SUPPLEMENTAL MATERIAL

lncExACT1 and DCHS2 Regulate Physiological and Pathological Cardiac Hypertrophy

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Supplemental Materials and Methods

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SUPPLEMENTAL METHODS

Animal models

All mice were maintained and studied using protocols in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by MGH Animal Care and Use Committees (protocol numbers 2015N000029, 2015N000070, and 2019N000105). Two- to three-month-old male C57BL/6J mice were purchased from the Jackson Laboratory. For experiments with adult zebrafish, animals ranging in age from 3 to 18 months were used. Approximately equal sex ratios were used for experiments. Adult density was maintained at 3-4 fish·l-1 for all experiments in Aquarius racks and fed twice daily. Water temperature was maintained at 28 ºC. Published strains used in this study include wild-type AB and Tg(ubb:Zebrabow-M)a131[54]. At least three independent founders of each line were isolated and tested to confirm the described expression patterns and phenotypes. All transgene sequences are available upon request. Rosa$^{Cas9GFP/Cas9GFP}$ mice were purchased from the Jackson Laboratory (No.026175)[31]. Eight to ten-week-old male mice were used for studies.

Human Subjects

Human studies were approved by the Partners Institutional Review Board or the Colorado Multicenter Institutional Review Boards. Informed consent was obtained from all transplant patients and study participants, and from the family member or authorized representative of all organ donors. Heart tissue was from transplant patients with non-ischemic cardiomyopathy and reduced systolic function (EF=22.3±9.0%) and nonfailing (unused donor) hearts (EF=67.7±7.2%) from otherwise similar subjects (50% female and mean age 57 years for both). Plasma was from patients with HF with preserved ejection fraction (HFpEF, EF=64.7±7.76%), HF with reduced ejection fraction (HFrEF, EF=22.3±8.92%) and patients without HF and with structurally normal hearts who presented with supraventricular tachycardia (SVT, EF=60.8±2.3%).

Running exercise protocol

Two-month-old mice were subjected to running exercise as previously described[10]. Briefly, mice were individually housed in plexiglass cages (36L x 20W x 15H cm) that contained a stainless steel running wheel (diameter 11.4 cm; Mini-Mitter, Starr Life Science, USA) equipped with a tachometer. Mice ran voluntarily. Mouse activity was recorded daily. The sedentary control mice were kept in the same cage system lacking running wheels.

Transverse aortic constriction

Sham and transverse aortic constriction (TAC) surgeries were performed as previously described[5, 55]. Briefly, thoracotomy was performed in anesthetized animals, and TAC was performed by ligating the transverse aortic arch with a 27-gauge needle between the innominate and left common carotid arteries. Subsequently, mice were followed weekly using echocardiography and sacrificed at 2 weeks or at fractional shortening lower than 30%.

Myocardial ischemia reperfusion

Sham and myocardial ischemia reperfusion (IRI) surgeries were performed as previously described[11]. Briefly, the left anterior descending artery (LAD) was ligated with 7-0 silk for 30 minutes. The LAD ligature was then released, and reperfusion was confirmed visually. Cardiac function was monitored by echocardiography after 24 hours and weekly for 7 weeks. Mice were then sacrificed, and hearts were collected for analyses. EdU (50 mg/kg, subcutaneously) was injected every 2 days for the first 2 weeks after reperfusion. Sham operated mice were used as controls. All surgeries and analyses were performed by investigators blinded to treatment.

Locked nucleic acid-GapmeR injection

Locked nucleic acid (LNA) treatment was performed as described[11, 56]. Briefly, 12-wk-old C57BL/6 male mice at baseline or 3 days before TAC surgery were injected subcutaneously with 10 mg/kg of LNA-GapmeR (Qiagen) targeting lncExACT1 (LNA-GapmeR, 5'-T*A*A*A*G*G*C*A*C*T*T*G*A*T*C*G-3') or scrambled control (LNA-Control, 5'-A*A*C*A*C*G*T*C*T*A*T*A*C*G*C-3') (*indicated an LNA modification) reconstituted in saline.

Mice were injected for three consecutive days followed by weekly injection throughout the experiments.

Echocardiographic studies

Cardiac echocardiography was performed on unanesthetized mice using a Vivid E90 cardiac ultrasound system (GE Healthcare) equipped with an L8-l8i-D transducer, as described [55, 57]. The heart was first visualized in long and short axis views followed by M-mode analysis of short axis. Care was taken to obtain symmetrical short axis images at the level of the papillary muscles. Images were analyzed using EchoPACS software (version 201, GE Healthcare). Relative wall thickness (RWT) was calculated as RWT=[(IVSd+LVPWd)/2]/(LVIDd/2). At least three measurements were obtained and averaged for every data point from each mouse.

RNA sequencing

RNA sequencing was performed by the MGH NextGen Sequencing Core. Total RNA was extracted from murine hearts using RNA prep kit. Libraries were constructed from polyA-selected RNA and sequenced on an Illumina HiSeq 2500 instrument in High-Output mode as paired end 100. Raw sequence files were converted to the FASTQ format using STAR aligner and were annotated using custom noncoding RNA annotation database combining list of lncRNAs from ENSEMBL, NONCODE, and lincRNAdb. R package DESeq2 was used for gene expression analysis. Genes were considered differentially expressed if fold-change >1.5 or < -1.5 with *p* value <0.05. RNA-seq raw data had been deposited to GEO with accession number GSE186081.

To generated principal component analysis (PCA) plot with all the lncRNAs, protein coding genes were filtered out of the matrix containing all 24 samples with normalized read count data. Data were then log2-transformed to normalize the variances of the read counts among samples. Scree plots were used to identify top principal components based on a proportion of percentage that explains the variance between different samples or cluster of samples of a dataset.

Quantitative real-time polymerase chain reaction

RNA was isolated using RNA prep kit (Zymo Research). Polymerase chain reaction (PCR) reactions were performed using SYBR green and standard amplification protocols. Gene expression was normalized to RPS18 or GAPDH and calculated using the $\Delta\Delta C_t$ method. Primer sequences are listed in **Supplemental Table IV**.

Droplet digital polymerase chain reaction

Droplet digital polymerase chain reaction (ddPCR) was performed in QX200 (Bio-Rad) as described[58]. Briefly, total RNA was isolated from human plasma using miRNease Serum/Plasma kit (Qiagen). Total amount of RNA extracted from plasma (500ul) was used for reverse transcription using standard amplification protocol from High Capacity Reverse Transcription (RT) Kit (Applied Biosystems). PCR reactions were performed using ddPCR Supermix for Probes (Bio-Rad), Taqman primers (listed in **Supplemental Table IV**) and RT reactions as template. Reactions (40ul) were loaded into DG8 cartridges with QX200 droplet Generation Oil for Probes (70ul, Bio-Rad) then subjected to QX200 droplet Generator for droplet generation. Droplet suspensions were then transferred to ddPCR plates. Readout was done using the QX200 Droplet Reader and Quantasoft Software (Bio-Rad) to determine the total number of droplets. Only genes that had at least 10,000 droplets were considered robustly detectable by ddPCR in plasma and subsequently underwent further analysis.

Cardiomyocytes isolation, culture, transfection, and treatments

Cardiomyocytes and non-cardiomyocytes were isolated from adult mice as previously described[11, 59]. Briefly, after Langendorff perfusion, left ventricles were harvested and dissected into small pieces to dissociate in transfer buffer. After filtering, cell solution was settled to sedimentation for several minutes in a Falcon tube. The cell pellet and supernatant were transferred to individual Falcon tubes for further separation. The cell pellet was resuspended in transfer buffer and settled to precipitation. After the second precipitation, the cell pellet was checked for typical rod-shaped morphology before RNA extraction to confirm expression of

cardiomyocyte markers (Troponin I and troponin T). The initial supernatant was centrifuged first at 50x g for 3 min and then 300x g for 5 min before confirmation of non-cardiomyocyte identity of pelleted cells by QRT-PCR for cardiomyocytes markers (Troponin I and troponin T).

Primary neonatal rat ventricular cardiomyocytes (NRVMs) were prepared as described[60]. Isolated NRVMs were purified by pre-plating and percoll gradient centrifugation[10]. NRVMs were plated in 60 mm dishes at $1x10^6$ cells *per* well and cultured in DMEM supplemented with 5%FBS and 10% horse serum for 24 hours. Before treatment, NRVMs were synchronized and cultured in DMEM containing 0.2% FBS. Twenty-four hours after plating, cells were transfected with negative control (20 mM; LG00000002-DDA, Qiagen) or LNA-GapmeR (20 mM; LG00235955-DDA for Gap #1; LG00197578-DDA for Gap #2, Qiagen); or scramble control (20 mM; 129351000, Invitrogen) or DCHS2 siRNA (20 mM; RSS318591, Invitrogen); or scramble controls or miR-222 inhibitor[11] (20 mM) or miR-222 precursor[11] (0.4 mM) for forty-eight hours, using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. After overnight serum starvation, NRVMs were treated with 200μmol/L phenylephrine to induce pathological hypertrophy or 100ng/mL IGF1 (291-G1-01M; R&D Systems) to induce physiological hypertrophy. For signaling inhibition experiments, 10μmol/L U0126 (19-147; Sigma) was used to inhibit MEK-Erk1/2 signaling; 10μmol/L SP600125 (S5567; Sigma) was used to inhibit JNK; 10μmol/L SB203580 (TLRL-SB20; Invivogen) was used to inhibit p38; 100nM Wortmannin (9951; Cell Signaling Technology) was used to inhibit PI3K; 10μmol/L FK506 (TLRL-FK5; Invivogen) was used to inhibit calcineurin.

