SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Please see the Major Resources Table in the Online Supplemental Materials.

Human studies. Discarded human right ventricle outflow tract specimens resected from non-syndromic tetralogy of Fallot patients or left ventricle outflow tract specimens resected from non-syndromic subaortic membrane patients were collected during clinically indicated cardiac operations. Primary genetic screening results of the participants including karyotype, fluorescence *in situ* hybridization (FISH), and SNP microarray, were normal. Control samples were obtained from heart donors who died from noncardiac causes and were nonsuitable for transplant. The specimens were immediately snap-frozen using liquid nitrogen. Total RNAs were isolated using standard methods and used for generating cDNA libraries. Deep, paired-end RNA sequencing (RNA-seq) was performed. Sequencing data were deposited in UCLA CHD-BioCore Genomic Data Repository. *SLIT3* gene expression (RNA-Seq data) were obtained from UCLA CHD-BioCore Genomic Data Repository (Touma unpublished data). The data were quantified using RPKB (reads per kilobase of transcript, per million mapped reads) Measure.

Experimental Animals

Both male and female mice were included in all experiments at a ratio of 1:1. *Slit3* knockout mice ($Slit3^{-/-}$) and their littermates wild type mice ($Slit3^{+/+}$) on the CD-1 background were used as previously described [1]. *Robo1* knockout mice ($Robo1^{-/-}$) on the CD-1 background have been described previously [2]. All other mice described below were on the C57BL/6 (B6) strain. $Slit3^{fl/fl}$ mice [3] were a gift from Dr. Sun-Kyeong Lee. *Tcf21*-MerCreMer mice [4] were a gift from Dr. Michelle Tallquist. *Tbx18*-CreERT2 mice [5] have been generated and characterized in detail previously. *Rosa26*-CreERT2 mice and *Myh6*-MerCreMer mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). $Robo1^{fl/fl}$ mice were generated by the University of Michigan Transgenic Animal Model Core. $Robo1^{fl/fl}$ mice was first created on a B6 SVJ background using an Easi-CRISPR approach [6] that first deleted exon3 of *Robo1* and then replaced it with a long single stranded DNA donor molecule that was exon3 flanked by *loxP* sites as well as loci for diagnostic restriction enzymes. These animals then completed six backcrosses onto the B6 strain prior to use in experiments. At the age of 7 weeks, *Rosa26*-CreERT2, $Slit3^{fl/fl}$

mice, *Tcf21*-MerCreMer, *Slit3*$^{fl/fl}$ mice, *Tbx18*-CreERT2, *Slit3*$^{fl/fl}$ mice, and *Myh6*-MerCreMer; *Robo1*$^{fl/fl}$ mice were injected with tamoxifen (100 mg/kg/day) for consecutive 5 days to induce recombination. Littermate mice containing *Slit3*$^{fl/fl}$ or *Robo1*$^{fl/fl}$ without the Cre transgene were the controls.

Mice were housed in a temperature-controlled environment with 12-hour light/dark cycles where they received food and water *ad libitum*. All protocols concerning the use of animals were approved by the University of Michigan.

Mice were genotyped by polymerase chain reaction with the following primers: *Slit3*$^{fl/fl}$: Forward primer: TGTGACTAGCATGTAGTAGG, Reverse primer: CCAGGCTCAGCCTTTTAGAG. *Robo1*$^{fl/fl}$: Forward primer: AGTAATGGATTCAGTTAGAGTCGGGGTAT, Reverse primer: TCCCGTAGTACTGTAGGGACAAGATTAAA. *Slit3*$^{-/-}$: Forward primer: GCGCCTCCTCGGGCTCCTCGTGTC, Reverse primer: TGCGGGGGATGCCCCGAGGAA. *Robo1*$^{-/-}$: Forward primer: TGGCACGAAGGTATATGTGC, Reverse primer: CCTCCGCAAACTCCTATTTC. *Rosa26*-CreERT2: Forward primer: CGTGATCTGCAACTCCAGTC, Reverse Primer: AGGCAAATTTTGGTGTACGG. *Tbx18*-CreERT2: Forward primer: GCCAGAGAAAGAGGAAACGGCAAA, Reverse primer: TCCCTGAACATGTCCATCAGGTTC. *Tcf21*-MerCreMer: Forward primer: TTCTCCAGGCTCAAGACCAC, Reverse primer: CAAACCCTAGCACAAATCACTCGC. *Myh6*-MerCreMer: Forward primer: TCTATTGCACACAGCAATCCA, Reverse primer: CCAGCATTGTGAGAACAAGG.

TAC Model

Transverse aortic constriction was accomplished using a 27-gauge needle as described elsewhere [7]. Mice subjected to the same surgical procedures as TAC mice but without being tied at the transverse aorta (sham mice) were used as the control mice. Animals were randomized to Sham or TAC surgery. Animals with inadequate gradients (<25 mmHg) on echocardiogram were excluded. We performed a power analysis to determine the appropriate number of animals in each group. Given our historical measurements (mean±SD) of these outcomes on control animals, our intention to detect a 20% difference between control and experimental groups, a probability of Type I error (α) of 0.05, and the risk of Type II error (β) of 0.2, we have determined the minimum number animals needed for each experiment: n=8 mice/group for HW/BW ratio measurement. Initial

group size was at least 11 animals to account for attrition or dropout rate from surgical mortality. Approximately 20% of animals were excluded from the study because of procedure mortality or inadequate TAC gradients.

Isolation of Cardiac Cells and Cell Culture

Adult mice cardiac myocytes and nonmyocytes were isolated with Langendorff-free method as previously published [8]. Briefly, mice aged 6-8 weeks were anesthetized and the heart was exposed via sternotomy. After transecting the descending aorta, 7 ml EDTA buffer was immediately flushed into the right ventricle within 1 minute. The heart was then transferred to a 60-mm dish containing fresh EDTA buffer. EDTA buffer, perfusion buffer, and collagenase buffer were injected sequentially into the left ventricle. Cardiac chambers were separated and gently pulled into 1-mm pieces using forceps. Then the suspension underwent debris dissociation, enzymatic activity termination, and gravity settling, after which, the fractions containing the cardiomyocytes or cardiac nonmyocytes were collected separately for further analysis. Adult mice cardiac fibroblasts (AMCFs) were obtained from the cardiac nonmyocyte fraction that was cultured on polydimethylsiloxane (PDMS)-coated plates with DMEM supplemented with 10% FBS, 1% penicillin-streptomycin (P/S). AMCFs at passage number 1 or 2 were used. Serum-free media were conditioned with cultured AMCFs with or without adenovirus infection for 48 hours.

Neonatal rat cardiomyocytes (NRCMs) were isolated from the ventricles of 2-days-old Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) according to the instruction of Pierce™ Primary Cell Isolation Kits (Invitrogen, 88280). Briefly, neonatal rat ventricles were removed from the chest, minced and rinsed in HBSS buffer and then digested with the complex enzymes in the 37°C incubator. After 30-35 minutes, digestion was stopped with FBS addition and then cellular dissociation was completed by gentle trituration. Cells resuspended were pre-plated for 1.5 hours to remove cardiac fibroblasts. NRCMs were plated at the density of $1.2x10^5/cm^2$ on the 1% gelatin-coated plates and cultured in Medium 199 supplemented with 1% ITS, 1% P/S, 10 μM cytosine 1-β-d-arabinofuranoside (Ara C) (to inhibit the proliferation of fibroblasts).

