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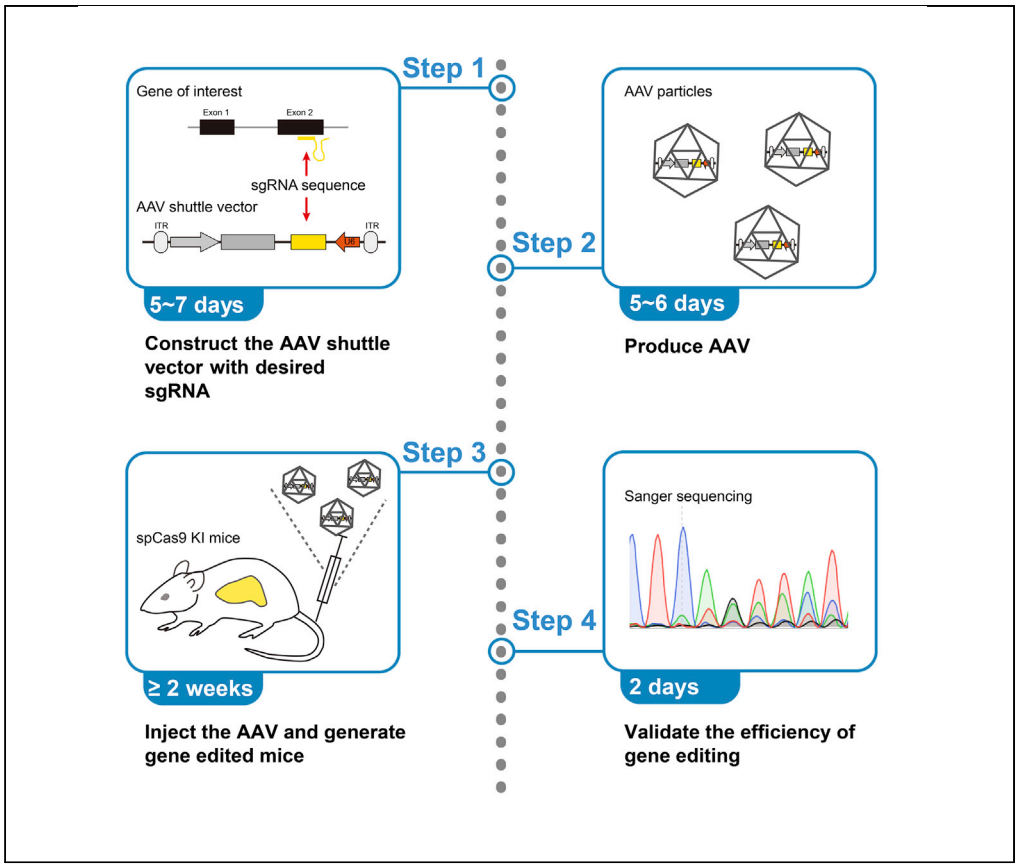
Acute gene inactivation in the adult mouse liver using the CRISPR-Cas9 technology



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

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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 (ROSA)26Sor$^{tm1(CAG-cas9*,-EGFP)Fezh}$/J (RRID:IMSR_JAX:026556) and B6J.129(B6N)-Gt(ROSA)26 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).

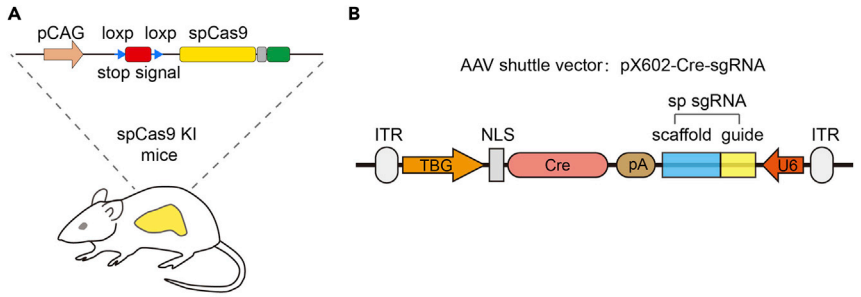

Figure 1. Scheme of CRISPR-mediated acute hepatic gene inactivation based on the ''silent'' spCas9 knockin mice

(A) The knockin cassette with spCas9 cDNA preceded of a LoxP-STOP-LoxP cassette.

