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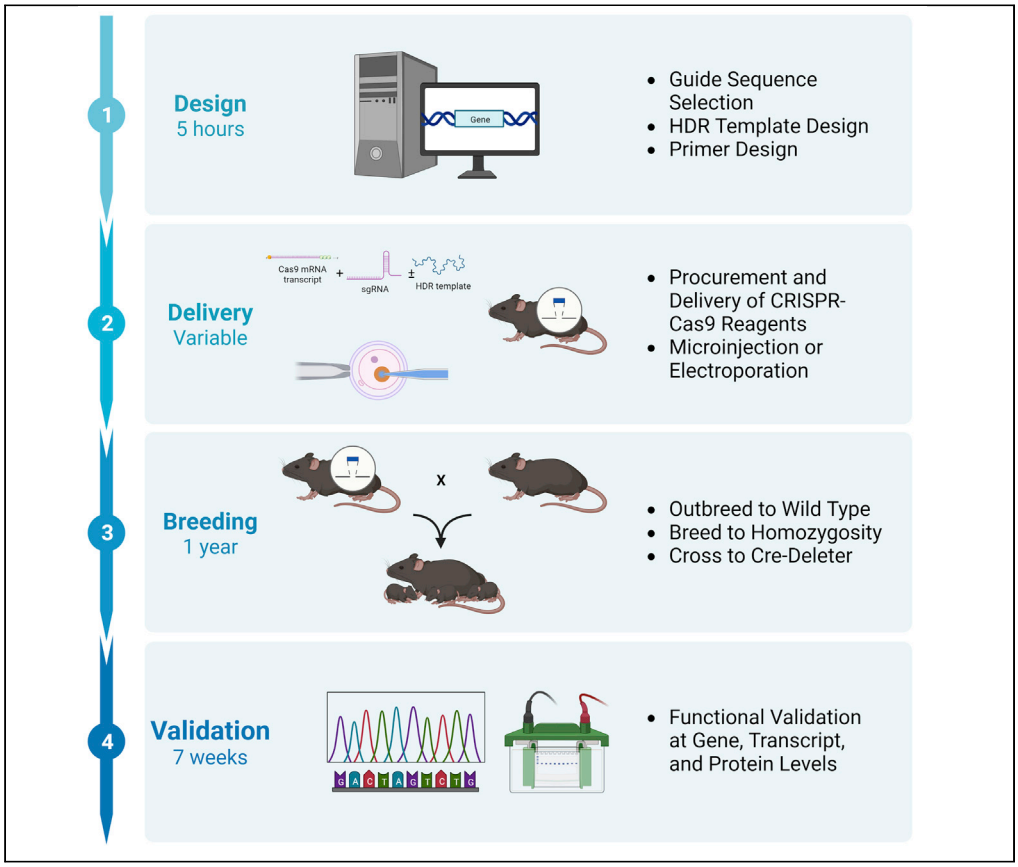
Protocol

CRISPR-Cas9-mediated insertion of a short artificial intron for the generation of conditional alleles in mice



In this protocol, we describe the generation of conditional alleles in mice using the DECAI (DEgradation based on Cre-regulated Artificial Intron) approach. We detail steps for the CRISPR-mediated insertion of the short DECAI cassette within exon 3 of Scyl1 and the functional validation of alleles at genomic, transcriptomic, and protein levels. This strategy simplifies the process of generating mice with conditional alleles.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Detailed procedures for the design of conditional alleles using short artificial introns

Optimized protocols for the generation of mouse models using artificial introns

Step-by-step procedures for the characterization at the genomic level

Comprehensive protocol for the functional validation of the allele

Cassidy & Pelletier, STAR Protocols 4, 102116
March 17, 2023
https://doi.org/10.1016/j.xpro.2023.102116

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CRISPR-Cas9-mediated insertion of a short artificial intron for the generation of conditional alleles in mice

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https://doi.org/10.1016/j.xpro.2023.102116

SUMMARY

In this protocol, we describe the generation of conditional alleles in mice using the DECAI (DEgradation based on Cre-regulated Artificial Intron) approach. We detail steps for the CRISPR-mediated insertion of the short DECAI cassette within exon 3 of Scyl1 and the functional validation of alleles at genomic, transcriptomic, and protein levels. This strategy simplifies the process of generating mice with conditional alleles.

For complete details on the use and execution of this protocol, please refer to Cassidy et al. (2022).[1]

BEFORE YOU BEGIN

This protocol describes the application of the DECAI approach for the generation of conditional alleles in mice, as an alternative to existing strategies. The DECAI approach consists of inserting a short 201 nucleotide-long cassette within an exon of a gene.[1,2] The cassette, here referred to as Artificial Intron version 4 (AIv4) as originally described,[1,2] contains sequences encoding a splice donor, essential intronic sequences flanked by two loxP sites, and a splice acceptor (Figure 1A). In the absence of Cre, the cassette is recognized as an intron by the splicing machinery and removed, allowing for proper translation of the protein. In the presence of Cre, recombination between the two loxP sites occurs and removes sequences critical for splicing. Consequently, the recombined cassette remains within the transcript, leaving a string of stop codons in all three frames. This results in early termination of translation and promotes degradation of the faulty mRNA transcript via the nonsense-mediated mRNA decay pathway (Figure 1B).

Here, the generation of a mouse model bearing a conditional allele of *Scyl1* (*Scyl1*^{AIv4}) - a gene essential for normal postnatal development and motor neuron viability[3,4] - and its functional validation are described. Considerations for the design of conditional alleles using the DECAI approach are also presented.

Institutional permissions

Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility and maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal experiments were reviewed and approved by the Indiana University Institutional Animal Care and Use Committee.

Scyl1^{AIv4} allele design

Timing: 1 h

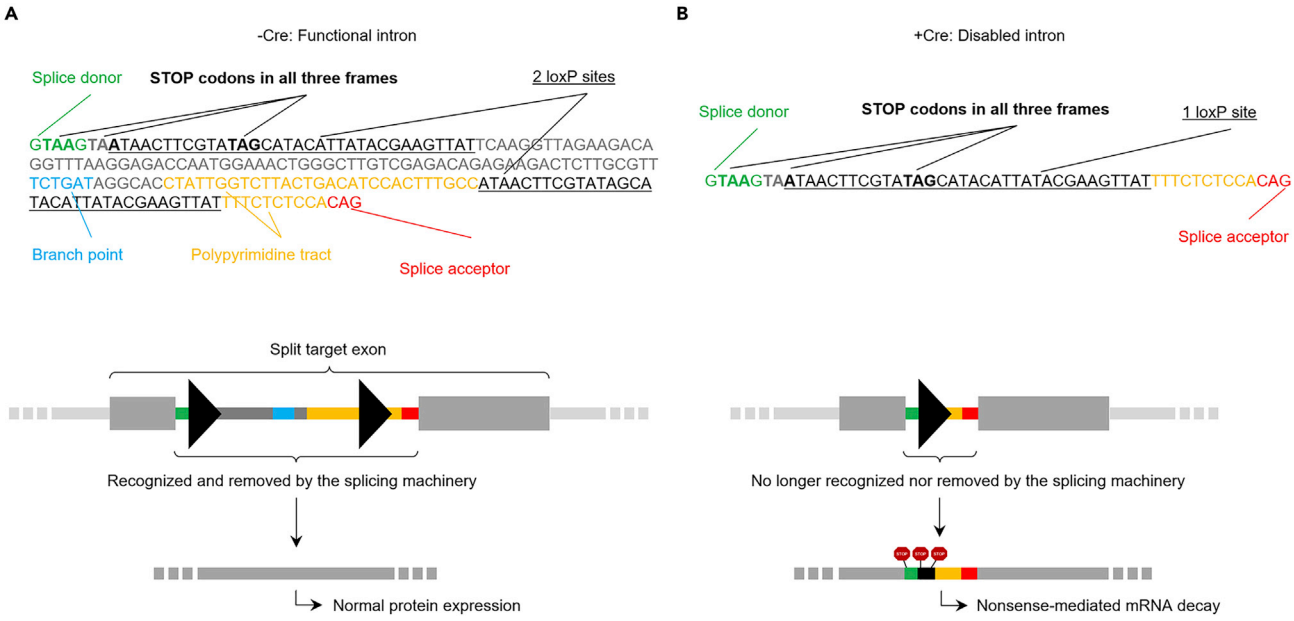

Figure 1. Generation of conditional alleles using the DECAI approach

(A) DNA sequence and schematic representation of the AIv4 cassette inserted within the exon of a target gene. In the absence of Cre, the cassette is recognized as an intron and removed by the splicing machinery, allowing expression of the gene.

(B) DNA sequence and schematic representation of the recombined AIv4 cassette within a target gene. In the presence of Cre, the recombined cassette is no longer recognized as an intron by the splicing machinery and no longer removed by the splicing machinery, resulting in early translation termination and, depending on the location of the stop codons within the target gene, degradation of the mRNA transcript via the nonsense-mediated mRNA decay pathway. The splice donor site is indicated in green. LoxP sites are shown in black. The branch point is shown in blue. The bipartite polypyrimidine tract is shown in yellow. The splice acceptor site is shown in red. In the presence of Cre, recombination between the two loxP sites occurs which removes the branch point and part of the polypyrimidine tract. Red octagons represent premature stop codons.

Exon 3 of the *Scyl1* gene was chosen as the insertion site for the AIv4 cassette for two main reasons. First, the presence of premature stop codons within the 5′ half of a gene and within the 5′ half of an exon is thought to promote mRNA degradation via the nonsense-mediated mRNA decay pathway.[1] Second, it was surmised that insertion of the short intron into a large exon would be better tolerated, allowing for proper splicing. To assist with the positioning of the AIv4 cassette within an exon, please refer to the "limitations" section of the manuscript. These considerations were based on findings obtained from the analysis of the *Scyl1*^{AIv4} allele.

The following steps describe the procedure to design the *Scyl1*^{AIv4} allele using SnapGene (https://www.snapgene.com/).

