SUPPLEMENTAL MATERIAL

In vivo Ryr2 editing corrects catecholaminergic polymorphic ventricular tachycardia

Xiaolu Pan MD, Leonne Philippen PhD , Satadru Lahiri PhD , Ciaran Lee PhD, So Hyun Park, Tarah A. Word PhD, Na Li PhD, Kelsey E. Jarrett MS, Rajat Gupta MS, Julia O. Reynolds BS, Jean Lin BS, Gang Bao PhD, William R. Lagor PhD, and Xander H.T. Wehrens MD PhD

DETAILED METHODS

Plasmid construction. The AAV-CRISPR vector, 1255_pAAV-U6-SA-BbsI-MluI-gRNA-CB-SACas9-HA-OLLAS-spA was generated by the Lagor lab (Addgene plasmid 109320) based on the SaCas9 transgene from px602, Addgene plasmid 61593 (gift from Dr. Feng Zhang).[1] The 1255 plasmid contains a site for cloning custom gRNA as well as the SaCas9 nuclease for packaging into a single AAV vector. A gRNA targeting exon 8 of the *Ryr2* R176Q allele was designed based on the presence of a canonical NNGRRT protospacer adjacent motif (PAM) in the target (R = G or A). This gRNA is adjacent to the R176Q mutation, and incorporates silent nucleotide changes relative to the wildtype sequence that were incorporated when the mouse was targeted.[2,3] Oligos for the gRNA were annealed and cloned into the BbsI site of the plasmid mentioned above: 5'-CACCgCCTTCGGACCGCTGCTTAGAGG-3', 5'-AAACCCTCTAAGCAGCGGTCCGAAGGC-3'. The final product pAAV-U6-Ryr2-R176Q-gRNA-CB-SaCas9 was sequenced and the integrity of the inverted terminal repeats (ITRs) was verified by restriction digests and Sanger sequencing.[4] To generate a reporter vector for editing with our gRNA, we designed a plasmid with the CB promoter upstream of a STOP cassette and an mCherry transgene. The STOP cassette is flanked by loxP sites (Cre activity is a positive control) as well as the gRNA target site on each side. Genome editing with SaCas9 and the gRNA will often result in excision of the STOP cassette, providing a useful surrogate for RyR2 editing activity. Additional restriction sites are included to allow for cloning of any SaCas9 gRNA target site around the STOP cassette. The mCherry transgene was synthesized by GeneWiz and cloned into a plasmid containing the ITRs from AAV2 to produce pAAV-CB-LSL-Ryr2SAgRNA4-mCherry-pA .

Production of adeno-associated virus type 9 (AAV9). AAV9 were packaged in the HEK293T cells (ATCC) by the triple transfection method of Xiao *et al*.[5] The Adenoviral helper plasmid pAdDeltaF6 (PL-F-PVADF6) and the AAV9 packaging vector pAAV2/9 (PL-T-P0008-R2) were obtained from the University of Pennsylvania Vector Core. Large-scale plasmid preps of these packaging vectors were generated by Puresyn, Inc. The constructs of BbsI-SaCas9, gRNA-SaCas9 and the "reporter" generated above were used for the AAV production, respectively. Briefly, 12 hours before transfection, cells were seeded in 150 mm plates (30-50% confluent) fed with DMEM (Lonza) containing 10% fetal bovine serum (HyClone) with L-Glutamine and Penicillin/Streptomycin (Gibco).

After 48-72 hours, cells were processed by TrypLE, collected in PBS (Corning) and resuspend in resuspension buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$). The cells are subjected to 3 freeze-thaw cycles in -80°C for 10 minutes, 37°C for 20 minutes. Cells were added with 3,000 U Benzonase (Sigma) and incubated at 37°C for 1 hour to digest cellular genomic DNA. The suspension was treated with 1/39th volume of 1M $CaCl_2$ solution and 2/3 volume of 20% PEG 8000/1.25N NaCl to remove the cell debris and precipitate AAV. AAV was then resuspended in HBS and purified by $CsCl_2$ gradient. After 2 round of $CsCl_2$ gradient in 45,000 rpm and 60,000 rpm, fractions were collected according to the desired refractive index, respectively, 1.3680-1.3750 for the first gradient and 1.3680-1.3750 for the second gradient.

