Supplemental Material

"Epicardial HDAC3 Promotes Myocardial Growth Through a Novel MicroRNA Pathway"

(Jang et al)

Supplemental Materials & Methods

Mice

Hdac3$^{flox/+}$ [31], *Tnnt2*$^{nGFP/+}$ [38], and *Wt1*$^{CreERT2/+}$ [10] mice were previously described. *Wt1*$^{CreERT2/+}$ (Stock #010912) and *R26*^{eYFP} mice (Stock #006148*)* were purchased from The Jackson Laboratory (Bar Harbor, ME). We got *Hdac3*$^{flox/+}$ mice from Dr. Mitchell A. Lazar at the University of Pennsylvania. *Hdac3*$^{flox/+}$ mice (Stock #024119) are also available at The Jackson Laboratory. All animal protocols were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Administration of tamoxifen and 5-bromo-2'-deoxyuridine (BrdU) in vivo

Tamoxifen (Sigma, Saint Louis, MO) was dissolved in corn oil and intraperitoneally (IP) given to pregnant mice (150 mg/kg body weight) at E8.5. BrdU (Sigma, Saint Lous, MO) was dissolved in phosphate-buffered saline (PBS) and given IP to pregnant mice (100 mg/kg body weight) at E11.5 and E12.5 (one dose per day).

Embryonic cardiomyocyte culture and proliferation assessment

E13.5 ventricular cardiomyocytes were isolated from dissected embryonic hearts and cultured as previously described[73]. Briefly, hearts from *Tnnt2*$^{nGFP/+}$ embryos were collected at E13.5 and digested in Trypsin solution (10 mM HEPES, 0.5 mM EDTA, 0.5% Trypsin in 1X HBSS solution) at 4°C for overnight on a rocker. On the following day, cells were spun down and dissociated in dissociation buffer (10% horse serum [w/v], 5% FBS, 10 mM HEPES, and 1X HBSS solution). After centrifugation, cell pellets were resuspended in rinse buffer (10% horse serum, 5% FBS, 1% P/S, and Ca^{2+} free DMEM) and filtered through 40 um cell strainers. After centrifugation, cells were resuspended in cultured media (Opti-MEM, 10% horse serum, 5% FBS, and 0.1% P/S) and cultured at 37°C in a humidified incubator with 5% CO_2. To eliminate adherent nonmyocytes, floating cells were replated on laminin-coated tissue culture dishes/chamber slides after one-hour culture. To assess cell proliferation, cardiomyocytes were fixed with 4% paraformaldehyde and stained with p-H3 antibody followed by Alexa 594-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA). The immunostaining was visualized and imaged on an EVOS FL Auto 2 microscope (Thermo Fisher Scientific, Waltham, MA). The percentages of p-H3+GFP+/GFP+ or total number of GFP+ cardiomyocytes were quantified using ImageJ software.

Histology and immunohistochemistry

All embryo specimens were fixed in 4% paraformaldehyde overnight, dehydrated through an ethanol series, paraffin embedded, and sectioned (6-7 μm). Primary antibodies (Major Resources Table) were incubated at 4°C overnight and secondary antibodies (Alexa Fluo 488, 555, or 647; Thermo Fisher Scientific, Waltham, MA) were incubated at room

temperature for 1 hour. The negative controls including isotypes (for antibody specificity) and secondary antibodies alone (for distinguishing genuine target staining from background) were tested for each antibody used. The stained slides were imaged on a Leica DM6 fluorescence microscope (Wetzlar, Germany). We quantified the number of p-H3+ cells, BrdU+ cells, TUNEL+ cells, EPDCs (eYFP+), and the staining intensity of FGF9 and IGF2 by using ImageJ software.

Cell culture, transient transfection, lentiviral infection, and luciferase assay

Mouse embryonic epicardial cells (MECs) originally generated by Dr. Sucov's group[74] were purchased from Millipore (Catalogue #SCC187, Burlington, MA). MECs were cultured in 10% fetal bovine serum (FBS) supplemented DMEM at 37°C in a humidified incubator with 5% CO_2. To generate stable *Hdac3* knockout (KO) MECs, we cloned *Hdac3* sgRNA into lentiCRISPR v2 vector (Addgene #52961; Watertown, MA). sgRNA primers (targeting on *Hdac3* Exon2) were 5'-CACCGCATAGCCTAGTCCTGCATTA-3' (forward) and 5'-AAACTAATGCAGGACTAGGCTATGC-3' (reverse). 70% confluent Lenti-X^{TM} 293T cells (Takara Bio USA, Inc., Mountain View, CA) were transfected with *Hdac3* lentiviral plasmids (*Hdac3* KO or empty vector control [EV]), PsPAX2 (Addgene #12260), and PMD2G (Addgene #12259). 72 hours after transfection, supernatants containing lentivirus were collected and filtered through a 40μm cell strainer. For *Hdac3* rescue experiments in MECs, plasmids expressing human *HDAC3* and *HDAC3^{Y298H}* have been previously described[75]. miR-322 mimics, miR-503 mimics, and scramble control mimics were custom-ordered from Dharmacon (Lafayette, CO). miR-322 mimics, miR-503 mimics, or control mimics (30 pmol) were transfected to MECs using Lipofectamine

