Acute gene inactivation in the adult mouse liver using the CRISPR-Cas9 technology

Genetic manipulation in mice allows the discovery of gene function and biological mechanisms in vivo. The widely used Cre/LoxP system usually takes months to years especially when starting with the production of floxed alleles of new a gene of interest (GOI). Here, we describe a protocol using the CRISPR-Cas9 system to acutely inactivate the GOI in adult mice. This protocol enables hepatocyte-specific gene editing within 4 weeks in adult mice and avoids compensatory effects of traditional gene inactivation initiated during various developmental stages.

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Highlights
Acute inactivation of a gene of interest in adult mouse using the CRISPR-Cas9 system

Description of sgRNA design, cloning, delivery, and validation

Generation of hepatocyte-specific gene-edited mice within 4 weeks
Acute gene inactivation in the adult mouse liver using the CRISPR-Cas9 technology

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SUMMARY

Genetic manipulation in mice allows the discovery of gene function and biological mechanisms in vivo. The widely used Cre/LoxP system usually takes months to years especially when starting with the production of floxed alleles of a new gene of interest (GOI). Here, we describe a protocol using the CRISPR-Cas9 system to acutely inactivate the GOI in adult mice. This protocol enables hepatocyte-specific gene editing within 4 weeks in adult mice and avoids compensatory effects of traditional gene inactivation initiated during various developmental stages.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2020).

BEFORE YOU BEGIN

To achieve gene inactivation in hepatocytes of adult mice, we employed knockin (KI) mice bearing a “silent” spCas9 allele, proceeded by a floxed stop codon (Figure 1A) (Platt et al., 2014). Expression of spCas9 can be turned on in a tissue-specific manner with Cre recombinases driven by tissue-selective promoters. In the case of hepatocytes, we used an AAV8 vector containing Cre driven by the liver specific Thyroid Hormone-binding Globulin (TBG) promoter and single guide RNA (sgRNA) (Figure 1B).

The spCas9 KI mice can be obtained from Jackson Lab. We recommend B6N.129(B6J)-Gt(Rosa26)Sor tm1(CAG-cas9*,-EGFP)Fezh/J (RRID:IMSR_JAX:026556) and B6J.129(B6N)-Gt(Rosa26)Sor tm1(CAG-cas9*,-EGFP)Fezh/J (RRID:IMSR_JAX:026175) on the C57BL/6N or C57BL/6J background, respectively. The mice bear spCas9 expressing cassette in the Rosa26 locus, where spCas9 is linked with EGFP by a self-cleaving peptide P2A. Upon treatment of Cre recombinases, the LoxP-flanked stop codon is removed and spCas9 expression can be turned on (Figure 1A). To achieve maximal expression of spCas9 for in vivo gene editing, homozygous alleles are recommended.

The AAV shuttle vector containing elements for expressing Cre and sgRNA in mouse hepatocytes should be obtained before the experiments. Here we use pX602 (Addgene, 61593) as the backbone. The original saCas9 cDNA is replaced with the Cre cDNA using the AgeI-EcoRI restriction sites, and the U6-BxpQI-sgRNA scaffold of spCas9 is cloned into the KpnI-NotI site, thereby generating the new vector pX602-AAV-TBG::NLS-Cre-bGHpA;U6::BxpQI-sgRNA (hereinafter, referred to as pX602-Cre-sgRNA, Figure 1B).
Design guide sequences

© Timing: 1 h

1. To design guide RNA sequences targeting a murine GOI, we use the Benchling platform (https://benchling.com/) according to the tutorials (https://help.benchling.com/en/articles/670980-design-guide-rnas-grnas). There are several critical points that should be considered.
   a. Align all known transcripts carefully and choose common exons proximal to the 5’ end as targets.
   b. Sort the sgRNA sequences using on-target scores. Select sgRNA sequences with on-target scores above 60 and off-target scores above 30 (higher off-target score means lower predicted off-target effects). A list of potential off-targets can also be visualized by clicking on the off-target score of specific sgRNAs. One can further examine this list of off-target genes as an additional consideration of sgRNA design. In essence, sgRNAs should not be selected, if their potential off-targets might influence biological processes under investigation (Figures 2A and 2B).
   c. There are 34 coding single nucleotide polymorphisms (SNPs) and 2 coding indels that have been identified to distinguish C57BL/6N from C57BL/6J (Simon et al., 2013). Benchling supports sgRNA design on the genome of C57BL/6J. When using spCas9 KI mice on C57BL/6N background, targeting genome region encompassing above genetic variants should be avoided.
   d. For each gene, select at least 2 sgRNA sequences which should be delivered individually. We usually select 3 to 4 sgRNA sequences to increase the success rate and minimize potential impacts of off-target effects.

