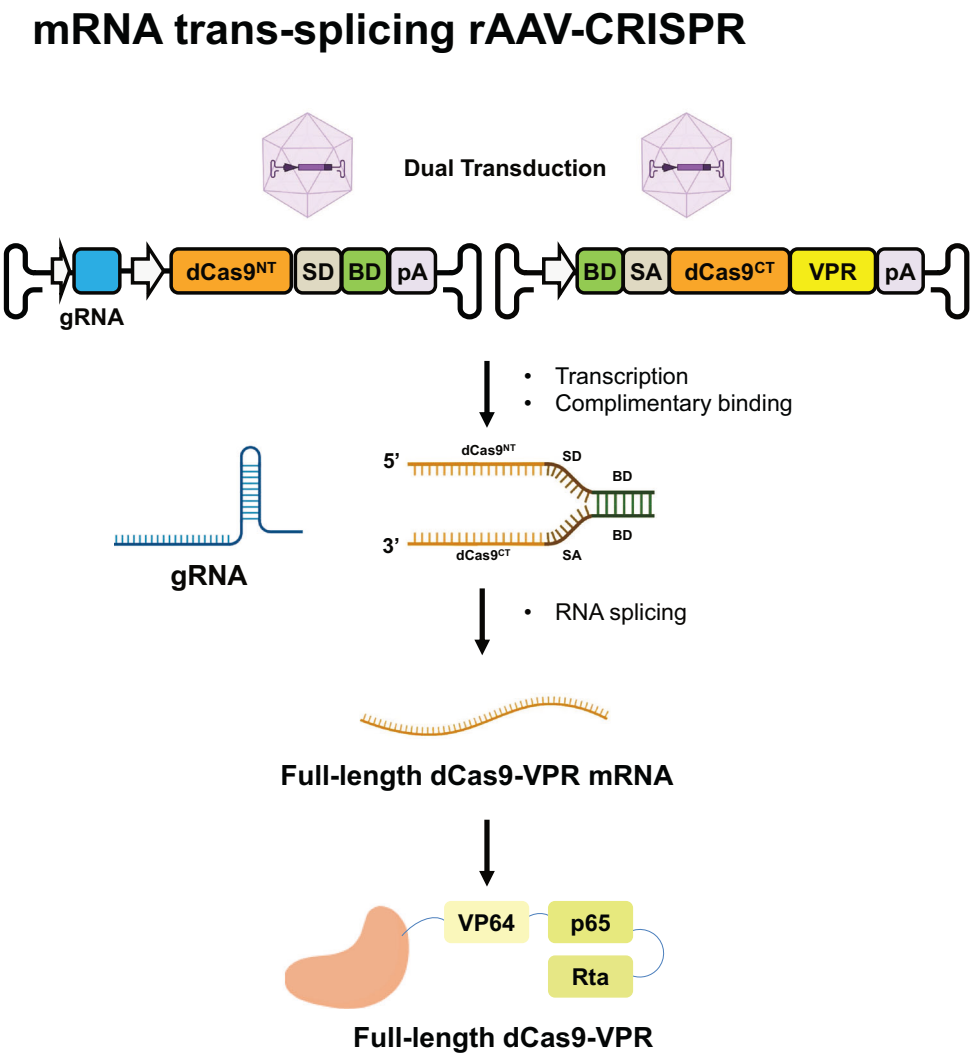


Fig. 4 mRNA trans-splicing rAAV-vectors for delivering split-CRISPR components. Split dCas9-VP64-p65-Rta (VPR) fragments are reassembled into full-length dCas9-VPR through mRNA trans-splicing, enabling transcriptional activation of target genes. The mRNA trans-splicing rAAV-dCas9-VPR vectors consist of two rAAV vectors encoding split dCas9 fused to the VPR transcription activation domain, binding domain (BD), SD, and SA.

to generate rAAV variants capable of evading pre-existing NAbs [55]. In addition, immunosuppressive regimens, such as corticosteroids or targeted immune modulators, have been employed to reduce host immune responses and extend vector persistence [56]. Finally, alternative delivery vehicles, including non-viral nanoparticles and extracellular vesicles, are emerging as immune-evasive platforms that can deliver CRISPR systems [57]. Collectively, these strategies address immune compatibility for expanding the applicability and durability of rAAV-CRISPR therapeutics in diverse patient populations.