RNAiMAX (Thermo Fisher Scientific, Waltham, MA). 72 hours after miR mimics treatment, supernatants were collected. miRZip anti-miR precursor constructs for miR-322 and miR-503 cluster (miRZip-322 and miRZip-503) and pGreenPuro Scramble Hairpin control constructs were purchased from System Biosciences (Palo Alto, CA), and lentiviruses were generated according to the manufacturer's protocol. MECs were co-transfected with SV40-renilla and pGL4.10-miR-322/miR-503 promoter using lipofectamine 2000. After collecting the cell lysates, Firefly and Renilla luciferase activity were measured by VICTOR X3 Multilabel plate reader (PerkinElmer, Waltham, MA) followed by Dual-Luciferase® Reporter Assay System (Catalogue #E1910, Promega, Madison, WI).

RNA isolation, quantitative reverse transcription-polymerase reaction (qRT-PCR), and bulk RNA-Seq

MECs and E13.5 hearts were used for RNA isolation. E13.5 hearts were microdissected in cold PBS and snap frozen in liquid nitrogen. The RNeasy Plus Mini Kit (QIAGEN, Germantown, MD) was used to extract total RNAs, and cDNAs were generated using the Superscript III kit (Thermo Fisher Scientific, Waltham, MA). To detect mRNA, SYBR Green qRT-PCR was performed. PCR primers for genes of interest are listed in Major Resources Table. To perform mature microRNA expression analyses, purified total RNAs were converted to cDNAs using TaqMan™ MicroRNA Reverse Transcription Kit (Catalogue #4366596, Thermo Fisher Scientific, Waltham, MA). qRT-PCR reactions were run on StepOne Plus Real-Time PCR System (Applied Biosystems, Waltham, MA). The probe sequences for mmu-miR-322-5p, mmu-miR-503-5p, and U6 snRNA were purchased from Thermo Fisher Scientific (Waltham, MA). For bulk RNA and miRNA-

Sequencing, samples were prepared following the provider's guidelines (Novogene Corporation Inc, Sacramento, CA) and sequenced on an Illumina NextSeq500 (San Diego, CA). Sequencing reads were aligned to the UCSC mm10 reference genome using tophat2 and bowtie2 in R. Differential expression of transcripts was calculated using the cufflinks suite in R analyses.

Chromatin immunoprecipitation assay

MECs were crosslinked for 8 minutes at room temperature by adding 1% final volumes of fresh 16% formaldehyde. The crosslink was then quenched by adding 1/10 volumes of 2.5 M glycine to cells and incubating at room temperature for 5 minutes. Nuclei were prepared according to Covaris truChIP™ Chromatin shearing kit protocol and sonicated to fragments (average fragment length: 300–500 bp) using M220 focused Ultrasonicator (Covaris, Wolburn, MA). Chromatin (10 uL) was saved for whole cell extract input. anti-H3K27Ac antibody or rabbit IgG antibody was conjugated to the Dynabeads™ protein G beads (Thermo Fisher Scientific, Waltham, MA). Chromatins (15 ug) were added to the bead solution and incubated overnight at 4ºC on a rotator. Beads were then collected and washed four times in wash buffer (20 mM HEPES pH 7.9, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2% Triton X-100, and 10% Glycerol). After removing the wash buffer, crosslinking was reversed at 65°C for 2 hours in proteinase K buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% SDS, 20 mM sodium butyrate, 200 µg/ml proteinase K). DNAs were purified with phenol/chloroform/isoamyl alcohol. Using purified precipitated DNAs, enrichment of the target sequences was measured by chromatin immunoprecipitation (ChIP)-qPCR using primers (Major Resources Table) designed against the murine miR-322/miR-503 promoter regions (-1.7kb to +1bp).