2. For cloning sgRNA sequences into the px602-Cre-sgRNA vector, order sgRNA oligos as standard oligos (Figures 3A and 3B). The forward oligos should have 5’-ACCG overhangs, while the reverse oligos have 5’-AAC and C-3’ overhangs, as illustrated in Figure 3B.

△ CRITICAL: The guide RNA sequence should be on the forward sequence.

Figure 2. Design sgRNA sequences by the Benchling platform
(A) Sorting sgRNA sequence targeting a coding region of mouse Surf4 by the on target scores.
(B) Potential off-target sequences of the selected gRNA in Figure 2A.
**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** |        |            |
| Stbl3 Competent Cell | TransGen Biotech | Cat# CD521-01 |
| AAV8 | In-house purified | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| BspQI | New England Biolabs (NEB) | Cat#R0712 |
| NEBuffer™ 3.1 buffer | NEB | Cat#B7203S |
| PNK | NEB | Cat#M0201 |
| T4 ligase | NEB | Cat#M0202 |
| 10× NEB T4 ligase buffer | NEB | Cat#B0202S |
| Ampicillin | Inalco | Cat#1758-9314 |
| Tryptone | Oxoid | Cat#Lp0042 |
| Yeast extract | Oxoid | Cat#Lp0021 |
| NaCl | Beihua | Cat#S0219 |
| KCl | Xilong | Cat#S21-1 |
| NaOH | Xilong | Cat#S0205 |
| MgCl₂ | AMRESCO | Cat#0288 |
| Tris-HCl | Sigma | Cat#V900483 |
| F188 | Sigma | Cat#P5556 |
| Optiprep Density Gradient Medium | Sigma | Cat#D1556 |
| Phenol red | Sigma | Cat#P0290 |
| Benzonase | Sigma | Cat#E1014 |
| PEI | Polysciences | Cat#23966 |
| DMEM | HyClone | Cat#SH30022.01B |
| PBS | HyClone | Cat#SH30256.01 |
| 10× PBS | CWBIO | Cat#CW0040S |
| Penicillin-Streptomycin Solution | Caisson | Cat#P0101 |
| Fetal bovine serum (FBS) | VISTECH | Cat#SE100-011 |
| DNsasel | Takara | Cat#2270A |
| 10× DNase buffer | Takara | Cat#SD4104 |

*(Continued on next page)*

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**Figure 3. sgRNA oligos for cloning into px602-Cre-sgRNA**

(A) Schematic representation of a guide-targeted sequence which is upstream of the PAM sequence.

(B) Guidelines for ordering sgRNA oligos.
Continued

| REAGENT or RESOURCE | SOURCE            | IDENTIFIER   |
|---------------------|-------------------|--------------|
| Proteinase K        | AMRESCO           | Cat#0706     |
| Glucose             | Beihua            | Cat#S1829    |
| Agarose             | Vetec             | Cat#V900500  |

Critical commercial assays

| REAGENT or RESOURCE | SOURCE            | IDENTIFIER   |
|---------------------|-------------------|--------------|
| OMEGA Gel Extraction Kit | Omega          | Cat#D2500-02 |
| OMEGA Plasmid Mini Kit | Omega          | Cat#D6943-02 |
| TIANGEN HighPure Maxi Plasmid Kit | TIANGEN      | Cat#DP116    |
| QIAGENaM Genomic DNA | TIANGEN           | Cat#DP304-03 |
| SuperReal PreMix Plus(SYBR Green) | TIANGEN      | Cat#FP205-03 |
| KOD-Plus-Neo kit    | Toyobo            | Cat#KOD-401  |

