CASAAV: A CRISPR-Based Platform for Rapid Dissection of Gene Function In Vivo

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In vivo loss-of-function studies are currently limited by the need for appropriate conditional knockout alleles. CRISPR/Cas9 is a powerful tool commonly used to induce loss-of-function mutations *in vitro*. However, CRISPR components have been difficult to deploy *in vivo*. To address this problem, we developed the CASAAV (CRISPR/Cas9/AAV-based somatic mutagenesis) platform, in which recombinant adeno-associated virus (AAV) is used to deliver tandem guide RNAs and Cre recombinase to Cre-dependent Cas9-P2A-GFP mice. Because Cre is under the control of a tissue-specific promoter, this system allows temporally controlled, cell type-selective knockout of virtually any gene to be obtained within a month using only one mouse line. Here, we focus on gene disruption in cardiomyocytes, but the system could easily be adapted to inactivate genes in other cell types transduced by AAV.

Keywords: CRISPR • Adeno-associated virus • Mouse model • Genetic mosaic • Cardiomyocyte

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INTRODUCTION

Generating loss-of-function mutations is a principal approach to elucidating gene function. The Cre/loxP system enables efficient conditional ablation of target genes, but generating the necessary loxP mice is time consuming, expensive, and very low throughput. The need to obtain appropriate mouse lines and mate them to homozygosity with the appropriate Cre is a further practical bottleneck.

Recent reports have shown that CRISPR-mediated genome editing can be effectively used *in vivo* to generate insertions and deletions (indels) that inactivate target genes in somatic cells (Carroll et al., 2016; Platt et al., 2014; Swiech et al., 2015). We therefore acquired Cre-dependent Cas9-P2A-GFP knock-in mice (Platt et al., 2014), hereafter referred to as *Rosa26*$^{Cas9\text{-}GFP}$, now available from The Jackson Laboratory, and designed an AAV9 vector that delivers both Cre and guide RNAs (gRNAs) to cardiomyocytes (CMs; Guo et al., 2017). Expression of gRNAs is controlled by the ubiquitous U6 promoter, while Cre expression is driven by a CM-specific cardiac troponin T (cTnT) promoter (Prasad, Xu, Yang, Acton, & French, 2011). We used two gRNAs to target the same gene to enhance the likelihood of generating inactivating mutations through frameshift mutation

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or excision of a key functional domain. Though AAV9 transduces multiple cell types, including CMs, the cTnT promoter enhances cardiomyocyte-selective Cre expression in our CASAAV system. The system can be adapted for other cell types by the selection of the appropriate AAV serotype and cell type-restricted promoter. Overall, the CASAAV system enables loss-of-function studies of nearly any gene, using the *Rosa26*$^{Cas9\text{-}GFP}$ mouse line and AAV vectors that are easy to engineer and deliver.

In vivo loss-of-function studies are often confounded by secondary effects that arise from whole-organ dysfunction. For instance, in studies of CM gene function, secondary changes arising from heart dysfunction have been misinterpreted to reflect cell autonomous gene function (Prendiville et al., 2015; Guo et al., 2017). A compelling advantage of CASAAV is that secondary effects can be minimized by employing a genetic mosaic approach in which AAV dosage is titrated and only a fraction of cells transduced (Guo et al., 2017). Thus, this powerful technique allows for a quick and cost-efficient method of obtaining mutant CMs from otherwise healthy heart tissue.

The following protocols provide guidelines for target and gRNA selection (Basic Protocol 1), construction and delivery of the AAV vector (Basic Protocol 2), and collection of mutant CMs (Basic Protocol 3). We also include instructions for immunostaining as a simple avenue to confirm gene depletion at the single-cell level (Basic Protocol 3), as well as investigating the effect of gene knockout. We've successfully used CASAAV to study the role of junctophilin-2 in cardiomyocyte maturation (Guo et al., 2017). With minor modifications, this system could be used to inactivate genes in other AAV-transduced cell types.

Strategic Planning

Many different experimental contexts require mutagenesis or deletion of a region of genomic DNA. For the purpose of these protocols, we assume that the target is a protein-coding region, for which gene disruption can be verified by assaying for loss of protein expression. Immunostaining is a useful way to examine the efficiency of gene depletion at the single-cell level, is particularly valuable and, therefore, we find it helpful to prioritize potential target genes based on the availability of suitable antibodies. This manuscript will focus on immunostaining-based validation, but other approaches could be used, including western blot analysis and targeted genome sequencing from purified transduced CMs (see Guo et al., 2017). If single-cell readouts are not essential for your particular application, then target selection need not depend on the availability or reliability of immunostaining-quality antibodies.