1. Download *Scyl1* gene (GenBank: NC_000085.7, Data S1) information form NCBI – Gene (https://www.ncbi.nlm.nih.gov/gene/):
   a. Type "Scyl1" in the query box and click "Search".
   b. From the list of results, select "Scyl1 from Mus musculus, ID: 78891".
   c. Scroll down to "Genomic regions, transcripts, and products" and click on: "GenBank".
   d. Click on "Send to" and select "Complete record", "File", "GenBank" and "Show GI." Click on "Create file." This will download the *Scyl1* GenBank file to your browser.
2. Open the file in SnapGene.
3. Generate the *Scyl1*^{AIv4} allele in silico.
   a. Locate the following guide sequence in exon 3: CATCCACAATAATGTCTGCA.

b. Using the "Design Synthetic Construct" function of SnapGene, insert the sequence of the AIv4 cassette two nucleotides upstream of the cut site: GTAAGTAATAACTTCGTATAGCATACAT TATACGAAGTTATTCAAGGTTAGAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCTTGT CGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCCACTTT GCCATAACTTCGTATAGCATACATTATACGAAGTTATTTTCTCTCCACAG.
c. Save the file under a new name (e.g., Scyl1_AIv4, Data S2).

Note: All nucleotide sequences in this manuscript are presented in a 5′ to 3′ orientation, unless otherwise specified.

⚠ CRITICAL: Although the AIv4 cassette was placed two nucleotides upstream of the cut site in this model, it would have been preferable to insert the cassette directly at the cut site for reasons that are discussed in the "limitations" section of this manuscript.

Guide sequence selection

Timing: 2 h

Successful manipulation of the mouse genome using CRISPR-Cas9 technology depends, for the most part, on the selection of efficient and selective guide sequences. These are selected based on 3 main criteria: the proximity of the cleavage site relative to the editing site, selectivity of the guide sequence, and efficiency of a guide sequence to cleave its target site. For more information about guide selection procedures, please see Cassidy et al.[1]

To select a guide sequence:

4. Go to UCSC Genome Browser (https://genome.ucsc.edu/).
5. From the list of options, select "BLAT".
6. Copy the target region from SnapGene and paste it into the query box.
   a. Under "Genome", select "Mouse".
   b. Under "Assembly", select "GRCm39/mm39" and click "Submit".
7. From the "BLAT Search Results" list, select the "browser" hyperlink with the highest "SCORE" value.
8. On the "CRISPR/Cas9 -NGG Targets, Whole genome" track, identify possible guide sequences.
   a. To engineer the *Scyl1*^{AIv4} allele, the following guide sequence was used: CATCCACAAT AATGTCTGCA.
9. After selecting the guide sequence, add annotations to your SnapGene files using the "Add feature" function.
10. Generate a list of potential off-target sites using Cas-OFFinder (http://www.rgenome.net/cas-offinder/).
    a. Navigate to the Cas-OFFinder website.
    b. Copy and paste your guide sequence in the box under "Query Sequences".
    c. Under "Mismatch Number", select 2.
    d. Under "PAM Type", Select "SpCas9 from Streptococcus pyogenes: 5′-NGG-3′".
    e. Under "Target Genome", for Organism Type select "Vertebrates" and then "Mus musculus (mm10) – Mouse".
    f. Click "Submit".
    g. After the list is generated, click on "Download result" under "Status".
    h. Keep the list of potential off-target sites for future analysis of potential off-target cleavage.

i. For the guide used to generate the *Scyl1*^{AIv4} allele, the following list of off-targets was identified:

OT	crRNA	DNA	Chromosome	Position	Direction	Mismatches
1	CATCCACAATAATGTCTGCANGG	CATCCACtATAgTGTCTGCATGG	chr1	123943090	–	2
2	CATCCACAATAATGTCTGCANGG	CATCCACAAcAgTGTCTGCATGG	chrX	100886302	–	2
3	CATCCACAATAATGTCTGCANGG	CATCCACAAcAcTGTCTGCATGG	chr18	81522627	–	2

Homology directed repair template design

Timing: 1 h

Designing homology directed repair (HDR) templates for the generation of alleles using the DECAI approach is relatively straightforward. These HDR templates contain the sequence of the AIv4 cassette flanked by homology arms of approximately 50 nucleotides.

⚠ CRITICAL: Although not considered when designing the *Scyl1*^{AIv4} allele, the placement of the cassette should ensure that any potential splicing events driven by the AIv4 splice donor with downstream exon(s) results in transcripts that would be out of frame. For more information on the positioning of the AIv4 cassette, please see the "limitations" section of the manuscript.

For the generation of the *Scyl1*^{AIv4} allele, the following HDR template encoding the AIv4 cassette sequence to be inserted and homology arms of 58 and 60 nucleotides (lower case) was used:

agatcgtgaaagccctcagcttcctggtcaacgactgcaacctcatccacaataatgtGTAAGTAATAACTTCGTATAGCAT
ACATTATACGAAGTTATTCAAGGTTAGAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCTTGT
CGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCCACTTTGCC
ATAACTTCGTATAGCATACATTATACGAAGTTATTTTCTCTCCACAGctgcatggccgctgtgtttgtggacagg
gctggcgagtggaaacttgggggtctggacta.

11. Once the HDR template is designed, order the template from IDT. Either ssDNA or dsDNA fragments can be used.
    a. For ssDNA fragments between 201 - 500 nucleotides, select:
        i. Megamer™ Single-Stranded DNA (ssDNA) Fragments,
        ii. 3 µg scale.
        iii. Fill out the Biohazard Disclosure.
    b. For dsDNA fragments between 201–500 nucleotides, select:
        i. Alt-R™ HDR Donor Blocks.
        ii. 3 µg scale.
        iii. Fill out the Biohazard Disclosure.

Primer design

Timing: 1 h

For the on-target site, design PCR primers to amplify ~500–800 bp amplicons, ensuring that the primers are located outside the homology arms of the HDR template. For off-target sites, PCR primers should be at least 200 nucleotides from the potential cleavage sites. Primers should have an annealing temperature of approximately 60°C. Please refer to Cassidy et al.[5] for a step-by-step procedure to design PCR primers using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). No additional changes were made under the advanced parameters tab in Primer-BLAST.

12. The following primer pair was selected for amplification of the on-target locus:
    a. S1E154D-F1: TACTCTCCTCAGGCCCTCAC.
    b. S1E154D-R1: GAAGCACCGAACACCCAAAC.
        i. The PCR genotyping banding pattern obtained from these primers are as follows:

Allele	S1E154D-F1 + S1E154D-R1
$Scyl1^{+}$	667 bp
$Scyl1^{Alv4}$	868 bp

Procurement of CRISPR-Cas9 reagents

Timing: variable

sgRNAs can be either synthesized according to Cassidy et al.[5] or purchased from IDT, Millipore-Sigma, ThermoFisher, and others. Similarly, Cas9 can be produced as an mRNA transcript according to Cassidy et al.[5] or purchased as a recombinant protein from IDT, Invitrogen, New England Biolabs, Sigma-Aldrich, and others.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-SCYL1 7645-AP (1:1,000)	Pelletier Lab	7645-AP
Anti-SCYL1 7691-AP (1:1,000)	Pelletier Lab	7691-AP
Anti-β-actin (1:3,000)	Sigma-Aldrich	A5441
Peroxidase AffiniPure Donkey Anti-Mouse IgG (1:10,000)	Jackson ImmunoResearch	715-035-151
Peroxidase AffiniPure Donkey Anti-Rabbit IgG (1:5,000)	Jackson ImmunoResearch	711-035-152
Bacterial and virus strains		
DH5α (high efficiency)	New England Biolabs	C2987H
Chemicals, peptides, and recombinant proteins		
Proteinase K	Invitrogen	25530031
dNTP Mix, PCR Grade (800 µL)	Qiagen	201901
Tail lysis buffer	Pelletier Lab	
UltraPure™ DNA Typing Grade™ 50× TAE Buffer	Invitrogen	24710030
Triton X-100 Lysis Buffer (NaCl, 150 mM; Triton X-100, 1% (v/v); Tris ph8.0, 50 mM)	Pelletier Lab	
PhosSTOP™ Phosphatase Inhibitor Cocktail tablets	Sigma-Aldrich	4906837001
cOmplete™, EDTA-free Protease Inhibitor Cocktail tablets	Sigma-Aldrich	4693132001
EcoRI	New England Biolabs	R3101L
Ponceau S	Sigma	P7170-1L
Tris-Buffered Saline with Tween®20 (TBST-10×)	Cell Signaling Technology	9997
Low EDTA TE 1 × 10 mM Tris-HCl 0.1 mM EDTA, pH 8.0	Quality Biological	351-324-721
Critical commercial assays		
Taq DNA Polymerase (1000 U)	Qiagen	201205
ExoSAP-IT™ PCR Product Cleanup Reagent	Invitrogen	78201
BigDye™ Terminator v3.1 Cycle Sequencing Kit	Applied Biosciences	4337456
BigDye XTerminator™ Purification Kit	Applied Biosciences	4376487
TOPO™ TA Cloning™ Kit for Subcloning, with One Shot™ TOP10 chemically competent E. coli cells	Invitrogen	K450001
QIAprep Spin Miniprep Kit	Qiagen	27106X4
Pierce™ BCA Protein Assay Kit	Thermo Fisher	23227
Clarity™ Max Western ECL Substrate	Bio-Rad	170506
RNeasy Maxi Kit	Qiagen	75162
RNase-Free DNase Set	Qiagen	79254

