

Reversible genome editing

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Genome editing has traditionally been regarded as permanent genomic alterations. While this durability underpins lasting therapeutic benefit, it also raises safety concerns due to the risk of potentially irreversible adverse effects. Here, we demonstrate the feasibility of reversible genome editing *in vitro* and *in vivo* using diverse platforms, including CRISPR-Cas9, base editors (BEs), and prime editors (PEs). Specifically, we show that a cytosine base editor (CBE) can restore normal splicing by reversing adenine base editor (ABE)-induced intron retention, and that PE can correct frameshift mutations generated by Cas9 or PE in animals. These genomic restorations led to sustained recovery of protein expression *in vivo*. Furthermore, we achieved reversible editing of clinically relevant human genes, including *ANGPTL3*, *SERPINA1*, *TTR*, and *PCSK9*, through targeted correction of diverse mutation types, providing an “antidote” to the genome editing events. Together, these findings establish a framework for reversible genome editing as a generalizable strategy to enhance the safety, flexibility, and therapeutic scope of genome editing.

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INTRODUCTION

Genome editing, which enables precise modification of the human genome, holds tremendous potential for treating a broad spectrum of diseases^{1,2}. Its therapeutic value was first demonstrated in clinical applications for sickle cell disease and transfusion-dependent β -thalassemia, with therapies now approved in the United States and several other countries³. To date, more than 200 genome editing-related clinical trials have been registered, with over 150 currently active, underscoring the rapid transition of this platform technology from preclinical studies to clinical application^{4,5}.

Metabolic diseases represent a major global health burden, with cardiovascular diseases remaining the leading cause of death worldwide despite the availability of effective treatments⁶. Therefore, clinical development of genome editing has largely focused on a set of targets for metabolic and cardiovascular diseases, with transthyretin amyloidosis and familial hypercholesterolemia repeatedly investigated across multiple trials (ClinicalTrials.gov)^{4,5}. For example, an ongoing clinical trial is evaluating lipid nanoparticles (LNPs) encapsulating Cas9 mRNA and gRNA for targeted delivery to the liver to suppress angiotensin-like 3 (*ANGPTL3*), a gene that regulates low-density lipoprotein (LDL) and triglyceride (TG) levels⁷. Initial results demonstrated more than 80% reductions in LDL and TG in patients with familial hypercholesterolemia (crisprtx.com). A parallel trial using a base editor to target *ANGPTL3* has also been initiated⁸ (ClinicalTrials.gov NCT06451770). Another major target under clinical investigation is proprotein convertase subtilisin/kexin type 9 (*PCSK9*), an essential factor for plasma cholesterol homeostasis, which

is disrupted *in vivo* using different base editors (BEs)^{9,10} (NCT06164730, NCT06461702).

The initial clinical applications of genome editing targeting metabolic regulators such as *ANGPTL3* and *PCSK9* have primarily focused on monogenic cardiovascular disorders, including familial hypercholesterolemia. However, the patient population of these types of diseases remains relatively small. The genome editing therapy for the same set of targets holds the potential to expand beyond monogenic cardiovascular diseases to a much larger population of patients with established cardiovascular diseases, and even to high-risk individuals as a form of preventive medicine. Its unique ability to provide a one-time intervention could fundamentally disrupt the current model of chronic disease management, which depends on lifelong adherence and substantial healthcare resources. Nevertheless, most genes exert pleiotropic effects within complex regulatory networks. Consequently, therapeutic strategies that permanently abolish gene expression may carry unforeseen risks. For instance, *ANGPTL3* plays critical physiological roles in metabolic adaptation during cold exposure, in cardiac energy supply, and in hepatic lipid metabolism¹¹⁻¹³. Similarly, while *PCSK9* inhibition is generally well tolerated, population-based studies suggest that long-term suppression may elevate the risk of type 2 diabetes, and genetic models indicate that complete, lifelong *PCSK9* deficiency can remodel cardiac metabolism and predispose to heart failure^{14,15}. Therefore, this permanence and irreversibility may also raise important concerns about drug safety and restrict therapeutic applications to a broader population, particularly when inhibition of a target could cause severe

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side effects in a subset of patients or in certain modified physiological and medical conditions.

Similar safety considerations of genome editing apply to siRNA-based therapies, which also induce long-term suppression of target genes. The dose intervals of siRNA therapeutics have advanced from weeks to months, and now to annual regimens¹⁶. Although siRNA does not achieve the permanent inhibition as genome editing, sustained gene inhibition has raised safety concerns. To address this, an siRNA “antidote” termed REVERSIR was developed to provide control over inhibitory effects¹⁷. An siRNA therapy targeting transthyretin (TTR) has been FDA-approved for treating TTR-related amyloidosis, and co-administration of REVERSIR has been shown to restore TTR protein expression and rapidly terminate siRNA-mediated inhibition in emergency situations¹⁷. Another example is angiotensinogen (AGT), the sole precursor protein in the renin-angiotensin-aldosterone system that is central to long-term blood pressure regulation and fluid-electrolyte homeostasis. AGT is primarily synthesized in the liver, and a single injection of siRNA targeting hepatic AGT production effectively lowers blood pressure for months¹⁸. However, AGT and its downstream effector, angiotensin II, are indispensable for maintaining arterial pressure and ensuring critical tissue perfusion during acute hypotensive crises, such as hemorrhagic or septic shock, major emergency surgery or pregnancy¹⁹. To mitigate potential risks associated with sustained AGT suppression, an AGT-targeted REVERSIR has been evaluated in a clinical trial (NCT06675565). In parallel, studies employing Cas9-mediated disruption of AGT showed a sustained decrease in blood pressure *in vivo*, supporting the therapeutic potential of a one-time, durable treatment for hypertension²⁰. A clinical trial pursuing this approach is imminent (crisprtx.com). Unlike siRNA, effective reversal strategies for genome editing remain elusive, and would be critically important in scenarios where restoration of blood pressure is required. Current genome editing offers no means to terminate or reverse its effects once delivered.

Therapeutic genome editing, which modifies the DNA sequences of human cells, has been regarded as a “one-way” ticket, as permanent and irreversible intervention. Here, we demonstrate that DNA genome editing can be reversible at the genomic level. The major genome editing strategies currently employed in clinical trials include Cas9 nucleases, BEs, and prime editors (PEs). We show that at the genomic level, single-base mutations generated by BE can be restored by using BE or PE, while indels introduced by Cas9 or PE can be largely reversed by PE, resulting in functional recovery at the protein and phenotypic levels, in both human cells and in animals. This capability to reverse genome editing offers a potential safeguard to terminate therapy under emergency situations, while also expanding the therapeutic scope of this technology.

RESULTS

Reversible genome editing in cultured cells

Reversible genome editing employs two genome editors: one to disrupt the target gene and another to restore protein function by reversing the induced mutation (Fig. 1a). Among various strategies for genome editing to disrupt protein-coding sequences, we initially focused on nonsense muta-

tions, missense mutations, and intron retention by base editors as they are used for therapeutic applications (Fig. 1b)^{21,22}.

We applied a HEK293T-EGFP stable cell line, and employed CBE to target the cytosine at codon 158 (EGFP site 1) in the coding region, aiming to switch it from CAG to TAG, thereby introducing a nonsense mutation that resulted in a premature stop codon (Fig. 1b, up). The percentages of EGFP negative cells were more than 60% after CBE treatment (Supplementary Fig. S1a, b). For reverse editing, we used ABE to reverse it from TAG to CAG. The efficiency of reverse editing in the single-cell clone derived HEK293T-EGFP cell lines with TAG genotype was near 60%, while the efficiency of reverse editing in bulk cell pool treated with the first genome editor was about 50% (Fig. 1c and Supplementary Fig. S1a). Consistent with genomic correction, more than 50% of the cells that were TAG genotype regained EGFP fluorescence after reverse editing treatment (Fig. 1d). The percentage of EGFP-positive cells in the bulk cell pool rose from 36% to 63% (Supplementary Fig. S1b). The efficiency gap of reverse editing between cells with TAG genotype and bulk cell pool was attributed to the bystander editing of the BEs. Nevertheless, these data indicate that reversible editing is feasible in cells.

To determine whether reversible editing can be achieved along with other deactivating strategies, we utilized ABE to introduce a missense mutation (TCC to CCC) by changing an A to a G in the complementary strand, generating an S31P EGFP mutant that lost fluorescence activity (Fig. 1b, middle). ABE-mediated knockout efficiency was high, achieving near 80% EGFP negative cells (Supplementary Fig. S1c, d). To reverse the mutation, we employed CBE to restore it from CCC to TCC. The efficiencies of reverse editing were about 50% and more than 30% in the cells with CCC genotype and bulk cell pool, respectively (Fig. 1e and Supplementary Fig. S1c). Consistent with genomic correction, about 67% of the cells with CCC genotype recovered EGFP fluorescence after reverse editing (Fig. 1f). The percentage of EGFP positive cells in the bulk cell pool rose from about 22% to 65% (Supplementary Fig. S1d).