BASIC PROTOCOL 1

TARGET AND gRNA SELECTION

This protocol describes how to most effectively pick a region within a desired gene for mutagenesis, and to select appropriate gRNAs to target the region. The CASAAV platform utilizes tandem gRNAs to disable gene expression by introducing frame-shifting indels via imprecise repair of Cas9-induced double-strand breaks at one or both gRNA sites. Optimally, the gRNAs should target regions towards the 5′ end of the coding sequence so that frameshift mutations will have the greatest likelihood to disrupt most of the encoded protein. Using tandem gRNAs provides the additional advantage that the intervening genomic DNA can be deleted if both gRNAs work efficiently. We therefore commonly design gRNAs to flank a protein domain predicted to be functional, so that deletion will disable the protein. Deletion is not 100% efficient, and in fact *in vitro* studies have shown that efficiency decreases as deletion size increases (Canver et al., 2014). So, if deletion is a desired outcome, the size of the deletion is important to consider. When targeting genes with many exons, or genes that produce alternatively spliced transcripts, design gRNA pairs that target exons shared by all splice variants. Finally, gRNAs must

be selected that have a high probability of on-target activity and low probability of off-target activity. There are now many computational tools for predicting gRNA activity. Here we describe the Broad Institute's Genetic Perturbation Platform (GPP) web tool (Doench et al., 2016), which we have used with great success. Recent work suggests that the sequence at the gRNA target site impacts the frequency of frameshifting indels (Bae, Kweon, Kim, & Kim, 2014), and a computational gRNA design tool that takes this into consideration is also available (*http://www.rgenome.net/cas-designer*).

Materials

Computer with internet connection

Select target region

1. Connect to the internet, go to *www.uniprot.org*, and in the top search bar, enter the name of the gene you wish to target. Click on the "Entry" link for the mouse ortholog of the gene.

   We will use the cardiac zinc-finger transcription factor GATA4 as an example. GATA4′s UniProt ID is Q08369.

2. In the left navigation pane, click on "Family & Domains". Uniprot will display a list of domains as well as a graphical depiction of where in the amino acid sequence they are located. Select an important domain, preferably towards the 5′ end of the gene.

   For example, GATA4 has two zinc finger domains that are known to be crucial for DNA binding. Therefore we selected the first zinc finger domain for deletion.

3. Click on the highlighted domain in the graphical view (red arrow in Fig. 31.11.1A) to retrieve the amino acid sequence of the domain. Copy the sequence to the clipboard.

   Example: CVNCGAMSTPLWRRDGTGHYLCNAC for the GATA4 zinc finger domain.

4. Navigate to genome.ucsc.edu, hover cursor over "Tools" on the menu bar, and select "Blat" from the resulting dropdown menu.

5. On next screen, use the drop-down arrow to change BLAT genome to "mouse" if necessary. Paste in the amino acid domain sequence from the clipboard and click "Submit" (Fig. 31.11.1B).

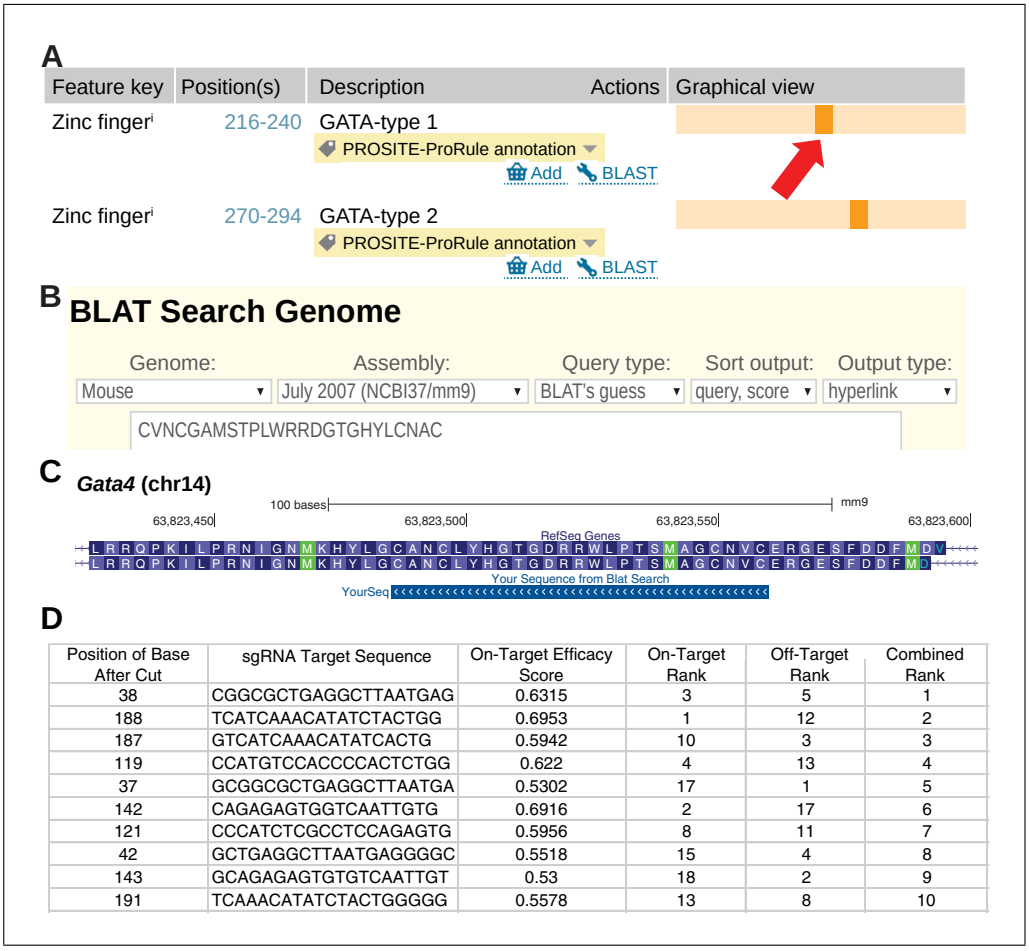
6. Select the entry corresponding to your gene of interest from the list of genomic loci with homology to the search query (most likely the top result).

