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Review Article

Gene Editing Frontiers: CRISPR-Cas9 as a Novel Therapeutic Approach for Autism Spectrum Disorder

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ABSTRACT

Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental condition characterized by deficits in social interaction and communication alongside repetitive behaviors and restricted interests. Despite substantial progress in identifying genetic and molecular contributors, effective disease-modifying therapies remain limited. The CRISPR-Cas9 gene-editing system has emerged as a powerful tool for unraveling the complex genetic architecture of ASD and developing targeted therapeutic strategies. This review explores the mechanistic principles of CRISPR-Cas9, including its molecular components, DNA repair pathways, and innovations in delivery systems for central nervous system targeting. We examine the CRISPR-mediated development of *in vitro* and *in vivo* ASD models spanning from iPSC-derived neurons to nonhuman primates and evaluate their translational relevance. Additionally, this review highlights the therapeutic applications of CRISPR in correcting pathogenic mutations, modifying epigenetic states, and modulating gene expression in both syndromic and nonsyndromic ASD. Critical challenges such as immune responses, mosaicism, and off-target effects are discussed, alongside emerging solutions, including anti-CRISPR proteins, base editing, and tissue-specific delivery systems. Finally, we assess the future prospects of integrating CRISPR technology with personalized medicine to advance ASD diagnosis, prevention, and treatment.

Keywords: Autism Spectrum Disorder (ASD), CRISPR-Cas9, Gene Editing, Therapeutic Strategies, Cellular Models.

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INTRODUCTION

Autism Spectrum Disorder (ASD) represents a complex neurodevelopmental condition with profound clinical and genetic heterogeneity, currently affecting approximately 1 in 31 children (3.2%) aged 8 years globally (Hwang & Lee, 2024; X. Jiang *et al.*, 2024). The disorder is characterized by two core symptom domains: persistent deficits in social communication and interaction and restricted, repetitive patterns of behaviors, interests, or activities (Calderoni, 2023; Hodges *et al.*, 2020). These core features encompass social deficits, including impaired reciprocal conversation, difficulty in forming age-appropriate friendships, and challenges in nonverbal communication, alongside repetitive behaviors such as stereotyped motor movements, insistence on sameness, highly restricted fixated interests, and unusual sensory reactivity (CDC, 2025; J. Tian *et al.*, 2022). The genetic architecture of ASD is extraordinarily complex, with de novo mutations playing a crucial role in its pathogenesis. Significant ASD-associated genes include *SHANK3*, which encodes a synaptic scaffolding protein critical for dendritic spine development, and *MECP2* (Redin *et al.*, 2014), a methyl-CpG-binding protein whose mutations cause Rett syndrome and are associated with ASD phenotypes (T. Wang *et al.*, 2021). Copy Number Variants (CNVs) represent another major genetic component, occurring in 10–20% of ASD cases, with notable examples including 16p11.2 deletions and duplications that affect multiple genes simultaneously (Abedini *et al.*, 2023; Zarrei *et al.*, 2019). The distinction between syndromic and nonsyndromic ASD is clinically important, as syndromic forms such as fragile X syndrome (affecting approximately 1 in 4,000 males) are caused by specific genetic mutations such as CGG repeats in the *FMR1* gene, whereas nonsyndromic cases often involve complex polygenic inheritance patterns ("Autism," n.d.). Epigenetic factors, particularly DNA methylation alterations, add another layer of complexity, with studies demonstrating aberrant methylation patterns in ASD-associated genes such as *GAD1*, *OXTR*, and *RELN*, suggesting that environmental factors may interact with genetic susceptibility through epigenetic mechanisms (Gallo *et al.*, 2022; Gholamalizadeh *et al.*, 2024; Pearson *et al.*, 2022; Ravaei *et al.*, 2023; Tremblay & Jiang, 2019).

Current therapeutic approaches for ASD remain largely symptom-focused rather than disease-modifying,

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highlighting significant limitations in addressing the underlying pathophysiology (J. Xu, 2023). Risperidone and aripiprazole represent the only FDA-approved medications for ASD; these medications specifically target irritability and aggression rather than core social communication deficits. Clinical studies have demonstrated that while risperidone is effective at reducing behavioral symptoms, with response rates showing significant improvement on the Aberrant Behavior Checklist, it is associated with substantial risks, including weight gain, metabolic disturbances, and extrapyramidal symptoms (da Silva *et al.*, 2023; Department of Psychiatry, The First Affiliated Hospital of Kunming Medical University, Kunming, China *et al.*, 2025; Moazen-Zadeh *et al.*, 2018; A. S. Mohamed *et al.*, 2023). The mechanism of action of medication through dopamine and serotonin receptor antagonism addresses secondary symptoms but does not target the fundamental neurodevelopmental processes underlying ASD ("Risperidone Use in Children with Autism Carries Heavy Risks," 2014). Behavioral interventions, including Applied Behavior Analysis (ABA), speech therapy, and occupational therapy, remain the cornerstone of treatment but require intensive, long-term implementation with variable outcomes depending on individual characteristics and severity. The lack of disease-modifying treatments reflects fundamental challenges in modeling ASD pathophysiology, stemming from the disorder's genetic heterogeneity, the complexity of neurodevelopmental processes, and the difficulty in recapitulating human-specific brain development in animal models (Beopoulos *et al.*, 2022; S. Xu *et al.*, 2024). Although traditional mouse models are valuable for studying individual gene functions, they fail to capture the polygenic nature of most ASD cases and the intricate gene–environment interactions that contribute to phenotypic heterogeneity (C.-C. Jiang *et al.*, 2022; S. Xu *et al.*, 2024). A conceptual framework summarizing the translational research process—from identifying risk factors to developing animal models, conducting multidimensional evaluations and mechanistic analyses, and deriving new therapeutic strategies—is illustrated in Figure 1.

The emergence of CRISPR-Cas9 as a precise gene-editing tool represents a revolutionary advancement in genomic medicine, offering unprecedented opportunities to address the genetic complexity underlying ASD (Ansori *et al.*, 2023; Asmamaw & Zawdie, 2021; Deneault, 2024; Tavakoli *et al.*, 2021). The CRISPR–Cas9 system utilizes a programmable RNA-guided nuclease that can introduce targeted double-strand breaks at specific genomic loci, enabling precise modifications through cellular DNA repair mechanisms, including Nonhomologous End Joining (NHEJ) and Homology-Directed Repair (HDR) (Lino *et al.*, 2018). This technology's remarkable precision, efficiency, and programmability have made it the preferred gene-editing platform capable of correcting point mutations, introducing specific genetic variants, and creating sophisticated disease models (Asmamaw & Zawdie, 2021; Ravichandran & Maddalo, 2023; Tavakoli *et al.*, 2021). The rationale for CRISPR application in polygenic disorders such as ASD stems from its unique capacity to address multiple genetic components simultaneously and create more representative disease models that capture the polygenic architecture of the disorder (Ingle *et al.*, 2025). Unlike traditional approaches that focus on single genes, CRISPR-Cas9 can be employed to study gene–gene interactions, model complex genetic backgrounds, and investigate the functional consequences of rare variants identified through large-scale sequencing studies (J. J. Li *et al.*, 2025). Recent innovations, including base editing and prime editing, have further expanded the toolkit, enabling precise single-nucleotide modifications without requiring double-strand breaks, thus reducing potential off-target effects while maintaining high editing efficiency (Sandhu *et al.*, 2023). This technology has already demonstrated success in ASD-related applications, including the correction of *SHANK3* mutations in patient-derived iPSCs, the restoration of *MECP2* function in Rett syndrome models, and the development of improved animal models that better recapitulate human ASD phenotypes (Ricci & Colasante, 2021). Furthermore, CRISPR–Gold delivery systems have shown promising results in reducing autism-like behaviors in Fragile X syndrome mouse models, demonstrating the potential for *in vivo* therapeutic applications. This convergence of technological precision with improved understanding of ASD genetics positions CRISPR-Cas9 as a transformative tool for both advancing mechanistic understanding and developing novel therapeutic strategies for this complex neurodevelopmental disorder (Hong & Iakoucheva, 2023).

This comprehensive review focuses on the multifaceted role of CRISPR in ASD research and therapeutics, examining its applications across four critical domains: model development, therapeutic strategies, delivery challenges, and future directions. The analysis encompasses CRISPR-mediated generation of sophisticated *in vitro* cellular models using patient-derived iPSCs and organoid systems that recapitulate key aspects of human neurodevelopment, alongside the creation of improved animal models that better capture the genetic and phenotypic complexity of ASD (S. Xu *et al.*, 2024). Therapeutic strategy discussions include direct gene correction approaches for monogenic ASD forms, epigenome editing for complex cases involving dysregulated gene expression, and innovative base editing techniques for precise modification of disease-causing variants (Ricci & Colasante, 2021). This review critically examines current delivery challenges, including the development of targeted delivery systems such as viral vectors, lipid nanoparticles, and biomimetic approaches necessary for safe and efficient *in vivo* gene editing (Sioson *et al.*, 2021). Finally, future directions encompass the integration of CRISPR technology with emerging precision medicine approaches, the potential for preventive interventions on the basis of early genetic screening, and the ethical considerations surrounding germline editing applications (Aljabali *et al.*, 2024). Through this comprehensive analysis, this study aims to provide

a roadmap for leveraging CRISPR–Cas9 technology to transform ASD research and treatment, ultimately advancing toward personalized therapeutic interventions that address the root causes of this complex neurodevelopmental disorder.