Primary human aortic vascular smooth muscle cells were purchased from Promocell GmbH (Heidelberg, Germany) and cultured with Promocell Smooth Muscle Cell Growth Medium 2 with supplement and 1% P/S and used between passages 2-5. Human induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs)

iCell® Cardiomyocytes were purchased from FUJIFILM Cellular Dynamics, Inc. (Madison, WI, USA) and cultured according to the manufacturer instructions. Cells were periodically assessed for *Mycoplasma* contamination.

Echocardiography

Transthoracic echocardiography of mice was performed under anesthesia with 2% isoflurane by the University of Michigan Physiology Phenotyping Core as previously described [1]. Animals were placed on a warming pad to maintain normothermia. Two-dimensional, M-mode, Doppler, and tissue Doppler echocardiography images were recorded using a Visual Sonics Vevo 2100 high-resolution in vivo microimaging system with an MS 550D transducer that has a center frequency of 40 MHz and a bandwidth of 22–55 MHz with the animal in a supine or lateral position. Measurements of the end-diastolic and end-systolic interventricular septum, posterior wall thickness, and LV internal diameter were conducted for each mouse. Then, the LV ejection fraction and fractional shortening were calculated. Echocardiogram parameters were assessed by a blinded technician.

Adenovirus Infection and siRNA Transfection

Adenovirus encoding for either GFP (AdGFP), *SLIT3* (AdSLIT3), or Cre (AdCre) were dissolved in pre-chilled, serum free media without P/S, and then added to plated cells at the MOI of 50. After 6 hours, media were changed to the media appropriate for the responding cells. For the knockdown of *SLIT3* in vascular smooth muscle cells, or the knockdown of *Robo1* in neonatal rat cardiomocytes, cells were transfected with siRNA-*SLIT3* or siRNA-*Robo1* or siRNA-Scramble purchased from Origene Technologies, Inc. (Rockville, MD, USA) using Lipofectamine RNAiMAX (Invitrogen) with the reverse transfection according to the manufacturer's instructions.

Western Blot Analysis

Cellular or tissue extracts were lysed in RIPA lysis buffer with the addition of phosphatase inhibitor and protease inhibitor. Equal amounts of proteins were loaded and separated on 4-12% SDS–PAGE before transfer to nitrocellulose blotting membranes. After blocked in 5% skimmed milk in TBST at room temperature for 1.5 hours, the membranes were incubated with primary antibodies at 4°C overnight. After TBST washing, membranes were incubated with fluorescence-conjugated secondary antibodies for 1 hour at room temperature.

After TBST washing, bands were scanned and analyzed by Li-Cor Odyssey imaging system (LiCor Bioscience, Lincoln, NE). The used antibodies are listed: anti-SLIT3 (1:1000, Thermo Fisher Scientific, Waltham, MA, USA), anti-phosphorylated-PKA substrates (1:1000, Abcam, Cambridge, UK), anti-phosphorylated-AKT (1:1000, Abcam), anti-total-AKT (1:1000, Abcam), anti-phosphorylated-PKAc (1:1000, Abcam), anti-total-PKAc (1:1000, Abcam).

qPCR Analysis

Total RNA was extracted from cells or tissue using RNeasy Mini Kit (QIAGEN, Hilden, Germany) followed by reverse transcription with SuperScript III kit (Thermo Fisher Scientific). mRNA was determined by Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad). The mRNA level was normalized to the internal control, GAPDH, and presented as the fold change to the control group. The qPCR primers used in this study are available upon request.

Histological Analysis

After euthanasia, mice were perfused with ice-cold saline through the left ventricle. The hearts were dissected, fixed in formalin, embedded in paraffin, and sectioned. The H&E staining was performed by the In-Vivo Animal Core at the University of Michigan. After hydration, antigen retrieval, permeabilization, and blocking, the slices were stained with anti-SLIT3 (1:100, Sigma Aldrich,), anti-ROBO1 (1:100, Abcam), anti-cTNT (1:100, Sigma-Aldrich, St. Louis, MO, USA), anti-POSTN (abcam, ab215199, 1:100), anti-Tdtomato (1:100, Abcam) at 4°C overnight, followed by Alexa Fluor-conjugated secondary antibody (1:100, Thermo Fisher Scientific) immunostaining. For the cardiomyocyte cross-sectional area analysis, slices were stained with Alexa Fluor488-conjugated wheat-germ agglutinin (WGA, Sigma Aldrich). The nuclei were stained with DAPI. Images were obtained with a fluorescence microscope and analyzed. For anti-SLIT3 and anti-ROBO1 antibodies, we utilized WT and KO tissue as our positive and negative controls, respectively. Secondary-only antibody controls were also performed with each staining experiment to determine the level of background staining.

Picrosirius red staining was used to visualize fibrillar collagen. For the staining, one LV short-axis section at the level of the mid-lower papillary muscles was selected per heart. For H&E staining, the images of sections were scanned and analyzed with Keyence BZ-X800 microscopy (Keyence). For staining with picrosirius red (Picro-Sirius

Red Stain Kit-Cardiac Muscle, Abcam, ab245887), Heart sections were deparaffinized with xylene and rehydrated through a graded ethanol series (100, 95, 80, 70 and 50%) at 5-minute intervals, then rinsed in deionized water and incubated in 0.2% phosphomolybdic acid solution for 2 min. After rinsed in deionized water, the heart tissue was then incubated with picrosirius red solution for 1 hour at room temperature. Afterward, the slide was washed in two changes of 0.5% acetic acid for a total 30 seconds, then hydrated with two changes of absolute ethanol for 30 seconds each, cleared in xylene (Fisher Chemical, X5-1) twice for a total 10 min and mounted in synthetic resin (Fisher Chemical , SP15) . The finished slides were imaged using the Olympus BX53 microscopy with the same light setting and pixel classifier. The production of mature fibrillary collagen in the heart tissues was indicated by the mean red channel signal intensity which was measured with ImageJ software (Version1.8.0).

Hydroxyproline Assay

To quantify the total tissue collagen content, the hydroxyproline assay kit (Hydroxyproline Assay Kit- Perchlorate Free, Sigma-Aldrich, MAK357) was used according to the manufacturer's protocol. For this assay, 10 mg heart tissues from the left ventricle near apex per heart were collected, mixing with 100 µL of ultrapure water, and thoroughly homogenized with Epishear probe sonicator (Active Motif).100 µL of sample homogenate was then transferred to a pressure-tight, screw-capped polypropylene vial and added with 100 µL of 10 M concentrated NaOH, followed by incubation at at 120 °C for 1 hour. Following alkaline hydrolysis, the vial was placed on ice to cool briefly before opening cap and mixed with 100 µL of 10 M concentrated HCl to neutralize residual NaOH. Then the vials were centrifuged at 10,000 × g for 5 minutes to pellet any insoluble debris that may remain following hydrolysis. 10 µL of each neutralized sample hydrolysate supernatants were transferred to 96-well plates, respectively. The samples were placed in a 65°C oven to be evaporated to dryness. Then 100 µL Oxidation Buffer/ Chloramine T mixture (provided in the kit) was added to each well followed by incubation at room temperature for 20 minutes. Then 50 µL of Developer Solution (provided in the kit) to each reaction well, followed by incubation at 37 °C for 5 minutes. Next, 50 µL of DMAB Concentrate solution (provided in the kit) was added to each reaction well, and then the plates were incubated in a 65°C oven for 45 minutes. After incubation, the absorbance of each well was immediately measured at 560 nm with a spectrophotometer (Multiskan SkyHigh Microplate Spectrophotometer, A51119500C).