Primary neonatal mouse ventricular cardiomyocytes were prepared using Pierce Primary Cardiomyocyte Isolation Kit (Thermo Scientific) according to the manufacturer's instruction.

Isolation of cytosolic and nuclear fractions

Isolated adult cardiomyocytes were suspended and washed with PBS and harvested for isolation of cytosolic and nuclear fractions according to the manufacturer's protocol as described in PARIS kit (Life Technologies) followed by RNA extraction and QRT-PCR. Cardiac tissues were

washed with PBS and harvested for isolation of cytosolic and nuclear fractions according to the manufacturer's protocol as described in Cytoplasmic & Nuclear RNA Purification kit (Norgen Biotek, 21000). Beta-actin was used as cytosolic marker and U6 was used as nuclear marker. Previously described lncRNA HOTAIR[61], predominantly expressed in the nucleus, was used as positive control.

Hearts from mice or zebrafish were cleared of blood by washing thoroughly in Tyrode buffer and aortic and atrial sections removed from the ventricles. Ventricular tissues were fractionated to isolate cytosolic and nuclear fractions according to the manufacturer's protocol as described in the Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific).

Gene copy number measurement

The absolute copy number of lncExACT1 per cell was quantified by using QRT-PCR as described using 200ng of RNA. Total RNA was extracted from $1*10^5$ cardiomyocytes or non-cardiomyocytes isolated from mouse hearts. A serial diluted known amount DNA fragment of synthesis full length of lncExACT1 (IDT) was used as templates to formulate standard curve, and then, the copy number of lncExACT1 per cell was calculated accordingly.

Generation and transfection of lentivirus overexpressing lncExACT1

The full-length lncExACT1 genomic sequence was cloned and inserted into a modified pLVX-Tight-Puro vector (TER promoter was replaced by EF1a promoter from pLVX-Tight-Puro) at *BclI* and *MluI* sites for generating lncExACT1 overexpression lentivirus vector. Lentiviral construct was transfected into the HEK293T cells along with lentiviral packaging plasmids psPAX2 and VSVG using Lipofectamine 3000 (Invitrogen). Virus-containing supernatant was collected and cellular debris was removed by syringe filtration (0.22 μm pore size; Millipore). Filtered supernatant was concentrated using lenti-X Concentrator (Clontech). The viral pellet was resuspended in serum-free DMEM. For infection in NRVMs, 24 hours after plating, NRVMs were incubated with DMEM medium containing lentivirus overexpressing lncExACT1 and polybrene (6 μg/ml, Millipore) for 48 hours.

CRISPR/dCAS9 and sgRNA construct design

For transcriptional activation of DCHS2 in NRVMs, single guide RNAs targeting the DCHS2 promoter were designed using IDT crRNA design tool and analyzed for specificity (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). Three guide RNAs targeting the DCHS2 promoter and one guide RNA not targeting rat genome (as control), sequences of guide RNAs were listed in **Supplemental Table IV**. Guide RNA was inserted into the lentiGuide-Puro vector (52963, Addgene)[62] at the *BsmBI* site. lentiGuide-Puro vector and lenti-dCAS-VP64_Blast vector (61425, Addgene)[63] were packaged as lentivirus using psPAX2 and VSVG. lentiGudie-Puro lentivirus and lenti-dCAS-VP64_Blast lentivirus were infected into NRVMs for 48 hours to activate DCHS2 transcription.

Protein coding potential

Transcripts of lncExACT1 full sequence and two small ORFs (sORFs) were synthesized by Intergrated DNA Technologies (IDT). These transcripts were cloned upstream of p3xFLAG-CMV-13 expression vector (Sigma, E4776) using EcoRI and BamHI restriction sites. In total, HEK293T cells were transfected with 1000ng plasmid using Lipofectamine 3000 (L3000015, Invitrogen) and protein lysate was isolated 72 hours after transfection, followed by immunoblotting for FLAG Tag (8146S, Cell Signaling Technology). RILPL1-Flag tag plasmid was used as positive control (shared by Dr. James Rhee, Department of Anesthesiology, MGH).

Transcripts of GFP (GFPwt), GFP with start codon (ATGGTG) mutated to ATTGTT (GFPmut), two sORFs with or without start codon (ATG) mutated to ATT, and 5′ untranslated (5'-UTR) of sORFs were synthesized by IDT. These transcripts were cloned into a pLV-EF1a-IRES-Puro (85132, Addgene[64]) using Gibson Assembly Cloning Kit (E5510S, NEB). HEK293T cells were transfected with 1000ng plasmid using Lipofectamine 3000 (L3000015, Invitrogen) followed by visualization and imaging of GFP fluorescence by ZOE Fluorescent Cell Imager (1450031, Bio-Rad) 72 hours after transfection.

Luciferase assays

Fragments containing the miR-876- and miR-222-binding sites of lncExACT1 were inserted downstream of Renilla luciferase in a dual luciferase reporter plasmid with firefly luciferase expression driven by a separate promotor (psiCHECK-2, Promega). Control constructs contained the same fragments but with the putative binding sites mutated. HEK293T cells were co-transfected with the reporter plasmid or the mutated construct (100 ng) and miR-876 precursor (1 μM, Qiagen), miR-222 precursor (1 μM, Qiagen) or scrambled control (1 μM, Qiagen) according to the lipofectamine 3000 transfection procedures (Invitrogen). Forty-eight hours after transfection, cells were lysed. Relative (Renilla/firefly) luciferase expression was measured on a multi-mode multi-format reader SpectraMax M5 (Molecular Devices). To examine the effect of lncExACT1 on DCHS2 transcription, the DCHS2 promoter region was divided into different fragments. Fragments were synthesized by IDT and inserted into the same dual luciferase reporter plasmid psiCHECK-2 (Promega). A scrambled control that did not match the mouse genome was inserted into psiCHECK-2 and used as negative control. Mouse neonatal cardiomyocytes were co-transfected with the reporter plasmid or the control construct (100 ng) with or without lncExACT1 LNA-GapmeR or lentivirus overexpressing lncExACT1 according to the lipofectamine 3000 transfection procedures (Invitrogen). Forty-eight hours after transfection, cells were lysed and relative luciferase expression was measured.

Generation and injection of adeno-associated virus

AAV9 vector with cardiomyocyte-specific troponin I promoter was provided by the Seidman Lab[65]. AAV9-lncExACT1 was constructed by subcloning the full-length mouse lncExACT1 genomic fragment into an AAV9 vector at *NcoI* and *BsrGI* sites. Recombinant AAV9-GFP and AAV9-lncExACT1 were produced using a triple transfection approach in HEK293T cells in 15cm dishes with polyethylenimine and were harvested after 60 hours incubation, purified by filtration and iodixanol gradient ultracentrifugation, and quantified as described[66]. A total amount of $2*10^{12}$ GC/mouse of AAV9-GFP or AAV9-lncExACT1 was delivered by tail vein injection to 12-

week-old C57BL/6J mice. For AAV9 mediated DCHS2 gene depletion, two pairs of gRNAs targeting either DCHS2 exon2 (5'-GATCGACCCACAGGATGGCC-3; 5'-TCCGGGCACCTTTGA TCTGC-3') or exon 3 (5'-GGCGTTTGTCCGGGTAGAAG-3'; 5'-TTCACCATTGACTCCACTAC-3') using the GPP Web Portal (Broad Institute). The gRNA sequences were synthesized as single-stranded oligonucleotides, annealed, and inserted into AAV-U6gRNA-U6gRNA-cTNT-Cre plasmids (Addgene #87682)[67] at SapI and NheI sites. AAV9 was produced as described above. $2*10^{12}$ GC/mouse AAV9 was injected into eight to ten-week-old $Rosa^{Cas9GFP/Cas9GFP}$ mice via tail vein.