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ProtoScript® II First Strand cDNA Synthesis Kit	New England Biolabs	E6560L
TaqMan 2× Master Mix	Applied Biosystems	4444557
Scyl1 TaqMan Gene Expression Assay Probe (FAM)	Applied Biosystems	Mm00452459_m1
Gapdh TaqMan Gene Expression Assay Probe (FAM)	Applied Biosystems	Mm99999915_g1
OneTaq One-Step RT-PCR Kit	New England Biolabs	E5315S
Experimental models: Organisms/strains		
Scyl1^{Alv4} mouse model	Pelletier Lab	Name of the allele: SCY1-like 1 (S. cerevisiae); targeted mutation 1.1, Stephane Pelletier Allele symbol: Scyl1$^{tm1.1Spel}$
Scyl1^{Alv44} mouse model	Pelletier Lab	Name of the allele: SCY1-like 1 (S. cerevisiae); targeted mutation 2, Stephane Pelletier Allele symbol: Scyl1^{tm2Spel}
Scyl1^{-} mouse model	Pelletier Lab	Name of the allele: SCY1-like 1 (S. cerevisiae); targeted mutation 1, Stephane Pelletier Allele symbol: Scyl1^{tm1Spel}
B6.C-Tg(CMV-cre)1Cgn/J mouse model	The Jackson Laboratory	006054
Oligonucleotides		
Scyl1_Alv4_E3_HDR	Integrated DNA Technologies	AGATCGTGAAAGCCCTCAGCTTCCTGGTCA ACGACTGCAACCTCATCCACAATAATGTGTA AGTAATAACTTCGTATAGCATACATTATACGA AGTTATTCAAGGTTAGAAGACAGGTTTAAGG AGACCAATAGAAACTGGGCTTGTCGAGACA GAGAAGACTCTTGCGTTTCTGATAGGCACCT ATTGGTCTTACTGACATCCACTTTGCCATAAC TTCGTATAGCATACATTATACGAAGTTATTTT CTCTCCACAGCTGCATGGCCGCTGTGTTTGT GGACAGGGCTGGCGAGTGGAAACTTGGGG GTCTGGACTA
S1E154D-F1	Integrated DNA Technologies	TACTCTCCTCAGGCCCTCAC
S1E154D-R1	Integrated DNA Technologies	GAAGCACCGAACACCCAAAC
Scyl1_Alv4_F51	Integrated DNA Technologies	GTGCCTCCACATCGTGACAG
Scyl1_Alv4_R52	Integrated DNA Technologies	CTCCGGGGGATCATACTGCT
Cre66	Integrated DNA Technologies	CCTGCGGTGCTAACCAGCGTT
Cre99	Integrated DNA Technologies	TGGGCGGCATGGTGCAAGTT
M13R	Integrated DNA Technologies	CAGGAAACAGCTATGAC
T7	Integrated DNA Technologies	TAATACGACTCACTATAGG
RT-Scyl1_F21	Integrated DNA Technologies	CGCAGTGTCCATCTTCGTGTA
RT-Scyl1_R51	Integrated DNA Technologies	CCCGGCAGTTCTGCAGGAA
Scyl1_Alv4_OT1_F1	Integrated DNA Technologies	CAAGTTATGTAAATGTGGACTCTCA
Scyl1_Alv4_OT1_R2	Integrated DNA Technologies	ACAGGACCAAGCACCTCTTC
Scyl1_Alv4_OT2_F1	Integrated DNA Technologies	GCCTAGAAGCTCAGAATACCCA
Scyl1_Alv4_OT2_R2	Integrated DNA Technologies	CACTTCCTGACCCTGGCATT
Scyl1_Alv4_OT3_F1	Integrated DNA Technologies	ATGGGCAACTGAGAGCAGAG
Scyl1_Alv4_OT3_R2	Integrated DNA Technologies	ACATCAGAAGATCCCGTGGC
Software and algorithms		
NCBI Gene		https://www.ncbi.nlm.nih.gov/gene/
UCSC Genome Browser		https://genome.ucsc.edu/
Cas-OFFinder		http://www.rgenome.net/cas-offinder/
Primer-BLAST		http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome
SnapGene		https://www.snapgene.com/
ImageJ		https://imagej.nih.gov/ij/
NEBioCalculator®		https://nebiocalculator.neb.com/
Other		
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems	4306737
SeqStudio™ Septa 96-Well	Applied Biosystems	A35641
10% Criterion™ XT Bis-Tris Protein Gels	Bio-Rad	3450112
Nitrocellulose Membranes	Bio-Rad	1620233

MATERIALS AND EQUIPMENT

Tail Lysis Buffer		
Reagent	Final concentration	Amount
KCl (2 M)	500 mM	12.5 mL
Tris pH8.3 (1 M)	100 mM	5.0 mL
Gelatin	0.1 mg/mL	5.0 mg
NP40 (100%)	1% (v/v)	0.5 mL
Tween20 (100%)	1% (v/v)	0.5 mL
Proteinase K (50 mg/mL)	500 μg/mL	500 μL
ddH_2O	N/A	31 mL
Total	N/A	50 mL

Note: Gelatin takes several hours to dissolve. Before adding Proteinase K, the solution can be autoclaved to help dissolve gelatin.

STEP-BY-STEP METHOD DETAILS

Delivery of CRISPR-Cas9 reagents

Timing: several weeks

This section discusses the delivery of SpCas9, the sgRNA, and the HDR template into zygotes. Delivery of these reagents occurs by two main routes: electroporation or microinjection of pronuclear stage zygotes. Before delivery can occur, the CRISPR-Cas9 reagents must be prepared and combined into a mix. While similar, mixes for electroporation and microinjection differ in the amounts of each reagent to be delivered, as shown in the tables below. Microinjection mixes with Cas9 mRNA transcript can be aliquoted and stored at −80°C until use.

Microinjection Mix using Cas9 mRNA transcript		
Reagent	Final concentration	Amount
Cas9 mRNA transcript	100 ng/μL	8 μg
sgRNA	50 ng/μL	4 μg
HDR template (100 μM)	1.25 pmol/μL	1 μL
Low EDTA TE (1 × 10 mM Tris-HCl 0.1 mM EDTA, pH 8.0)	N/A	To 80 μL
To assist with molar to mass conversion, please visit NEBioCalculator® version 1.15.0 at https://nebiocalculator.neb.com/.		

Microinjection Mix using Cas9 Protein		
Reagent	Final concentration	Amount
Cas9 Protein	0.33 μM	26.4 pmol
sgRNA	50 ng/μL	4 μg
HDR template (100 μM)	1.25 pmol/μL	1 μL
Low EDTA TE 1× (10 mM Tris-HCl 0.1 mM EDTA, pH 8.0)	N/A	To 80 μL

Electroporation Mix using Cas9 mRNA transcript		
Reagent	Final concentration	Amount
Cas9 mRNA transcript	200 ng/μL	2 μg
sgRNA	400 ng/μL	4 μg
HDR template (100 μM)	20 μM	2 μL
TE Buffer 1× (10 mM Tris-HCl 1 mM EDTA, pH 7.4)	N/A	To 10 μL

Electroporation Mix using Cas9 Protein		
Reagent	Final concentration	Amount
Cas9 Protein	8 μM	80 pmol
sgRNA	400 ng/μL	4 μg
HDR template (100 μM)	20 μM	2 μL
5× RNP Buffer + 5 mM TCEP[6]	1×	2 μL
TE Buffer 1× (10 mM Tris-HCl 1 mM EDTA, pH 7.4)	N/A	To 10 μL

Electroporation and/or microinjection of pronuclear stage zygotes are usually performed by transgenic core facilities. For detailed procedures on electroporation or microinjection, please refer to the following publications: Efficient mouse genome engineering by CRISPR-EZ technology[6] and Manipulating the Mouse Embryo; A Laboratory Manual, Fourth Edition.[7] For the generation of *Scy-I1*^{Alv4} mice, microinjection was used. Additional models are being generated using both microinjection and electroporation with similar results.

PCR optimization

Timing: 2 days

This section describes the procedure to optimize PCR reactions for the characterization of potential founders. For all genotyping procedures, Qiagen Taq Polymerase (Qiagen, 201205) is used. Control genomic DNA can be obtained from a wild-type mouse tail biopsy. Tail biopsies are prepared as follows:

1. Prepare Tail Lysis Buffer, as shown in the "materials and equipment" section.
2. Extract genomic DNA by adding 300 μL of Tail Lysis Buffer to the biopsy and incubating at 55°C for 4–16 h.
3. The next day, incubate the digested biopsies at 95°C for 10 min to heat inactivate the Proteinase K.

To optimize PCR genotyping conditions, the optimal annealing temperature of a primer pair can be determined by testing identical reactions across a range of annealing temperatures and two concentrations of $MgCl_2$. This procedure can be performed prior to obtaining potential founders so that when mice are obtained, genotyping can be performed quickly.

4. For a given pair of primers, prepare, on ice, separate PCR mixes with low and high $MgCl_2$ concentrations, as detailed below.

	Low $MgCl_2$	High $MgCl_2$
Reagents	Volume (μL) per Reaction	Volume (μL) per Reaction
10× CoralLoad PCR Buffer	5	5
5× Q-Solution	10	10
$MgCl_2$	–	2
dNTP (10 mM each)	1	1
Forward primer (100 μM)	0.1	0.1
Reverse primer (100 μM)	0.1	0.1
Taq DNA Polymerase	0.5	0.5
Wild-type genomic DNA (~50 ng/μL)	0.5	0.5
ddH_2O to 50 μL	32.8	30.8

5. Distribute 50 μL of master mix per reaction in 8 wells of a 96 well plate so that each well corresponds to a different annealing temperature.

6. Use the cycling conditions displayed in the table below.

Step	Number of cycles	Temperature	Time
1	1	94°C	4 min
2	35	94°C	30 s
		50°C–70°C	30 s
		72°C	60 s
3	1	72°C	10 min
4	1	4°C	Hold

7. Load 10 μL of PCR product on a 1%–2% agarose gel at 120 V for approximately 40 min in 1× TAE Buffer.
   a. Image gel and save picture for your records.
8. Select the best amplification condition.

On-target PCR genotyping

Timing: 1 week

This procedure describes the characterization, at the genomic level, of potential founders. To analyze on-target gene editing, the guide target region is PCR amplified and analyzed by gel electrophoresis and direct Sanger sequencing. Founders with potential insertion of the AIv4 cassette are further analyzed by TOPO® TA cloning® and Sanger sequencing (Figures 2 and 4).