AAV were dialyzed against PBS in 10,000 MWCO Slide-A-Lyzer Cassettes (0.5–3.0 mL), and concentrated using Amicon 100 kDa MWCO centrifugal filtration device (EMD Millipore Cat# UFC910008) prior to storage at -80°C. After DNAse digestion, AAV was titered by Q-PCR as described using primers to targeting SaCas9: forward: 5'-CCGTCGTGAAGAGAAGCTTCATC-3', reverse: 5'-CCACCTCATAGTTGAAGGGGTTG-3', or for the reporter to target mCherry: forward: 5'-cctgtcccctcagttcatgt-3', reverse: 5'-cccatggtcttcttctgcat-3.[4] Specifically, purified virus was digested with DNAse I (Sigma) to remove unencapsidated DNA from the prep. This was followed by proteinase K digestion to free the DNA template from the AAV capsid. AAV transgene plasmids were serially diluted to generate standard curves, and the genomic copies numbers per mL were calculated for each virus.

EP studies. *In vivo* electrophysiology studies were performed at 5-6 weeks post-injection in R176Q/+ and wildtype littermates as previously described.[6,7] Briefly, a 1.1 F octapolar catheter (EPR-800, Millar, USA) was inserted into right atrium and right ventricle via the external jugular vein of the anesthetized mouse. Simultaneous recording of surface ECG and intracardiac electrograms was acquired by IOX2.4 software (EMKA, USA). The body temperature was maintained at 36.5-37.0 °C by a heating device (Indus, Houston, TX) connected to the ECG board. Baseline ECG parameters (RR, HR, PR, QRS, and QTc) were measured in R176Q/+ and WT mice that were treated with either control virus (AAV9-SaCas9/Bbsl) or editing virus (AAV9-SaCas9/gRNA). To mimic the adrenergic stimulation, mice were injected with isoproterenol (0.5 mg/kg, i.p.) and caffeine (120 mg/kg, i.p.). To determine the inducibility of ventricular tachycardia (VT) after adrenergic stimulation, programmed intracardiac stimulation was performed 10 minutes after the injections. All mice were subjected to the S1-S2 extra stimuli pacing at cycle length of 70 ms. The incidence of inducible VT was calculated by the number of mice with reproducible VT divided by the total number of mice studied in each group.

Echocardiography. Mice were anesthetized with 2% Isoflurane and underwent transthoracic echocardiography using the VisualSonics Vevo 2100 Imaging System with a high frequency (30 Mhz) probe. Briefly, mice chests were depilated using Nair crème, and mice were placed supine on a heated board with embedded ECG leads. Temperature was strictly maintained between 36.5 ºC and 37.5 ºC, and heart rate was maintained above 400 beats per minute to void confounding effects of hypothermia and bradycardia. B-mode short axis images and M-mode scans were collected, and analyzed following completion of the data acquisition.

Western blotting. Whole mouse ventricles were isolated from anesthetized mice and flash frozen in liquid nitrogen. Frozen tissues were resuspended in a modified radioimmunoprecipitation assay (RIPA) buffer containing 1% CHAPS, phos-stop and complete mini protease inhibitor cocktail (Roche), 20mM Sodium Fluoride (NaF), 1mM Sodium Orthovanadate (Na_3VO_4) and further homogenized with steel beads using a 4^0 C electric homogenizer (Tissue Lyser LT - Qiagen) at 50 1/s for 5 minutes. These samples were then sonicated 3 times for 1 second each on ice, and centrifuged at 14,000 rpm for 20 minutes. Supernatants were collected as the tissue lysates and processed for western blot experiment. Western blotting was performed as described.[8] Sample concentration was measured using Pierce BCA protein assay kit (23227, Thermo Scientific). 75 μg of each samples was further denatured in 2x Laemmli sample buffer (1610737, Biorad) with 5% 2-Mercaptoethanol (M3148, Sigma) for 30 minutes at room temperature and loaded in SDS gel alongside precision plus protein dual color standards (Biorad).