Experimental models: Organisms/strains

| Organism/strain                  | SOURCE                      | IDENTIFIER     |
|----------------------------------|-----------------------------|----------------|
| SpCas9 mice on C57BL/6N         | Jackson Laboratory          | JAX-026556, RRID:IMSR_JAX-026556 |
| SpCas9 mice on C57BL/6J         | Jackson Laboratory          | JAX-026175, RRID:IMSR_JAX-026175 |

Oligonucleotides

| Oligonucleotide | Description | SOURCE |
|-----------------|-------------|--------|
| TBG F: ATTCTGCCTGCTGAAGACACTCTCT | This paper | N/A |
| TBG R: CCCACAGCATTAACCCTGGGATG | This paper | N/A |

Recombinant DNA

| REAGENT or RESOURCE | SOURCE            | IDENTIFIER   |
|---------------------|-------------------|--------------|
| pRC2/8              | Addgene           | Cat#112864   |
| PHelp4              | Addgene           | Cat#112867   |
| pX602-Cre-sgRNA     | This paper        | N/A          |

Software and algorithms

| SOFTWARE or ALGORITHM | SOURCE |
|-----------------------|--------|
| Benchling             | Benchling [https://benchling.com/](https://benchling.com/) |
| ICE                   | Synthego [https://ice.synthego.com/#/](https://ice.synthego.com/#/) |

Other

| REAGENT or RESOURCE | SOURCE            | IDENTIFIER   |
|---------------------|-------------------|--------------|
| 33 mL Optiseal tube | Beckman           | Cat#361625   |
| Type 70 Ti Fixed-Angle Titanium Rotor | Beckman | Cat#337922 |

**MATERIALS AND EQUIPMENT**

**SOC medium**

| REAGENT     | Final concentration | Amount |
|-------------|----------------------|--------|
| Tryptone    | 20 g/L               | 20 g   |
| Yeast extract | 5 g/L               | 5 g    |
| NaCl        | 0.5 g/L              | 0.5 g  |
| 250 mM KCl  | 2.5 mM               | 10 mL  |
| 5 M NaOH    | N/A                  | Adjust pH to 7.0 |
| ddH2O       | N/A                  | Bring up to 1 L |
| Total       | N/A                  | 1 L    |

Combine above and Sterilize by autoclaving, then add the reagents below:

| REAGENT | Final concentration | Amount |
|---------|---------------------|--------|
| *2 M MgCl2 | 10 mM             | 5 mL   |
| *1 M glucose | 20 mM             | 20 mL  |

The SOC medium can be stored at 4°C for a couple of months.

**LB medium**

| REAGENT     | Final concentration | Amount |
|-------------|----------------------|--------|
| Tryptone    | 10 g/L               | 10 g   |
| Yeast extract | 5 g/L               | 5 g    |
| 5 M NaOH    | N/A                  | Adjust pH to 7.0 |
| ddH2O       | N/A                  | Bring up to 1 L |
| Total       | N/A                  | 1 L    |

The LB medium can be stored at 4°C for a couple of months.
### AAV lysis buffer

| Reagent                   | Final concentration | Amount     |
|---------------------------|---------------------|------------|
| 5 M NaCl                  | 150 mM              | 15 mL      |
| 1 M Tris-HCl, pH 8.0      | 20 mM               | 10 mL      |
| ddH₂O                     | N/A                 | Bring up to 500 mL |
| **Total**                | N/A                 | 500 mL     |

The buffer can be stored at 20°C–25°C for a couple of months.

### 17% iodixanol solution

| Reagent                     | Final concentration | Amount     |
|-----------------------------|---------------------|------------|
| 10x PBS                     | 1x                  | 5 mL       |
| 1 M MgCl₂                   | 1 μM                | 50 μL      |
| 1 M KCl                     | 2.5 μM              | 125 μL     |
| 5 M NaCl                    | 1 M                 | 10 mL      |
| Optiprep                    | 17%                 | 12.5 mL    |
| ddH₂O                       | N/A                 | Bring up to 50 mL |
| **Total**                  | N/A                 | 50 mL      |

The solution should be freshly made.

### 25% iodixanol solution

| Reagent                     | Final concentration | Amount     |
|-----------------------------|---------------------|------------|
| 10x PBS                     | 1x                  | 5 mL       |
| 1 M MgCl₂                   | 1 μM                | 50 μL      |
| 1 M KCl                     | 2.5 μM              | 125 μL     |
| Optiprep                    | 25%                 | 20 mL      |
| Phenol red                  | N/A                 | 100 μL     |
| ddH₂O                       | N/A                 | Bring up to 50 mL |
| **Total**                  | N/A                 | 50 mL      |

The solution should be freshly made.