7. The following screen will display your search query aligned to a portion of an exon from your gene. Use the menu buttons to zoom out until the intron-exon boundaries can be observed (Fig. 31.11.1C). To select and zoom in on the exon, press and hold the "Shift" key while you click at one end of the exon and drag the mouse across to the other end.

8. Hover over "View" on top menu, and select "DNA". Fill in the appropriate boxes to add 16 extra nucleotides 5′ and 3′ of the selected exon sequence. Click on "get DNA".

9. Copy the resulting DNA sequence to a text file for record keeping. Determine which nucleotides correspond to the protein domain to be targeted for deletion, and make a note.

   For example: we retrieved 200 bp centered on Gata4 exon 3; zinc finger 1 corresponds to nucleotides 77 to 151.



Position of Base After Cut	sgRNA Target Sequence	On-Target Efficacy Score	On-Target Rank	Off-Target Rank	Combined Rank
38	CGGCGCTGAGGCTTAATGAG	0.6315	3	5	1
188	TCATCAAACATATCTACTGG	0.6953	1	12	2
187	GTCATCAAACATATCACTG	0.5942	10	3	3
119	CCATGTCCACCCCACTCTGG	0.622	4	13	4
37	GCGGCGCTGAGGCTTAATGA	0.5302	17	1	5
142	CAGAGAGTGGTCAATTGTG	0.6916	2	17	6
121	CCCATCTCGCCTCCAGAGTG	0.5956	8	11	7
42	GCTGAGGCTTAATGAGGGGC	0.5518	15	4	8
143	GCAGAGAGTGTGTCAATTGT	0.53	18	2	9
191	TCAAACATATCTACTGGGGG	0.5578	13	8	10

Figure 31.11.1 gRNA design. (**A**) Uniprot.org user interface showing GATA4 domains and domain location. Red arrow indicates where to click to retrieve sequence for gRNA targeting. (**B**) Alignment of GATA4 zinc-finger domain sequence to gene sequence. (**C**) Retrieval of gene sequence flanking target domain. (**D**) Top scoring *Gata4* gRNAs and their cut positions.

Select gRNAs and prepare for cloning

10. Navigate to the Broad Institute GPP Web Portal at *http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design*.

11. Select "mouse" as Target Taxon, and paste the target exon sequence into the input field. Click "Submit".

12. When job is complete click on the "sgRNA Picking Results" link, download the text file, and open the file in Microsoft Excel.

13. Find the columns labeled "Position of Base After Cut" and "Combined Rank", which takes into account both on-target and off-target predicted activity (Fig. 31.11.1D), and identify the three highest ranking gRNAs targeting positions upstream of the protein domain and the three highest ranking gRNAs targeting positions downstream of the domain. Copy these top sequences—from the "sgRNA Target Sequence" column—to your notes.

    For Gata4, we identified three gRNAs targeting positions 17 to 76, and three targeting positions 152 to 184.

    If there are too few high-quality gRNAs (i.e., on-target efficacy score >0.5) targeting the desired region, you may wish to expand your search to include gRNAs that target within the protein domain, or a different domain entirely.

