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CASAAV: a CRISPR based platform for rapid dissection of gene function *in vivo*

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Abstract

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, the CRISPR components have been difficult to deploy *in vivo*. To address this problem, we developed CASAAV (CRISPR/Cas9-AAV-based somatic mutagenesis), a platform in which recombinant adeno-associated virus (AAV) is used to deliver tandem guide RNAs targeting a gene of interest and Cre, driven by a cell type selective promoter, to Rosa^{fsCas9GFP} postnatal mice. Using this system, within one month it is possible to induce temporally controlled cell type-selective knockout of virtually any gene using only one mouse line. Here, we focus on knockout of genes in cardiomyocytes, although with minimal modifications the system could be adapted in inactivate genes in other AAV-transduced cell types.

Keywords

CRISPR; Adeno-associated virus; Mouse model; Genetic mosaic; Cardiomyocyte

Introduction

Loss-of-function is one of the principal approaches to elucidating gene function. The Cre/loxP system enables efficient conditional ablation of target genes; however, 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 the Cas9/gRNA genome editing system can be effectively used *in vivo* to generate inactivating insertions and deletions (indels) within somatic cell targets (Carroll et al., 2016; Platt et al., 2014; Swiech et al., 2015). To that end we acquired Cre-activatable Cas9-P2A-GFP knock-in mice (Platt et al., 2014), now available from The Jackson Laboratory, and designed a single AAV vector that

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delivers both Cre and gRNAs to cardiomyocytes (CMs). 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 maximize the likelihood of generating an inactivating mutation through frameshift mutation or excision of a key functional domain. While AAV9 transduces several cell types including CMs, in our CASAAV system the cTnT promoter enhances cardiomyocyte-selective Cre expression. Within the heart, the system is only active in CMs. However, low level activity does occur in the liver (Prendiville et al., 2015). Overall the CASAAV system enables loss-of-function studies of nearly all genes, using one mouse line (Rosa26$^{Cas9\text{-}P2A\text{-}GFP}$) and easily engineered and delivered AAV vectors.

In vivo loss-of-function studies are often confounded by secondary effects that arise from whole-organ dysfunction. For instance, studies of CM gene function are prone to misinterpretation of cell autonomous gene function as a result of secondary changes that result from heart dysfunction resulting from whole-organ gene inactivation (Prendiville et al., 2015). A compelling additional advantage of using CASAAV is that these secondary effects can be avoided by employing a genetic mosaic approach in which AAV dosage is titrated such that only a fraction of cells are transduced. Thus this powerful technique allows for a quick and cost-efficient method of obtaining mutant CMs from hearts that are healthy at the organ level. To this end, in the following protocols we provide guidelines for target selection (Basic Protocol 1), selection of appropriate gRNAs (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. With minor modifications, this system could be used to inactivate genes in other AAV-transduced cell types.

Strategic Planning

There are many different experimental contexts that require mutagenesis or deletion of a region of genomic DNA. However, for the purpose of these protocols we assume that the target is a protein-coding region. This allows for validation of the knockout by assaying gene expression at the protein level. We have found that the use of immunostaining to confirm gene depletion efficiency at the single-cell level is particularly valuable, and therefore when picking gene targets we find it helpful to prioritize genes that have suitable antibodies available. This manuscript will focus on immunostaining-based validation, however, additional approaches such as western blot analysis and targeted genome sequencing using purified transduced CMs are also useful. If single-cell readouts are not as important to your particular application, then you do not need to consider the need for reliable immunostaining-quality antibodies in target selection.