To examine whether reversible editing can be achieved with the intron retention mediated knockout strategy, we targeted endogenous *Pcsk9* gene as a proof-of-concept. We first utilized ABE to generate an A to G mutation in the splicing site adjacent to exon 1 (*Pcsk9* site 1), changing GT to GC, a modification known to induce intron retention^{23,24} (Fig. 1b). This mutation has been reported to decrease serum levels of PCSK9 and LDL cholesterol in mice and cynomolgus monkeys^{23,24}. ABE converted this GT to GC with an efficiency of over 70% in the mouse hepatocyte cell line Hepa1-6 (Supplementary Fig. S1e). The expression of *Pcsk9* mRNA levels reduced approximately 65% accordingly (Supplementary Fig. S1f). We applied CBE to successfully revert the edited C to T, with an average editing efficiency of about 56% in cells with GC genotype, and the reverse editing was also significant in bulk cells (Fig. 1g and Supplementary Fig. S1e). Notably, approximately 7% of unintended base conversions (GG/GA alleles) were detected during reverse editing, underscoring the need for more precise BEs in future applications. Consistent with genomic allele correction, the expression of both mRNA and protein levels of *Pcsk9* were significantly

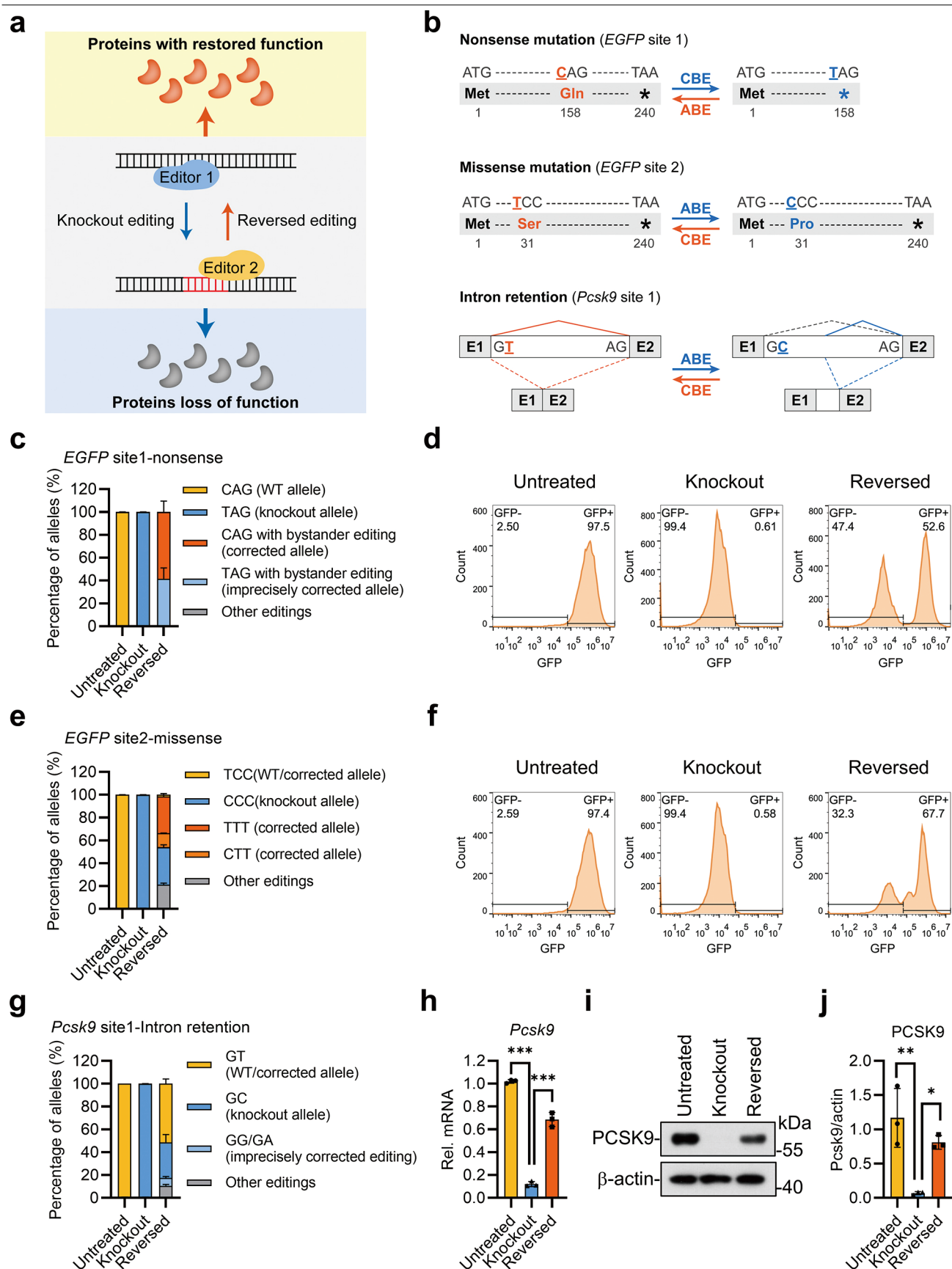


Fig. 1 Reversible genome editing in cultured cells. **a** Schematic of reversible genome editing. Editor 1 introduces disruptive lesions in genomic DNA, leading to loss of protein function. Editor 2 mediates reverse editing, restoring protein expression and function. **b** Design principles for reversible genome editing. Three effective editing strategies of gene disruption and corresponding reverse methods are depicted. **c**, **d** Deep sequencing analysis of reverse editing efficiencies (**c**) and flow cytometric quantification of EGFP functional impairment and recovery

(d) in HEK293T-EGFP cells harboring pre-edited nonsense mutations at EGFP site 1. CAG, wild-type allele encoding EGFP protein with normal fluorescence activity. TAG, knockout allele carrying a nonsense mutation that produces a truncated, non-fluorescent EGFP protein. CAG with bystander editing, corrected allele encoding EGFP with the W160G mutation and retaining normal fluorescence activity. TAG with bystander editing, imprecisely corrected allele producing a truncated, mutant EGFP protein with no fluorescence activity. e, f Deep sequencing analysis of reverse editing efficiencies (e) and flow cytometric quantification of EGFP functional impairment and recovery (f) in HEK293T-EGFP cells harboring pre-edited missense mutations at EGFP site 2. TCC, wild-type/corrected allele encoding serine at codon 31, producing EGFP with normal fluorescence activity. CCC, knockout allele encoding EGFP carrying the S31P mutation with abolished fluorescence activity. TTT/CTT, corrected alleles encoding fluorescent EGFP with the S31F and S31L mutations, respectively. g Deep sequencing analysis of reverse editing efficiencies in Hepa1-6 cells harboring pre-edited GT to GC mutation at *Pcsk9* site 1. GT, wild-type/corrected allele encoding a fully functional *Pcsk9* protein. GC, knockout allele that causes intron retention and abolishes protein function. GG/GA, imprecisely corrected alleles that cause intron retention and abolish protein function. h qRT-PCR quantification of *Pcsk9* mRNA level in above cells. i, j Representative western blot analysis of PCSK9 (i) and quantification of the relative protein levels normalized to β -actin (j). Data are represented as mean $\pm$ SD of $n = 3$ independent biological replicates for c, e, g, h, and j, and $n = 3$ independent experiments with similar results for d and f. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

boosted in those cells after reverse editing (Fig. 1h–j and Supplementary Fig. S1f). These data collectively demonstrated that genome editing is reversible for multiple knock-out strategies.

Reversible genome editing by BEs *in vivo*

To demonstrate reversible editing is feasible *in vivo*, we targeted *Pcsk9* site 1 (Fig. 2a), and encapsulated mRNA encoding ABE and corresponding gRNA into LNPs and delivered intravenously to mice, mimicking the recent clinical trial using BEs^{23,24}. Serum levels of PCSK9 and LDL cholesterol (LDL-C) were assessed 1.5 weeks post-injection to evaluate the efficiency of loss-of-function editing (Fig. 2b). At week 8, the mice underwent reverse editing, followed by evaluation of editing outcomes at week 9.5 or week 13. After knockout with ABE, the proportion of wild-type alleles in the mouse liver decreased to about 33%, while this proportion was restored to 57% after reverse editing (Fig. 2c). The efficiencies of reverse editing at week 13 were consistent with those at week 9.5 (Fig. 2d). Serum PCSK9 and LDL-C levels exhibited a significant decrease at 1.5 weeks and remained reduced until reverse editing, after which they significantly recovered and remained stable at both week 9.5 and week 13 (Fig. 2e, f). These results demonstrate that the reversible genome editing *in vivo* is feasible.