5-Ethynyl-2'-deoxyuridine incorporation labeling

Isolated NRVMs were plated into a 6 cm diameter BD Primaria tissue culture dish. Twenty-four hours after plating, cells were subjected to various treatments. Forty-eight hours after treatments, cells were labeled with 20 μM 5-Ethynyl-2'-deoxyuridine (EdU, Invitrogen) for 24 hours. Collected cells were stained using the Click-iT Plus EdU Pacific Blue Flow Cytometry Assay protocol (Thermo Fisher Scientific) with cardiac troponin T (Alexa Fluor 647, BD Bioscience, 565744) as cardiomyocyte marker**,** or CD140α (APC, Invitrogen, 17-1401-81) as fibroblast marker, or CD31 (PE, BD Bioscience, 555027) as endothelial cell marker, or CD11b (APC, BioLegend, 201809) as leukocytes marker. Stained cells were analyzed in a 5-laser LSR II machine in the MGH flow cytometry core facility. A total of 15,000 events were recorded by flow cytometry in each treatment. Flowjo 10 was used to analyze flow data. To quantify cardiomyocyte proliferation *in vivo* in mice, EdU (50 mg/kg) was subcutaneously injected every 2 days for the last two weeks after LNA-GapmeR or AAV injections. At the end of experiments, hearts were snap frozen in OCT in liquid nitrogen, sectioned and processed for EdU staining according to the of Click-iT EdU Flow

Cytometry Assay (Invitrogen) protocol with PCM1 (HPA023370, Sigma-Aldrich) as cardiomyocyte marker.

Histology and immunohistochemistry

Mid-ventricular sections were stained with Wheat Germ Agglutinin (WGA), Alexa Fluor™ 594 (W11262, Thermo Fisher Scientific) for cell size measurement and Masson's Trichrome staining was carried out for fibrosis analysis, according to manufacturer's instruction (HT15, Sigma-Aldrich). WGA and Masson's Trichrome stained slides were scanned by a digital slide scanner, NanoZoomer 2.0-RS (Hamamatsu, Japan). For cardiomyocyte proliferation analysis, immunofluorescent double staining was performed. Anti-PCM1 antibody (HPA023374, Sigma-Aldrich, cardiomyocyte specific marker[17]) was incubated at 4°C overnight and a biotinylated secondary antibody followed by streptavidin-DyLight 594 (BA1000 and SA-5594, Vector Laboratories) were used for cardiomyocyte identification. For a proliferation marker, Click-iT™ Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye (C10337, Thermo Fisher Scientific) was used. As another proliferation makers, anti-Ki67 antibody (clone: SolA15, 14-5698-82, Thermo Fisher Scientific) and Alexa Fluor 488 goat anti-rat IgG secondary antibody (A-11006, Thermo Fisher Scientific) or anti-pHH3 (Ser10) (Alexa Fluor 488 conjugate; 3465S, Cell Signaling Technology) were applied. Nuclei were counterstained with DAPI (D21490, Thermo Fisher Scientific) and the slides were imaged on a Leica DM500B Microscope. Cardiomyocyte cross-sectional area (~250 cells per heart) was measured from six randomly selected sections per heart using ImageJ (NIH). Fibrosis, EdU and PCM1 double positive cardiomyocytes, and Ki67 and PCM1 double positive cardiomyocytes were quantified from full mid-ventricular section (Keyence BZ-X Analyzer for fibrosis, ImageJ for others). Image analysis was blinded.

Immunoblotting

Protein was isolated using radioimmunoprecipitation assay (RIPA) buffer and processed using standard Western blotting protocols. Image quantification was performed with the ChemiDoc system (Bio-Rad). Cytosolic phosphorylation of Yap1 protein expression was

normalized to GAPDH, nuclear total Yap1 protein expression was normalized to Histone H3. The following primary antibodies were used: rabbit-anti-mouse phosphor-Yap1 (Ser127) (1:2000; 13008, Cell Signaling Technology), rabbit-anti-mouse Yap1 (1:2000; NB110-58358, Novus Biologicals), rabbit-anti-mouse GAPDH (1:3000; 2118, Cell Signaling Technology), rabbit-anti-mouse Histone H3 (1:5000; 9715, Cell Signaling Technology), rabbit-anti-mouse DSCR1 (1:2000; D6694, Sigma-Aldrich), rabbit-anti-mouse calcineurin A (1:2000; ab71149, Abcam), anti-goat NFATc3 (1:1000, AF5834, R&D Systems), rabbit-anti-mouse phosphor-MST1 (Thr183)/MST2 (Thr180) (1:500; 49332, Cell Signaling Technology), rabbit-anti-mouse MST1 (1:500; 3682S, Cell Signaling Technology).

RNA pulldown and chromatin oligo-affinity precipitation (ChOP)

RNA pulldown was performed as previously described[68]. Briefly, a mixture of twenty-five 3'-end-labelled DNA oligonucleotide probes targeting lncExACT1 was synthesized (IDT), listed in **Supplemental Table V**. Forty-eight hours after infection with lncExACT1-overexpressing lentivirus, mouse neonatal cardiomyocytes were rinsed with PBS, fixed with 1% paraformaldehyde, and cross-linked under agitation for 10 min at room temperature, then quenched by adding 1/10 volume of glycine. After two PBS washes, cells were collected. Cells were then lysed (50 mM Tris-HCl pH 7.0, 10 mM EDTA, 1% SDS supplemented with 200 U/mL of a RNAse inhibitor solution (Invitrogen, 10777019), and a cocktail of protease inhibitors 5 µL/mL (Thermo Scientific, 78442)) and processed to a series of sonication. Supernatants were then hybridized with biotinylated probes for 6 hours under moderate agitation at room temperature followed by incubation with magnetic streptavidin beads (Invitrogen) supplemented with 200U/ml of RNAse inhibitor and protease inhibitor cocktail, overnight at room temperature. Beads were collected using a magnetic stand and washed five times with wash buffer (0.5% SDS, SSC 2x) and digested with proteinase K for 45min at 50°C, then 10min at 95°C. Beads were then removed and supernatant was processed for RNA extraction using standard protocol. ChOP was performed as previously described[69]. Briefly, mouse neonatal cardiomyocytes were rinsed with

PBS, fixed with paraformaldehyde and cross-lined as described above. Chromatin was extracted according to the manufacturer's protocol as described in the Chromatin Extraction Kit (Abcam, ab117152) followed with incubation with DNA oligonucleotide probes targeting lncExACT1 was synthesized (IDT, **Supplemental Table V**), RNA-DNA complex was then precipitated using magnetic streptavidin beads followed with DNA extraction using standard protocol.

Construction of cmlc2:hGFP and cmlc2:hGFP-DCHS2 zebrafish lines

Construction of *cmlc2:hGFP:* To generate the *cmlc2:hGFP* transgenic line, a construct containing the following DNA elements was assembled by Gibson cloning: (1) a 0.9-kb *cmlc2* promoter to drive specific expression in cardiomyocytes; (2) a GFP fused to the histone H2B; and (3) an SV40 polyadenylation signal. The entire construct was flanked with Tol2 sites to facilitate transgenesis. In this line, all cardiomyocytes constitutively express H2B-GFP. The full name of this line is *Tg(cmlc2:H2B-EGFP)*^{fb501Tg}.

Construction of *cmlc2:hGFP-DCHS2*: To generate the *cmlc2:hGFP-DCHS2* transgenic line, a construct containing the following DNA elements was assembled by Gibson cloning: (1) a 0.9-kb *cmlc2* promoter to drive specific expression in cardiomyocytes; (2) a bicistronic cassette encoding H2B-GFP and human DHCS2, separated by a P2A sequence (Kim et al., 2011), and (3) an SV40 polyadenylation signal. The transcript ENST00000339452.2 from DCHS2 was amplified from clone MHS6278-213246293 (GE Dharmacon). To facilitate detection of the protein, six copies of the myc-tag were incorporated at the N-terminus of DCHS2. The construct was flanked with Tol2 sites to facilitate transgenesis. Before injection, this transgene was sequenced using the Complete Plasmid Next Generation Sequencing service of the MGH CCIB DNA Core. In this line, all cardiomyocytes constitutively express H2B-GFP and DCHS2. The full name of this line is *Tg(cmlc2:H2B-EGFP-P2A-Hs.DCHS2)*^{fb502Tg}.

Zebrafish cardiac injuries

Regeneration experiments were conducted using adult zebrafish as described[70]. Briefly, fishes were anesthetized in tricaine, placed with their ventral side up on a sponge, and a small

incision was created to expose the apex of the ventricle. A Kimwipe was applied to the surface of the heart to remove the excess of water from the animal. Next, a platinum filament precooled in liquid nitrogen was applied to the apex of the ventricle to induce rapid freezing of approximately 20% of the ventricle. After this procedure, animals were retrieved to a fish tank and revived by gently directing water to their gills using a plastic Pasteur pipette.