Western blotting

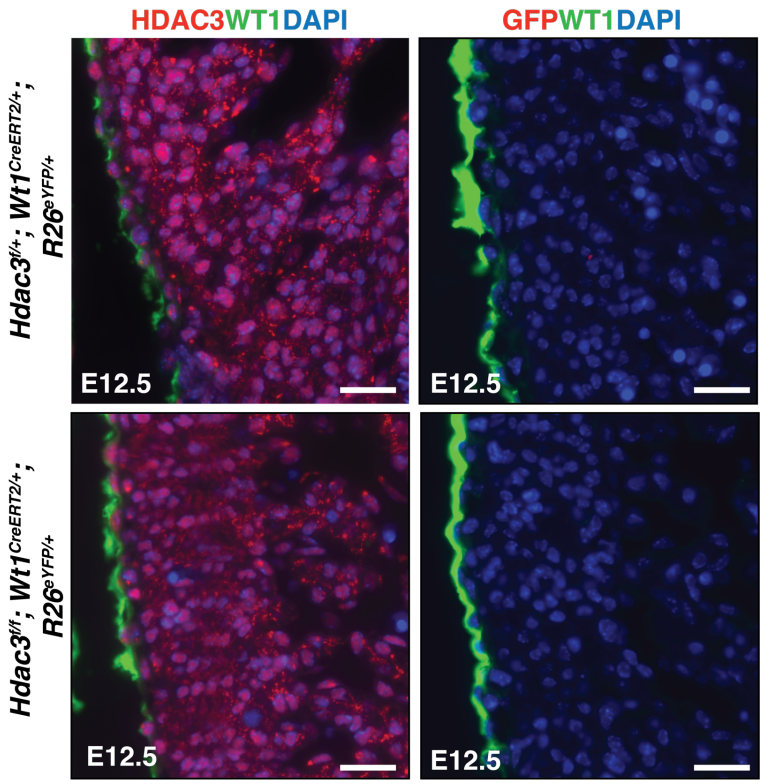
Cell or tissue lysates were prepared in lysis buffer (20 mm Tris-HCl [pH 7.5], 15 mm NaCl, 1 mm Na_2EDTA, 1 mm EGTA, 1% Triton X-100, 1 μg/ml leupeptin, 2.5 mm sodium pyrophosphate, 1 mm Na_3VO_4, and 1 mm β-glycerophosphate) with protease inhibitor cocktail (Roche, Indianapolis, IN) and 1 mM phenylmethylsulfonyl fluoride. Protein samples were resolved on 4–12% SDS-PAGE acrylamide gel before transferring to polyvinylidene fluoride membranes. Primary antibodies were visualized by chemiluminescence using HRP-conjugated secondary antibodies. Western blots were normalized to loading controls and densitometric analysis was performed using Image J software.

Statistical analysis

All experiments were independently repeated at least three times, and the number of samples (n) is stated in figure legends. Results are reported as the mean ± SD. For statistical analyses, normal distribution of data was assessed by Shapiro-Wilk's test (when $n \geq 6$). For data with small sample size ($n < 6$), non-parametric tests were applied. For parametric data with normal distribution, the statistical significance of the difference between means was assessed using the unpaired two tailed Student *t* test between two groups and the Tukey or Dunnett post hoc test for 1-way ANOVA. For non-parametric data or data with small sample size ($n < 6$), the Mann-Whitney U test and Kruskal-Wallis test for 1-way ANOVA followed by the Dunn post hoc test were performed for