### 40% iodixanol solution

| Reagent                     | Final concentration | Amount     |
|-----------------------------|---------------------|------------|
| 10x PBS                     | 1x                  | 5 mL       |
| 1 M MgCl₂                   | 1 μM                | 50 μL      |
| 1 M KCl                     | 2.5 μM              | 125 μL     |
| Optiprep                    | 40%                 | 33.3 mL    |
| phenol red                  | N/A                 | 25 μL      |
| ddH₂O                       | N/A                 | Bring up to 50 mL |
| **Total**                  | N/A                 | 50 mL      |

The solution should be freshly made.

### 60% iodixanol solution

| Reagent                     | Final concentration | Amount     |
|-----------------------------|---------------------|------------|
| 1 M MgCl₂                   | 1 μM                | 50 μL      |
| 1 M KCl                     | 2.5 μM              | 125 μL     |
| Optiprep                    | 60%                 | 50 mL      |
| Phenol red                  | N/A                 | 25 μL      |
| ddH₂O                       | N/A                 | Bring up to 50 mL |
| **Total**                  | N/A                 | 50 mL      |

The solution should be freshly made.
STEP-BY-STEP METHOD DETAILS

Insert guide oligos into pX602-Cre-sgRNA

© Timing: 5–7 days

This step is used to generate the AAV shuttle vector containing the sgRNA sequence.

1. Digest the pX602-Cre-sgRNA vector with BspQI.
   a. Set up reactions as shown below.

| Reagents          | Amounts       |
|-------------------|---------------|
| pX602-Cre-sgRNA   | 5 μg          |
| NEBuffer™ 3.1 buffer | 5 μL        |
| BspQI             | 0.5 μL (5 units) |
| ddH₂O             | Bring up to 50 μL |

b. Incubate at 50°C for 1 h.

c. Run a 0.8% agarose gel and cut out the band containing the linearized vectors (about 5 kb).
d. Purify the digested vector from gels using a commercially available kit. We use OMEGA Gel Extraction Kit (D2500-02) and perform the purification following the manufacturer’s instructions (https://www.omegabiotek.com/wp-content/uploads/2018/07/D2500.D2501-PROTOCOL-E.Z.N.A.-Gel-Extraction-Kit.pdf).

2. Anneal each pair of oligos.
   a. Mix the following reagents in PCR tubes

| Reagents          | Amounts       |
|-------------------|---------------|
| 100 μM Forward guide oligo | 1 μL          |
| 100 μM Reverse guide oligo | 1 μL          |
| 10X NEB T4 ligase buffer | 1 μL          |
| NEB PNK           | 0.5 μL        |
| ddH₂O             | Bring up to 10 μL |

b. Anneal in a thermocycler using the following program.

| Temperature | Time  |
|-------------|-------|
| 37°C        | 30 min|
| 95°C        | 5 min |
| Ramp down to 25°C at 5°C/min |

3. Ligate annealed oligos to the digested pX602-Cre-sgRNA vector.
   a. Centrifuge the PCR tube briefly.
   b. Dilute the annealed oligos at the ratio of 1:200 by ddH₂O.
   c. Set up the ligation reaction as shown below.

| Reagents          | Amounts       |
|-------------------|---------------|
| Digested pX602-Cre-sgRNA | 50 ng        |
| Diluted annealed oligo duplex | 1 μL        |
| 10X NEB T4 ligase buffer | 1 μL          |
| NEB T4 ligase     | 0.5 μL        |
| ddH₂O             | Bring up to 10 μL |
4. Transformation and colony picking
   a. Transform 2 μL of each ligation reaction mixture using 50 μL of Stbl3 competent cell.
   b. Add 500 μL of SOC medium and allow the transformed cells to recover at 37°C for 30 min with 220 rpm shaking.
   c. Plate transformed cells on agar dishes containing 100 μg/mL ampicillin. Incubate the plate at 37°C for 12–14 h.
   d. Pick 2 to 4 colonies into 3 mL of LB medium containing 100 μg/mL ampicillin and let them grow at 37°C with 220 rpm shaking for 12–14 h (no more than 16 h)
   e. Perform plasmid minipreps using a commercially available kit. We use OMEGA Plasmid Mini Kit (D6943-02) and perform the purification following the manufacturer’s instructions (https://www.omegabiotek.com/wp-content/uploads/2013/05/D6942.D6943.D6945-January-2017-Online.pdf).
   f. Verify guide oligo insertion by sequencing.
   g. Perform plasmid maxipreps using a commercially available kit to obtain enough plasmids (>100 μg) for following AAV preparation. We use TIANGEN HighPure Maxi Plasmid Kit (DP116) following the manufacturer’s instructions (http://www.tiangen.com.cn/asset/imsupload/up0004885001433129475.pdf).