CRISPR-CAS9: MECHANISM AND WORKFLOW

Molecular Components

The *Streptococcus pyogenes* Cas9 endonuclease is organized into two major lobes, an α-helical Recognition (REC) lobe and a Nuclease (NUC) lobe connected by an arginine-rich bridge helix that also contributes to guiding RNA binding. High-resolution cryo-EM and molecular dynamics studies have revealed three REC subdomains (REC1--3) that undergo extensive conformational rearrangements upon guide RNA (gRNA) and DNA binding: REC1 initially "senses" base pairing in the seed region, whereas REC2 and REC3 amplify R-loop formation through allosteric coupling and concertedly "lock" the HNH nuclease domain onto the target strand cleavage site (Nishimasu *et al.*, 2014; Pacesa *et al.*, 2022; Palermo *et al.*, 2018). The NUC lobe comprises the HNH domain responsible for cleaving the DNA strand complementary to the gRNA and the RuvC domain, which possesses three split motifs (RuvC I--III) that cleave the noncomplementary strand. PAM recognition by the C-terminal PI domain initiates local DNA unwinding and coordinates R-loop propagation with REC-mediated conformational checkpoints, ensuring that off-target sites with incomplete heteroduplexes (<17 bp) do not trigger cleavage (Nishimasu *et al.*, 2014).

Guide RNA design is equally critical for target specificity and editing efficiency. In nature, a dual-RNA complex of an ~20 nt CRISPR RNA (crRNA) and an ~75 nt transactivating crRNA (tracrRNA) hybridizes via an anti-repeat duplex, recruits Cas9, and directs site recognition (F. Jiang & Doudna, 2017; Ui-Tei *et al.*, 2017). Synthetic single-guide RNAs (sgRNAs) fuse crRNA and tracrRNA into a continuous RNA containing a 20 nt spacer and a structured scaffold with essential hairpins (tetraloop, stem loops 1-3) that stabilize Cas9 binding and R-loop formation. Empirical and computational analyses reveal that sgRNA secondary structure integrity avoids spacer scaffold mispairing and that intramolecular hairpins in the seed region are paramount: misfolded guides often exhibit <35% cleavage efficiency, whereas optimally folded sgRNAs within a thermodynamically narrow free-energy window (ΔG_self-folding ~-10--5 kcal/mol) achieve high on-target activity. sgRNA sequence features also modulate transcription and loading: homopolymers (e.g., poly-T) cause premature Pol III termination, whereas a balanced GC content (40-80%) ensures adequate R-loop stability without overstabilizing guide folding. Furthermore, emerging studies highlight the importance of maintaining an unpaired 3′ seed region (positions 18--20) and intact stem–loop 1 for efficient cleavage, as these elements govern initial DNA interrogation and high-affinity Cas9 engagement (Jung *et al.*, n.d.). A simplified schematic of the CRISPR-Cas9 editing mechanism-including double-strand break formation and subsequent repair via nonhomologous end joining or homology-directed repair—is shown in Figure 2.

Mechanism of Action

Genome editing by *Streptococcus pyogenes* Cas9 (SpCas9) occurs via three intimately linked stages: PAM-mediated target recognition, concerted strand cleavage by the HNH and RuvC nuclease domains, and cellular Double-Strand Break (DSB) repair by competing pathways. While foundational studies have characterized each stage, emerging structural, biophysical, and cellular data highlight nuanced allosteric controls, off-target considerations, and repair pathway biases that shape editing outcomes.

PAM-Dependent Recognition and R-Loop Initiation

SpCas9 locates its target through interrogation of a short Protospacer Adjacent Motif (PAM), canonically 5′-NGG-3′, on the nontarget DNA strand. Recognition of NGG by two invariant arginines in the PAM-Interacting (PI) domain induces local DNA unwinding and a pronounced ~30°-35° kink in the target strand, which catalyzes R-loop formation. Crystallography and molecular dynamics revealed that PAM binding allosterically reorganizes the REC and NUC lobes into an "induced-fit" conformation, funneling the adjacent 20-nt protospacer into the guide RNA channel. Broad PAM promiscuity and weak recognition of NAG, NGA, and other NGH motifs under high enzyme concentrations can drive off-target cleavage, underscoring the trade-off between target scope and specificity. Efforts to engineer PAM-relaxed variants (e.g., SpCas9-NG, xCas9, SpRY) expand targetable sequences but often exhibit reduced cleavage fidelity, necessitating rigorous genome-wide specificity profiling (Collias & Beisel, 2021; W. Zhang *et al.*, 2021).

Allosteric Coordination of HNH and RuvC Cleavage

Following R-loop stabilization, SpCas9's HNH domain cleaves the target strand, whereas the RuvC domain cuts the nontarget strand, generating a blunt DSB three base pairs upstream of the PAM. Time-resolved cryo-EM, NMR, and single-molecule FRET studies have revealed a dynamic "cross-talk" pathway from the REC2 lobe through HNH to RuvC: upon PAM and guide-target duplex engagement, the HNH domain rotates >20 Å into a catalytic orientation, docking via L1/L2 linker remodeling and coordinating a single divalent metal ion in its active site (East *et al.*, 2020). Allosteric coupling ensures near-synchronous cleavage, yet kinetic analyses reveal parallel pathways—either HNH-first or RuvC-first—whose relative flux depends on guide-target complementarity, bridge-helix integrity, and local magnesium coordination (Babu *et al.*, 2021). Off-target single-strand nicks, arising from partial HNH or RuvC misactivation, can evade detection in bulk assays yet provoke mutagenic repair *in vivo*,

highlighting the need for high-fidelity and nickase variants with reshaped allosteric networks.

DSB Repair: Balancing NHEJ and HDR

Once Cas9 induces DSB, mammalian cells choose between Nonhomologous End Joining (NHEJ) and Homology-Directed Repair (HDR). NHEJ predominates throughout the cell cycle, ligating DNA ends within minutes and often introducing small indels that disrupt reading frames ideal for gene knockout applications. In contrast, HDR is restricted to S/G2 phases, requires a homologous donor template, and yields precise gene corrections, albeit at lower efficiency (typically <20% without intervention) (Yang *et al.*, 2020). Repair-pathway choice is governed by DNA-end resection: minimal resection favors NHEJ via Ku70/80 and DNA-PKcs activation, whereas extensive 5′→3′ resection mediated by CtIP, Mre11, and exonucleases commits to HDR by recruiting BRCA1/2 and Rad51 loading complexes. Small-molecule inhibitors of NHEJ factors (e.g., the DNA-PKcs inhibitors NU7441 and SCR7) or cell cycle synchronization agents (e.g., nocodazole and thymidine) can modestly shift repair toward HDR but often at the cost of cell viability or increased genotoxicity. The fusion of Cas9 to HDR-promoting factors such as CtIP or Rad52 via protein or RNA scaffolds localizes the resection machinery to the DSB and can increase HDR frequencies up to 6- to 15-fold in select cell types; however, locus-specific variability persists (Shams *et al.*, 2022).

Despite transformative utility, Cas9 editing faces persistent challenges:

- PAM specificity vs. targeting breadth: Engineering PAM-relaxed variants expands editing landscapes but demands parallel advances in high-throughput off-target mapping and allosteric redesign to maintain fidelity.
- Allosteric dynamics and nicking byproducts: Detailed mapping of HNH–RuvC energy landscapes via time-resolved structural methods could inform the design of highly coordinated nuclease variants that minimize aberrant single-strand breaks.
- HDR enhancement strategies: Combining NHEJ suppression, cell cycle control, donor format optimization, and Cas9 fusion strategies yields incremental improvements; however, *in vivo* applicability hinges on minimizing toxicity and immune responses.
- Predictive modeling of repair outcomes: Machine learning frameworks trained on large-scale indel and HDR datasets show promise for guiding guide RNA and donor design but require further validation across diverse genomic contexts.

In summary, the CRISPR–Cas9 mechanism involves an orchestrated interplay between molecular recognition, dynamic allostery, and cellular repair. Integrating high-resolution structural insights with advanced engineering and predictive biology will be essential to realize next-generation genome-editing tools with increased precision and therapeutic potential.

Delivery Strategies

Efficient delivery of CRISPR–Cas9 components is paramount to maximize editing efficacy while minimizing off-target effects and immunogenicity. *In vitro*, electroporation remains the gold standard for many cell types, enabling direct cytosolic entry of Cas9 Ribonucleoprotein (RNP) complexes with high efficiency and minimal vector-associated integration risk, although cell viability and throughput can be limiting factors (Fajrial *et al.*, 2020). Viral vectors, including lentivirus and recombinant AAV (rAAV), offer robust transduction: lentiviral systems accommodate large payloads and transduce dividing or nondividing cells effectively but carry insertional mutagenesis concerns unless integrase-deficient designs (NILVs) are employed; rAAVs exhibit lower immunogenicity and precise episomal persistence yet are constrained by ~4.7 kb packaging limits, often necessitating split-Cas9 strategies or orthologous small-Cas9 variants to fit within the capsid. *In vivo*, Lipid Nanoparticles (LNPs) and polymeric nanocapsules functionalized with targeting ligands (e.g., angiopep-2) have emerged as nonviral platforms capable of systemic delivery, tissue-specific tropism, and controlled release: LNPs encapsulating Cas9 mRNA/sgRNA achieve efficient hepatic editing, whereas disulfide-crosslinked nanocapsules (~30 nm) demonstrate blood–brain barrier penetration and up to 38% tumor editing in orthotopic glioblastoma models with negligible off-target activity (X. Liu *et al.*, 2025). Despite these advances, challenges persist in balancing payload capacity, circulation half-life, and immunogenicity, as well as manufacturing scalability and regulatory approval. Crucially, germline versus somatic editing presents distinct trade-offs: germline modifications enable heritable changes but raise profound ethical and safety concerns, whereas somatic editing confines alterations to individual patients, reducing long-term population risk yet necessitating repeated or localized delivery and careful assessment of tissue-specific off-target effects and immunotoxicity (Polcz & Lewis, 2016). Future optimization must integrate high-fidelity Cas9 variants, sophisticated delivery vehicles tailored to target tissues, including the central nervous system, and predictive models of repair outcomes to achieve precise, safe, and ethically responsible genome editing.

