Chapter 42

In Vivo Gene Editing in Lipid and Atherosclerosis Research

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Abstract

The low-density lipoprotein receptor (*Ldlr*) and apolipoprotein E (*Apoe*) germline knockout (KO) models have provided fundamental insights in lipid and atherosclerosis research for decades. However, testing new candidate genes in these models requires extensive breeding, which is highly time and resource consuming. In this chapter, we provide methods for rapidly modeling hypercholesterolemia and atherosclerosis as well as testing new genes in adult mice through somatic gene editing. Adeno-associated viral (AAV) vectors are exploited to deliver the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 genome editing system (AAV-CRISPR) to the liver. This tool enables rapid and efficient editing of lipid- and atherosclerosis-related genes in the liver.

Key words CRISPR/Cas9, AAV, Gene editing, Ldlr, Hypercholesterolemia, Atherosclerosis, Lipid, Mouse model

1 Introduction

The low-density lipoprotein receptor (*Ldlr*) and apolipoprotein E (*Apoe*) germline knockout (KO) models are the most used mouse models of atherosclerosis and have provided fundamental insights into pathogenic factors and mechanisms contributing to this disease [1]. *Ldlr* encodes the primary liver receptor for apolipoprotein B (apoB)-containing lipoproteins and mutations in the human *LDLR* gene have been associated with familial hypercholesterolemia [2, 3]. *Apoe* encodes a lipoprotein ligand for lipoprotein receptors such as Ldlr and Ldlr-related proteins (Lrp), thereby promoting the hepatic uptake of lipoproteins from the circulation [4]. As a consequence of the disrupted clearance of atherogenic lipoproteins by the liver, both models develop severe hypercholesterolemia and atherosclerosis when fed a high-fat diet, providing the background for investigating the role of a candidate gene in this disease. However, testing a new gene in atherosclerosis is highly

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time- and resource-consuming. First, a KO model for the gene of interest has to be generated. Then, extensive backcrossing to the *C57BL/6J* genetic background is required, followed by breeding to homozygosity with *Ldlr*- or *Apoe*-KO mice, before the actual experiment can begin [1]. This severely limits the rate at which candidate genes can be investigated and is further complicated when conditional alleles and reporter genes are required. Therefore, there is a compelling need to develop new, higher-throughput in vivo models that provide greater flexibility, lower costs and faster completion times to advance our understanding of this complex disease.

Adeno-associated viral (AAV) vectors are a leading platform for overexpressing transgenes as well as delivering the somatic genome editing tools to the liver, making them ideal for investigating candidate genes in lipid and atherosclerosis research [5]. Recombinant AAV are composed of a small, nonenveloped, single stranded DNA genome that can accommodate up to 4.9 kb of exogenous DNA, flanked on either side by hairpin like structures called inverted terminal repeats (ITRs). Following entry into the nucleus, the single stranded AAV genomes are converted to double-stranded episomes, which provide strong and stable expression of the transgene [6]. In addition to this, AAVs are well-tolerated and show no toxicity and modest immune response in mice, contrary to other commonly used viral vectors [5].

AAVs are currently being used to overexpress human or mouse proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gain-of-function variants for generating atherosclerosis in mice. PCSK9 is secreted by the liver and promotes degradation of LDLR by preventing recycling to the cell surface [7]. As a consequence of the increased Ldlr turnover, this tool enables the rapid development of hypercholesterolemia in adult mice, bypassing the need of time-consuming crossing to *Ldlr* or *Apoe* KO background [8–10]. However, PCSK9 is overexpressed at supraphysiological levels by this method, which may not be ideal for all applications. For instance, it has been reported that at least a fraction of circulating PCSK9 resides on lipoprotein particles [11–13] and this could interfere if the gene of interest is expected to alter lipoprotein functions.

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing tool is derived from a naturally occurring bacteriophage defense system in bacteria [14]. This technology consists of a nuclease (Cas9) and a ~22-nucleotide synthetic guide RNA (gRNA), which guides the Cas9 to a complementary target sequence in genomic DNA, in immediate proximity to a protospacer adjacent motif (PAM). Cas9 induces a double-strand break (DSB) that, in mammalian cells, can be repaired by (1) non-homologous end-joining (NHEJ), which is the error-prone dominant repair pathway in nondividing cells that results in insertions and/or deletions of nucleotides (referred as “indels”), or

(2) homology directed repair (HDR), which uses a DNA template to repair DSB through homologous recombination, functioning most efficiently in dividing cells [15]. By providing an exogenous donor template with homology to the targeted site, it may eventually be possible to use CRISPR/Cas9 to correct a pathogenic mutation or insert a therapeutic transgene in a predetermined genomic site [16, 17]. On the other end, when an open reading frame is targeted, the NHEJ-derived indels induce frameshift mutations and premature stop codons that may be used for knocking out the gene of interest. Theoretically, any gene can be edited with CRISPR/Cas9 by simply modifying the gRNA design to be complementary to the targeted site.

The CRISPR/Cas9 system can be efficiently delivered to the liver for somatic genome editing. In this case, a double-stranded break introduces a myriad of mutations at a defined site in the genome. Although the liver will be mosaic with different mutations across alleles and target cells, the net effect is near complete ablation of expression of the target protein. One of the first in vivo applications of the CRISPR/Cas9 system in the setting of lipid and atherosclerosis research involved the somatic disruption of the *Pcsk9* gene. In the original study, adenovirus was used to deliver *Streptococcus pyogenes* (Sp) Cas9 to the liver, where increases in liver Ldlr and decreases in plasma cholesterol were observed [18]. However, adenovirus promotes undesirable immune responses and turnover of adeno-infected hepatocytes, unlike AAV particles. In a later study, Ran et al. used an AAV vector encoding the smaller *Staphylococcus aureus* (Sa) Cas9 and a gRNA targeting *Pcsk9*, which resulted in a high-rate of NHEJ-derived indels and significant reductions in circulating Pcsk9 and plasma cholesterol [19].

Recently, our group showed that the CRISPR/Cas9 system can be used for generating hypercholesterolemia and atherosclerosis in adult mice. First, we used AAVs to deliver two gRNAs targeting the *Ldlr* and *Apob* genes in the liver of adult Cas9-transgenic mice. We showed efficient disruption of *Ldlr* resulting in severe hypercholesterolemia and atherosclerosis, which could be rescued by the concomitant disruption of *Apob* [20]. Then, we used an all-in-one AAV-CRISPR system to deliver both SaCas9 and a gRNA targeting *Ldlr* in the liver of adult *C57BL/6J* mice (referred as *Ldlr* AAV-CRISPR). Similar to AAV-PCSK9, our *Ldlr* AAV-CRISPR tool induced severe hypercholesterolemia and atherosclerosis at extent comparable to *Ldlr* KO mice, but without requiring extensive crossing to *Ldlr* or *Apoe* KO background [21]. As a further example of application in lipid research, we recently exploited the AAV-CRISPR system for investigating the function of a branch point enzyme of the cholesterol biosynthetic pathway—dehydrodolichyl diphosphate synthase (DHDDS)—in the liver of adult mice, bypassing the need for generating new floxed animals [22].

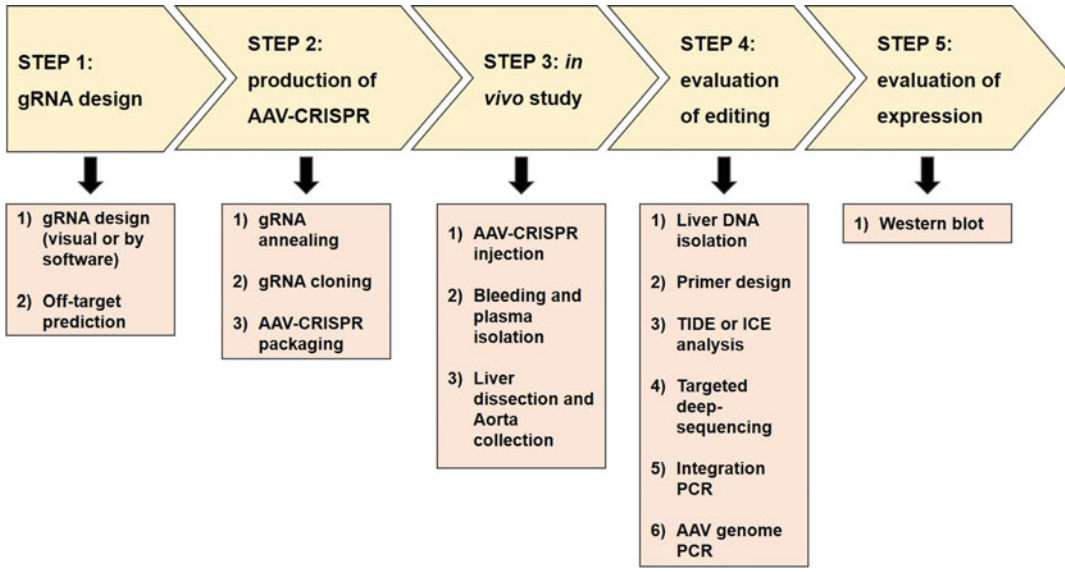


Fig. 1 Process flow diagram for somatically editing a gene in the liver using AAV-CRISPR. A flow diagram indicates the steps to follow for somatically editing a gene in the liver. The major methods covered in this Chapter are enlisted beneath the corresponding step

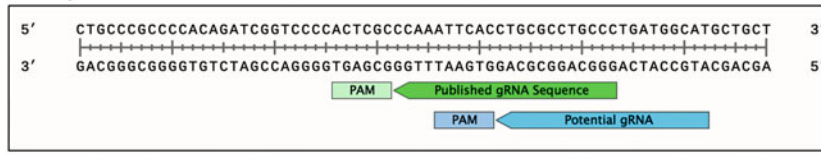
In this chapter, we provide a detailed protocol for using AAV-CRISPR for lipid and atherosclerosis research, through somatic editing of genes in the liver. Our protocol includes five general steps, as schematized in Fig. 1: (1) design of the gRNA for targeting the gene of interest; (2) generating AAV-CRISPR; (3) injection of AAV-CRISPR and in vivo study; (4) evaluation of editing at the targeted locus; (5) evaluation of expression level of the targeted gene. In this chapter, we do not cover methods for evaluating lipids and atherosclerosis, which can be found in other chapters of this book. We also do not cover AAV packaging, which can be performed by many academic or commercial cores, and has been published in a previous edition of Methods in Molecular Biology [6]. An overview of methods and expected outcomes is described in Subheadings 1.1 and 1.2.

1.1 AAV-CRISPR for Editing Candidate Genes in the Liver

gRNAs are designed manually or using CRISPOR for targeting the gene of interest. Off target analysis by COSMID or CRISPOR provides a list of potential off-target sites in the genome other than the targeted site that may be edited based on the gRNA sequence similarity and proximity to a PAM sequence (Fig. 2). gRNAs are annealed and cloned in 1313_pAAV-U6-SA-BbsI-MluI-gRNA-HLP-SACas9-HA-OLLAS-spA acceptor vector (Figs. 3 and 4) and the obtained plasmids need to be sequenced for verifying the insert and enzymatically digested for verifying that the ITRs are intact (Fig. 5). The final plasmids are used for generating AAVs based on serotype 8 for targeting the liver, by a triple

A

Excerpt from *Ldlr* Exon 14:



B

COSMID Output

Processing input tag: GGGCAGGCGCAGGTGAATTTGGNNGRR
Search in target database: mm10
Length: 27
searching for no indel hits allowing up to 3 mismatch(es) ... Done
searching for 1b-deletion hits allowing up to 2 mismatch(es) ..... Done
searching for 1b-insertion hits allowing up to 2 mismatch(es) ..... Done

Result	Query type	Mismatch	Hit ends in RG	chr position	Strand	Cut site	Score
GGGCAGGCGCAGGTGAATTTGGGCGAG -- hit NN RR -- query	No indel	0	Yes	Chr9:21744315-21744341	-	21744323	0
^GGCAGGCGCAGGTGAATTTGGGCGAG -- hit G NN RR -- query	Del 20, or Del 21, or Del 22	0	Yes	Chr9:21744315-21744340	-	21744323	0.61
GGC^AGGCGCAGGTGAATTTGGGCGAG -- hit GC NN RR -- query	Del 19	1	Yes	Chr9:21744315-21744340	-	21744323	0.76
GGCA^GGCGCAGGTGAATTTGGGCGAG -- hit GCA NN RR -- query	Del 18	2	Yes	Chr9:21744315-21744340	-	21744323	0.91
GGGCAGG^GCAGGTGAATTTCTGTGAG -- hit C GGNN RR -- query	Del 15	2	Yes	Chr2:126498613-126498638	+	126498630	11.72
GGGGAGGAGCA^GTGAATTTGGGGGAG -- hit C C G NN RR -- query	Del 10, or Del 11	2	Yes	ChrX:36067528-36067553	+	36067545	1.35
GGGCAGGCGCAGGTGAA^TTTGGGCGA -- hit T G NNGRR -- query	Del 3, or Del 4, or Del 5	2	No	Chr9:21744316-21744341	-	21744324	27.81

C

Predicted guide sequences for PAMs

Ranked by default from highest to lowest specificity score (Hsu et al., Nat Biot 2013). Click on a column title to rank by a score.
If you use this website, please cite our paper in NAR 2018. Too much information? Look at the CRISPOR manual.