Cell Immunofluorescence Staining

Cardiomyocytes were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-

100 for 10 min. After a preincubation in 3% BSA for 1h, myocytes were incubated with an anti-α-sarcomeric actinin (1:200, Abcam) at 4°C overnight, followed by Alexa Fluor conjugated secondary antibody or Alexa Fluor488-conjugated WGA (1:100, Thermo Fisher Scientific). The nuclei were stained with DAPI. Images were analyzed using ImageJ software.

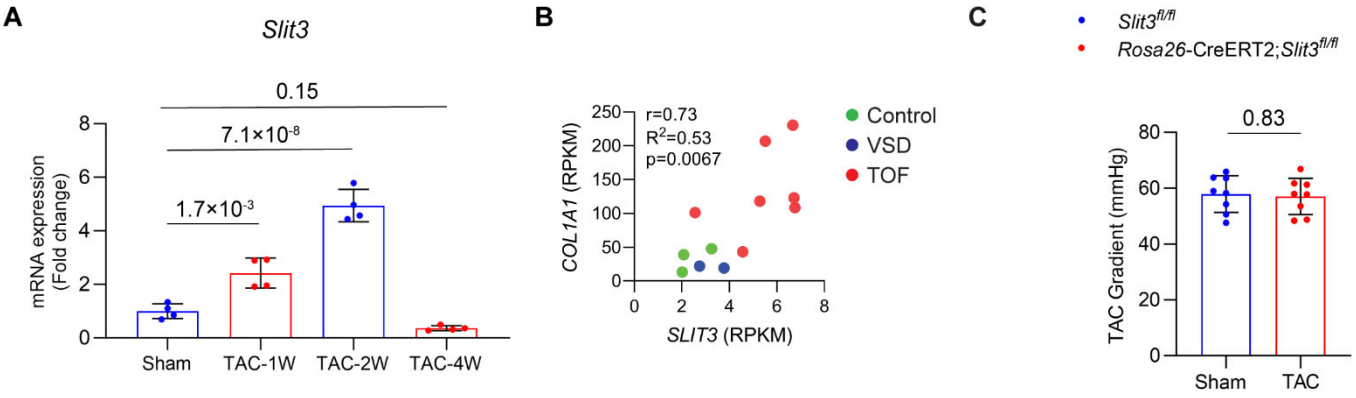
Statistical Analysis

All data are presented as mean ± SEM. Statistical significance of genotype, surgery (Sham or TAC), and their interactions on echocardiography parameters, HW/BW, histological changes, and gene expression were analyzed for planned comparisons using 2-way ANOVA with Tukey's multiple comparison test. Where appropriate, the Student's t test or Mann Whitney test were used to compare data between two groups. For results with n≥6, we have used the D'Agostino-Pearson (omnibus K2) test to confirm normality prior to comparison with a parametric test. For data n<6 or data that were n≥6 and not normal, we have used a non-parametric test. A linear regression model with cluster option and robust standard errors was used to analyze the picosirius red polarized micrscopy data. All statistical analyses were performed using GraphPad Prism 9.4.1, except for the picosirius red polarized microscopy data, which was analyzed using Stata 18.0 (StataCorp LLC, College Station, TX, USA). A p value less than 0.05 was considered significant. Images selected for the figures were chosen to most closely represent the average of the experimental findings. The corresponding author (MS) had full access to all the data in the study and takes responsibility for its integrity and the data analysis.

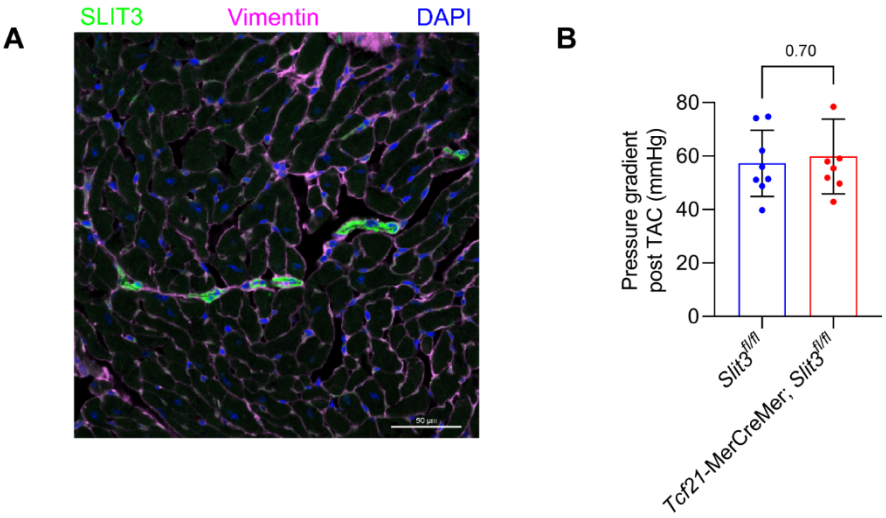
Study Approval

Pediatric patients with clinical diagnosis of a congenital heart defect were enrolled in the Congenital Heart Defect Bio-banking core (UCLA CHD-BioCore) using a protocol approved by the UCLA Institutional Review Board (UCLA IRB 13-000646). Written informed consents were obtained from all participants. All animal research was conducted under protocol #PRO00008538 that was approved by the University of Michigan Institutional Animal Care and Use Committee.