Challenges

Despite the remarkable progress in rAAV vector-mediated CRISPR delivery systems, several challenges remain that must be addressed to ensure clinical safety and therapeutic precision. One of the primary concerns arises from the intrinsic properties of vector-related risks. Recent reports of patient deaths following high-dose systemic administration of rAAV vectors have underscored persistent safety concerns, including fatalities in trials for Danon disease [58], Rett syndrome [59], X-linked myotubular myopathy [60], and DMD [61, 62]. These cases, often associated with immune-mediated toxicities, liver failure, or systemic inflammatory responses, highlight the urgent need for strategies such as AAV capsid engineering with improved tropism and safety profiles, optimized dosing regimens, and transient immunosuppression. Moreover, evolving regulatory responses by updated FDA communications and guidance reflect an increased emphasis on long-term safety monitoring and risk–benefit assessment in ongoing and future trials. Addressing these developments is critical to provide a balanced and current perspective on the safety challenges of systemic rAAV vector-based gene therapy.

CONCLUSIONS

rAAV vector-based delivery of CRISPR-Cas systems holds tremendous potential for in vivo therapeutic applications. Innovations in vector design, including compact Cas nucleases, dual-vector systems, and trans-splicing approaches, have significantly expanded the scope of rAAV vector-mediated genome editing by overcoming key limitations associated with vector packaging capacity and targeting precision. These advancements have enabled precise genome editing in diverse preclinical models, providing a strong foundation for translating these technologies into clinical settings. Despite these advances, immune responses to rAAV vectors and CRISPR components remain a major obstacle to widespread clinical implementation. Pre-existing immunity to

AAV serotypes can reduce vector transduction efficiency, while immune activation following vector administration may limit the duration of therapeutic effects. Additionally, immune responses against the Cas9 protein can result in the clearance of the edited cells, diminishing therapeutic outcomes. To overcome these challenges, ongoing efforts focus on the development of less immunogenic rAAV serotypes, the implementation of transient immunosuppression protocols, and the engineering of immune-evading Cas9 variants. These strategies are crucial for ensuring the safety, efficacy, and long-term success of rAAV-CRISPR therapies. Continued interdisciplinary research and clinical validation are vital to overcoming these barriers and unlocking the full therapeutic potential of this platform.