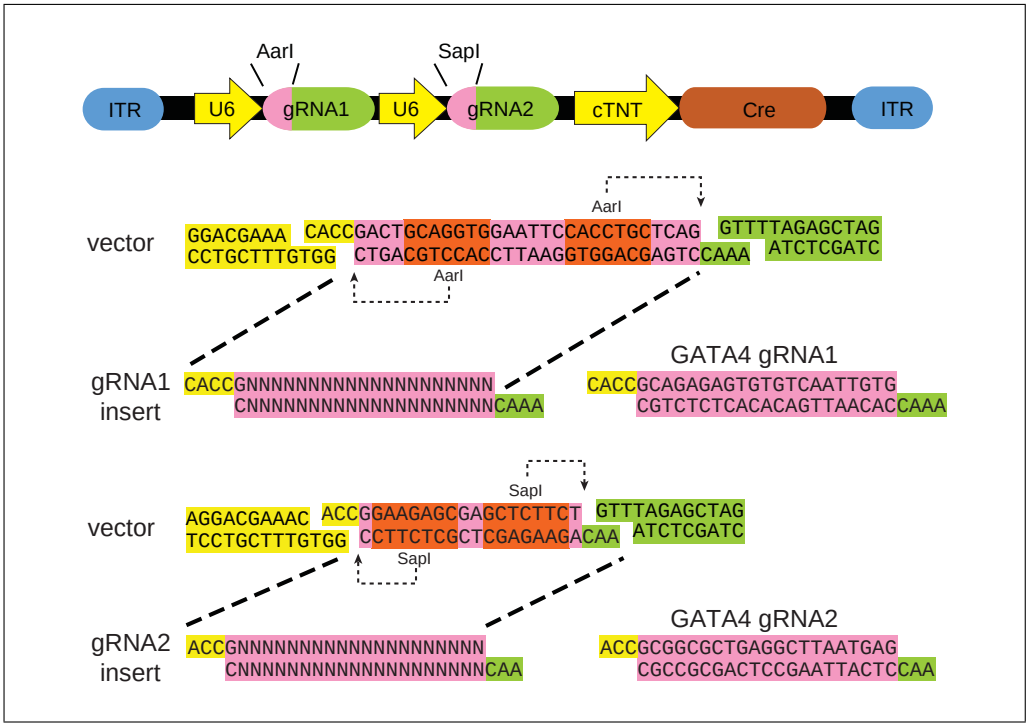


Figure 31.11.2 Insertion of gRNA sequences into AAV vector. gRNAs are inserted into AAV plasmid containing U6 promoters and cardiac troponin T (cTNT) promoter-driven Cre recombinase. Annealed gRNA1 oligonucleotides are cloned between AarI sites, and gRNA2 oligonucleotides are cloned between SapI sites. The entire construct is contained within AAV inverted terminal repeat sequences, allowing for packaging into AAV capsids. Examples of gRNAs designed against *Gata4* are shown.

14. Add a guanine nucleotide to the 5′ end of each gRNA sequences that starts with A, C, or T.

    Optimal RNA expression from the U6 promoter requires that the first nucleotide of the transcript be guanine (Das et al., 1988; Paule, 2000).

15. Obtain the reverse complement sequence for each of the six gRNA sequences.

    This can be done using a number of web-based tools, including http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html.

    Forward and reverse oligonucleotides carrying the gRNA sequence will be annealed during cloning.

16. To each upstream gRNA, add AarI overhang sequences: i.e., add CACC to the 5′ end of the gRNA forward sequence, and AAAC to the 5′ end of the reverse complement sequence.

    See gRNA1 in Figure 31.11.2.

17. To each downstream gRNA, add SapI overhang sequences: i.e., add ACC to the 5′ end of the gRNA forward sequence, and AAC to the 5′ end of the gRNA reverse complement sequence.

    See gRNA2 in Figure 31.11.2.

18. Oligonucleotides can now be synthesized by your preferred vendor. Twelve oligonucleotides should be synthesized to make three pairs of gRNAs.

    Standard purity/desalting (UNIT 2.11; Ellington & Pollard, 2001) is sufficient.

CONSTRUCTION AND DELIVERY OF AAV9 VECTOR

In vivo delivery of tandem gRNAs designed in Basic Protocol 1 is achieved by cloning the gRNA sequences into an AAV genome vector. The vector that we have designed (AAV-U6gRNA1-U6gRNA2-TnT-Cre) contains two distinct insertion sites downstream of ubiquitous U6 promoters. Digestion with AarI restriction enzyme removes a small placeholder fragment and opens up the insertion site for gRNA1. Annealed gRNA1 oligonucleotides, with 5' AarI overhangs, are then ligated into the vector. The resulting vector containing gRNA1 is digested with SapI to remove a placeholder fragment in the gRNA2 insertion site, and then ligated to annealed gRNA2 oligonucleotides with 5' SapI overhangs. In this way, two gRNAs that target positions flanking the desired domain can be quickly designed and cloned into a single AAV vector. Once both gRNAs are cloned into the vector, the vector can be packaged into AAV via a standard triple transfection of 293T cells with vector and two helper plasmids (*UNIT 23.16*; Wakimoto, Seidman, Foo, & Jiang, 2016). Iodixanol density gradient-based purification of the AAV from transfected cells results in concentrated, highly pure virus, sufficient for robust transduction of neonatal mouse myocardium. The protocol is written for cloning of a single gRNA pair, but the protocol can be scaled to conveniently clone multiple gRNAs in parallel.

NOTE: This protocol uses live mice. All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

gRNA oligonucleotides (from Basic Protocol 1)
Annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA)
AAV-U6gRNA1-U6gRNA2-TnT-Cre vector (Addgene, cat. no. 87682)
AarI enzyme and buffer (Thermo Fisher, cat. no. ER1581)
SapI enzyme and buffer (NEB, cat. no. R0569L)
6× DNA loading dye (NEB, cat. no. B7024S)
Agarose (GeneMate, cat. no. E-3120-500)
50× TAE (Boston BioProducts, cat. no. BM250)
Ethidium bromide (Sigma-Aldrich, cat. no. E7637)
Gel purification kit (Thermo Fisher Scientific, cat. no. K210012)
Chemically competent cells, e.g., MAX Efficiency Stbl2 Competent Cells (ThermoFisher, cat. no. 10268019).

Recombination-resistant bacteria are essential.

Quick ligation kit (NEB, cat. no. M2200L)
Ampicillin (Thermo Fisher, cat. no. 11593027)
LB agar plates with 100 µg/ml Ampicillin (Teknova, cat. no. L1004)
LB media (40 g/liter H_2O, Teknova, cat. no. L9115)
Miniprep kit (Thermo Fisher Scientific, cat. no. K210011)
TnT-R sequencing primer, AGGGACTTCGGGCACAATCG
Midiprep kit (Thermo Fisher Scientific, cat. no. K210014)
Isoflurane (Baxter, cat. no. 10019-360-40)

PCR tubes
Thermal cycler
37°C incubator
37°C shaking incubator
Gel electrophoresis chamber
UV transilluminator (365 nm)

42°C heat block or waterbath
Miniprep culture tubes (Corning, cat. no. 352059)
Nanodrop spectrophotometer (Thermo Fisher Scientific, cat. no. ND-1000)
Isoflurane anesthesia setup (anesthesia machine, induction chamber, scavenging system).
0.3-ml syringes (XELINT, cat. no. 26200)
Rosa26$^{Cas9\text{-}GFP}$ mice (Jackson Laboratory, cat. no. 026175)

Additional reagents and equipment for AAV production and purification (*UNIT 23.16*; Wakimoto, et al., 2016).