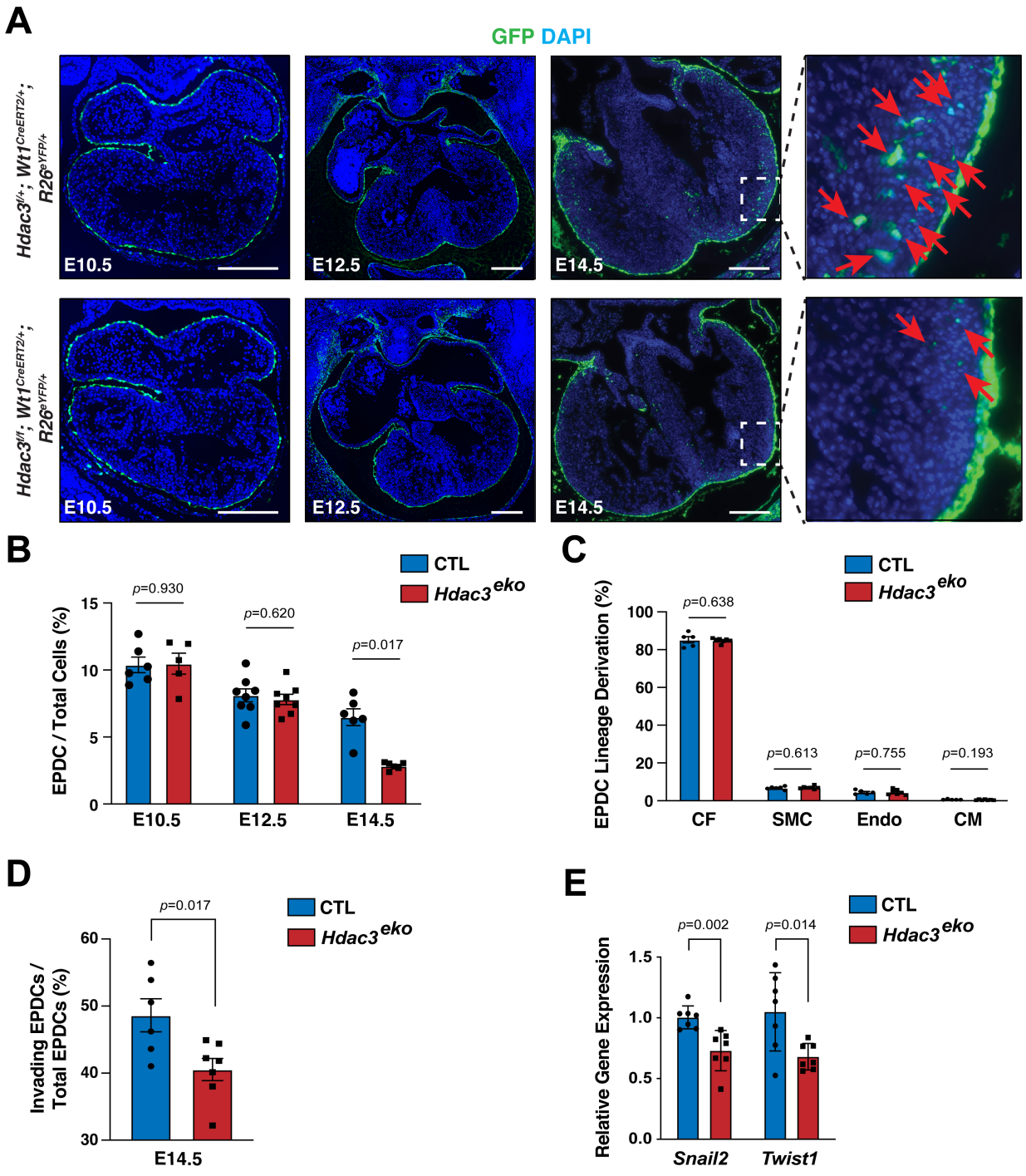
comparisons between two groups. Specific statistical tests are stated in figure legends. The RNA sequencing data were background-corrected, variance-stabilized, normalized and count matrix extracted through quality control using lumi package for the R programming language (http://bioconductor.org/packages/release/bioc/html/lumi.html), and deposited in the NCBI GEO database repository (https://www.ncbi.nlm.nih.gov/gds) and the access number is 22753645. Differential expression analysis was performed using DESeq2 R package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html)[76]. The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes or miRs with an adjusted *p*-value<0.05 found by DESeq2 were assigned as differentially expressed. Gene ontology (GO) enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources (https://david.ncifcrf.gov/). For relative gene expression analysis of qRT-PCR data, the expression of a gene of interest relative to an internal loading control gene was calculated using the comparative CT method (-deltadelta CT)[77]. The relative protein expression on western blot data was quantified by densitometry, The fold changes for relative gene or protein expression were derived through division of values in the experimental group by the mean values from the control group. A *p*-value<0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism 8 (GraphPad Prism software, Inc). The images with the value closest to the mean value were selected as representative images.

Online Figures



Online Figure S1. No leakage of *Wt1*$^{CreERT2/+}$.

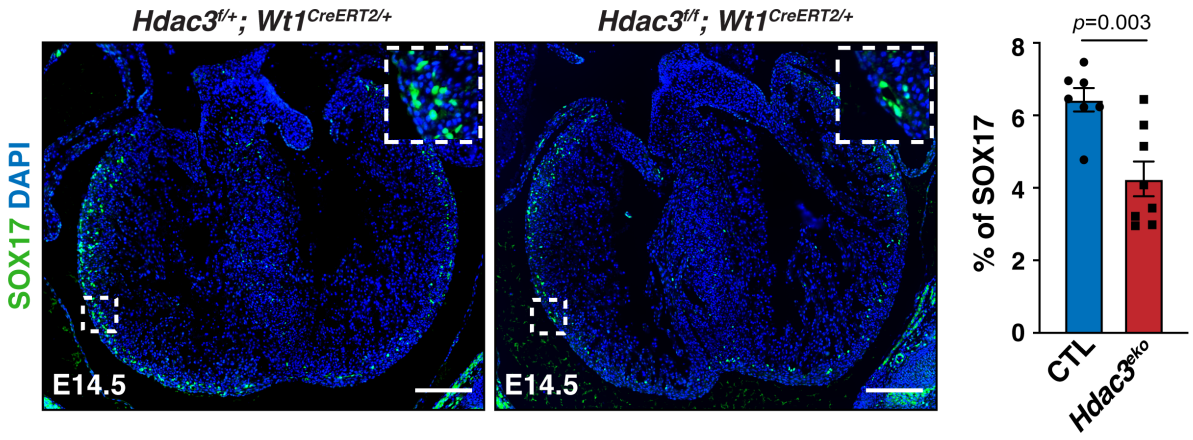
Representative micrographs of HDAC3 and GFP immunofluorescence staining of E12.5 *Hdac3*$^{f/+}$*; Wt1*$^{CreERT2/+}$*; R26R*$^{eYFP/+}$ and *Hdac3*$^{f/f}$*; Wt1*$^{CreERT2/+}$*; R26R*$^{eYFP/+}$ hearts. Corn oil was given to dams intraperitoneally (150 mg/kg body weight) at E8.5. Scale bars: 25 μm. eYFP immunosignal was detected by a GFP antibody. In the absence of tamoxifen administration, there was neither *Hdac3* deletion nor eYFP reporter activity in the epicardium.