REFERENCES

1. Frangoul H, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK, et al. CRISPR-Cas9 gene editing for sickle cell disease and beta-Thalassemia. N Engl J Med. 2021;384:252–60.
2. Musunuru K, Grandinette SA, Wang X, Hudson TR, Briseno K, Berry AM, et al. Patient-specific in vivo gene editing to treat a rare genetic disease. N Engl J Med. 2025;392:2235–43.
3. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337:816–21.
4. Song M, Koo T. Recent advances in CRISPR technologies for genome editing. Arch Pharm Res. 2021;44:537–52.
5. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 2016;533:420–4.
6. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature. 2017;551:464–71.
7. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. 2019;576:149–57.
8. Issa SS, Shaimardanova AA, Solovyeva VV, Rizvanov AA. Various AAV serotypes and their applications in gene therapy: an overview. Cells. 2023;12:785.
9. Zhao Q, Peng H, Ma Y, Yuan H, Jiang H. In vivo applications and toxicities of AAV-based gene therapies in rare diseases. Orphanet J Rare Dis. 2025;20:368.
10. Huang J, Li J, Xu X, Li K. Adeno-associated virus vectors in retinal gene therapy: challenges, innovations, and future directions. Biomolecules. 2025;15:940.
11. Wang JH, Gessler DJ, Zhan W, Gallagher TL, Gao G. Adeno-associated virus as a delivery vector for gene therapy of human diseases. Sig Transduct Target Ther. 2024;9:78.
12. Choi E, Koo T. Muscular Dystrophy Therapy Using Viral Vector-based CRISPR/Cas. In: Yun YH, Yoder KE, editors. Biotechnologies for Gene Therapy: RNA, CRISPR, Nanobots, and Preclinical Applications. Cham: Springer International Publishing; 2022. p. 61–83.
13. Ronzitti G, Gross DA, Mingozzi F. Human immune responses to adeno-associated virus (AAV) vectors. Front Immunol. 2020;11:670.
14. Gonzalez-Sandoval A, Pekrun K, Tsuji S, Zhang F, Hung KL, Chang HY, et al. The AAV capsid can influence the epigenetic marking of rAAV delivered episomal genomes in a species dependent manner. Nat Commun. 2023;14:2448.
15. Ford JL, Karatza E, Mody H, Nagaraja Shastri P, Khajeh Pour S, Yang TY, et al. Clinical pharmacology perspective on development of adeno-associated virus vector-based retina gene therapy. Clin Pharmacol Ther. 2024;115:1212–32.
16. Zhou X, Liu J, Xiao S, Liang X, Li Y, Mo F, et al. Adeno-associated virus engineering and load strategy for tropism modification, immune evasion and enhanced transgene expression. Int J Nanomed. 2024;19:7691–708.
17. Pierce EA, Aleman TS, Jayasundera KT, Ashimatey BS, Kim K, Rashid A, et al. Gene editing for CEP290-associated retinal degeneration. N Engl J Med. 2024;390:1972–84.
18. Jo DH, Koo T, Cho CS, Kim JH, Kim JS, Kim JH. Long-term effects of in vivo genome editing in the mouse retina using Campylobacter jejuni Cas9 expressed via adeno-associated virus. Mol Ther. 2019;27:130–6.
19. Koo T, Lu-Nguyen NB, Malerba A, Kim E, Kim D, Cappellari O, et al. Functional rescue of dystrophin deficiency in mice caused by frameshift mutations using Campylobacter jejuni Cas9. Mol Ther. 2018;26:1529–38.
20. Maalouf KE, Frederick DM, Sharma N, Haidar EA, Xiao T, Han JS, et al. Non-invasive detection of allele-specific CRISPR-SaCas9-KKH disruption of TOR1A DYT1 allele in a xenograft mouse model. Mol Ther Nucleic Acids. 2025;36:102466.
21. Guo R, Li Z, Li G, Zhang H, Zhang C, Huo X, et al. In vivo treatment of tyrosinaemia with hypercompact Cas12f1. Cell Discov. 2023;9:73.
22. Cui T, Cai B, Tian Y, Liu X, Liang C, Gao Q, et al. Therapeutic In vivo gene editing achieved by a hypercompact CRISPR-Cas12f1 system delivered with all-in-one adeno-associated virus. Adv Sci. 2024;11:e2308095.
23. Zhang H, Bamidele N, Liu P, Ojelabi O, Gao XD, Rodriguez T, et al. Adenine base editing in vivo with a single adeno-associated virus vector. GEN Biotechnol. 2022;1:285–99.
24. Guo R, Sun X, Wang F, Han D, Yang Q, Gao H, et al. Engineered IscB-omegaRNA system with improved base editing efficiency for disease correction via single AAV delivery in mice. Cell Rep. 2024;43:114973.