Quantification of cardiomyocyte proliferation during zebrafish heart regeneration

To quantify cardiomyocyte proliferation after injury, adult zebrafish were used to perform ventricular cryoinjury. At 7 days post-injury (dpi), hearts were extracted and processed for analysis. To calculate cardiomyocyte proliferation indices, ventricular sections were immunostained with anti-nkx2.5 to label cardiomyocyte nuclei and anti-PCNA to identify proliferating cells (see above). Nuclei were counterstained with DAPI. For each heart, three ventricular sections containing the largest injury areas were imaged. nxk2.5^{+}PCNA^{+} and total nkx2.5^{+} cells were counted manually using Fiji/ImageJ software in defined regions (200 mm x 424.55 mm) that include the injury area and border zone. The percentages of nxk2.5^{+}PCNA^{+}/nxk2.5^{+} cells from individual sections were averaged to establish a cardiomyocyte proliferation index for each animal.

Zebrafish histological analysis and imaging

Adult zebrafish were euthanized by immersion in 0.16% tricaine (Sigma) and hearts dissected as described [70]. Samples were fixed overnight at 4 ºC in 4% paraformaldehyde (PFA) in PBS, included in paraffin and sectioned following conventional histological procedures. Immunofluorescence in paraffin sections were performed as described [71]. Primary antibodies used were mouse anti-tropomyosin (clone CH1, Developmental Studies Hybridoma Bank; 1:50), chicken anti-GFP (AVES, 1:500), mouse anti-GFP (clone B-2, Santa Cruz Biotechnology; 1:200), mouse anti-PCNA (clone PC10, Santa Cruz Biotechnology, 1:500), rabbit anti-nkx2.5 (GTX128357, GeneTex, 1:500). Alexa conjugated secondary antibodies (Life Technologies, 1:500) were used to detect primary antibody signals. Nuclei were counterstained with DAPI

(Invitrogen), and slides were mounted in FluorSave (Millipore). A Nikon A1 confocal microscope was used to image immunostained sections.

Acid fuchsin-orange G (AFOG) stain was used to detect fibrotic tissue, as described [72]. Muscle, fibrin/cell debris, and collagen were stained brown, orange, red, and blue, respectively. Imaging was performed on a DM6 Leica Scope with a motorized stage. Multi-image stitching was performed using the LAS X Navigator Software.

Cardiomyocyte isolation from zebrafish ventricles

Cardiomyocyte dissociations were performed as described previously[72]. Briefly, adult zebrafish hearts were dissected in ice-cold PBS supplemented with 0.3% bovine serum albumin and 20 mM glucose. Three ventricles were pooled per biological replicate. After bisecting the ventricles, tissues were washed twice in dissection buffer to remove blood. Then, the ventricles were digested for 15 min in a solution of 0.2% trypsin, 0.8 mM EDTA (25200-056, Gibco) supplemented with 20 mM glucose and 10 mM 2,3-butanedione monoxime (B0753, Sigma). This incubation was performed on ice and using gentle agitation. Next, the digestion solution was removed, the ventricles were washed three times using dissection buffer supplemented with 10 mM BDM and then digested for 45 min at room temperature in Accumax (SCR006, EMD Millipore) supplemented with 20 mM glucose and 10 mM BDM under mild agitation. Tissue fragments were dissociated by gentle pipetting, and cell suspensions were immediately fixed in 10% neutral buffered formalin (HT501128, Sigma) for 1 hour at room temperature. Cells were pelleted by centrifugation at 400 x *g* for 5 minutes, resuspended in PBS, spread in Superfrost Plus slides (1255015, ThermoFisher Scientific), and air-dried.

To facilitate quantifications, *cm:hGFP-DCHS2* (experimental) and age-matched *ubb:Zebrabow* (control) ventricles were pooled and dissociated in the same tube, as previously described [72]. In these spreads, cardiomyocytes overexpressing DCHS2 are identified based on nuclear GFP expression, while control cardiomyocytes strongly express the red fluorescent protein mCherry.

Cardiomyocyte size quantification in cell spreads

Cardiomyocyte spreads were rehydrated in PBS and permeabilized for 45 minutes in 0.1% IGEPAL CA-630 (I8896, Sigma), 3% BSA in PBS. Cardiomyocytes were then stained using anti-tropomyosin (clone CH1, Developmental Studies Hybridoma Bank; 1:200) for 1 hour at room temperature. Alexa conjugated secondary antibodies (Life Technologies, 1:500) were used to detect primary antibody signals. Nuclei were counterstained with DAPI dihydrochloride (14.3 mM in permeabilization solution, Invitrogen) and slides were mounted in FluorSafe.

A Nikon A1 confocal microscope was used to image cardiomyocyte spreads. Binary masks containing cardiomyocyte areas were manually generated in Adobe Photoshop (Adobe Systems Incorporated). Only intact cardiomyocytes were analyzed, and damaged cells were excluded. Independent binary masks were generated according to the expression of fluorescent proteins to distinguish cell populations. Masks were then quantified using Fiji/ImageJ. Values were exported and collected in an Excel file.

Statistical analysis

Data are presented as mean±SEM unless otherwise indicated and analyzed using GraphPad Prism 8 (GraphPad Software). Unpaired, two-tailed Student's t-test or, when assessing multiple groups, one-way ANOVA with Tukey's post hoc test, were used as indicated. In Figure 1E, pairwise Wilcoxon rank sum test with Bonferroni correction was used. In Figure 4K, repeated measures ANOVA was used. $p<0.05$ was considered significant.

SUPPLEMENTAL TABLES

Supplemental Table I. Echocardiography analysis of cardiac function of mice 16 weeks after AAV infection

	Con (Male, n=7)	lncExACT1 (Male, n=7)
IVSd (mm)	1.01 ± 0.04	1.29 ± 0.04*
IVSs (mm)	1.79 ± 0.05	1.86 ± 0.04
LVIDd (mm)	3.29 ± 0.04	2.99 ± 0.06*
LVIDs (mm)	1.52 ± 0.05	1.61 ± 0.06
LVPWd (mm)	1.14 ± 0.03	1.25 ± 0.04*
LVPWs (mm)	1.81 ± 0.03	1.92 ± 0.03*
FS (%)	54.59 ±0.81	46.30 ±1.44*

IVSs: systolic interventricular septum; IVSd diastolic interventricular septum; LVIDd: left ventricular end diastolic internal dimension; LVIDs: left ventricular end systolic dimension; LVPWd: left ventricular end diastolic posterior wall; LVPWs: left ventricular end systolic posterior wall dimension; FS (%): fractional shortening. *p<0.05 by Student's t test. Data are shown as mean±SEM.

Supplemental Table II. Echocardiography analysis of cardiac function of mice 2 weeks after GapmeR injection

	Con (Male, n=6)	GapmeR (Male, n=6)
IVSd (mm)	0.98 ± 0.03	1.27 ± 0.04*
IVSs (mm)	1.82 ± 0.03	2.05 ± 0.05*
LVIDd (mm)	2.71 ± 0.10	2.61 ± 0.05
LVIDs (mm)	1.14 ± 0.07	0.93 ± 0.01*
LVPWd (mm)	1.10 ± 0.04	1.39 ± 0.05*
LVPWs (mm)	1.87 ± 0.05	2.27 ± 0.06*
FS (%)	57.67 ± 1.48	64.00 ± 0.77 *

IVSs: systolic interventricular septum; IVSd diastolic interventricular septum; LVIDd: left ventricular end diastolic internal dimension; LVIDs: left ventricular end systolic dimension; LVPWd: left ventricular end diastolic posterior wall; LVPWs: left ventricular end systolic posterior wall dimension; FS (%): fractional shortening. *p<0.05 by Student's t test. Data are shown as mean±SEM.

Supplemental Table III. Echocardiography analysis of cardiac function of mice 6 weeks after TAC

	Con (Male, n=6)	TAC (Male, n=6)	TAC+GapmeR (Male, n=6)
IVSd (mm)	1.24 ± 0.13	0.98 ± 0.07*	1.22 ± 0.08#
IVSs (mm)	1.51 ± 0.04	1.37 ± 0.08*	1.69 ± 0.07#
LVIDd (mm)	2.39 ± 0.11	4.12 ± 0.33*	3.51 ± 0.34*#
LVIDs (mm)	1.05 ± 0.08	3.20 ± 0.34*	2.41 ± 0.36*#
LVPWd (mm)	1.23 ± 0.07	1.02 ± 0.08*	1.23 ± 0.07#
LVPWs (mm)	1.26 ± 0.03	1.01 ± 0.03*	1.21 ± 0.05#
FS (%)	55.50 ± 1.85	21.25 ± 2.85*	33.23 ± 3.36*#

IVSs: systolic interventricular septum; IVSd diastolic interventricular septum; LVIDd: left ventricular end diastolic internal dimension; LVIDs: left ventricular end systolic dimension; LVPWd: left ventricular end diastolic posterior wall; LVPWs: left ventricular end systolic posterior wall dimension; FS (%): fractional shortening. *p<0.05. vs Con; #p<0.05 vs TAC by one-way analysis of variance (ANOVA) with post hoc Tukey. Data are shown as mean±SEM.