CRISPR FOR MODELING ASD PATHOPHYSIOLOGY

CRISPR technology with cellular models, including induced Pluripotent Stem Cell (iPSC)-derived neurons and 3D brain organoids, can provide potential options for studying the

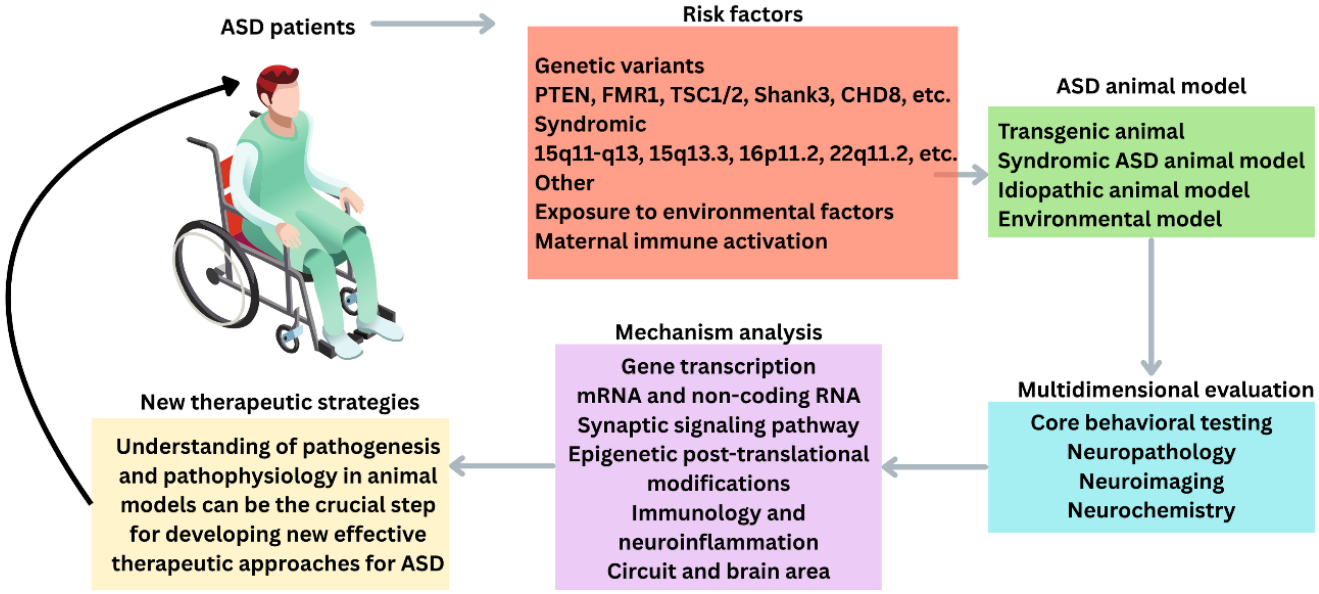

Figure 1: Overview of the translational research pipeline for ASD therapy development. Risk factors guide the creation of animal models, which undergo multidimensional evaluation and mechanism analysis to uncover disease pathways and enable new therapeutic strategies for ASD patients.

pathophysiology of Autism Spectrum Disorder (ASD). These models allow researchers to examine the impact of specific genetic mutations on neurodevelopment and neuronal function. iPSC-Derived Neurons: Because iPSCs are derived from ASD patient fibroblasts, it is possible to produce large amounts of iPSC-derived neurons, which will be invaluable in investigating the cell-autonomous activity of ASD-related genes. The use of CRISPR-Cas9 allows us to correct the mutations in these cells derived from patients and introduces isogenic controls, which are identical to each other except that the mutation has been repaired (Revankar *et al.*, 2018). This approach minimizes confounding variables and allows for a more accurate assessment of the impact of the mutation on neuronal phenotypes. For example, FMR1 mutations that cause Fragile X syndrome, a common single-gene cause of ASD, may be addressed with CRISPR correction. With the help of corrected and uncorrected neuron comparisons, it is possible to reveal a set of cellular and molecular alterations related to FMR1 mutation, including changes connected with synaptic activity, dendritic formation and gene regulation (Zaslavsky *et al.*, 2019).

Cellular models

3D brain organoids: Brain 3D organoids are human pluripotent-derived stem cells that provide a more complex and physiologically relevant brain development model than 2D cell culture brains do (Kiaee *et al.*, 2021; Zaslavsky *et al.*, 2019). These are 3D organoids in which some features of the brain 3D cytoarchitecture, such as cortical layering, are recapitulated and the organoids can be used to examine neurodevelopmental processes in a more natural environment. ASD-associated mutations, including CNTNAP2 Knockout (KO), can be introduced into organoids via CRISPR-Cas9, allowing the investigation of brain development in response to the mutated variant (Jang *et al.*, 2023). CNTNAP2 is strongly associated with ASD, and its disruption has been linked to altered cortical layering and other ASD-related phenotypes. CNTNAP2-KO organoid studies have revealed that neural migration is disrupted, the lateral specificity of neuron differentiation is not efficiently shaped, and the balance between excitatory and inhibitory neurons is altered, which is the main cause of epilepsy (Jourdon *et al.*, 2022).

Neurons developed from iPSCs and 3D brain organoids are highly advantageous for the study of ASD pathophysiology, but their limitations should also be recognized. iPSC-derived neurons can reflect only early stages of development and are likely unable to reveal the complexity of neurons in the adult brain ("Using iPSC-Based Models to Understand the Signaling and Cellular Phenotypes in Idiopathic Autism and 16p11.2 Derived Neurons," 2020). While brain organoids are more complex than 2D cultures are, they still lack the vascularization and immune components of the native brain, which can affect their maturation and functionality (Hossain *et al.*, 2024). Furthermore, organoid-to-organoid variability can be a challenge, making it difficult to compare specific gene regulatory pathways across different organoids. Moreover, organoid-to-organoid variables can prove to be challenging, and it may not be easy to compare certain gene regulatory pathways among different organoids. Despite these limitations, the combination of CRISPR technology and cellular models provides a powerful approach for dissecting the complex genetic and cellular mechanisms underlying ASD (Chiola *et al.*, 2022; Pintacuda *et al.*, 2021). Further studies should aim to improve these *in vitro* models to more faithfully recapitulate *in vivo* neurodevelopmental dynamics and use high-throughput screening capabilities in the discovery of individualized treatments.

Animal models

Animal models play an essential role in the study of the intricate neurobiological processes of neurodevelopmental disorders, such as Autism Spectrum Disorder (ASD), and in the evaluation of possible therapeutic strategies (Twining *et al.*, 2017). Each model offers unique advantages and limitations, making the selection of an appropriate species essential for addressing specific research questions (Padmanabhan & Götz, 2023).

Rodent models, especially mice and rats, are widely used because they are inexpensive to maintain, reproduce quickly and are easily genetically manipulated. They have been instrumental in identifying ASD risk genes and investigating their impact on synaptic function (Chung *et al.*, 2012). For example, in Shank3 mutant rodent models, defects in synaptic transmission and synaptic protein expression have been shown in relevant brain areas implicated in ASD, including the striatum, hippocampus and prefrontal cortex. However, rodents have limitations in replicating the complex social behaviors characteristic of ASD in humans (F. Zhu *et al.*, 2024). The fairly basic structure of society and poor cortical development of these individuals decrease the applicability of the findings associated with social deficiencies to translation problems (Karsenty & Olson, 2016). Despite these limitations, adequate neurobehavioral and neurological tests have been developed in rodents.

Despite these limitations, adequate neurobehavioral and neurological tests have been developed in rodents in which SHANK3 is knocked out in macaques via CRISPR/Cas9 technology, which recapitulates the behavioral features of autism, changes in neuroconnectivity, and synaptic abnormalities related to ASD. These models can provide insights into the neuronal and cognitive disruptions linked to *SHANK3* mutations that rodent models may not fully capture. However, NHP models are ethically challenging and expensive, limiting their widespread use (González-González *et al.*, 2024). The high cost associated with procurement and maintenance can be a major barrier for research institutions.

Pigs are emerging as valuable large animal models for studying synaptic plasticity and neurobehavioural disorders. They have a larger and structurally more similar brain to the human brain than rodents do, enabling similar-sized equipment and neuroimaging modalities to be used. Pigs exhibit complex social behaviors and cognitive abilities, making them suitable for modeling aspects of neurological and psychiatric disorders. While pig models are less ethically complex and less expensive than NHPs are, they still require more resources and space than rodent models do.