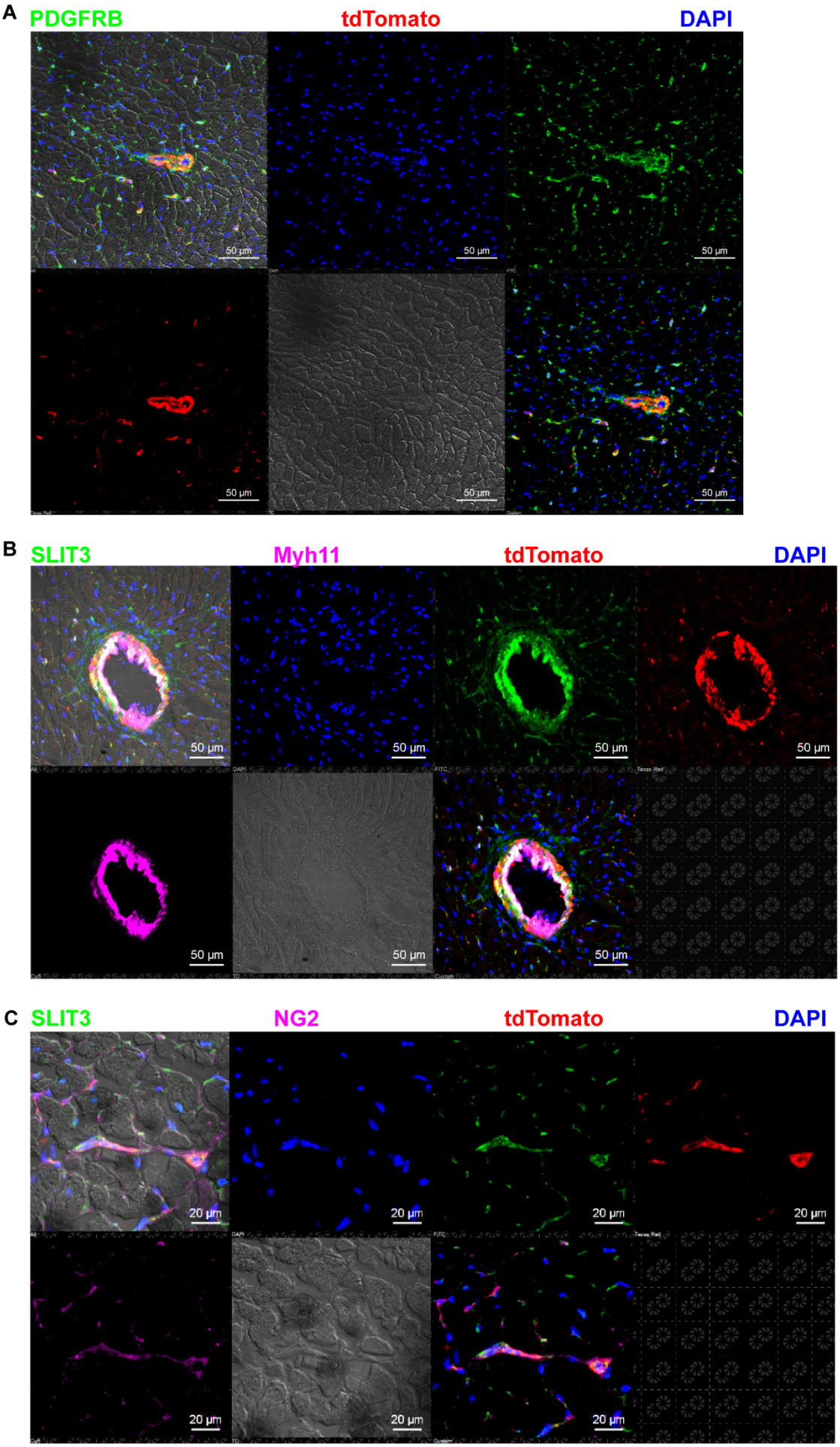
SUPPLEMENTAL FIGURES



Supplemental Figure 1. A. *Slit3* transcription determined by qPCR in wild type mouse hearts after sham surgery or 1, 2, and 4 weeks after TAC. One-way ANOVA with Dunnett's multiple comparisons test. N=4 mice/time point. **B**. Correlation of *COL1A1* and *SLIT3* transcription in myocardial tissue samples from human control hearts, patients with ventricular septal defects (VSDs, a volume overload defect), and tetralogy of Fallot (TOF, a pressure overload defect). **C.** Mean peak pressure gradient of aorta measured by echocardiography at 3 days after TAC surgery in *Slit3*$^{fl/fl}$ and *Rosa26*-CreERT2; *Slit3*$^{fl/fl}$ mice. Student's t test and assuming application of the Central Limit Theorem. N=8 mice/group..

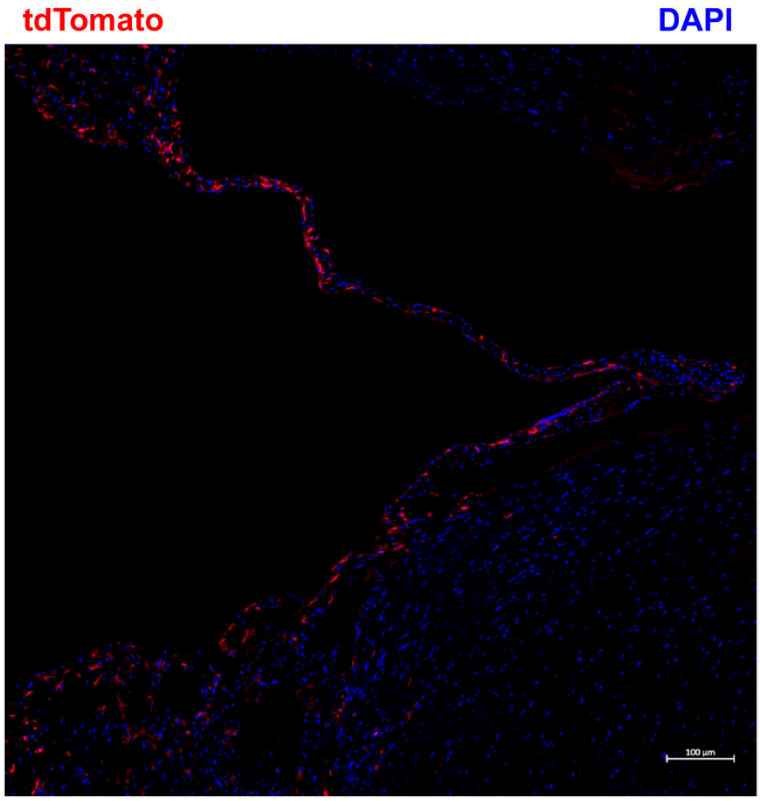


Supplemental Figure 2. A. Overlay immunofluorescence image of wild type mouse heart stained with anti-SLIT3 (green) and anti-vimentin (magenta) antibodies and DAPI nuclear counterstain. Scale bar = 50 μm. **B.** Mean peak pressure gradient of aorta measured by echocardiography at 3 days after TAC surgery in *Slit3*$^{fl/fl}$ and *Tcf21*-MerCreMer; *Slit3*$^{fl/fl}$ mice. Student's t-test assuming application of the Central Limit Theorem. N=8 mice/group. B.

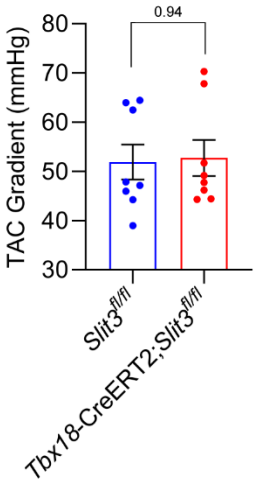

A
PDGFRB
tdTomato
DAPI
50 μm
B
SLIT3
Myh11
tdTomato
DAPI
50 μm
C
SLIT3
NG2
tdTomato
DAPI
20 μm

Supplemental Figure 3. Immunofluorescence staining of myocardial cryosections from *Tbx18*-CreERT2, *Rosa26*-tdTomato mice, which had been injected with tamoxifen 6 weeks prior. **A.** Immunofluorescence staining using anti-PDGFRα antibody (green), anti-tdTomato antibody (red), and DAPI (blue); **B.** Immunofluorescence staining using anti-SLIT3 antibody (green), anti-MYH11 antibody (purple), anti-tdTomato antibody (red), and DAPI (blue). **C.** Immunofluorescence staining using anti-SLIT3 antibody (green), anti-NG2 antibody (purple), anti-tdTomato antibody (red), and DAPI (blue).