Anneal gRNAs

1. Suspend the oligonucleotides in H_2O to a concentration of 1 µg/µl.
2. For each gRNA pair, mix 2 µg forward oligonucleotide and 2 µg reverse complement oligonucleotide in 46 µl annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) in a PCR tube, and place the tube in a thermal cycler.
3. Set the thermal cycler to denature the oligonucleotides at 95°C for 2 min, and anneal by gradually cooling to 25°C over 45 min. Alternatively, oligonucleotides can be denatured in a 95°C hot block for 2 min, and annealed by turning the block off and letting it cool to 37°C. Annealed oligos can be stored at 4°C for several weeks.

Linearize AAV vector

4. Prepare the following AarI restriction digest to linearize the AAV-U6gRNA1-U6gRNA2-TnT-Cre vector:

   3 µg AAV-U6gRNA1-U6gRNA2-TnT-Cre vector
   2.5 µl 10× AarI buffer
   H_2O to 18.5 µl
   Mix
   Add 1 µl (2 units) of AarI enzyme
   Mix and incubate for at least 3 hr at 37°C.

5. Gel-purify the AarI-linearized vector from uncut plasmid and the 30-bp placeholder: add 5 µl 6× loading dye and fractionate the sample on a 0.8% agarose gel (1× TAE, 0.5 µg/ml ethidium bromide) at 5 V/cm for 30 min.
6. Excise the band containing the digested vector from the gel, and extract the DNA from the gel slice using a gel purification kit. Follow standard directions and elute in 20 µl H_2O.
7. Measure the DNA concentration using a Nanodrop spectrophotometer.
8. Remove competent cells (50 µl) from freezer, and place directly on ice. Proceed with next step while the cells thaw.

Clone gRNA into AAV vector

9. Ligate of gel-purified AarI-digested vector to the annealed gRNA1 oligos:

   10 µl 2× Quick Ligase Buffer
   25 ng digested vector
   1 µl annealed gRNA1 oligonucleotides, diluted 1:100
   H_2O to 9 µl
   Mix
   Add 1 µl Quick Ligase
   Mix and incubate at room temperature for 5 min

NOTE: *Also perform a control ligation in which the annealed oligo is omitted.*

10. Add 10 µl of ligation reaction to the competent cells on ice. Incubate on ice for 20 min. Store the remaining 10 µl of the ligation reaction at –20°C as a backup.

11. Heat-shock the competent cells and ligation mixture at 42°C for 30 sec.

12. Add 300 µl LB media and incubate for 45 min in a 37°C shaking incubator. During this incubation, warm LB agar plates containing ampicillin at 37°C.

13. Remove transformation reactions from incubator, resuspend the bacteria in the medium, and spread 100 µl of the bacterial culture on a pre-warmed LB agar plates containing 100 µg/ml ampicillin. Incubate the plate at 37°C incubator overnight. The remainder of the transformation reaction can be discarded.

14. The next day remove dishes and estimate the number of colonies on each plate.

    A successful ligation of the vector to the annealed gRNA oligonucleotides should yield many more colonies than the vector-only ligation.

15. Place 3 ml LB medium containing 100 µg/ml ampicillin into numbered miniprep culture tubes. Use a sterile pipet tip to pick colonies. Gently spot each picked colony onto a fresh LB-amp agar plate using a numbered grid, and then deposit the tip into the culture tube. Incubate cultures overnight at 37°C in a shaking incubator (liquid cultures) or standard incubator (plates).

    Three colonies is usually enough to find a positive clone. You may need to screen more colonies if the ligations with and without gRNA1 oligonucleotides yield similar numbers of colonies.

16. Store grid plate at 4°C, and isolate AAV plasmid DNA from bacterial cultures using a DNA mini-prep kit, following the manufacturer's instructions.

    The grid plate serves as an emergency backup after gRNA cloning, and to scale up the DNA prep.

17. Confirm successful insertion of gRNA1 by sequencing the miniprep DNA. Use primer TnT-R (AGGGACTTCGGGCACAATCG), which sequences backward from the TnT promoter.

    Using the TnT-R primer, we usually obtain sequence reads long enough to verify the sequence of each gRNA insertion site. Alternatively, the primer gSITE2-R (CAGAAGAGCTCGCTCTTCCG) can be used to sequence gRNA1, if nothing has yet been inserted into gRNA site 2.

18. Clone the 3′ gRNA (gRNA2) into a gRNA1-containing clone by following steps 4 to 17, but linearize the gRNA1-containing vector with SapI in step 4, and ligate to gRNA2 in step 9.

19. Once a clone with both gRNAs has been acquired and sequence-verified, prepare a DNA midi-prep (>100 µg) using a kit, according to manufacturer's instructions. Use the corresponding bacterial colony from the grid plate to start the culture for the midi-prep.

