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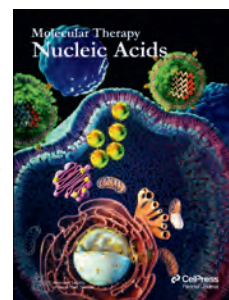
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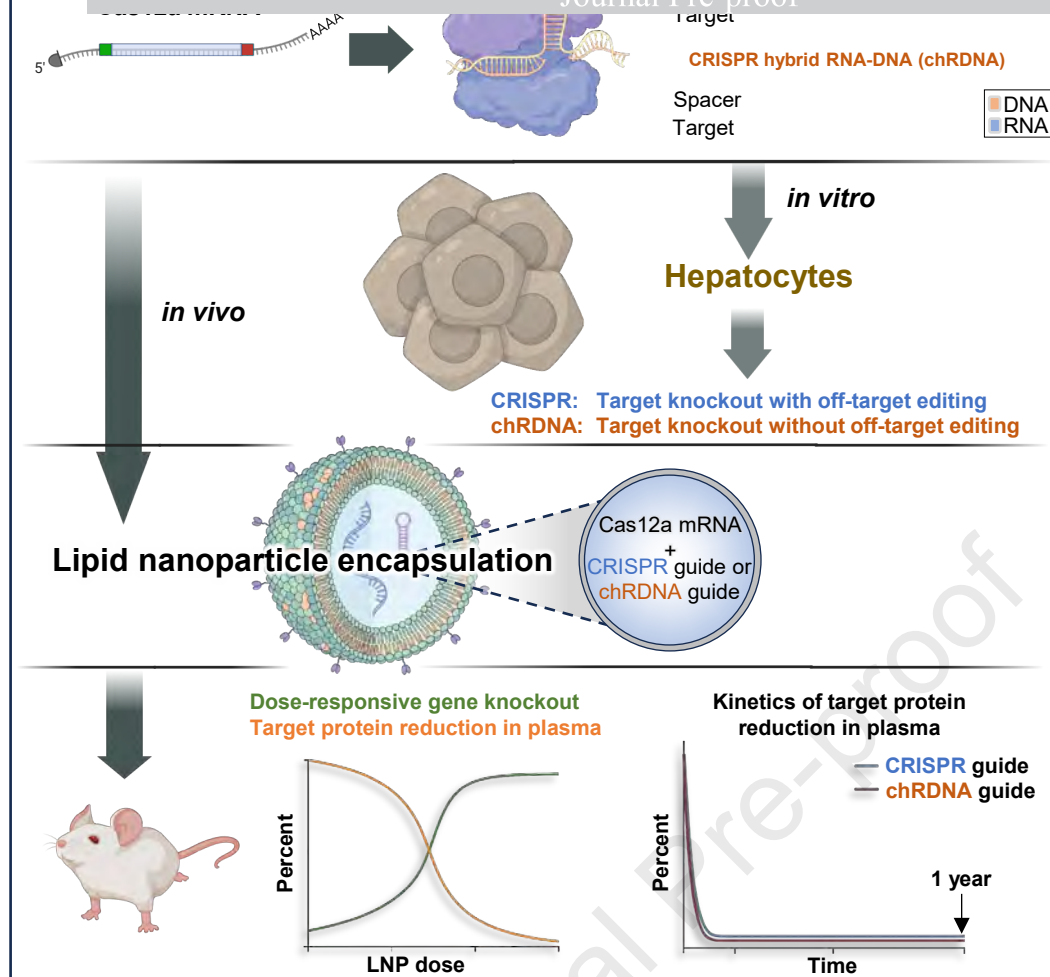
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Cas12a chRDNA-mediated *in vivo* genome editing for high specificity functional gene disruption

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Short title: Cas12a chRDNA-mediated *in vivo* genome editing

ABSTRACT

CRISPR hybrid guides containing a combination of RNA and DNA nucleotides (CRISPR hybrid RNA DNA or chRDNA) enhance both Cas9 and Cas12a nuclease specificity and reduce off-target editing *in vitro*. CRISPR-Cas9 with all-RNA crRNA guides has been implemented for *in vivo* intervention of familial hypercholesterolemia, an inherited autosomal dominant disorder exhibiting high circulating low-density lipoprotein cholesterol, and transthyretin amyloidosis, a progressive life-threatening disease characterized by accumulation of misfolded transthyretin protein leading to neurodegeneration or cardiomyopathy. We targeted three clinically validated genes, *Pcsk9*, *Angptl3*, and *Ttr*, that address these indications using our Cas12a chRDNA genome-editing technology delivered in lipid nanoparticles. A single intravenous dose of LNP encapsulated Cas12a mRNA with a chRDNA guide led to high editing efficiencies without detectable off-target editing, corresponding with near complete reduction of all three targeted plasma proteins in wild-type mice. Additionally, *Pcsk9* and *Angptl3* knockouts were accompanied by reductions of plasma cholesterol. No liver toxicity, chronic cytokine release, off-target editing, or on-target editing in non-hepatic tissue was observed in rodents administered high doses. We propose that LNP-delivered Cas12a mRNA and chRDNA is a safe, highly specific, and powerful tool for genome editing *in vivo*.

INTRODUCTION

Genome engineering for human therapeutic benefit has evolved rapidly over the last decade, including the emergence of enabling technologies that have fostered precise editing and improved delivery modalities. Programmable clustered regularly interspaced short palindromic repeats (CRISPR) Cas9-based systems have led the field recently¹⁻³, and hold promise for use in multiple disease areas, including settings where a gene knockout (KO), mutant gene correction, or a therapeutic gene insertion called a knock-in (KI) is warranted⁴⁻⁷. Delivery of these agents as therapies has faced technical, efficacy, and safety hurdles, generally where viral vectors such as adeno-associated viruses (AAV) have been implemented⁸⁻¹⁰. Nonviral strategies have gained popularity given their adaptation to delivery of payloads of different sizes and origins, with fewer concomitant issues of immunogenicity and adverse clinical events.

Delivery modalities that [i] reduce toxicologic events, including CRISPR-based insertional mutagenesis of the host genome, [ii] increase payload capacity, [iii] offer multi-dosing potential, [iv] are scalable for manufacturing, [v] have reduced complexity and cost, and [vi] expand tissue specificity are critical for therapeutic applications. Lipid nanoparticle (LNP)-based systems are nonviral vehicles that offer these advantages¹¹⁻¹⁸, and have been implemented to deliver mRNAs as vaccines, as well as CRISPR payloads for knocking out liver-expressed genes¹⁹⁻³⁰. LNPs consist of four major elements, including a phospholipid, a pegylated lipid, an ionizable lipid, and cholesterol, to which an anionic payload is added^{11-13,24,25,31-34}. Limitations of LNP tropism have prohibited their use across multiple tissues, but ongoing work to formulate them with alternate lipid compositions and/or directed ligands in the form of peptides or antibodies has enabled targeting outside of liver tissue^{25,29,35-44,45}. Issues of guide RNA instability and poor

expression of genome editors have plagued LNP delivery^{26,46,47}, but these challenges may be overcome by next-generation modalities.

Our prior work delivering recombinant Cas proteins complexed with guides using transfection systems to cells *in vitro* demonstrated the high efficiency and specificity of the chRDNA technology, in which CRISPR guides containing both RNA and DNA residues improved editing specificity⁴⁸. We showed that both Class 2 Type II Cas9 (*S. pyogenes*) and Class 2 Type V Cas12a (*Acidaminococcus sp.*) enzymes elicit high efficiency gene KO and KI capabilities, where Cas12a exhibited higher efficiency gene KI levels compared to Cas9^{49,50}. The use of mRNA for expression of a Cas enzyme enables increased protein expression in the target cell relative to RNP nucleofection, which is limited by low negative charge density and rapid protein degradation^{13,23-28,33,34,44,51}. For this reason, we chose to implement mRNA to the Cas12a enzyme together with our chRDNA guide technology in LNPs to evaluate its potential as a modality for liver-targeted gene KO (and correction). The chRDNA technology offers enhanced specificity *in vitro* when combined with Cas9^{48,49,52}, and when used together with Cas12a exhibits high efficiency and precise genome editing⁵⁰. Chemical modification of guide RNAs is a key enabling strategy to improve their molecular stability, reduce potential innate immune activation, and support robust *in vivo* editing^{46,52,53}. Optimization of both Cas12a mRNA and chRDNA guide chemistries are essential for the development of safe and effective CRISPR-based therapeutics.