To assess the potential hepatic toxicities associated with reverse editing, we measured serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AKP). No significant differences in these liver damage markers were identified among the untreated, knockout and reversed groups (Supplementary Fig. S2a–c). To assess the gRNA-dependent off-target effects of knockout and reverse editing in mouse liver, we performed CHANGE-seq using a randomly fragmented and circularized mouse genomic DNA library²⁵. We sequenced the amplicons of the top 75 or 79 CHANGE-seq-nominated sites for knockout- or reversed-sgRNAs using genome of livers from untreated, knockout-edited, and reverse-edited mice (Supplementary Fig. S3a, b). Notably, while discernible editing was observed at on-target sites, only minimal off-target editing was observed at a small subset of predicted sites. No statistically significant differences in off-target editing frequencies were identified between sgRNA-treated groups and untreated controls (Supplementary Fig. S3c, d). Nevertheless, the possibility of gRNA-independent DNA off-target editing by BEs persists in reversible editing, but we have not evaluated such

editing owing to the challenge of achieving clonal expansion of single primary mouse hepatocytes *in vitro*. In addition to potential DNA off-target effects, BEs are also known to induce sgRNA-independent RNA editing via the deaminase activities^{26,27}. To assess the off-target effects from RNA editing, RNA sequencing was conducted on mouse liver samples 48 h post LNP delivery. Compared to livers treated with Cas9, livers treated with ABE7.10 RA (knockout editing) or ABE7.10 RA followed by A3A(N57Q)-BE3 (reverse editing) did not exhibit higher off-target effects, with no significant differences observed between the knockout and reverse editing groups (Supplementary Fig. S4a, b). The minimal off-target profiles of ABE and CBE observed in this study align well with previously published findings^{23,28}. The low frequency of RNA off-target editing is likely attributable to both the transient expression of BEs in mouse livers and the use of BE variants, ABE7.10 RA and A3A(N57Q)-BE3, which are known to exhibit reduced off-target propensity^{29,30}. Further analysis of RNA off-target editing sites showed that they were broadly distributed across the transcriptome, with the highest proportions found in 3' untranslated regions, downstream regions, and transcripts targeted for nonsense-mediated decay (Supplementary Fig. S4c–e). These results suggest that despite the involvement of two sequential genome editing processes for reversible editing using different BEs, the off-target effects remain low.

Reversible genome editing using Cas9 and PE *in vivo*

BEs can reverse point mutations but are constrained to specific base conversions and cannot repair Cas9-induced indels. Given that Cas9-mediated knockout editing may produce insertion or deletion (indel) products, we selected PE to perform reverse editing due to its capacity to address various editing outcomes (Fig. 3a). Although CRISPR-Cas9-mediated editing typically generates random indels, appropriate sgRNA selection allows the preferential generation of 1-bp insertions as the predominant editing outcome *in vivo*^{31,32}. This preference results from staggered double-strand breaks that promote precise 1-bp insertions, and these breaks are favored by specific nucleotides such as a guanine at position 18³¹. We therefore screened sgRNAs by amplicon deep sequencing to identify target sites that predominantly yield 1-bp insertions. Using Cas9, we introduced a single thymine insertion (insT) at codon 250 of the *Pcsk9* gene, and induced a frameshift mutation that disrupted the open reading frame and abolished PCSK9 expression (Fig. 3a–e and Supplemen-

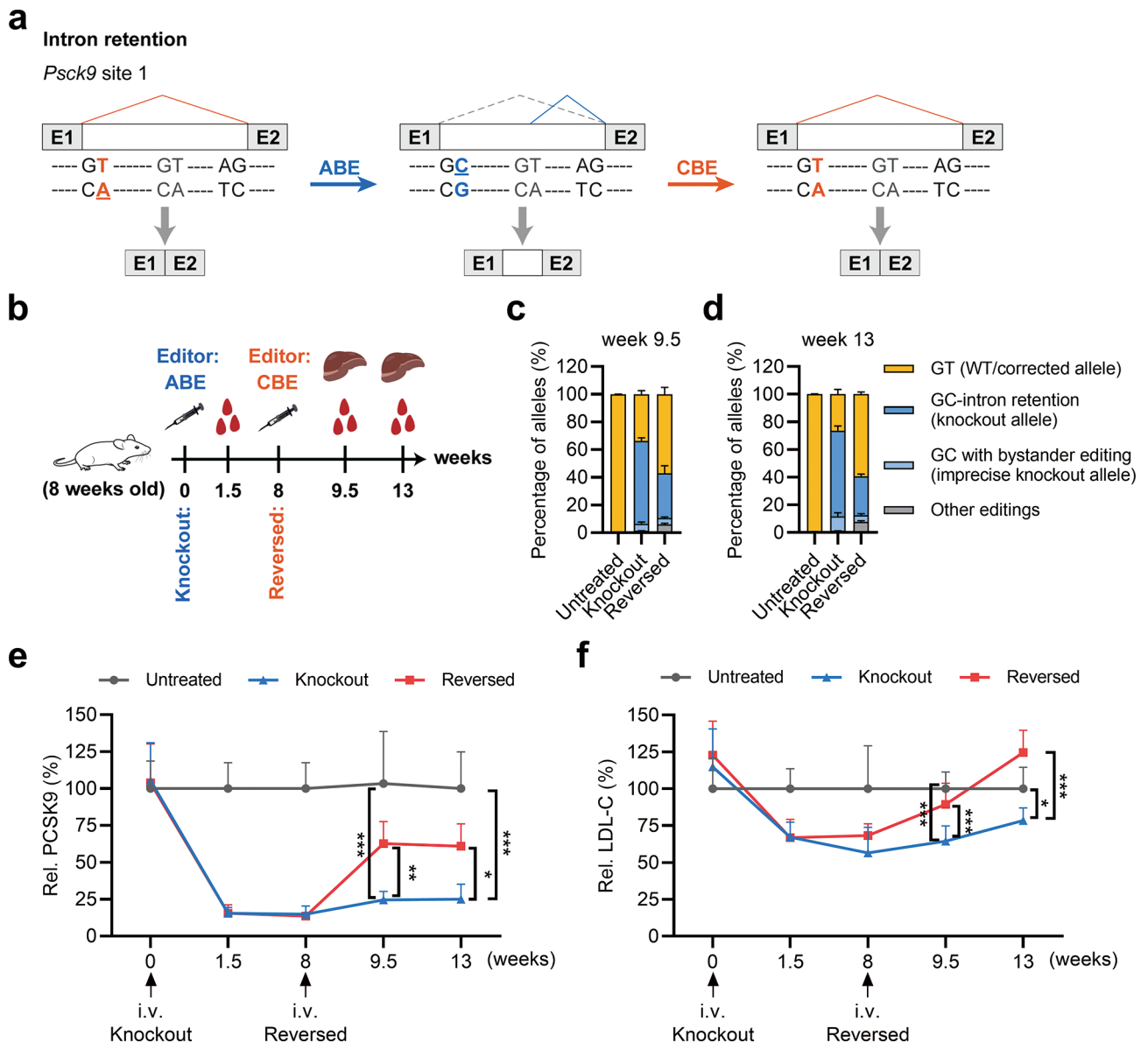


Fig. 2 Reversible genome editing by BEs *in vivo*. **a** Schematic illustration of the reverse editing strategy for *Pcsk9* site 1. Edited nucleotides are labeled in colors. **b** Timeline and workflow of *in vivo* experiments. **c**, **d** Deep sequencing analysis for genomic editing efficiencies in the mouse livers at weeks 9.5 and 13. GT, wild-type/corrected allele encoding a fully functional PCSK9 protein. GC, knockout allele that causes intron retention and abolishes protein function. GC with bystander editing, imprecise knockout allele harboring bystander edits that also induces intron retention and produces no functional protein. **e** Serum PCSK9 levels of mice at each time point. **f** Serum LDL-C levels of mice at each time point. Data are represented as mean $\pm$ SD. For panel (c): $n = 4$ mice for untreated and knockout groups, $n = 6$ mice for the reversed group. For panel (d): $n = 6$ mice for untreated and knockout groups, $n = 4$ mice for the reversed group. For panels (e, f): $n = 10$ mice per group at 0, 1.5, and 9.5 weeks; $n = 6$ mice for untreated and knockout groups, $n = 4$ mice for the reversed group at 8 and 13 weeks. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

tary Fig. S5a). Restoration was then achieved by PE to correct this insT genotype (Fig. 3a). To discriminate correctly restored editing products from wild-type sequences and improve editing efficiency, we engineered a molecular barcode by introducing synonymous point mutations in the codon 248 (PAM site) and codon 250 (Fig. 3a). To improve editing efficiency and precision, we screened nick sgRNAs. On the basis of its superior reverse editing efficiency among different nick sgRNAs tested, nick 6 sgRNA was chosen for all following editing experiments (Supplementary Fig. S5a). Deep sequencing analysis indicated that PE effectively reverted the frameshift mutation with an average efficiency of approximately 80% in

the single-cell clone derived Hepa1-6 cells with insT genotype (Fig. 3b). Consistent with the genomic changes, *Pcsk9* mRNA and protein levels showed a dramatic decrease in knockout Hepa1-6 cells and were restored following reverse editing (Fig. 3c-e).