Supplemental Table IV. List of primers used in QPCR and ddPCR, and guide RNA sequencing used in CRISPR/dCAS9 system

Primer name	Forward	Reverse
mlncExACT1	GCGAGCCTCGGTTCTGTA	CCCATCAGCGTCACTGTCT
rlncExACT1	TGGCCAGCCATATGTAGGGA	TTTTGTCAGGGGTACAGGGTG
hlncExACT1	GGCCAAGTGCCAAGTGATGA	AGCTATGCATCGGCCTTTGA
mlncExACT2	GGCATAAAGGGAACGAGGGA	GCACAATTCTGAGCTTGGACC
mlncExACT3	CGTGGAAGGACCCTAGTGAAG	TGCAAGGGACAGAACACCAT
mlncExACT4	TGCAGTGGGAAGAGCATACC	CAGTTCCCTTGTGCAGGGTT
mlncExACT5	TCTGCAGGACTGATTGTTTTATGCT	TGTCTGTCTGTCTCTCTCTCTCTCT
mDCHS2	CATCTCTTCAGATTATCCTGCTC	TCATGTGAAAGGATCGAGGT
rDCHS2	TGCAGGTAAAAGCCTCGGAC	CTCCACTTCCACCCGAACAA
hDCHS2	GAGTTTGAAAGGCCTAAGTACAC	CTGAGGAGTTCAAGGGCTC
ANP	GTAGGATTGACAGGATTGGA	TCCTCCTTGGCTGTTATC
BNP	GCTGCTGGAGCTGATAAGAGAA	GTTCTTTTGTAGGGCCTTGGTC
αMHC	ACATCAGTCAGCAGAACA	TTCCTCTAGCCTCTCACT
βMHC	GCTGTTATTGCTGCCATT	TTATCATTCCGAACTGTC
PGC1α	TGATGTGAATGACCTGGACACAGACA	GCTCATTGTTGTACTGGTTGGATATG
mC/EBPβ	ATATGAATTCGCCACCATGCACCGCC	GCACCTCGAGTCAAGTCCCGAAACC
	TGCTGGCCTGGGACGCA	CGGTGCGCTGTG
ETS1	CCTGCAGAAAGAGGATGTG	TAATCCGAGGTGTAACGGG
MCM10	GCAAGGGACTGTCATAGGG	TCGATAGATAAGCACACCTCC
MYBL1	TATTGAATCGGATCCTGTAGC	GTAGACTTGACAGGAGAAGC
BUB1	ACAACAATACAGGCTATTCCAG	CTATGCAAAGGTTCTGAGGT
CDCA8	TTGACTACTTTGCCCTAGGA	TGTTTATCTCTGTGATGTCTCG
KLF23	AGAATCGCCAACTGGTAGTC	GTCCATTCAGACTCTGTACCA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT
hGAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
RPS18	CATGCAGAACCCACGACAGTA	CCTCACGCAGCTTGTTGTCTA
hRPS18	CAACACCAACATCGATGGGC	GGTGATCACACGTTCCACCT
Beta-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
HOTAIR	CTTTCAAGGCCTGTCTCCTG	CAACATTCTAGCTGCACGGA
zfRPS11	GATGGCGGACACTCAGAAC	CCAATCCAACGTTTCTGTGA
zfANP	CAAGCGCACGCGTTGA	TCTTGAGCTTGGCCATGTTG
zfBNP	CATGGGTGTTTTAAAGTTTCTCC	CTTCAATATTTGCCGCCTTTAC
CHOP 500bp	GGAACAGATGTAAAAGGAATAG	GGAGCCTCTTCAGTATTAAGCC
CHOP 1000bp	GGAGCCATGGGCAGGCTCGCTG	GAATTCCAACCTGCGAGCCTCGG
CHOP 1500bp	CCCTGCAAGGTTCTTCTCATAG	AGCAGCGTGCTAGTGTCTTGTC
hlncExACT1-ddPCR	GGCCAAGTGCCAAGTGATGA Probe:CCGTCCCTCTGCAGGCCTGAC	AGCTATGCATCGGCCTTTGA
Guide RNA for DCHS2	AAACCCATTGAGCACTGAGGAGTCC	
Guide RNA for Control	GCACTCACATCGCTACATCA	

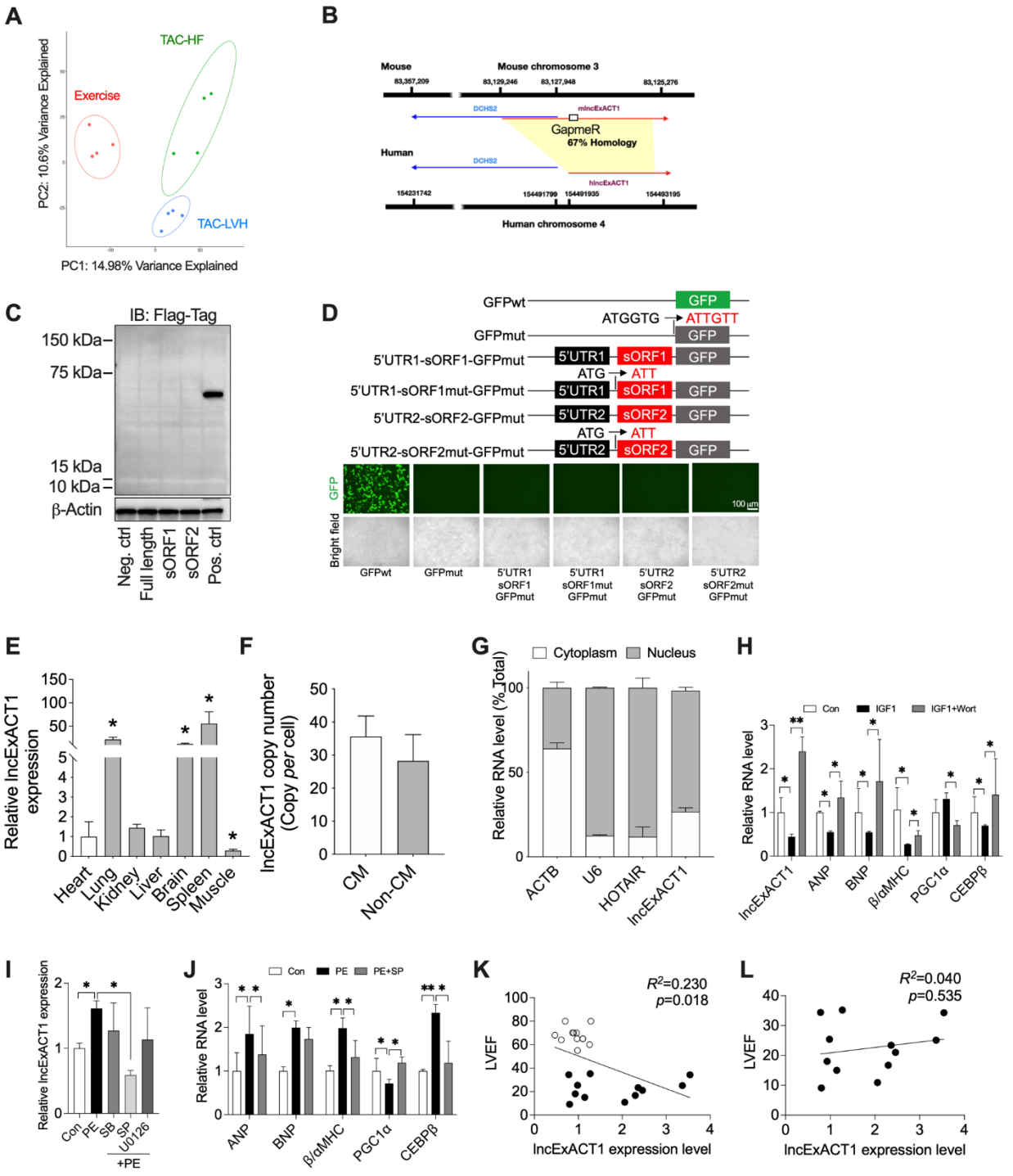
Supplemental Table V. List of probes for RNA pulldown and ChOP

ID	Antisense	Sense
	Biotinylated modification at the 3’ end for each probe	
1	TCCTAAAGAGCTGGAGGAGA	CTGAGATCCCAATACAAGCT
2	GATGGGACTCCCTTAAAGAC	AGCCGAGCAGAACAAGGAAC
3	TCCTCCTTTTTGGCAAAAGA	TTTCCTCCTTTTTGGCAAAA
4	GTACACTTTGCACGCAAAGC	CTGTTCTGAGGATGGAGAGA
5	CTGTTCTGAGGATGGAGAGA	GCACAAGTTTGTCTCCTTAA
6	GGCATAATAAAGCGTGCCAT	GTGTCACAGATTTTTTCGGG