Here we describe the targeting of proprotein convertase subtilisin/kexin type 9 (*Pcsk9*), angiopoietin-like 3 (*Angptl3*), and transthyretin (*Ttr*), three clinically validated liver-expressed gene KO targets for the treatment of familial hypercholesterolemia (FH) or transthyretin amyloidosis (ATTR), using LNP Cas12a mRNA chRDNA genome-editing to evaluate its safety,

efficacy, and durability in rodents. Upon identification of high-activity gene targeting chRDNA guides, optimization of the Cas12a mRNA sequence, and development of a new biodegradable lipid nanoparticle (LNP) formulation, we show that a tolerable, single intravenous dose of LNP Cas12a mRNA chRDNA led to 60-70% gene disruption in bulk liver tissue and up to 98% reduction in circulating blood protein levels in rodent models. Importantly, targeting *Pcsk9* or *Angptl3* for gene KO via Cas12a chRDNA resulted in the functional outcome of a 30% reduction of plasma cholesterol in wild-type mice. No detection of significant liver toxicity or chronic cytokine release was observed in either mice or rats, and no peripheral organ on-target genome editing other than liver was detected following tissue isolation and next-generation sequencing (NGS) analysis. Evaluation of *in silico* predicted off-target sites by NGS did not identify off-target genome editing using this system. Further, gene correction of *Angptl3* was observed when including a 2-nucleotide altered single-stranded DNA oligonucleotide in the LNP Cas12a mRNA chRDNA system. These studies demonstrate that LNP delivery of fully-modified Class 2 Type V Cas12a mRNA in conjunction with an optimized chRDNA guide is a safe and effective tool for efficient, high specificity functional genome editing in liver, offering a promising platform for high specificity genome-editing.

RESULTS

Cas12a CRISPR hybrid RNA-DNA (chRDNA) guides exhibit improved *in vitro* editing specificity while maintaining activity

To determine if chRDNA guides function in the context of chemical modifications to promote activity while maintaining molecular integrity, we first generated a fully modified mRNA to Cas12a to protect it from degradation and maximize expression for ultimate use *in vivo*. Cas12a mRNA was optimized to enhance stability, increase translation efficiency, and reduce innate immune activation⁵⁴⁻⁵⁸. The Cas12a mRNA construct was mammalian codon optimized, uridine-depleted, fully substituted with N1-methylpseudouridine, and included UTRs and a 120-nucleotide poly(A) tail for enhanced performance *in vivo* (**Figure S1**). The codon-optimized amino acid sequence of Cas12a (*Acidaminococcus sp.*) is shown (**Figure S2**). Next, we generated an all-RNA guide (crRNA) to *Ttr* and demonstrated its KO activity in mouse hepatocytes *in vitro* (**Figure 1A**). Two different sets of chemical modifications were incorporated into the guides which led to reduced activity (**Figure S3**), whereas in the context of a chRDNA background, activity was restored (**Figure 1A**). Comparison of the all-RNA guide to the chRDNA guide by off-target analysis using amplicon sequencing showed improved specificity of the chRDNA guide, where off-targets were detectable using crRNA but undetectable with chRDNA guides (**Figure 1B**). Editing efficiency of five optimized chRDNA designs was evaluated across the three gene targets (*Pcsk9*, *Ttr*, and *Angptl3*). Several chemically-modified guide designs (**Figure S4**) achieved $\geq 95\%$ relative activity compared to their all-RNA counterparts, demonstrating robust performance across targets (**Figure 1C**). Additional analysis in mouse hepatocytes *in vitro* demonstrated that Cas12a mRNA combined

with multiple individual crRNAs targeting different genes showed that the majority achieved 30-80% disruption efficiency (**Figure S5**).

Generation of functional lipid nanoparticles (LNPs) was critical for the evaluation of Cas12a chRDNA guides against liver targets in mice. For an initial formulation strategy, we evaluated a previously described LNP system based on the ionizable lipid ALC0315⁵⁹, an extensively characterized formulation for *in vivo* delivery that served as a benchmark platform. LNPs were evaluated in the context of Cas12a mRNA comparing crRNA guides to chRDNA guides against the 3 therapeutically-relevant target genes. LNP size (**Figure 1D**), polydispersity (**Figure 1E**), and encapsulation efficiency (**Figure 1F**) were all consistent when switching from all-RNA guides to chRDNAs for all evaluated targets.

LNP formulation of ALC-0315 achieves superior mRNA delivery and protein expression compared to commercial LNPs

To identify an effective LNP formulation, we compared four distinct formulations, including three previously implemented (ALC0315, SM102, and MC3)⁶⁰ and one commercially sourced (GenVoy). Evaluation of all 4 LNP formulations in mice at 1 mg/kg encapsulating fLuc mRNA with the same 5' cap, UTRs, polyA, and N1-methylpseudouridine modifications as in Cas12a mRNA (**Figure 2A**) demonstrated similar kinetics over the 8-day evaluation period (**Figure 2B**). ALC0315 drove significantly higher luciferase expression than the commercial LNP formulations, including at the critical editing timeframe of 24 hours post-infusion (**Figure 2C**). RNA encapsulation efficiency was evaluated across multiple target payloads, demonstrating

consistency of encapsulation of the Cas12a mRNA and guides using a unique ALC0315 LNP formulation (**Figure S6**). All subsequent *in vivo* studies used this ALC0315 formulation.

Efficient *in vivo* Cas12a-mediated editing of *Ttr*, *Angptl3*, and *Pcsk9* leads to reduced plasma protein and functional outcomes

Evaluation of an ALC0315 LNP encapsulating the optimized Cas12a mRNA and a crRNA guide or a chRDNA guide (identical sequence) targeting *Ttr* demonstrated >60% KO editing efficiency in the bulk liver tissue of wild-type mice, where the chRDNA activity was equivalent to that of the crRNA (**Figure 3A**). Total liver tissue was evaluated, and with hepatocytes representing 70-80% of cells in the liver, the level of genome editing was near saturating levels. Thus, the effect of dosing on the circulating blood level of TTR protein was near complete (**Figure 3A**). The dosing level was high (3 doses of 2 mg/kg), as these studies were performed to establish the *in vivo* activity of LNP Cas12a mRNA chRDNA in our model system, and we subsequently performed dose-response studies with a single dose to identify the optimal dosing strategy. Further, we evaluated the *Pcsk9* and *Angptl3* guides with the same LNP Cas12a mRNA chRDNA system and observed up to 70% genome editing in mouse liver, with 50-80% reductions in circulating protein levels, and a functional outcome of up to 30% reduction in circulating blood cholesterol (**Figures 3B,3C**).

Dose-dependence, durability, and specificity of plasma TTR reduction following Cas12a and guide delivery *in vivo*

To gain an understanding of the optimal dosing strategy with a single dose administration of Cas12a mRNA and the *Ttr* guide, a dose-response study was implemented evaluating both editing efficiency and circulating blood TTR protein levels. A single dose of 1 mg/kg achieved maximal editing (>60%) with a corresponding >95% reduction in the TTR protein level (**Figure 4A**). Next, the duration of the effect was evaluated at both the 1 mg/kg dose and the 2 mg/kg dose comparing the crRNA guide to the chRDNA. A near complete reduction (>98%) in the circulating blood TTR level was observed for 12 months following a single administration of either the 1 or 2 mg/kg dose of the crRNA or the chRDNA (**Figure 4B**). This long-term effect prompted us to evaluate the editing specificity of the administration. We compared the *Ttr* crRNA to the *Ttr* chRDNA at the repeated high dose level (**Figure 3**) and assessed *in vitro* editing in mouse hepatocytes at the top 10 *in silico* predicted off-target sites identified by guide sequence homology. Off-target sites were predicted using Cas-OFFinder⁶¹ to identify candidate loci genome-wide on the basis of sequence similarity to the on-target protospacer. From this prediction, we sub-selected the top 10 candidate off-target loci (four with 3 mismatches, and six with 4 mismatches), designed locus-specific primers for each, and assessed editing at these sites by targeted (amplicon) deep sequencing (**Figure S7**). On-target editing was observed at the expected rate (>60%), whereas no off-target editing for multiple potential off-target sites was observed (**Figure 4C**). Additionally, we evaluated mice in a dose-response study for off-tissue genome editing, and observed the expected activity in liver tissue, with only negligible levels in spleen, heart, kidney, or lung tissues (**Figure 4D**).