(B) Schematic of the AAV shuttle vector pX602-Cre-sgRNA.

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.

A

Position	Strand	Guide Sequence	PAM	On-Target Score	Off-Target Score
9449	-	ACATGCTCTTCCCTTCTGAG	CGG	71.3	32.5
9559	-	GCTGGCATCAAAGTGAAGGA	GGG	65.9	33.9
9518	+	AAGCAGTACATGCAGCTTGG	AGG	65.7	39.0
9502	-	AAGCTGCATGTACTGCTTGG	GGG	63.3	38.6
9450	+	TGGCAGAATCCCGCTCAGAA	GGG	62.6	43.6
9563	-	AGAAGCTGGCATCAAAGTGA	AGG	61.8	35.6

B

Off-Target Sites				
Sequence	PAM	Score	Gene	Chromosome
CCTTTCTCTTCCCTTCTGGG	AGG	0.639582		chr3
CTCTGCTCTTCCTTTCTGAG	TGG	0.644259		chr11
ACATACTGTGCTCTTCTGAG	GAG	0.675432		chr2
ACATTCTGTGCTCTTCTGAG	GAG	0.675432		chr16
AGAAGCTCTGCCCTTCAGAG	AGG	0.690278	ENSMUSG00000032657	chr3
CTGTGCTCTTCCCTTCGGAG	TAG	0.723188		chr7
AAAAGCTTTTCCCTTCAGAG	CGG	0.733456		chr5
ACCTGCTGTGCCCTTCTGAT	CAG	0.753658		chr6
GCATGCTCTTCCCTTCAGAA	AAG	0.7645		chr10
ACATTCCCTTCCCTTCTGGG	TGG	0.787621		chr6

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.

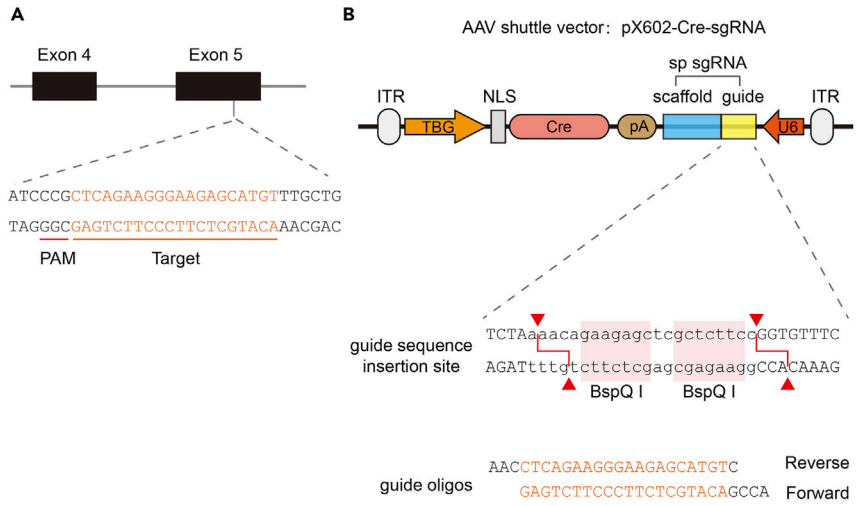

Figure 3. sgRNA oligos for cloning into px602-Cre-sgRNA

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

(B) Guidelines for ordering sgRNA oligos.

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 Bioloabs (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#* 21-1
NaOH	Xilong	Cat#S0205
$MgCl_2$	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#PSL01
Fetal bovine serum (FBS)	VISTECH	Cat#SE100-011
DNaseI	Takara	Cat#2270A
10× DNase buffer	Takara	Cat#SD4104