7	TGGATGCCTAAAAAGGTCCG	CGCTGCTAAATAGTAGCTCG
8	TTGGATTCAACCCTAAGCTA	TGGATGCCTAAAAAGGTCCG
9	GCATACTTTATTAGAGGCCA	TTGGATTCAACCCTAAGCTA
10	CCTAGCACAGAACAGTATCT	TCAGTTTCCATCTATAGCTG
11	ATGCATAGGCCACTCAATAT	CCTAGCACAGAACAGTATCT
12	TGGTTACATACAACACTGCC	ATGCATAGGCCACTCAATAT
13	GTAGTTGTGGTGCTTTGTAA	ATGCCTTTGCATTAAGTTGG
14	GATTCTTCAATGAGCAGGGA	GTAGTTGTGGTGCTTTGTAA
15	CTCAGTTGGCATCTTACATG	AAAGGCTTAGTGTTGGCTTT
16	TGTGAGGATCTAGGTTTACC	GAAGAGCAAGGTGGGCAATT
17	CAAGCTGATCTCAGGTACTT	CTCAGTTGGCATCTTACATG
18	ACCAAACACTCTCTCAGACT	CTAGAAGGAGGGTAGTCAGG
19	TAGTTGTGGTTTCCAAGTTC	TGTGAGGATCTAGGTTTACC
20	CAAAGCTGGGACTCAGTACA	CAAGCTGATCTCAGGTACTT
21	ACCTCTGTTAACAAAGTCCG	GGGTTAAAGCAGCGCTTTAA
22	CTGAGATCCCAATACAAGCT	ACCAAACACTCTCTCAGACT
23	AGCCGAGCAGAACAAGGAAC	TAGTTGTGGTTTCCAAGTTC
24	TCCTCCTTTTTGGCAAAAGA	CAAAGCTGGGACTCAGTACA

SUPPLEMENTAL FIGURES

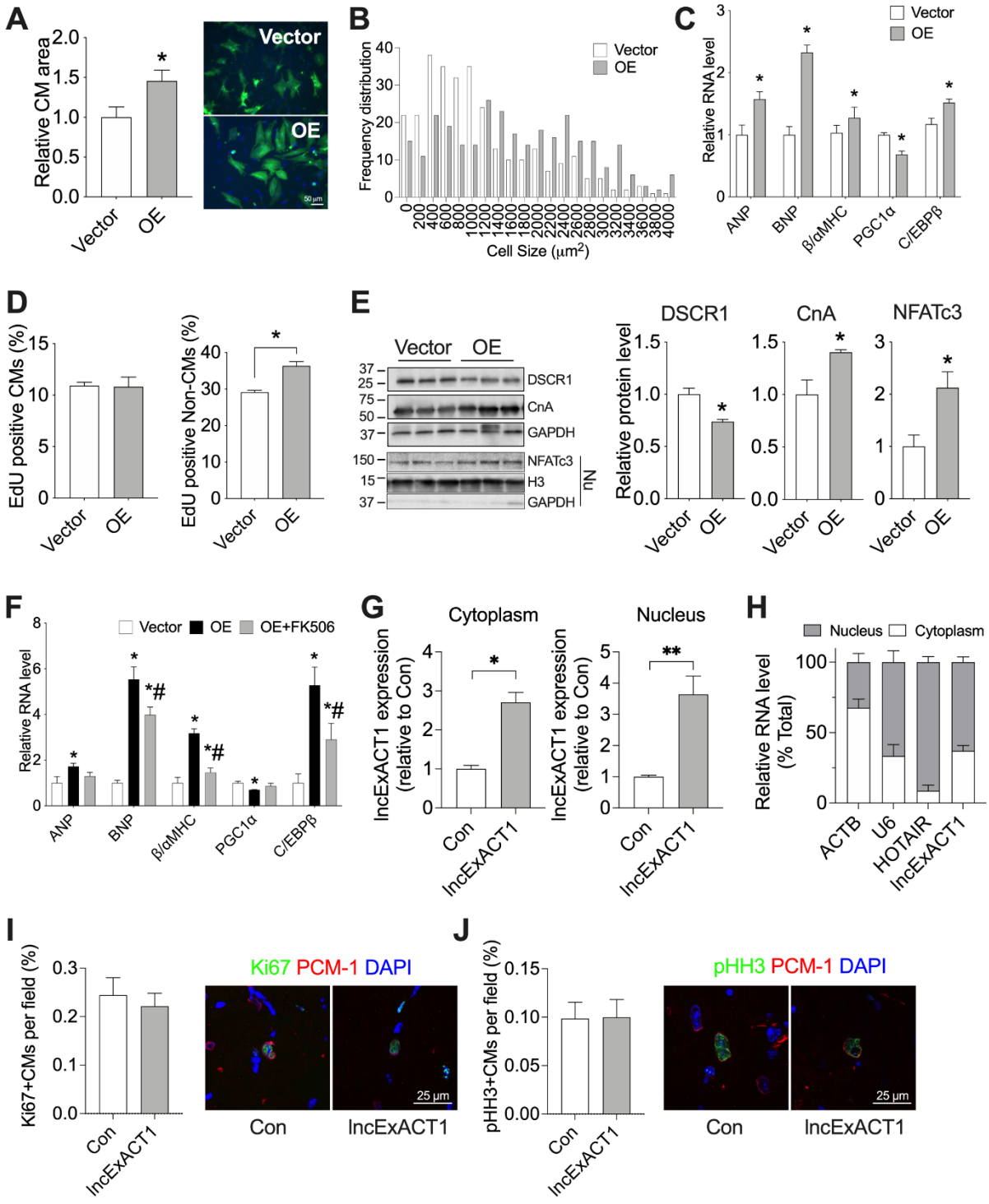
Supplemental Figure I



Supplemental Figure I. Characterization of lncExACT1. A. PCA plot of lncRNAs from mice with voluntary wheel exercise training (Exercise), or transvers aortic construction (TAC)-induced left ventricular

hypertrophy (TAC-LVH) or heart failure (TAC-HF). **B.** Schematic diagram of lncExACT1 in mouse and human genomes. **C.** Representative image of western blot from HEK293T transfected with negative control (neg. ctrl), full length of lncExACT1 (Full length), small ORF1 or 2 identified within lncExACT1 (sORF1 and sORF2), or positive control (pos. ctrl). **D.** Diagram of the GFP fusion constructs used for transfection and representative images of GFP fluorescence in HEK293T cells 72 hours after transfection. **E.** Relative lncExACT1 mRNA expression in different organs in mice. n=3 mice/group. **F.** Gene copy number of lncExACT1 in cardiomyocytes (CMs) and non-CMs isolated from mouse hearts. n=3 independent replicates/group. **G.** Relative expression of Beta-Actin (ACTB), U6, HOTAIR, and lncExACT1 RNA in CM cytosolic and nuclear fractions. **H.** Expression of lncExACT1 and hypertrophy gene markers in neonatal rat ventricular cardiomyocytes (NRVMs) treated with control (Con), IGF1, or IGF1 and Akt inhibitor wortamanin (IGF1+Wort). **I.** Expression of lncExACT1 in NRVMs treated with control (Con), phenylephrine (PE), PE and p38 inhibitor SB203580 (SB), PE and JNK inhibitor SP600125 (SP), or PE and MEK1/2 inhibitor U0126. **J.** Expression of hypertrophic markers. **K and L.** Correlation of lncExACT1 expression and left ventricular ejection fraction (LVEF) in human cardiac tissues (open circle: non-heart failure patients, black circle: heart failure patient). n=3 independent replicates/group. *p<0.05 vs Heart by one-way analysis of variance (ANOVA) with post hoc Tukey in E, *p<0.05, **p<0.01. Data are shown as mean±SEM.