Titration of Cas12a mRNA and a *Ttr* guide in separate LNP formulations demonstrated that editing in mouse liver was sensitive to a reduction in guide payload, but less impacted by

diminution of Cas12a mRNA LNP material (**Figure S8**). To evaluate residual activity of Cas12a protein post-administration, we tested for the activity of the enzyme by including a guide to *Ttr* at the time of LNP Cas12a mRNA administration, followed separately by a second LNP guide to a different gene (*Angptl3*), either 24, 48, 72, or 96 hours later (**Figure S9**). Editing in the liver for *Ttr* was the same for all conditions 7 days after the initial administration, where editing of *Angptl3* was diminished only after 48 hours, demonstrating the durability of the Cas12a mRNA effect (**Figure S9**). Further, evaluation of splitting the dosing of the Cas12a mRNA and guide into separate LNPs showed similar editing levels as when combined into a single LNP, and was achieved with reduced amounts of Cas12a mRNA payload (**Figure S9**). Together, these data demonstrate that the Cas12a protein expressed from a single LNP mRNA dose remained functional for at least 48 hours, allowing for sequential multiplex editing, such that editing of a second target (*Angptl3*) did not require additional Cas12a mRNA dosing.

chRDNA-mediated *in vivo* editing achieves high efficiency and tolerability in rats

Safety is a major consideration for therapeutic applications of genome editors *in vivo*. To understand the implications of LNP Cas12a mRNA chRDNA administration *in vivo*, we evaluated both a single low dose (0.7 mg/kg) and a single high dose (3 mg/kg) in Sprague-Dawley rats. Liver biopsies showed a high degree of editing ensuring that the material trafficked to the liver and was functional (**Figures 5A and 5B**). No significant impact on body weight was observed over the course of 15 days (**Figure 5C**). Evaluation of blood chemistries, including bilirubin, albumin, liver enzymes AST and ALT, BUN, creatinine, and cytokines TNF- α , IFN- γ and IL-6 remained relatively unchanged over the 15-day period, except for the expected transient spikes observed on day 1 that resolved quickly (**Figures 5D-5L**). No hematologic changes were

noted in WBC, RBC, or platelet counts (**Figures 5M-5O**). Similarly, evaluation of different doses of LNP Cas12a guides in wild-type mice did not lead to major impacts on body weight, liver enzymes, or cytokine levels (**Figure S10**).

Single-stranded DNA oligodeoxynucleotide elicits homology directed gene correction by Cas12a mRNA chRDNA

Programmable CRISPR systems have flexibility for eliciting gene modification, including knockouts, corrections, and insertions. To understand the capability of our system to mediate gene correction, a single-stranded DNA oligonucleotide with end modifications and a 2-nucleotide substitution (**Figure S11**) was included in the LNP Cas12a mRNA guide system targeting *Angptl3* (**Figure S12A**). Gene knockout in the liver was observed at the expected rate of >60% (**Figure S12B**), and gene correction by perfect homology-directed repair (HDR) at the 2-nucleotide site at a modest but detectable level also was observed (**Figure S12C**). This CRISPR delivery system has potential for adaptable and optimizable therapeutic implementation.

DISCUSSION

Lipid-based nanoparticles (LNPs), or nanospheres^{8,11,12,18,25,26,30}, have been in development for therapeutic application for decades, and commercially have been implemented for both targeted siRNA therapy (ONPATRO[®]) and SARS-CoV-2 vaccines⁶². Upon human administration, LNPs capture a protein complex on their surface that includes the ApoE protein that facilitates their uptake by hepatocytes⁶³. Further, current LNP formulations include lipids that promote [i] increased circulation time, [ii] reduced innate immune reactivity, [iii] enhanced cargo encapsulation, [iv] diminution of toxicity, [v] altered tropism for targeted delivery to specific cell types, and [vi] uptake by targeted cells. These observations have advanced LNPs as a viable delivery modality for more sophisticated payloads, including CRISPR-Cas systems^{7,8,11,14-20,22,23,25,29,30,64}. In this report, we implemented our Cas12a chRDNA technology⁵⁰ in biodegradable LNPs to demonstrate its target specificity and capability *in vivo* for genome KOs and gene correction.

Previously, our Cas12a chRDNA technology was implemented using electroporation of recombinant Cas12a protein complexed with chRDNA guides *in vitro* to generate allogeneic BCMA-specific human CAR-T cells (CB-011) for therapeutic use in multiple myeloma⁵⁰. We demonstrated that Cas12a chRDNA complexes could be used to both knock out and knock in different genes critical for the development and manufacturing of CB-011. Here we utilized a fully modified Cas12a mRNA together with chRDNA guides as payloads to target hepatic genes to recapitulate the high specificity of the Cas12a chRDNA system we observed in primary T cells *in vitro*, but using LNPs as the delivery modality and targeting hepatocytes *in vivo*. The LNP Cas12a mRNA chRDNA system enabled significant KO of multiple liver-expressed genes *in*

vivo, durability of the KO effect for at least one year of evaluation, high specificity with respect to a lack of off-targets (within the limits of the assay and based on predicted sites) and limited on-target off-tissue targeting, minimal toxicity in both mice and rats, and the ability to correct a gene mutation using a ssDNA template. Cas12a generates a staggered double-stranded break which may increase the potential for efficient HDR rates. The HDR efficiency reported here (approximately 0.13% perfect correction with approximately 60% concurrent indel formation at the *Angptl3* locus, corresponding to an edit-to-indel ratio of approximately 1:500) is not therapeutically sufficient for most monogenic disorders that require precise sequence alteration. Two complementary paths could advance this platform toward viable *in vivo* gene correction. The first is direct optimization of ssODN-mediated HDR. Asymmetric ssODN designs complementary to the non-target strand have been shown to increase HDR by up to 60% in human cells⁶⁵, and terminal chemical modifications, length tuning, and co-delivery of HDR-enhancing factors further improve donor utilization. Adapting these designs to LNP co-formulation is a defined engineering path, with the edit-to-indel ratio as the critical metric the field will need to address before *in vivo* HDR becomes therapeutically viable. The second path is the use of base editing⁶⁶ and prime editing⁶⁷, which installs precise sequence changes without requiring a double-strand break or a donor template. Both modalities have been delivered to the liver by LNPs. One study using ABE8.8 mRNA targeting *Pcsk9* reported approximately 70% liver base editing in mice and durable editing with corresponding LDL cholesterol reduction in cynomolgus macaques⁶⁸, and another report demonstrated *in vivo* adenine base editing of *Pcsk9* in non-human primates⁶⁹. Recent LNP-based prime editing has demonstrated single-dose hepatic editing in mice⁷⁰. The chRDNA chemistry described here is mechanism-agnostic with respect to nuclease state, such that the spacer DNA substitutions that confer biochemical specificity⁴⁸ are

equally applicable to base-editor and prime-editor scaffolds based on Cas nickase or dead-Cas variants. Adapting the LNP Cas12a chRDNA platform to these precision editing modalities is the most direct path to therapeutically meaningful *in vivo* gene correction. Overall, this technology platform provides both flexibility and selectivity for direct *in vivo* applications.