To assess the *in vivo* feasibility of this strategy, we utilized Cas9 to introduce an insT mutation in mouse livers via LNP delivery, and then the mice underwent reverse editing using PE by LNP delivery (Fig. 3f). The *in vivo* editing efficiencies by LNP delivery of PE were low or moderate³³. To meet the high efficiency demands of reversible *in vivo* prime editing, we utilized our recently developed densely modified

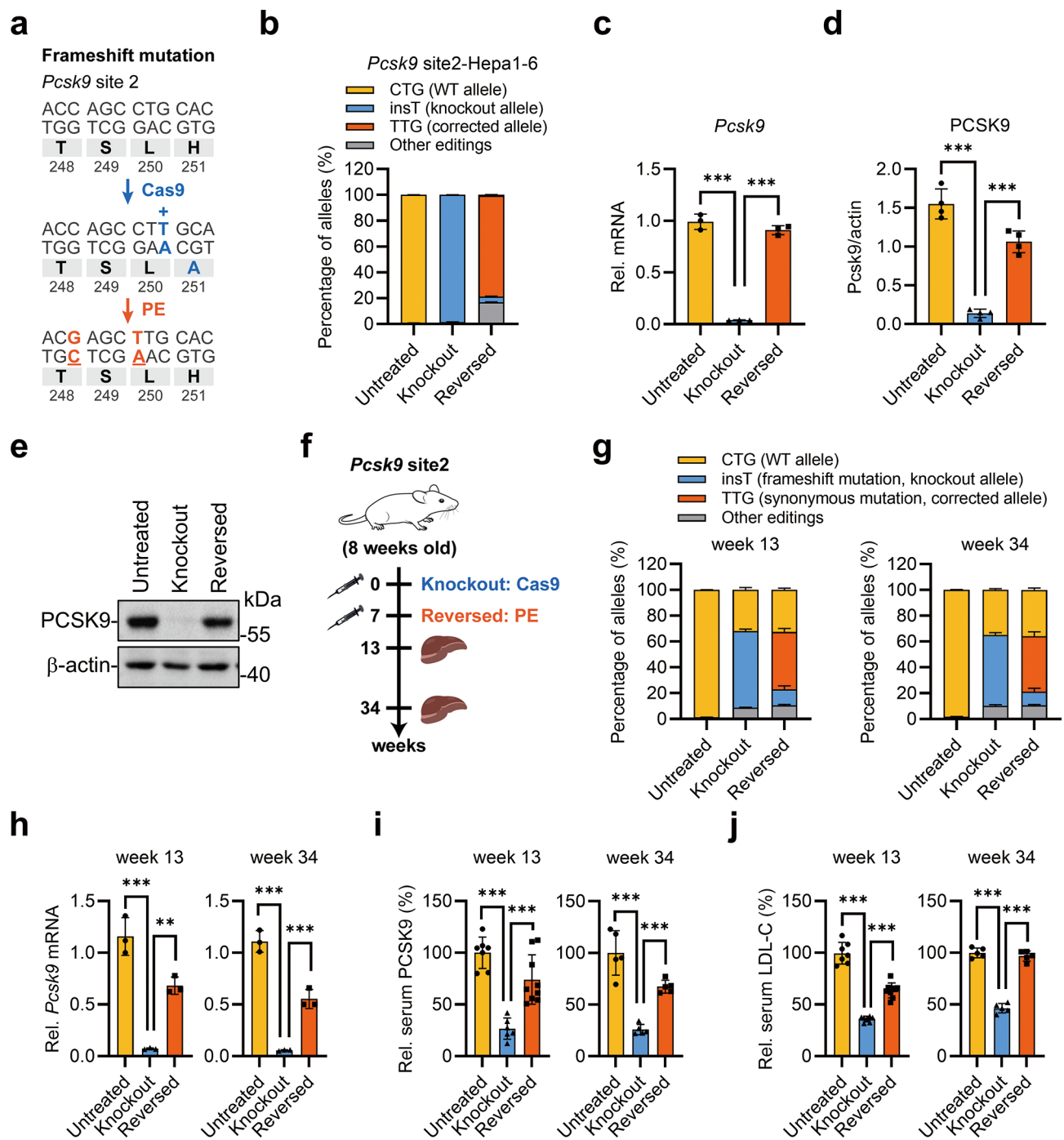


Fig. 3 Reversible genome editing using Cas9 and prime editor *in vivo*. **a** Schematic illustration of the editing strategy for *Pcsk9* site 2. Edited nucleotides are indicated in colors. **b** Deep sequencing analysis of editing efficiencies in Hepa1-6 cells harboring pre-edited insT mutation in the *Pcsk9* site 2. CTG, wild-type allele encoding PCSK9 with Leu250. insT, knockout allele that causes frameshift mutation and abolishes PCSK9 expression. TTG, corrected allele with synonymous mutations, encoding functional PCSK9. **c–e**, qRT-PCR and western blot quantification of *Pcsk9* mRNA (**c**) and its protein expression levels (**d, e**) in Hepa1-6 cells. **f** Timeline and workflow of *in vivo* experiments. **g** Deep sequencing analysis of genomic knockout and reverse editing efficiencies by Cas9 and prime editor in mouse liver. The meanings of the product genotypes and protein functions are the same as in panel (**b**). **h** qRT-PCR quantification of *Pcsk9* mRNA levels in mouse liver. **i, j** Serum PCSK9 and LDL-C levels of mice. Data are represented as mean $\pm$ SD of $n = 3$ independent biological replicates for **b** and **c**, $n = 4$ independent biological replicates for **d** and **e**. For panel (**g**): $n = 6$ mice per group at week 13, and $n = 5$ mice per group at week 34. For panel (**h**): $n = 3$ mice per group at both week 13 and week 34. For panel (**i**): $n = 7$ mice for the untreated group, $n = 6$ mice for the knockout group, and $n = 9$ mice for the reversed group at week 13; $n = 5$ mice per group at week 34. For panel (**j**): $n = 7$ mice for the untreated and knockout groups, and $n = 9$ mice for the reversed group at week 13; $n = 5$ mice per group at week 34. 34. ** $P < 0.01$; *** $P < 0.001$.

epgRNA^{33–35}. We previously demonstrated that systemic LNP delivery of the PE mRNA and this densely modified epgRNA reached almost 70% prime editing efficiency in bulk mouse

liver, corresponding to an approximately 80-fold increase over conventional end-modified pegRNAs, supporting efficient *in vivo* PE editing via non-viral delivery^{33,34}. Cas9-mediated

ated knockout editing yielded approximately 70% knockout alleles, with the precise insT allele comprising about 60% (Fig. 3g). The efficiencies of reverse editing by PE remained consistent at weeks 13 and 34, with the barcoded synonymous mutation accounting for 44% and 43% of total alleles, respectively (Fig. 3g). Consistent with the allele changes, *Pcsk9* mRNA levels, as well as serum PCSK9 and LDL-C levels, showed a dramatic decrease in the knockout group and were restored in the reverse-edited group at both time points, indicating that the restored PCSK9 expression and associated serum phenotypes were stably maintained over the long term (Fig. 3h–j).