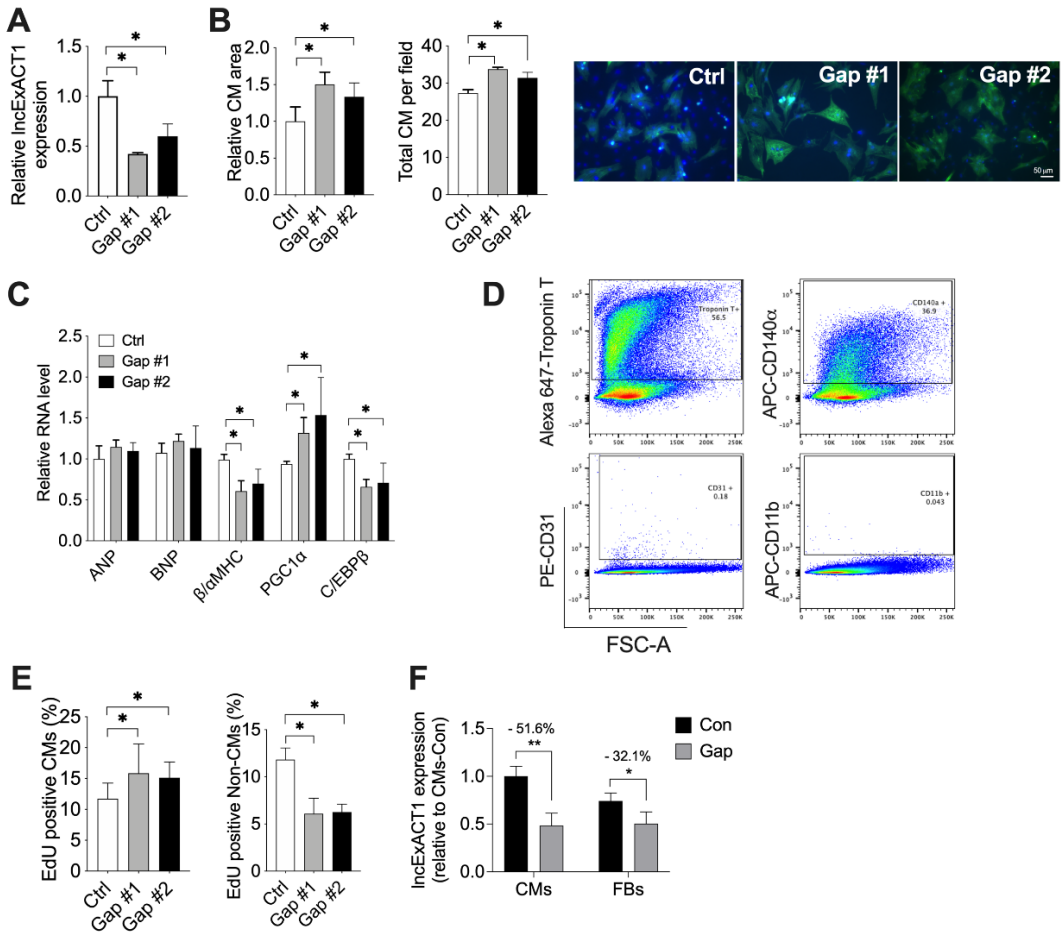
Supplemental Figure II



Supplemental Figure II. Overexpression of lncExACT1 induces pathological hypertrophy in cardiomyocytes *in vitro*. A. Quantification of cardiomyocyte (CM) area for neonatal rat ventricular

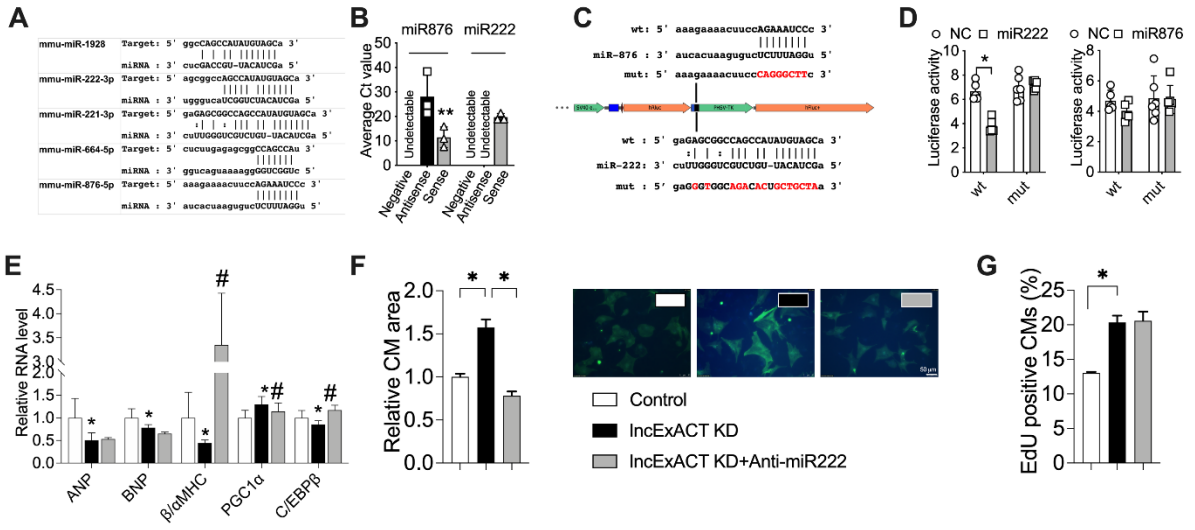
cardiomyocytes (NRVMs) stained with α-Actinin (green) and DAPI (blue) after infection with empty vector (Vector) or lncExACT1-overexpressing lentivirus (OE). **B.** Distribution of CM area from NRVMs treated with vector and OE. **C.** Expression of hypertrophy gene markers. **D.** Quantification of EdU-positive CMs and non-CMs. **E.** Quantification of DSCR1 and CnA protein expression, and nuclear NFATc3 protein expression. **F.** Expression of hypertrophy gene markers in NRVMs treated with vector, lncExACT1 without (OE) or with calcineurin inhibitor FK506 (OE+FK506). **G** and **H.** Relative expression of beta-Actin (ACTB), U6, HOTAIR, and lncExACT1 RNA in cytosolic and nuclear fractions from cardiomyocytes isolated from mice treated with control- (Con) or lncExACT1- (lncExACT1) AAV9. **I.** Quantification of Ki67 and PCM1 double-positive CMs in heart sections. **J.** Quantification of pHH3 and PCM1 double-positive CMs in heart sections. n=3 independent replicates/group in A-H, n=6-7 in I and J. **p*<0.05 vs Vector by Student's *t* test. **p*<0.05 vs Vector, #*p*<0.05 vs OE in A, B, and F. Data are shown as mean±SEM.

Supplemental Figure III



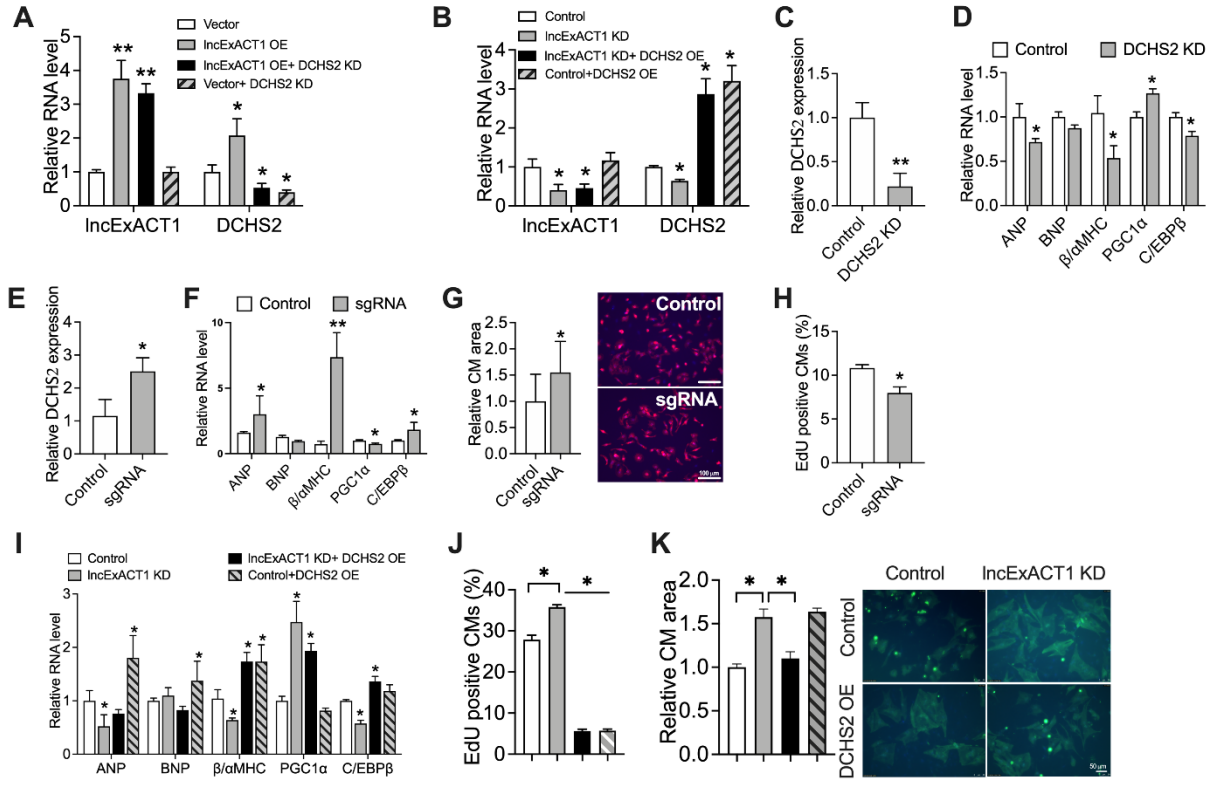
Supplemental Figure III. Inhibition of lncExACT1 induces physiological hypertrophy in cardiomyocytes *in vitro*. A. Relative lncExACT1 expression in NRVMs transfected with scramble control (Ctrl) or two LNA-GapmeRs targeting different regions of lncExACT1 (Gap #1 and Gap #2). **B.** Quantification of cardiomyocyte (CM) area and α-Actinin positive (CMs) cells for NRVMs stained with α-Actinin (green) and DAPI (blue). **C.** Expression of hypertrophy markers. **D.** Representative images of NRVMs flow cytometry from cells isolated from neonatal rat hearts stained with troponin T (Alexa 647) for cardiomyocytes, or CD140α (APC) for fibroblasts, or CD31 (PE) for endothelial cells, or CD11b (APC) for leukocytes. **E.** Quantifications of EdU-positive CMs and non-CMs. **F.** Expression of lncExACT1 in cardiomyocytes (CMs) and cardiac fibroblasts (FBs) isolated from mice treated with control- (Con) or lncExACT1-GapmeR (Gap). n=3 independent replicates/group. *p<0.05 vs Ctrl by one-way analysis of variance (ANOVA) with post hoc Tukey. Data are shown as mean±SEM.