Finally, the type of animal model is subject to the research question to be answered and the resources available. Rodents

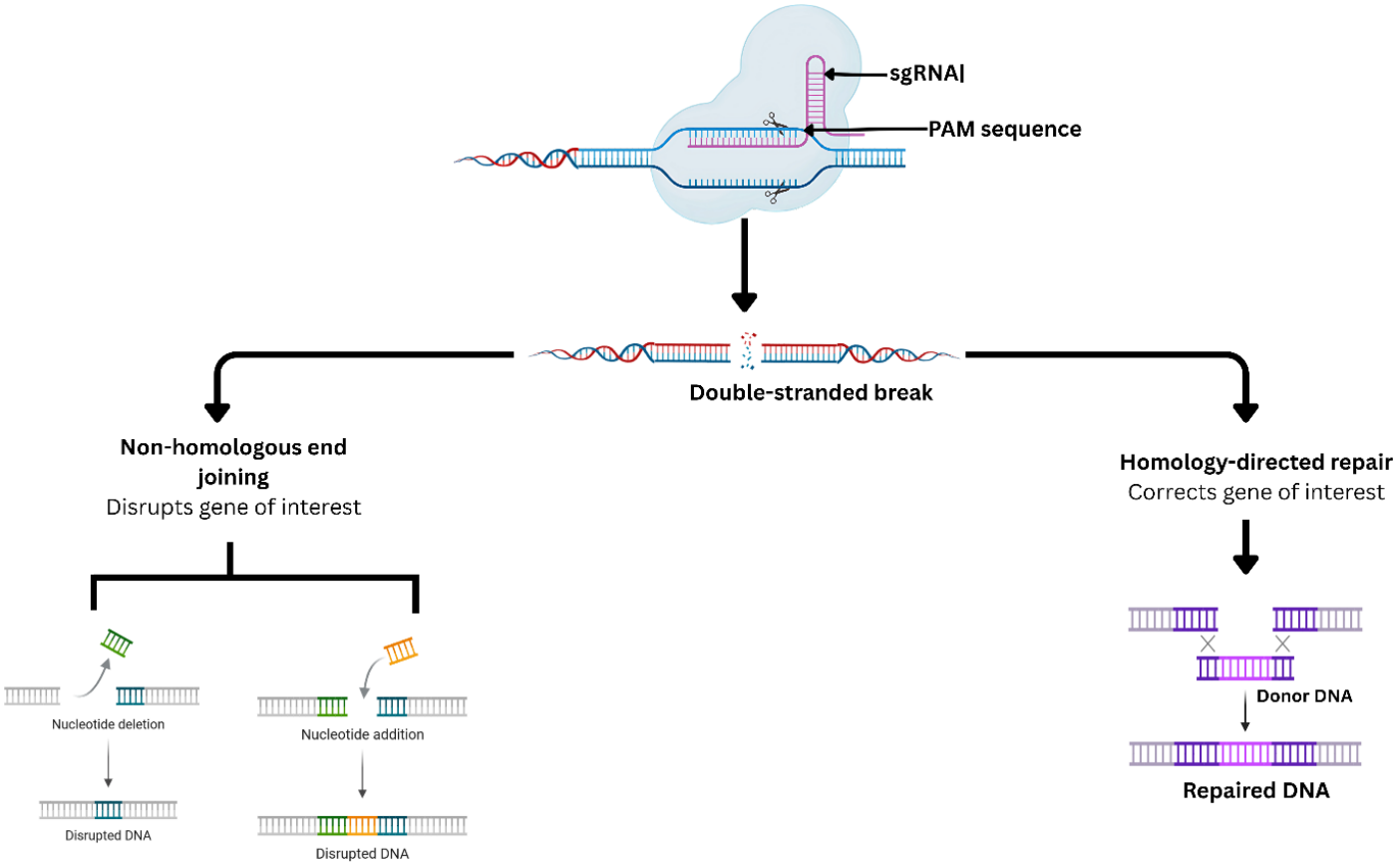

Figure 2: CRISPR-Cas9 genome editing workflow.

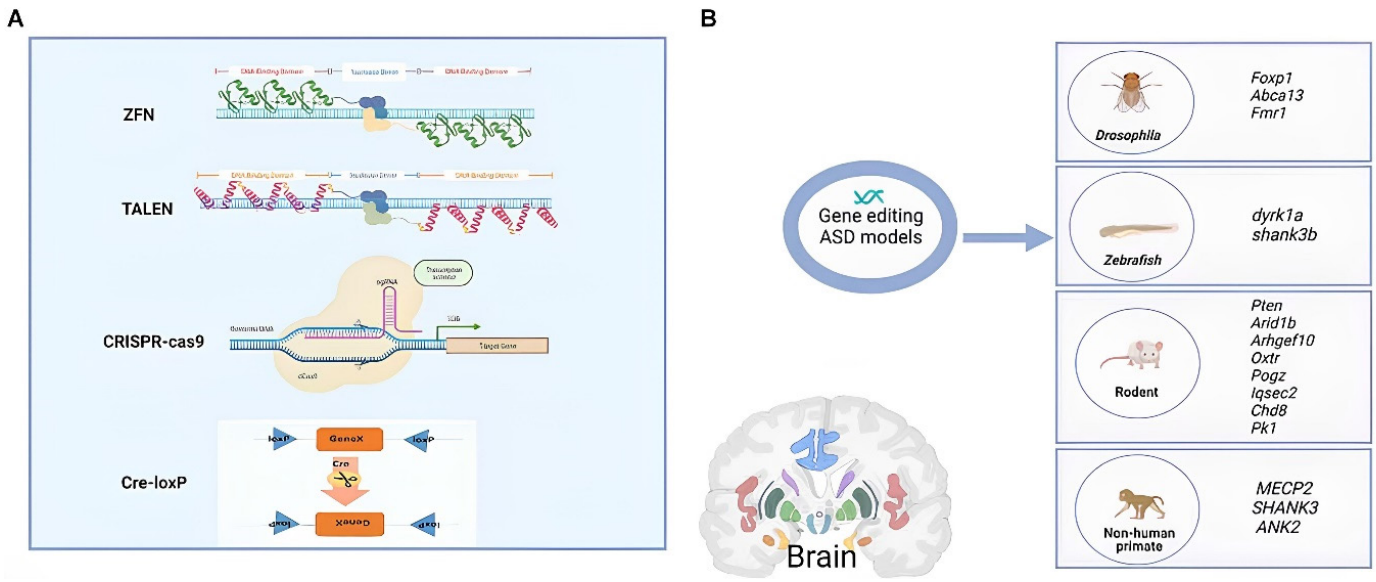

Figure 3: A] Comparative overview of genome editing technologies used in ASD research. Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and the CRISPR-Cas9 system represent successive advancements in genome editing precision and efficiency. The Cre–loxP system, although widely used for conditional gene knockout in developmental studies, lacks the programmable versatility of CRISPR. Among these, CRISPR-Cas9 has become the most widely adopted tool because of its simplicity, scalability, and high specificity. B] CRISPR-generated ASD models across species. Gene-editing technologies have been applied to a range of model organisms to investigate ASD-related genes. These genes include Foxp1, Abca13, and Fmr1 in Drosophila; dyrk1a and shank3b in zebrafish; and a broad panel of genes, including Pten, Arid1b, Chd8, and Oxtr, in rodent models. Nonhuman primates provide the closest physiological relevance, with targeted editing of MECP2, SHANK3, and ANK2 for studying human-like ASD phenotypes. (adapted from Wang *et al.* under the terms and conditions of the Creative Commons Attribution (CC-BY) license (CC-BY 4.0)). (F) Available at (N. Wang *et al.*, 2022).

have become a cost-effective and genetically tractable model for investigating basic synaptic mechanics, and NHPs represent a more translationally relevant model of complex social behaviors and neural circuitry. Pigs have the potential to serve as a beneficial intermediate model to facilitate the transition between rodent and NHP studies. The enhancement of such models and the development of innovative behavioral and neuroimaging measures may also be needed to enhance their translational relevance and to pursue the progress of effective treatment delivery in individuals with neurodevelopmental disorders (Padmanabhan & Götz, 2023).

Functional Insights from CRISPR Models

CRISPR models provide practical information on neurodevelopmental illnesses, especially those linked to synaptic dysfunction, impaired neurogenesis, and network hyperexcitability (Figure 3). CRISPR/Cas9 technology has been exploited to alter genes such as SHANK3, which encodes a scaffolding protein with an essential role in glutamatergic synapse organization and performance, in many model systems. Studies utilizing CRISPR-engineered SHANK3 mutant dogs have revealed autism-like behaviors and impaired synaptic function in the prefrontal cortex. Similarly, research on SHANK3-deficient rats revealed altered synaptic ultrastructures in the prefrontal cortex, further underscoring the importance of SHANK3 in synaptic organization and function (Jacot-Descombes *et al.*, 2020). A study of patient-derived and CRISPR-engineered neurons from Phelan–McDermid Syndrome (PMDS) patients reported that a decrease in SHANK3 expression contributes to neuronal hyperdifferentiation, augmented synapses and reduced neuronal activity. Furthermore, studies using human neurons with hemizygous SHANK3 deletion engrafted in the mouse prefrontal cortex have demonstrated defective AMPA-mediated synaptic transmission and morphology (Chiola *et al.*, 2021). Furthermore, studies using human neurons with hemizygous SHANK3 deletion engrafted in the mouse prefrontal cortex have demonstrated defective AMPA-mediated synaptic transmission and morphology. These models indicate that SHANK3 is essential for synaptic transmission, plasticity and, in general, the functioning of neuronal networks (Arons *et al.*, 2016; Jaramillo *et al.*, 2016). A summary of key CRISPR-generated ASD models and their associated phenotypic outcomes is presented in Table 1.

An additional vital route in which CRISPR models offer crucial knowledge is impaired neurogenesis or the development of new neuron production. The use of CRISPR to model disruptions in neurodevelopmental genes such as those that affect cell signaling and transcriptional regulation can help in understanding how these genes may operate during neurogenesis (Huang *et al.*, 2019). Research has demonstrated that SHANK3 knockdown in human-based induced Pluripotent Stem Cells (iPSCs) disrupts neurodevelopment processes, which influences neuronal differentiation and maturation. Alterations in neurogenesis can have profound effects on brain structure and function, contributing to cognitive and behavioral deficits. Alterations in neurogenesis can have profound effects on brain structure and

function, contributing to cognitive and behavioral deficits (Haniff *et al.*, 2024).