The chRDNA s used in this study were designed with both DNA substitutions throughout the spacer and chemical modifications at the 5' and 3' termini. The exonuclease resistance conferred by such terminal modifications has been demonstrated directly for the Cas12a (Cpf1) system⁵² where it was shown that crRNAs with phosphorothioate and 2'-O-methyl substitutions at the termini were resistant to exonuclease activity while retaining gene-editing activity comparable to natural crRNA. Because the chRDNA s in this report share the same terminal chemistry and include DNA substitutions throughout the spacer, the prior study⁵² provides a direct Cas12a-specific mechanistic precedent for *in vivo* guide persistence.

Any modification to the guide, whether a chemical modification or a DNA substitution, has the potential to alter guide folding which may either increase or decrease R-loop formation with the target depending on the position and sequence context of the modification. The net effect on activity is not predictable in advance and must be determined empirically through structure-activity relationship (SAR) screening. In the case of the *Ttr* example in Figure 1, the chemical modifications we evaluated disrupted folding or R-loop formation that reduced activity to near baseline at this site. Introducing DNA substitutions identified through SAR screening re-established favorable folding and R-loop formation and restored activity. Cas12a target selection is constrained by PAM availability, thus rescuing activity at this site through backbone modification was preferable to abandoning the guide. The *Ttr* guide illustrates one case within the broader principle that at the sites we tested, replacing the chemical modification with a

smaller, less bulky DNA nucleotide rather than a larger substituent, restored activity. DNA substitution does not generally outperform bulkier modifications, but rather it is a case-specific outcome that reinforces why SAR screening remains critical for each guide and modification pattern.

Human liver comprises ~80% hepatocytes, with the remainder including cholangiocytes, Kupfer cells, stroma, and endothelial cells⁷¹. We observed ~60-70% genome editing in whole liver samples, and we estimate that LNP Cas12a mRNA chRDNA edits 75-99% of rodent hepatocytes in whole liver, thus corresponding to the observed near complete reductions in serum proteins expressed by the targeted genes. These observations suggest the possibility of high efficiency multiplexing with guide arrays using this targeting system. A single administration of our LNP Cas12a mRNA chRDNA formulation did not lead to off-tissue targeting, and did not require multi-dosing to achieve sufficient potency, unlike a prior report using an earlier LNP formulation with a canonical CRISPR-Cas9 system, all of which may have influenced the outcome relative to our approach²². Previously, others implemented LNPs formulated with canonical CRISPR-Cas9 to target hepatic genes, with varying degrees of efficacy and toxicity. Optimization is critical for all components of these complex therapies, and our use of full modification of the Cas12a enzyme mRNA and modified chRDNA guides promoted genome editing enabling a single-dose administration, durability, and low toxicity. Variability in outcomes across prior LNP-based Cas9 studies targeting liver-expressed genes reflects multiple optimization axes that compound rather than operate independently. LNP composition varies widely. Finn *et al.*²² used the biodegradable LNP-INT01 with a heavily chemically-modified sgRNA to achieve approximately 70% liver editing and greater than 97% serum TTR reduction at 3 mg/kg in mice. Qiu *et al.*²³ used a bio-reducible tail-branched lipidoid (306-O12B) to achieve a median 38.5% liver editing

of *Angptl3* with a 65.2% reduction in serum ANGPTL3 at 3 mg/kg in mice. The clinical formulation NTLA-2001¹⁹ achieved approximately 87% serum TTR reduction at 0.3 mg/kg in patients. mRNA modification chemistry is a second axis. End-capped, codon-optimized mRNAs with full N1-methylpseudouridine substitution, as used here and in clinical-stage candidates, produced higher protein expression with reduced innate immune activation than less-optimized counterparts⁵⁴⁻⁵⁸. Guide chemistry is a third axis. Heavily end- and internally-modified all-RNA sgRNAs, as used by Finn *et al.* and in NTLA-2001, differ from the chRDNA used in this study, which combine DNA substitutions throughout the spacer with terminal chemical modifications and confer exonuclease resistance⁵² and reduced biochemical off-target editing⁴⁸. Intrinsic guide-target sequence context contributes a fourth axis through its effect on off-target proclivity, independent of nuclease choice. The durable single-dose efficacy reported in this study reflects simultaneous optimization across all of these axes, and direct attribution of the observed outcomes to chRDNA chemistry or Cas12a nuclease identity would require controlled head-to-head comparisons in the future. The co-packaging requirements of the platform and the kinetics of multiplex targeting were optimized using crRNAs, and we believe that chRDNA will provide similar outcomes in future optimization efforts. Base modification of the RNA components may be insufficient for their stability and enhanced Cas enzyme expression, and as such others have shown that LNP lipid chemistry contributes to overall efficacy^{11-13,18,24-26,28,30-32,34,36,39,40-44}. The improvements in LNP formulations that we implemented likely contributed to the effects we observed with our Cas12a chRDNA technology. Further, an unbiased off-target screen (the SITE-Seq[®] assay, GUIDE-seq, CIRCLE-seq, CHANGE-seq, or ChIP/DISCOVER-Seq) is an important next step for fully characterizing chRDNA specificity, particularly as the platform advances toward clinical evaluation. The combination of these refinements will ultimately

provide a foundation for the development of durable and reproducible therapeutics to impact diseases with significant unmet medical need. Current approaches in the clinic have included LNPs with CRISPR-Cas9 that have made inroads in achieving disease modification^{19,20}, and further evaluation of alternative platforms is needed to identify optimal next-generation therapeutics for specific indications. The studies presented here demonstrate that biodegradable LNP delivery of fully-modified Class 2 Type V Cas12a mRNA in conjunction with an optimized chRDNA guide is a safe and effective tool for efficient, high specificity functional genome editing in hepatocytes, offering a promising platform for direct genome editing *in vivo*.

MATERIALS AND METHODS

Nucleic acid preparation

Cas12a mRNA was synthesized by TriLink Biotechnologies and capped using CleanCap Reagent M6, which incorporates a 3'-O-methyl modification on the m⁷-guanosine and an additional methyl group at position 6 of the +1 adenosine (m⁶A). Cas12a mRNA included complete N¹-methyl-pseudouridine substitution and a 120-nucleotide poly(A) tail. Coding sequences were codon-optimized using the mRNAid algorithm (Dichlab; <https://mrnaid.dichlab.org>) prior to synthesis. CRISPR guide RNAs (crRNAs) and CRISPR hybrid RNA-DNA guides (chRDNA) were synthesized as custom RNA oligonucleotides by Integrated DNA Technologies (IDT).

Lipid nanoparticle (LNP) formulation

Lipid nanoparticles were formulated at an amine-to-RNA phosphate (N:P) ratio of 4.5. Most formulations used in-house LNP formulations with ionizable lipid ALC0315. All lipids were sourced from Cayman Chemical. Briefly, lipids (ALC0315, DSPC, cholesterol, and ALC-0159) were dissolved in 100% ethanol at a molar ratio of 46.3:9.4:42.7:1.6 (ionizable lipid:DSPC:cholesterol:PEG-lipid). Alternative formulations used in Figure 2: 1) DLin-MC3-DMA (molar ratio of 50:10:38.5:1.5 with DLin-MC3-DMA:DSPC:cholesterol:DMG-PEG(2000)); 2) SM-102 (molar ratio of 50:10:38.5:1.5 with SM102:DSPC:cholesterol:DMG-PEG(2000)); 3) GenVoy-ILM reagent (Cytiva). In all cases, the nucleic acid cargo consisting of mRNA and gRNA were mixed at a 1:1 weight ratio (except as indicated otherwise) was diluted in 50 mM acetate buffer (pH 5). LNPs were assembled by microfluidic mixing using a NanoAssemblr instrument (Precision Nanosystems). The lipid (ethanol) and aqueous (RNA) phases were loaded at a 3:1 flow-rate ratio and mixed at a total flow rate of 10 mL/min. The

freshly formed LNPs were immediately diluted 40-fold in PBS (pH 7.4) and subsequently buffer-exchanged and concentrated into PBS (pH 7.4) using 10 kDa MWCO centrifugal concentrators.