Online Figure S2. Reduction of EPDCs in *Hdac3^{eko}* hearts.

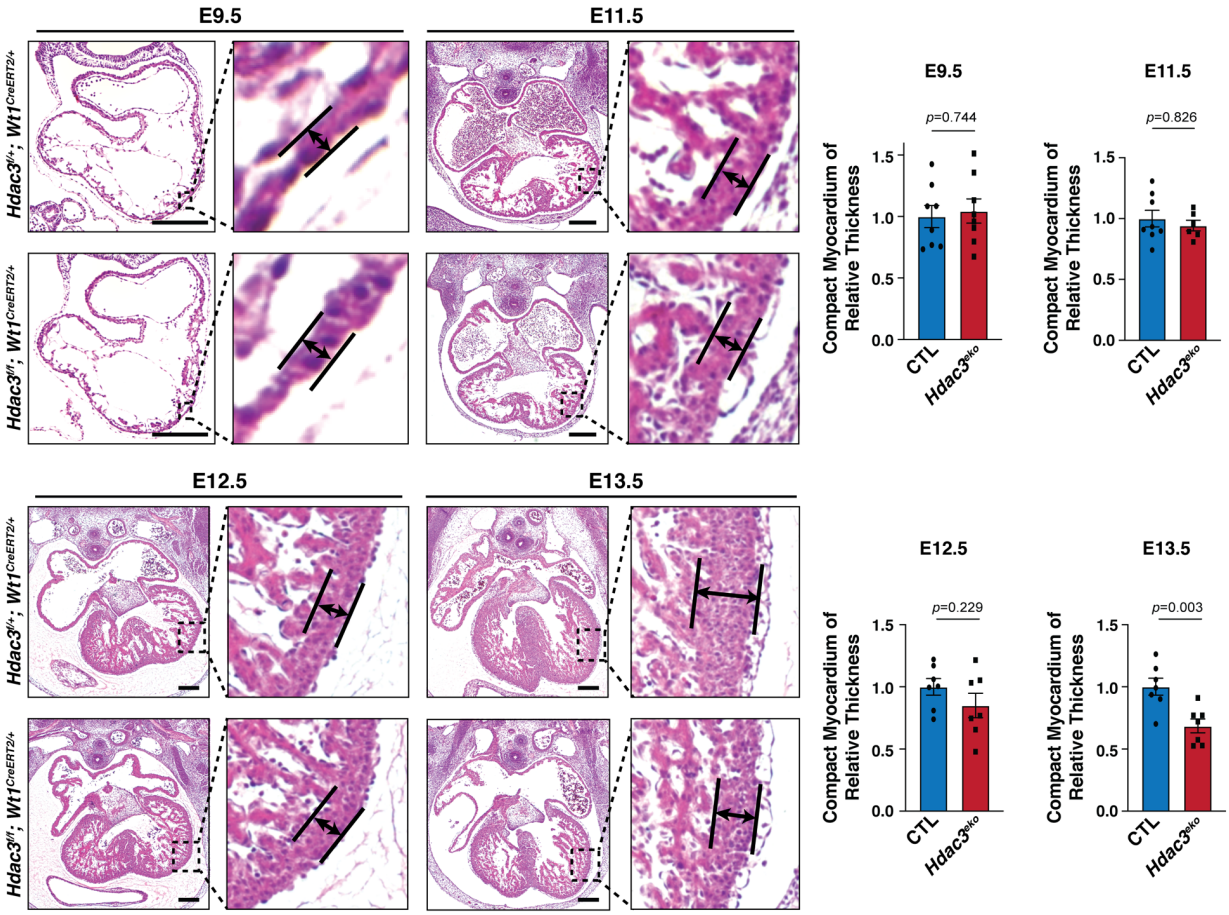
(A) Representative micrographs of GFP immunofluorescence staining of E10.5, E12.5 and E14.5 *Hdac3$^{f/+}$; Wt1$^{CreERT2/+}$; R26R$^{eYFP/+}$* (control [CTL]) and *Hdac3$^{f/f}$; Wt1$^{CreERT2/+}$; R26R$^{eYFP/+}$* (*Hdac3^{eko}*) hearts. Scale bars: 250 µm. **(B)** Quantifications of EPDCs DCs at