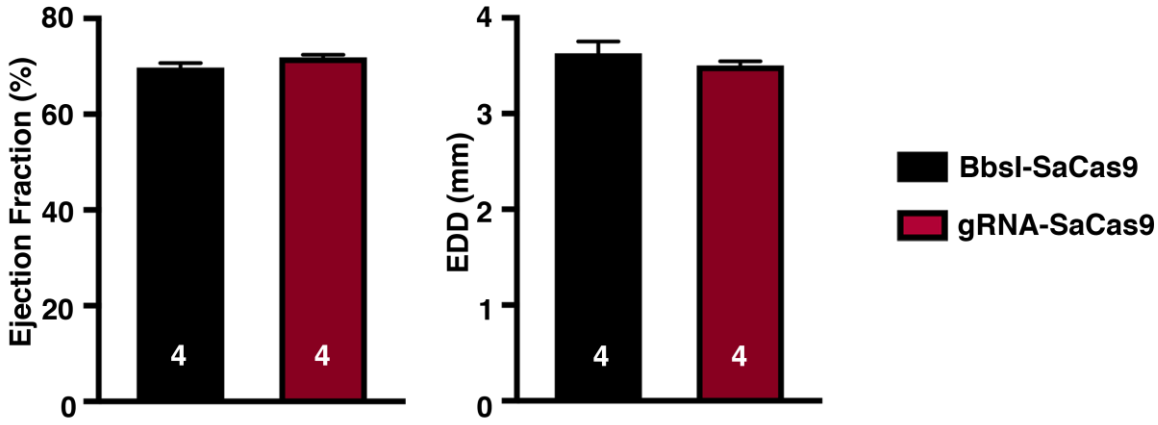
SDS gel electrophoresis was done on 5-12% gradient acrylamide gel with 5% top and 12% bottom resolving gels at constant 80 Volts. Proteins were further transferred onto 0.45 micron polyvinylidene fluoride (PVDF) membranes overnight at constant 20 Volts in cold room.

Membranes were blocked for 1-hr at room temperature with Odyssey® blocking buffer-TBS (LICOR) and incubated overnight at 4ºC with primary antibodies diluted in blocking buffer and 0.1% tween-20. Antibodies for following proteins were used to probe the membranes; RYR2 (1:2000, Custom, Yenzyme, Rabbit), JPH2 (1:2000, Custom, Yenzyme, Rabbit), CSQ (1:1,000; PA1-913, Thermo Scientific, Rabbit) and GAPDH (1:10,000; MAB-374, Millipore, Darmstadt, Germany, Mouse). Membranes were washed with TBST (0.1% tween-20) for 3x 10 minutes each and incubated in secondary goat-anti-mouse-Alexa-Fluor-680 (1:10,000, A-21057, Invitrogen, Thermo Fisher Scientific) and goat-anti-rabbit-IR800 (1:10,000, 611-145-122, Rockland Immunochemicals), for 1hr at room temperature. After washing 3x 10 minutes each in TBST; membranes were developed using LICOR odyssey infrared imager. Bands were quantified using the ImageJ software.

Analysis of next generation deep sequencing data. Processed reads were aligned to reference sequences and analyzed using a custom Matlab script. First, WT and R176Q reads were identified and binned based on unique SNPs. Indels were identified by variation in sequence length in a 800-bp window surrounding the gRNA cut site. All indel reads were sorted by length and sequence type to identify common indels in each mouse, as described.[9] Reported indel frequencies are presented as an average of four treated mice.