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Proteinase K	AMRESCO	Cat#0706
Glucose	Beihua	Cat#S1829
Agarose	Vetec	Cat#V900500
Critical commercial assays		
OMEGA Gel Extraction Kit	Omega	Cat#D2500-02
OMEGA Plasmid Mini Kit	Omega	Cat#D6943-02
TIANGEN HighPure Maxi Plasmid Kit	TIANGEN	Cat#DP116
QIANamp 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		
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		
TBG F: ATTCTGCCTGCTGAAGACACTCT	This paper	N/A
TBG R: CCCCAGCATTAACCCTGGGATG	This paper	N/A
Recombinant DNA		
pRC2/8	Addgene	Cat#112864
PHelper	Addgene	Cat#112867
pX602-Cre-sgRNA	This paper	N/A
Software and algorithms		
Benchling	Benchling	https://benchling.com/
ICE	Synthego	https://ice.synthego.com/#/
Other		
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
ddH_2O	N/A	Bring up to 1 L
Total	N/A	1 L
Combine above and Sterilize by autoclaving, then add the reagents below		
*2 M $MgCl_2$	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
ddH_2O	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
ddH_2O	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
10× PBS	1×	5 mL
1 M $MgCl_2$	1 μM	50 μL
1 M KCl	2.5 μM	125 μL
5 M NaCl	1 M	10 mL
Optiprep	17%	12.5 mL
ddH_2O	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
10× PBS	1×	5 mL
1 M $MgCl_2$	1 μM	50 μL
1 M KCl	2.5 μM	125 μL
Optiprep	25%	20 mL
Phenol red	N/A	100 μL
ddH_2O	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
10× PBS	1×	5 mL
1 M $MgCl_2$	1 μM	50 μL
1 M KCl	2.5 μM	125 μL
Optiprep	40%	33.3 mL
ddH_2O	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 $MgCl_2$	1 μM	50 μL
1 M KCl	2.5 μM	125 μL
Optiprep	60%	50 mL
Phenol red	N/A	25 μL
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)
ddH_2O	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
10× NEB T4 ligase buffer	1 μL
NEB PNK	0.5 μL
ddH_2O	Bring up to 10 μL

   b. Anneal in a thermocycler using the following program.

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 ddH_2O.
   c. Set up the ligation reaction as shown below.

Reagents	Amounts
Digested pX602-Cre-sgRNA	50 ng
Diluted annealed oligo duplex	1 μL
10× NEB T4 ligase buffer	1 μL
NEB T4 ligase	0.5 μL
ddH_2O	Bing up to 10 μL

d. Incubate at 20°C–25°C for 1 h.

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 (assume 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 × *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°C.

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

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

13. Add 5 μL of 1 M $MgCl_2$ and 5 μL of 25 kU/mL Benzonase to the cell lysate. Mix well and incubate at 37°C for 15 min.
14. Spin at 3200 × *g* for 30 min at 4°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 × *g* (Type 70Ti) at 14°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 × *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 × *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 μL. 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 μg/mL stock to get 1 μg/mL, 10^{-1} μg/mL, 10^{-2} μg/mL, 10^{-3} μg/mL and 10^{-4} μg/mL standards. Calculate the vector genomes (Vg) of your standards according to the following formula:

$$Vg \Big/ ml = \frac{6.02 \times 10^{23} \times 2}{Length\ of\ the\ vector\ (bp) \times 6.6 \times \frac{10^8 \mu g}{mol \times bp}} \times Concentration(\mu g / ml)$$

Length of pX602-Cre-sgRNA: 5036 bp

So the vector genome concentration of the standards are 3.6×10^{11}Vg/mL, 3.6×10^{10}Vg/mL, 3.6×10^{9}Vg/mL, 3.6×10^{8}Vg/mL and 3.6×10^{7}Vg/mL respectively.