AAV production

Timing: 4–5 days

AAV8 is one of the most widely used AAV serotypes for in vivo gene delivery. Efficient transduction to the liver by a single tail vein injection has been well demonstrated(Sands, 2011). Outstanding liver tropism of AAV8 combined with a hepatocyte-specific promoter can specifically introduce Cre recombinase in hepatocytes of the spCas9 KI mouse. In this section, we will describe how to produce AAV in a step by step manner.

5. HEK293T cells are cultured in high glucose DMEM supplemented with 10% FBS and 1% Pen/strep. 20–24 h before transfection, five 15 cm plates of cells with 80%–90% confluency are split to ten 15 cm plates such that the cells will reach ~80% confluency the next day.

6. Prepare transfection mixture: add the plasmid DNA as shown below into 49 mL of serum-free, antibiotic-free DMEM. Mix well, then add 1360 μL of 1 mg/mL PEI (assumed the ratio of DNA:PEI is 1:4). Mix immediately. Incubate the mixture at 20°C–25°C for 15 min.

| Plasmids          | Stock concentration (μg/μL) | Volume (μL) |
|-------------------|-----------------------------|-------------|
| pRC2/8            | 1                           | 70          |
| PHelper           | 1                           | 200         |
| pX602-Cre-sgRNA   | 1                           | 70          |

△ CRITICAL: The ratio of DNA: PEI needs to be tested for each batch of PEI.

7. Add 5 mL of transfection mixture to each 15 cm plate in a drop by drop fashion.
8. About 14 h after transfection, change the media with DMEM supplemented with 1% Pen/strep.

Note: FBS could be omitted in this step without lowering virus yield.

9. After 60 h after transfection, scrape and transfer the cells with medium to several 50 mL tubes. Spin at 200 × g for 10 min and discard the supernatant.
10. Re-suspend and combine the cell pellets by 20 mL of PBS. Spin at $200 \times g$ for 10 min and discard the supernatant.

11. Re-suspend the cell pellet by 5 mL of AAV lysis buffer.

**Pause point:** The cell suspension can be stored at $-80^\circ C$.

12. Freeze-thaw the cells three times between liquid nitrogen and water bath ($37^\circ C$) to lyse the cells.

*Note:* Make sure to freeze and thaw the cells completely.

13. Add 5 $\mu$L of 1 M MgCl$_2$ and 5 $\mu$L of 25 kU/mL Benzonase to the cell lysate. Mix well and incubate at $37^\circ C$ for 15 min.

14. Spin at 3200 $\times g$ for 30 min at $4^\circ C$. Carefully transfer the supernatant (lysate) which contains the virus to a new 15 mL tube.

15. Prepare iodixanol gradient solution as shown in KRT.

16. Prepare a discontinuous 17%-25%-40%-60% gradient in a 33 mL Optiseal tube (Beckman). Add from the bottom first 6 mL of the 17% iodixanol solution, then underlay 6 mL of the 25% iodixanol solution, then 5 mL of the 40% iodixanol solution and then 4 mL of the 60% iodixanol solution (Figure 4A). Mark the interface between the 60% layer and 40% layer. (Figure 4B)

17. Transfer the lysate on the top layer gently and fill up the tube by AAV lysis buffer until the liquid level reaches the neck of the tube.

18. Centrifuge for 2 h 40 min at 289, 100 $\times g$ (Type 70Ti) at $14^\circ C$.

19. Harvest viral fraction (40% fraction): use a 10 mL syringe and insert the needle at the interface between 60% layer and 40% layer which has been marked before centrifugation (Figure 4C). The total volume collected is 3.5–4 mL.