Basic Protocol 1: Target and gRNA selection

This protocol describes how to most effectively pick a region within a desired gene for mutagenesis, as well as select appropriate gRNAs to target the region. The CASAAV platform utilizes tandem gRNAs to disable gene expression primarily through introduction

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of frame-shifting indels via Cas9-induced double-strand breaks at one or both gRNA sites. Optimally, the gRNAs should target regions towards the 5' end of the gene so that frameshifts that do not induce nonsense-mediated decay will disrupt most of the encoded protein. An additional advantage of using tandem gRNAs is that if both gRNAs work efficiently the intervening genomic DNA can be deleted. As a result, we commonly design gRNAs to flank a protein domain predicted to be crucial for function, so that deletion will disable the protein. Deletion does not occur at 100% efficiency, and in fact *in vitro* studies have shown that deletion efficiency decreases as deletion size increases (Canver et al., 2014), thus deletion size is an important consideration if deletion is a desired outcome. As frameshifting indels are the primary gene-inactivation mechanism, gRNAs designed to flank an upstream domain should always target within the coding sequence. When targeting genes with many exons, or genes having alternative splicing variants, it is critical to design gRNA pairs that target exons shared by all splicing variants. Finally, gRNAs must be selected that have a high probability of on-target activity and low probability of off-target activity. While there are now many computational tools for predicting gRNA activity, here we describe use of 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 et al., 2014), and a computational gRNA design tool that incorporates this consideration is also available (http://www.rgenome.net/cas-designer).

Materials

Computer with internet connection

Oligonucleotide vendor

Target selection

1. Connect to the internet and go to www.uniprot.org. 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. Here, the cardiac zinc-finger transcription factor GATA4 is used 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 Figure 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. On next screen use drop down arrow to change BLAT genome to mouse if necessary. Paste in the amino acid

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domain sequence from the clipboard and click "Submit" (Figure 1B). 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). 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 (Figure 1C). While holding down shift, click and hold on one end of the exon. Drag across to the other end to select and zoom in on the intervening exon. Hover over "View" on top menu and select "DNA". Fill in the appropriate boxes to add 16 extra bases 5' and 3' of the selected exon sequence. Click on "get DNA". Copy the resulting DNA sequence to a new document. In this document determine which base pairs correspond to the protein domain that is being targeted for deletion, and make a note (Example: 200 bp centered on Gata4 exon 3 was retrieved; zinc finger 1 corresponds to position 77–151).

gRNA selection and preparation for cloning

5 Navigate to the BROAD Institute GPP Web Portal at http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design. Select "mouse" as Target Taxon and paste the target exon sequence into the input field. Click "Submit". When job is complete click on the "sgRNA Picking Results" link and download the text file. Open the file with Microsoft Excel.

6 Find the columns labeled "Position of Base After Cut" and "Combined Rank", which takes into account both on-target and off-target predicted activity (Figure 1D). Select the "sgRNA Target Sequence" from the three highest ranking gRNAs targeting positions upstream of the protein domain and the three highest ranking gRNAs targeting positions downstream of the domain (for *Gata4*: three gRNAs targeting positions 17–76, and three for 152–184). Copy these sequences to your notes. If there are not enough quality gRNAs targeting the desired region (on-target efficacy score >0.5), you may wish to expand your search to include gRNAs that target within the protein domain, or a different domain entirely.

7 The six sequences selected above need to be optimized for expression from the dual U6 promoters in the AAV vector. Optimal RNA expression from the U6 promoter requires that the first nucleotide of the transcript be guanine (Das et al., 2000). Therefore, add a guanine nucleotide to the 5' end of all gRNA sequences that start with A, C, or T.

8 Forward and reverse oligos carrying the gRNA sequence will be annealed during cloning. Obtain the reverse complement sequence for each of the six gRNA sequences. One helpful webtool among many can be found at http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html.

9 AarI overhang sequences must be added to gRNAs corresponding to regions upstream of the target domain. This can be achieved by simply adding CACC 5' of the gRNA forward sequence, and AAAC 5' of the reverse complement sequence. See Figure 2: gRNA1 for a graphical depiction.

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10. Similarly, SapI overhang sequences must be added to gRNAs corresponding to regions downstream of the target domain. This is achieved by adding ACC 5' of the gRNA forward sequence, and AAC 5' of the gRNA reverse complement sequence. See Figure 2: gRNA2.

11. Oligonucleotides can now be synthesized by your preferred vendor. Twelve oligos should be synthesized for three pairs of gRNAs. Standard purity/desalting is sufficient.

Basic Protocol 2: Construction and Delivery of AAV9 vector

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.