Download as Excel tables: Guides / Off-targets / Saturating mutagenesis assistant

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes + Variants Only G- Only GG- Only A-	MIT Specificity Score	Predicted Efficiency Najm 2018	Out-of-Frame	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score exons only chr9 only
95 / rev	ATGCCATCAGGGCAGGCGCAG *GTGAAT* Enzymes: *HinP1I, AsuHPI, MluCI, XapI* **Cloning / PCR primers**	97	76	60	0 - 0 - 0 - 0 - 5 0 - 0 - 0 - 0 - 0 5 off-targets	4:intergenic:Schip1-Gm22073 4:intron:Klhl18 4:intron:Col14a1 show all...
85 / rev	GGCAGGCGCAGGTGAATTTGG *GCGAGT* Enzymes: *XapI* **Cloning / PCR primers**	60	82	71	0 - 0 - 0 - 2 - 94 0 - 0 - 0 - 0 - 0 96 off-targets	3:intergenic:Ccdc18-Dr1 3:intergenic:Swi5-Swi5/Golga2 4:intron:Ubac2 show all...

Fig. 2 gRNA design. (**a**) Excerpt of *Ldlr* Exon 14 with previously published gRNA and a second possible gRNA annotated. Sequence annotated in SnapGene. (**b**) Sample output from COSMID for the published gRNA sequence in (**a**). (**c**) Sample output from CRISPOR. Outputs for (**b**) and (**c**) were generated on 12-15-2020 and have been cropped for clarity

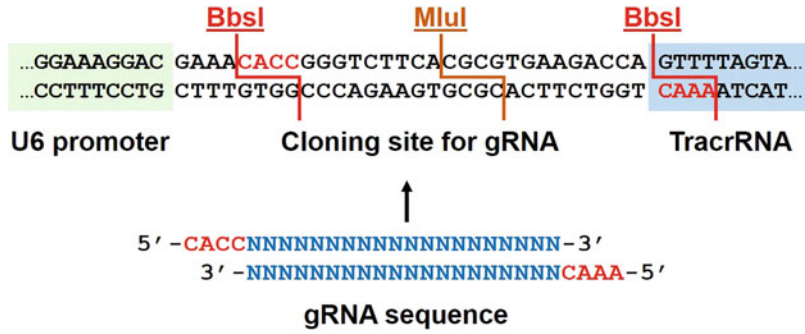


Fig. 3 Scheme of gRNA sequence and cloning. The gRNA sequence is composed of 20–22 double-strand nucleotides complementary to the targeted site, flanked by a BbsI restriction site overhang at each 5′ terminal. Annealed gRNAs are cloned into BbsI-linearized 1313 acceptor vector. The cloning site for gRNA in 1313 acceptor vector is shown with BbsI and MluI restriction sites indicated by elbow connectors

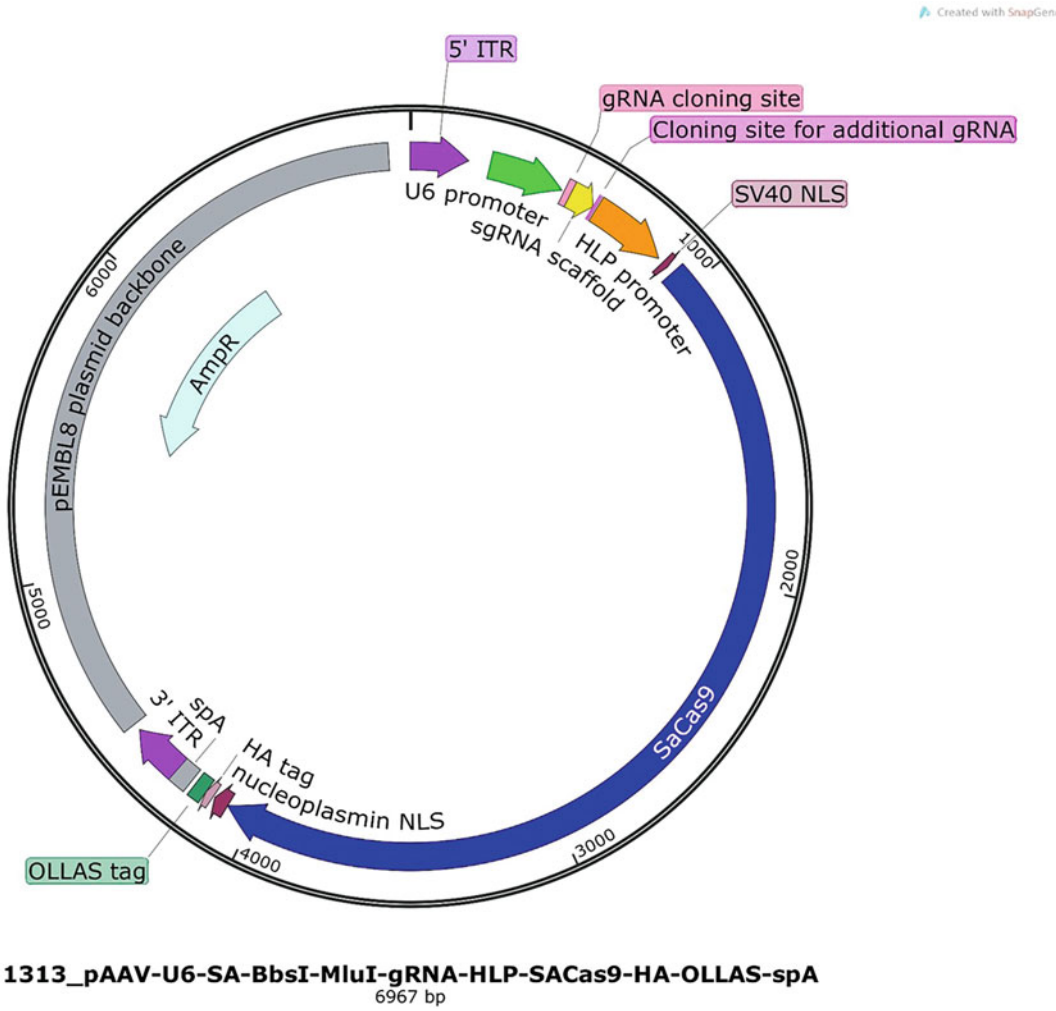


Fig. 4 Map of 1313 acceptor vector for gRNA cloning (Addgene 109,314). A gRNA cloning site is downstream of a U6 promoter and upstream of a sgRNA scaffold. The liver specific HLP promoter drives the expression of SaCas9. gRNA and SaCas9 expression cassettes are flanked by two complementary ITR sequences. Map generated with SnapGene

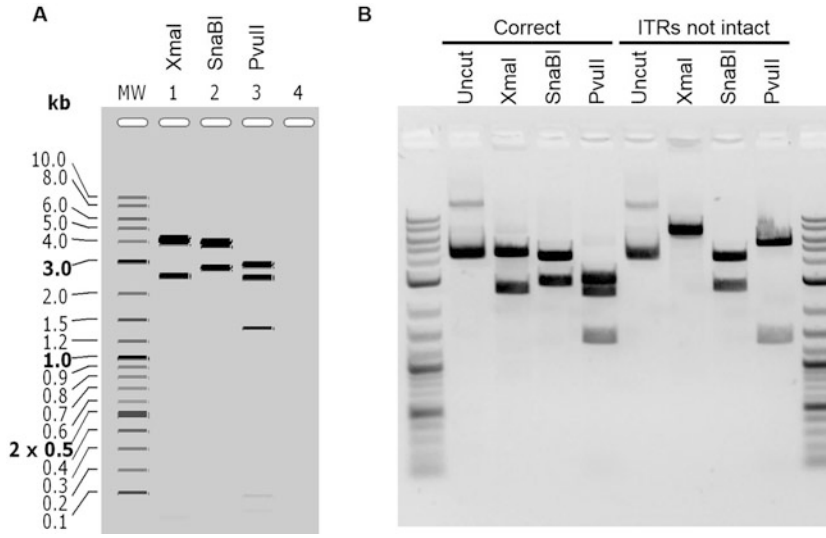


Fig. 5 Diagnostic enzymatic digestion of ITRs. (**a**) Prediction of diagnostic enzymatic digestions of ITRs in an AAV-CRISPR plasmid. Generated with SnapGene. (**b**) Gel electrophoresis of AAV-CRISPR plasmids digested with XmaI, SnaBI, and PvuII for checking the integrity of ITRs. Lanes 1 and 10 are loaded with a 1 kb DNA ladder. Lanes 2–5 represent a plasmid with intact ITRs, matching the predicted restriction patterns shown in (A). Lanes 6–9 represent a plasmid with damaged ITRs, where XmaI and PvuII show incorrect band sizes by restriction digest

transfection method. In our hands, an average yield in the order of 10^{12} to 10^{13} genome copies per lot is expected by using this method [6]. However, cores and companies can produce AAVs at a large scale. It is recommended to inject mice starting at 6–8 weeks of age. Editing is expected to be more efficient in adult mice, as a result of predominance of the error prone NHEJ-mediated repair in postmitotic tissues. A dose of 5×10^{11} genome copies is a good starting point for reaching high levels of editing at the desired locus [21–24]. However, a higher dose (up to 1×10^{12} genome copies) can be used for increasing editing levels, or when female mice are injected, due to the reported sexual dimorphism [21]. Based on our data, editing is detectable starting at 7 days postinjection. Integration PCR is the easiest method for qualitatively assessing editing, resulting in the PCR amplification of AAV-genome insertion events in the DSB site (Fig. 6). ICE and TIDE analyses, or deep sequencing provide the spectrum and frequency of indel formation (Fig. 6). It is important to amplify and analyze predicted off-target sites by deep sequencing to rule out any nonspecific effects on phenotype. When testing new gRNAs, a good gRNA candidate should show an indel frequency of at least 20–30% and no off target activity. The effects of gene editing on protein expression is verified by western blot on liver lysate or plasma. Ideally, efficient gene editing should result in undetectable protein expression levels of the targeted gene. The effects of gene editing are expected to be permanent if