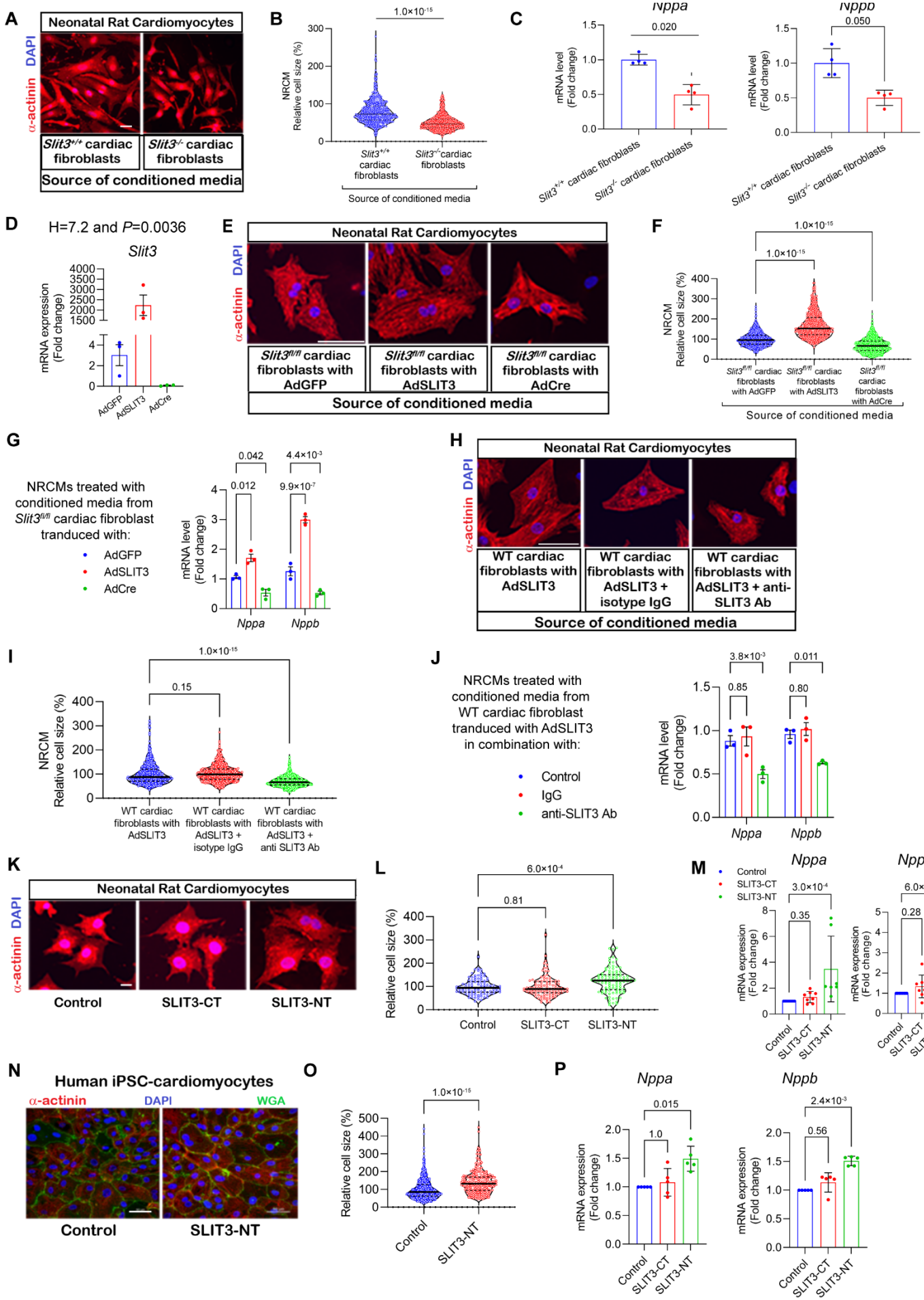
A



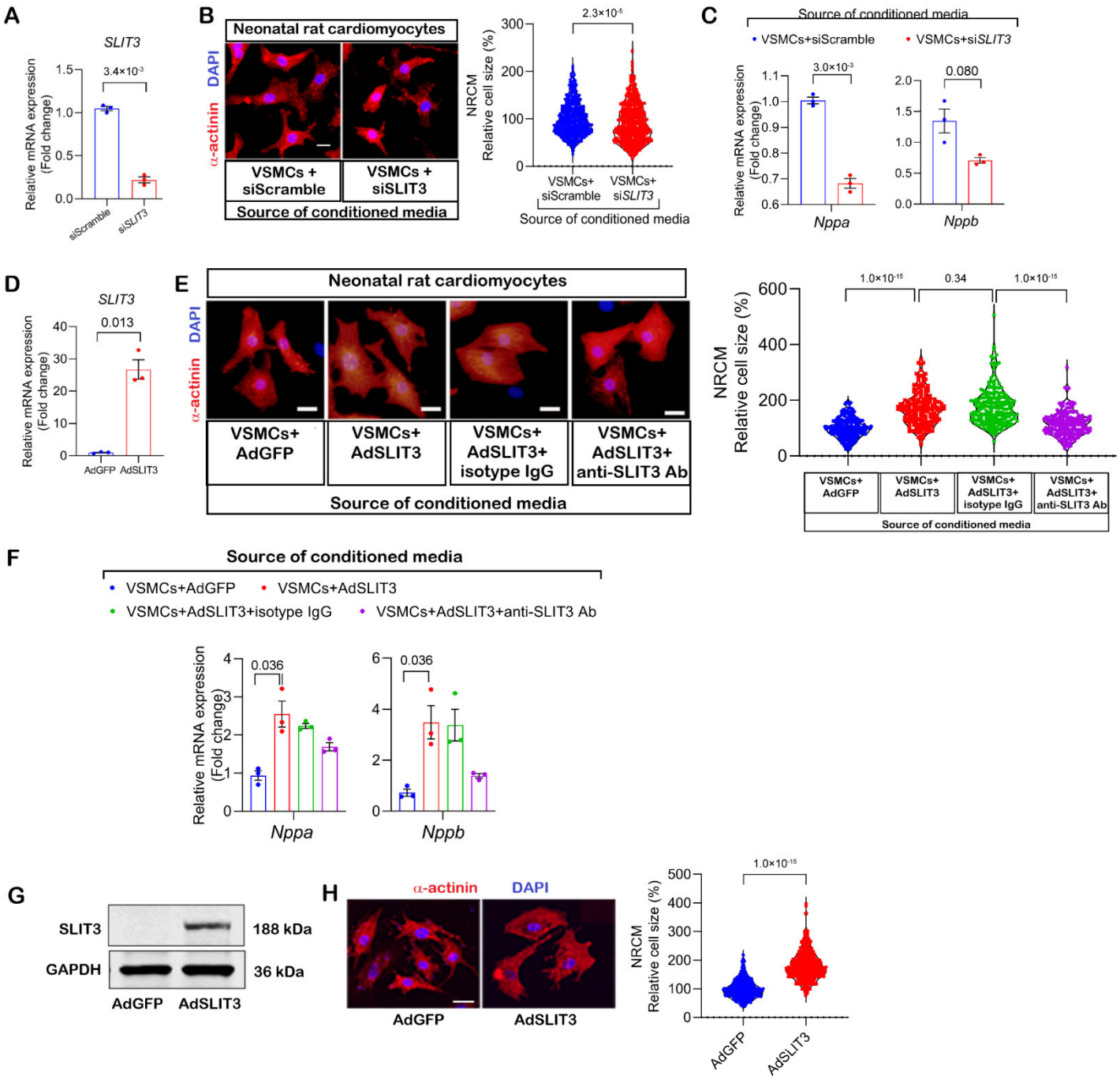
B



Supplemental Figure 4. Immunofluorescence staining of myocardial sections from *Tcf21*-MerCreMer; *Rosa26*-tdTomato reporter mice (**A**), which had been injected with tamoxifen 6 weeks prior. Anti-tdTomato antibody (red) and DAPI (blue). Note tdTomato signal in the interstitial area and valve tissue. Scale bars = 100 µm. **B.** TAC gradient in *Slit3*$^{fl/fl}$ and *Tbx18*-CreERT2;*Slit3*$^{fl/fl}$ groups and comparison made with Student's t test assuming application of the Central Limit Theorem.