We further validated this reversible editing strategy at the *Ttr* site 1 in mouse liver (Supplementary Fig. S5b, c). Cas9 was used to generate a frameshift mutation via a single adenine insertion (insA) at codon 45, disrupting the open reading frame and abolishing *Ttr* expression. Subsequent PE correction restored the frame (Supplementary Fig. S5b, c). A synonymous CCT to CCG change at codon 44 served as a molecular barcode to distinguish corrected from wild-type alleles (Supplementary Fig. S5b). Cas9-mediated knockout editing generated the insA allele at a frequency of nearly 62% (Supplementary Fig. S5d). Subsequent PE-mediated reverse editing resulted in the barcoded synonymous mutation accounting for about 34% and 40% of total alleles at weeks 10 and 54, respectively (Supplementary Fig. S5d). Consistent with the genomic changes, *Ttr* mRNA and protein levels dropped after knockout and recovered following PE-mediated correction (Supplementary Fig. S5e–g).

We also performed reversible editing for *Pcsk9* site 3 and Inhibin β E (*Inhbe*) in Hepa1-6 cells using Cas9 as knockout tool. INHBE has emerged as an attractive target for genome editing-based therapeutic intervention against obesity, and siRNA targeting INHBE is under clinical investigation^{36,37}. Although Cas9 tends to produce dominant 1-bp insertion alleles *in vivo*, its editing profiles in dividing cell lines are more heterogeneous and diverse^{31,32}. Despite this limitation, PE can reverse a major portion of the 1-bp deletion in cells (Supplementary Fig. S5h, i). Collectively, these results indicated that PE can precisely restore the knockout editing products generated by Cas9.

In addition, given that Cas9-mediated editing frequently generates a heterogeneous spectrum of indels rather than a single uniform genotype, we assessed the feasibility of multi-pegRNA reverse editing at mouse *Pcsk9* site 4 in Hepa1-6 cells (Supplementary Fig. S5j, k). Cas9 knockout predominantly produced delG (64.6%), insG (7.6%), and delGG (4.4%) alleles (Supplementary Fig. S5j, k). We designed individual reverse pegRNAs targeting each product and compared the correction efficiencies of single-pegRNA versus multi-pegRNA strategies. Deep sequencing revealed that the delG-specific pegRNA alone achieved a correction rate of 30% in total alleles, whereas the insG-specific pegRNA alone yielded only about 0.5% corrected alleles. Notably, the delGG-specific pegRNA alone reverted both delGG and delG alleles, achieving a total correction efficiency of 27% (Supplementary Fig. S5k). Co-delivery of the delG-specific pegRNA with either the insG- or delGG-specific pegRNA, or of all three pegRNAs together, did not significantly improve the correction efficiency of the delG allele, nor did it enhance the overall correction frequency

across all mutant alleles (Supplementary Fig. S5k). Collectively, these results indicate that multiplexing additional pegRNAs confers no additional benefit under our experimental conditions.

Reversible genome editing using an all-PE-based platform *in vivo*

PE demonstrates significant potential for gene therapy, which is primarily attributed to its high precision and operational versatility. To harness this capability, we developed an all-PE-mediated reversible editing strategy. For direct comparison with Cas9 knockout, we applied this system to the same *Ttr* site 1, where PE was used to introduce the insA frameshift mutation (Fig. 4a). Restoration of the reading frame was achieved via PE-mediated precise deletion of the inserted adenine (Fig. 4a). A synonymous mutation (CCT to CCG) in codon 44 was introduced to barcode the corrected alleles (Fig. 4a). Deep sequencing analysis demonstrated that PE6c effectively reverted the frameshift, with a mean correction efficiency of about 36% in insA-genotype Hepa1-6 cells (Fig. 4b).

We further utilized PE to introduce an insA mutation in mouse livers via LNP delivery, and six weeks later, the mice underwent reverse editing using PE (Fig. 4c). PE-mediated knockout editing achieved an insA editing efficiency of about 60% in the liver. Subsequent PE-mediated reverse editing introduced the barcoded synonymous mutation into about 29% and 37% of total alleles at weeks 10 and 43, respectively (Fig. 4d). Consistent with the genomic changes, *Ttr* mRNA and protein levels exhibited a dramatic decrease and nearly full restoration in knockout and reversed groups, respectively (Fig. 4e–g). Collectively, these data validate the feasibility of all-PE-based reversible editing strategy, while also demonstrating that PE can be employed for the high-fidelity restoration of knockout edits generated by diverse editors.

Reversible genome editing at therapeutic-related human genomic sites

We have shown reversible editing *in vitro* and in animals. To demonstrate that reversible editing is feasible in human cells, we selected human *ANGPTL3*, serpin family A member 1 (*SERPINA1*), *TTR* and *PCSK9*, which are major targets for metabolic and cardiovascular diseases (Fig. 5a–i). Genome editing to disrupt these genes is either in clinical trials or about to enter clinical trials. We applied nonsense mutation or intron retention to knockout these genes, and used BE or PE for reverse editing (Fig. 5a and Supplementary Fig. S6a–h). We utilized CBE to target codon 171 of *ANGPTL3* gene and codon 180 of *SERPINA1* for nonsense mutation, and applied ABE to successfully reverse the genomic sequences and gene expression levels (Fig. 5b–e and Supplementary Fig. S6a–e). We utilized ABE to target the splicing site of *TTR* exon 3, or that of *PCSK9* exon 1, and applied CBE or PE for effective reversing (Fig. 5f–i and Supplementary Fig. S6f–h). This side-by-side comparison indicates PE generates less undesired bystander editing than BE during reverse process (Fig. 5h). These data indicate that reversible editing strategy can be applied for various therapeutically relevant targets, and PE-mediated reversed editing is more precise and versatile than BE (Fig. 5h–j).