In vivo delivery of tandem gRNAs is achieved by cloning the gRNA sequences designed in Basic Protocol 1 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 (Addgene #87682). Digestion with AarI restriction enzyme removes a small placeholder fragment and opens up gRNA insertion site one. Annealed oligonucleotides which correspond to the upstream gRNA sequences, designed with AarI sticky ends, are then ligated in. To add gRNA2 the process is repeated using SapI restriction enzyme and annealed oligos corresponding to the downstream gRNA sequences, designed with SapI sticky ends. In this way, two gRNAs that target positions flanking the desired domain can be quickly designed and cloned into a single AAV vector. After obtaining the final plasmid, the vector is packaged into AAV via a standard triple transfection of 293T cells with vector and two helper plasmids. 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, but the protocol can be scaled to conveniently clone multiple gRNAs in parallel.

Materials

AAV-U6gRNA1-U6gRNA2-TnT-Cre Vector (Addgene #87682)

gRNA sequences from Basic Protocol 1/DNA oligos

Thermocycler

AarI enzyme and buffer (ThermoFisher ER1581)

SapI enzyme and buffer (NEB R0569L)

37°C incubator

37°C shaking incubator

Agarose (GeneMate E-3120-500)

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50× TAE (Boston BioProducts BM250)

Ethidium bromide (Sigma E7637)

DNA loading dye (NEB B7024S)

Gel electrophoresis chamber

UV transilluminator (365 nm)

Gel purification kit (Invitrogen K210012)

Quick ligation kit (NEB M2200L)

Chemically competent cells such as ThermoFisher MAX Efficiency Stbl2 Competent Cells (Cat# 10268019). Recombination resistant bacteria are essential.

42°C heat block or waterbath

Ampicillin (ThermoFisher 11593027)

LB agar plates with 100 μg/mL Ampicillin (Teknova L1004)

LB media (40 g/L H_2O, Teknova L9115)

Miniprep culture tubes (Corning 352059)

Miniprep kit (Invitrogen K210011)

Midiprep kit (Invitrogen K210014)

Nanodrop spectrophotometer (Thermo Fisher Scientific ND-1000)

Materials for AAV production and purification (see Current Protocols in Molecular Biology, Volume 115, Unit 23.16, Basic Protocol 2 (Wakimoto, Seidman, Foo, & Jiang, 2016)).

Isoflurane (Baxter 10019-360-40)

Isoflurane anesthesia setup (anesthesia machine, induction chamber, scavenging system).

0.3 ml syringes (XELINT 26200)

$R26^{fsCas9GFP/fsCas9GFP}$ mice (Jax Stock #026175)

Cloning gRNAs into AAV vector

1. Prior to cloning, the synthesized oligos must be annealed to produce the gRNA insert. Resuspend oligos at 1 μg/μl in H_2O. For each of the six gRNAs, mix 2 μg forward oligo with 2 μg reverse complement oligo in 46 μl annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA). Place the mixed oligos in a thermocycler programmed to start at 95°C for 2 minutes, then gradually cool to

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25°C over 45 minutes. Alternatively, oligos can be annealed by placing the mixed oligos in a 95°C hot block for 2 minutes, then turning the block off and letting it cool to 37°C before removing samples. Annealed oligos can be stored at 4°C for several weeks.

2. Prepare AAV-U6gRNA1-U6gRNA2-TnT-Cre for cloning the 5' gRNA (gRNA1) by restriction digest with AarI as directed below:

3 μg of vector

2.5 μl of 10× AarI buffer

H_2O to 18.5 μl

mix

Add 1 μl (2 units) of AarI

Mix and incubate at 37°C for at least 3 hrs.

After digestion is complete, separate the linearized vector from uncut plasmid and the 30 bp placeholder by adding 5 μl of 6× loading dye and running on a 0.8% agarose gel (1× TAE, 0.5 μg/ml ethidium bromide) at 5 V/cm for 30 min.

3. Excise the digested vector band and recover DNA using a gel purification kit. Follow standard directions and elute in 20 μl H_2O. Measure concentration using a Nanodrop spectrophotometer.