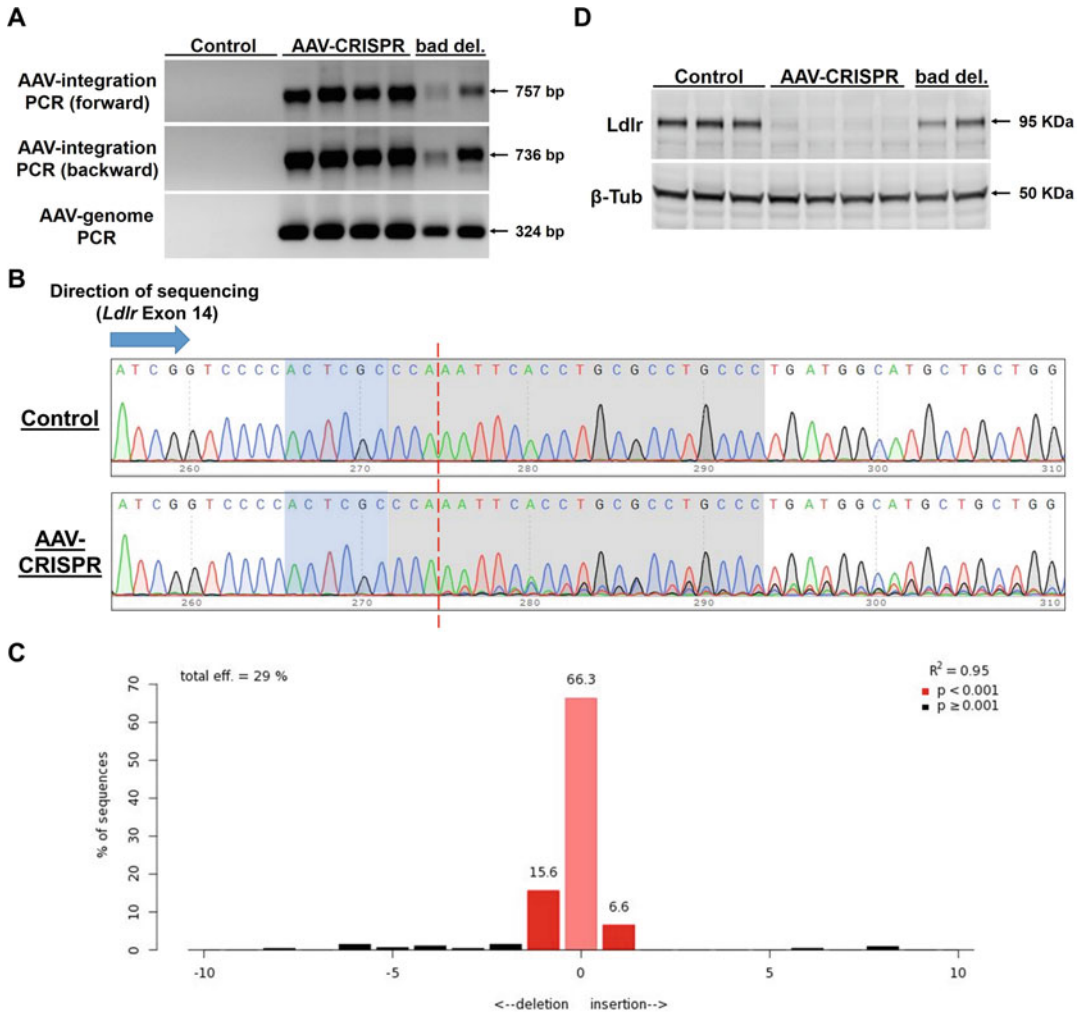


Fig. 6 Analysis of editing at the *Ldlr* locus and effects on Ldlr expression in the liver. (**a**) Integration PCRs detecting the AAV-genome forward and backward integration in the *Ldlr* locus by NHEJ in *Ldlr* AAV-CRISPR-injected mice. AAV genome PCR showing AAV genomes in the liver of AAV-CRISPR mice. Control mice were injected with sterile saline solution. Mice in the last two lanes show reduced delivery of AAVs to the liver, resulting in lower editing at the *Ldlr* locus (bad del.: bad delivery). (**b**) Representative DNA sequencing chromatograms showing multiple sequence traces in exon 14 of *Ldlr* in the liver from an *Ldlr* AAV-CRISPR-injected mouse compared to a control. The PAM and gRNA sequences are respectively highlighted in blue and grey. A dashed red line indicates the expected nuclease cut site. An arrow indicates the sequencing direction. (**c**) Indel spectrum by TIDE showing the relative percentage of unedited and edited sequences (with insertions or deletions) at the targeted locus. (**d**) Ldlr western blot on liver lysates with beta tubulin (β-Tub) used as loading control (1:500, mouse, University of Iowa Developmental Studies Hybridoma Bank E7). Mice in the last two lanes (bad del.) show marginally decreased Ldlr levels as a result of AAV bad delivery. A homemade antibody was used for detecting Ldlr at 1:5000 dilution (gift from Gene Ness)

the targeted gene is not essential for hepatocyte survival. In case of essential genes, cell death of targeted hepatocytes and regeneration of unedited hepatocytes may result in loss of the edited alleles over

time [22]. Moreover, the time frame between gene editing and protein knockdown along with phenotype development depends on the abundance and half-life of the targeted protein. Therefore, we recommend assessing editing and expression level of the candidate gene at different time points postinjection (e.g., at 2, 4, and 6 weeks). The effects of gene editing on lipid metabolism can be monitored by measuring plasma lipids at different time points and analyzing the liver at endpoint. Overall, AAV-CRISPR enables one to rapidly knock out genes in the liver, bypassing the need for floxed mice and complicated breeding schemes, and avoiding any developmental compensation.

1.2 AAV-CRISPR for Modeling Hypercholesterolemia and Atherosclerosis

A mouse model of hypercholesterolemia and atherosclerosis is generated by somatic editing of *Ldlr* in the liver of adult mice. A single dose of *Ldlr* AAV-CRISPR (5×10^{11} genome copies in male and 1×10^{12} genome copies in female mice injected at 6–8 weeks of age) is expected to edit exon 14 of *Ldlr* by NHEJ-mediated indel formation and AAV-genome insertion at the DSB site. AAV insertion events at the *Ldlr* locus can be easily detected by integration PCR as a first qualitative assessment of editing. An AAV genome PCR is useful for identifying mice with incomplete or failed delivery of AAV to the liver as a result of bad injection, and excluding them from the study (Fig. 6). TIDE and ICE analyses or deep sequencing provide quantitative information on the frequency of indels [21]. The genetic disruption of *Ldlr* is permanent resulting in near-complete loss of hepatic Ldlr throughout the atherosclerosis study. Mice with ~30% of indel formation rate are expected to show undetectable levels of Ldlr in liver lysates by western blot (Fig. 6). On the contrary, failed injections result in incomplete editing with only marginally decreased Ldlr levels (Fig. 6). *Ldlr* disruption is expected to gradually increase plasma cholesterol levels at extent comparable to the gold standard $Ldlr^{-/-}$ germline mouse model—for example, plasma cholesterol: 1408 ± 473 mg/dL versus 1966 ± 412 mg/dL in male mice after 20 weeks of western diet [21]. Measuring cholesterol at intermediate time points during the study can give an idea on the efficiency of *Ldlr* editing. Moreover, plasma cholesterol is expected to distribute mostly in the very low–density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and LDL fractions, similar to what was observed in $Ldlr^{-/-}$ mice. As a consequence of severe hypercholesterolemia, *Ldlr* AAV-CRISPR-injected mice fed a western diet develop severe atherosclerotic lesions, showing lesion area in the range of 6–10% by en face Oil Red O staining of aortae in male mice after 20 weeks of western diet feeding [21] (Fig. 7).

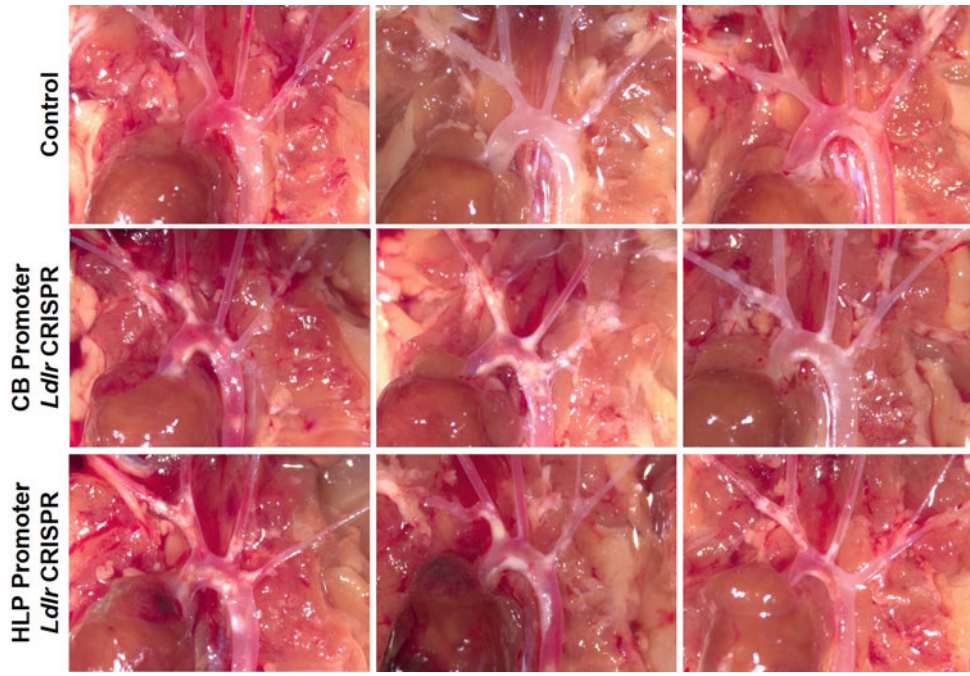


Fig. 7 Atherosclerosis development following *Ldlr* editing with *Ldlr* AAV-CRISPR. 8-week-old *C57BL/6 J* mice were injected with 5×10^{11} genome copies of *Ldlr* AAV-CRISPR expressing SaCas9 from the chicken β-actin (CB) promoter [21], *Ldlr* AAV-CRISPR expressing SaCas9 from the liver-specific HLP promoter or saline (control). Mice were placed on Western diet and followed for 20 weeks. Aortae were dissected and raw images were taken. The HLP vector results in atherosclerosis development at similar extent to the CB vector (*see* **Note 55**)

2 Materials

All buffers are made in Milli-Q double distilled water and stored at room temperature, unless stated otherwise.

2.1 General Materials and Equipment

1. Sterile PCR tubes.
2. Sterile 1.5 and 2 mL tubes.
3. Sterile 15 and 50 mL tubes.
4. Micropipettes.
5. Filtered tips.
6. Electronic pipettor.
7. Serological pipettes.
8. Heat block with 1.5 mL tube insert.
9. Thermocycler.
10. Molecular grade (nuclease-free) water.
11. Milli-Q double distilled water.

12. Oligonucleotides/primers (*see* **Note 1**).
13. Electrophoresis power supply.
14. Gel electrophoresis apparatus.
15. Gel tweezers.
16. Shaker.
17. Agarose, molecular biology grade: 1% (w/v) in 1× tris–acetate–EDTA (TAE) buffer (*see* **Note 2**).
18. 1× TAE buffer: 40 mM tris–acetate, 1 mM EDTA, pH 8.3.
19. Ethidium bromide, 10 mg/mL (*see* **Note 3**).
20. 1 kb DNA ladder for gel electrophoresis.
21. 6× DNA loading buffer for gel electrophoresis: 6× TAE buffer, 30% glycerol, 0.02% Bromophenol Blue.
22. Gel extraction kit (any commercially available kit).
23. UV transilluminator and gel imager (*see* **Note 4**).
24. Ice.
25. Tabletop centrifuge.
26. Centrifuge for 15, 50, and 250 mL centrifuge tubes.
27. Laboratory balances.
28. Bead mill homogenizer.
29. Steel beads.
30. UV-Vis spectrophotometer.
31. Vortex.
32. Sharp disposal container.
33. −80 °C freezer.
34. 70% ethanol.
35. 1× phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, and 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.

2.2 gRNA Design

1. Gene annotation software (e.g., SnapGene) (*see* **Note 5**).
2. Genomic and mRNA sequence files for gene(s) of interest downloaded from NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/) or UCSC Genome Browser (https://genome.ucsc.edu/) and edited in annotation software.
3. CRISPR Off-target Sites with Mismatches, Insertions and Deletions (COSMID) website for off-target prediction: https://crispr.bme.gatech.edu [25].
4. CRISPOR website for gRNA design and off-target prediction: http://crispor.tefor.net/ [26].