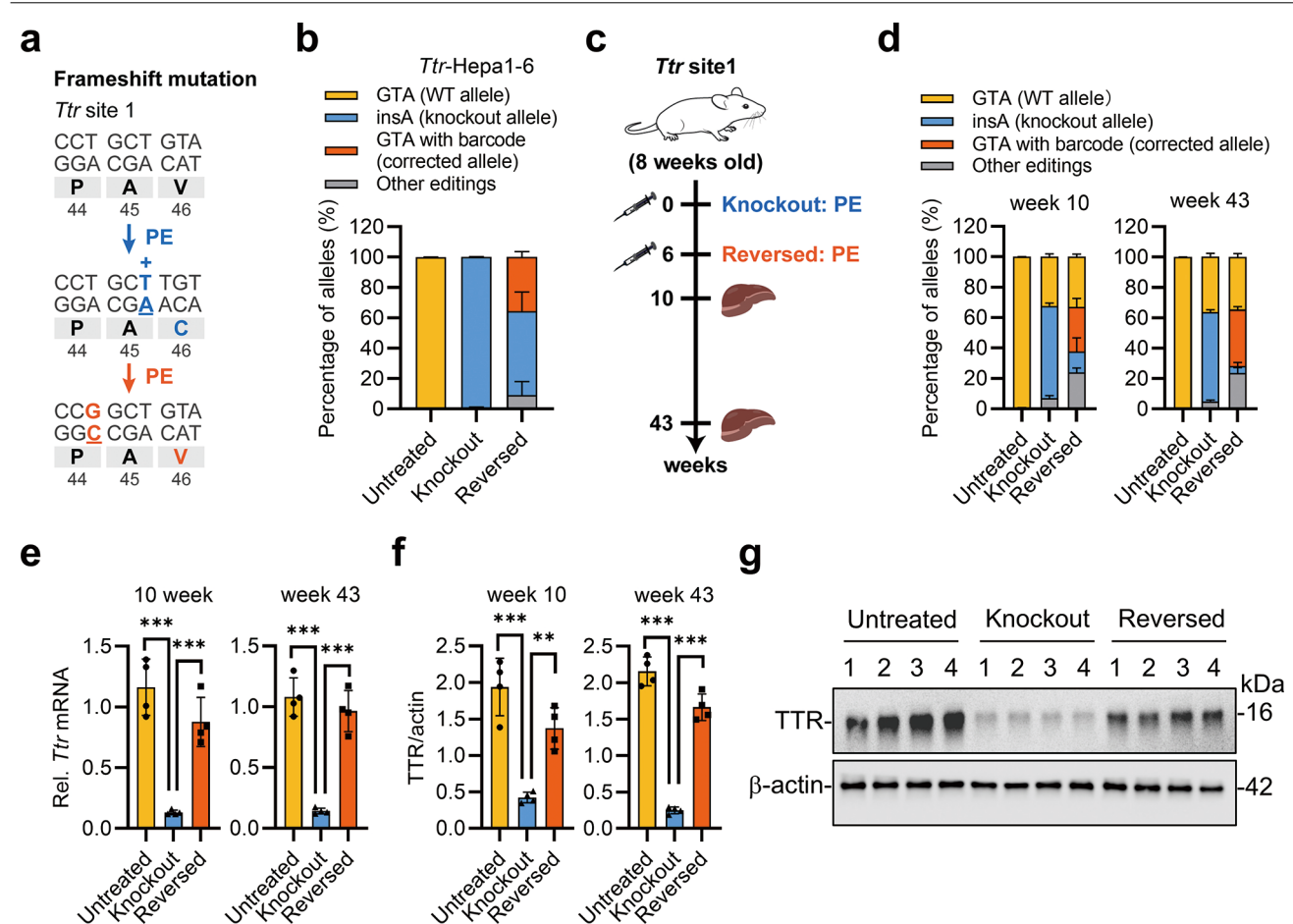


Fig. 4 Reversible genome editing using an all-PE platform *in vivo*. **a** Schematic illustration of the editing strategy for *Ttr* locus. Edited nucleotides are indicated in colors. **b** Deep sequencing analysis of reverse editing efficiency of the prime editor in Hepa1-6 cells harboring pre-edited insA mutation in the *Ttr* locus. GTA, wild-type allele encoding TTR with Val46. insA, knockout allele that causes the frameshift mutation and abolishes *Ttr* expression. GTA with the barcode, corrected allele with synonymous mutations, encoding functional TTR. **c** Timeline and workflow of *in vivo* experiments. **d** Deep sequencing analysis of genomic knockout and reverse editing efficiencies by the PE in mouse liver. The meanings of the product genotypes and protein functions are the same as in panel (b). **e–g** qRT-PCR and western blot quantification of *Ttr* mRNA (**e**) and its protein (**f**, **g**) levels in mouse liver. Data are represented as mean $\pm$ SD. Panel (b), $n = 3$ independent biological replicates. Panel (d), $n = 6$ mice per group at week 10 and $n = 5$ mice per group at week 43. Panels (e–g), $n = 4$ mice per group. ** $P < 0.01$; *** $P < 0.001$.

DISCUSSION

In this study, we demonstrated that genome editing can be reversed, thereby mitigating the risks of lifelong and life-threatening adverse effects, and broadening the range of recipient population and potential therapeutic targets, in particular for metabolic and cardiovascular diseases. Across multiple knockout strategies, including nonsense and missense mutations, intron retention, and frameshift mutations, and using widely applied genome editors such as Cas9, ABE, CBE, and PE, we show that protein deactivation can be reversed at both genomic and expression levels with precision editors. Furthermore, reversal was achieved *in vitro*, *in vivo*, and in human cells. Our findings shift the paradigm of therapeutic genome editing by introducing a route for emergency termination of gene-based therapies, expanding their safety, versatility, and potential clinical applications, underscoring the reversibility of genome editing across various targets for indications of common diseases beyond monogenic disorders. This advancement enables therapeutic genome editing to move from a small patient population and

clinical targets toward a much broader spectrum, promoting a wide range of clinical applications.

Current genome editing tools, including BEs and PEs, can precisely introduce specific types of desired edits and can also be used to reverse mutations introduced by themselves, with their application for either mutation induction or reversion depending largely on experimental design. Nevertheless, there is a fundamental distinction between previous investigations and the present study. Earlier studies that employed genome editors to generate disease models or correct pathogenic mutations generally adopted zygotic editing to establish genetically modified animal lines, followed by therapeutic intervention across subsequent generations. Such approaches essentially represent germline modification and intergenerational intervention, which differ substantially from somatic clinical gene therapy. By contrast, our work establishes sequential *in vivo* somatic knockout and reverse editing within the same adult individual, faithfully recapitulating a clinically relevant paradigm of iterative somatic genome editing. This strategy enables targeted genetic alteration and

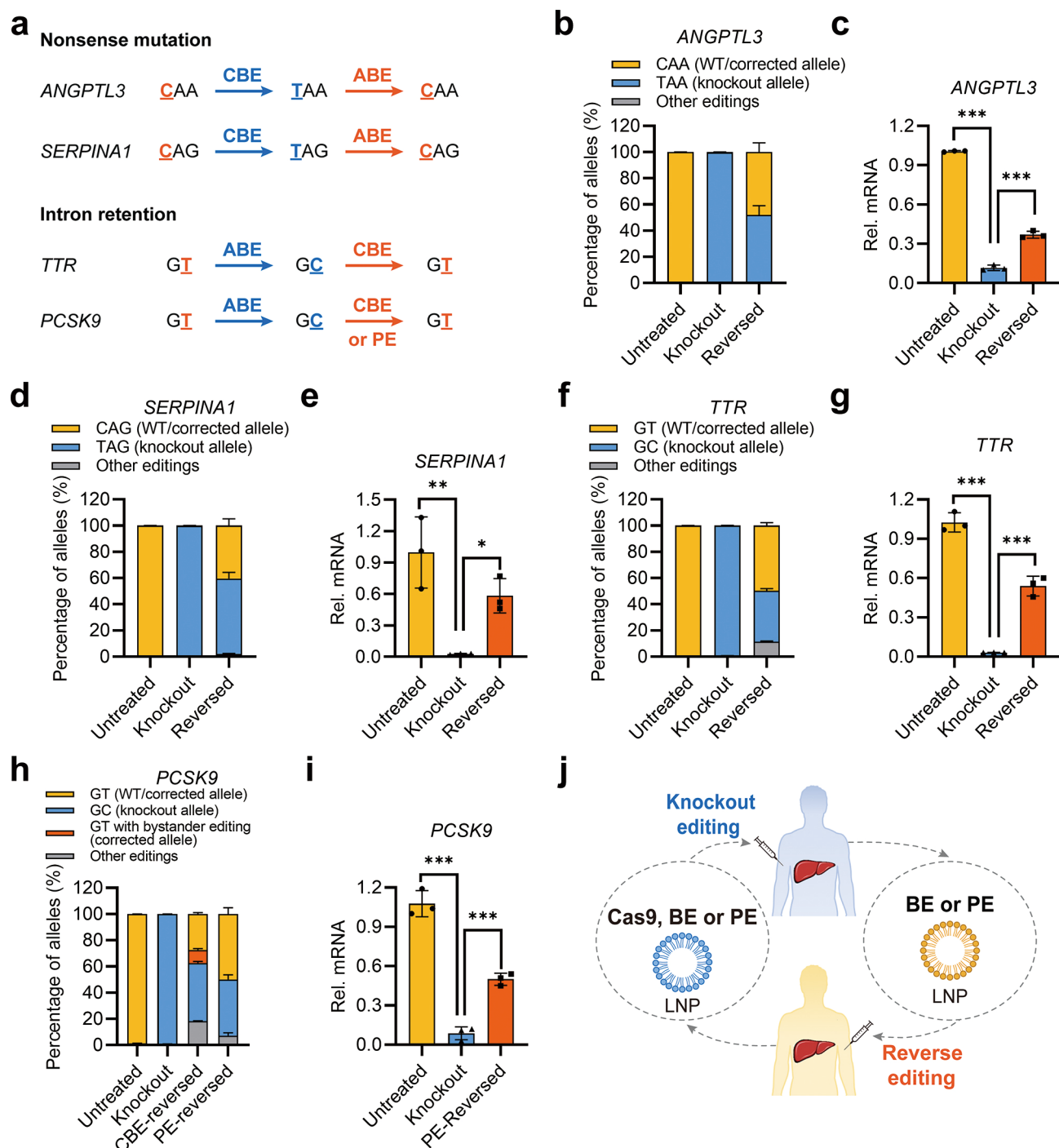


Fig. 5 Reversible genome editing at therapeutic-related human genomic sites. a Schematic illustration of reversible genome editing. The disruption strategies were nonsense mutations for *ANGPTL3* and *SERPINA1* loci and intron retention for *TTR* and *PCSK9*. Disruption was mediated by CBE or ABE, and the reverse editing was introduced by ABE, CBE, or PE. b, c Deep sequencing analysis of reverse editing efficiencies (b) and qRT-PCR quantification of mRNA levels (c) in Huh-7 cells harboring the pre-edited mutation at the *ANGPTL3* locus. CAA, wild-type/corrected allele encoding functional *ANGPTL3* proteins. TAA, knockout allele carrying a nonsense mutation that produces non-functional *ANGPTL3* proteins. d, e Deep sequencing analysis of reverse editing efficiencies (d) and qRT-PCR quantification of mRNA levels (e) in Huh-7 cells harboring the pre-edited mutation at the *SERPINA1* locus. CAG, wild-type/corrected allele encoding functional *SERPINA1* proteins. TAG, knockout allele carrying a nonsense mutation that produces non-functional *SERPINA1* proteins. f, g Deep sequencing analysis of reverse editing efficiencies (f) and qRT-PCR quantification of mRNA levels (g) in Huh-7 cells harboring the pre-edited mutation at the *TTR* locus. GT, wild-type/corrected allele encoding fully functional *TTR* proteins. GC, knockout allele that causes intron retention and abolishes protein functions. h, i Deep sequencing analysis of reverse editing efficiencies (h) and qRT-PCR quantification of mRNA levels (i) in Huh-7 cells harboring the pre-edited mutation at the *PCSK9* locus. GT, wild-type/corrected allele encoding fully functional *PCSK9* proteins. GC, knockout allele causing intron retention and abolished protein function. GT with bystander editing, corrected allele carrying bystander edits that produces functional proteins. j Illustration of reversible editing using precision genome editors. Data are represented as mean $\pm$ SD of $n = 3$ independent biological replicates, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

precise restoration in a single adult organism, providing a straightforward and clinically translatable proof-of-concept that is mechanistically distinct from conventional zygote-based model construction and intergenerational mutation correction.