Network hyperexcitability, an imbalance between excitatory and inhibitory neurotransmission, has also been investigated via CRISPR models (Berecki *et al.*, 2023). Inducing gene modifications that influence ion channel activity or transmission across synapses, researchers have developed models with increased neuronal excitability and increased chances of seizures. Computational research of the Epileptor model has evaluated the relationship between the placement and connectivity of an epileptogenic region within a mouse brain and focal seizures, which provides analytical insight into the concept of seizure onset and spread. Furthermore, research has indicated that SCN1A gain-of-function variants can lead to interneuron hyperexcitability and network instability. Hyperexcitability in a network can also be the result of maladaptation of homeostatic mechanisms of plasticity that are normally stabilizing neural networks. This can result in a variety of neurological and psychiatric disorders.

Critical analysis of CRISPR models reveals both their strengths and limitations. The precision of CRISPR/Cas9 technology allows for targeted gene editing and the creation of highly specific models that can recapitulate aspects of human disease. These models, however, might be insufficient for modeling human diseases because species provide variation in the function and regulation of genes. There is also an issue in the interpretation of the results due to off-target activity caused by CRISPR editing and the compensatory mechanism seen in knockouts (Delling *et al.*, 2024). Additionally, many neurodevelopmental disorders are polygenic and involve numerous genes and the environment, and this is challenging to model in single-gene CRISPR efforts. Despite these limitations, CRISPR models remain a powerful tool for dissecting the molecular and cellular mechanisms underlying synaptic dysfunction, impaired neurogenesis, and network hyperexcitability, providing valuable insights into the pathogenesis of neurodevelopmental disorders and potential therapeutic targets.

CRISPR-BASED THERAPEUTIC STRATEGIES FOR ASD

The current direction of CRISPR-based therapeutic interventions provides exciting prospects for taking care of Autism Spectrum Disorder (ASD) as a complex neurodevelopmental issue that has significant heritability (Yenkoyan *et al.*, 2024). ASD is characterized by impaired social interaction and communication, as well as restricted and repetitive behaviors. ASD is a heterogeneous disorder caused by a few genetic and environmental factors, which presents a major challenge in the formulation of effective treatments. The most recent developments in the fields of genetics and genomics have revealed the existence of hundreds of genes connected to ASD, indicating the potential of gene-editing techniques such as CRISPR to focus on underlying genetic factors that lead to the disorder (Ayhan & Konopka, 2018). CRISPR-Cas9 systems can be employed to

Table 1: CRISPR-Generated ASD Models.

Model Type	Target Gene	Phenotype	Supporting Research
iPSC Neurons	FMR1	Dendritic spine defects	iPSC-derived neurons allow for the study of ASD-relevant phenotypes in human cells (Deneault *et al.*, 2018). Using iPSC-derived neurons, researchers can model synaptic dysfunction, including dendritic spine abnormalities, seen in ASD (Lin *et al.*, 2023).
Macaque	SHANK3	Social withdrawal, synaptic loss	CRISPR/Cas9-edited beagle dogs targeting *SHANK3* offer a large animal model for ASD, which may better mimic the human condition compared to rodent models. *SHANK3* mutations are strongly associated with ASD, and modeling these mutations in animals can help elucidate the underlying mechanisms (R. Tian *et al.*, 2023).
Mouse Organoid	CHD8	Altered GABAergic signaling	CRISPR/Cas9-mediated knockout of *CHD8* in cerebral organoids allows for the study of its role in neural differentiation and transcriptional networks (P. Wang *et al.*, 2017). *CHD8* is a high-risk mutated gene in ASD, and its loss-of-function affects early neuroectoderm differentiation. This can lead to altered GABAergic signaling, which is implicated in ASD (Ding *et al.*, 2021).

modify the expression of genes implicated in ASD, offering a potential therapeutic approach to correct or compensate for the effects of disease-causing mutations (Chrzanowski & Batra, 2024; Tamura *et al.*, 2022). CRISPR activation focused on enhancers has been used to rescue haploinsufficient autism susceptibility genes, suggesting that increasing the expression of functional alleles may be an effective therapeutic strategy (Chen *et al.*, 2024). This potential is illustrated in various model organisms, from Drosophila to non-human primates, where CRISPR-Cas9 has been employed to edit ASD-associated genes and study their functional impact (see Figure 4).

Gene Correction and Knockout

CRISPR-based therapeutic strategies for Autism Spectrum Disorder (ASD) are being explored through gene correction and knockout methods, including allele-specific editing and the rescue of synaptic gene expression. One potential gene editing application is allele-specific editing, which would be useful for targeting mutations within a gene such as MECP2 that causes the neurodevelopmental disorder Rett syndrome, which resembles ASD in some ways. MECP2 is a gene that encodes a protein that plays a vital role in transcriptional regulation and chromatin modification, and any form of mutation in this gene may trigger numerous neurological symptoms (Liyanage *et al.*, 2019). Using CRISPR-Cas9, it may be possible to correct the mutated *MECP2* allele while leaving the normal allele intact, thereby restoring proper gene function (Qian *et al.*, 2023). Such a strategy should be strictly targeted to prevent off-target effects and allow modification of the precise activity of the disease-causing allele.

The expression of synaptic genes, which are highly dependent on the NRXN1 and NLGN3 genes and are rescued via CRISPR therapy, is restored in the context of synaptic loss or disease. Mutations in these genes have been linked to ASD, and correcting their expression could improve synaptic transmission and neuronal connectivity. The next CRISPR treatment option is tNRXN1, which encodes neurexin 1, and NLGN3, which encodes neuroligin 3; both proteins are implicated in cell adhesion and communication in synapses (Marcó De La Cruz *et al.*, 2024). Liprin-α proteins, for example, are master regulators of human presynapse assembly and could be targets to enhance synaptic function. Deregulation in ASD is believed to be due to an imbalance between excitatory and inhibitory neurotransmission, which results when the expression or activity of synaptic proteins is disrupted. CRISPR-based therapies have the potential to rectify imbalances in the synaptic setup by overriding the expression of key synaptic genes or fixing mutations that disrupt their performance (Z.-H. Zhang *et al.*, 2024).

Among the factors that must be resolved concerning the use of CRISPR-Cas9 technology in the treatment of ASD, multiple limitations need to be mentioned. The major barrier is how to efficiently and safely deliver the CRISPR components to the brain, since the blood–brain barrier might be restrictive to the delivery of therapeutic molecules (Christensen *et al.*, 2019). Viral vectors, such as Adeno-Associated Viruses (AAVs), are commonly used for gene delivery, but they can elicit immune responses and have limited packaging capacity (S.-H. Wu *et al.*, 2021). Nonviral delivery methods, such as magnetic nanoparticles, are also being explored to increase gene-editing efficiency and biocompatibility. The other issue is the challenge of off-target effects where CRISPR–Cas9 edits at unintended locations in the genome, causing unintended effects. Careful design of guide RNAs and thorough screening for off-target effects are necessary to minimize this risk (Venema *et al.*, 2024). Furthermore, ethical considerations surrounding gene editing in the brain, particularly in the context of neurodevelopmental disorders, need to be carefully evaluated.

Gene Regulation

CRISPRa and CRISPRi regulate gene expression without creating double-strand breaks (DSBs), while base and prime editing allow precise single-base modifications, offering strong potential as targeted therapeutic tools. CRISPRa/i use catalytically inactive Cas9 (dCas9) with sgRNAs to up- or downregulate genes, reducing risks linked to DSBs. For instance, CRISPRa could enhance OXTR expression to improve neurological or social functions (Loth *et al.*, 2025). Yet, challenges remain in achieving precise control and minimizing off-target effects. Base editors, such as Cytidine (C>T) and Adenine (A>G) editors, and prime editors—which allow all base conversions plus small indels—enable correction of pathogenic variants like those in SCN2A (Antoniou *et al.*, 2021). These approaches avoid limitations of traditional HDR-based CRISPR, but safety, specificity, and protein function impacts require careful evaluation (Saber Sichani *et al.*, 2023).

CRISPR technologies now extend to epigenetic editing and reversible gene expression control. Druggable CRISPRa/i systems, using Estrogen Receptor (ERT2) domains responsive to estrogen analogs, allow temporal regulation across species (Sui *et al.*, 2025). AI integration could further improve design and accuracy. Base and prime editing have shown promise in correcting cardiac mutations, as well as in treating blood disorders and mucopolysaccharidoses (Shang *et al.*, 2024).

Beyond human therapies, CRISPR-based tools have advanced plant research by enabling precise amino acid changes, stop codon insertions, and regulatory modifications, improving yields and stress tolerance. Viral vectors provide efficient delivery in crops, supporting practical applications in breeding (Shen *et al.*, 2024).