25. Xiao Q, Li G, Han D, Wang H, Yao M, Ma T, et al. Engineered IscB-omegaRNA system with expanded target range for base editing. Nat Chem Biol. 2025;21:100–8.
26. Marquart KF, Mathis N, Mollaysa A, Muller S, Kissling L, Rothgangl T, et al. Effective genome editing with an enhanced ISDra2 TnpB system and deep learning-predicted omegaRNAs. Nat Methods. 2024;21:2084–93.
27. Pickar-Oliver A, Gough V, Bohning JD, Liu S, Robinson-Hamm JN, Daniels H, et al. Full-length dystrophin restoration via targeted exon integration by AAV-CRISPR in a humanized mouse model of Duchenne muscular dystrophy. Mol Ther. 2021;29:3243–57.
28. Lim CKW, Gapinske M, Brooks AK, Woods WS, Powell JE, Zeballos CM, et al. Treatment of a mouse model of ALS by in vivo base editing. Mol Ther. 2020;28:1177–89.
29. Levy JM, Yeh WH, Pendse N, Davis JR, Hennessey E, Butcher R, et al. Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. Nat Biomed Eng. 2020;4:97–110.
30. Jo DH, Jang HK, Cho CS, Han JH, Ryu G, Jung Y, et al. Visual function restoration in a mouse model of Leber congenital amaurosis via therapeutic base editing. Mol Ther Nucleic Acids. 2023;31:16–27.
31. Davis JR, Banskota S, Levy JM, Newby GA, Wang X, Anzalone AV, et al. Efficient prime editing in mouse brain, liver and heart with dual AAVs. Nat Biotechnol. 2024;42:253–64.
32. Liu Z, Chen S, Davis AE, Lo CH, Wang Q, Li T, et al. Efficient rescue of retinal degeneration in Pde6a mice by engineered base editing and prime editing. Adv Sci. 2024;11:e2405628.
33. Qin H, Zhang W, Zhang S, Feng Y, Xu W, Qi J, et al. Vision rescue via unconstrained in vivo prime editing in degenerating neural retinas. J Exp Med. 2023;220:e20220776.
34. Truong DJ, Kuhner K, Kuhn R, Werfel S, Engelhardt S, Wurst W, et al. Development of an intein-mediated split-Cas9 system for gene therapy. Nucleic Acids Res. 2015;43:6450–8.
35. Wang H, Wang L, Zhong B, Dai Z. Protein splicing of inteins: a powerful tool in synthetic biology. Front Bioeng Biotechnol. 2022;10:810180.
36. Davis JR, Wang X, Witte IP, Huang TP, Levy JM, Raguram A, et al. Efficient in vivo base editing via single adeno-associated viruses with size-optimized genomes encoding compact adenine base editors. Nat Biomed Eng. 2022;6:1272–83.
37. Ryu SM, Koo T, Kim K, Lim K, Baek G, Kim ST, et al. Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. Nat Biotechnol. 2018;36:536–9.
38. Gee MS, Kwon E, Song MH, Jeon SH, Kim N, Lee JK, et al. CRISPR base editing-mediated correction of a tau mutation rescues cognitive decline in a mouse model of tauopathy. Transl Neurodegener. 2024;13:21.
39. Jang H, Jo DH, Cho CS, Shin JH, Seo JH, Yu G, et al. Application of prime editing to the correction of mutations and phenotypes in adult mice with liver and eye diseases. Nat Biomed Eng. 2022;6:181–94.
40. Riedmayr LM, Hinrichsmeyer KS, Thalhammer SB, Mittas DM, Karguth N, Otify DY, et al. mRNA trans-splicing dual AAV vectors for (epi)genome editing and gene therapy. Nat Commun. 2023;14:6578.
41. Lau CH, Ho JW, Lo PK, Tin C. Targeted transgene activation in the brain tissue by systemic delivery of engineered AAV1 expressing CRISPRa. Mol Ther Nucleic Acids. 2019;16:637–49.
42. Weinmann J, Weis S, Sippel J, Tulalamba W, Remes A, El Andari J, et al. Identification of a myotropic AAV by massively parallel in vivo evaluation of barcoded capsid variants. Nat Commun. 2020;11:5432.
43. Tabebordbar M, Lagerborg KA, Stanton A, King EM, Ye S, Tellez L, et al. Directed evolution of a family of AAV capsid variants enabling potent muscle-directed gene delivery across species. Cell. 2021;184:4919–38.e22.
44. Eid FE, Chen AT, Chan KY, Huang Q, Zheng Q, Tobey IG, et al. Systematic multi-trait AAV capsid engineering for efficient gene delivery. Nat Commun. 2024;15:6602.
45. Nierzwicki L, East KW, Morzan UN, Arantes PR, Batista VS, Lisi GP, et al. Enhanced specificity mutations perturb allosteric signaling in CRISPR-Cas9. Elife. 2021;10:73601.