In addition, imprecise editing products, including mutated RNAs, partial peptides (pre-stop codon) or alternative splicing products, and off-target editing by Cas9 and BEs, may all give rise to toxic gene editing outcomes. We suggest that reversible editing also holds the potential to reverse such outcomes, particularly in the case of PE-mediated reversal, which may enable simultaneous correction of diverse unwanted editing products. Therefore, the direct experimental verification of genome editing reversibility in our study not only offers a viable reversal strategy for gene therapy, but also suggests a potential approach to mitigate adverse genome editing outcomes, through the reversal of such byproducts generated during treatment. Of note, the current reversible editing approach cannot achieve complete restoration of the wild-type allele, and genomic mosaicism inevitably persists. Nevertheless, our work establishes a proof-of-concept framework for reversible genome editing. Future optimization of editor architectures, target design, and delivery systems will further improve the efficiency and precision of reversible editing and reduce the persistence of mosaicism.

Despite the high efficiency and precision of BEs, they are prone to bystander edits³⁸. We found that while ABE and CBE can reverse each other's editing outcomes, substantial bystander editing persists. Moreover, BEs are limited in mediating insertions and deletions, restricting their capacity for broad reverse editing. In contrast, PE can mediate all types of base substitutions, in addition to insertions and deletions³⁹. We demonstrated that PE can efficiently reverse edits introduced by Cas9, BEs, or PE itself, with markedly reduced bystander editing compared to BEs, in both cells and animals. These results position PE as a versatile platform for broad therapeutic target selection and precise reversal of diverse genome edits.

As mentioned above, our two-step reversible editing strategy may result in genomic mosaicism in the final editing products. This is primarily attributed to the inherent imprecision or incompleteness of the editing events, which causes reverse editing to generate a mosaic of genetic states rather than a full restoration to the wild-type allele. Such genomic mosaicism may lead to unpredictable functional consequences and is associated with an increased cumulative mutational burden. This elevated mutational burden, in turn, raises theoretical long-term safety concerns, including an increased risk of abnormal clonal expansion and tumorigenesis. Importantly, genomic mosaicism can also result in cell population chimerism, further contributing to potential safety hazards. Specifically, cell population chimerism, characterized by the coexistence of unedited cells, correctly edited cells, and cells harboring unintended editing by-products, may trigger immune responses via the production of aberrant proteins and complicate both the traceability and correction of off-target editing events. To address these safety considerations and improve the clinical translatability of our reversible editing strategy, future studies should focus on target site selection and optimization of genome editor vari-

ants to minimize off-target effects and on-target byproducts to reduce the occurrence of genomic mosaicism. Furthermore, long-term observational studies will be necessary to systematically evaluate the safety risks associated with genomic mosaicism, thereby providing a more comprehensive understanding of the long-term safety profile of the proposed strategy. Although our current reversible editing system has not yet achieved complete restoration of the wild-type DNA sequence, the achieved genome-level correction is sufficient to recover the corresponding protein function. Therefore, reversibility can be defined at the functional level rather than requiring complete restoration of the wild-type genotype.

In developing genome-editing-based therapeutics, commonly used selection criteria focus on editing efficiency and the potential off-target effects of sgRNAs, resulting in a large pool of candidate guide sequences. Here, we propose that an additional criterion should be the reversibility of genome editing, with companion guide sequences for reverse editing evaluated alongside the primary guides. This consideration is particularly important for applications in metabolic diseases, which often affect large and genetically diverse patient populations and may often require treatment termination during a patient's lifetime.

We demonstrated that reverse editing can be achieved when genome editor expression is transient. Suitable delivery platforms include LNPs, virus-like particles, or short-lived viral vectors such as adenovirus. In contrast, when genome editing components are delivered via long-term expressing viral vectors such as adeno-associated virus (AAV), reverse editing may be impaired unless the original guide sequence is deactivated. Previous studies have shown that deactivating the guide sequence in AAV can reduce off-target effects⁴⁰. We propose that this strategy could also enable the subsequent delivery of a second guide sequence for reversal, particularly in cases where the same genome editor such as PE is employed for both knockout and reversal.

In this study, we established the feasibility of reversible genome editing, offering a transformative approach that broadens the scope of therapeutic target selection while reducing the potential long-term risks associated with permanent genomic alterations. Our study paves the way for clinical applications that extend beyond rare monogenic diseases to encompass more prevalent and complex conditions, including cholesterol dysfunction and cardiometabolic diseases. The ability to reverse genetic changes enhances the safety profile of genome editing, making previously considered high-risk targets accessible for therapeutic intervention, expanding the range of conditions that could benefit from genome editing-based therapies to unlock its full potential for improving human health.

MATERIALS AND METHODS

Materials

Quant-iT™ RiboGreen™ kit was purchased from Thermo Fisher Scientific Inc. (Cleveland, OH, USA). T7 High Yield RNA Transcription kit, N1-methylpseudouridine (N1-me-Ψ) and trinucleotide cap1 analog were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and Cholesterol were purchased

from A.V.T. Pharmaceutical Co., Ltd. (Shanghai, China). DMG-PEG2000 was purchased from Xiamen Sinopeg Biotech Co., Ltd. (Xiamen, China). Lipid29 was synthesized by WuXi AppTec. (Wuhan, China). The rNTP mix and acetate buffer were purchased from Novoprotein Scientific Inc. (Shanghai, China) and Coolaber Science & Technology (Beijing, China), respectively.

Plasmids

DNA fragments were amplified via PCR with Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd.). The vector fragments and corresponding inserted fragments were assembled using one-step Gibson Assembly (Vazyme Biotech Co., Ltd.). The pLenti-ABE7.10 RA (Addgene Cat#. 112675), pCMV-hyA3A-BE4max (Addgene Cat#.157943), pCMV-ABE8.8-m (Addgene Cat#. 136294), pCMV-PEmax plasmid (Addgene Cat#. 174820), and pCMV-PE6c plasmid (Addgene Cat#. 207853) were purchased from Addgene. The plasmids were optimized to meet specific editing requirements. Sequences of pegRNAs and sgRNAs are listed in Supplementary Table S1.

Cell culture and transfection

HEK293T (CRL-3216), Hepa1-6 (CRL-1830) and Huh-7 (PTA-4583) cells were purchased from the American Type Culture Collection. HEK293T, Hepa1-6 and Huh-7 cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Corning). All cell types were cultured and maintained under 37 °C with 5% CO₂, and were routinely tested to confirm the absence of mycoplasma contamination. Before transfection, Hepa1-6 or Huh-7 cells were seeded in 24-well plates at a density of 1×10^5 cells per well. Following 16–24 h, cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) at approximately 80–90% confluency. For BEs, 750 ng ABE/CBE plasmid, 350 ng sgRNA plasmid and 100 ng pMax-GFP plasmid were transfected per well. For prime editing, 800 ng PE6c plasmid, 250 ng pegRNA plasmid, 83 ng nick sgRNAs and 100 ng pMax-GFP plasmid were transfected per well. Three to five days after transfection, cells were harvested and sorted via flow cytometry to obtain GFP positive cells. The genomic DNA from sorted cells was extracted.

Real-time qPCR

Total RNA was extracted from cells using RNA isolation kit (Magen Biotechnology). After reverse transcription (Vazyme), the cDNA products were subjected to real-time qPCR to measure mRNA levels of targeted genes. Gene-specific primer sequences for PCR were collected in Supplementary Table S2.