Multigene editing approaches

Multigene editing provides a powerful approach to study epistatic networks, such as PTEN and TSC1 in the mTOR pathway, which

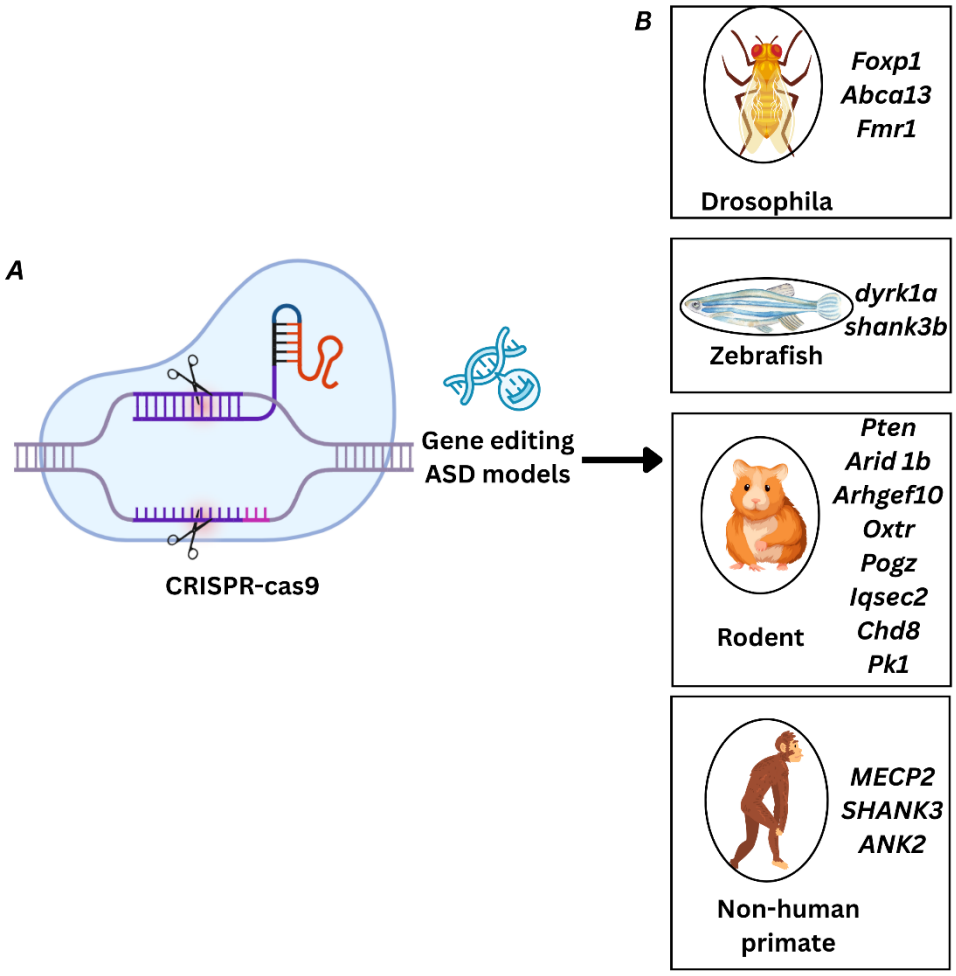

Figure 4: CRISPR-Cas9-based gene editing in ASD animal models. (A) Schematic representation of the CRISPR-Cas9 system used to introduce targeted modifications in genomic DNA. (B) Application of CRISPR gene editing across various model organisms to generate Autism Spectrum Disorder (ASD) models by targeting specific ASD-associated genes. These include *Foxp1, Abca13*, and *Fmr1* in Drosophila, *dyrk1a* and *shank3b* in Zebrafish, and a broader panel of genes such as *Pten, Arid1b, Arhgef10, Oxtr, Pogz, Iqsec2, Chd8*, and *Pk1* in rodent models. Additionally, non-human primates have been used to model ASD through CRISPR-mediated modification of *MECP2, SHANK3*, and *ANK2*.

is frequently dysregulated in cancers (Laes *et al*., 2017; Nguyen *et al*., 2023).

PTEN is a phosphatase that antagonizes PI3K/AKT signaling by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3), a lipid second messenger that recruits and activates AKT. By reducing PIP3 levels, PTEN inhibits AKT activation, thereby dampening downstream signaling to mTOR. On the other hand, TSC1 in complex with TSC2 acts in conjunction with TSC2 in the generation of GTPase-Activating Protein (GAP) activity expressed by Rheb, a GTPase that directly activates mTORC1. The TSC1/TSC2 complex also favors the hydrolysis of Rheb-GTP to Rheb-GDP, inactivating Rheb and suppressing the mTORC1 signaling pathway (Magdalon *et al*., 2017). Disruption of *PTEN* or *TSC1* leads to hyperactivation of the mTOR pathway, resulting in increased cell growth, proliferation, and survival. PTEN antagonizes PI3K/AKT signaling by reducing PIP3 levels, thereby inhibiting AKT-mediated mTOR activation. Conversely, TSC1/TSC2 inactivates Rheb, suppressing mTORC1 activity.

Multiple gene editing strategies (including CRISPR-Cas9) permit the concomitant loss of both PTEN and TSC1, permitting researchers to parse their epistatic associations and look into the joint consequences of their malfunction. For example, researchers can generate isogenic cell lines via CRISPR-Cas9 by generating single (PTEN or TSC1 knockout) or combined (PTEN and TSC1 knockout) strains and determine the effects of these genetic variants on the activity of the mTOR pathway, cellular growth, proliferation, apoptosis, and other phenotypes of interest (Izumi *et al*., 2020). These experiments may be used to determine the combined synergistic or antagonistic effects of PTEN and TSC1 loss on mTOR signaling and the cellular response.

In vivo vs. *Ex vivo* Strategies

Therapeutic approaches to gene editing can be broadly defined as *ex vivo* (organized outside of the body) and *in vivo* (within the body), which have relative benefits and limitations. The principles behind *ex vivo* gene editing include the isolation of cells, modification, and expansion of patient cells *ex vivo* before they are finally transplanted back into the same patient (Rosanwo & Bauer, 2021).

The benefit of this method is tight control of the editing process and possible fogging of the off-target effects and stringent quality testing prior to administration. For example, Hematopoietic Stem Cells (HSCs) may be sampled from a patient, engineered to counteract a mutation, and redosed into the patient to ensure the presence of functioning blood cells (George *et al.*, 2024). *Conversely, in vivo* gene editing involves the direct delivery of gene-editing components into the body, targeting specific cells or tissues for modification. In the context of brain delivery, this can be achieved through intracranial injection or systemic vectors. The *in vivo* method avoids cell transplantation and therefore may have wider applicability and simpler implementation; however, it suffers greatly in terms of delivery efficacy, specificity and possible immune reactions (T. Wei *et al.*, 2020).

The option between *ex vivo* and *in vivo* approaches is largely dictated by the disease, target tissue and treatment objectives. *Ex vivo* methods are especially useful for treating blood disorders and those related to the immune system, as the manipulated cells can be reinfused in breadth and grafted. In the treatment of neurological disorders where direct use of the brain is of the utmost need, *in vivo* methods are promising but are limited by successful transportation across the blood–brain barrier (Haasbroek-Pheiffer *et al.*, 2023). Both strategies are becoming increasingly sophisticated as delivery technologies, including those involving viral vectors, lipid nanoparticles and cell-penetrating peptides, are developing toward greater efficiency and selectivity. Gene editing therapies can only be successful in the long term by ensuring the safety of the processes, streamlining delivery, and enhancing the accuracy of introducing a change in the genes with the thin end of the wedge (Volodina & Smirnikhina, 2025).

In vivo and *ex vivo* gene therapies can also potentially enhance organ transplantation. For example, *Ex vivo* Lung Perfusion (EVLP) is being used with gene therapy to produce better lungs for use as transplant organs (Nykänen *et al.*, 2024). This would include developing therapeutic targets, the use of gene therapies in EVLP and a cycle of improvement. Similarly, *in vivo* gene modification has been investigated in 11784 cellular therapies as a possible remedy for 11784 hemoglobinopathy, providing the potential to change the genetics of patients' hematopoietic stem cells at a locus without the allogeneic immune response or the danger of insertion mutagenesis. *Ex vivo* gene therapy has successfully been used to treat the Central Nervous System (CNS), where neurodegenerative diseases and posttraumatic recovery after CNS injury can be averted by the delivery of genes through the use of viral vector systems ("*Ex vivo* Gene Therapy in the Central Nervous System," 2002). This approach leverages the clinical knowledge of hematopoietic stem cell transplantation and gene transfer. Hydrogel-based formulations have supported the local administration and combined delivery of immunotherapies, which have improved the efficacy of the treatment and reduced the systemic toxicity of immunotherapy as an oncological agent (Erfani *et al.*, 2023). For intracranial cancers, hydrogels can bypass the blood–brain barrier, offering a direct and efficient delivery method.

In summary, *ex vivo* and *in vivo* gene editing approaches have immeasurable potential for treating myriads of diseases, and more studies are being conducted on how to make these methods more efficient, specific, and safe in the long run. The advancement of gene therapy can be characterized by the development of an increasing number of clinical trials and approvals by the FDA, which signifies an important transformative change in the direction of acknowledgment of gene therapy as a remedy (Delshad *et al.*, 2025). Future research directions include studies

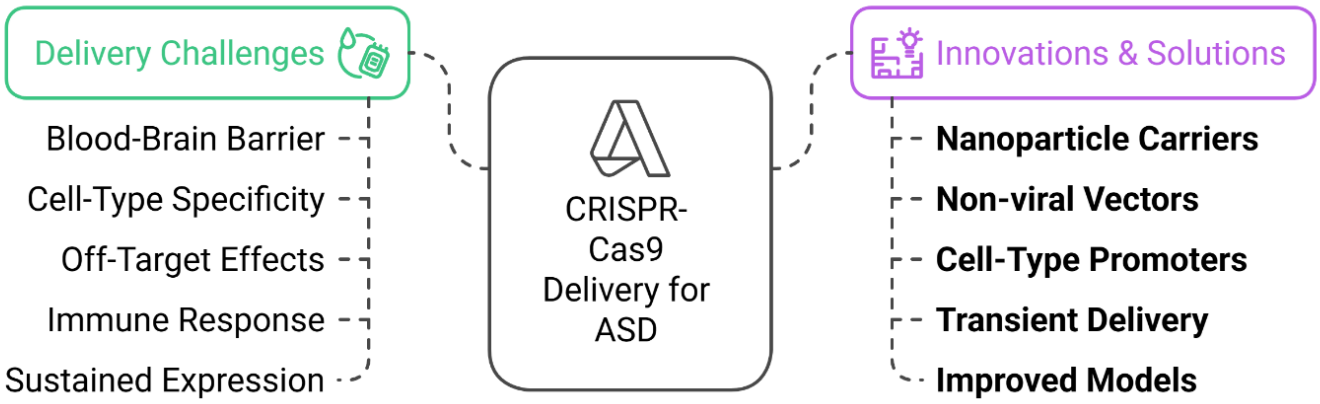

Figure 5: CRISPR-Cas9 Delivery Challenges and Solutions for ASD.