2.3 Plasmid Construction

1. Tris–EDTA (TE) buffer: 10 mM Tris–HCl pH 7.5, and 1 mM EDTA in molecular grade water.
2. 10× Annealing Buffer: 100 mM Tris–HCl pH 7.5, 1 M NaCl, and 10 mM EDTA in molecular grade water.
3. Plasmid 1313_pAAV-U6-SA-BbsI-MluI-gRNA-HLP-SACas9-HA-OLLAS-spA (Addgene 109314).
4. BbsI restriction enzyme.
5. 10× Enzyme buffer: 500 mM potassium acetate, 200 mM Tris–acetate, 100 mM magnesium acetate, 1 mg/mL BSA, pH 7.9. Store at −20 °C.
6. 2× Rapid Ligation Buffer: 132 mM Tris pH 7.6, 20 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 15% PEG. Store at −20 °C.
7. T4 DNA ligase.
8. Stable competent *E. coli* (NEB, C3040H): F′ *proA+B+ lacI*^{q} *Δ(lacZ)M15 zzf::Tn10* (Tet^R)*/Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- Φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1 Δ(mrr-hsdRMS-mcrBC)*.
9. 500 mL—1 L Erlenmeyer flasks.
10. LB (Luria–Bertani) broth: 25 g per L of Milli-Q double distilled water (*see* **Note 6**).
11. 50 mg/mL ampicillin, store at −20 °C.
12. LB Agar: 37 g per L in Milli-Q double distilled water (*see* **Note 7**).
13. 10 cm petri dish.
14. 42 °C water bath.
15. 37 °C incubator.
16. Laminar flow hood.
17. TE buffer: 10 mM Tris–HCl, pH 8.0. 1 mM EDTA.
18. 37 °C bacterial shaker equipped for 500 mL flasks and 15 mL tubes.
19. Miniprep kit (any commercially available kit).
20. Maxiprep kit (any commercially available kit).
21. XmaI restriction enzyme.
22. SnaBI restriction enzyme.
23. PvuII restriction enzyme.

2.4 In vivo Study

1. *C57BL/6J* mice.
2. Sterile PBS solution for injection.
3. Insulin syringes with 25–27G needles.
4. 10 mL syringes with 23G needles.

5. Isoflurane for animal use.
6. Anesthesia machine and charcoal filters.
7. Western diet containing 0.21% (w/w) cholesterol and 21% (w/w) fat.
8. Sterile heparinized capillary tubes.
9. Sterile gauze sponges.
10. Set of surgical instruments for animal dissection.
11. Razor blades.
12. Glass bead sterilizer.
13. 10% formalin.
14. Optimal cutting temperature (OCT) compound.
15. Specimen molds.
16. Liquid nitrogen.
17. Cryogenic microtubes.

2.5 Analysis of On-Target and Off-Target Nuclease Activity

1. DNA isolation kit (any commercially available kit).
2. Qubit 4 Fluorometer (Thermo Scientific).
3. 2 U/μL Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific).
4. 10 mM Deoxynucleotide triphosphate (dNTPs).
5. Herculase II Fusion DNA Polymerase (Agilent Technologies).
6. Agencourt AmPure XP (Beckman Coulter).
7. Apex 2× Taq Red Master Mix (Genesee Scientific).
8. 96-well plates.
9. Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/).
10. Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).
11. Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi).
12. Tracking of Indels by DEcomposition (TIDE) web-based tool (http://shinyapps.datacurators.nl/tide/) [27].
13. Inference of CRISPR Edits (ICE) web-based tool (https://ice.synthego.com/#/) [28].
14. CRISPResso2 web-based tool (https://crispresso.pinellolab.partners.org/submission) [29].

2.6 Evaluation of Gene Knockdown by Western Blot

1. RIPA buffer: 50 mM Tris pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 150 mM sodium chloride. Store at −20 °C.
2. Protease inhibitor cocktail.

3. BCA protein assay kit.
4. 4× NuPAGE™ LDS Sample Buffer (Thermo Scientific) or equivalent.
5. Beta-mercaptoethanol.
6. XCell SureLock™ Mini-Cell (Thermo Scientific) or equivalent.
7. NuPAGE™ 4–12%, Bis-Tris, 1.0 mm, Mini Protein Gel (Thermo Scientific) or equivalent.
8. 20× NuPAGE™ MES SDS Running Buffer (Thermo Scientific) or equivalent.
9. Protein Marker.
10. 0.45 μm polyvinylidene fluoride (PDVF) membrane.
11. Tweezers.
12. Sponges.
13. 20× NuPAGE™ Transfer Buffer (Thermo Scientific) or equivalent.
14. Methanol.
15. XCell II™ Blot Module (Thermo Scientific) or equivalent.
16. Filter papers.
17. PBS-T: PBS supplemented with 0.05% Tween 20.
18. Western blot boxes.
19. Odyssey blocking buffer (PBS) (Licor): mix 2 parts of blocking buffer with 1 part of PBS-T.
20. Antibody buffer: 1% BSA in 0.05% PBS-T.
21. Anti Ldlr antibody (Abcam ab52818, 1:1000–1:2500).
22. Anti-housekeeping protein antibody (e.g., β-actin).
23. Goat anti-rabbit 680 nm (1:15000) (Rockland).
24. Goat anti-mouse 800 nm (1:15000) (Rockland).
25. Odyssey infrared imaging system (Licor).

3 Methods

3.1 gRNA Design

3.1.1 Target Gene Annotation

1. Navigate to NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/) and search for your gene of interest (e.g., mouse *Ldlr*) (*see* **Note 8**).
2. Select the mouse ortholog of your gene of interest, scroll down to "NCBI Reference Sequences" and select the FASTA Genomic Build.
3. Select "Send to → Complete Record → File" and "GenBank (full)." Click "Create File" and open the downloaded file in SnapGene.

4. Repeat the process for any mRNA isoforms.
5. Using SnapGene, align mRNA sequences to confirm which exons are present in all mRNA isoforms. Annotate these exons in the mRNA and genomic files. Also note essential domains (*see* **Note 9**).

3.1.2 gRNA Design—Visual Selection

1. Open the sequence file for the target gene and search for 5′-NNGRRT-3′ (N is any nucleotide and R is A or G), which matches the PAM motif for SaCas9 (*see* **Note 10**). SnapGene will show PAM motifs on either strand. The gRNA will be 5′-(20-22N)NNGRRT-3′ (*see* Fig. 2a for example).
2. Highlight gRNA sequences 20–22 bp 5′ of the PAM. Annotate potential gRNAs early in the coding sequence and near important protein domains.
3. Note gRNAs for further consideration if they meet the following criteria (not including the PAM).
   (a) Melting temperature (Tm) between 55 and 70 °C.
   (b) Few repetitive bases (note that quadruple repeats of T will terminate U6 promoter expression).
   (c) 45–70% GC content.
   (d) The region within 3–5 bp from the PAM should be within the protein-coding region. Otherwise indel formation from editing may not knock out protein expression.
   (e) A gRNA starting with a G at the 5′ end is preferred for proper expression from the U6 promoter. Add a G at the 5′ when it is not present before proceeding to cloning.

3.1.3 Off-Target Prediction Using COSMID

1. Open the genomic sequence file for the target gene and copy the gRNA sequence to be assessed from 5′ to 3′. The PAM would be at the 3′ end of this sequence but should not be copied with the gRNA sequence. Make sure to copy the correct strand of the DNA. For example, if the gRNA is on the bottom strand, the sequence must be copied from the bottom strand from 5′ to 3′.
2. Navigate to https://crispr.bme.gatech.edu (*see* **Note 11**) and select the following parameters:
   (a) Target Genome: Mus Musculus GRCm38 (mm10).
   (b) Query Sequence: Paste the gRNA sequence 5′ to 3′, excluding the PAM.
   (c) Search Options:
   - Add PAM suffix NNGRR—exclude the T to allow the search to be slightly less stringent, returning more off-targets.

- Allowed indels and mismatch: Set to the least stringent search terms with the highest number of mismatches for each option: No Indels = 3, 1-base Del = 2 and 1-base Ins = 2.

(d) PCR Design Options: If you check the box, it will design a pair of primers for off-targets (*see* **Note 12**).

3. Hit submit. The page may take several minutes to load.
4. Analyze output:
   (a) Result: Shows the hit versus the sequence that was queried with mismatches from the hit in red text. These, minus ambiguous bases like "N" are totaled in the "Mismatch" column.
   (b) Query Type: The type of alteration required for binding (e.g., no indel, deletions or insertions in the gRNA or deletions or insertions in the PAM).
   (c) Hit ends in RG: This is for SpCas9 systems and is not applicable to the SaCas9 system that is being used here. Ignore this column.
   (d) Chr Position: This is the chromosomal location of the gRNA binding site for the on- or off-target. This is clickable and navigates to UCSC Genome Browser.
   (e) Score: The closer to 0 the score, the more likely the off-target site will be edited by that gRNA sequence. The on-target will ideally be the only site with a low score.
5. Download the Excel spreadsheet summary.
6. Duplicate the results tab and sort by chromosomal location. Some hits, especially for the on-target site, will be duplicates. Delete duplicates of the same site, keeping the lowest scoring duplicate. The final list should include any off-targets as well as your on-target.
7. Click the links for chromosomal position to determine if any off-targets reside in exons or near important gene regulatory regions. If off-targets are near these locations, do not choose this gRNA for testing. Choose final gRNAs with the fewest off-targets and preferably no predicted off-targets in exons. Off-targets with high scores are less likely to have off-target editing than those with lower scores. An example of COSMID output is shown in Fig. 2b.

3.1.4 gRNA Design and Off-Target Prediction Using CRISPOR

1. Open the genomic sequence file for the target gene and copy the region of interest, usually an early exon or essential region.
2. Navigate to http://crispor.tefor.net and paste the sequence into the text box (*see* **Note 13**).
3. Make the correct selections:

(a) For the genome, select Mus Musculus—Mouse (reference—UCSC Dec, 2011 (mm10 = C57BL/6J); and

(b) For the PAM, select 21 bp-NNG(A/G)(A/G)T—Cas9 S. Aureus.

4. Hit submit and review the outputs. The best gRNAs selected by CRISPOR will be noted with green to the left of the gRNA output (Fig. 2c) and do not consider gRNAs that are yellow or red. The parameters to look at are as follows

(a) Specificity score—How specific is the gRNA to the target? This is rated from 0–100, with 100 being the best, most specific score. gRNAs with a specificity score above 75 should be considered.

(b) Efficiency score—How quickly and effectively does the gRNA disrupt the target? Faster disruption is preferred. On the scale of 0–100, where 100 is the best, consider efficiency scores of greater than 75.

(c) Out of Frame—How likely is it that cutting with this gRNA will result in indel formation? Indels are needed both for loss of the protein of interest and for detection of editing, so higher Out of Frame scores are preferred. Select gRNAs with Out of Frame scores of greater than 50.

(d) Off-targets for 0-1-2-3-4 mismatches—This column lists how many off-targets are predicted for a given gRNA for 0–4 mismatches to the gRNA sequence. Off-targets that are perfect matches or have only single mismatches for the gRNA sequence exclude that gRNA from consideration. Select gRNAs that have the fewest number of off-targets and which have off-targets that have 3+ mismatches. There is a gray, second set of numbers in this column denoted as "+ next to PAM." These numbers show the number of off-targets with 0–4 mismatches that have no mismatches within 12 bp of the PAM. These gRNAs are more likely to have off-target effects at sites like these and should be avoided.

(e) Genome browser links to matches sorted by cutting frequency determination (CFD) off-target score—This is the list of off-targets predicted for a given gRNA. Select gRNAs with the lowest number of possible off-targets, but this list allows for further narrowing of gRNA candidates. Off-targets are listed as intron, intergenic or exon. Exclude gRNAs with off-targets in exons. The links for each off-target are clickable and direct to the UCSC Genome Browser to show the potential off-target cut site.

(f) Other points to consider:

- Pay attention to notes in the "Guide Sequence + PAM" column—CRISPOR notes gRNAs that have quadruple repeats of T, which halt transcription from the U6 promoter.
- Generally, try to avoid multiple repeats (3+) of the same base.
- Aim for gRNAs that have similar GC content across the gRNA sequence.
- If a gRNA does not start with a G at the 5′ end, one will need to be added before cloning for the gRNA to be properly expressed from the U6 promoter. Rerun the gRNA sequence of interest through COSMID with the G added.

