Supplementary Materials for

Allele-specific Silencing of Mutant *Myh6* Allele in Mice Suppresses Hypertrophic Cardiomyopathy

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Materials and Methods

Mouse protocols. All mice were maintained and studied using protocols approved by the Animal Care and Use Committee of Harvard Medical School. Studies used male heterozygous $MHC^{403/+}$ mice that were in 129SvEv background or heterozygous $MHC^{403/+}$ F1 offspring male mice on the 129SvEv and FVB background. Viruses were injected via a single 50ul bolus, using a 30G needle inserted through the diaphragm by a subxiphoid approach into the inferior mediastinum, avoiding the heart and the lung. Cyclosporine A (CsA, Sandimmune (100mg cyclosporine capsule), Novartis, NY, USA)) was administered via oral chow that contains CsA (1mg/g). We studied male $MHC^{403/+}$ mice that more consistently develop HCM than do female littermates.

Cell culture and transfection. 293T cells were cultured at 37 °C with 5% CO_2 and maintained in DMEM, supplemented with 10% heat-inactivated FBS, 0.1 mM MEM nonessential amino acid, 5,000 units per ml penicillin–streptomycin. Transfection of RNAi constructs and overexpression plasmids was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

AAV-9 production and purification. AAV vectors were packaged into AAV-9 capsid by the triple transfection method using helper plasmids pAdΔF6 and plasmid pAAV2/9 (Penn Vector Core). Fifty μg of plasmid DNA was used per 15 cm cell culture plate. Three days after transfection, AAV vectors were purified by Optiprep density gradient medium (D-1556, Sigma) by centrifugation and stored at −80°C.

RNAi vector construction. Constructs for *Myh6* R403Q were designed to target 21 base-pair gene-specific regions. Oligonucleotides were cloned into pCAG-miR30 (kindly provided by Dr. Constance Cepko). The sequences targeted by RNAi are as follow: 403m RNAi, ccctcaggtgagggtggggac; 403i, cactcaggtgagggtggggac; 129i, ccactttggagctactggaaa. The sequence against LacZ, gactacacaaatcagcgattt was used as control RNAi. The miR-30 cassette was inserted 3' of the EGFP gene in AAV vector.

RNA-seq and analysis. Hearts from mouse (strain 129SvEv) were rapidly isolated, placed in room temperature PBS to evacuate blood, and then immersed in RNALater (Qiagen) at room temperature. Two micrograms of total ventricular RNA was used to construct RNAseq sequencing libraries as described (*19*). In brief, polyA RNA is selected on oligo-dT magnetic beads, converted to cDNA with reverse transcriptase, made double stranded DNA, flush ended, and ligated to double strand Illumina sequencing adapters. Size selected 150-250 bp fragments were isolated from acrylamide gels before amplification and sequenced (50 base, paired ends) using the Illumina HiSeq2000.

Quantification of myocardial fibrosis. Hearts were excised from isoflurane- euthanized mice, washed in PBS, fixed overnight in 4% paraformaldehyde, and embedded in paraffin. After serial sectioning of hearts (apex to base), eight evenly distributed 5μm sections were stained with Masson trichrome. Heart sections were scanned by BZ-9000 Generation II (Keyence). Fibrosis areas within sections were measured by software BZ-II Analyzer (Keyence). The percentage of total fibrosis area was calculated as the summed blue-stained areas divided by total ventricular area.