△ CRITICAL: Bubbles should be avoided in step 16 and step 19.

△ CRITICAL: The volume of collected viral fraction should be no more than 4 mL. Contamination of 25% fraction will cause AAV impurity.

20. Equilibrate the 100K Centrifugal filter (Millipore, Amacon 100k-UFC 910096): add 5 mL of 1× PBS containing 0.01% F188, and centrifuge at 2500 $\times g$ for 5 min.

21. Transfer the viral fraction into the 100K Centrifugal filter and fill with 1× PBS containing 0.01% F188. Mix well and centrifuge at 2500 $\times g$ for 20 min. Discard the flow through and fill the column with 1× PBS containing 0.01% F188 again. Repeat spin and add PBS for 3 times.

22. Concentrate the virus to about 500–1000 $\mu$L. Determine the purity of the virus by SDS-PAGE gel (Figure 4D). Troubleshooting 1

23. Prepare standards using pX602-Cre-sgRNA. Make serial 10-fold dilutions of the 1 $\mu$g/mL stock to get 1 $\mu$g/mL, $10^{-1}$ $\mu$g/mL, $10^{-2}$ $\mu$g/mL, $10^{-3}$ $\mu$g/mL and $10^{-4}$ $\mu$g/mL standards. Calculate the vector genomes (Vg) of your standards according to the following formula:

$$Vg = \frac{6.02 \times 10^{23} \times 2 \times \text{Length of the vector (bp)} \times \frac{10^{\mu g}}{\mu l} \times \text{Concentration(\mu g / ml)}}{6.6 \times \frac{\text{mol}}{\text{bp}}}$$

Length of pX602-Cre-sgRNA: 5036 bp

So the vector genome concentration of the standards are $3.6 \times 10^{11} \text{Vg/mL}$, $3.6 \times 10^{10} \text{Vg/mL}$, $3.6 \times 10^{9} \text{Vg/mL}$, $3.6 \times 10^{8} \text{Vg/mL}$ and $3.6 \times 10^{7} \text{Vg/mL}$ respectively.
24. Extract genome DNA from the virus. Prepare a mixture as shown below.

Mix well and incubate at 37°C for 15 min, followed by incubation at 95°C for 10 min. Then add the following components

| Reagents                | Amounts |
|-------------------------|---------|
| AAV virus               | 5 µL    |
| 10×DNase buffer         | 5 µL    |
| DNase (Takara, 5 U/µL)  | 2 µL    |
| ddH₂O                   | 38 µL   |

Mix well and incubate at 37°C for 15 min, followed by incubation at 95°C for 10 min.

Note: this step will dilute the virus 20-fold.

25. Set up the qPCR reaction as shown below.
26. Run the qPCR reaction using the following thermocycler program.

| Reagents               | Amounts  |
|------------------------|----------|
| 2x SYBR mixture        | 10 µL    |
| TBG F (10 µM)          | 0.5 µL   |
| TBG R (10 µM)          | 0.5 µL   |
| Standards or virus DNA | 2 µL     |
| PCR grade water        | 7 µL     |

| PCR cycling conditions |
|------------------------|
| **Steps**               | **Temperature** | **Time** | **Cycles** |
| Initial Denaturation    | 95°C           | 15 min   | 1          |
| Denaturation            | 95°C           | 10 s     | 40         |
| Annealing               | 60°C           | 20 s     |            |
| Extension               | 72°C           | 30 s     |            |

27. Analyze the qPCR data to get a calibration curve. Calculate the AAV titer in vector genomes per mL (Vg/mL) as described in “quantification and statistical analysis”. Troubleshooting 2

28. Adjust the concentration of AAV to $4 \times 10^{12}$ Vg/mL by PBS.

**Viral injection**

*© Timing: 2 weeks or longer*

29. Inject 6- to 8-week-old male spCas9 KI mice with AAV-Cre-sgRNA via lateral tail vein injection. We recommend administering AAV at $4 \times 10^{11}$ total genome copies to each mouse (100 µL of $4 \times 10^{12}$ Vg/mL stock) (Figure 5D).