5. Download the "Guides" and "Off-targets" files. The off-targets file will be necessary for primer design for assessing off-target editing later (*see* Subheadings 3.4.2 and 3.4.4).
6. Annotate and name gRNAs in the genomic and RNA files.
7. Repeat this process for other exons or regions of interest.
8. Check gRNAs as a final step using COSMID, which uses different off-target prediction algorithms compared to CRISPOR. Ideal gRNAs will have less than 10 off-targets for both COSMID and CRISPOR.
9. Select the top 2 to 5 gRNAs and proceed to Plasmid Construction (Subheading 3.2). An example of CRISPOR output is shown in Fig. 2c.

3.2 Plasmid Construction and AAV Production

3.2.1 Oligonucleotides Annealing

1. Each gRNA is designed as a ~20–22 double stranded nucleotide sequence complementary to the targeted gene, flanked by a BbsI restriction site overhang at each 5′ terminal. Do not include the PAM sequence in the gRNA sequence used for cloning. In order to generate annealed oligonucleotides that can be ligated into the sticky ends left by BbsI restriction, 5′-CACC-3′ must be added to the 5′ end of the top oligonucleotide sequence and 5′-AAAC-3′ must be added to the 5′ end of the bottom oligonucleotide (Fig. 3). Top and bottom oligonucleotides can be ordered from any commercial DNA synthesis company (*see* **Note 14**).
2. Dilute each oligonucleotide stock solution to 1 μM working solution in TE Buffer.
3. In a 1.5 mL tube, mix 10 μL of 10× Annealing Buffer, 8.3 μL of 1 μM top oligonucleotide solution, 8.3 μL of 1 μM bottom oligonucleotide solution, and 73.4 μL of molecular grade water so the final volume is 100 μL.
4. Vortex and then centrifuge at 2000 × *g* for 1 min.

5. Heat the annealing reactions at 100 °C for 2 min using a heat block.
6. Move the tubes on a bench and let them slowly cool down to room temperature for at least 30 min.
7. Centrifuge at 2000 × *g* for 1 min and place the tubes in ice (*see* **Note 15**).

3.2.2 Cloning and Analysis

1. To digest the 1313_pAAV-U6-SA-BbsI-MluI-gRNA-HLP-SACas9-HA-OLLAS-spA acceptor vector (Addgene 109314; *see* **Note 16** and Fig. 4), mix in a PCR tube 6 μg of 1313 vector, 8 μL of 10× Enzyme buffer, 2 μL of 20 U/μL BbsI, and molecular grade water to a final volume of 80 μL.
2. Set the following program in a thermocycler: 6 h at 37 °C; 15 min at 65 °C; and hold at 4 °C (*see* **Note 17**).
3. Prepare a 1% agarose gel containing ethidium bromide in a gel electrophoresis apparatus and load the whole volume of digestion from **step 2** supplemented with 1× DNA loading buffer. Include a lane loaded with 5 μL of 1 kb DNA ladder for estimating the size of concurrently analyzed samples.
4. Separate the digested vector by gel electrophoresis using 1× TAE buffer (150 V for 30 to 45 min). Check DNA ladder and digestions using an UV gel imager.
5. Cut out the digested vector with a pair of gel tweezers using a UV transilluminator for visualizing the DNA.
6. Gel purify the digested vector using a gel extraction kit, following the manufacturer's instructions.
7. Measure DNA concentration with a UV-Vis spectrophotometer.
8. Ligate the annealed gRNA in 50 ng of digested vector using a 10:1 (insert:vector) molar ratio. Include a mock-ligation reaction, which contains no insert. Use the following formula for calculating the amount of gRNA needed: ng gRNA = 50 ng vector × (kb insert/kb vector) × (10:1 ratio insert:vector). Set up ligations in a PCR tube with *X* μL of linearized 1313 vector (final amount of 50 ng), calculated *X* μL of gRNA, 10 μL of 2× Rapid ligation buffer, 1 μL of T4 DNA ligase and molecular grade water to a final volume of 21 μL total.
9. Mix and centrifuge at 2000 × *g* for 1 min.
10. Incubate at room temperature for 15 min.
11. Proceed to transformation or store at −20 °C.
12. For each transformation include the following plates as controls: (1) bacteria transformed with water as a negative control for ampicillin; (2) bacteria transformed with the mock-ligation as control for undigested or self-ligated vectors; and

(3) bacteria transformed with 5 ng of undigested vector, as positive control for transformation efficiency of the *E. coli*.

13. Thaw stable competent *E. coli* on ice.
14. Add 4 μL of ligation (or controls) to each aliquot of 50 μL bacteria and mix gently by pipetting.
15. Incubate in ice for 10 min.
16. Heat shock at 42 °C in a water bath for 45 s.
17. Incubate in ice for 2 min.
18. Add 500 μL of sterile LB medium to each tube and mix by pipetting.
19. Incubate 1 h in a 37 °C bacterial shaker at 200 rpm.
20. Seed 80 μL of bacteria on sterile LB agar plates containing ampicillin.
21. Incubate the plates at 37 °C for at least 14 h.
22. On the following day, proceed to colony screening. The water plate should show no colonies, whereas the positive control plate should show colonies in the order of 10^2 to 10^3. The mock-ligation plate should show no or very few colonies. If these conditions are met, pick three to five colonies from the ligation plate with sterile pipette tips and place each of them in 15 mL tubes filled with 5 mL of sterile LB medium supplemented with ampicillin (0.1 mg/mL; LB-Amp). Include a tube with LB-Amp medium only, as control of the correct functioning of the antibiotic. Store the agar plates at 4 °C for few weeks.
23. Incubate the 15 mL tubes from **step 22** in a shaker for 12–18 h (200 rpm at 37 °C). There should not be bacteria growth in the negative control medium after incubation. If this medium is turbid, discard all the tubes and repeat with fresh ampicillin stocks.
24. After overnight incubation, save 0.5 mL of culture from each tube and store at 4 °C for up to 1 week.
25. Centrifuge the remaining 4.5 mL of culture for 20 min at 3500 × *g*.
26. Purify recombinant DNA from the pellets using a miniprep kit, following the manufacturer's instructions. Elute plasmid DNA with 30–50 μL of molecular grade water or TE buffer.
27. Measure plasmid DNA concentration using a UV-Vis spectrophotometer.
28. Check whether ITRs are still intact by enzymatic digestion with XmaI, SnaBI and PvuII restriction enzymes (*see* **Note 18**). Include a reaction with no restriction enzymes (undigested control). In a PCR tube, mix 2 μg of plasmid DNA, 3 μL of

10x Enzyme buffer, 1 μL of 20 U/μL restriction enzyme, and molecular grade water to a final volume of 30 μL.

29. Set the following program in a thermocycler: 6 h at 37 °C; 15 min at 65 °C; and hold at 4 °C.
30. Prepare a 1% agarose gel containing ethidium bromide and load 1 kb DNA ladder and half volume of each digestion or undigested control supplemented with 1× DNA loading buffer. Perform gel electrophoresis using 1× TAE buffer (150 V for 30–35 min). Visualize DNA bands using an UV gel imager (*see* **steps 3** and **4**).
31. Compare the size of the observed bands with those predicted on SnapGene or other vector mapping software, as shown in Fig. 5.
32. If ITR integrity is verified, send plasmid DNA out for sequencing the gRNA insert using U6ForSeq primer: 5′- CCTTCA TATTTGCATATACGATACAAGGCTGTTAG-3′.
33. Select the clone that contains the gRNA insert and intact ITRs and proceed to maxiprep preparation.
34. Add the previously saved 0.5 mL of culture (**step 24**) to 250 mL of sterile LB-Amp medium and incubate in a shaker for 16–20 h (200 rpm at 37 °C).
35. The day after, centrifuge down the culture at 6000 × *g* for 30 min and isolate plasmid DNA using a maxiprep kit, following the manufacturer's instructions. Elute plasmid DNA with ~300 μL of molecular grade water or TE buffer.
36. Measure plasmid DNA concentration using a UV-Vis spectrophotometer.
37. Check gRNA and ITRs as described above (*see* **steps 28–32**) before proceeding to transfection.
38. Store plasmid DNA at −20 °C until use.

3.2.3 AAV Packaging

Use recombinant AAV vectors based on serotype 8 for targeting the liver. For the vast majority of academic labs, it is more cost- and labor-effective to have AAV packaged by an academic or commercial core. Therefore, AAV packaging will not be covered in this chapter. For reference, primers for determining AAV titers for these vectors are given in **Note 19**. For labs interested in performing their own AAV packaging, an excellent protocol exists in a previously published chapter [6]. AAV should be aliquoted and stored at −80 °C until ready for use. Avoid repeated freeze-thaw cycles.

3.3 In vivo Study

3.3.1 AAV Preparation and Injection

A protocol for AAV preparation and intraperitoneal injection in *C57BL/6J* mice is described below (*see* **Note 20**). During AAV preparation, work in sterile conditions under a laminal flow in a biosafety hood.

1. Calculate a master mix for injecting n mice (+10% of volume), each one with 5×10^{11} genome copies in a final volume of 300 μL of sterile PBS (*see* **Note 21**).
2. Thaw AAV on ice.
3. Dilute AAV in the calculated volume of sterile PBS in a 2 mL tube.
4. Slowly load a sterile insulin syringes (25 to 27G needle) with 300 μL of the specific AAV dilution. Avoid the formation of air bubbles.
5. Manually restrain the mouse in head-down position with the abdomen side up facing the operator (*see* **Note 22**).
6. Insert the needle in the animal's right abdominal quadrant at about 30° angle.
7. Aspirate to be sure that the needle has not penetrated a blood vessel, the intestines or the urinary bladder. If any fluid is aspirated, discard the needle and syringe and prepare a fresh injection. If no fluid is aspirated, slowly inject the AAV dilution or sterile PBS in control mice.
8. Dispose the syringe in a sharp container and place the animal back in the cage. Injection groups are randomly assigned within cages when experimental design allows.
9. For atherosclerosis studies, feed mice with a Western diet for ~20 weeks with free access to food and water, and standard light–dark cycles.

3.3.2 Bleeding and Plasma Isolation

To assess plasma lipids, blood is collected at time 0 (right before the injection) and every 4 weeks after 5 h of fasting (*see* **Note 23**). For retroorbital bleeding (*see* **Note 24**), follow these steps:

1. Induce anesthesia by delivering isoflurane in an isolated sealed chamber connected to a precision vaporized device, using a rate of 3–4% isoflurane in oxygen.
2. Place the anesthetized mouse on its side on a work surface and control the animal reactivity by toe pinch. If no reflexes are shown, open the eye so that the eyeball is slightly protruding. Insert the appropriate sterile heparinized capillary tube at the medial or lateral canthus of the eye, at a 45° angle and avoiding the eyeball.
3. Puncture the retroorbital plexus by gently rotating the capillary tube and applying light pressure.

4. Once blood is visualized entering the capillary tube, raise the mouse relative to the tube, which helps the blood flow into the tube.
5. Remove the capillary tube and ensure hemostasis by applying gentle pressure to the eye with a sterile gauze sponge.
6. Place blood in a 1.5 mL tube in ice.
7. Place the animal back in the cage and monitor its recovery postanesthesia.
8. For isolating plasma, centrifuge blood at 10,000 × *g* for 20 min at 4 °C. Carefully, collect plasma and store samples at −80 °C until further use (*see* **Note 25**).

3.3.3 Liver Dissection

To assess editing and expression levels of the targeted genes, collect liver samples at the endpoint of the experiment. This procedure takes approximately 30 min per mouse. For atherosclerosis studies, collect also heart and aorta (*see* **Note 26**).

1. Fast mice for at least 5 h or overnight before dissection.
2. Induce anesthesia by delivering isoflurane in an isolated sealed chamber connected to a precision vaporized device, using a rate of 4–5% isoflurane in oxygen.
3. Collect blood by retroorbital bleeding as described in Subheading 3.3.2.
4. Euthanize the mouse by cervical dislocation.
5. Sanitize the abdomen with 70% ethanol. Then, cut the skin and the underneath abdominal wall with scissors to expose the caudal surface of the diaphragm. Cut the diaphragm and open the thoracic cavity. After exposure of the heart, perfuse the left ventricle with 10 mL of sterile PBS using a syringe with a 23G needle. Apply a cut on the right atrium. After a good perfusion, the liver should pale considerably as it is cleared of blood.
6. Harvest the liver and measure liver weight with a laboratory balance.
7. Section the largest lobe with a razor blade for collecting the samples in cryogenic microtubes: a ~20, 10 and 50 mg piece provides enough material respectively for DNA, RNA and protein isolation and downstream analyses. Snap freeze the samples in liquid nitrogen.
8. Collect a transverse slice for OCT embedding. Soak the slice in a mold filled with OCT, avoiding bubble formation and freeze the OCT block in a −80 °C freezer.
9. Collect a transverse slice for formalin fixation and paraffin embedding for immunohistochemistry. Incubate the slice in 10% formalin shaking overnight at room temperature, then

dehydrate samples with 70% ethanol. Samples can be stored at room temperature until proceeding with immunohistochemistry.