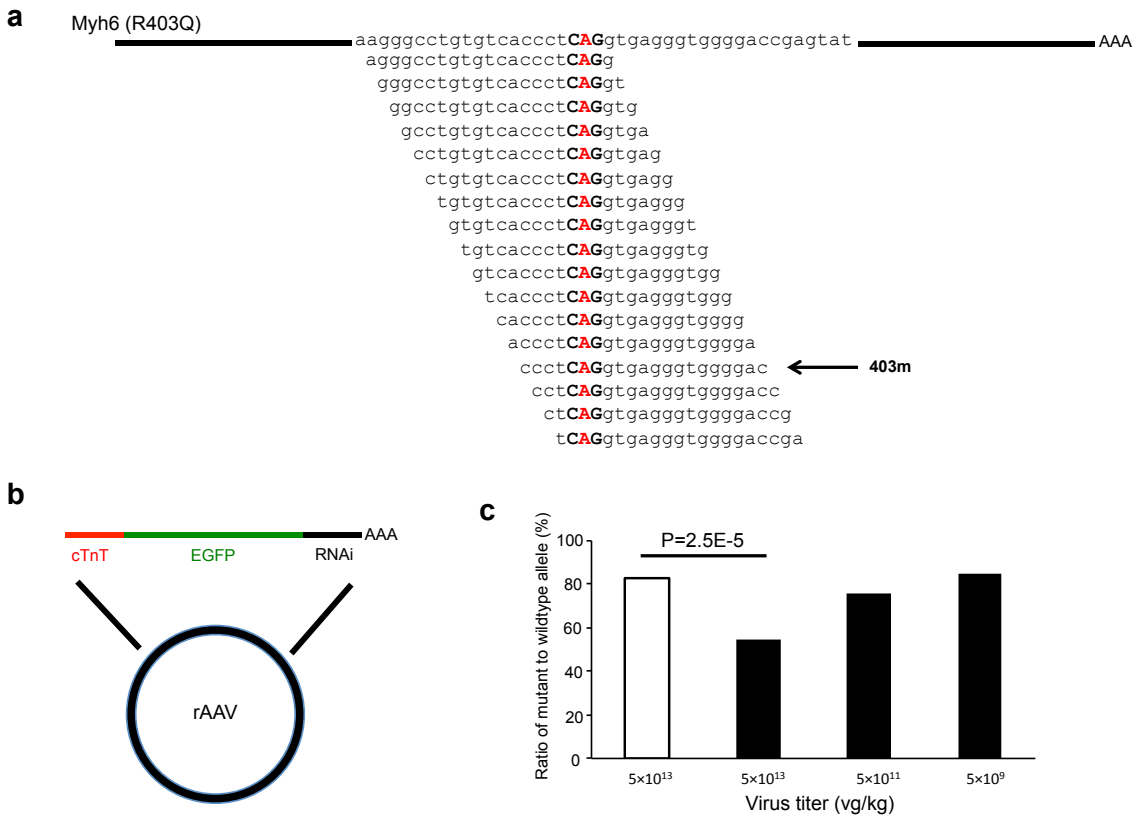
Immuno-staining and Analysis. Histochemical analyses were performed on heart sections fixed in 4% paraformaldehyde overnight. Sections were treated with xylene (to remove paraffin), re-hydrated, and permeabilized in 0.1%(v/v) Triton-X100 in PBS.

Sections were incubated with primary antibodies applied at 1:200 dilution (unless otherwise indicated) in 0.1%(w/v) BSA in PBS overnight at 4ºC and non-specific antibody binding was blocked by 1.5%(v/v) donkey serum in PBS. Primary antibodies included: cardiac troponin-I (goat anti-Tnni3, Abcam ab56357, 1:200), GFP (chicken anti-GFP, Abcam ab13970, 1:200).

Echocardiogram and surface electrocardiogram (ECG). Echocardiogram data were obtained using Vevo 770 High-Resolution In Vivo Micro-Imaging System and RMV 707B scan-head (VisualSonics Inc.) as previously described(*13*). The images were acquired as 2D and M-mode (left parasternal long and short axes) and measurements were averaged from 3 consecutive heart beats of M-mode tracings as recommended by the American Society of Echocardiography's Guidelines (*20*) . LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and wall thickness (LVWT) were attained by short axis image and left atrial diameter (LA) and aortic root diameter (Ao root) were measured by long axis image. LV fractional shortening (%) was calculated as follows: (LVEDD – LVESD)/LVEDD × 100. Surface ECG were recorded with GE/Marquette CardioLab 7000 EP recording system.