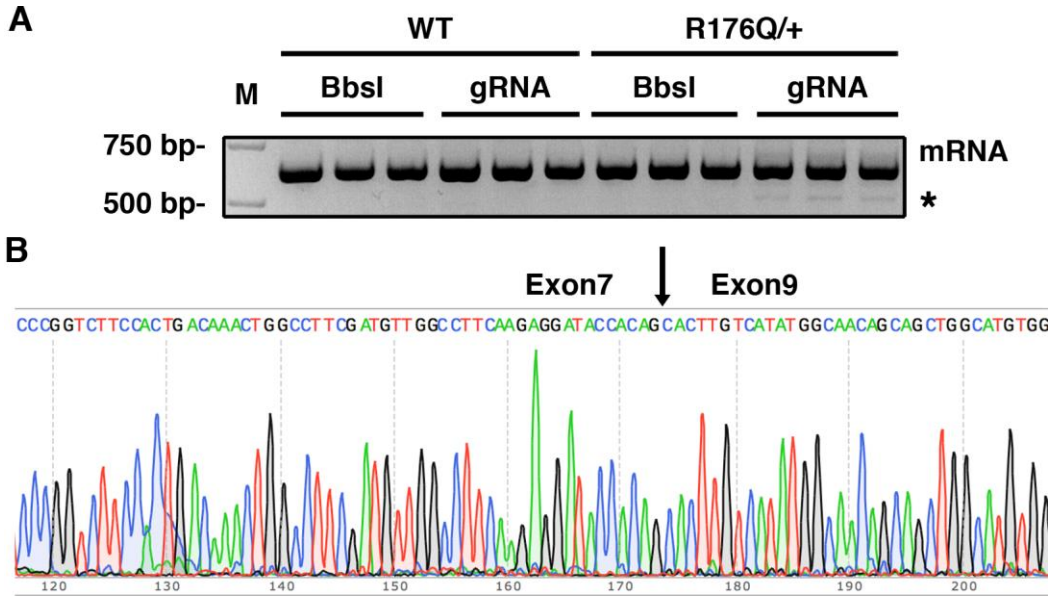
AAV-CRISPR Integration PCR. To detect forward and reverse integrations of AAV-CRISPR in Ryr2, PCRs were completed with one primer located within endogenous genomic sequence and a second primer located within AAV-CRISPR. 50 µg of genomic DNA was subjected to 40 cycles of PCR (APEX TaqRed, 42-138). The target bands were resolved by gel electrophoresis.

Online Figure I



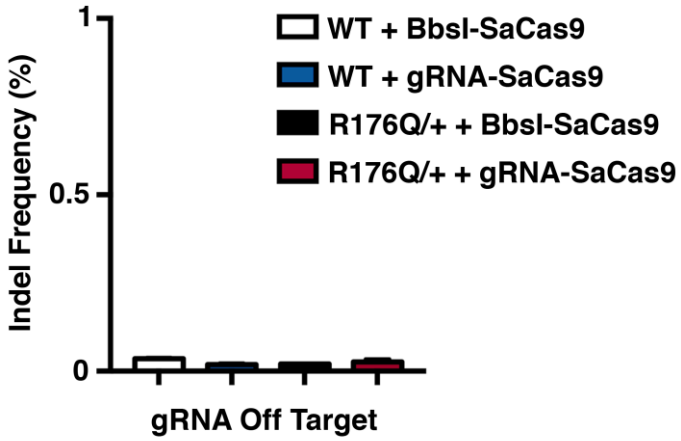
Echocardiographic assessment of cardiac contractility and dimensions in AAV9-treated R176Q/+ mice. Echocardiograms were performed on R176Q/+ mice treated with either gRNA-SaCas9 or BbsI-SaCas9. There was no significant difference in ejection fraction (EF) or end-diastolic left ventricular diameter in diastole (EDD) comparing both groups.