20. Before proceeding with AAV production, perform a restriction analysis with XmaI or SmaI to ensure that the AAV ITRs did not recombine and delete the AAV genome (i.e., gRNA and TnT-Cre cassettes) during plasmid production.

    If both ITRs are intact, SmaI or XmaI digestion will excise the AAV genome. If the ITRs recombine, SmaI or XmaI digestion will only linearize the vector.

Package the vector into AAV and inject mouse pups

21. For robust production or AAV9 vector, we recommend following Basic Protocol 2 of *UNIT 23.16* (Wakimoto et al., 2016). The titer of the AAV should be determined by quantitative PCR, as described. We use insert-specific primers (5′-tcgggataaaagcagtctgg-3′ and 5′-cccaagctattgtgtggcct-3′) that amplify a fragment of the TnT promoter, and express the titer as viral genomes per ml (vg/ml).

22. Dilute the AAV in sterile saline solution, so that the desired number of genomes will be delivered in 50 µl. Load the diluted AAV into a 300 µl syringe.

    The dose will need to be titrated to suit the desired application. As an approximate starting point, injection of P1 neonatal Rosa26$^{Cas9\text{-}GFP}$ mice with 1×10^{10} viral genomes per gram of body weight typically will transduce approximately 50% of CMs, whereas a dose of 1×10^{11} viral genomes per gram of body weight should transduce >90% of CMs.

23. To reliably deliver AAV to neonatal pups subcutaneously, anesthetize them with isoflurane to avoid loss of injected AAV when pups strain following the injection. Place pups into an isoflurane chamber and titrate the isoflurane (2% to 3%) until they become unresponsive to toe pinch.

24. Hold the anesthetized pup belly up, position the syringe needle over the sternum, pointed towards the head and nearly parallel to the skin. Gently insert the needle under the skin over the chest, and dispense the AAV.

    When done correctly you will observe a liquid bubble under the skin.

    NOTE: *If individual pups need to be marked, at the time of injection use a pair of sharp, fine dissecting scissors to remove the tip of individual digits, distal to the last interphalangeal joint.*

IMMUNOSTAINING ISOLATED CARDIOMYOCYTES

BASIC PROTOCOL 3

The ability to create genetic mosaics by delivery of low-dose AAV is a powerful feature of the CASAAV system. However, the genetic mosaic approach requires a way to identify transduced cells (marked by GFP, expressed from the *Rosa26*$^{Cas9\text{-}GFP}$ allele), and the subset of transduced cells that have undergone gene inactivation. Furthermore, it inherently requires single-cell phenotypic readouts. To address these experimental goals, we commonly employ immunostaining of isolated CMs. This technique allows to visualize GFP^{+}-transduced CMs, assess knockout efficiency, and assess the expression level and sub-cellular localization of many proteins. Prior to starting the staining protocol, it is necessary to perform cardiac dissociation by retrograde collagenase perfusion. Multiple variations of this procedure have been described, but we recommend a reliable protocol by O'Connell, Rodrigo, and Simpson (2005). In this protocol, we describe a simple procedure for adhering isolated CMs to laminin-coated coverslips, followed by fixation, permeabilization, and immunostaining that results in high quality images (Fig. 31.11.3).

NOTE: This protocol uses live mice. All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

1 mg/ml laminin (Corning, cat. no. 354239) in PBS
PBS (Thermo Fisher Scientific, cat. no. 10010-023)
AAV-treated *Rosa26*$^{Cas9\text{-}GFP}$ mice (from Basic Protocol 2)
Blebbistatin (EMD Millipore, cat. no. 203390)

Dulbecco's modified Eagle's medium (DMEM), high glucose (Gibco, cat. no. 11995-065)
4% paraformaldehyde (PFA; Electron Microscopy Sciences, cat. no. 15710), diluted in PBS
PBST: PBS containing 0.1% Triton X-100 (Sigma-Aldrich, cat. no. T9284)
Block solution: PBS containing 4% BSA (Sigma-Aldrich, cat. no. A3912-1006)
Prolong Diamond Antifade Mountant (Thermo Fisher Scientific, cat. no. P36961)
Primary antibody
Secondary antibody

Round coverslips (Fisher Scientific, cat. no. 50-121-5159)
24-well dishes (Corning, cat. no. 3526)
Coverglass forceps (Fine Science Tools, cat. no. 11074-02)
27-gauge needle (BD Biosciences, cat. no. 305136)
37°C, humidified CO_2 incubator
Langendorf perfusion apparatus and materials for collagenase perfusion (O'Connell et al., 2005).

Coat coverslips with laminin

1. Add a coverslip to each well of a 24-well tissue culture plate
2. Dilute 100 µl 1 mg/ml laminin stock solution in 7.2 ml PBS, and add 300 µl of diluted laminin solution to each well of the 24-well tissue culture plate containing coverslips (~2 µg laminin/cm^2).
3. Incubate at least 30 min at 37°C.
4. Aspirate the laminin solution and use immediately, or store at 4°C for up to 2 weeks.

Isolate CMs, attach to coverslips, and fix

5. Dissociate heart tissue from AAV-treated *Rosa26*$^{Cas9\text{-}GFP}$ mice by retrograde collagenase perfusion.

   We recommend the protocol by O'Connell et al. (2005).