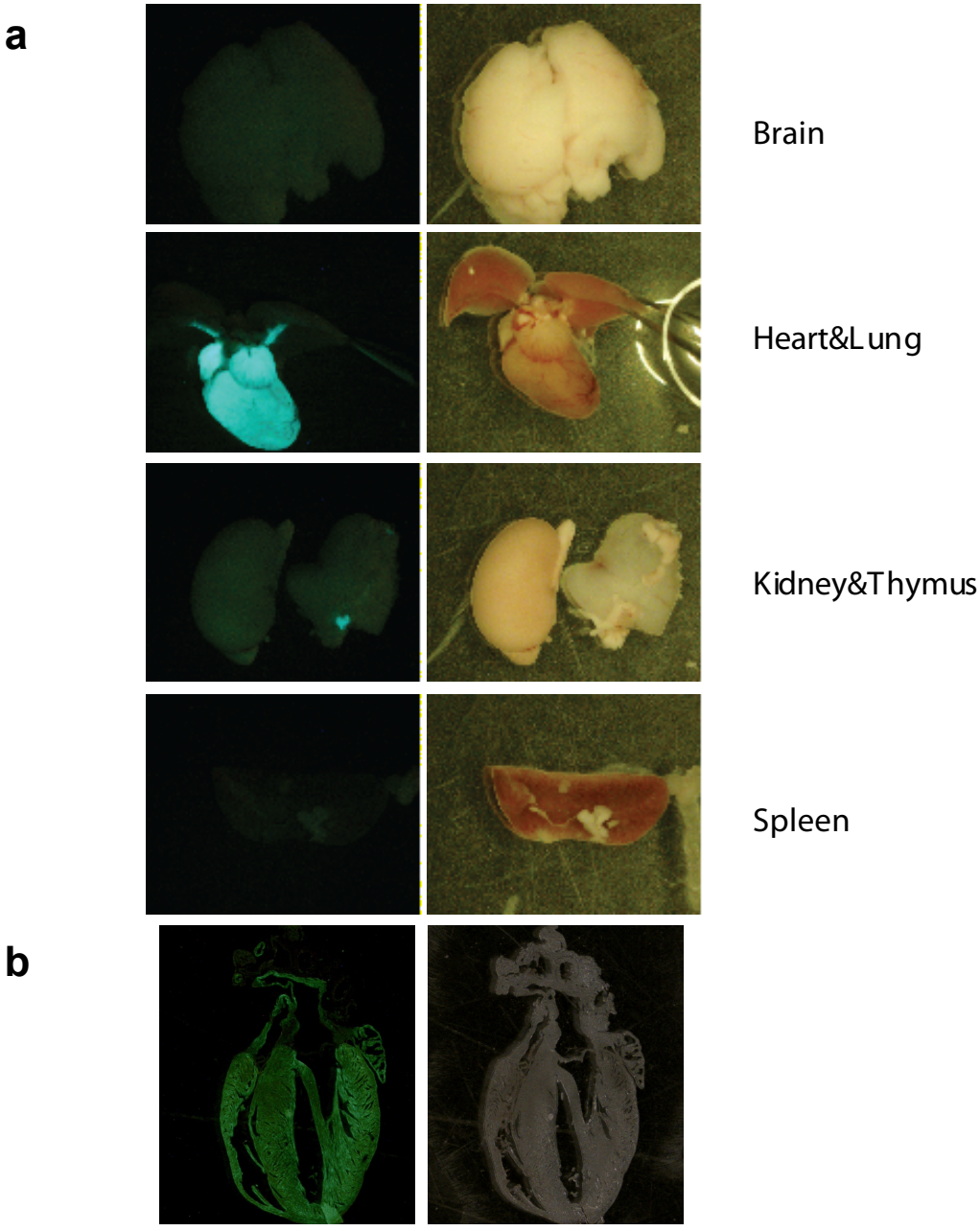
Statistical Analyses. Statistical analysis of data was performed by t-test, false discovery rate (FDR) and ANOVA for multiple comparisons.

Fig. S1.