4. Remove competent cells (50 μl) from freezer and place directly on ice. Proceed with next step as cells thaw.

5. Set up ligation of gel purified digested vector and annealed oligos.

10 μl 2× Quick Ligase Buffer

25 ng digested vector

1 μl annealed oligos diluted 1:100

H_2O to 9 μl

Mix

Add 1 μl Quick Ligase

Mix and incubate at room temperature for 5 minutes

Perform a control ligation in which the annealed oligo is omitted.

6. Add 10 μl of ligation product to the competent cells on ice. Incubate on ice for 20 minutes. Store the remaining 10 μl of ligation product at −20°C as a backup.

7. Heat shock competent cells and ligation mix at 42°C for 30 seconds. Add 300 μl LB media and incubate for 45 minutes in a 37°C shaking incubator. Place LB-amp agar petri dishes in 37°C incubator to warm.

8. Remove transformation reactions from incubator, resuspend bacteria and remove 100 μl of culture. Spread bacterial culture on a prewarmed petri dish and return

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dish to 37°C incubator overnight. The remaining unused bacteria can be discarded.

9. The next day remove dishes and estimate the number of colonies on each plate. If ligation is successful the vector plus annealed oligos ligation should yield several fold more colonies than the vector-only ligation. Place 3 ml LB-amp (10 μγ/ml) 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. We find that three colonies is generally sufficient to find a positive clone. If the difference between ligations with and without oligos is small, it may be necessary to screen more colonies. Incubate cultures overnight at 37°C in a shaking incubator. Incubate grid plate overnight in standard 37°C incubator.

10. The following day store grid plate at 4°C and isolate AAV plasmid vector from bacterial cultures by following mini-prep kit manufacturer instructions. The grid plate is used as emergency backup after gRNA1 cloning, and as the source of bacteria to produce a larger scale DNA prep after gRNA2 cloning.

11. Confirm successful insertion of gRNA1 by Sanger sequencing miniprep DNA. Use primer TnT-R (AGGGACTTCGGGCACAATCG), which sequences backward from the TnT promoter. Using this primer we generally get a sequencing read long enough to verify the sequence of both insert sites. Alternatively, the primer gSITE2-R (CAGAAGAGCTCGCTCTTCCG) can be used to sequence gRNA1 if nothing has yet been inserted into gRNA site 2.

12. To clone the 3' gRNA (gRNA2), repeat steps 2–11 using mini-prep DNA from a sequence-confirmed clone. At Step 2 the vector should be digested with SapI for gRNA2 insertion, and likewise, annealed oligos corresponding to gRNA2 should be used at step 5.

13. After acquiring and verifying the sequence of a clone with successful insertion of both gRNAs, prepare a midi-scale DNA prep (>100 μg) according to manufacturer instructions. Use the bacteria from the grid plate corresponding to the correct clone to start the midi-scale bacterial culture.

14. Before proceeding with AAV production confirm that the AAV ITRs have not recombined during plasmid production, resulting in deletion of the AAV genome (gRNA and TnT-Cre cassettes). This is achieved by digestion with XmaI or SmaI. If recombination has occurred, either enzyme will only linearize the plasmid, whereas if both ITRs are intact digestion will excise the AAV genome.

15. For AAV9 production we recommend a robust protocol previously published in CPMB (Wakimoto et al., 2016; See Basic Protocol 2 for details). The titer of the AAV should be determined by qPCR, as outlined in Basic Protocol 2. We use insert-specific primers that amplify a fragment of the TnT promoter, and express the titer as viral genomes per ml (vg/ml).

16. After purification of the virus, dose will need to be titrated to suit the desired application. As an approximate starting point, injection of P1 neonatal

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Rosa26$^{fsCas9GFP/fsCas9GFP}$ 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 transduces the large majority (>90%) of CMs. To reliably deliver AAV to neonatal pups subcutaneously, it is best to anesthetize them with isofluorane to avoid loss of injected AAV when pups strain following the injection. Dilute the AAV in sterile saline solution so that the desired will be delivered in 50 μl. Load the diluted AAV into a 300 μl syringe. Place pup into an isoflurane chamber and titrate the isoflurane (2–3%) so that pups become unresponsive to toe pinch. While holding the anesthetized pup belly up position the syringe needle over the sternum, pointed towards the head at an angle nearly parallel to the skin. Gently insert needle under the skin over the chest and dispense AAV. When done correctly you will observe a liquid bubble under the skin. 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.