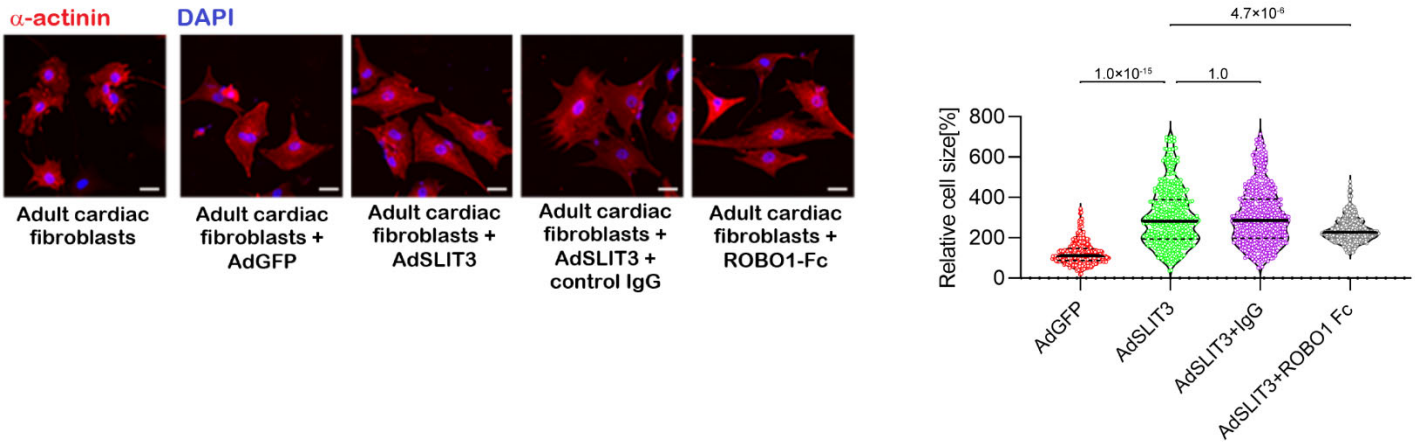

A
Neonatal Rat Cardiomyocytes
DAPI
α-actinin
Slit3+/+ cardiac fibroblasts
Slit3-/- cardiac fibroblasts
Source of conditioned media
B
1.0×10-15
NRCM Relative cell size (%)
Slit3+/+ cardiac fibroblasts
Slit3-/- cardiac fibroblasts
Source of conditioned media
C
Nppa
0.020
mRNA level (Fold change)
Slit3+/+ cardiac fibroblasts
Slit3-/- cardiac fibroblasts
Nppb
0.050
D
H=7.2 and P=0.0036
Slit3
mRNA expression (Fold change)
AdGFP
AdSLIT3
AdCre
E
Neonatal Rat Cardiomyocytes
Slit3fl/fl cardiac fibroblasts with AdGFP
Slit3fl/fl cardiac fibroblasts with AdSLIT3
Slit3fl/fl cardiac fibroblasts with AdCre
Source of conditioned media
F
1.0×10-15
NRCM Relative cell size (%)
Source of conditioned media
G
NRCMs treated with conditioned media from Slit3fl/fl cardiac fibroblast tranduced with:
AdGFP
AdSLIT3
AdCre
0.042
4.4×10-3
0.012
9.9×10-7
mRNA level (Fold change)
Nppa
Nppb
H
Neonatal Rat Cardiomyocytes
WT cardiac fibroblasts with AdSLIT3
WT cardiac fibroblasts with AdSLIT3 + isotype IgG
WT cardiac fibroblasts with AdSLIT3 + anti-SLIT3 Ab
Source of conditioned media
I
1.0×10-15
0.15
NRCM Relative cell size (%)
WT cardiac fibroblasts with AdSLIT3
WT cardiac fibroblasts with AdSLIT3 + isotype IgG
WT cardiac fibroblasts with AdSLIT3 + anti SLIT3 Ab
J
NRCMs treated with conditioned media from WT cardiac fibroblast tranduced with AdSLIT3 in combination with:
Control
IgG
anti-SLIT3 Ab
3.8×10-3
0.011
0.85
0.80
mRNA level (Fold change)
Nppa
Nppb
K
Neonatal Rat Cardiomyocytes
Control
SLIT3-CT
SLIT3-NT
L
6.0×10-4
0.81
Relative cell size (%)
Control
SLIT3-CT
SLIT3-NT
M
Control
SLIT3-CT
SLIT3-NT
Nppa
3.0×10-4
0.35
Nppb
6.0×10-4
0.28
mRNA expression (Fold change)
N
Human iPSC-cardiomyocytes
α-actinin
DAPI
WGA
Control
SLIT3-NT
O
1.0×10-15
Relative cell size (%)
Control
SLIT3-NT
P
Nppa
0.015
1.0
Nppb
2.4×10-3
0.56
mRNA expression (Fold change)
Control
SLIT3-CT
SLIT3-NT

Supplemental Figure 5. Paracrine-mediated SLIT3 from nonmyocytes can directly induce hypertrophy in cardiomyocytes. **A**. Immunofluorescence using anti-a-actinin antibody with DAPI counterstain of neonatal rat cardiomyocytes (NRCMs) that were exposed to conditioned media (CM) generated by culturing cardiac fibroblasts derived from adult wild type (*Slit3*$^{+/+}$) or *Slit3* knockout (*Slit3*$^{-/-}$) mice. **B**. NRCM size quantification after being cultured with CM for 72 hours, N=500 cells/group and comparison made with Mann-Whitney test. **C**. Transcription of hypertrophy-associated genes (*Nppa* and *Nppb,* normalized to *Gapdh* mRNA levels) in NRCMs were assessed by qPCR after culture with CM for 48 hours. Paired Student's t test. N=3 independent experiments. **D**. *Slit3* transcript levels (normalized to *Gapdh* mRNA levels) in adult cardiac *Slit3*$^{fl/fl}$ fibroblasts infected with AdGFP, AdSLIT3, or AdCre. Kruskal-Wallis test demonstrated significant differences in *Slit3* transcription amongst the three groups (H=7.2 and *P*=0.0036). Dunn's multiple comparisons test did not reveal any significant relationships. N=3 independent experiments. **E**. Immunofluorescence of NRCMs that were exposed to CM generated by culturing adult cardiac *Slit3*$^{fl/fl}$ fibroblasts infected with AdGFP, AdSLIT3, or AdCre. **F**. NRCM size quantification after being cultured with CM for 72 hours. Kruskal-Wallis test with Dunn's multiple comparisons test. N=500 cells/group. **G**. Transcription of *Nppa* and *Nppb*, normalized to *Gapdh* mRNA levels, in NRCMs were assessed by qPCR after culture with CM for 48 hours. Two-way ANOVA with Tukey's multiple comparisons test. N=3 independent experiments. **H**. Immunofluorescence of NRCMs that were exposed to CM generated by culturing adult wild type cardiac fibroblasts infected with AdSLIT3 in combination with isotype control IgG or an anti-SLIT3 antibody. **I**. NRCM size quantification after being cultured with CM for 72 hours. One-way ANOVA with Dunnett's multiple comparisons test. N=500 cells/group. **J**. Transcription of *Nppa* and *Nppb*, normalized to *Gapdh* mRNA levels, in NRCMs was assessed by qPCR after culture with CM for 48 hours. Two-way ANOVA with Tukey's multiple comparisons test. N=3 independent experiments. **K**. Immunofluorescence of NRCMs that were treated with recombinant SLIT3-C terminal fragment (SLIT3-CT) or recombinant SLIT3-N terminal fragment (SLIT3-NT). **L**. NRCM size quantification after being cultured for 72 hours. Kruskall-Wallis test and Dunn's multiple comparisons test. N=500 cells/group. **M**. Corresponding transcription of *Nppa* and *Nppb*. Kruskall-Wallis test and Dunn's multiple comparisons test. N=7 independent experiments. **N**. Immunofluorescence using WGA, anti-a-actinin antibody and DAPI counterstain of human iPSC-derived cardiomyocytes that were treated with SLIT3-NT. **O**. Human iPSC-derived cardiomyocyte size quantification after being cultured for 72 hours. Mann-Whitney test. N=250 cells in each group. **P**. Transcription of *NPPA* and *NPPB* of human iPSC-derived cardiomyocytes was assessed with qPCR. Kruskall-Wallis test and Dunn's multiple comparisons test. N=3 independent experiments.