9. After receiving biopsies, extract genomic DNA by adding 300 μL of Tail Lysis Buffer to each sample and incubating at 55°C for 4–16 h.
10. The next day, incubate the digested biopsies at 95°C for 10 min to heat inactivate the Proteinase K.
11. For each biopsy, set up PCR reactions on ice using previously established PCR conditions. The table below describes how to make the PCR mix with optional $MgCl_2$. Also include:
    a. A positive control (wild-type mouse genomic DNA).
    b. A negative control (no DNA).

	Low $MgCl_2$	High $MgCl_2$
Reagents	Volume (μL) per Reaction	Volume (μL) per Reaction
10× CoralLoad PCR Buffer	5	5
5× Q-Solution	10	10
$MgCl_2$	–	2
dNTP (10 mM each)	1	1
Forward primer (100 μM)	0.1	0.1
Reverse primer (100 μM)	0.1	0.1
Taq DNA Polymerase	0.5	0.5
Wild-type genomic DNA (~50 ng/μL)	0.5	0.5
ddH_2O to 50 μL	32.8	30.8

12. Use the cycling conditions, as illustrated below.

Step	Number of cycles	Temperature	Time
1	1	94°C	4 min
2	35	94°C	30 s
		58°C (or as determined from the PCR Optimization step)	30 s
		72°C	60 s

(*Continued on next page*)

Continued

Step	Number of cycles	Temperature	Time
3	1	72°C	10 min
4	1	4°C	Hold

13. Following amplification, put aside:
    a. 10 μL to run on a 2% agarose gel.
    b. 15 μL for TOPO® TA cloning® and Sanger sequencing.
    c. 25 μL for sample preparation using ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix, 78200) and direct Sanger sequencing.

TOPO® TA cloning®

Timing: 1 week

14. To the 15 μL PCR product set aside for TOPO® TA cloning® in step 13.b, add 1 μL Taq DNA Polymerase (Qiagen, 201205) and 1 μL dNTP Mix (Qiagen, 201901).
    a. Incubate at 72°C for 10 min.
15. Using the TOPO® TA Cloning® Kit for Subcloning (Invitrogen, K450001), set up the TOPO® TA cloning® reaction(s) at room temperature, as described in the table below.

Reagent	Volume
PCR Product	4 μL
Salt Solution	1 μL
TOPO® Vector	1 μL

16. Gently mix the reaction and allow to sit at room temperature for 1 h.
17. Transform DH5α competent *E. coli* (New England Biolabs, C2987H), according to the TOPO® TA cloning® kit user guide using blue/white selection (https://tools.thermofisher.com/content/sfs/manuals/topota_man.pdf).
18. Pick 30 white colonies per plate and culture them overnight in LB medium containing 50 μg/mL ampicillin.
19. Isolate plasmid DNA using QIAprep Spin Miniprep Kit (Qiagen, 27106X4) (https://www.qiagen.com/us/resources/resourcedetail?id=22df6325-9579-4aa0-819c-788f73d81a09&lang=en).
20. Digest 5 μL of each plasmid using EcoRI (New England Biolabs, R3101L).
21. Run digested plasmids on a 2% agarose gel at 120 V for 1 h.
    a. PCR fragments of various sizes should be observed. The larger fragment will likely contain the AIv4 cassette, whereas shorter fragments will likely be wild type or contain indels.An example of digested plasmids containing various fragment sizes is presented in Figure 4B).
    b. Plasmids containing PCR products with the larger fragment (corresponding to the AIv4 cassette) should be sequenced using the M13R and T7 primers, following the protocol in the "sanger sequencing" section of this manuscript.

Sanger sequencing

Timing: 1 week

Direct sequencing of post-PCR products and sequencing of TOPO® TA cloning® plasmids are both important for the characterization of mouse models generated using the DECAI approach. The former allows for the detection of editing events and to determine the rate of editing in

potential founder animals. The latter is used to confirm the proper arrangement of the allele (Figures 2 and 4).

22. For direct sequencing of post-PCR products, set up ExoSAP-IT PCR Product Cleanup (Affymetrix, 78200) reactions as follows:
    a. Combine 25 μL of a PCR product with 1 μL of ExoSAP-IT reagent (Affymetrix, 78200) in a well of a 96-well plate or 200 μL microfuge tube.
    b. Incubate at 37°C for 30 min to degrade remaining primers and nucleotides, then at 80°C for 15 min to inactivate ExoSAP-IT reagent.
23. Set up sequencing reactions using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences, 4337456) in a 96-well plate, as demonstrated below.

Reagent	Volume (μL) per reaction
BigDye™ Terminator v3.1	0.5
5× Sequencing Buffer	2
Primer (2 μM)	2
Template (PCR product or plasmid)	5.5 (~100 ng)
ddH_2O	To 10 μL

    a. Spin down the 96-well plate to ensure all reagents are at the bottom of the well.
    b. Transfer the 96-well plate to a thermal cycler for cycle sequencing, using the conditions below.

Step	Number of cycles	Temperature	Time
1	1	96°C	1 min
2	25 (Ramp at 1°C/s)	96°C	10 s
		50°C	5 s
		60°C	1 min
3	1	4°C	Hold

    c. When cycle sequencing has finished, centrifuge the 96-well plate at 1,000 × *g* for 1 min.
    d. Premix the BigDye XTerminator™ Solution and SAM™ Solution (Applied Biosystems, 4376493), as shown below:
        i. Make sure the BigDye Xterminator™ Solution (Applied Biosystems, 4376493) is homogenous before pipetting.

Reagent	Volume (μL) per reaction
BigDye™ XTerminator Solution	10
SAM Solution	45

    e. Dispense 55 μL of the premixed solution per well of a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, 4306737).
    f. Transfer the total volume (10 μL) of each cycle sequencing reaction from the 96-well plate into the MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, 4306737) with the premixed XTerminator solution.
    g. Seal the MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, 4306737) with clear adhesive film and vortex the plate for 30 min.
    h. When the plate has finished shaking, centrifuge at 1,000 × *g* for 2 min.
    i. Without disturbing the pellet, carefully remove the adhesive film and replace it with a SeqStudio™ Septa 96-Well (Applied Biosystems, A35641) on the plate. Immediately transfer the MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, 4306737) to the SeqStudio™ Genetic Analyzer (Applied Biosystems, A35644) and begin the capillary electrophoresis run.

24. Analyze sequencing data using SnapGene.

Off-target PCR genotyping

Timing: 1 week

One downside of using CRISPR-Cas9 technology for genome editing is the potential to introduce mutations at off-target locations. However, the use of a hit-and-run strategy, as described here, where Cas9 mRNA transcript or Cas9 proteins are used, rarely results in the introduction of off-target mutations. Nevertheless, it is recommended to analyze a few potential off-target sites for genetic alterations.

Off-target analysis should be performed on genomic DNA from each potential founder. For each potential off-target, setup PCR reactions and direct sequencing using specific off-target primer pairs, as described in the "On-target PCR genotyping" and "sanger sequencing" sections.

Animal breeding

Timing: 1 year

After characterization of founder animals, mice bearing the AIv4 cassette must be outbred to wild-type mice for at least two subsequent generations. From this generation, heterozygous mice can be crossed together to produce mice homozygous for the artificial intron (*Scyl1*$^{Alv4/Alv4}$) (Figures 3 and 4). These mice are then bred to B6.C-Tg(CMV-cre)1Cgn/J mice to produce mice that are CMV-Cre+ and heterozygous for the recombined allele (CMV-Cre+;*Scyl1*$^{+/Alv4\Delta}$). Finally, mice homozygous for the recombined allele are produced from a cross between CMV-Cre+;*Scyl1*$^{+/Alv4\Delta}$ and *Scyl1*$^{Alv4/Alv4}$ mice (Figure 3).

For the routine genotyping of mice bearing *Scyl1*^{Alv4} or *Scyl1*$^{Alv4\Delta}$ alleles, the following primers were used:

Scyl1_AIv4_F51: GTGCCTCCACATCGTGACAG.

Scyl1_AIv4_R52: CTCCGGGGGATCATACTGCT.

Allele	Scyl1_AIv4_F51 + Scyl1_AIv4_R52
Scyl1^{-}	304 bp
Scyl1^{Alv4}	505 bp
Scyl1$^{Alv4\Delta}$	358 bp

For the routine genotyping of CMV-Cre mice, the following primers were used:

Cre66: CCTGCGGTGCTAACCAGCGTT.

Cre99: TGGGCGGCATGGTGCAAGTT.

Allele	Cre66 + Cre99
CMV-Cre+	470 bp
CMV-Cre-	0 bp

Functional validation by western blotting

Timing: 1 week

The functionality of both *Scyl1*^{Alv4} and *Scyl1*$^{Alv4\Delta}$ alleles must be demonstrated at the protein level. To this end, tissues are collected from these animals and SCYL1 expression is examined by western blotting (Figure 5).

The following steps describe the procedure to collect tissues and analyze SCYL1 expression by western blot.

25. Euthanize selected mice using CO_2 and cervical dislocation.
26. Collect the tissues of interest and snap freeze in liquid nitrogen. Store at −80°C until use.
    a. Thaw tissue samples on ice before lysis.
27. Lyse the tissues using Triton X-100 (Sigma-Aldrich, T8787-250ML) Lysis Buffer (see table below) supplemented with PhosSTOP™ Phosphatase Inhibitor Cocktail tablets (Sigma-Aldrich, 4906837001) and cOmplete™, EDTA-free Protease Inhibitor Cocktail tablets (Sigma-Aldrich, 4693132001).