46. Matsumoto D, Matsugi E, Kishi K, Inoue Y, Nigorikawa K, Nomura W. SpCas9-HF1 enhances accuracy of cell cycle-dependent genome editing by increasing HDR efficiency, and by reducing off-target effects and indel rates. Mol Ther Nucleic Acids. 2024;35:102124.
47. Lv J, Xi H, Lv X, Zhou Y, Wang J, Chen H, et al. Two high-fidelity variants: efSaCas9 and SaCas9-HF, which one is better?. Gene Ther. 2022;29:458–63.
48. Richter MF, Zhao KT, Eton E, Lapinaite A, Newby GA, Thuronyi BW, et al. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. Nat Biotechnol. 2020;38:883–91.
49. Zhang G, Song Z, Huang S, Wang Y, Sun J, Qiao L, et al. nCas9 engineering for improved target interaction presents an effective strategy to enhance base editing. Adv Sci. 2024;11:e2405426.
50. Fei J, Zhao D, Pang C, Li J, Li S, Qiao W, et al. Mismatch prime editing gRNA increased efficiency and reduced indels. Nat Commun. 2025;16:139.
51. Gough V, Gersbach CA. Immunity to Cas9 as an obstacle to persistent genome editing. Mol Ther. 2020;28:1389–91.
52. Li A, Tanner MR, Lee CM, Hurley AE, De Giorgi M, Jarrett KE, et al. AAV-CRISPR gene editing is negated by pre-existing immunity to Cas9. Mol Ther. 2020;28:1432–41.
53. Raghavan R, Friedrich MJ, King I, Chau-Duy-Tam Vo S, Strebinger D, Lash B, et al. Rational engineering of minimally immunogenic nucleases for gene therapy. Nat Commun. 2025;16:105.
54. Ibraheim R, Tai PWL, Mir A, Javeed N, Wang J, Rodriguez TC, et al. Self-inactivating, all-in-one AAV vectors for precision Cas9 genome editing via homology-directed repair in vivo. Nat Commun. 2021;12:6267.
55. Barnes C, Scheideler O, Schaffer D. Engineering the AAV capsid to evade immune responses. Curr Opin Biotechnol. 2019;60:99–103.
56. Prasad S, Dimmock DP, Greenberg B, Walia JS, Sadhu C, Tavakkoli F, et al. Immune responses and immunosuppressive strategies for adeno-associated virus-based gene therapy for treatment of central nervous system disorders: current knowledge and approaches. Hum Gene Ther. 2022;33:1228–45.
57. Geng G, Xu Y, Hu Z, Wang H, Chen X, Yuan W, et al. Viral and non-viral vectors in gene therapy: current state and clinical perspectives. EBioMedicine. 2025;118:105834.
58. Community Letter: Danon Disease Gene Therapy Trial Update. Rocket Pharmaceuticals. 2025. Accessed September 2, 2025. https://ir.rocketpharma.com/news-releases/news-release-details/rocket-pharmaceuticals-provides-update-phase-2-clinical-trial-rp.
59. Community Letter: NGN-401 Clinical Trial Update for the Rett Community. Neurogene Inc. 2024. Accessed September 2, 2025. https://reverserett.org/site/assets/files/18874/21nov2024_rett_community_letter_final.pdf.
60. Community Letter: In analysis of gene therapy deaths, Astellas points to underlying liver issues. 2023. Accessed September 2, 2025 https://www.fiercebiotech.com/biotech/astellas-posts-analysis-gene-therapy-deaths-pinning-blame-exacerbation-underlying-liver.
61. Community Letter: ELEVIDYS Safety Update. Sarepta Therapeutics. 2025. Accessed April 3, 2025. https://www.sarepta.com/community-letter-elevidys-safety-update.
62. Community Letter. Roche. 2025. Accessed April 3, 2024. https://www.duchenneuk.org/wp-content/uploads/2025/04/March-2025-Roche-DMD-Temporary-Clinical-Study-Halt-Community-Letter_31Mar25.pdf.

AUTHOR CONTRIBUTIONS

TK designed the study and supervised the research. TK, JG, and SL provided conceptualization and wrote the manuscript. JG generated the figures and tables.

FUNDING

This work was supported by a grant from Kyung Hee University in 2023 [KHU-20230920].

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Taeyoung Koo.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.