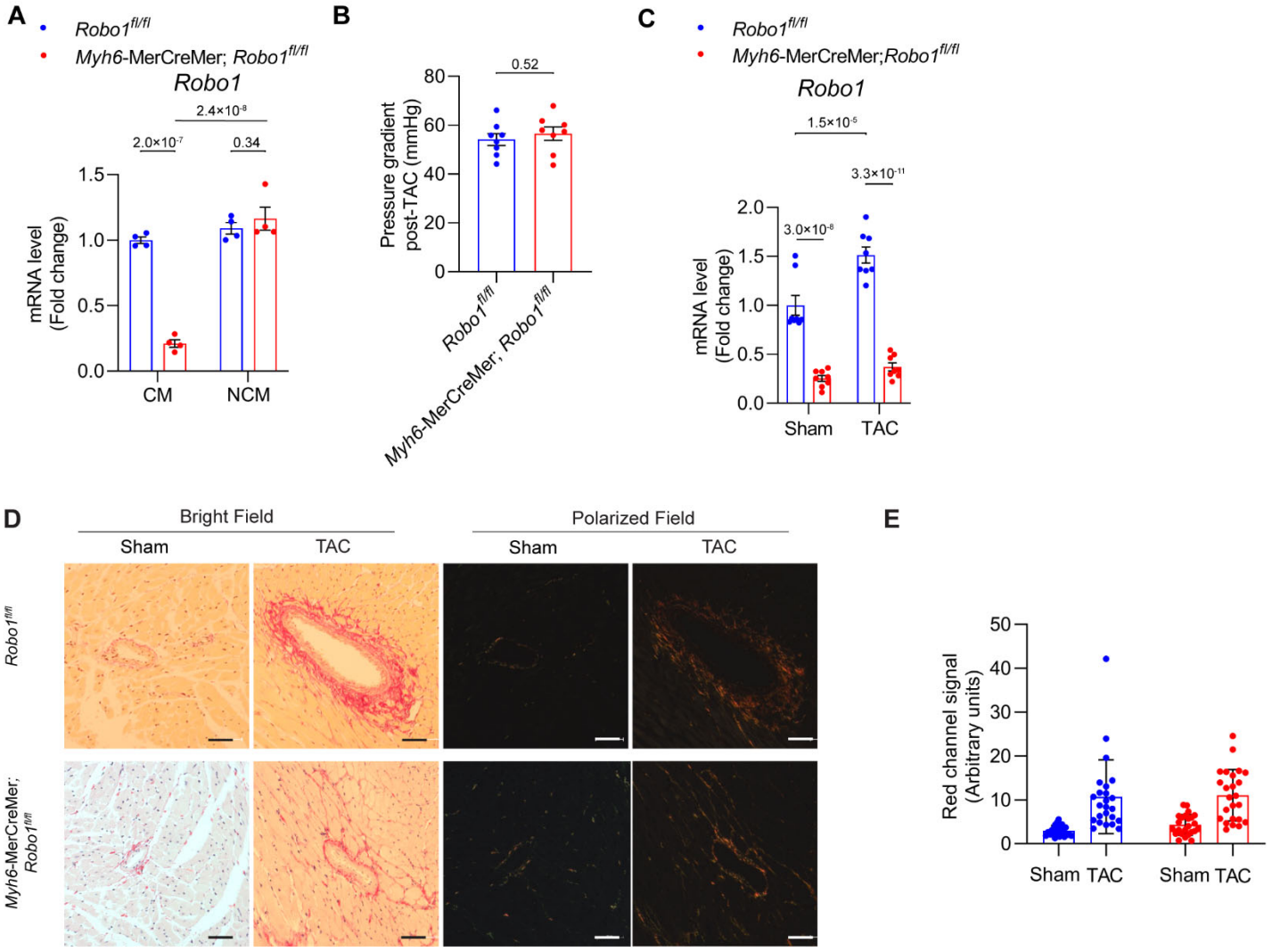

A
SLIT3
Relative mRNA expression (Fold change)
3.4×10-3
siScramble
siSLIT3
B
Neonatal rat cardiomyocytes
DAPI
α-actinin
VSMCs + siScramble
VSMCs + siSLIT3
Source of conditioned media
NRCM Relative cell size (%)
2.3×10-5
VSMCs+ siScramble
VSMCs+ siSLIT3
Source of conditioned media
C
Source of conditioned media
VSMCs+siScramble
VSMCs+siSLIT3
3.0×10-3
0.080
Nppa
Nppb
D
SLIT3
0.013
AdGFP
AdSLIT3
E
Neonatal rat cardiomyocytes
VSMCs+ AdGFP
VSMCs+ AdSLIT3
VSMCs+ AdSLIT3+ isotype IgG
VSMCs+ AdSLIT3+ anti-SLIT3 Ab
Source of conditioned media
1.0×10-15
0.34
1.0×10-15
F
Source of conditioned media
VSMCs+AdGFP
VSMCs+AdSLIT3
VSMCs+AdSLIT3+isotype IgG
VSMCs+AdSLIT3+anti-SLIT3 Ab
0.036
0.036
Nppa
Nppb
G
SLIT3
188 kDa
GAPDH
36 kDa
AdGFP
AdSLIT3
H
α-actinin
DAPI
AdGFP
AdSLIT3
1.0×10-15
AdGFP
AdSLIT3