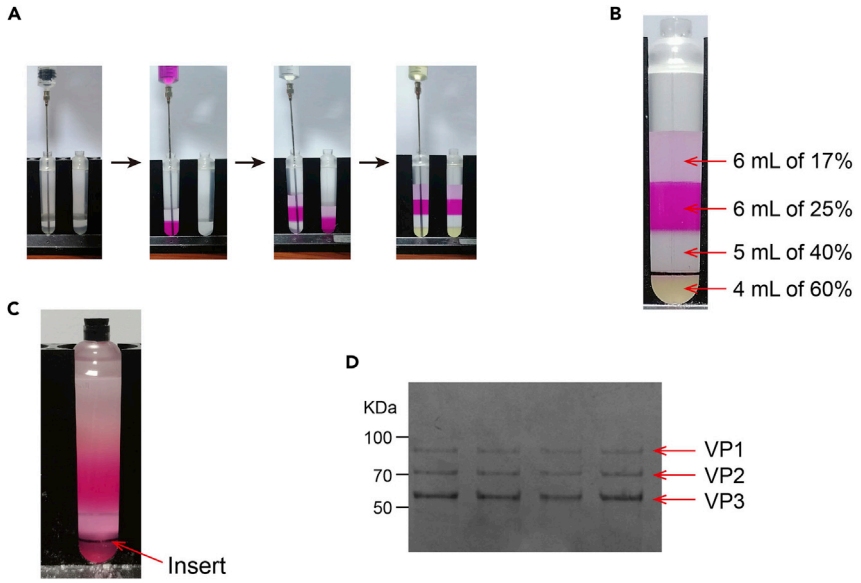

Figure 4. Purification of AAV by density gradient centrifugation

(A) Steps for preparing discontinuous iodixanol 17%-25%-40%-60% gradient as described in step 16.

(B) Discontinuous iodixanol gradient prepared from Figure 4A before ultracentrifugation. The interfaces of different gradients are visualized by phenol red. The interface between 60% and 40% is marked.

(C) Iodixanol gradient after ultracentrifugation. The marked line (also indicated by the arrow) indicating the position where the needle insert in for harvesting AAV fraction.

(D) SDS-PAGE gel of purified AAV followed by Coomassie brilliant blue staining. VP1, VP2, VP3 are capsid proteins of AAV.

24. Extract genome DNA from the virus. Prepare a mixture as shown below.

Reagents	Amounts
AAV virus	5 μL
10×DNase buffer	5 μL
DNase (Takara, 5 U/μL)	2 μL
ddH_2O	38 μL

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
10×DNase buffer	5 μL
Proteinase K (20 mg/mL)	1 μL
ddH_2O	44 μL

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

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

25. Set up the qPCR reaction as shown below.

Reagents	Amounts
2× 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

26. Run the qPCR reaction using the following thermocycler program.

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 4×10^{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 4×10^{11} total genome copies to each mouse (100 μL of 4×10^{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°C.