6. Collect CMs by centrifugation for 4 min at 20 × *g*, room temperature, remove the supernatant containing non-cardiomyocytes, and suspend the pellet containing CMs in 700 µl PBS.

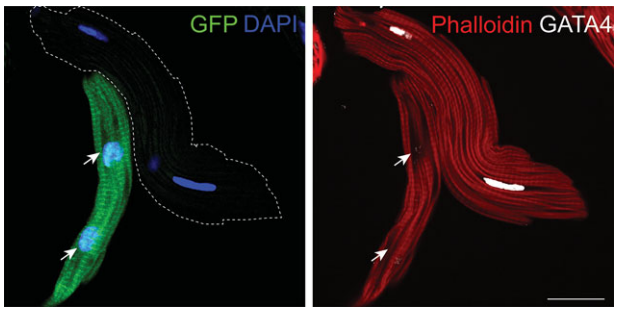


Figure 31.11.3 Immunostained CMs featuring CASAAV-mediated knockout of *Gata4*. *Rosa26*$^{Cas9\text{-}GFP}$ CMs transduced with AAV9-U6-gRNA(Gata4)-TNT-Cre express GFP and Cas9. Immunostaining confirmed successful depletion of GATA4. Phalloidin (red) labels cardiomyocytes. GATA4 nuclear staining (white nuclei) was observed in a non-transduced (GFP–) CM, but not in nuclei of a transduced (GFP+) CM (white arrows). Scale bar = 20 µm.

7. Add 12 µl 10 mM blebbistatin to 12 ml DMEM that was pre-warmed to 37°C, mix, and add 500 µl to each well of a 24-well tissue culture plate.

   Blebbistatin is a myosin inhibitor that improves CM survival and morphology during the brief culture period.

8. Using a wide-bore 200-µl pipet tip, add 30 µl of CMs to each well.

   Wide bore pipet tips are used to ensure that CMs are not sheared during pipetting. A wide bore tip can be creating by snipping off the end of a standard 200 µl tip with scissors.

9. Allow the CMs to attach to the coverslips for 30 min at 37°C.

   Do not culture CMs for longer than 60 min! CM cytoarchitecture rapidly remodels during in vitro culture.

NOTE: In all subsequent steps, cells should be protected from light as much as possible, by covering the plate with aluminum foil.

10. Remove the media and fix cells for 10 min in 200 µl 4% PFA, at room temperature.

    Add PFA gently to avoid detaching cells.

11. Remove the fixative and dispose properly. Add 200 µl PBST to each well, and incubate for 10 min.

12. Remove PBST, add 400 µl block solution (4% BSA in PBS) to each well, and incubate 1 hr at room temperature. Cells are ready for antibody staining (steps 13 to 18). Alternatively, plates containing fixed cells can be wrapped with Parafilm and aluminum foil and stored at 4°C for several weeks.

Immunostain CMs

13. Remove block solution, and add 200 µl primary antibody diluted in fresh block solution to each well, and incubate overnight at 4°C.

    A 1:300 dilution works well with many antibodies, but the optimal dilution should be empirically determined for each antibody.

14. Wash each well three times with 300 µl block solution, 5 min each.

15. Remove the last wash, add 200 µl secondary antibody diluted 1:500 in block solution to each well, and incubate for 1 hr at room temperature.

    DAPI nuclear stain may be added to the secondary antibody solution if desired.

16. Rinse each well three times with 300 µl block solution, 2 min each.

17. Remove the coverslips from each well and mount onto slides.

    To remove coverslips, use forceps to bend the tip of a 27-gauge needle at a right angle. Use this needle to hook and pull each coverslip up off the bottom of the dish. Carefully grasp the coverslip with forceps and place cell-side down onto a single drop of Prolong Diamond Antifade Mountant on a slide.

18. Allow the mountant to cure for several hours at room temperature before imaging. Keep slides protected from light.

COMMENTARY

Background Information

The CASAAV system permits Cas9-mediated somatic gene inactivation *in vivo*. The main advantage is that the entire procedure—from target selection to in vivo phenotype—can be done in as little as 4 weeks, without the need to generate, import, or breed a different mouse line for each target gene. The system is also well-suited to genetic mosaic analysis of single-cell phenotypes, a

powerful strategy to separate direct, cell-autonomous gene function from indirect effects that result from gene inactivation throughout an organ. The CASAAV system can easily be integrated with Cre-loxP conditional alleles so that inactivation of one gene can be evaluated on a mutant background achieved by Cre-mediated inactivation of floxed alleles. Here we apply CASAAV to the inactivation of genes in cardiomyocytes, but with minor modifications, the system could be used to inactivate genes in other cell types that are transduced by AAV.

The CASAAV system uses dual gRNAs to enhance the frequency of gene inactivation primarily through frameshift mechanisms. Dual gRNAs can also excise sequences between the gRNA target sites that encode critical protein domains. Although one gRNA can induce efficient somatic gene inactivation (Platt et al., 2014), in our experience using a pair of gRNAs increases the reliability and efficiency of gene inactivation.