LNP characterization

Particle size and polydispersity index (PDI) were measured by dynamic light scattering (UNCLE instrument, Unchained Labs). RNA encapsulation efficiency was determined using the Quant-iT RiboGreen assay (Thermo Fisher Scientific) by comparing fluorescence in detergent-treated versus untreated samples. Preparations intended for *in vivo* studies met the following predefined quality criteria: average particle diameter of 60-100 nm, PDI < 0.3, and RNA encapsulation efficiency >70%. Representative LNP characterization parameters used throughout the study are shown in Figure 1D-F.

ssODN donor design

A single-stranded oligodeoxynucleotide (ssODN) was designed as a symmetric homology-directed repair (HDR) donor template for the *Angptl3* locus, consisting of two 60-nucleotide homology arms (120 nt total homology) flanking a central 2-nucleotide substitution corresponding to the intended edit. To improve stability and nuclease resistance, both the 5' and 3' termini were chemically protected with three 2'-O-methyl (2'-OMe) modified residues and two phosphorothioate (PS) internucleotide linkages at each end. The full ssODN sequence, with all modifications annotated, is provided in the supplemental Figure S11. For *in vivo* delivery in mice, Cas12a mRNA, the crRNA, and the ssODN donor were co-encapsulated within a single LNP at a 1:1:1 mass ratio (w:w:w), formulated with ALC0315.

Cell culture and transfection

H2.35, Hepa1-6, and AML12 cell lines were obtained from ATCC and maintained according to the manufacturer's recommended culture conditions. For primary mouse hepatocyte isolation, mouse livers were dissected and immediately processed using the Miltenyi Biotec Liver Perfusion Kit (mouse/rat; 130-128-030). Briefly, livers were isolated from BALB/c mice and perfused *in situ* via the gentleMACS Perfuser system with pre-warmed perfusion buffer to remove blood, followed by digestion buffer containing the supplied collagenase (Enzyme D) to dissociate the tissue into single cells. The digested liver was then gently dissociated, filtered through a 100 µm smart strainer, washed, and pelleted. The resulting hepatocyte-enriched suspension was plated directly for culture. Cell lines and primary cells were seeded and transfected at 50-60% confluency. For nucleic acid delivery, cells were transfected using Lipofectamine MessengerMAX (Thermo Fisher Scientific) following the manufacturer's protocol in a 96- or 24-well format. Cells were lysed with QuickExtract DNA Extraction Solution for genomic DNA isolation.

LNP delivery *in vivo*

All procedures for animal experimentation were approved by the Caribou Biosciences Institutional Animal Care and Use Committee and conducted in accordance with their guidelines. All animals were ordered from Charles River or Jackson Laboratories. Female BALB/c mice aged 6-9 weeks were used for most studies. LNPs were administered via lateral tail vein injection at a dose volume of 0.2 mL per mouse. Saline-treated animals served as negative controls. Animals were euthanized at designated time points by CO₂ inhalation followed by cervical dislocation. Female Sprague-Dawley rats ranging from 5-8 weeks of age were used in safety

studies. LNPs were administered via lateral tail vein injection at a dose volume of 2-3 mL per rat. Saline-treated animals served as negative controls. For *in vivo* editing rodent studies, liver tissue was collected from the median and/or left lateral lobe for DNA extraction and analysis. Genomic DNA was extracted using a commercial extraction kit (Qiagen DNeasy Blood and Tissue) following the manufacturer's instructions. Blood was collected into serum separator tubes for ELISA-based quantification, as well as immunogenicity and toxicity assessments.

ELISA Assays

Plasma levels of ANGPTL3 and PCSK9 were quantified using mouse ELISA kits ((R&D Systems, Cat. MANL30 and Cat. MPC900, respectively) following the manufacturer's instructions. Transthyretin (TTR) concentrations were measured using a mouse pre-albumin ELISA kit (Aviva Systems Biology, Cat. OKIA00111) according to the supplier's protocol. Serum samples were diluted as recommended by each manufacturer. Absorbance was measured at 450 nm using a SpectraMax M5 plate reader (Molecular Devices). Serum concentrations were calculated in GraphPad Prism v10 using a four-parameter logistic curve fit from the standard curve, and final values were adjusted for assay dilution.

Immunogenicity and toxicity analysis

Acute toxicity was evaluated by measuring circulating AST and ALT levels in serum by IDEXX BioAnalytics. Cytokine release was assessed as a measure of immune activation at 6 hours and 10-15 days following LNP administration. Serum levels of TNF- α , IFN- γ , and IL-6 were quantified using the following Quantikine ELISA kits (R&D Systems): Mouse TNF- α (Cat. MTA00B), Mouse IFN- γ (Cat. MIF00), and Mouse IL-6 (Cat. M6000B), according to the

manufacturer's instructions. All liver chemistry panels and complete blood counts were analyzed by IDEXX BioAnalytics.

Quantification of genome editing

Cas12a on- and off-target sites were amplified in a two-step PCR reaction. In brief, genomic DNA was used as a template for PCR amplification with Q5 Hot-Start High Fidelity DNA Polymerase (NEB) and unique primer pairs containing an internal locus-specific region and an outer Illumina-compatible adaptor sequence. A second PCR reaction targeting the outer-adaptor sequence was performed to append unique indices to each amplicon. The resulting amplicons were pooled, purified using SPRIselect (Beckman Coulter) beads, and quantified by Qubit (Thermo Fisher). Library size distribution was determined by Bioanalyzer (Agilent) and normalized to 4 nM. Libraries were sequenced on an Illumina MiSeq instrument according to the manufacturer's instructions. The reads were aligned to the mouse or rat reference genome after eliminating those having low quality scores. For each site, indels were tallied if they occurred within 3 nucleotides of the putative Cas12a cut site, and editing efficiencies were calculated by subtracting the percentage of indels in saline or nontreated controls from the percentage of indels in dosed animals or cells. Depth of coverage was ~5,000–20,000 reads per target region, and all samples with <500 reads aligning to the predicted target were discarded. The lower limit of detection (e.g., $\geq 0.1\%$) was determined by titration of NIST genomic standards and assessment of expected versus measured values.

Off-target analysis

Off-target sites were predicted using Cas-OFFinder⁶¹ to identify candidate loci genome-wide on the basis of sequence similarity to the on-target protospacer. From this prediction, top candidate off-target loci (>3 mismatches) were identified, locus-specific primers were designed for each, and they were assessed for editing at these sites by targeted (amplicon) deep sequencing.

Statistical methods

All data were analyzed using Prism v10 (GraphPad). For all editing outcomes, liver tissue from each animal was assessed in technical triplicates via NGS. Each data point is the mean of the three technical replicates from each mouse. The mean and SD are plotted per biological group. Figure 1 includes n=3 biological replicates per condition. Figure 2 includes n=3 animals dosed per group with each LNP formulation. Unpaired t-test (Prism) was used in pairwise comparison of each in-house LNP formulation compared to commercial GenVoy lipid mix. Figure 3A-C represents n= 4 animals per group dosed with mRNA and *Ttr/Angptl3* guides (either crRNA or chRDNA). Figure 3D includes n= 8 animals per group dosed with mRNA and *Pcsk9* guides (either crRNA or chRDNA). Figure 4 includes n=4 animals per group both for dose-response (panel A) and durability assessment (panel B). Figure 4C-D includes at least n=3 biological replicates per condition. Figure 5 represents n=2 animals per group at 0.7mg/kg dose with either crRNA or chRDNA, n=1 animal in crRNA group at 3mg/kg, and n=3 in chRDNA group.

DATA AVAILABILITY STATEMENT

The authors confirm that materials and protocols will be available upon request, subject to appropriate agreements.

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AUTHOR CONTRIBUTIONS

M.R., M.L., and S.B.K. conceptualized the studies. M.L. supervised, designed experiments, performed experiments, and analyzed data. P.R., M.S., M.C., A.M., V.G., B.T., R.G., G.C., A.L.G.O., and J.T., conducted experiments and/or analyzed data. M.T., Z.A., P.D.D., S.G., M.R., and S.B.K. supervised, designed experiments, and/or analyzed data. The manuscript was drafted by S.B.K. and revised by M.L., S.G., M.T., and M.R. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

All authors are, or were, employees of Caribou Biosciences, Inc. and received compensation in the form of salary and stock options. Some of the work in this manuscript is related to patent applications filed by Caribou Biosciences, Inc. S.B.K. is a consultant with Specific Biologics and an advisor to FinalDose, companies focusing on the gene therapy space.