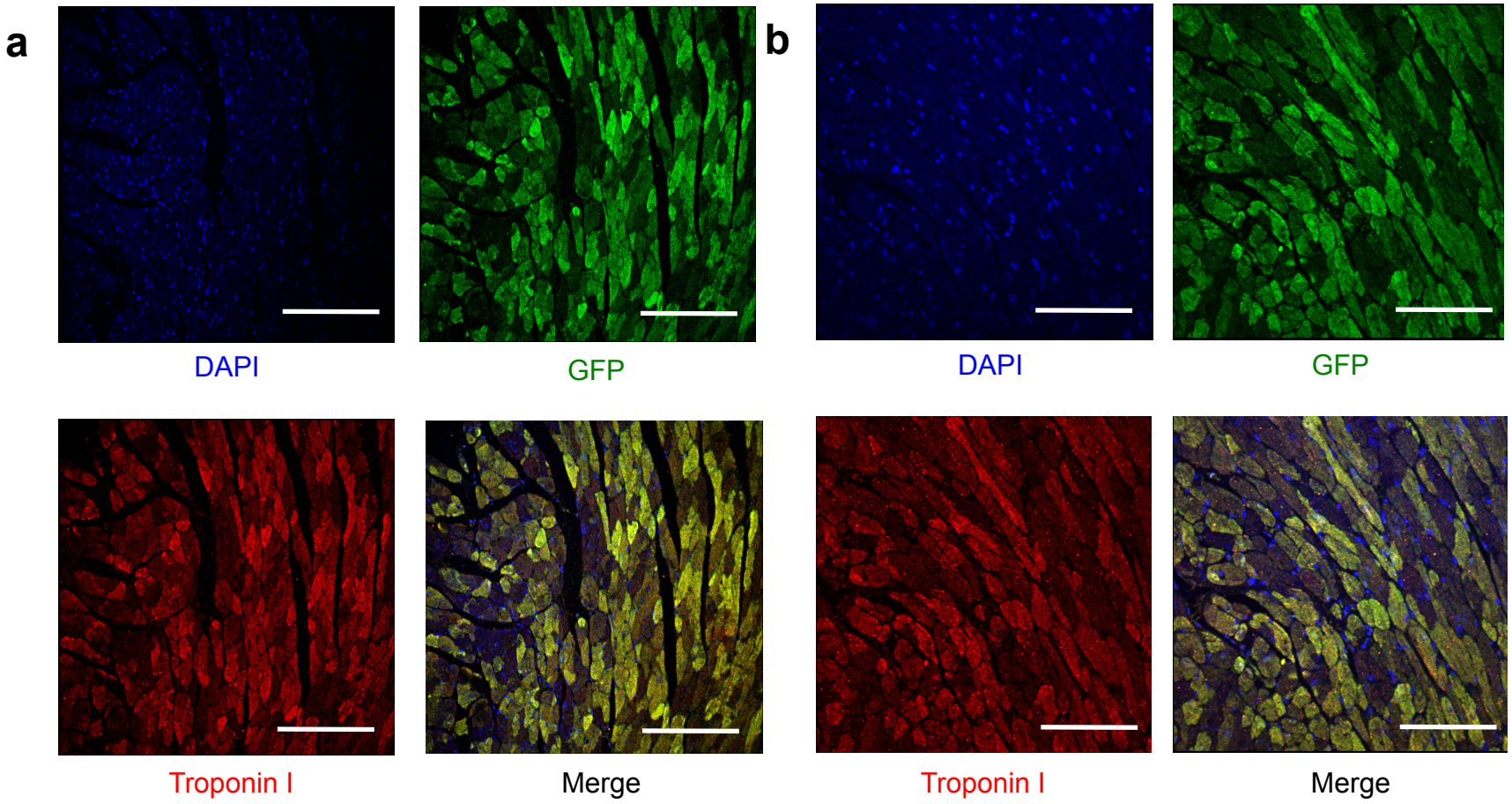
RNAi molecules used to suppress *Myh6* R403Q. (a) Schematic representation of mutant (R403Q) transcript and RNAi sequences. (**b**) Schematic representation of AAV vector including cTnT, cardiac troponin promoter; EGFP, enhanced green fluorescent protein; and RNAi, RNAi cassette. (**c**) Relative expression of mutant *Myh6* R403Q transcripts to wild-type transcripts (quantified by RNAseq) in hearts from 14-day old $MHC^{403/+}$ mice injected at day one with AAV-9 control virus (white) or AAV-9-403i virus (black) at the titers indicated.

Fig. S2.