Reagent	Final concentration	Amount
5 M NaCl	150 mM	30 mL
Triton X-100 (100%)	1%	10 mL
1 M Tris (pH 8.0)	50 mM	50 mL
ddH_2O	N/A	910 mL
Total	N/A	1 L

    a. Add 5 volumes of Triton X-100 Lysis Buffer to the sample (approximately 5 mL for mouse cerebrum).
    b. Shred the tissue using a tissue homogenizer on ice.
    c. Incubate the homogenate on ice for 30 min.
    d. Aliquot the homogenate in microfuge tubes and centrifuge for 5 min at 12,000 rpm at 4°C.
    e. Transfer the supernatant to new microfuge tubes and store at −80°C until use.
28. Quantify protein concentration using Pierce™ BCA Protein Assay Kit (ThermoFisher, 23227) according to the manufacturer's recommendations (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf). For mouse cerebrum, the concentration of total protein should be approximately 1 mg/mL.
29. Analyze protein expression by western blotting.
    a. Load 5 μg of total protein extract on 10% Criterion™ XT Bis-Tris Protein Gels (Bio-Rad, 3450112).
    b. Run at 200 V for 60 min at room temperature.
    c. Transfer proteins onto Nitrocellulose Membranes (Bio-Rad, 1620233) using the Trans-Blot® Turbo™ Transfer system (Bio-Rad, 17001915) using the following procedure:
        i. Under "Select Protocol", select "List".
        ii. From the list of "Protocols", select "Bio-Rad".
        iii. Under "Gels Per Cassette", select "2 midi or 1 midi".
        iv. From the "Bio-Rad Protocol List", select "StandardSD 25V – 1.0A – 30M" and press "Run".
    d. Rinse membranes twice with ddH_2O.
    e. Remove ddH_2O and add 10–20 mL of Ponceau S (Sigma, P7170-1L, 0.1% (w/v) in 5% acetic acid). Incubate for 5 min on rocker to stain membranes.
    f. Remove Ponceau S and wash twice more with ddH_2O.
        i. After washing, image the membrane and save the picture for your records.

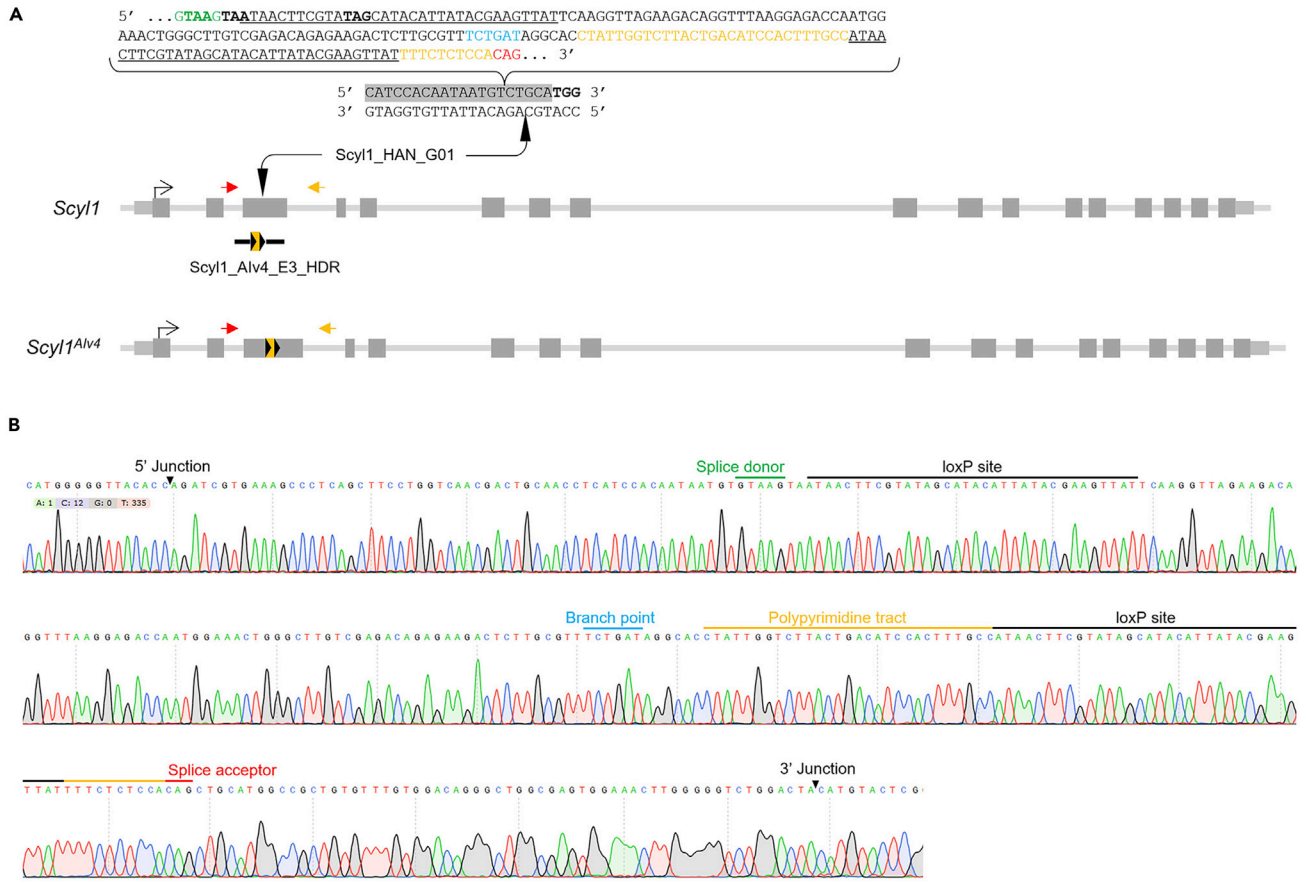

Figure 2. Targeting strategy to engineer the *Scyl1*^{Alv4} allele

(A) Schematic representation of the *Scyl1* gene, the sgRNA target site within exon 3 of Scyl1 and the HDR template containing sequences encoding the Alv4 cassette flanked by homology arms of 58 and 60 nucleotides. A black arrowhead indicates the location of the Cas9 cleavage site in exon 3. Grey boxes indicate exons. Gray lines indicate introns. The yellow box flanked by two back triangles represents the Alv4 cassette. The black arrow represents the translation start site. Yellow and red arrows represent the genotyping primers S1E154D-F1 and S1E154D-R2.

(B) Sanger sequencing chromatogram of the TOPO-TA cloned PCR fragment obtained from a founder animal. 5′ and 3′ junctions are indicated by black arrowheads. The splice donor site is indicated in green. loxP sites are shown in black. The branch point is shown in blue. The bipartite polypyrimidine tract is shown in yellow. The splice acceptor site is shown in red.

g. After transfer, Ponceau S staining and imaging, wash nitrocellulose membrane with 25 mL 1× TBST (Cell Signaling Technology, 9997, 137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20, pH 7.6) for 5 min at room temperature.
h. Incubate membrane in 25 mL of blocking buffer for 1 h at room temperature.
i. Blocking buffer is composed of 1× TBST with 5% w/v nonfat dry milk.
    i. Incubate membrane in 10 mL primary antibody dilution buffer (7645-AP 1:1,000; 7691-AP 1:1,000; β-actin 1:3,000) with gentle agitation overnight at 4°C. For multiple antibody visualizations, separate blots need to be run. It is not recommended to strip membranes.
j. Wash three times for 5 min each with 25 mL of 1× TBST.
k. Incubate membrane with the species appropriate HRP-conjugated secondary antibody at a 1:10,000 dilution in 20 mL of blocking buffer with gentle agitation for 1 h at room temperature.
l. Wash three times for 5 min each with 25 mL of 1× TBST.
m. For protein detection using Bio-Rad Clarity™ Western ECL Substrate, #170506:
    i. Immediately before use, mix 3 mL of Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent together in a 15 mL conical tube.
    ii. Incubate the membrane in the substrate for 2 min.