Table 2: Delivery systems for CNS editing.

Method	Description	Advantages	Limitations	Considerations
Adeno-Associated Virus (AAV) Vectors	Viral vectors with various serotypes engineered for CNS tropism (Hui *et al.*, 2022; D. Zhu *et al.*, 2021). Can be delivered via direct intraparenchymal injection, into the Cerebrospinal Fluid (CSF), or intravenously.	High transduction efficiency long-term therapeutic gene expression, ability to transduce nondividing cells like neurons (Shaikhutdinov *et al.*, 2024), broad tissue, and reduced immunogenicity with capsid engineering. Systemic delivery has the potential to broadly transduce the CNS.	Limited cargo capacity (~4.7 kb), potential for off-target expression and heightened immune response with systemic delivery, can result in significant systemic biodistribution depending on the dose. Preexisting immunity to AAV serotypes in many.	Choice of serotype, delivery route, and dose are critical for safety and efficacy. Capsid engineering can improve transduction efficiency, reduce immunogenicity, and enhance BBB (Kingwell, 2023). AAV9 is considered efficient at targeting the blood–brain barrier. Novel AAV vectors (e.g., AAV-AS) show improved CNS transduction after systemic administration.
Lipid Nanoparticles (LNPs)	Nonviral vectors composed of lipids that encapsulate gene-editing components. Can be modified with targeting ligands to enhance delivery to specific. Can be administered intravenously or intrathecally.	Low immunogenicity, high delivery, can protect gene editing components against biological barriers and release them into the cytoplasm (F. Wu *et al.*, 2025), can deliver mRNA to the blood–brain barrier, can deliver Cas9 mRNA/sgRNA complex for effective genome editing in the brain.	Variable Blood–Brain Barrier (BBB) penetration (Gao *et al.*, 2024), delivery to the CNS has been, can face challenges in delivering large donors, lack of restriction by cell-type/ tissue-specific promoters for Cas9 expression.	Optimization of LNPs is needed for brain delivery. New LNP platforms (e.g., MK16 BLNP) can deliver mRNA to the brain via intravenous injection by taking advantage of natural transport mechanisms within the BBB (D. Wang *et al.*, 2020). Brain-targeting Lipids (BLs) can be incorporated into LNPs to generate brain-targeting lipid nanoparticles.
Ex vivo Editing	Cells are extracted from the patient, genetically modified *in vitro*, and then transplanted back into the patient. Can involve various cell types, including T cells, Hematopoietic Stem Cells (HSPCs), induced Pluripotent Stem Cells (iPSCs), and Neural Stem Cells (NSCs).	Precise cell targeting, more controlled editing compared to *in vivo* approaches, reduced risk of GVHD and graft rejection, offers the possibility of manipulating the cell cycle to improve HDR efficiencies (Mwema *et al.*, 2023).	Invasive extraction required, requires large, clean populations of genome-edited cells, may require enrichment for the edited cell population, relies on efficient plasmid uptake and suitability as a template donor for eukaryotic cell repair mechanisms.	The choice of cell type depends on the specific disease and therapeutic goals. Can be combined with CRISPR-Cas9 or TALEN reagents for gene editing.

of new gene-editing technologies, such as prime editing and new delivery systems, as ways of addressing current limitations.

DELIVERY CHALLENGES AND INNOVATIONS

Blood–Brain Barrier (BBB) penetration

The barrier to overcome is the Blood–Brain Barrier (BBB), which poses an enormous challenge to the delivery of any therapeutic agent into the brain and leads to the development of novel methods to overcome it (Deisseroth, 2013). The three types of approaches, which are viral vectors, Focused Ultrasound (FUS), and Trojan horse liposomes, have advantages and limitations, and all have the same aim, namely, to either break through or circumnavigate the BBB (Fu & McCarty, 2016). The altered adeno-associated virus AAV-PHP.eB effectively penetrates the BBB in mice, although its mode of action is not fully understood, and its efficacy and safety in humans have not been determined (Xie *et al.*, 2021).

FUS, especially when used together with microbubbles, is a noninvasive approach for temporarily opening the BBB in a controlled way, which allows effective delivery into a specific part of the brain. Trojan horse liposomes rely on receptor-mediated transcytosis to deliver drugs through the BBB; however, they may have limited receptor target supply and may cause undesired off-target activities (Y. Wei *et al.*, 2023a).

AAV-PHP.eB takes advantage of the natural transduction property of viruses, and its application presents concerns both the immune response and off-target effects. It involves the use of concentrative waves of ultrasound in the presence of microbubbles of air, which scavenge and break the tight junctions between the cells of the endothelium, increasing BBB permeability. Recent studies have highlighted the potential of FUS in treating neurodegenerative diseases by enhancing the delivery of neuroprotective agents. A previous study demonstrated that FUS technology augmented with microbubbles and GDNF-based therapeutics can be used to access the blood–brain barrier temporarily to introduce neuroprotective agents as a treatment for human neurological disorders (Zhao *et al.*, 2024). The acoustic pressure, intensity of the ultrasound, and concentration of microbubbles should be properly controlled in terms of BBB opening and nondestructive cracking of the brain tissue in the surrounding environment (Mehkri *et al.*, 2023). Upon binding to the receptor, the liposomes are internalized via endocytosis and transported across the BBB.

In one paper, combining chitosan and pApoE2 to improve penetration of the BBB via dual-modified liposomes was reported in a mouse model (Chakraborty *et al.*, 2024). Trojan horse liposomes are dependent on a number of factors related to efficient activity, such as the selection of ligands, the receptor density on the BBB, and the propensity of liposomes to escape endosomes subsequent to internalization (Y. Wei *et al.*, 2023b). Furthermore, potential limitations include off-target effects and the risk of triggering immune responses.

Each of these BBB penetration strategies presents unique advantages and disadvantages. AAV-PHP.eB offers high transduction efficiency but raises safety concerns. FUS provides a noninvasive and targeted approach but requires careful optimization of treatment parameters. Trojan horse liposomes offer a targeted delivery system but face challenges in achieving efficient transcytosis and avoiding off-target effects. Future research may focus on combining these strategies to create synergistic effects and overcome the limitations of individual approaches. For example, FUS could be used to enhance the delivery of Trojan horse liposomes, or AAV-PHP.eB vectors could be engineered to incorporate targeting ligands for improved specificity. Multimodal strategies may be necessary to revolutionize CNS therapeutics (Ghaznavi *et al.*, 2024; Lei *et al.*, 2024).

Enhancing specificity

The specificity of CRISPR-Cas9 genome editing is further improved with high-fidelity variants of Cas9 and the optimization of sgRNAs to reduce off-target effects (Matsumoto *et al.*, 2024). SpCas9-HF1 is an engineered version of the Cas9 protein designed for increased specificity while maintaining on-target activity. Modification of nonessential residues that participate in DNA interactions decreases nonspecific binding without affecting the capacity to cut the target sequence. It is essential to optimize single-guide RNAs (sgRNAs) to increase target recognition followed by efficiency. There are strategies of engineered iterations to omit the repetitions likely to cause off-target binding, the addition of chemical modifications, or shorter sgRNAs that are designed to lower the tolerance of mismatches. Although high-fidelity Cas9 variants are highly useful for reducing off-target effects, they must be utilized to determine universal reliability by testing their applications in various genomic contexts. Precision-targeting SgRNAs can be designed systematically and computationally, allowing precision targeting; however, unpredictable off-target mutations require advanced algorithms that reflect genomic heterogeneity learning (Yuan, 2024). Integrating sgRNA optimization and protein engineering is the key to having fully specific CRISPR-Cas9-based gene-editing systems without sacrificing the efficacy of the therapeutic.

The unintended mutations that result from off-target effects are a major issue of concern in CRISPR-Cas9 technology and undermine the validity of a given study and its therapeutic safety. Some methods have been created to overcome these impacts, such as high-fidelity versions of Cas9, e.g., SpCas9-HF1, which increases specificity. The other method involves the optimization of sgRNA design, including features such as the specificity of a designed sequence, its GC content, and secondary structure,

to increase its binding efficiency and suppress its off-target interactions. The technology of bioinformatics is also important in reducing off-target effects, including predicting possible off-target effects, as well as in improving the specificity of sgRNA planning. These tools enable researchers to reveal parts with high similarity to the target site, thus allowing them to avoid or adjust sgRNAs that can potentially bind to them. Furthermore, the delivery method of CRISPR–Cas9 components influences the efficiency and specificity of genome editing. Ribonucleoprotein (RNP) delivery, in which the preassembled Cas9-sgRNA complex is used directly by cell entry, is more specific and efficient than the delivery of plasmid DNA or mRNA into cells (Alsaiari *et al*., 2024).

The CRISPR-Cas9 method is based on the use of Cas9 to cut target DNA guided by specific RNA (sgRNA), but off-target effects and mutations that are uncontrollable can occur (Guo & Zhen, 2020). Major considerations that can enhance CRISPR sgRNA effectiveness are the specificity of the sequence, the GC content, the formation of second molecules and how close the sequence is to the Protospacer Adjacent Motif (PAM). The PAM sequence, typically NGG, is essential for Cas9 recognition and binding (H. I. Mohamed *et al*., 2024).

The Cas9-Sgrna complex breaks the DNA strands to form Double-Strand Breaks (DSBs) that need to be unrestricted to interact with DSB pathways (S.-C. Liu *et al*., 2022). Target affinity the affinity of Cas9-sgRNA for target binding depends on its target interaction, which regulates the time at which its targets remain at the target site after cleavage and influences the definition of DSB repair pathways, which are a source of mutational heterogeneity. High-fidelity SpCas9-HF1 increases the accuracy of cell cycle-dependent editing because it improves HDR and minimizes off-target effects and on-target mutagenic consequences (Matsumoto *et al*., 2024). Nanomaterial-mediated Cas9 delivery and highly specific RNA molecules can further prevent off-target editing.