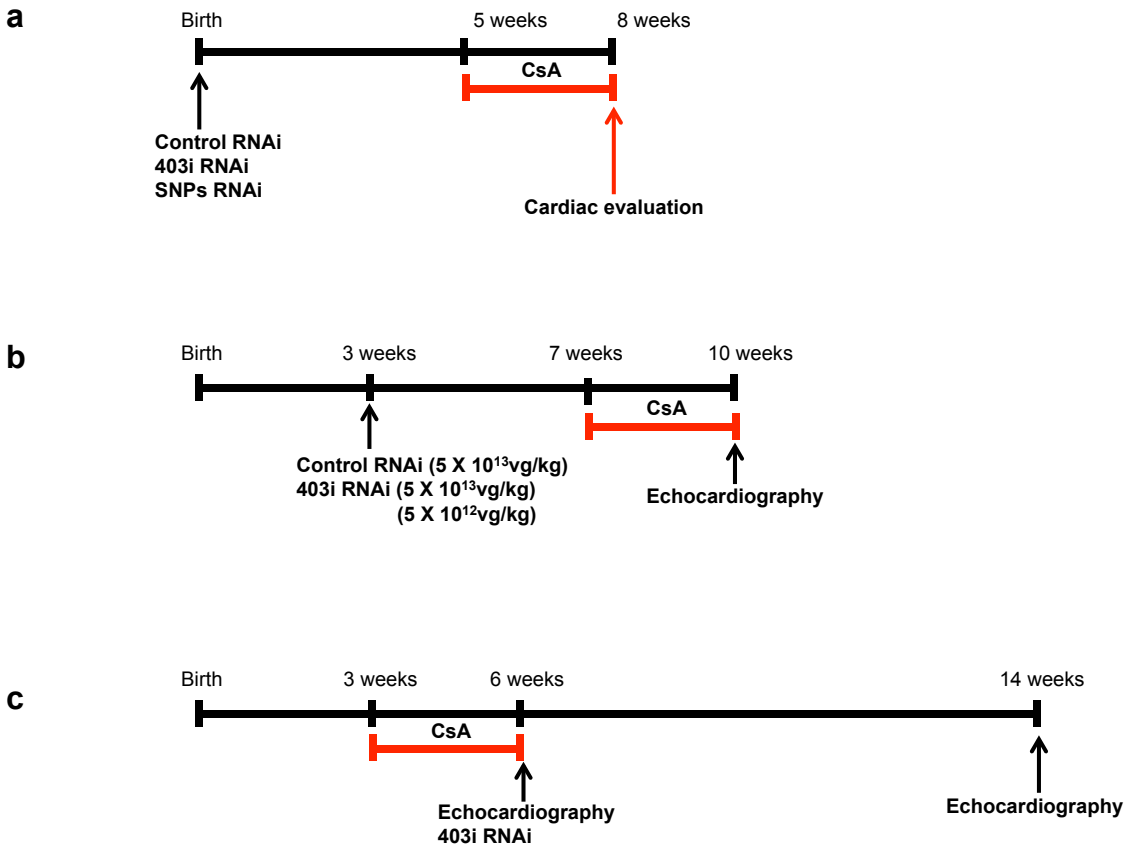
AAV-9 and cTnT promotes selective expression of EGFP in the heart. GFP signals were visualized with fluorescence (left) and light (right) microscopy to assess expression in organs isolated from mice at three weeks **(a)** or three months **(b)** after transduction with AAV-9 encoding EGFP under the control of the cTnT-promoter.

Fig. S3.



Long-term EGFP expression in myocytes. Confocal micrographs of myocytes in mouse hearts studied 5 months **(a)** and 12 months **(b)** after transduction. DAPI (blue), GFP (green), troponin I (red). Bar = 40 μm.

Fig. S4.



RNAi silencing protocols. **(a)** $MHC^{403/+}$ mice transduced with RNAi on day 1 of life and subsequent treated with CsA to accelerate hypertrophic remodeling from age 5 weeks through age 8 weeks, at which time cardiac evaluations were performed. **(b)** $MHC^{403/+}$ mice were transduced with RNAi (5×10^{12}vg/kg or 5×10^{13}vg/kg) on day 21 of life and then treated with CsA from ages 7 weeks through age 10 weeks at which time cardiac evaluations were performed. **(c)** $MHC^{403/+}$ mice were treated with CsA for 3 weeks, beginning 21 day of life. At 6 weeks of age, mice were transduced with RNAi and cardiac evaluations were performed at age 14 weeks.