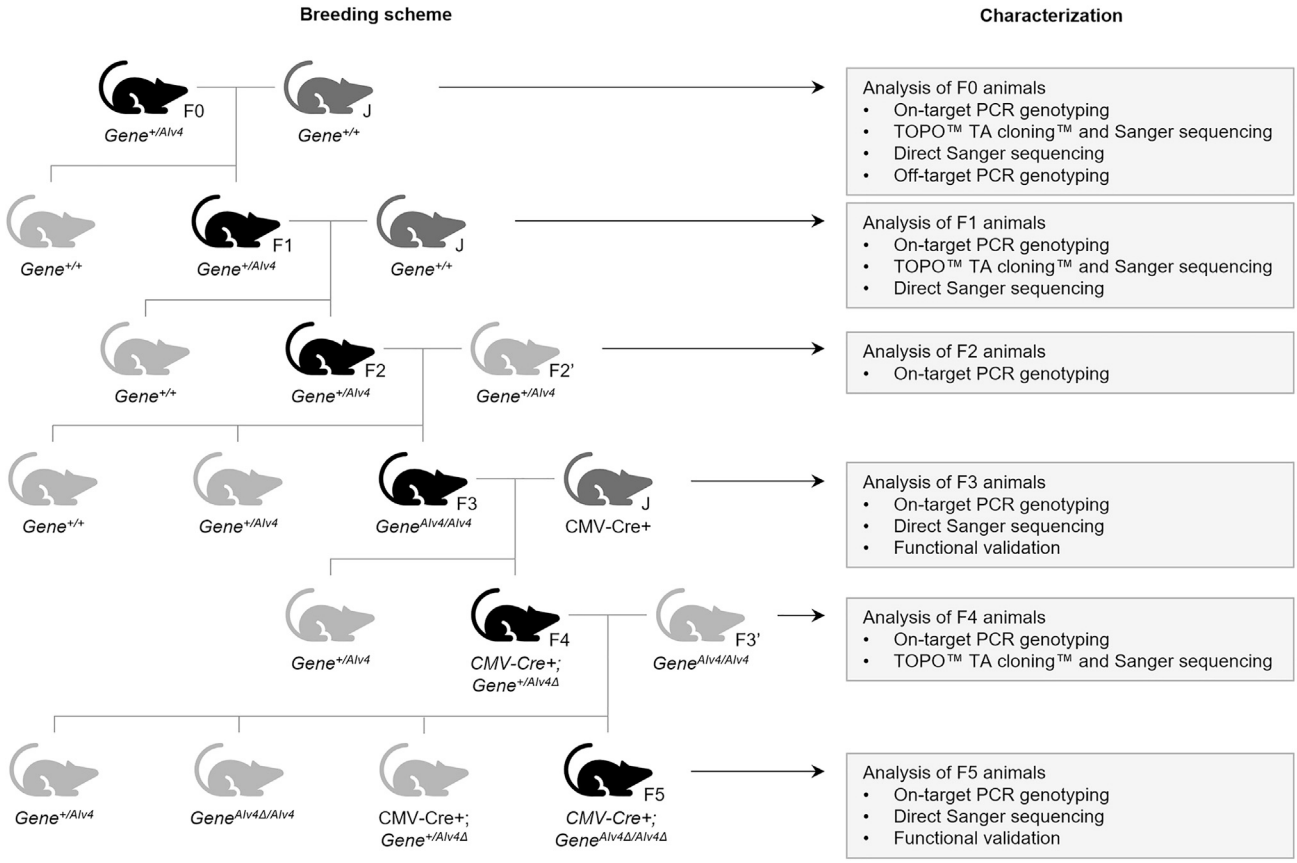

Figure 3. Breeding scheme and validation steps

Breeding scheme involved in the generation of *Scyl1*$^{Alv4/Alv4}$ and CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice. F0, founder mouse; F1, mice obtained from the outbreed of F0 mice with wild-type mice (J) from a commercial source (e.g., Jackson Laboratory); F2, mouse obtained from a second outbreeding step between F1 mice with wild-type mice from a commercial source (J); F3, mice obtained from interbreeding of F2 and F2′ animals from distinct parents and grand-parents; F4, mice obtain from the breeding of CMV-Cre+ mice from an outside source (Jackson Laboratory) with F3 mice; F5, mice obtained from the breeding of CMV-Cre+; *Scyl1*$^{+/Alv4\Delta}$ with *Scyl1*$^{Alv4/Alv4}$ (F3) mice. Founder animals must be characterized at the genomic level using PCR amplification, TOPO® TA cloning® and Sanger sequencing. F1 and F2 mice must be characterized at the genomic level using PCR amplification, TOPO® TA cloning® and Sanger sequencing to confirm germline transmission of the proper allele. F3 mice must be genotype at the genomic and protein level using PCR amplification, direct Sanger sequencing and western blotting. F4 mice must be characterized at the genomic level using PCR amplification and direct Sanger sequencing. The *Scyl1*^{Alv4} allele should also be characterized by TOPO® TA Cloning® and Sanger sequencing. F5 mice must be characterized at the genomic and protein levels using PCR amplification, direct Sanger sequencing and western blotting.

iii. Image membrane using chemiluminescence program of the ChemiDoc™ MP Imaging System (Bio-Rad, 17001402).

30. Quantify protein expression using ImageJ (https://imagej.nih.gov/ij/).
    a. Upload the image into ImageJ and invert.
        i. Under the "Edit" tab select "Invert".
    b. Draw a small box around the band corresponding to SCYL1 and measure the mean gray value.
        i. Under the "Analyze" tab select "Measure".
    c. Move the box without altering its dimensions and measure the mean gray value of the background.
    d. Subtract the mean gray value of the background from the mean gray value of the band.
    e. Repeat steps 30.a–d for the β-actin western blot.
    f. Divide the mean gray value obtained in step iv for SCYL1 by the mean gray value of the β-actin band to determine the relative SCYL1 expression.
    g. Average the values of the relative SCYL1 expression compared to β-actin.

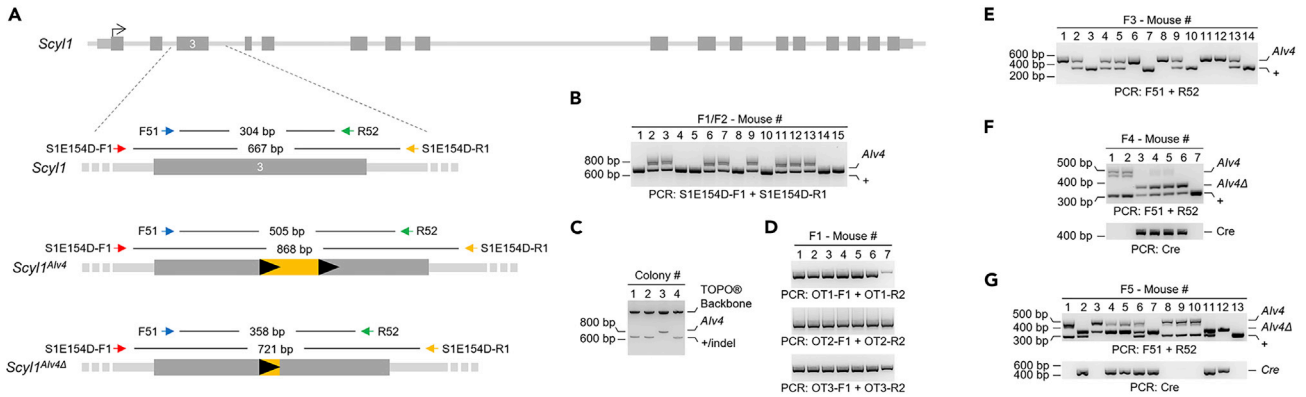

Figure 4. Representative PCR based genotyping and TOPO® TA Cloning® results obtained from each breeding step

(A) Schematic representation of the *Scyl1* gene highlighting the location and banding pattern of PCR genotyping primers for the characterization of founder animals and routine genotyping. S1E154D-F1 (red) and S1E154D-R1 (yellow) were used for genotyping F0, F1, and F2 mice. Using this primer pair, amplicons of 667 bp, 868 bp, and 721 bp are obtained for wild-type, $Scyl1^{AIv4}$, and $Scyl1^{AIv4\Delta}$ alleles, respectively. For routine genotyping, a second primer pair, Scyl1_AIv4_F51 (blue) and Scyl1_AIv4_R52 (green), were designed to more easily distinguish the wild-type allele from the $Scyl1^{AIv4\Delta}$ allele. Using this second set of primers, amplicons of 304 bp, 505 bp, and 358 bp are obtained for wild-type, $Scyl1^{AIv4}$, and $Scyl1^{AIv4\Delta}$ alleles, respectively.

(B) PCR genotyping of $Scyl1^{+/+}$, $Scyl1^{+/AIv4}$, F1 or F2 animals obtained from F0 or F1 outbreeds to wild-type C57BL/6J mice. Amplicons of 667 bp correspond to the wild-type allele, whereas amplicons of 868 bp correspond to the $Scyl1^{AIv4}$allele.

(C) Representative image of digested plasmids obtained from TOPO® TA Cloning® of PCR products obtained from founder animals. The ~868 bp upper band likely contains a PCR product corresponding to the AIv4 allele, while the shorter fragments likely correspond to the wild-type allele or contain indels. This is further confirmed by Sanger sequencing.

(D) PCR genotyping of potential off-target cleavage sites in F1 animals. Three potential off-target sites were identified, as described in the "guide sequence selection" section of this manuscript. Primer pairs were designed to amplify each site: Scyl1_AIv4_OT1-F1 and Scyl1_AIv4_OT1-R2 generate a 504 bp fragment corresponding to off-target site 1, Scyl1_AIv4_OT2-F1 and Scyl1_AIv4_OT2-R2 generate a 563 bp fragment corresponding to off-target site 2, and Scyl1_AIv4_OT3-F1 and Scyl1_AIv4_OT3-R2 generate a 474 bp fragment corresponding to off-target site 3. Direct Sanger sequencing of these PCR products was used to confirm that no off-target editing occurred at these sites.

(E) PCR genotyping of $Scyl1^{+/+}$, $Scyl1^{+/AIv4}$, and $Scyl1^{AIv4/AIv4}$ F3 animals obtained from F2 outbreeds. Amplicons of 304 bp correspond to the wild-type allele, whereas amplicons of 505 bp correspond to the AIv4 allele.

(F) PCR genotyping of mice obtained from CMV-Cre+ mice crossed with $Scyl1^{AIv4/AIv4}$ mice. Amplicons of 304, 358, and 505 bp correspond to the wild-type, $Scyl1^{AIv4\Delta}$, and $Scyl1^{AIv4}$ alleles, respectively. In the lower panel, CMV-Cre+ mice are identified by a separate PCR reaction: CMV-Cre+ mice produce a 470 bp amplicon, whereas wild-type mice do not produce a PCR product.

(G) PCR genotyping of $Scyl1^{+}$, $Scyl1^{AIv4}$, and $Scyl1^{AIv4\Delta}$ alleles. Amplicons of 304, 358, and 505 bp correspond to the wild-type, $Scyl1^{AIv4}$, and $Scyl1^{AIv4\Delta}$ alleles, respectively. A separate PCR reaction identifies CMV-Cre+ mice, which produce a 470 bp amplicon, whereas wild-type mice do not produce a PCR product.

h. Graph the values obtained in the previous step for each genotype.

Functional validation by RT-qPCR

Timing: 1 week

Because inactivation of the allele is thought to occur via the nonsense-mediated mRNA decay pathway, its functionality can also be assessed at the RNA transcript level. To this end, total RNA is extracted from control and $Scyl1^{AIv4\Delta/AIv4\Delta}$ mice and examined by RT-qPCR (Figure 6).

The following steps describe the procedure to extract RNA from tissues and analyze *Scyl1* transcript expression by RT-qPCR.

31. Extract total RNA from the cerebellum of CMV-Cre+;$Scyl1^{+/+}$ and CMV-Cre+;$Scyl1^{AIv4\Delta/AIv4\Delta}$ mice using the RNeasy Maxi Kit (Qiagen, 75162) with the RNase-Free DNase Set (Qiagen, 79254), according to the manufacturer's protocol (https://www.qiagen.com/us/resources/resourcedetail?id=a58c7b03-a25f-4ba7-9825-8fa473ba784f&lang=en).