Online Figure II



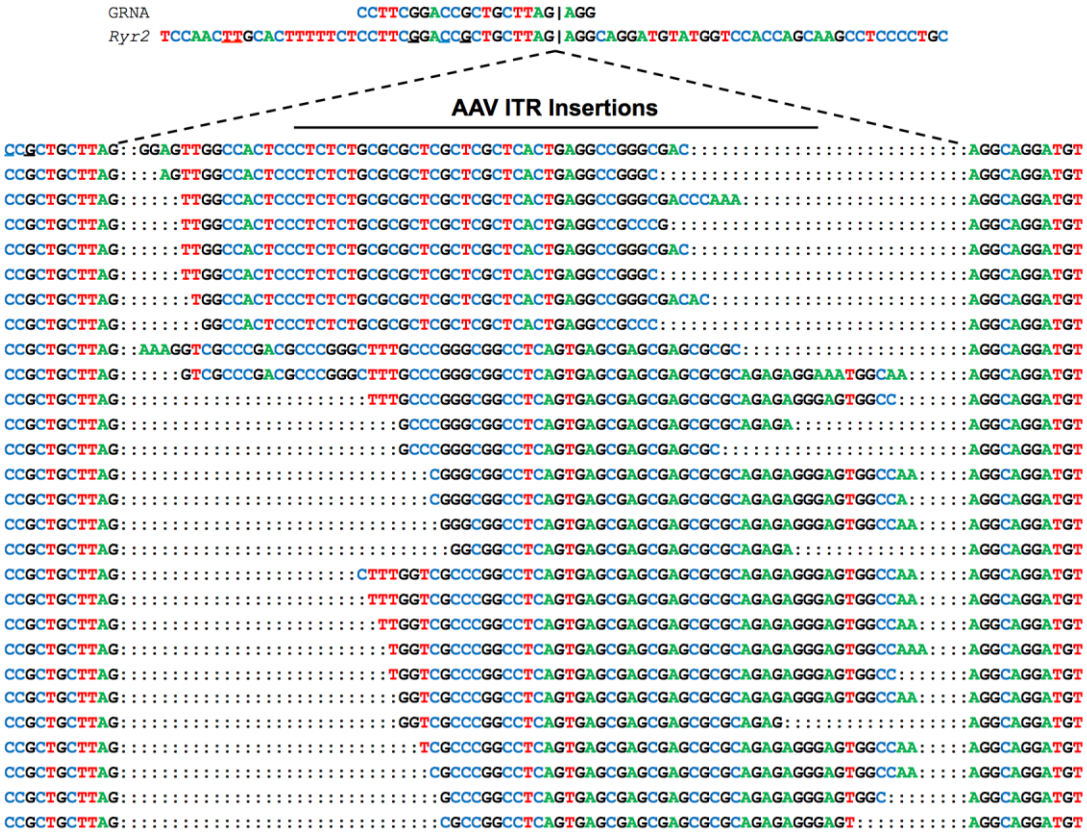
Exon 8 skipping in gRNA-SaCas9 R176Q/+ model. A, RT-PCR Analysis of cDNA extracted from WT and R176Q/+ mice treated with gRNA-SaCas9, * marks the alternative spliced sequence lacking exon 8. **B,** Sanger sequencing analysis of the smaller RT-PCR product in RT-PCR analysis. All data is representative of two experimental samples.