Basic Protocol 3: Immunostaining isolated cardiomyocytes

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.

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$^{Cas9\text{-}P2A\text{-}GFP}$ 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 for visualization of GFP^{+}-transduced CMs, assessment of knockout efficiency, and assessment of 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. As multiple variations of this procedure have been previously described we do not include instructions, but recommend a reliable protocol by O'Connell et al. (2005). In the following section we present a simple procedure for adhering isolated CMs to laminin-coated coverslips, followed by fixation, permeabilization, and immunostaining that results in high quality images (Figure 3).

Materials

AAV-treated Rosa26$^{Cas9\text{-}P2A\text{-}GFP}$ mouse.

24-well dishes (Corning 3526)

Round coverslips (Fisher Scientific 50-121-5159)

Coverglass forceps (Fine Science Tools 11074-02)

DMEM High Glucose (Gibco 11995-065)

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Blebbistatin (EMD Millipore 203390)

Laminin (Corning 354239)

BSA (Sigma A3912-1006)

PBS (Gibco 10010-023)

Triton X-100 (Sigma-T9284)

PBST (PBS + 0.1% Triton X-100)

4% PFA (Diluted from 16%, Electron Microscopy Sciences 15710)

Prolong Diamond antifade mountant (Invitrogen, 36961)

27 gauge needle (BD 305136)

37°C humidified CO2 incubator

Langendorf perfusion apparatus and materials for collagenase perfusion, as described elsewhere (O'Connell et al., 2005).

Laminin-coated coverslips (see reagents and solutions, below).

Primary antibody

Secondary antibody

Attachment and pre-processing of isolated CMs

1. Dissociate heart by retrograde collagenase perfusion. We recommend the protocol by O'Connell et al. (2005).
2. Collect CMs by centrifugation at 20 × g for 4 min at room temperature. Remove the supernatant, which contains non-cardiomyocytes. Resuspend CMs in 700 μl PBS.
3. Add 12 μl 10 mM Blebbistatin to 12 ml DMEM prewarmed to 37°C. Mix and add 500 μl to each well. Note: Blebbistatin is a myosin inhibitor that improves CM survival and morphology during the brief culture period.
4. Using a wide bore 200 μl pipet tip, add 30 μl of CMs to each well. Note: wide bore pipet tips are used to ensure CMs are not damaged by shear stress during pipetting. A wide bore tip can be creating by snipping off the end of a standard 200 μl tip with scissors.
5. Incubate at 37°C for 30 minutes to allow CMs to attach. Do not culture CMs for longer than 60 minutes as CM cytoarchitecture rapidly remodels during in vitro culture. Note: In all subsequent steps cells should be protected from light when possible. This can be achieved by covering cells with aluminum foil.

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6. Remove the media and fix cells with 4% PFA diluted in PBS, 200 μl per well at room temperature for 10 minutes. Add fixative gently to avoid detaching cells.
7. Remove the fixative and replace with 200 μl PBST (PBS+0.1%Triton). Incubate for 10 minutes. Dispose of the fixative properly.
8. Remove PBST and replace with 400 μl 4% BSA/PBS. After 1 hour at room temperature, proceed to antibody staining (next section). Alternatively, plate containing cells can be wrapped in parafilm and aluminum foil and stored at 4°C for several weeks.

Antibody Staining

9. Remove block solution and replace with 200 μl 4% BSA/PBS containing diluted primary antibody. Incubate at 4°C overnight. Note: A 1:300 dilution works well with many antibodies, however, optimal dilution should be empirically determined for each antibody.
10. Rinse with 300 μl 4% BSA/PBS three times, 5 minutes each.
11. Incubate with secondary antibody diluted 1:500 in 4%BSA/PBS (200 μl per well) for 1 hour at room temperature. Note: DAPI nuclear stain may be added to the secondary antibody solution if desired.
12. Rinse with 300 μl 4% BSA/PBS three times, 2 minutes each.
13. Remove coverslips from 24-well dishes and mount on 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.
14. Allow several hours for mountant to cure at room temperature before imaging. Keep slides protected from light.