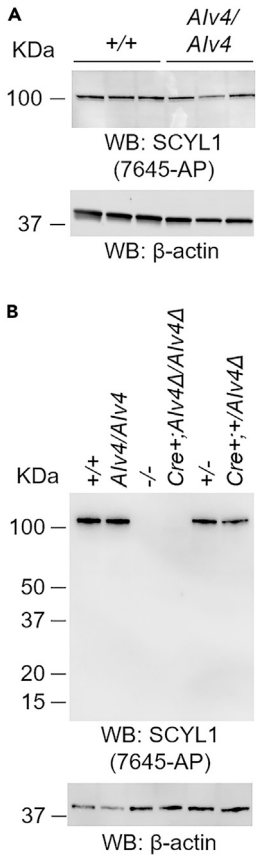

Figure 5. SCYL1 expression in *Scyl1*$^{Alv4/Alv4}$ and *Scyl1*$^{Alv4\Delta/Alv4\Delta}$

(A) SCYL1 expression in the brain of *Scyl1*$^{+/+}$ and *Scyl1*$^{Alv4/Alv4}$ mice, as detected by western blotting (top panel). β-actin western blot (bottom panel) serves as loading control.

(B) SCYL1 protein expression in the brain of *Scyl1*$^{+/+}$, *Scyl1*$^{Alv4/Alv4}$, *Scyl1*$^{-/-}$, CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$, *Scyl1*$^{+/-}$, and CMV-Cre+;*Scyl1*$^{+/Alv4\Delta}$ mice, as detected by western blotting (top panel), using the anti-SCYL1 7645-AP antibody, which detects the C-terminal segment of SCYL1. Note the absence of SCYL1 proteins or any truncated isoforms in *Scyl1*$^{-/-}$, CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice. β-actin western blot (bottom panel) serves as loading control.

32. Quantify the RNA using the ThermoScientific™ NanoDrop™ Spectrophotometer (ThermoFisher, 13-400-519). For mouse cerebella, approximately 80 μg of total RNA is obtained. A ratio of absorbance at 260 nm and 280 nm for a high-quality RNA preparation should be around 2.0.
33. Assess the quality of the RNA using the Agilent TapeStation system (Agilent, G2992AA), according to the manufacturer's recommendations (https://www.agilent.com/cs/library/usermanuals/public/G2964-90000_TapeStation_USR_ENU.pdf).
34. Using the ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs, E6560L) with the d(T)$_{23}$ VN primer, produce cDNA from 100 ng of total RNA, as described by the manufacturer (https://www.neb.com/protocols/2013/01/23/first-strand-cdna-synthesis-protocols-e6560).
35. Measure *Scyl1* transcript expression using TaqMan 2× Master Mix (Applied Biosystems, 4444557) and TaqMan Gene Expression Assay Probe Mm00452459_m1(FAM). Normalize against *Gapdh* expression using TaqMan Gene Expression Assay Probe Mm99999915_g1 (FAM).
36. Quantify the relative expression of *Scyl1* using the ΔΔCt method.
37. Graph the values obtained in the previous step for each genotype.

Alternative splicing analysis

Timing: 1 week

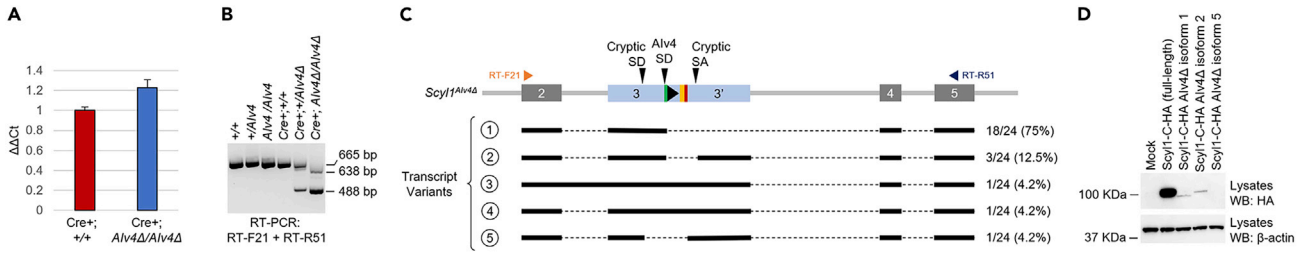

Figure 6. Alternative splicing events produced from the recombined Alv4 allele

(A) *Scyl1* transcript expression in the cerebella of CMV-Cre+;*Scyl1*$^{+/+}$ and CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice. Data is expressed as mean ± SEM. RNA extracts from 3 CMV-Cre+;*Scyl1*$^{+/+}$ and 3 CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice were analyzed in triplicates.

(B) RT-PCR products generated from cerebellar total RNA extracts of *Scyl1*$^{+/+}$, *Scyl1*$^{+/Alv4}$, *Scyl1*$^{Alv4/Alv4}$, CMV-Cre+;*Scyl1*$^{+/+}$, CMV-Cre+;*Scyl1*$^{+/Alv4\Delta}$, and CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice. Amplicons of 665, 638, and 488 bp, corresponding to the mature form of the *Scyl1* transcript, as well as shorter transcript variants 1 and 2 were obtained. These shorter transcripts were observed only in CMV-Cre+;*Scyl1*$^{+/Alv4\Delta}$ and CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice.

(C) Schematic representation of the five most abundant RNA transcripts observed in CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice (transcripts variants labeled 1–5). RT-PCR products from CMV-Cre+;*Scyl1*$^{Alv4\Delta/Alv4\Delta}$ mice were TOPO cloned. Plasmid DNA was recovered from 24 clones and analyzed by Sanger sequencing. The frequency of each transcript is illustrated to the right of the transcript. 18 out of 24 clones were obtained for transcript variant 1; 3 out of 24 clones were obtained for transcript variant 2; and 1 out of 24 clones were obtained for transcript variants 3, 4, and 5. Transcript variant 1 resulted from the splicing of the Alv4 splice donor site into the splice acceptor site of exon 4. Transcript variant 2 resulted from the splicing of the Alv4 splice donor site into a cryptic splice acceptor site within the 3′ half of exon 3. Transcript variant 3 contained intronic sequences from intron 2 and the recombined Alv4 cassette as well as sequences encoding exon 2–5. Transcript variant 4 contained the expected sequence of the recombined Alv4 cassette. Transcript variant 5 resulted from the splicing of a cryptic splice donor site within the 5′ half of exon 3 into the Alv4 splice acceptor site.

(D) Ectopic expression of HA tagged versions of wild type, transcript variant 1, 2 and 5 under the control of the CMV promoter. All three isoforms generated from transcript variants 1, 2 and 5 were expressed at a much lower level than their wild-type, full length, counterpart. The images are representative of 3 independent experiments.

When engineering genetic modifications, there is a chance that the newly engineered allele behaves unexpectedly. This is particularly true for conditional alleles where the location of loxP sites within introns, promoters, or 3′ untranslated regions, may affect expression or splicing events resulting in reduced expression of a gene or generation of aberrant transcripts. Similarly, generation of conditional alleles using reversible cassettes like COIN or CRIPSR-FLIP which expose a strong splice acceptor site followed by a STOP cassette upon Cre-mediated recombination, may also produce aberrant transcripts.[8,9] To test whether abnormal transcripts are produced from the engineered *Scyl1*^{Alv4} and *Scyl1*$^{Alv4\Delta}$ alleles, RNA transcripts can be analyzed by RT-PCR, TOPO® TA cloning®, and Sanger sequencing (Figure 6). The following section describes a step-by-step procedure for the identification and analysis of transcripts produced from the *Scyl1*^{Alv4} and *Scyl1*$^{Alv4\Delta}$ alleles.

38. For multiple exon genes, design a primer pair to amplify a cDNA fragment encompassing the targeted exon, the exon immediately upstream, and one or more exons downstream of the targeted exon. In the case of single exon genes, primers should be designed to amplify the entirety of the targeted exon, if possible. For *Scyl1*, the following primers were designed to amplify the region from exon 2 to exon 5.
    a. RT-Scyl1_F21: CGCAGTGTCCATCTTCGTGTA.
    b. RT-Scyl1_R51: CCCGGCAGTTCTGCAGGAA.
39. Extract total RNA from the cerebellum of mice with control, Alv4, and Alv4Δ alleles using the RNeasy Maxi Kit (Qiagen, 75162) with the RNase-Free DNase Set (Qiagen, 79254), according to the manufacturer's protocol (see step 31).
40. Quantify and assess the quality of the RNA according to steps 32 and 33.
41. Set up and run the RT-PCR reaction following the OneTaq One-Step RT-PCR Kit (New England Biolabs, E5315S) procedure using RT-Scyl1_F21 and RT-Scyl1_R51.
42. Load 10 μL of RT-PCR product on a 2% agarose gel at 120 V for approximately 90 min in 1× TAE Buffer.
    a. Image gel and save picture for your records.

43. Analyze the nature of the transcripts using TOPO® TA cloning® and Sanger sequencing, as described previously in this manuscript. Alternatively, this analysis could be performed using RNA sequencing.

EXPECTED OUTCOMES

Insertion of the 201-nucleotide-long AIv4 cassette within exon 3 of Scyl1 occurred in 17% of edited mice (Figure 2). In three other projects using this strategy, insertion rates in edited mice using microinjection were found to be 50%, 100% and 60%. These results suggest that this method may be more efficient than conventional strategies to engineer conditional alleles in mice.

Recombination between the two loxP sites within the AIv4 cassette is highly efficient despite their proximity. On average, nearly 85% recombination was obtained in CMV-Cre+;*Scyl1*$^{+/AIv4\Delta}$ mice, ranging from approximately 40% in some mice to complete recombination in others.[10] This incomplete recombination rate is likely due to the mosaic expression of the Cre recombinase itself, rather than the functionality of the AIv4 cassette. Animals in which nearly 100% recombination was observed were used for breeding and to determine the expression levels of SCYL1 (Figures 3 and 4).

Functionally, protein expression should be unaffected in mice homozygous for the AIv4 allele and abrogated in mice homozygous for the recombined allele (Figure 5).