The challenges of CRISPR/Cas9-mediated genome editing can be divided into four categories: regulatory concerns, constraints of the PAM sequences, economic aspects, and specific unwanted effects at various sites. Novel promoters, optimized transformation techniques, and improved T-DNA delivery can improve outcomes in medicinal plants. Ribonucleoprotein (RNP) usage is a more accurate and nonimmunogenic way to edit the genome. Multiomic approaches can be used to investigate valuable genes and pathways, enabling more targeted gene editing (Borah *et al*., 2024).

The CRISPR/Cas9 system holds promise for clinical applications, but off-target effects remain a major challenge. Deleting specific residues from HNH linkers can create SpCas9 variants with high fidelity and efficiency (G. Wang *et al*., 2023). High-throughput *in vitro* specificity profiling can compare the cleavage activities of different Cas9 variants (Murugan *et al*., 2020). SWOffinder is an effective tool for finding CRISPR off-target sequences, including bulges with the implementation of Smith–Waterman alignment. RNA and DNA bulges, mismatches, and PAM sequences contribute to off-target effects (Yaish *et al*., 2024).

Immune and Safety Concerns

Despite having a revolutionary effect on gene editing, CRISPR-Cas9 technology has very profound immune and safety issues, including the threat of anti-Cas9 antibodies, inflammation, and mosaicism of edited tissues (Crudele & Chamberlain, 2018). The bacterial characteristic of Cas9 is the possibility of causing an immune response in humans, thus affecting its medical effectiveness and leading to adverse consequences ("Safety Aspects of Genome Editing," 2022a). Moreover, mosaicism (occurring owing to the content of genetically dissimilar cell groups in edited tissues) hinders the provision of therapeutic results in the form of consistency between edits of various individuals (Mehravar *et al*., 2019).

Preexisting immunity to Cas9 proteins in humans is a significant hurdle for CRISPR-Cas9 therapies. Humans can create antibodies against Cas9 resulting from past exposure to bacteria ("Safety Aspects of Genome Editing," 2022b). These preexisting antibodies can neutralize Cas9, reducing the efficiency of gene editing. The presence of these antibodies can lead to the rejection of CRISPR–Cas9-based therapies, limiting their effectiveness (A. Li *et al*., 2020). On the one hand, antibodies, cytokines, and resident immune cells are present in the eye, although they lack T cells, and intraocular inflammation, which can be fuelled by Cas9, is also a highly recognized cause of vision loss (Toral *et al*., 2022).

Inflammation is another significant safety concern associated with CRISPR–Cas9 gene editing. The immune response to Cas9 can induce inflammation, both locally and systemically. The immune response to Cas9 can induce inflammation, both locally and systemically. This inflammatory condition may cause destruction of organs and tissues with negative health effects. Given the setting of cancer treatment, although CRISPR-Cas9 may be employed to knock out IL-6 in CAR-T cells to reduce Cytokine Storm Syndrome (CRS), the inflammatory reaction itself must be tightly controlled. Immunosuppressants might be required to manage inflammation; however, intervention can also reduce the effectiveness of gene editing treatment (Z. Wang, 2025).

One problem that is widely encountered in the context of CRISPR-Cas9-based gene editing is mosaicism (the case of cells with distinct genetic constitutions in the same organ tissue). Mosaicism arises because gene editing may not occur uniformly across all cells. This may yield certain cells that are edited and other cells that are not edited, resulting in an impure cell culture. In human embryos, the common Loss of Heterozygosity (LOH) in CRISPR-Cas9-modified clones is observed, resulting in

mosaicism (Alanis-Lobato *et al.*, 2021). This mosaicism can reduce the therapeutic efficacy of gene editing, as unedited cells may continue to express disease-causing genes. Furthermore, mosaicism can lead to unpredictable and variable outcomes, making it difficult to assess the safety and efficacy of CRISPR-Cas9 therapies. In sperm genome editing, mosaicism and off-target edits remain challenges.

These immune and safety concerns are subject to the development of several strategies to overcome. Depending on the route of delivery, it is possible to limit the duration of Cas9 expression (e.g., Cas9 delivery via mRNA or incorporation into a Ribonucleoprotein (RNP) complex), minimizing the likelihood of prompt activation of an immune response (Chew, 2018). An overview of various delivery systems designed to increase CRISPR–Cas9 precision and reduce immune-related risks in the central nervous system is summarized in Table 2. Another approach is the use of humanized Cas9 versions, which have been engineered so that they are less immunogenic. To limit off-target effects during Cas9 applications, Cas9 can be regulated by anti-CRISPR proteins (Acrs), which are naturally existing inhibitors of CRISPR–Cas systems. Such tissue-restricted delivery systems as microRNA-blockable anti-CRISPR proteins have the potential to limit gene editing to labeled tissues and reduce the possibility of off-target effects or inflammation (Lee *et al.*, 2019). The accuracy of genome targeting via CRISPR–Cas can also be enhanced with computational engineering of Acrs. Moreover, methods to standardize editing, which reduces the risks associated with mosaicism and increases clinical safety and efficacy, are needed (Marsiglia *et al.*, 2024).

Advancement of next-generation allogeneic CAR-T-cell products with multiple edits, such as CTX112, is an illustration of an attempt to enhance efficacy and avoid prevalent failure mechanisms. With all of these advanced treatments, CRISPR/Cas9 editing technology is used to disarm genes that induce T-cell exhaustion and promote the specificity of CAR-T cells (Ghobadi *et al.*, 2024). In hematopoietic stem cell transplantation, CRISPR/Cas9 is used to modify HSCs to treat hematological malignancies and severe autoimmune diseases. Moreover, CRISPR-Cas9-induced knockout of genes has been applied to enhance host-to-egg immunity in Schistosoma japonicum, thus confirming the ability of CRISPR-Cas9 to manipulate immune responses (L. Zhang *et al.*, 2022). CRISPR-Cas9 is an engineering tool that is being used in plants where disease-resistant crops are developed so that food safety is improved worldwide. In xenotransplantation, the genetic engineering of pigs to minimize the immunogenicity of pig organs used in the transplant is being performed via CRISPR-Cas9 genetic engineering (Galli, 2025). The various applications emphasize the flexibility and potential of CRISPR-Cas9 technology, as well as the necessity of discussing related immune and safety issues (Figure 5) (Tyagi *et al.*, 2021).

FUTURE DIRECTIONS

Advancements in research are paving the way for more precise and personalised approaches to understanding and treating diseases. New technologies, like prime editing, have shown an unmatched refinement in genome engineering directly writing new genetic data to a defined DNA location in a form not producing unwanted byproducts by using double-strand breaks in the DNA (Anzalone *et al.*, 2019). In addition, CRISPR-dCas9 systems, with the maturity of epigenetic editing, can give chances to study reversible gene control in disease processes through transcription stimulation and inhibition (Rajanathadurai *et al.*, 2024). Individualised medicine solutions, such as patient-specific *in vitro* screening with organoids give new avenues to predict individual response to therapies and therefore do advance treatment until the treatment itself can be customized (Martel *et al.*, 2020). As these methods leveraged the power of collaborative and data-sharing initiatives worldwide, like SFARI Gene, which can quicker discover the targets and feasibly develop more effective methods of handling and treatment of various diseases through resources and experience shared (Majchrzak-Celińska *et al.*, 2021). Together, these strategies bridge genetic insights with therapeutic applications, promising a future of more targeted and effective treatments.

CONCLUSION

CRISPR-Cas9 technology represents a transformative platform in ASD research and therapy, offering unprecedented precision in modeling disease mechanisms and targeting genetic aberrations. By enabling the generation of patient-specific cellular models, humanized animal systems, and direct *in vivo* gene correction, CRISPR bridges the gap between genetic discovery and therapeutic intervention. However, critical barriers such as immunogenicity, off-target effects, delivery limitations, and ethical considerations must be overcome to fully harness its clinical potential. Continued innovation in guide RNA design, delivery vectors, and immune evasion strategies is essential. As our understanding of the genetic and epigenetic complexity of ASD has deepened, CRISPR-based interventions hold promise not only for treating monogenic forms but also for addressing the multifactorial nature of idiopathic ASD. With rigorous preclinical validation and ethical foresight, CRISPR technology may soon shift the paradigm from symptom management to precision-guided, etiology-based interventions in autism spectrum disorder.

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ABBREVIATIONS

ASD: Autism Spectrum Disorder; **CRISPR:** Clustered Regularly Interspaced Short Palindromic Repeats; **Cas9:** CRISPR-associated protein 9; **FDA:** Food and Drug Administration; **ABA:** Applied Behavior Analysis; **iPSC:** Induced Pluripotent Stem Cell; **HDR:** Homology-Directed Repair; **NHEJ:** Nonhomologous End Joining; **sgRNA:** Single-guide RNA; **crRNA:** CRISPR RNA; **tracrRNA:** Trans-activating CRISPR RNA; **DSB:** Double-strand Break; **PAM:** Protospacer Adjacent Motif; **NHP:** Nonhuman Primate; **AAV:** Adeno-Associated Virus; **LNP:** Lipid Nanoparticle; **NSC:** Neural Stem Cell; **HSPC:** Hematopoietic Stem and Progenitor Cell; **FXS:** Fragile X Syndrome; **KO:** Knockout; **ERT2:** Mutated Human Estrogen Receptor Domain; **BLNP:** Brain-targeting Lipid Nanoparticle; **EVLP:** *Ex vivo* Lung Perfusion; **BBB:** Blood–Brain Barrier; **GVHD:** Graft-versus-host Disease.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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