Reagents and Solutions

Laminin Coated Coverslips

1. Prepare laminin stock solution by dissolving laminin in PBS at 1 mg/ml. Freeze in 100 μl aliquots.
2. Add a coverslip to each well of a 24-well dish
3. Dilute 100 μl laminin stock solution in 7.2 ml PBS and add 300 μl to each well of a 24 well dish (~2 μg/cm^2).
4. Incubate at least 30 minutes at 37°C.
5. Aspirate the laminin solution and advance to next section, or store at 4°C for up to two weeks.

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COMMENTARY

Background Information

The CASAAV system permits Cas9-mediated somatic gene inactivation *in vivo*. Compared to Cre-loxP gene inactivation, the main advantage is that one can go from target selection to in vivo phenotype 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; however, 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 maximize the frequency of gene inactivation primarily through frameshift mechanisms. Dual gRNAs may also excise critical protein domains encoded by DNA between the two gRNA sites, which further enhances knockout efficiency. 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 system for CRISPR-based cardiac somatic mutagenesis has been reported (Carroll et al., 2016). In that study the authors generated transgenic mice in which Cas9 expression and a fluorescent label is driven by the cardiomyocyte-specific *Myh6* promoter. Similar to CASAAV, gRNA was delivered by AAV along with a second fluorescent marker. Some practical disadvantages of this system are that the transgenic mouse line is not widely available, it consumes two fluorescent channels, and its application 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 availability of suitable gRNAs targeting it. While designing gRNAs it is also useful to know where the epitopes of available antibodies are located, as analysis with an antibody that targets an epitope upstream of the gRNA target region may be confounded by detection of the mutant protein.

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 adhered to. An antibody directed against GFP might also enhance GFP detection.

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Troubleshooting

Ligation of annealed oligos 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 extending the AarI/SapI digestions, using more enzyme, or less vector. In addition, the placeholder insert for AarI contains a unique EcoRI site, while the SapI placeholder contains a unique SacI site. These restriction sites 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 the appropriate restriction enzyme. This will cut recircularized vector that lacks insert, 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 over- or under-fix, minimize exposure to light, minimize changes in sample temperature after fixation, use PBS+4%BSA for all washes, and utilize 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 a gene of interest does not have appropriate antibodies for immunofluorescence, an alternative approach is to use FACS to sort out GFP^+ CMs and detect protein depletion by western blot.

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 (Figure 3). More often, we observe that approximately 60–70% 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 quantitatively measure

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the fluorescence level for 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 qPCR, western blotting, and next generation sequencing. These methods should be used to corroborate immunostaining results.

Time Considerations

Basic Protocol One: 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.

Basic Protocol Two: Construction and delivery of AAV vector generally takes 2–3 weeks. After receiving oligos ordered after Basic Protocol 1, it will take approximately 1 week to clone both gRNAs into the vector, and another week to produce the AAV.

Basic Protocol Three: Immunostaining isolated cardiomyocytes can easily be conducted any time after pups reach 3 weeks of age, and takes 2 days to complete. Time to complete subsequent analysis will vary.

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A

Feature key	Position(s)	Description	Actions	Graphical view
Zinc finger[i]	216-240	GATA-type 1 PROSITE-ProRule annotation	Add BLAST	
Zinc finger[i]	270-294	GATA-type 2 PROSITE-ProRule annotation	Add BLAST	

B

BLAT Search Genome

Genome: Mouse
Assembly: July 2007 (NCBI37/mm9)
Query type: BLAT's guess
Sort output: query, score
Output type: hyperlink

CVNCGAMSTPLWRRDGTGHYLCNAC

C

***Gata4* (chr14)**

100 bases mm9
63,823,450 63,823,500 63,823,550 63,823,600
RefSeq Genes
L R R Q P K I L P R N I G N M K H Y L G C A N C L Y H G T G D R R W L P T S M A G C N V C E R G E S F D D F M D V
L R R Q P K I L P R N I G N M K H Y L G C A N C L Y H G T G D R R W L P T S M A G C N V C E R G E S F D D F M D
Your Sequence from Blat Search
YourSeq

D

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 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.