10. Collect all the remainder liver in a cryogenic microtube for further analyses and snap freeze it in liquid nitrogen.
11. Store liver samples in a −80 °C freezer.

3.4 Analysis of On-Target and Off-Target Nuclease Activity

Genome editing can be evaluated by (1) Sanger sequencing-based methods, (2) targeted deep sequencing and (3) integration PCR. The first two methods enable the detection of small indels at the DSB site. The third method enables detection of large insertions derived from the NHEJ-mediated integration of viral genomes in the DSB site. Furthermore, an AAV-genome PCR can be performed for diagnostic detection of the AAV-CRISPR genome in the liver. All these methods require genomic DNA isolation, primer design and PCR-amplification.

3.4.1 Liver DNA Isolation and Quantification

To assess editing at *Ldlr*, other targeted loci, or predicted off-target sites, we isolate genomic DNA from the liver (*see* **Note 27**).

1. Homogenize a small piece of liver (~20 mg) in 180 μL of lysis buffer (provided with the DNA isolation kit) with 1 steel bead in the bead mill homogenizer (4 cycles at 2500 rpm, 30 s each).
2. Incubate the homogenate with proteinase K (provided with the DNA isolation kit) at 37 °C for 30 min.
3. Isolate and purify DNA using silica-based membranes following the manufacturer's instructions.
4. Quantify DNA using any UV-Vis spectrophotometer that reads absorbance at 260 nm (*see* **Note 28**).
5. DNA can be stored at −20 °C until further use.

3.4.2 Primers Design for Sanger Sequencing-Based Methods

The targeted locus needs to be PCR-amplified and then sequenced by Sanger sequencing.

For this, design PCR-primers with the help of primer design software tools, such as Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) or Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (*see* **Note 29**).

1. Select the targeted locus containing the gRNA cut-site flanked by around 400 bp of genomic DNA on each side.
2. Design the forward and reverse primer to anneal at least 200 bp from the guide RNA cut-site to allow for optimal sequencing quality across the edit.
3. Design the primers to have an amplicon ranging between 500 and 800 bp.

4. As general criteria for designing primers, select primers with a T_m of ~60 °C (range: 58–62 °C), length around 18–22 bp and about 50% of GC content.
5. Check the specificity of each primer pair by using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi).

3.4.3 TIDE and ICE Analyses

1. Amplify the targeted locus in control and edited DNA samples (*see* **Note 30**). Perform a PCR on 100 ng of genomic DNA in a total volume of 50 μL using 10 μL of 5× reaction buffer (for chosen Taq DNA polymerase), 1 μL of 10 mM dNTPs, 0.5 μM of forward primer, 0.5 μM of reverse primer, 0.5 μL of 2 U/μL Taq DNA polymerase, 100 ng of DNA, and molecular grade water to a final volume of 50 μL (*see* **Notes 31** and **32**).
2. Set the following program in a thermocycler: Initial denaturation for 1 cycle at 98 °C for 3 min; 35–40 cycles of denaturation (30 s at 98 °C), annealing at temperature dependent on the Tm of the primers (e.g., 60–62 °C) for 1 min and extension (1 min for amplicon <1 kb at 72 °C); final extension for 5 min at 72 °C and hold at 4 °C till required.
3. Perform agarose gel electrophoresis as described in **steps 3** and **4** of Subheading 3.2.2, loading the whole volume of each PCR reaction supplemented with 1× DNA loading buffer. Visualize PCR products by an UV gel imager (*see* **Note 33**).
4. Cut out the band of interest and purify the PCR product as described in **steps 5** and **6** of Subheading 3.2.2.
5. Measure DNA concentration using a UV-Vis spectrophotometer and prepare samples for Sanger sequencing as required by the sequencing company.
6. Once the sequencing chromatograms have been obtained, proceed using TIDE (http://shinyapps.datacurators.nl/tide/) or ICE (https://ice.synthego.com/#/) tool (*see* **Note 34**). Both tools require to insert the gRNA sequence without PAM and upload the control and edited chromatograms as .ab1 or .scf files. The analysis provides the spectrum and frequency of indels at the cut site. The R^2 value should be greater than 0.9 to ensure accurate estimation of CRISPR activity. An example of output from TIDE is shown in Fig. 6 (*see* **Note 35**).

3.4.4 Targeted Deep Sequencing

While Sanger sequencing-based methods successfully indicate on-target editing, deep sequencing is a highly sensitive and accurate method for qualitative and quantitative analysis of indels formation at both the targeted locus and at predicted off-target sites (*see* **Note 36**).For the Illumina system, this is how we have prepared DNA for deep sequencing editing analyses in the past.

1. Design primers for amplifying the targeted regions using primer design software tools following the general criteria described in **step 4** of Subheading 3.4.2. It is required that the gRNA target site is included at the center of the amplicon, whose length should be between 200 and 300 bp. Add the following adapter sequences to the 5′ of each primer pair.

   Forward primer adapter: 5′-TCTACAGTCCGACGATCA-3′.

   Reverse primer adapter: 5′-GACGTGTGCTCTTCCGATC-3′.

   Check each primer pair for specificity with Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) (*see* **Note 37**).

2. PCR-amplify the targeted locus and predicted off-target sites using a high-fidelity Taq Polymerase (*see* **Note 38**). For a single reaction, mix 100 ng of genomic DNA, 10 μL of 5× reaction buffer (for chosen DNA polymerase), 1.25 μL of 10 mM dNTPs, 0.25 μM of forward primer, 0.25 μM of reverse primer, 0.5 μL of 5 U/μL Herculase II Fusion DNA polymerase, and molecular grade water to a final volume of 50 μL total (*see* **Note 32**).

3. Set the following program in a thermocycler: Initial denaturation for 1 cycle at 95 °C for 3 min; 30–35 cycles of denaturation (30 s at 95 °C), annealing at temperature dependent on the Tm of the primers (e.g., 60–62 °C) for 30 s and extension (30 s at 72 °C); final extension for 3 min at 72 °C and hold at 4 °C till required.

4. Perform agarose gel electrophoresis as described in **steps 3** and **4** of Subheading 3.2.2, loading 5 μL of each PCR reaction supplemented with 1× DNA loading buffer. Visualize DNA using an UV gel imager (*see* **Notes 33** and **39**).

5. In case of successful PCR, use each PCR reaction as template for the barcoding PCR (*see* **Note 40**). For each experimental group, prepare a reaction mix using the specific P5 forward primer by mixing 10 μL of 5× reaction buffer (for chosen DNA polymerase), 1.25 μL of 10 mM dNTPs, 2.5 μL of 5 μM P5 forward primer (Cf: 0.25 μM), 0.5 μL of 5 U/μL Herculase II Fusion DNA polymerase and molecular grade water to a final volume of 45.5 μL total. For preparing multiple reactions, make a master mix by scaling up all the reagent volumes. Then, aliquot the master mix in a 96 well plate.

6. Add 2.5 μL of specific 5 μM P7 reverse primer to the appropriate well (Cf: 0.25 μM).

7. Add 2 μL of the appropriate PCR product (on- or off-target PCRs generated in **steps 2–4**). Include a reaction with no DNA as negative control for any reagent contamination.

8. Set a touch down PCR protocol in a thermocycler as follows: Initial denaturation for 1 cycle at 95 °C for 3 min; 7 cycles (delta −1 °C for annealing temperature at each cycle) of denaturation (30 s at 95 °C), annealing (30 s starting at 67 °C) and extension (30 s at 72 °C); then, 29 cycles of denaturation (30 s at 95 °C), annealing (30 s at 60 °C) and extension (30 s at 72 °C); final extension for 3 min at 72 °C and hold at 4 °C till required.
9. Perform agarose gel electrophoresis as described in **steps 3** and **4** of Subheading 3.2.2, loading 5 μL of each PCR reaction supplemented with 1× DNA loading buffer. Visualize DNA using an UV gel imager (*see* **Note 33**).
10. Purify each PCR reaction using Agencourt AmPure XP magnetic beads following the manufacturer's instructions.
11. Use Qubit fluorimeter for measuring the concentration of each purified DNA product (*see* **Note 41**).
12. Generate equimolar pools of the purified targets based on the requirements for the sequencing reaction (*see* **Note 42**).
13. Submit the equimolar, pooled barcoded DNA library to appropriate facility or company for sequencing (*see* **Note 43**).

3.4.5 Integration PCR

This method consists of PCR amplification of the targeted locus using a first primer complementary to a genomic region upstream or downstream of the targeted site and a second primer annealing to the ITR or other viral sequences (*see* **Note 44**). An example of integration PCRs at the *Ldlr* locus is shown in Fig. 6.

1. Design primers for amplifying the targeted locus using primer design software tools following the general criteria described in **step 4** of Subheading 3.4.2 (*see* **Note 45**).
2. Perform the PCR using 100 ng of genomic DNA using Apex 2× Taq Red Master Mix following the manufacturer's protocol. For a PCR reaction use 0.4 μM of each primer, 12.5 μL of 2× Taq Red Master Mix, 100 ng of genomic DNA, and molecular grade water to a final volume of 25 μL (*see* **Notes 32** and **46**).
3. Set the following program in a thermocycler: Initial denaturation for 1 cycle at 95 °C for 5 min; 35 cycles of denaturation (30 s at 95 °C), annealing at temperature dependent on the Tm of the primers (e.g., 60–62 °C) for 1 min and extension at 72 °C (1 min for <1 kb amplicon); final extension for 5 min at 72 °C; and hold at 4 °C till required.
4. Perform agarose gel electrophoresis as described in **steps 3** and **4** of Subheading 3.2.2, loading half volume of each PCR reaction supplemented with 1× DNA loading buffer. Visualize PCR products using an UV gel imager (*see* **Note 33**).

3.4.6 AAV Genome PCR

1. For an AAV genome PCR reaction (*see* **Note 47**) use 0.4 μM of primer WRL_0064 (5′ CCGTCGTGAAGAGAAGCTTCATC-3′), 0.4 μM of primer WRL_0070 (5′- CCACCTCATAGTT GAAGGGGTTG-3′), 12.5 μL of 2× Taq Red Master Mix, 100 ng of genomic DNA and molecular grade water to a final volume of 25 μL (*see* **Notes 32** and **46**).
2. Set the following program in a thermocycler: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation (30 s at 95 °C), annealing (1 min at 62 °C) and extension (1 min at 72 °C); final extension at 72 °C for 5 min; and hold at 4 °C till required.
3. Perform agarose gel electrophoresis as described in **steps 3** and **4** of Subheading 3.2.2, loading half volume of each PCR reaction supplemented with 1× DNA loading buffer. Visualize PCR products using an UV gel imager (*see* **Note 33**).

3.5 Evaluation of Gene Knockdown by Western Blot

The effect of genome editing on the expression level of *Ldlr* or other targeted genes can be evaluated by western blot on liver lysates (or plasma if the protein is secreted) (*see* **Note 48**). An example of Ldlr western blot is shown in Fig. 6.