261

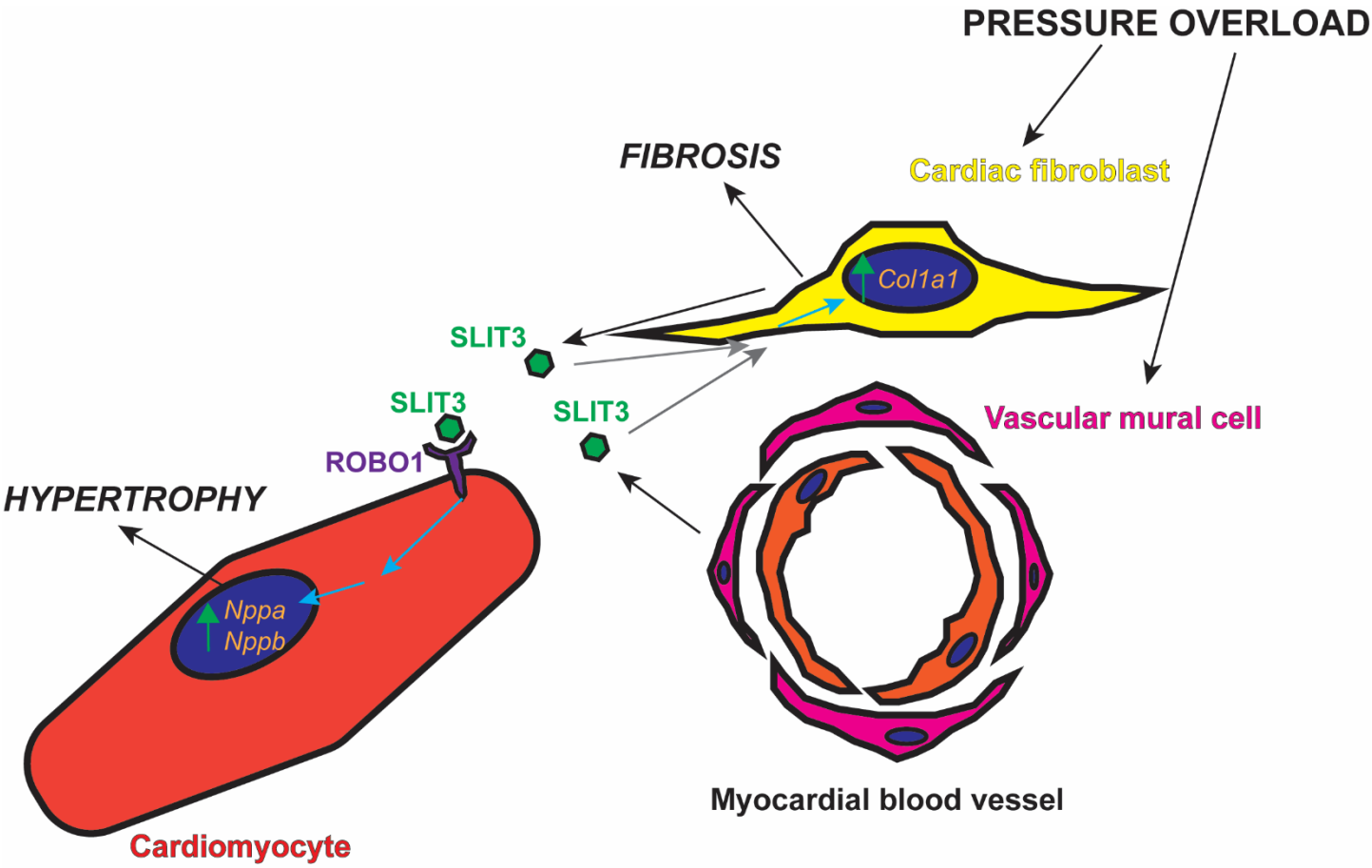
Supplemental Figure 6. **A.** *Slit3* transcription in primary human vSMCs transfected with siScramble or si*SLIT3* was determined by qPCR and compared by two-tailed student's t test assuming application of the Central Limit Theorem. Results are from n=3 independent experiments. **B.** Immunofluorescence of NRCMs treated for 72 hours with conditioned media from vSMCs in (**A**) with anti-α-actinin and DAPI counterstain with cell size quantification. **C.** qPCR-determined transcription of *Nppa* and *Nppb* in NRCMs treated with conditioned media from vSMCs from (**A**) for 48 hours and compared by two-tailed student's t test assuming application of the Central Limit Theorem. **D.** SLIT3 transcript levels measured by qPCR in primary human vSMCs that were transduced with AdGFP or AdSLIT3 and compared by paired Student's t test assuming application of the Central Limit Theorem. **E.** NRCMs were incubated with conditioned media from vSMCs in (**D**) along with control isotype IgG or an anti-SLIT3 antibody for 48 hours and immunofluorescence was performed and NRCM cell size was measured. Kruskal-Wallis test revealed significant differences amongst the four groups (H=262, P<0.0001) and Dunn's multiple comparisons test. **F.** Transcription of *Nppa* and *Nppb* in NRCMs treated with conditioned media and antibodies as in (**E**) for 48 hours. Kruskal-Wallis test revealed significant differences amongst the four groups (H=9.35, P=0.002) and Dunn's multiple comparisons test. **G.** Western blot of SLIT3 protein in NRCMs that were transduced with AdGFP or AdSLIT3 (MOI of 50). **H.** Immunofluorescence of NRCMs from (**G**) with cell size quantification and comparison with Mann-Whitney test. **I.** Transcription of *Nppa* and *Nppb* in NRCMs from (**G**) was assessed with qPCR. All results are representative of 3 independent experiments. Cell size quantification analysis was performed on data from all 3 independent experiments (N=500 cells analyzed/group). Scale bars = 20 μm. **J.** Transcription of *Nppa* and *Nppb* in NRCMs treated with SLIT3-NT (2.5 mg/ml) or phenylephrine (PE, 20 μM) for 48 hours. Kruskal-Wallis test revealed significant differences amongst the three groups (H=7.448, P=0.0036)



Supplemental Figure 7. A. Immunofluorescence with an anti α-actinin antibody and DAPI counterstain of NRCMs that were treated with conditioned media (from adult wild type cardiac fibroblasts transduced with AdGFP or AdSLIT3) in addition to isotype control IgG or ROBO1-Fc chimeric protein for 72 hours. NRCM cell size was quantified. Kruskal-Wallis test with Dunn's multiple comparisons test. N=500 cells/group.



Supplemental Figure 8. A. qPCR analysis of *Robo1* (normalized to *Gaphd* mRNA) in cardiomyocytes (CM) or non-cardiomyocytes (NCM) isolated from *Robo1*$^{fl/fl}$ or *Myh6*-CreER^{T2}; *Robo1*$^{fl/fl}$ mice, which had been injected with tamoxifen 6 weeks prior. Two-way ANOVA with Tukey's multiple comparisons test. N=4 mice/group. **B.** Peak pressure gradient of aorta measured by echocardiography 3 day after TAC in *Robo1*$^{fl/fl}$ and *Myh6*-CreER^{T2}; *Robo1*$^{fl/fl}$ mice. Student's t-test assuming the application of the Central Limit Theorem. N=8 mice/group. **C.** *Robo1* mRNA levels assayed by qPCR in *Robo1*$^{fl/fl}$ and *Myh6*-CreER^{T2}; *Robo1*$^{fl/fl}$ mice subjected to Sham or TAC surgery. Two-way ANOVA with Tukey's multiple comparisons test. **D**. Picosirius red staining of myocardial sections from *Robo1*$^{fl/fl}$ and *Myh6*-MerCreMer;*Robo1*$^{fl/fl}$ mice after sham or TAC surgery and visualized under brightfield microscopy and polarized light. Representative images shown and scale bar = 100 mm. **E**. Quantification of red channel signal under polarized light of picosirius red stained myocardial sections from **D**. Data from N=3 animals/group with 6-7 high power fields analyzed per animal. A linear regression model with cluster option was used to evaluate the data in **E**. The comparison of surgery (Sham vs. TAC) was found to be significant ($p=1.0\times10^{-4}$). The comparison of genotype (*Robo1*$^{fl/fl}$ vs. *Myh6*-MerCreMer;*Robo1*$^{fl/fl}$) and the interaction between genotype and surgery both yielded no significant difference (p=0.12 and p=0.34, respectively).



Supplemental Figure 9. Summary figure and working model of how SLIT3 signaling stimulates cardiomyocyte hypertrophy and cardiac fibrosis in response to pressure overload via a stromal cell:cardiomyocyte and stromal cell:cardiac fibroblastaxes.