Supplemental Figure IV



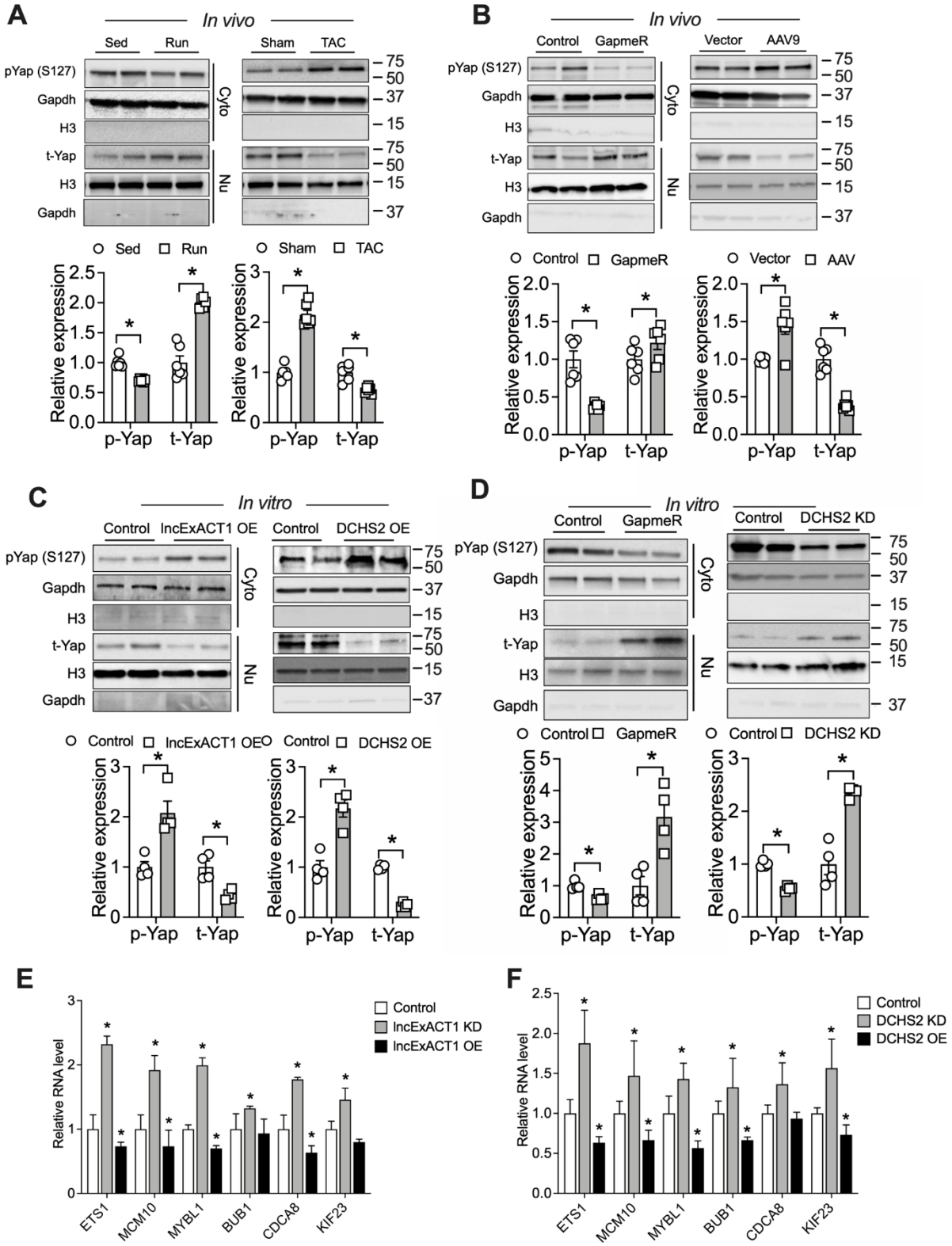
Supplemental Figure IV. lncExACT1 binds to miR-222 in cardiomyocytes *in vitro*. A. List of miRNAs predicted to bind lncExACT1. **B.** Ct values for miR-876 and miR-222 confirm lncExACT1 binding in pulldown with probes targeting lncExACT1 antisense or sense sequence, or negative control. **C.** Representation of luciferase vector construction. **D.** Luciferase activity in 293T cells transfected with wild-type (wt) or mutant (mut) lncExACT1 reporter with/without miRNA mimics. **E.** Expression of hypertrophy gene markers in NRVMs treated with control GapmeR (Con), lncExACT1 GapmeR (lncExACT1 KD), or lncExACT1 GapmeR with miR222 inhibitor (lncExACT1 KD+Anti-miR222). **F.** Quantification of cardiomyocyte (CM) area for NRVMs stained with α-Actinin (green) and DAPI (blue). **G.** Quantifications of EdU-positive CMs. n=3 independent replicates/group in E-G. *p<0.05, **p<0.01 in other panels by one-way analysis of variance (ANOVA) with post hoc Tukey in B, and by Student's t test in D. Data are shown as mean±SEM.

Supplemental Figure V



Supplemental Figure V. lncExACT1 regulates DCHS2 in cardiac hypertrophy *in vitro*. A. Relative expression of lncExACT1 and DCHS2 transcripts in NRVMs with lncExACT1 overexpression (OE) in the presence or absence of DCHS2 siRNA (KD). **B.** Relative mRNA expression of lncExACT1 and DCHS2 in NRVMs with lncExACT1 inhibition (KD) in the presence or absence of DCHS2 overexpression (OE). **C.** Relative DCHS2 transcript levels in NRVMs with DCHS2 siRNA (KD). **D.** Relative mRNA expression of hypertrophy gene markers in NRVMs with DCHS2 KD. **E.** Relative DCHS2 transcript levels in NRVMs with DCHS2 overexpression using CRISPR/dCAS9 system with single guide RNA (sgRNA). **F.** Relative mRNA expression of hypertrophy markers in NRVMs with DCHS2 overexpression (sgRNA). **G-H.** Quantification of cell size and EdU positive in NRVMs with DCHS2 overexpression (sgRNA). I**.** Relative mRNA expression of ANP, BNP, β/αMHC ratio, PGC1α, and C/EBPβ in NRVMs with lncExACT1 KD and/or DCHS2 OE. **J** and **K.** Quantification of EdU positive cardiomyocytes and cell size in NRVMs treated with scrambled control (Control), or lncExACT1 KD with or without DCHS2 OE. **p*<0.05, ***p*<0.01 vs Control/Vector in A-H. **p*<0.05 in J and K. A, B, I, J, and K by one-way analysis of variance (ANOVA) with post hoc Tukey; C, D, E, F, G, and H by Student's *t* test. n=3 independent replicates/group. Data are shown as mean±SEM.

Supplemental Figure VI



Supplemental Figure VI. lncExACT1 regulates Hippo pathway. A. Representative images and quantification of phosphorylated Yap1 (pYap) and total Yap (t-Yap) in hearts from sedentary mice (Sed), or mice subjected to voluntary wheel running (Run), sham surgery or TAC. n=6 mice/group **B.** Representative images and quantification of pYap and t-Yap in hearts from mice treated with LNA-GapmeR targeting lncExACT1 (GapmeR) or AAV9 overexpressing lncExACT1 (AAV). n=6 mice/group **C.** Representative images and quantification of pYap and t-Yap from NRVMs with lncExACT1 OE or DCHS2 OE. n=4 independent replicates/group. **D.** Representative images and quantification of pYap and t-Yap from NRVMs with lncExACT1 KD or DCHS2 KD. n=4 independent replicates/group. **D.** Relative mRNA expression of Yap1 downstream targets (ETS1, MCM10, MYBL1, BUB1, CDCA8, and KLF23) in NVRMs with lncExACT1 KD and OE. **E.** Relative mRNA expression of Yap1 downstream targets in NVRMs with DCHS2 KD and OE. n=3 independent replicates/group in E-F. **p*<0.05, ***p*<0.01 vs Control/Vector by Student's *t* test in A-D; by by one-way analysis of variance (ANOVA) with post hoc Tukey in E and F. Data are shown as mean±SEM.