Online Figure III



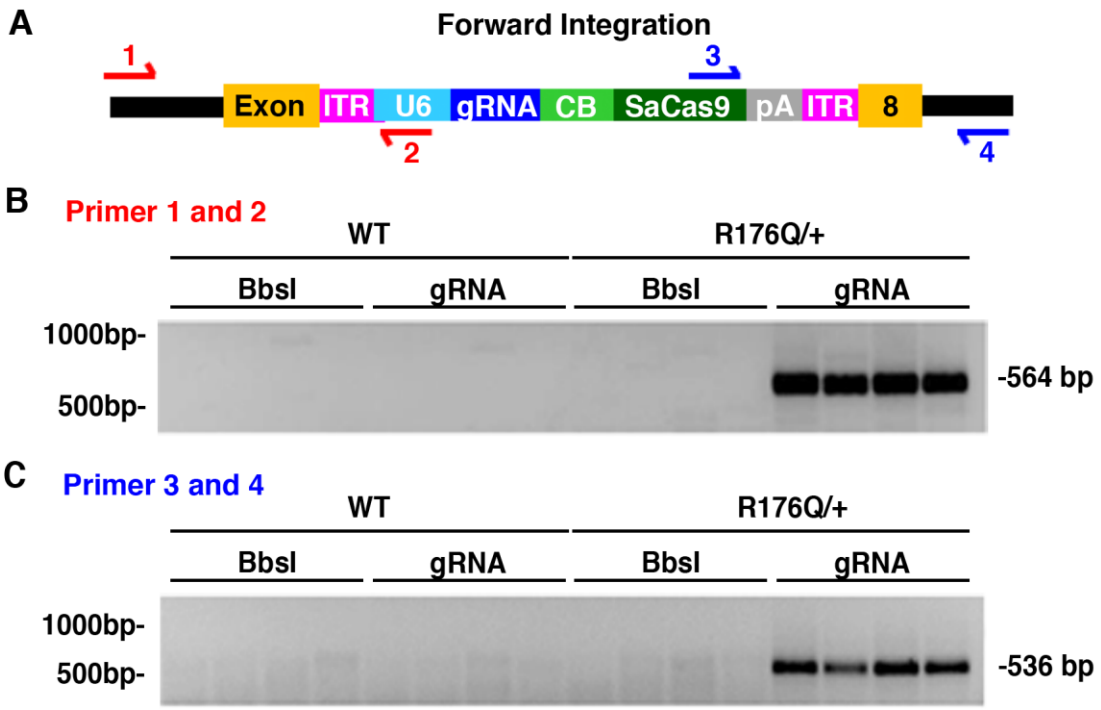
Absence of off-target indels. Next generation deep sequencing was used to quantify the frequency of indels in genomic DNA from mouse heart tissue samples obtained from WT and R176Q/+ mice receiving Bbsl-SaCas9 (n=4) or gRNA-SaCas9 (n=4), respectively. Data represented as mean +/- SEM.

Online Figure IV



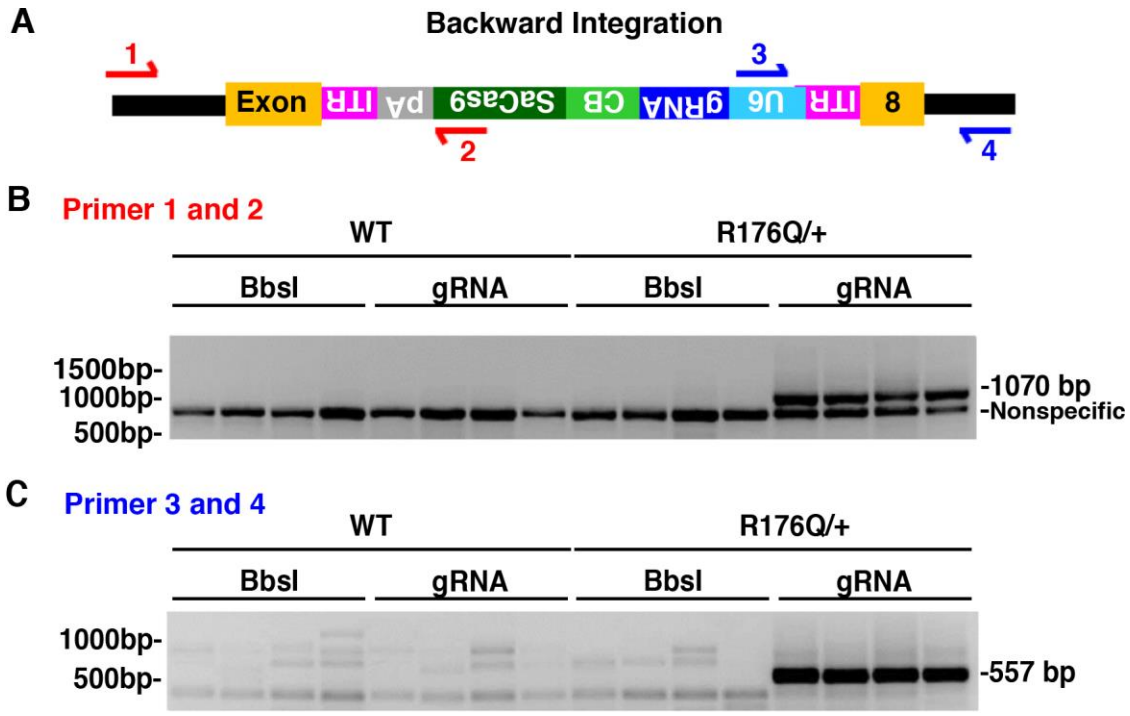
Insertion of AAV-ITR sequences at CRISPR/SaCas9 target site. Next generation deep sequencing of genomic DNA obtained from a R176Q/+ mouse treated with gRNA-SaCas9. Short ITR sequences were detected at the gRNA cut site 3 base pairs upstream of the PAM sequence (CAGGAT). Representative sequencing reads are shown.