E10.5, E12.5 and E14.5 are shown on the lower left. CTL: n=6 (E10.5), n=8 (E12.5) and n=6 (E14.5); *Hdac3*^{eko}: n=5 (E10.5), n=8 (E12.5) and n=7 (E14.5). *P*-values were determined by the Mann-Whitney test (E10.5) and unpaired two tailed Student *t* test (E12.5 and E14.5). **(C)** Quantifications of percentage of derivation percentage of each cell type in E14.5 *Hdac3*^{eko} and CTL hearts. CTL: n=5 (CF, Endo and CM) and n=6 (SMC); *Hdac3*^{eko}: n=7 in each group. *P*-values were determined by the Mann-Whitney test (CF, Endo and CM) and unpaired two tailed Student *t* test (SMC). CF, cardiac fibroblast (Vimentin+); SMC, smooth muscle cells (smMHC11+); Endo, endothelial cells (CD31+); CM, cardiomyocytes (ACTC1+). **(D)** Quantification of invading EPDCs in E14.5 *Hdac3*^{eko} and CTL hearts. CTL: n=6, *Hdac3*^{eko}: n=7. *P*-values were determined by unpaired two tailed Student *t* test. **(E)** Quantification of the expression of *Snail2* and *Twist1* in E13.5 *Hdac3*^{eko} and CTL hearts by qRT-PCR. n=7 in each group. *P*-values were determined by unpaired two tailed Student *t* test.



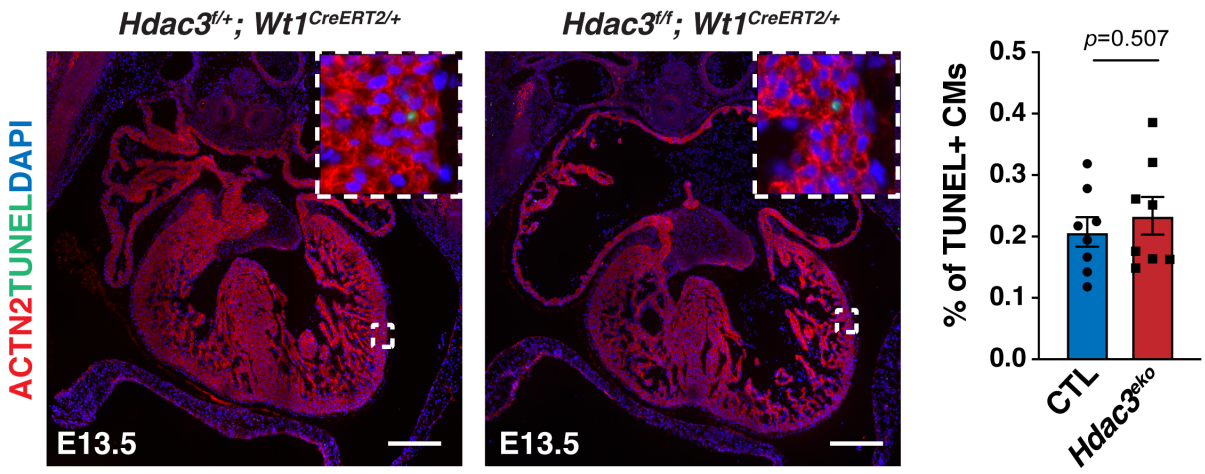
Online Figure S3. Reduction of coronary vascular cells in *Hdac3^{eko}* hearts.

Representative micrographs of SOX17 immunofluorescence staining of E14.5 *Hdac3$^{f/f}$; Wt1$^{CreERT2/+}$* (*Hdac3^{eko}*) and *Hdac3$^{f/+}$; Wt1$^{CreERT2/+}$* (CTL) hearts. Scale bars: 200 μm. Quantification of SOX17+ cells is shown on the right. CTL: n=7, *Hdac3^{eko}*: n=8. *P*-values were determined by unpaired two tailed Student *t* test.