1. Thaw liver and plasma samples on ice.
2. For isolating liver proteins, add 10 volumes of RIPA buffer (supplemented with protease inhibitor cocktail) relative to liver weight (i.e., 500 μL for a 50 mg piece).
3. Add 1 steel bead to each tube.
4. Homogenize in a bead mill homogenizer (4 cycles at 2500 rpm, 30 s each).
5. Place lysed livers on ice for 30 min, and then vortex.
6. Centrifuge at 13,800 × *g* for 20 min at 4 °C.
7. Collect supernatant to a new 1.5 mL tube.
8. Dilute each sample 1:10 with Milli-Q double distilled water and use 2 μL of dilution in a final volume of 100 μL for measuring protein concentration (*see* **Note 49**). Once the concentration is determined, proceed to SDS-PAGE or store at −20 °C until further use.
9. Prepare liver lysates or plasma samples for SDS-PAGE in a final volume of 15 μL of loading mix per lane using 50–80 μg of liver lysate (or 1 μL of plasma sample), 3.75 μL of 4× LDS Sample Buffer, 0.75 μL of Beta-Mercaptoethanol (Cf: 5%) and water to 15 μL (*see* **Note 50**).
10. Heat samples to 95 °C for 10 min using a heat block or thermocycler, vortex, centrifuge at 2000 × *g* for 1 min to collect condensate and cool samples to room temperature before loading.

11. For most western blot applications, use 4–12% Bis-Tris gels with the appropriate number of lanes.
12. Dilute the 20× MES running buffer to 1× with Milli-Q double distilled water (*see* **Note 51**).
13. Assemble up to two gels in a mini-protein gel electrophoresis system.
14. Fill inner chamber with 1× running buffer and check for leaks.
15. Fill outer chamber about 80% with 1× running buffer.
16. Load the first lane with 3 μL of protein marker.
17. Load the following lanes with 15 μL of each sample.
18. Perform electrophoresis at 150 V (if desired gels can be run slower).
19. Shut off power supply when the dye front reaches about 1 cm from the bottom of the gel and proceed to transfer using the wet transfer method (*see* **Note 52**).
20. Dilute 20× transfer buffer to 1× with Milli-Q double distilled water and add methanol to 20% final concentration.
21. Soak 5 sponges and 4 filter papers per blot module in 1× transfer buffer.
22. Activate 0.45 μm PDVF membrane with 100% methanol for few minutes and then soak the membrane in 1× transfer buffer. Use gel tweezers for handling the membranes at each step.
23. Assemble the blot sandwich as following: FRONT (flat plate of the blot module); Sponge; Sponge; Filter paper; PDVF membrane 2; Gel 2; Filter paper; Sponge; Filter paper; PDVF Membrane 1; Gel 1; Filter paper; Sponge; Sponge; BACK (Deep plate of the blot module).
24. Avoid bubbles by rolling the sandwich with a serological pipette after the addition of each layer.
25. Put the sandwich in the blot module.
26. Close the blot module and place it in the mini-protein gel electrophoresis system, then secure with clamp.
27. Add 1× transfer buffer to the inner chamber.
28. Tap the gel box several times for removing any air bubbles.
29. Add ice water to the outer chamber.
30. Transfer at 35 V for 90 min.
31. Remove the membranes from transfer sandwich.
32. Block the membranes with blocking buffer by gently rocking for 2 h on a shaker (40 rpm at room temperature).

33. Dilute primary antibodies in antibody buffer following the dilution recommended by the manufacturer (*see* **Note 53**). Incubate on a shaker overnight (40 rpm at 4 °C).
34. The day after, wash membranes three times with PBS-T on a shaker, 10 min each (40 rpm at room temperature).
35. Dilute secondary antibodies, goat anti-rabbit 700 nm and goat anti-mouse 800 nm, 1:15,000 with antibody buffer (*see* **Note 54**). Incubate the membranes with the secondary antibodies gently rocking for 1 h at room temperature (40 rpm).
36. Wash membranes three times with PBS-T on a shaker, 10 min each (40 rpm at room temperature).
37. Image the membranes with Odyssey infrared imaging system using the following settings: Resolution: 169 μm; Quality: Low; Intensity for 700 nm channel: 5.0; Intensity for 800 nm channel: 5.0.

4 Notes

1. Resuspend oligonucleotides/primers in molecular grade water at a stock concentration of 100 μM. Store at −20 °C.
2. Bring the solution to boil by using a microwave oven for dissolving the agarose.
3. Add 3–4 μL per 150 mL of agarose in 1× TAE buffer, only after dissolving agarose. Handle ethidium bromide with caution because it is a mutagen.
4. Use a protective shield while using the UV transilluminator because UV radiation is harmful to both skin and eyes.
5. We use SnapGene (from Insightful Science; available at https://www.snapgene.com). SnapGene Viewer can also be used, but functionality is limited. However, other annotation software can be used.
6. Autoclave and store at room temperature for a short period.
7. Autoclave, cool down to 50 °C and add the 50 mg/mL Ampicillin stock to a final concentration of 0.1 mg/mL. Pour in 10 cm petri dishes and let it solidify. Store at 4 °C.
8. Before proceeding with gRNA design, a target file should be generated for both the genomic sequence and for the mRNA sequences for the target. The files should be annotated to highlight exons included in all mRNA isoforms, as well as essential domains or other regions of interest that would be detrimental to protein function if disrupted. gRNAs will be annotated in the files as they are generated. Later, primers for detection of editing can be added to these files. We use

SnapGene to annotate genomic, RNA and plasmid files, but other programs can be used.

9. We recommend designing gRNAs that target the first coding exons of the gene of interest when possible. Editing early exons is expected to increase the likelihood of frameshift mutations and premature stop codons resulting in aberrant proteins. If a target gene expresses multiple transcripts, we recommend targeting the exons conserved among the transcripts.
10. Until recently, most gRNA design tools did not include SaCas9, which has a 5′-NNGRRT-3′ PAM. Because of this, gRNAs were designed manually through visual inspection of the target genes. While tools that will be discussed later now exist (*see* Subheading 3.1.4), some gRNAs still need to be designed by in this way for complicated targets.
11. A limitation of the CRISPR/Cas9 system is the potential nuclease activity at genomic sites other than the targeted locus that show similarity with the gRNA sequence and are in close proximity to a PAM sequence (referred to as off-target sites). Off-target prediction allows selection of gRNAs that have the lowest probability for undesired edits at off-target sites. For predicting off-targets, we use the COSMID web-based tool that searches genomes for potential off-target sites (http://crispr.bme.gatech.edu) [25]. In addition, using COSMID website, both the gRNA target (on-target) and off-targets will be scored. The closer to zero this score is, the more likely there is to be cutting at the off-target site.
12. However, we generally download sequences for the off-targets for later primer design based on the chromosomal position (*see* Subheading 3.4.2).
13. Like COSMID, CRISPOR will predict off-targets though it uses different parameters and prediction algorithms, so the off-targets it predicts are slightly different. For details on the tool refer to Concordet et al. [26]. CRISPOR also selects potential gRNA sequences from a sequence input, so candidate gRNAs do not need to be designed by eye. CRISPOR ranks potential gRNAs, scores them based on specificity and efficiency and provides additional parameters for assessing gRNAs for the desired application. Using this tool is easier and faster than designing gRNAs by hand.
14. The oligonucleotide sequences for the *Ldlr* gRNA as follows: top: 5′-CACCGGGCAGGCGCAGGTGAATTTGG-3′ and bottom 5′-AAACCCAAATTCACCTGCGCCTGCCC-3′, with the gRNA sequence underlined.
15. Oligonucleotides are now annealed at a final concentration of 1.3 ng/μL (0.083 μM). Annealed oligonucleotides are ready

for direct ligation into the predigested acceptor vector or can be stored at −20 °C until further use.

16. Annealed gRNAs with BbsI overhangs are then cloned into BbsI-linearized 1313_pAAV-U6-SA-BbsI-MluI-gRNA-HLP-SACas9-HA-OLLAS-spA acceptor vector (Addgene 109314) [22, 23]. SnapGene or other vector mapping software can be used to design and map the vector, plan cloning, and predict enzymatic digestions. The 1313 acceptor vector contains the following components (Fig. 4).
    (a) Two complementary ITR sequences located at the 5′ and 3′ terminal ends of AAV genome, which are required for intermolecular recombination and circularization.
    (b) The U6 promoter, which drives the expression of the gRNA.
    (c) The gRNA cloning site containing BbsI and MluI restriction sites (Fig. 3).
    (d) A gRNA scaffold required for the Cas9:gRNA complex formation, composed of the crRNA direct repeat portion, a synthetic tetraloop and a Cas9 nuclease-recruiting sequence (TracrRNA).
    (e) A multiple cloning site, containing XbaI and HindIII restriction sites.
    (f) The hepatocyte specific hybrid liver promoter (HLP) [30], which enables a liver-restricted expression of Cas9, resulting in specific editing in the liver without any extrahepatic activity [22].
    (g) The SaCas9 coding sequence fused to the following elements: (a) the nuclear localization signal (NLS) from SV40 large T antigen in the 5′ end; (b) the bipartite NLS from nucleoplasmin in the 3′ end; (c) the human influenza hemagglutinin (HA) epitope tag in the 3′ end; (d) the *Escherichia coli* OmpF Linker and mouse Langerin fusion Sequence (OLLAS) epitope tag fused to the 3′ end upstream of the stop codon.
    (h) A synthetic polyadenylation signal (spA).
    (i) The pEMBL8 backbone including an ampicillin resistance gene (AmpR).
17. The dephosphorylation step is not required because BbsI generates sites that do not self-ligate as they are not complementary.
18. ITRs cannot be easily sequenced due to the repetitive nature and the hairpin structure. Hence, restriction digestions constitute the preferred diagnosis tool for checking the ITR integrity.

19. For determining the titer of AAV-CRISPR we recommend using primers WRL_0064 (5′-CCGTCGTGAAGAGAAGCTT CATC -3′) and WRL_0070 (5′- CCACCTCATAGTT GAAGGGGTTG -3′), which target the SaCas9 coding sequence generating a 324 bp product.
20. For *in vivo* studies, we use *C57BL/6J* mice, but the AAV-CRISPR tool can be applied to other mouse strains and genetic backgrounds. Somatic editing in mice on genetic backgrounds other than *C57BL/6J* may require a preliminary experiment for optimizing the dose of AAV-CRISPR. Older mice might also need a higher dose and different mouse models of liver disorders (e.g., liver steatosis or fibrosis) showing liver malfunction or toxicity may require dose optimization. We recommend titrating AAV-CRISPR from 5×10^{11} to 1×10^{12} genome copies per mouse for identifying the most efficient dose before starting the experiment. For targeting the liver, AAV8 vectors can be injected either intraperitoneally or intravenously. We generally practice intraperitoneal injection, which is highly effective in targeting the liver and is also easily executed with minimal training. When looking for editing a candidate gene, we find that an "n" of 6 to 8 age- and sex-matched mice per group is a good starting point. For atherosclerosis studies, we recommend enrolling 15 to 20 mice per group. Control mice will be injected with the same volume of sterile PBS or saline solution. Alternatively, control mice can be injected with an AAV-CRISPR with no gRNA. AAV injection can be performed only once because of the elicited immune response to the AAV capsid proteins and Cas9 [24, 31, 32].
21. In our experience, editing is more efficient in male *C57BL/6J* mice injected at an age of at least 6 to 8 week old. A dose of 5×10^{11} genome copies of *Ldlr* AAV-CRISPR enables efficient and permanent editing of the targeted gene [21]. We observed a sexual dimorphism in female mice injected with the same dose of *Ldlr* AAV-CRISPR, resulting in less efficient Ldlr knockdown and atherosclerosis development as compared to male mice [21]. In case of enrollment of female mice in a study, test 5×10^{11} and 1×10^{12} genome copies per mouse to ensure efficient editing of the target locus. The administered volume (300 μL) is much lower than 1.5 mL, which is the maximum volume allowed for a safe intraperitoneal injection to a ~20 g mouse.
22. Perform all the animal experiments in accordance with IACUC and institutional guidelines.
23. Fasting is important for clearance of food-derived lipids.