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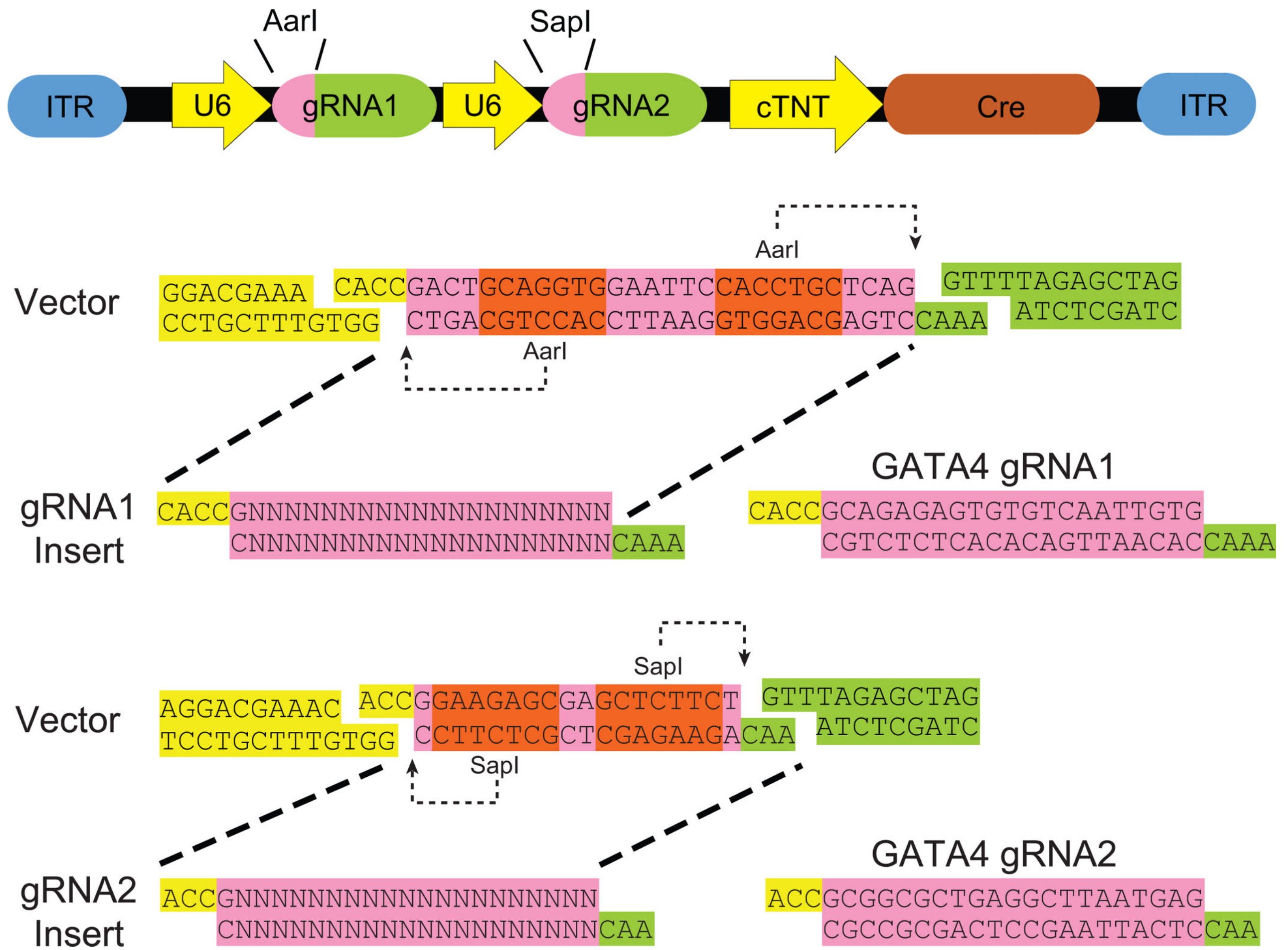

Figure 2. Insertion of gRNA sequences into AAV vector

gRNAs are inserted into AAV plasmid containing U6 promoters and cardiac troponin T (cTNT) promoter-driven Cre recombinase. Annealed oligos corresponding to the targeting portions of gRNA1 and gRNA2 are cloned between AarI and SapI sites, respectively. 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.