LIMITATIONS

One possible limitation to the application of this strategy is the possibility that the HDR template be incorporated randomly into the genome. Although this has been reported in other studies,[11] random insertion analysis of the *Scyl1*^{AIv4} cassette using primers within the HDR template failed to reveal insertion in F1 mice other than the ones identified as having the proper insertion.[1] Although random integration of HDR template remains a possibility, outbreeding of these insertional events is simple.

Another potential limitation is the inability to obtain single stranded HDR templates from commercial sources due to sequence complexity. As an alternative, dsDNA templates can be obtained commercially and used to produce models using this approach. However, the insertion rate between ssDNA and dsDNA templates may differ.

Finally, insertion of an artificial intron within an exon may have unpredictable effects on splicing. Indeed, the presence of a splice donor and splice acceptor can drive unpredictable splicing events that may result in transcripts encoding mutant forms of the protein. These proteins may not be folded properly, and thus degraded, leading to inactivation of the allele. Alternatively, folded proteins may possess aberrant properties, resulting in biologically irrelevant phenotypes. From our findings, RT-PCR and western blotting data obtained from *Scyl1*$^{AIv4/AIv4}$ mice showed that no such events occurred (Figure 6). However, aberrant splicing events were observed in mice bearing the recombined form of the allele. Five transcript variants were observed, three of which were in-frame (Figure 6C, variants, 1, 2, and 5). These variants represented more than 85% of all transcripts produced from the recombined allele. Transcript variant 1 (Figure 6) resulted from the splicing of the splice donor of the AIv4 cassette into the splice acceptor of exon 4. Transcript variant 2 resulted from the splicing of the splice donor of the AIv4 cassette into a cryptic splice acceptor in the 3′ half of exon 3. Transcript variant 5 resulted from a cryptic splice donor in the 5′ half of exon 3 into the splice acceptor of the AIv4 cassette. Although it is very likely that proteins are produced from these variants, western blotting failed to reveal the presence of these isoforms in *Scyl1*$^{AIv4\Delta/AIv4\Delta}$ mice, suggesting that, if produced, these variants are likely unstable. This was confirmed by ectopic expression of isoforms 1, 2, and 5 into *SCYL1*-deficient Hek293T cells (Figure 6D).[5,12] At the protein level, expression of these isoforms was drastically lower than that of the wild-type isoform

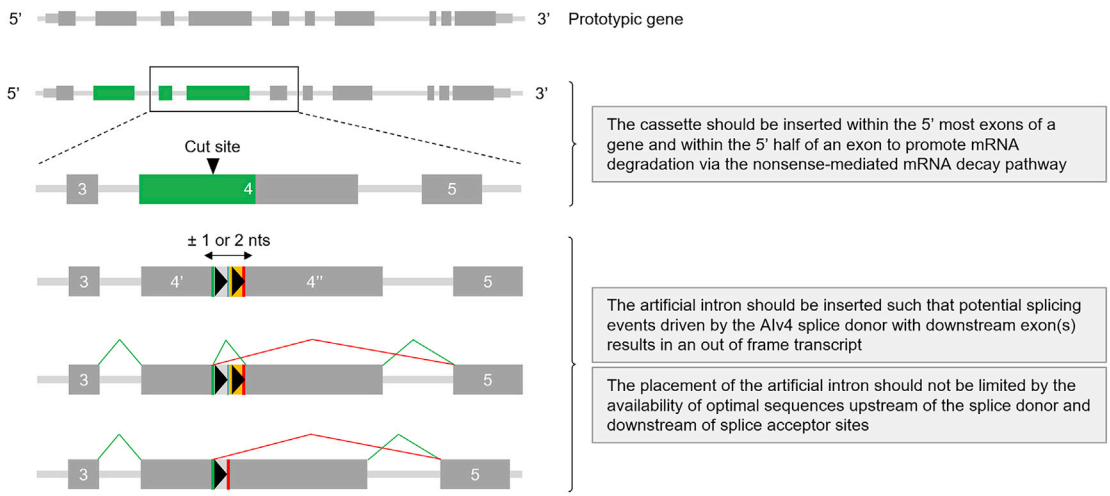

Figure 7. Design considerations

Schematic representation of a prototypic gene and AIv4 cassette insertion site to promote nonsense mediated mRNA degradation upon Cre-mediated recombination of the internal loxP sites and to avoid in-frame splicing events between the AIv4 splice donor and downstream acceptor site. The AIv4 cassette should be inserted within the 5′ most exons (green boxes) of a gene and within the 5′ half of an exon (exon 4, highlighted in green) to promote mRNA degradation via the nonsense-mediated mRNA decay pathway (Popp and Maquat[13]). The artificial intron should be inserted such that potential splicing events driven by the AIv4 splice donor with downstream exon(s) results in an out of frame transcript (red line). Since the location of the AIv4 cassette does not depend on the availability of optimal sequences upstream of the splice donor and downstream of splice acceptor sites, the emplacement of the cassette can be slightly shifted avoid potential in-frame splicing events with downstream exons to occur. Grey boxes, exons; grey lines; introns; green boxes, target exons; green line, proper splicing events; red lines, undesirable in-frame splicing events.

(Figure 6D). The two remaining out of frame transcript variants were significantly underrepresented, suggesting that these transcripts are likely sent for degradation via the nonsense-mediated mRNA decay pathway.[10] From these results, design considerations for the generation of conditional alleles using this technology are proposed.

The successful application of this approach to engineer conditional alleles in mice resides, for the most part, on the positioning of the artificial intron within a gene. Studies on the nonsense-mediated mRNA decay pathway have shown that the introduction of a premature stop codon within the first exon of a gene nearby the ATG, within the second half of an exon, or within the last exon of a gene does not promote RNA degradation. Conversely, insertion of premature stop codons within the first half of an exon, excluding the first exon, and the first half of a gene is more likely to promote nonsense-mediated mRNA decay.[13] Thus, insertion of the AIv4 cassette early in a gene and within the first half of an exon is likely to promote inactivation of the allele by degradation of the transcript (Figure 7).

From our findings, aberrant splicing events may occur from the AIv4 splice donor into downstream exons. If these events are in-frame, this may result in the generation of mutant proteins with unpredictable properties. To avoid this, the cassette should be positioned such that splicing emanating from the AIv4 splice donor site into downstream exons is out of frame. Moreover, to ascertain the inactivation of the allele, not only at the transcriptional or translational level, but also at the post-translational level, the cassette should be inserted within a region of the gene that encodes a critical structural feature of the protein, if possible. This way, the gene product may become unstable and readily degraded, as shown in.[1]

The preference of GT-AG introns for specific sequences upstream of the splice donor and downstream of the splice acceptor site has been viewed as a potential limitation to the widespread application of this approach. These introns are thought to prefer sequences containing a CAG or AAG upstream of the splice donor, and an A or G immediately downstream of the splice acceptor to promote optimal splicing. However, at the time of engineering the *Scyl1*^{AIv4} allele, this notion was overlooked,

and the cassette was inserted into suboptimal sequences, more specifically at a TGT-C. Despite this, the intron was found to be properly and efficiently removed, allowing for expression of the SCYL1 protein. From this data, it appears that knocking in the AIv4 cassette within specific sequences is not required for its functionality. Additional models are needed to confirm this observation.

The following design considerations for the generation of conditional allele in mice using the DECAI approach are illustrated in Figure 7. The cassette should be inserted within the 5′ most exons of a gene and within the 5′ half of an exon to promote mRNA degradation via the nonsense-mediated mRNA decay pathway.[13] The artificial intron should be inserted such that potential splicing events driven by the AIv4 splice donor with downstream exon(s) results in out of frame transcripts. The placement of the artificial intron should not be limited by the availability of optimal sequences upstream of the splice donor and downstream of splice acceptor sites.

TROUBLESHOOTING

Problem 1

No PCR products are obtained when optimizing PCR reactions (steps 1–8).

Potential solution

Make sure PCR mixes are prepared on ice to prevent mispriming. Consider designing a new primer pair.

Problem 2

Low editing frequency (steps 9–24).

Potential solution

If indel frequency is abnormally low (less than 10% indel rates in newborn pups) for your microinjection facility, consider using a sgRNA that targets a different site. A new HDR template will also have to be engineered.

Problem 3

No white colonies observed following TOPO® Cloning and transformation (steps 14–21).

Potential solution

TOPO® TA Cloning described here requires the presence of adenine at the 3′ ends of the PCR product. Make sure you perform the extra step described in step 14 of this protocol and use fresh PCR products.

Problem 4

Partial recombination between loxP sites within the cassette (animal breeding section).

Potential solution

The CMV-Cre mouse model used in this study express Cre in all tissues although cellular mosaicism is frequently observed.[14] Mice with partial recombination should be excluded from subsequent breeding to ensure complete inactivation of the allele.

Problem 5

Off-target editing observed (off-target PCR genotyping section).

Potential solution

Mice with off-target editing should be outbred to wild-type to breed out the undesired mutation(s). If the off-target mutation(s) segregate with the on-target mutation because both are on the same allele, these mice should be excluded from subsequent breeding and re-targeting is recommended.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephane Pelletier, spellet@iu.edu.

Materials availability

Mice (Scyl1^{-}, Scyl1^{Alv4}, Scyl1$^{Alv4\Delta}$), plasmids, and antibodies generated and/or used in this study are available from Dr. Pelletier's laboratory, upon request.

Data and code availability

This study did not generate or analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102116.

ACKNOWLEDGMENTS

The authors would like to thank the St. Jude Children's Research Hospital Transgenic/Gene Knockout Shared Resource for microinjection and members of the Indiana University Genome Editing Center (IUGEC) for rederiving the *Scyl1*^{Alv4} mouse model. Funding supporting this work was provided by the Indiana University School of Medicine and the Department of Medical and Molecular Genetics. The graphical abstract was adapted from "Timeline 4 Steps (Layout 4×1)", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

AUTHOR CONTRIBUTIONS

A.C. and S.P. generated and analyzed data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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