30. Let the mice recover for 2 weeks and validate gene editing and phenotype.

**CRITICAL:** AAV packaging sgRNA targeting a gene not existing in the mouse genome should be included as the negative control. sgRNAs targeting LacZ are usually recommended.

**Validate gene editing efficiency after AAV injection**

*© Timing: ~2 days*

To validate gene editing, liver samples from AAV injected mice are harvested. Detection of protein levels is necessary with specific antibodies against proteins produced by GOI (in the case of SURF4, a home-made antibody is used, Figure 5D). In some cases, this procedure is limited by the lack of specific antibodies, so we also describe a semi-quantitative method for validating gene editing on the genomic level which relies on Sanger sequencing and data analysis by software ICE.

31. Isolate primary hepatocytes from control mice (injected with AAV-Cre-control sgRNA) and experimental mice (injected with AAV-Cre-GOI sgRNA). Please refer to (Charni-Natan and Goldstein, 2020) for step-by-step instructions.

**Optional:** Sacrifice the mice by decapitation via the ethical and approved method and collect the livers. However, non-parenchymal cells make up 30%–40% of total cell population in the liver, so that using liver samples will underestimate the knockout efficiency (Seo and Jeong, 2016).

**Pause point:** Hepatocytes or tissues can be snap frozen in liquid nitrogen and stored at $-80^\circ$C.
32. Extract genomic DNA using QIANamp Genomic DNA (DP304-03) kit following manufacturer’s instructions (http://tiangen.com/asset/imsupload/up0875005001348194139.pdf).

33. Design PCR primers to amplify 400 to 600 bp amplicons containing the sgRNA targeted region.

34. Perform PCR using KOD-Plus-Neo kit. Set up PCR reactions as shown below.

35. Run the PCR reaction using the following thermocycler program.
36. Run the PCR products on 1.5% agarose gel and purify the amplicons using OMEGA Gel Extraction Kit (D2500-02) following the manufacturer’s instructions (http://tiangen.com/asset/imsupload/up0875005001348194139.pdf). Troubleshooting 3 and 4.

37. Sequence the amplicons by a commercial sequencing facility. The Sanger sequencing in our lab is supported by TSINGKE Biological Technology. The reaction is set up as shown below.

38. Upload guide RNA sequence, sequencing results of control amplicons and sequencing results from experimental amplicons onto the software ICE (https://ice.synthego.com/#). Troubleshooting 5.

**EXPECTED OUTCOMES**

Typically, the efficiency of gene editing in isolated primary hepatocytes ranges from 60% to 90%. If the edited efficiency is very low (e.g., <50%), this protocol needs to be repeated using new guide sequences. An example analyzed by ICE software is presented in Figures 5A–5C.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Calculation of AAV titer is performed by Microsoft Excel. The qPCR from step 27 will offer raw data of Ct value. Standard curves are plotted using Log10 Vg of standards (y) as a function of the Ct value of standards (x). AAV titer can be calculated according to the standard curve. For example, as shown below, the Ct value of the AAV sample is 6.11 and the standard curve is y = -0.2507x + 13.164 (Figure 6), so Vg in the AAV sample is $4 \times 10^{11} (10^{-0.2507 \times 6.11 + 13.164})$ Vg/mL. After multiplying the dilution factor of 20, the original AAV titer is $8 \times 10^{12}$ Vg/mL.

**LIMITATIONS**

One major limitation of this protocol concerns with potential off-target effects of CRISPR. To minimize the impact of off-target effects on investigating the GOI function, we use at least three sgRNAs targeting different coding regions of the same gene. Furthermore, we highly recommend rescue
experiments using sgRNA-resistant cDNAs to validate the on-target phenotypes (troubleshooting 6). Additionally, the acute gene editing system, in theory, cannot achieve 100% inactivation. Hence, analysis of the phenotypic outcomes needs to be correlated with the penetration of the gene inactivation. Another limitation is that validating gene editing by Sanger sequencing does not directly reflect functional inactivation of the interested gene. If commercial antibodies specific to the protein encoded by the interested gene are available, analyzing the knockout efficiency at the protein level is necessary.

TROUBLESHOOTING

Problem 1
Purified AAV containing large amounts of protein impurities (step 22)

Potential solution
In vivo administration of AAV containing large amounts of impurities can result in extra immune response and low transduction efficiency. In order to improve the purity of the purified AAV, cell debris in step 14 should be removed completely. In addition, when collecting AAV enriched fraction after ultracentrifugation (40% iodixanol gradient layer), contamination with 60% iodixanol gradient layer and 25% iodixanol gradient layer should be carefully avoided.