Generation of HEK293T-EGFP cell line

The EGFP expression sequence was constructed into lentiviral vector with EF1 α promoter and puromycin resistance. The pLenti-EF1 α -EGFP-PuroR plasmid and lentiviral helper plasmids (pVSV-G and psPAX2) were co-transfected into HEK293T cells to package lentivirus. After 48 h, cell culture supernatant containing lentivirus was collected and filtered with 0.45- μ m filter. Then, the supernatant was diluted in a gradient to infect HEK293T cells. Cells with a green fluorescence-

positive rate of approximately 20% were selected as cell lines containing a single-copy insertion of EGFP and subsequently selected with puromycin.

Genomic DNA extraction

The cells were subjected to centrifugation at 300 $\times$ g for 5 min and the cell pellet was resuspended in 100 μ L lysis buffer (0.05% SDS, 10 mM Tris-HCl, pH 8.0) with 1 μ L proteinase K (Thermo Fisher Scientific). The mixture was incubated at 50 °C for 30 min, followed by 95 °C for 10 min, and then used as a template for subsequent PCR reaction.

Animal studies

All mice were maintained under specific pathogen-free conditions at the animal facilities of Wuhan University Medical Research Institute. Mice had free access to food and water, and were raised under a 12 h light/ 12 h dark cycle with controlled humidity (50 $\pm$ 10%) and temperature (20–25 °C). All mouse studies received approval from the Animal Care and Ethical Committee at Wuhan University. Eight-week-old female C57BL/6 mice were used. In all *in vivo* experiments, each mouse first received knockout editing. After a defined interval, the same animal then received reverse editing. The LNPs encapsulating mRNA and gRNA/pegRNA were injected through mouse via tail vein. Before injection, the LNP was adjusted to 150–200 μ L for each mouse. The total doses of LNP in Fig. 2 were about 1 mg/kg for *Pcsk9* knockout editing and 3 mg/kg for reverse editing. The total doses of LNP in Fig. 3 (*Pcsk9* site 2) were 2 mg/kg for Cas9-mediated knockout editing, 2 mg/kg for PE-mediated reverse editing. The PE mRNA was delivered twice to improve reverse efficiency in Fig. 3. The total doses of LNP in Fig. 4 (*Ttr* site 1) were 3 mg/kg for PE-mediated knockout and reverse editing. The total doses of LNP in Supplementary Fig. S5 (*Ttr* site 1) were 0.75 mg/kg for Cas9-mediated knockout editing, 2 mg/kg for PE-mediated reverse editing. Before and at specific time points after injection, blood samples were collected and serum was separated via centrifugation under 1,000 $\times$ g at 4 °C for 15 min. Serum levels of PCSK9 and LDL-cholesterol were measured using ELISA Kit (Proteintech Group, Inc) and LDL-cholesterol kit (Shanghai Kehua Bio-Engineering Co., Ltd), respectively. Serum ALT, AST and AKP were measured using corresponding diagnostic kits (Beijing Boxbio Science & Technology Co., Ltd). The mouse livers were cryogenically ground into powder, and the total RNA and genomic DNA were extracted, respectively.

RNA production

BE, Cas9, PE and MLH1-SB mRNAs used in this study were produced via *in vitro* transcription (IVT)⁴¹. The coding sequences were cloned into pUC18 plasmid. Template DNA containing a 110-nt poly A tail was generated using high-fidelity DNA polymerase. The IVT mixture containing template DNA, T7 RNA polymerase, ATP, CTP, GTP, N1-methylpseudouridine (N1-me- Ψ) and RNase inhibitor (Vazyme) was incubated at 37 °C for 2 h. Co-capping of mRNA was performed using trinucleotide cap1 analog (Vazyme). Then the template DNA was digested with DNase for 15 min at 37 °C. RNA was subjected to purification with a Monarch® RNA Cleanup Kit (New England Biolabs), after which dsRNA was removed by

cellulose purification (Sigma-Aldrich)³³. Chemically modified RNAs were synthesized (Genscript), and pegRNAs were produced via RNA ligation as described previously³³.

LNP formulation

The gRNA and mRNA were encapsulated into lipid nanoparticles by microfluidic techniques⁴². Briefly, the following reagents were dissolved in ethanol at the indicated molar ratios: lipid 29 at 50%, DSPC at 10%, cholesterol at 38.5%, and DMG-PEG2000 at 1.5%. mRNA and gRNA (1.5:1 weight ratio) were dissolved in sodium acetate buffer (6.25 mM, pH 5.0). Lipid mixture and RNA cargo were formulated by NanoAssembler microfluidic mixing device at 1:3 volume ratio. The collected LNP was dialyzed with PBS (pH 7.4) overnight at 4 °C. RNA encapsulation efficiency and particle size of LNPs were characterized for quality control.

DNA library preparation and sequencing

Genomic targets of interest were amplified by a two-round PCR and sequenced using an Illumina sequencing platform (Novaseq 6000). Genomic DNA mixtures were used as templates in the first round of PCR (PCR 1) and were amplified using specific primers containing Illumina adaptors and 6-bp barcodes (listed in Supplementary Table S3) for 25 cycles. Then, 5 µL of unpurified PCR 1 mixture was used as templates for the second round of PCR (PCR 2) and amplified with primers containing Illumina adaptors and an 8-bp index for another 14 cycles. All the PCR reactions were performed following the manufacturer's instructions (Vazyme). The PCR 2 products were subjected to purification using a HiPure PCR Pure Mini kit (Magen). Then, DNA concentration was measured using a Nanodrop according to the manufacturer's protocols.

CHANGE-Seq

CHANGE-seq was performed to assess the off-target effects of knockout and reverse editing in mouse liver. Genomic DNA from wild-type mice was fragmented by Tn5 transposase and circularized using T4 DNA ligase. The circular DNA was then incubated with Cas9 ribonucleoprotein (RNP) complexes assembled with either knockout or reverse sgRNAs. Among the cleavage products, only DNA linearized by Cas9 could be end-repaired, A-tailed, and ligated to sequencing adaptors using the VAHTS Universal DNA Library Prep Kit (Vazyme). The adaptor-ligated DNA was PCR-amplified with adapter specific primers, and the resulting libraries were subjected to high-throughput sequencing. Based on CHANGE-seq results, the top 75 candidate off-target sites for the knockout sgRNA were selected for validation. We performed amplicon-based NGS sequencing on these sites using liver genomic DNA from untreated, knockout-edited, and reverse-edited mice. Similarly, the top 79 sites nominated for the reverse sgRNA were validated using liver genomic DNA from untreated and reverse-edited mice.

RNA sequencing and analysis

To assess RNA off-targeting effects of reversible editing, total RNA from mouse liver was extracted by homogenizing tissue in TRIzol reagent (TIANGEN) on dry ice and then processed according to the manufacturer's instructions. RNA-seq library construction and RNA sequencing were carried out by the

Novogene. Over 50 million pair-end reads were achieved per library. Raw RNA-seq reads were checked by FastQC for quality control purpose. RNA editing sites were identified using the RADAR pipeline^{28,43}. To adapt the RADAR pipeline for mouse RNA editing analysis, we modified the configuration file to include the *Mus musculus* GRCh39 reference genome and Ensembl Release 110 gene annotations. Genome indices for HISAT2, BWA-MEM, BLAT, and GATK were constructed based on GRCh39, and an additional BWA-MEM index was built for the mouse 45S ribosomal DNA (Rn45s.fa) reference, to identify and filter rRNA reads. SNP annotation was performed using the Ensembl Variation Release 115 database, and the corresponding GATK VCF index and chromosome-specific annotation files were generated. Gene structure annotations (refFlat) and RepeatMasker annotations for mm39 were obtained from the UCSC Genome Browser and processed with the RADAR annotation preparation script to generate gene transcription strand, intronic splice-site, genome-wide repetitive element, and simple tandem repeat annotations. As the mouse genome lacks Alu elements, repetitive regions were annotated using All_repetitive.txt without distinguishing Alu and non-Alu repeats.

Statistical analysis

GraphPad Prism 9 software was used to analyze the data. Two-tailed Student's *t*-tests were used for comparisons of intergroup differences, and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed for multiple-group comparisons.

DATA AVAILABILITY

The data underlying the findings of this study are available in this manuscript, and have been deposited in the National Center for Biotechnology Information under BioProject accession number PRJNA1010992.

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AUTHOR CONTRIBUTIONS

H.Y. conceived, designed, and supervised the project. H.-X.Z. and J.D. performed most experiments with X.Lei and Q.C., Ying Z. provided conceptual advice, and Yizhou Z. performed bioinformatic analysis. M.D., D.C., X.Liu, L.W. and G.W. performed experiments. H.-X.Z. and H.Y. analyzed the data. H.Y. and H.-X.Z. wrote the paper with valuable input from the authors.

COMPETING INTERESTS

The authors declare no competing interests.

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ADDITIONAL INFORMATION

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