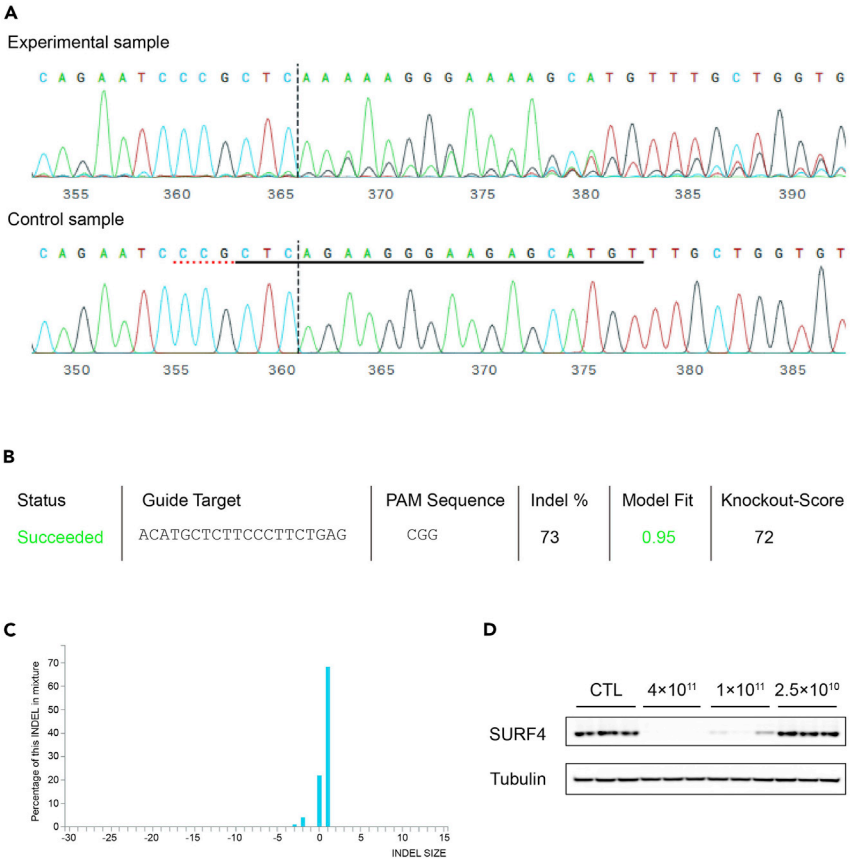

Figure 5. Representative results of gene editing efficiency analysis

(A) Sanger sequencing results of regions around the guide sequence. Primary hepatocytes isolated from spCas9 KI mice receiving AAV8-Cre-LacZ sgRNA or AAV8-Cre-*Surf4* sgRNA are subjected to DNA extraction followed by PCR amplification and Sanger sequencing. The guide sequence is underlined by the black line. The PAM sequence is underlined by the red dashed line. The predicted cut site is indicated by the vertical black dashed line.

(B) The summary of editing efficiency analyzed by ICE.

(C) The Indel plot displays the inferred distribution of indels in the edited gene region.

(D) The SURF4 knockout efficiency of spCas9 KI mice receiving different doses of AAV8-Cre-*Surf4* sgRNA validated by immunoblotting.

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.

Reagents	Amounts
10x buffer	5 μL
2 mM dNTPs	5 μL
25 mM $MgSO_4$	3 μL
10 μM Primer F	0.75–1.5 μL
10 μM Primer R	0.75–1.5 μL
Genomic DNA	≤ 200 ng
KOD-Plus-Neo	1 μL (1 unit)
PCR grade water	Up to 50 μL

35. Run the PCR reaction using the following thermocycler program.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	Tm of primers	30 s	
Extension	68°C	30 s	
Hold	16°C	forever	

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/up08750050013481941 39.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.

Reagents	Amounts
Purified PCR Product (adjust to 20 ng/μL)	1 μL
BigDyeTM Terminator v3.1 reaction mix	2 μL
0.36 μM Primer F in step 34	1 μL
ddH_2O	6 μL

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 $Log_{10}Vg$ 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 $4\times10^{11}(10^{-0.2507\times6.11+13.164})$ Vg/mL. After multiplying the dilution factor of 20, the original AAV titer is 8×10^{12} Vg/mL.

	Ct			Ct average	Vg	LogVg
standard1	5.79	6.74	6.99	6.51	3.60E+11	11.56
standard2	10.00	10.50	9.80	10.10	3.60E+10	10.56
standard3	14.87	14.57	14.54	14.66	3.60E+09	9.56
standard4	18.42	18.24	18.63	18.43	3.60E+08	8.56
standard5	22.54	21.66	22.59	22.26	3.60E+07	7.56
AAV sample	6.14	6.33	5.90	6.11		

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

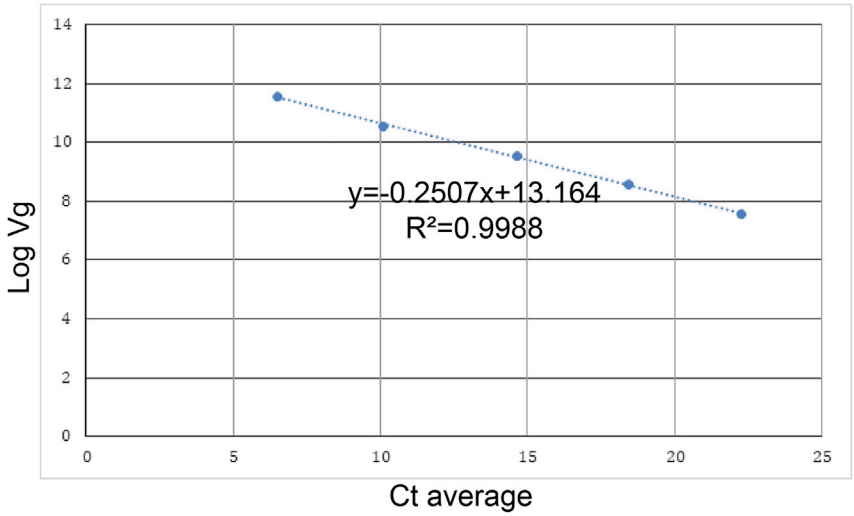

Figure 6. An example of the standard curve described in "quantification and statistical analysis"

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 4×10^{11} Vg for each mouse) together with AAV8-TBG-cDNA (usually around 1×10^{11} 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, 105535) 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.

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