Problem 2
Low yield of AAV production (step 27)

Potential solution
Healthy HEK293T and high transfection efficiency are critical for optimal AAV production. We maintain the HEK293T cells below 90% confluence and passage the cells less than 30 times. To improve the transfection efficiency, the DNA vectors should be endotoxin free (use endotoxin-free plasmid isolation kit) and the PEI: DNA ratio should be optimized for each batch of newly prepared PEI stock solution.

Problem 3
No band from PCR (step 36)
Potential solution
The yield of PCR reaction can be improved by lowering the annealing temperature, and increasing the cycle numbers (by 2 to 5 cycles). In the case of high GC contents (>60%) in target sequences, supplying 2%-5% DMSO to the PCR reaction may help.

Problem 4
Multiple bands from PCR (step36)

Potential solution
The specificity of PCR reaction can be improved by increasing the annealing temperature, decreasing the cycle numbers, reducing the enzyme concentration (from 1 unit/50 μL to 0.5 unit/50 μL) and reducing the amount of DNA template. If above solutions do not solve the problem, design a new pair of PCR primer and re-run step34 to step36.

Problem 5
Low editing efficiency (step 38)

Potential solution
One possibility is that the sgRNA used has a poor Cas9-mediated DNA cleavage activity, therefore, a few more guide sequences should be designed and tested. Another possibility is loss of function of the targeted gene leads to cell death over time. In this case, earlier analyses of the edited mice or hepatocytes are recommended.

Problem 6
Concerns about off-target effects (section of “Limitations”)

Potential solution
To minimize the impact of off-target effects, we use at least three sgRNAs targeting different coding regions of the same gene. In addition, we perform rescue experiments using sgRNA-resistant cDNAs to validate the on-target phenotypes. For the rescue experiment, AAV8-TBG-Cre-sgRNA (still 4 × 10¹¹ Vg for each mouse) together with AAV8-TBG-cDNA (usually around 1 × 10¹¹ Vg for each mouse) are injected into each spCas9 KI mouse simultaneously. The shuttle vector for packaging AAV8-TBG-cDNA can be generated from pAAV-TBG-GFP (Addgene, 10553) by replacing the GFP sequence with respective cDNA. The PAM sequence in the cDNA of the corresponding sgRNA should be synonymously mutated by standard mutagenesis procedure.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for reagents and resources will be fulfilled by the lead contact, Xiao-Wei Chen (xiaowei_chen@pku.edu.cn).

Materials availability
Plasmids generated in this study are available from the lead contact upon request.

Data and code availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
X.W., B.-L.X., and X.-W.C. conceptualized the study, designed experiments, and analyzed data. X.W. and B.-L.X. performed experiments. X.W. and X.-W.C. wrote the paper. All authors approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Charni-Natan, M., and Goldstein, I. (2020). Protocol for primary mouse hepatocyte isolation. STAR Protoc. 1, 100086.

Platt, R.J., Chen, S., Zhou, Y., Yim, M.J., Siewiech, L., Kempton, H.R., Dahlman, J.E., Parnas, O., Eisenhaure, T.M., Jovanovic, M., et al. (2014). CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159, 440–455.

Sands, M.S. (2011). AAV-mediated liver-directed gene therapy. Methods Mol. Biol. 807, 141–157.

Seo, W., and Jeong, W.I. (2016). Hepatic non-parenchymal cells: master regulators of alcoholic liver disease? World J. Gastroenterol. 22, 1348–1356.

Simon, M.M., Greenaway, S., White, J.K., Fuchs, H., Gailus-Durner, V., Wells, S., Sorg, T., Wong, K., Bedu, E., Cartwright, E.J., et al. (2013). A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. Genome Biol. 14, R82.

Wang, X., Wang, H., Xu, B., Huang, D., Nie, C., Fu, L., Zajac, G.J.M., Yan, H., Zhao, J., Shi, F., et al. (2020). Receptor-mediated ER export of lipoproteins controls lipid homeostasis in mice and humans. Cell Metab. 33, 350–366.e7.