Online Figure V



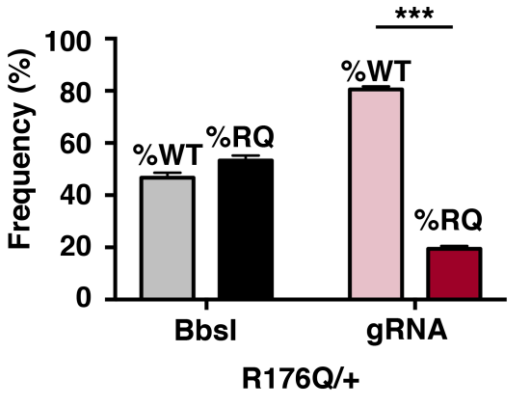
Forward AAV Vector genome insertions at the CRISPR cut site in *Ryr2*. A, Schematic diagram of the Exon 8 of *Ryr2* with forward integration of AAV-CRISPR. Red forward primer 1 is located in *Ryr2* intron 7 and red reverse primer 2 is located in the U6 promoter of the AAV-CRISPR vector. These primers amplify a product only when the front portion of AAV-CRISPR vector integrates in the forward direction with products shown in **B**, Blue forward primer 3 is located within SaCas9 in the AAV-CRISPR vector and blue reverse primer 4 is located in *Ryr2* intron 8 of the genomic sequence. These primers amplify a product only when the back portion of AAV-CRISPR vector integrates in the forward direction with products shown in **C**, Four representative samples are shown for each group tested.

Online Figure VI



Reverse AAV Vector genome insertions at the CRISPR cut site in *Ryr2*. A, Schematic diagram of the Exon 8 of *Ryr2* with backward integration of AAV-CRISPR. Red forward primer 1 is located in *Ryr2* intron 7 and red reverse primer 2 is located in SaCas9 within the AAV-CRISPR vector. These primers amplify a product only when the back portion of AAV-CRISPR vector integrates in the backward direction with products shown in **B**. Blue forward primer 3 is located within the U6 promoter in the AAV-CRISPR vector and blue reverse primer 4 is located in *Ryr2* intron 8 of the genomic sequence. These primers amplify a product only when the front portion of AAV-CRISPR vector integrates in the backward direction with products shown in **C**. Four representative samples are shown for each group tested.

Online Figure VII



Relative amounts of mRNA transcripts encoding the WT or R176Q mutant RyR2. RT-PCR was performed to generate cDNA from mRNA in heart samples obtained from R176Q/+ mice treated with Bbsl-SaCas9 (n=3) or gRNA-SaCas9 (n=4), respectively. Next generation deep sequencing was performed to quantify the relative proportions of mRNA from the WT or mutant allele. Data represented as mean +/- SEM. *** $p<0.001$.