KEY WORDS

Lipid nanoparticle, LNP, CRISPR, Cas12a, chRDNA, genome editing, liver, gene correction

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FIGURE LEGENDS

Figure 1. Cas12a CRISPR hybrid RNA-DNA guides (chRDNA) exhibit improved *in vitro* editing specificity while maintaining activity.

(A) Chemically modified guides that contain RNA and DNA residues (chRDNA) targeting *Ttr* restore editing activity that is lost with certain base modifications in all-RNA guides (crRNA). (B) chRDNA guides targeting *Ttr* exhibit improved specificity, with potent on-target activity and reduced off-target editing compared to all-RNA crRNAs. Editing efficiencies are shown as mean $\pm$ SD across replicates. (C) Guide identification strategy for *Ttr*, *Pcsk9*, and *Angptl3* targets included systematic screening of repeat and spacer regions followed by iterative structure–activity relationship (SAR) optimization. Guide variants with chemical modifications were assessed for editing activity. Normalized editing efficiency of five chRDNA designs across three gene targets, with several designs achieving 95% or more of the all-RNA crRNA baseline (dotted line). (D–F) Analysis of lipid nanoparticle (LNP) formulations co-encapsulating Cas12a mRNA together with either a crRNA or a chRDNA guide, for the three targets (*Ttr*, *Pcsk9*, and *Angptl3*) as indicated in the color legend. All formulations contain Cas12a mRNA; the color legend refers to the co-formulated guide. (D) The Z-average diameter of the LNPs was measured by dynamic light scattering (DLS). Dashed blue line indicates the upper size limit above which immunogenic responses are observed *in vivo*. (E) The polydispersity index (PDI) of the LNPs was measured by DLS to assess size homogeneity. Dashed blue line indicates the acceptable PDI range for *in vivo* use. (F) Total nucleic acid encapsulation efficiency (Cas12a mRNA plus guide combined) was determined using the Quant-iT RiboGreen assay; since both cargoes are co-encapsulated in the same particle, this assay reports the combined encapsulation efficiency of the formulation rather than separate values for the mRNA and the guide. Dashed blue line indicates the desired

encapsulation efficiency for *in vivo* evaluation; dashed black line indicates the minimum acceptable encapsulation efficiency.

Figure 2. LNP formulation of ALC-0315 achieves superior mRNA delivery and protein expression compared to commercial LNPs.

Mice were intravenously dosed with 1 mg/kg firefly luciferase (ffLuc) mRNA formulated with various LNPs: ALC0315, SM102, MC3, or commercial GenVoy. (A) Representative *in vivo* bioluminescence images show liver-restricted luciferase expression at 24 hours post-dose. (B) Longitudinal quantification of bioluminescent signal over 8 days shows highest peak expression with ALC0315 LNPs, within the editing-relevant time window. (C) Quantification at 24 hours confirms higher protein expression from ALC0315 compared to GenVoy ($p \leq 0.01$). Data represent mean $\pm$ SEM ($n = 3$ per group). Statistical significance was determined using ANOVA with multiple comparisons.

Figure 3. Efficient *in vivo* Cas12a-mediated KO editing of *Ttr*, *Angptl3*, and *Pcsk9* leads to reduced plasma protein and functional outcomes.

Wild-type BALB/c mice were administered a single intravenous dose of LNP-formulated Cas12a mRNA and either crRNA or chRDNA guides. Editing efficiency (left-hand panels: (A) *Ttr*, (B) *Angptl3*, or (C) *Pcsk9*) and plasma protein levels (center panels: (A) TTR, (B) ANGPTL3, or (C) PCSK9), and plasma cholesterol levels (right-hand panels) were measured 20 days (B, *Angptl3*) and 22 days (C, *Pcsk9*) post-injection. Both guide formats induced robust editing and statistically significant decreases in plasma protein levels. Functional knockdown of *Pcsk9* and *Angptl3* was

associated with a reduction in total plasma cholesterol. Statistical significance of editing efficiencies was compared between the crRNA and chRDNA groups by an unpaired two-tailed t-test (GraphPad Prism). Data are shown as mean $\pm$ SD, n = 4–10 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$.

Figure 4. Dose-dependence, durability, and specificity of plasma TTR reduction following Cas12a and guide delivery *in vivo*.

(A) Mice were intravenously dosed with LNP-formulated Cas12a and an all-RNA guide (*Ttr*-crRNA) at varying doses ranging from 0.125 to 2 mg/kg. Plasma TTR protein levels (left y-axis, light blue circles) and *Ttr* gene editing efficiency in liver tissue (right y-axis, dark blue bars) were measured. Increasing doses led to a progressive reduction in plasma TTR, reaching near-complete knockdown at 1 and 2 mg/kg, which correlated with >60% editing efficiency. Data are shown as mean $\pm$ SEM (n = 4 per group). (B) Mice received a single intravenous dose of LNP-formulated Cas12a mRNA and guide targeting the *Ttr* gene at 1 or 2 mg/kg. Four groups were tested: *Ttr*-crRNA (1 or 2 mg/kg) and *Ttr*-chRDNA (1 or 2 mg/kg). Plasma TTR protein levels were measured monthly by ELISA for up to 12 months post-dose. Saline-treated animals served as controls. All treatment groups showed rapid and sustained reduction of plasma TTR, with levels remaining near the limit of detection throughout the study. Previously reported TTR levels following CRISPR-Cas9 editing in wild-type mice at 1 mg/kg were ~ 375 $\mu\text{g/mL}$ ²². Data are presented as mean $\pm$ SEM (n = 4 per group). (C) Liver tissue was analyzed for editing at the on-target site and ten top bioinformatically-predicted off-target sites following three doses of LNP-formulated Cas12a mRNA and *Ttr* guides (crRNA or chRDNA, 3×2 mg/kg). Significant editing

was detected only at the on-target locus, with all off-target sites remaining below 0.2% editing. (D) Heatmap showing tissue-specific editing across five organs following a single dose of LNP-formulated Cas12a mRNA and *Ttr*-crRNA at doses from 0.125 to 2 mg/kg. Editing was restricted to the liver in a dose-dependent manner, with negligible activity in spleen, heart, kidney, or lungs. Data represent mean % editing (n = 4 per group).

Figure 5. chRDNA-mediated *in vivo* editing achieves high efficiency and tolerability in rats.

In vivo editing outcomes in Sprague-Dawley rats following liver-targeted delivery of LNPs containing Cas12a mRNA with either crRNA or chRDNA targeting *Ttr* (0.7 or 3 mg/kg [mpk]). (A) Editing was observed in liver tissue, with dose-dependent increases in editing (up to ~70%) across multiple liver tissue punches. (B) Plasma TTR protein levels were reduced by >98% by day 15 post-dose at 0.7 mg/kg (central data points) and 3 mg/kg (right-side data points). (C) Body weight change remained within a normal range following treatment. (D-O) Liver function biomarkers on Day 0 (D0), Day 1 (D1), Day 7 (D7), and Day 15 (D15), where legend in panel D is the same for all panels D-O, including (D) bilirubin, (E) AST, (F) ALT, and (G) albumin, revealed only transient elevations in transaminases (AST, ALT) at Day 1 post-dose, with values returning to baseline by Day 15. Renal function markers (H) BUN and (I) creatinine remained within normal ranges, with transient BUN elevation observed in some animals on Day 1. Cytokine levels, including (J) TNF- α , (K) IFN- γ , and (L) IL-6, showed transient increases on Day 1 post-treatment but normalized by Day 7–15, indicating an acute inflammatory response. Hematological parameters, including (M) white blood cell counts, (N) red blood cell counts, and (O) platelet counts were stable over time.