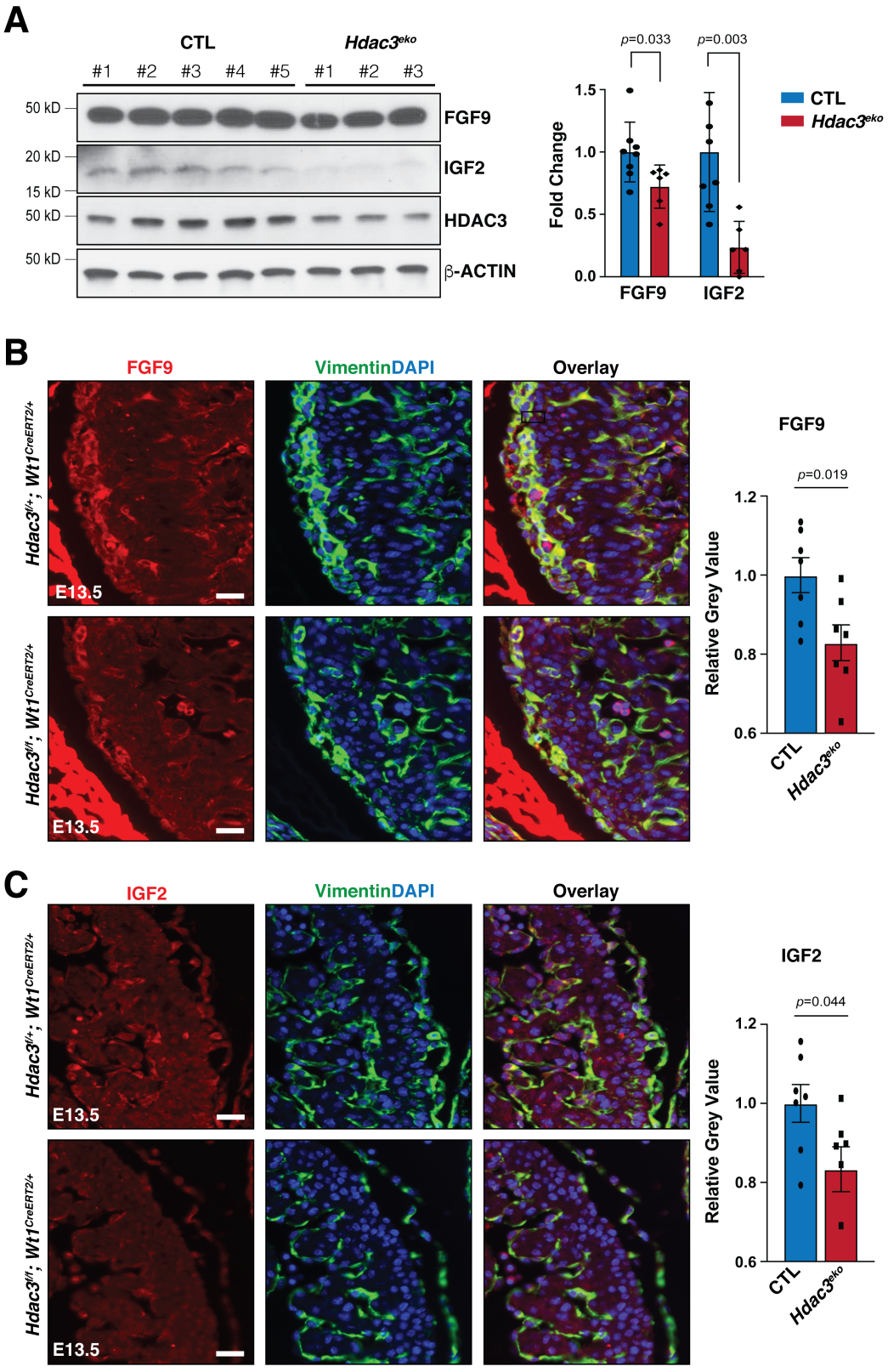
Online Figure S4. Ventricular wall morphogenesis in *Hdac3*^{eko} hearts.

Representative hematoxylin and eosin staining of *Hdac3*$^{f/f}$*; Wt1*$^{CreERT2/+}$ (*Hdac3*^{eko}) and *Hdac3*$^{f/+}$*; Wt1*$^{CreERT2/+}$ (CTL) hearts at E9.5, E11.5, E12.5 and E13.5. Scale bars: 200 μm. Quantifications are shown on the right. CTL: n=8 (E9.5), n=6 (E11.5), n=7 (E12.5) and n=7 (E13.5); *Hdac3*^{eko}: n=8 (E9.5), n=8 (E11.5), n=7 (E12.5) and n=7 (E13.5). The relative thickness of compact wall for each sample was derived by dividing its actual measured thickness by the mean measured thickness value of the CTL group. *P*-values were determined by unpaired two tailed Student *t* test.