Another CRISPR-based system for somatic mutagenesis of cardiac tissue has been reported (Carroll et al., 2016), in which the authors generated transgenic mice that express Cas9 and a fluorescent label under the control of the cardiomyocyte-specific *Myh6* promoter. Similar to CASAAV, gRNA was delivered by AAV along with a second fluorescent marker. This system has a few practical disadvantages; e.g., the transgenic mouse line is not widely available, it consumes two fluorescent channels (which limits the availability of fluorescent channels for other experimental readouts), and mutagenesis is confined to CMs. This system is also difficult to integrate with existing Cre/loxP mice.

Critical Parameters

Special attention should be given to the gRNA design phase of the CASAAV workflow, as all subsequent steps depend on correct design. It may take some effort to determine which protein domains constitute the most promising targets for deletion, taking into account both the hypothesized function of the domain and the identification of suitable gRNA target sites that flank the domains. While designing gRNAs, sequences that encode epitopes of available antibodies would be useful to know, as staining with an antibody that targets an epitope that lies upstream of a targeted region could detect the mutant protein, confounding the analysis.

An additional parameter to pay special attention to is GFP fluorescence of transduced cells. We have found that the GFP signal from Cas9-P2A-GFP can be easily lost if cells are not processed properly. To minimize this issue, CMs should be immunostained and imaged soon after fixation, and all immunostaining steps should be strictly followed. An antibody directed against GFP might also enhance GFP detection.

Troubleshooting

Ligation of annealed oligonucleotides into AAV vector fails

The most common cause of ligation failure is incomplete digestion of the vector, resulting in a large number of "empty vector" clones, in which the placeholder insert is not removed. This problem can be solved by digesting with AarI/SapI for a longer period of time, using more enzyme, or using less vector. In addition, the AarI placeholder fragment for contains a unique EcoRI site, and the SapI placeholder contains a unique SacI site. Digestion with EcoRI or SacI can be used to reduce the background of "empty vector" clones: prior to transformation, heat-inactivate the ligase, and then digest the ligation product with EcoRI or SacI, as appropriate, to linearize vectors that lack gRNA inserts, thereby preventing them from transforming bacteria.

Low GFP fluorescence of immunostained CMs

In our experience, the GFP signal from Cas9-P2A-GFP is sensitive and degrades over time. To maximize signal use fresh cells, do not fix for too long or too short a time, minimize exposure to light, minimize changes to sample temperature after fixation, use block solution (4% BSA in PBS) for all washes, and use Prolong Diamond Antifade Mountant, which is optimized for preservation of weak GFP signals. Immunodetection of GFP might also increase detection sensitivity.

No reduction in gene expression is observed

Not all gRNA pairs will be effective. To increase the likelihood of obtaining an effective gRNA, we recommend cloning at least three distinct pairs of gRNAs in parallel. Optimally, two of the gRNAs will be effective, so that both AAVs can be used to show that any observed phenotype is not due to random off-target effects.

Immunofluorescence-grade antibodies are not available

When antibodies for immunofluorescence are unavailable, GFP^+ CMs can be isolated by FACS, and protein depletion can be analyzed

by western blot if suitable antibodies are available. Other methods to detect target depletion are described in Guo et al. (2017). If a suitable antibody is not available, amplicon sequencing can be used to determine the fraction of CMs affected by truncating mutations (Guo et al., 2017).

Statistical Analyses

For quantitation of immunostaining, the number of cells that need to be imaged to observe statistically significant results will vary depending on how dramatic the observed phenotype is. Generally, we find that 50 cells is sufficient, but more subtle phenotypes may require more cells, thus in most cases 100 cells per group, split between at least three animals, will be a good experimental plan.

Understanding Results

The first step in interpreting results is to determine the efficiency of gene inactivation by CASAAV. For our best gRNAs, over 90% of transduced CMs (GFP^+) lose immunoreactivity to target-specific antibody (Fig. 31.11.3). More often, we observe that approximately 60% to 70% of transduced CMs lose immunoreactivity. Importantly, the fraction of transduced cells that undergo gene inactivation is relatively insensitive to the AAV dose, an observation that is fundamental to the mosaic gene inactivation strategy.

Immunostaining can be scored in a variety of ways (expression level, localization, etc.). For expression level, we recommend using ImageJ or similar software to quantify the fluorescence level in each cell. When interpreting results, it is important to have control groups from mice transduced with AAV carrying scrambled gRNAs, or no gRNAs at all. While we have presented a robust protocol for immunostaining, the CASAAV technique is compatible with several other approaches to measuring gene expression. Suspensions of dissociated CMs are ideal for flow cytometry, which allows for collection and pooling of mutant CMs. Pooled cells can then be utilized for quantitative PCR measurement of gene expression, western blot measurement of protein expression, and next generation sequencing to profile transcript levels. These methods should be used to corroborate immunostaining results (Guo et al., 2017).

Time Considerations

Basic Protocol 1: Target and gRNA selection can easily be completed in one day. However, this is perhaps the most crucial step in the CASAAV workflow and should not be rushed. Ordering and synthesis of oligonucleotides usually takes only a couple days.

Basic Protocol 2: Construction and delivery of AAV vector generally takes 2 to 3 weeks. It will take approximately 1 week to clone both gRNAs into the vector, and another week to produce the AAV.

Basic Protocol 3: Immunostaining can be completed in 2 days and can be conducted any time after pups reach 3 weeks of age. Time to complete subsequent analyses will vary.

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