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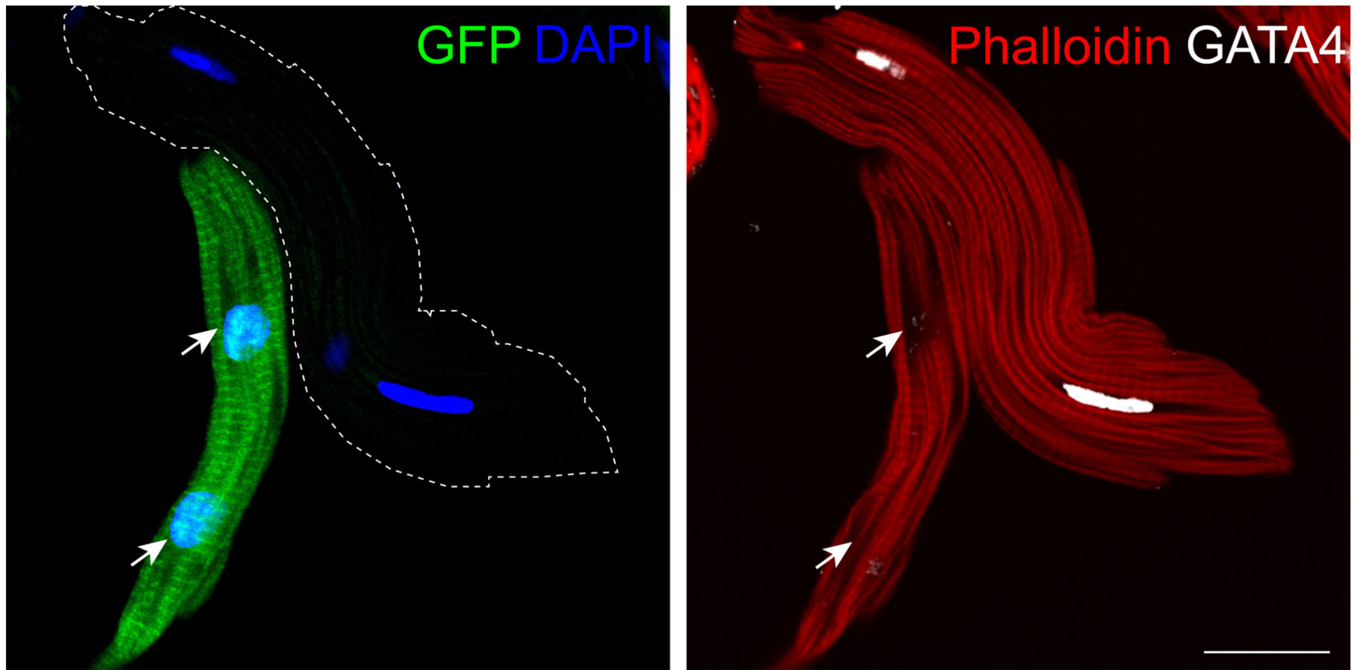

Figure 3. Immunostained CMs featuring CASAAV mediated KO of GATA4

Rosa26$^{fsCas9GFP/fsCas9GFP}$ CMs transduced with AAV9-U6-gRNA(Gata4)-cTNTCre express GFP and Cas9. Immunostaining confirmed successful depletion of GATA4. Phalloidin labels cardiomyocytes. GATA4 nuclear staining was observed in a non-transduced (GFP–) CM, but not in nuclei of transduced (GFP+) CM (white arrows). Scale bar = 20 μm.