Online Table I

Name	Sequence (5' - 3') Forward	Sequence (5' - 3') Reverse
Deep _Sequencing_ On target	**TCTACAGTCCGACGATCA**GAAGC CATGTCCCCACACTT	**GACGTGTGCTCTTCCGATC**GAGTG GCGTCATCTGGAGAG
Deep _Sequencing_ Off Target	**TCTACAGTCCGACGATCA**TCCAA GTGCCACTATAGGGG	**GACGTGTGCTCTTCCGATC**TGGTG AAGAGCATTGTGGGA
Deep _Sequencing_ cDNA_On target	**TCTACAGTCCGACGATCA**ACCGG ACACTCCTCTATGGA	**GACGTGTGCTCTTCCGATC**ACACG CTGACCAAGATGAGA
qRT-PCR exon 3-6	CGGACCTGTCTATCTGCACC	GGCGTGTCCATAGAGGAGTG
qRT-PCR exon 11-13	GGTGGTGCCGTATCAGTTCA	CGACTTCTTTCCTCACCCC
RT-PCR for RyR	AAAGTCAGAAGGGCAGGTGG	ATGGCTGGCCCCATCTTATG
GAPDH	TGTGTCCGTCGTGGTCTGA	CCTGCTTCACCACCTTCTTGAT

Supplemental References

1. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using staphylococcus aureus Cas9. *Nature*. 2015;520:186-191.
2. Kannankeril PJ, Mitchell BM, Goonasekera SA, Chelu MG, Zhang W, Sood S, Kearney DL, Danila CI, De Biasi M, Wehrens XH, Pautler RG, Roden DM, Taffet GE, Dirksen RT, Anderson ME, Hamilton SL. Mice with the R176 cardiac ryanodine receptor mutation exhibit catecholamine-induced ventricular tachycardia and cardiomyopathy. *Proc Natl Acad Sci U S A*. 2006;103:12179-12184.
3. Mathur N, Sood S, Wang S, van Oort RJ, Sarma S, Li N, Skapura DG, Bayle JH, Valderrabano M, Wehrens XH. Sudden infant death syndrome in mice with an inherited mutation in RyR2. *Circ Arrhythm Electrophysiol*. 2009;2:677-685.
4. Lagor WR, Johnston JC, Lock M, Vandenberghe LH, Rader DJ. Adeno-associated viruses as liver-directed gene delivery vehicles: Focus on lipoprotein metabolism. *Methods Mol Biol*. 2013;1027:273-307.œœ
5. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol*. 1998;72:2224-2232.
6. Chelu MG, Sarma S, Sood S, Wang S, van Oort RJ, Skapura DG, Li N, Santonastasi M, Muller FU, Schmitz W, Schotten U, Anderson ME, Valderrabano M, Dobrev D, Wehrens XH. Calmodulin kinase II-mediated sarcoplasmic reticulum Ca2+ leak promotes atrial fibrillation in mice. *J Clin Invest*. 2009;119:1940-1951.
7. Li N, Wehrens XH. Programmed electrical stimulation in mice. *J Vis Exp*. 2010:1730.
8. Li N, Chiang DY, Wang S, Wang Q, Sun L, Voigt N, Respress JL, Ather S, Skapura DG, Jordan VK, Horrigan FT, Schmitz W, Muller FU, Valderrabano M, Nattel S, Dobrev D, Wehrens XHT. Ryanodine receptor-mediated calcium leak drives progressive development of an atrial fibrillation substrate in a transgenic mouse model. *Circulation*. 2014;129:1276-1285.
9. Lee CM, Cradick TJ, Bao G. The neisseria meningitidis CRISP-Cas9 system enables specific genome editing in mammalian cells. *Mol Ther*. 2016;24:645-654.