24. We collect blood from the retroorbital plexus, but other blood collection techniques can be applied. Seek training and ensure protocol approval before attempting blood collection. Mice have to be anesthetized during the procedure and the retroorbital puncture must be performed by skilled personnel for limiting the risk of injury to the eye and surrounding structures. In agreement with IACUC regulations, approximately 10% of the total blood volume can be safely removed every 2 to 4 weeks (around 110–140 μL of blood for a 20 g mouse).
25. For measuring plasma total cholesterol, we use a commercial kit (WAKO cholesterol E reagent) following the manufacturer protocol and 5 μL of 1:10 dilution of plasma samples. For measuring plasma triglycerides, we use a commercial kit (Infinity Triglycerides Reagent and Control Serum I) following the manufacturer protocol using 10 μL of plasma.
26. To quantify atherosclerosis development, the heart and aorta are collected for immunohistochemistry analysis of the aortic sinus and en face Oil Red O staining, respectively. These techniques enable to quantify the lipid burden and characterize the morphology, cellularity, and pathophysiology of the atherosclerosis lesions. For heart and aorta dissection and analysis protocols we refer to other relevant chapters of this book and to standard protocols already published [1, 33]. Wash and sterilize surgical instruments with a glass bead sterilizer after each dissection.
27. We use the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol, though any other commercial kit can be used. Phenol–chloroform extraction can also be used.
28. For downstream analyses that require a more accurate and sensitive measurement, such as deep sequencing, we recommend using a fluorometric quantification (e.g., Qubit fluorometer). This method specifically quantifies DNA molecules via intercalating dyes resulting in higher specificity and sensitivity than absorbance-based methods, which do not discriminate DNA from RNA and nucleotide contaminants.
29. We recommend designing two to three primer pairs and test them for efficient amplification of the expected PCR product and absence of any nonspecific amplification. We use the same primers for sequencing the PCR-amplicon by Sanger sequencing. Otherwise, nested primers can be designed using the same primer design software tools.
30. We recommend using a high-fidelity Taq DNA polymerase for avoiding the generation of any random mutations that can bias the editing analysis. We use the Phusion High-Fidelity DNA Polymerase (Thermo-Scientific), following the manufacturer's protocol.

31. For amplifying the *Ldlr* locus, we recommend using primers *Ldlr* Ex14For1 (5′- CCGGAGGACATTGTCCTGTTC -3′) and *Ldlr* Ex14Rev1 (5′- CTCAGGGAATCTGCTTCAG CAAC-3′), which generate a PCR product of 638 bp.
32. For preparing multiple PCRs, make a master mix by scaling up all the reagent volumes except the template. Then, aliquot the master mix in the PCR tubes and finally add genomic DNA to each tube. Include a reaction with no DNA as negative control for any reagent contamination.
33. Discard tubes and repeat PCRs using new reagents if any amplification is detected in the negative control. Optimize the primer design or PCR protocol in case of no or multiple PCR products.
34. TIDE and ICE are free and easy-to-use web-based software tools that offer fast analysis of CRISPR editing data. Both methods require the PCR amplification of the targeted locus followed by Sanger sequencing. Moreover, both methods require an unedited sequence of the targeted site as a control sequence. TIDE aligns the control to the edited sequence, which shows a mixture of signals starting at the cut site and consisting of the unmodified DNA plus the sequences that are modified by insertions and deletions of nucleotides. Then, the TIDE software decomposes the composite sequence trace into its individual traces and estimates the relative abundance of every possible indel. Some parameters like the alignment and decomposition windows and the detected indel size range can be manually adjusted and optimized. Details on the algorithm are described in Brinkman et al. [27]. ICE has been generated by Synthego for implementing TIDE. Similarly to TIDE, ICE aligns the control to the edited sequence and quantifies the traces with indels. Distinct from TIDE, ICE enables multiple editing analyses at once and there is no requirement for parameter adjustments [28]. ICE and TIDE were designed for SpCas9 indel frequency calculation and do not allow specification of the PAM sequence. Because of this, when using ICE for SaCas9 gRNAs, there is often a notation that there is no PAM detected, which can be ignored. The editing results are still accurate. In our experience, TIDE and ICE result in comparable editing efficiency rates. The reliability of these methods depends on the purity of the PCR products and the quality of the sequence reads. Highly repetitive sequences around the target site can hamper the decomposition process. TIDE can detect indels with a sensitivity of ~1–2% across various targets [34].
35. In our experience, an editing frequency of at least 30% at the *Ldlr* locus is sufficient for reaching a ~90–95% knockdown

level [21]. The editing efficiency obtained by ICE, TIDE and deep sequencing underestimates actual editing. This is because analysis is performed using total liver DNA, which includes DNA derived from nonparenchymal cells (~15% of liver cells), which are not transduced by AAV. In addition, large insertions and deletions are not captured by sequencing-based methods, which involve small PCR amplicons. Finally, AAV-genome insertion at the DSB site is a recurring event and contributes to gene editing. For detecting AAV genome insertion at the DSB site, we perform an integration PCR as described in Subheading 3.4.5 in this chapter.

36. Deep sequencing can detect indels with a sensitivity of ~0.1% [23], making it ideal for assessing off-target editing events. The targeted locus and CRISPOR- or COSMID- predicted off-targets are amplified through two rounds of PCR, which add adaptors and barcoding sequences for sequencing by the Illumina system. Despite the high sensitivity of the method, the major limitations for this technique are the availability of Illumina system as well as the cost of the service. However, deep sequencing cores and companies can offer this service. Preparation of libraries of DNA samples may vary by facility, so confirm which barcoding paradigm they require for sequencing before proceeding.
37. If these barcode primers are used, for amplifying the *Ldlr* locus, we recommend using primers KEJ_0462 (5′-TCTACAGTCC GACGATCACTTGACTCTGCCCGTCATCA -3′) and KEJ_0463 (5′- GACGTGTGCTCTTCCGATCCTGGA CATCTGGTCAGCCTC-3′), which generates a PCR product of 318 bp.
38. We use the Herculase II Fusion DNA Polymerase (Agilent Technologies). However, other high-fidelity Taq Polymerases can be used.
39. Gel extraction can be performed for purifying the expected PCR product when multiple products of different size are detected.
40. The aim of this PCR reaction is to add barcodes to each PCR amplicon by using a unique combination of P5 forward and P7 reverse primers pair. A unique P5 forward primer (P5_1 or P5_2, etc.) is used for barcoding the specific experimental group. A unique P7 primer (P7_1 or P7_2, etc.) identifies a specific mouse in the experimental group. For instance, P5_1 and P5_2 are respectively used for barcoding “control” and “knockout” group. Then, each of the 3 “control” mice are barcoded combining P5_1 respectively with P7_1, P7_2 and P7_3. We use Herculase II Fusion DNA Polymerase (Agilent

Technologies) and 2 μL of the on- or off-target PCR reactions as template in a final 50 μL reaction.

41. Ideally the final concentration should be greater than 20 ng/μL.
42. For a previous sequencing reaction, we diluted the products to 40 nM and pooled equal volumes of all barcoded products to a final volume of greater than 200 μL.
43. Paired-end reads can be analyzed for genome editing using many different methods using Python or R, but extensive knowledge of computational methods is not required. Resources like CRISPResso2 (https://crispresso.pinellolab.partners.org/submission) allow direct submission of Fastq files, amplicon and gRNA information via a web form to determine editing efficiency [29]. CRISPResso2 is also open-source and can be run in the command line with no limitations (https://github.com/pinellolab/CRISPResso2).

    If needed CRISPResso2 can also filter low quality reads, trim adapters, and align reads. More information can be found here: https://crispresso.pinellolab.partners.org/help.

    For the web version of CRISPResso2, assign the center of the quantification window to −3, which is the most common SaCas9 cut site relative to the PAM. The Quantification Window Size determines how large the window around the cut site is for calling mutated bases. By default this is set to 1, but may need to be adjusted for different gRNA sequences.

    In the liver, because insertions and deletions larger than 2–3 bp are missed by standard editing pipelines, editing events greater than 25–30% suggest a large proportion of hepatocytes have been successfully edited.
44. ICE, TIDE, and deep sequencing enable detection of small editing events at the cut site. However, we and others have observed the NHEJ-mediated integration of partial or whole AAV genomes in the DSB site, which cannot be resolved with the sequencing-based methods due to their size [21–23, 35, 36]. Integration PCR is a simple and rapid method for detecting these large insertion events. We usually use this method as quick and qualitative assessment of genome editing at the targeted locus.
45. For detecting AAV integration at the DBS in exon 14 of *Ldlr*, we recommend using the following primers: *Ldlr* Ex14For1 (5′-CCGGAGGACATTGTCCTGTTC-3′) and U6RevSeq (5′-CTTTCAAGTTACGGTAAGCATATGATAG-3′) for detecting AAV forward integration (expected product size: 757 bp) or *Ldlr* Ex14For1 (5′-CCGGAGGACATTGTCCTGTTC-3′) and WRL_0067 (5′-CAGACATTCTGGGCAACCTGTA-3′) for detecting AAV backward integration (expected

product size: 736 bp). An example of integration PCRs at the *Ldlr* locus is shown in Fig. 6.

MDG_0151 (5′- GATAAGTAGCATGGCGGGTT -3′) primer anneals to the ITR sequence and can be used in pair with any other compatible primer for detecting integration of ITRs at the cut site, regardless of the viral genome orientation.

46. We use Apex 2X Taq Red Master Mix. However, other Taq Polymerases can be used. High-fidelity Taq Polymerases are not required for this PCR. It is recommended to perform an additional PCR in parallel using primer pairs already validated, as control of the DNA quality and PCR protocol.
47. When testing new gRNAs, it may be possible to observe low or no editing at the targeted locus. We recommend assessing in parallel, the expression of the targeted gene at protein level. Low levels of detectable editing levels due to high levels of AAV integration may still result in efficient protein knockdown. However, results showing low levels of both editing and knockdown may be explained by either poor design/specificity of the gRNA or a bad injection, which results in reduced or no delivery of the CRISPR/Cas9 tool to the liver. An AAV genome PCR on liver DNA provides rapid and qualitative information on the presence of AAVs in the liver, and therefore is very useful for troubleshooting. For detecting AAV genomes, we recommend using WRL_0064 (5′-CCGTCGTGAA GAGAAGCTTCATC -3′) and WRL_0070 (5′-CCACCTCATAGTTGAAGGGGTTG-3′) primers, which are designed to amplify the SaCas9 coding sequence generating a 324 bp PCR product. An example of AAV genome PCR is shown in Fig. 6.
48. Liver samples are mechanically homogenized producing liver lysates. Then, liver proteins (50–80 μg) are separated by SDS-PAGE, transferred to a PDVF membrane and probed for Ldlr (or the targeted protein), along with a constitutively expressed protein used as loading control. In the case of plasma, 1 μL of plasma is diluted in loading mix. As loading control for plasma samples, blots can be stained with Ponceau Red or probed for a constitutive expressed plasma protein (e.g., albumin, alpha-1 antitrypsin) [22]. We use an infrared-based detection system (Odyssey, Licor) that enables the simultaneous detection of two differently labeled proteins on a single blot. Other labeling and detection systems can be used. In the absence of working antibodies, qPCR analysis can be performed to investigate the knockdown of the targeted gene at RNA level [22].
49. We use a BCA assay following the manufacturer's protocol. Any other commercial kit can be used.

50. For preparing multiple loadings, make a master mix by scaling up LDS Sample Buffer and beta-mercaptoethanol. Then, aliquot the mix in 1.5 mL tubes and finally add the liver lysate or plasma dilution to each tube.
51. One mini-protein gel electrophoresis system requires about 800 mL of 1× running buffer.
52. Alternative transfer methods can be used.
53. For probing Ldlr, we recommend ab52818 (Abcam) at a 1:1000–1:2500 dilution.
54. Other secondary antibodies and labeling and detection systems can be used.
55. The *Ldlr* AAV-CRISPR described in Jarrett et al. [21] (1375_pAAV8-U6-SA-WTmLdlrEx14-gRNA2-N22-CB-SACas9-HA-OLLAS-spA, Addgene 109311) has been generated using 1255_ pAAV-U6-SA-BbsI-MluI-gRNA-CB-SACas9-HA-OLLAS-spA (Addgene 109320) as acceptor vector for the *Ldlr* gRNA. We recommend using 1313_pAAV-U6-SA-BbsI-MluI-gRNA-HLP-SACas9-HA-OLLAS-spA (Addgene 109314) as acceptor vector for the *Ldlr* gRNA, which implements 1255 vector by replacing the constitutive chicken β-actin (CB) promoter with the hepatocyte specific HLP promoter for driving the expression of SaCas9 (Fig. 3). We showed that the change of promoter does not affect *Ldlr* editing and disruption [24], as well as atherosclerosis development as documented in Fig. 7.

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