Figure 1

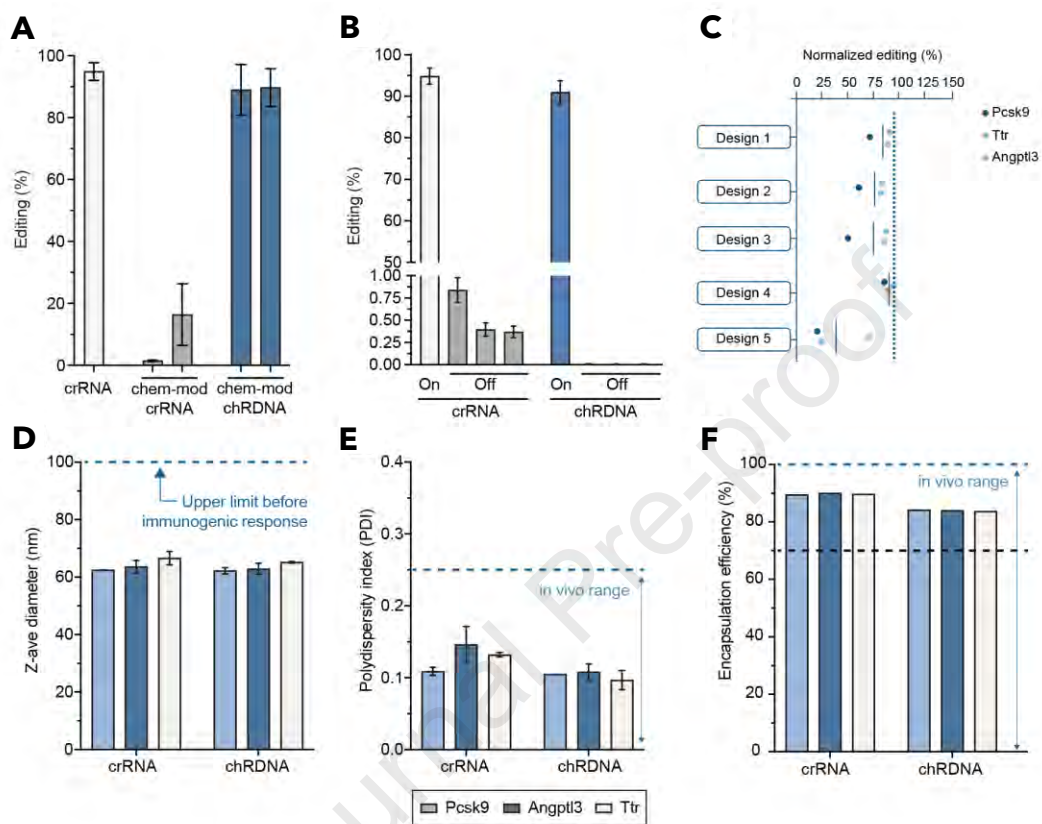


Figure 2

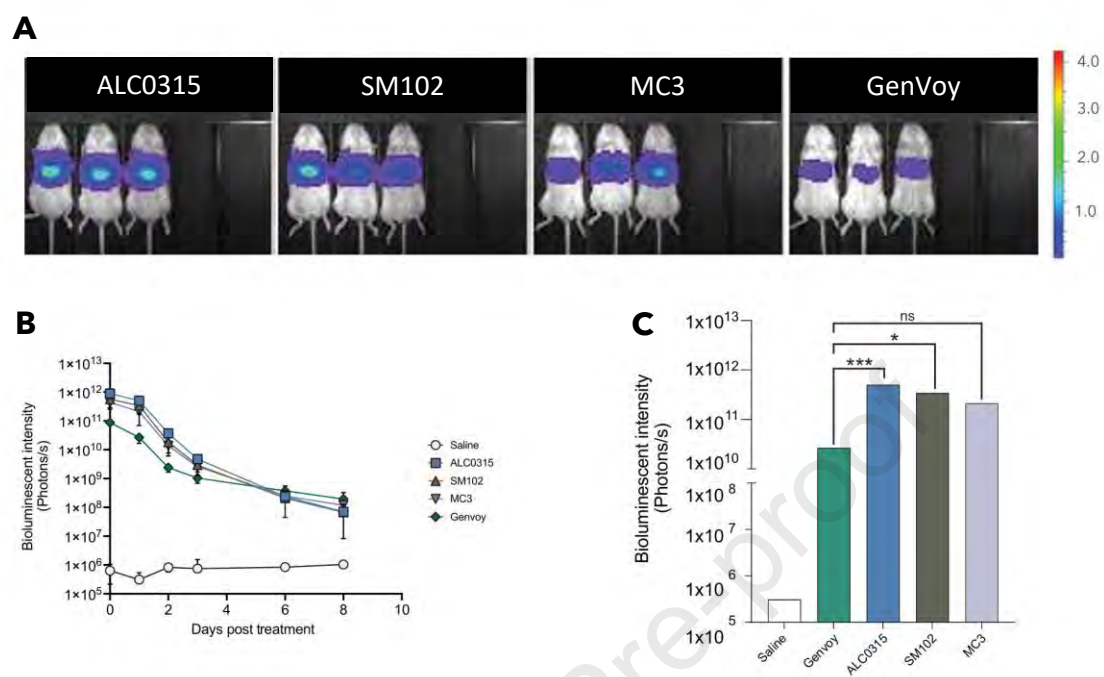


Figure 3

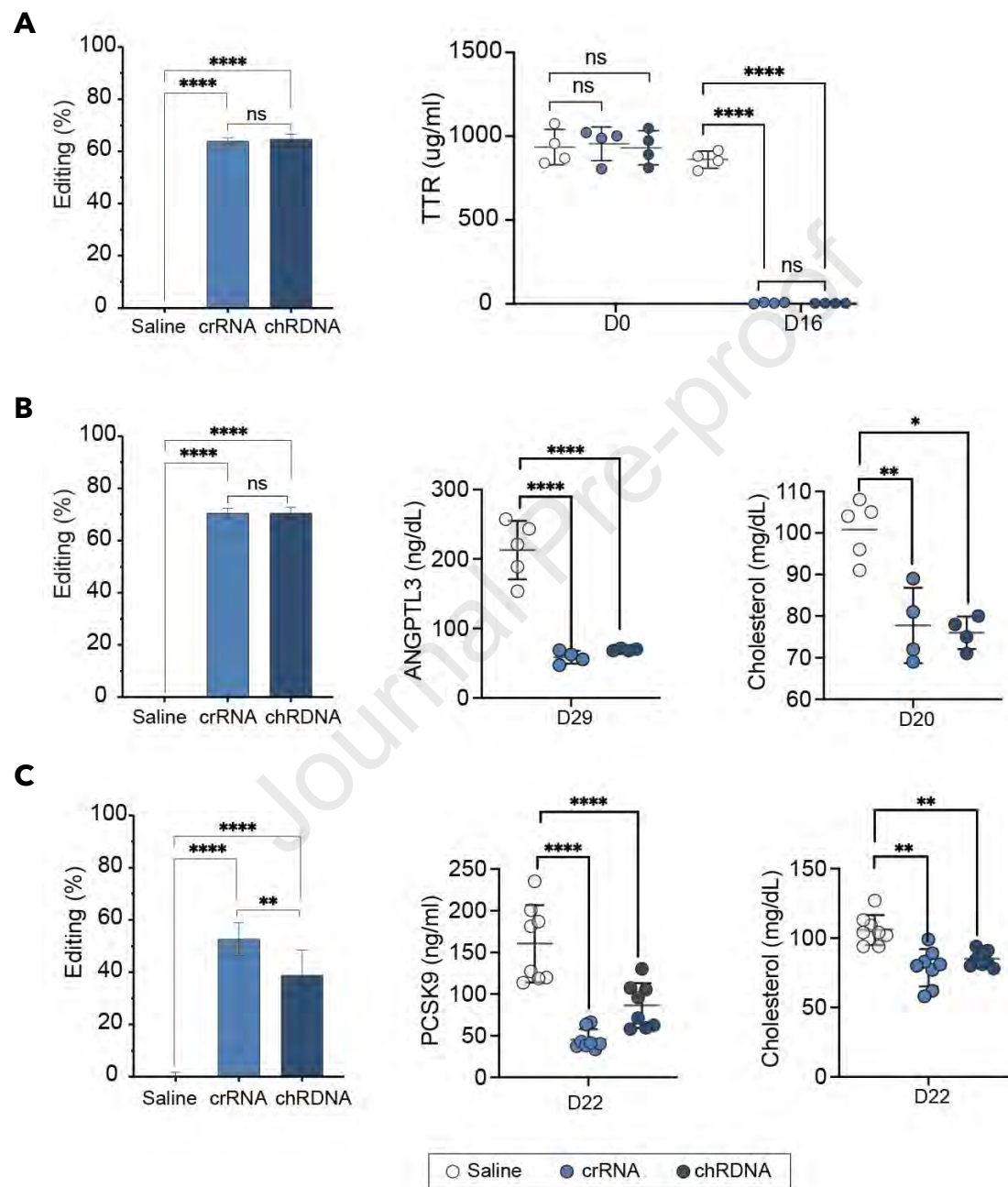


Figure 4

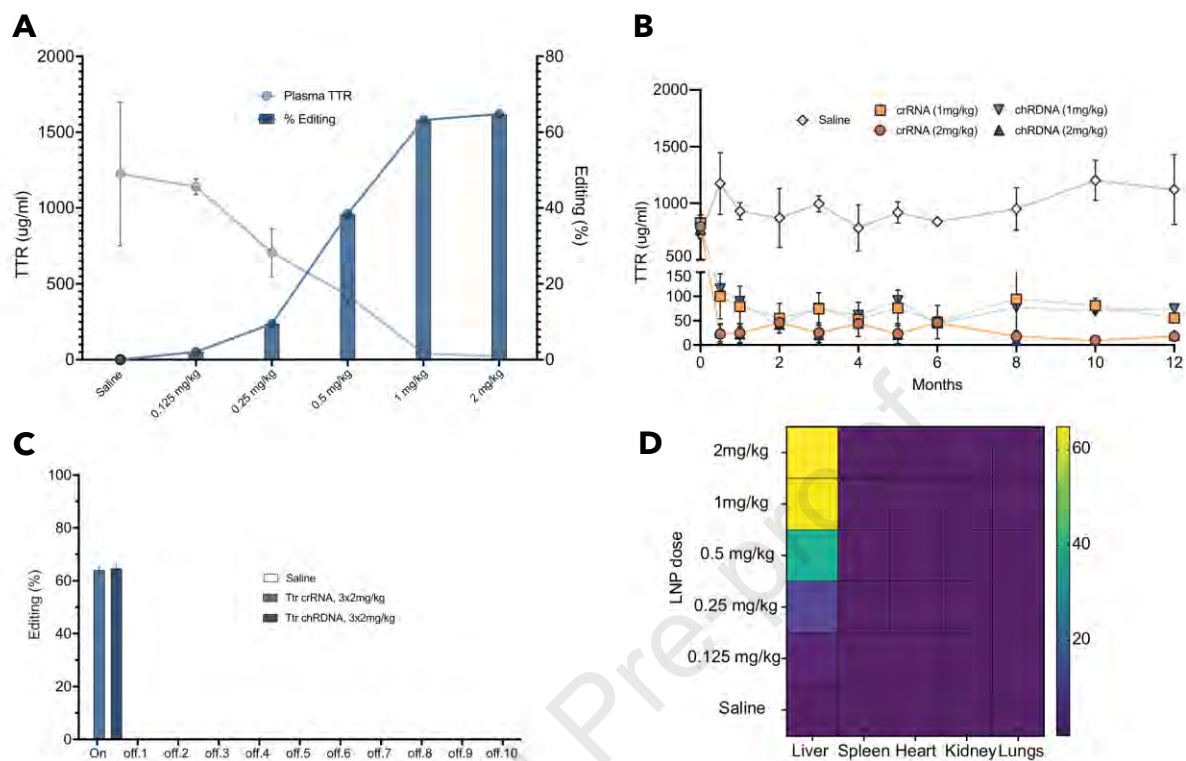
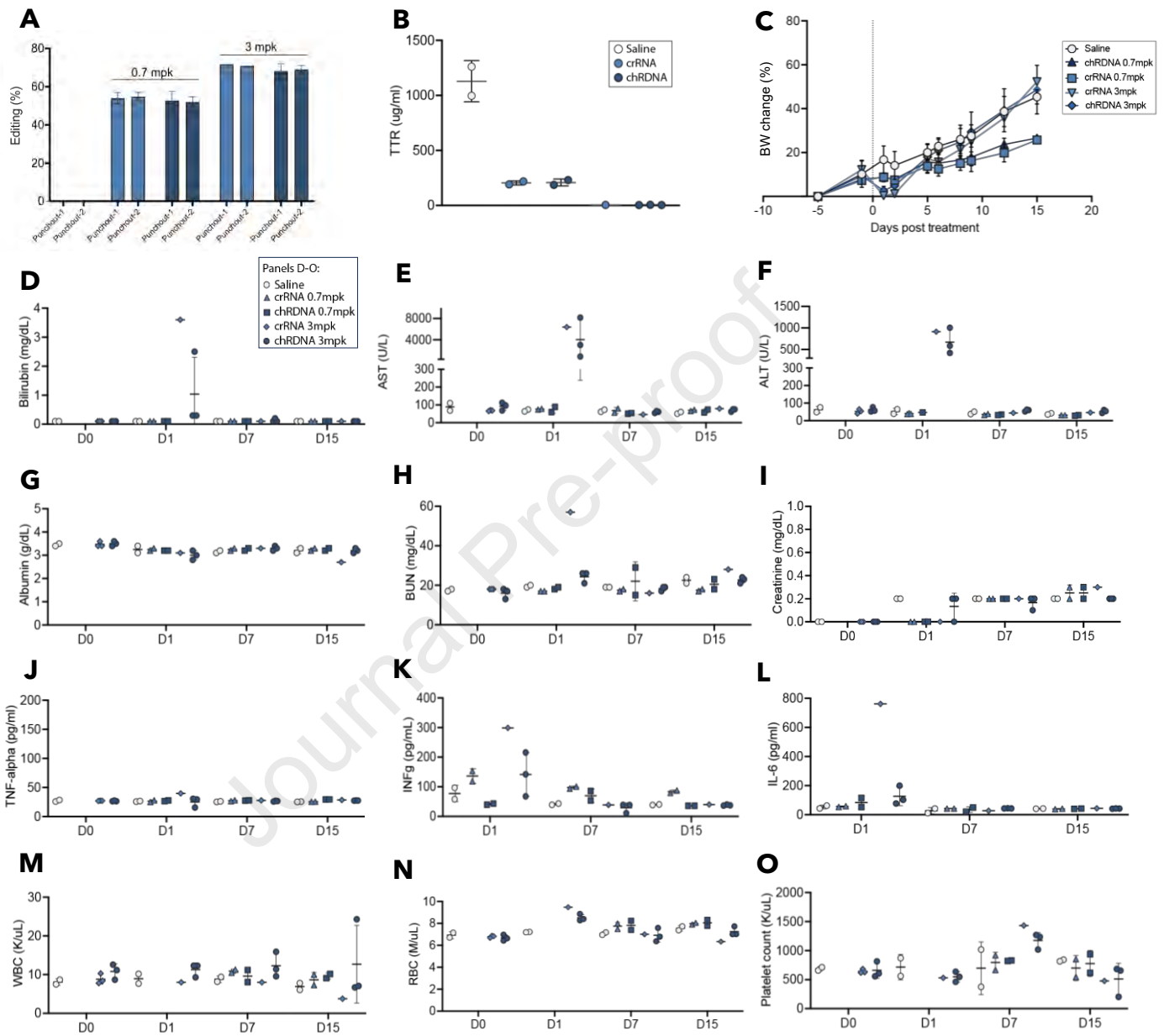


Figure 5



CRISPR hybrid guides containing RNA and DNA nucleotides (chRDNA) enhance Cas nuclease specificity by reducing off-target editing. Intravenous administration of LNP-encapsulated Cas12a mRNA with a chRDNA guide in mice led to dose-responsive hepatic genome editing without detectable off-target editing and near elimination of the targeted plasma protein for one year.