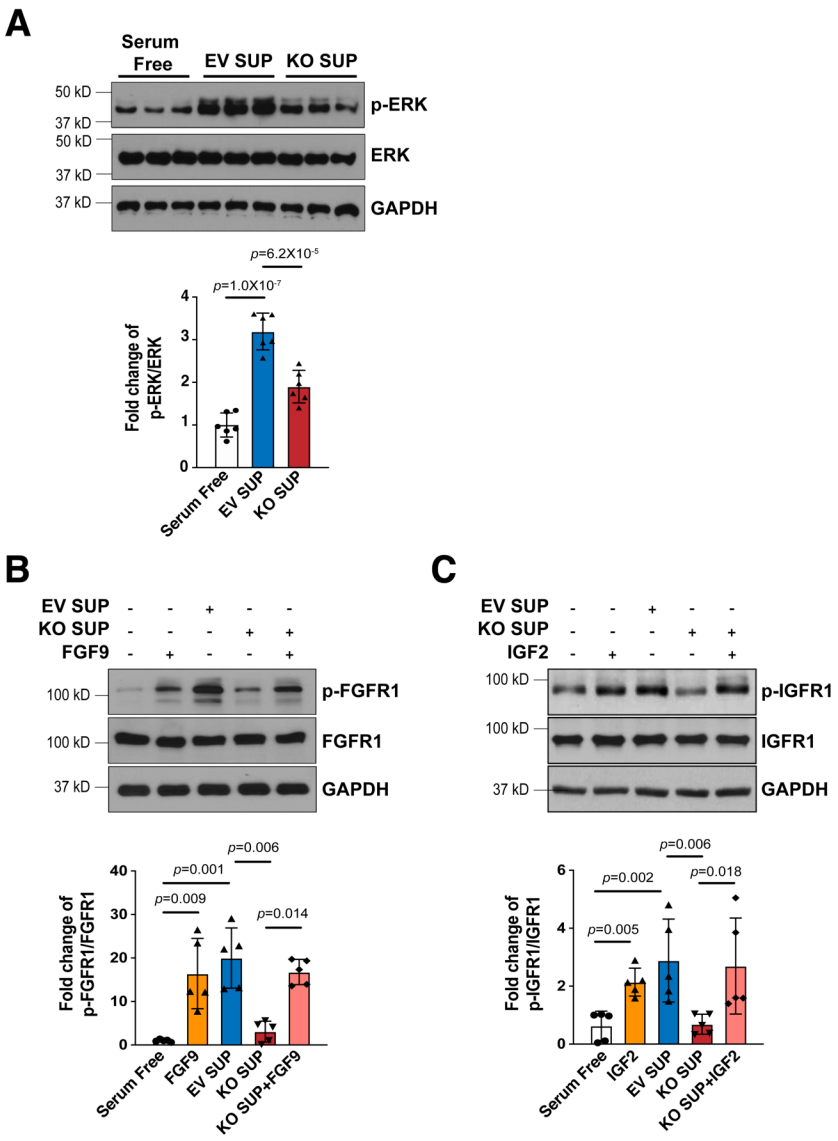
Online Figure S5. Cell death in *Hdac3*^{eko} hearts.

Representative micrographs of TUNEL immunofluorescence staining of E13.5 *Hdac3*$^{f/f}$*; Wt1*$^{CreERT2/+}$ (*Hdac3*^{eko}) and *Hdac3*$^{f/+}$*; Wt1*$^{CreERT2/+}$ (CTL) and hearts. TUNEL+ signals are in green. Scale bars: 250 μm. Quantification of TUNEL+ cardiomyocytes (CMs) is shown on the right. n=8 in each group. *P*-values were determined by unpaired two tailed Student *t* test.



Online Figure S6. Reduced expression of FGF9 and IGF2 in *Hdac3*^{eko} hearts.

(A) Quantification of FGF9 and IGF2 in E13.5 *Hdac3*$^{f/f}$*; Wt1*$^{CreERT2/+}$*; R26R*$^{eYFP/+}$ and (*Hdac3*^{eko}) *Hdac3*$^{f/+}$*; Wt1*$^{CreERT2/+}$*; R26R*$^{eYFP/+}$ (CTL) hearts by western blot. CTL: n=8, *Hdac3*^{eko}: n=6. **(B and C)** Representative immunofluorescence staining of FGF9 and IGF2 on E13.5 *Hdac3*^{eko} and CTL hearts. Vimentin was used to mark cardiac fibroblasts, cardiac endothelial cells and the epicardium. Scale bars: 25 μm. Quantifications of immunofluorescence intensity of FGF9 and IGF2 are shown on the right. n=7 in each group. *P*-values were determined by unpaired two tailed Student *t* test for **(A)**, **(B)** and **(C)**.



Online Figure S7. The downstream signaling of FGF9 or IGF2 in cultured cardiomyocytes.

Representative western blots of **(A)** p-ERK, **(B)** p-FGFR1, or **(C)** p-IGFR1 in serum-starved cultured E13.5 cardiomyocytes after treatment of MEC supernatants and/or mouse recombinant FGF9 or IGF2 proteins (Final concentration: 100 ng/ml). Quantifications are shown below. **(A),** n=6 in each group; **(B)** and **(C)**, n=5 in each group. *P*-values were determined by 1-way ANOVA followed by the Tukey post hoc test for **(A)**, Kruskal-Wallis test followed by the Dunn post hoc test for **(B)** and **(C)**.

Full unedited gels for

Figure 2A

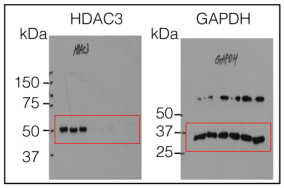


Figure 2E

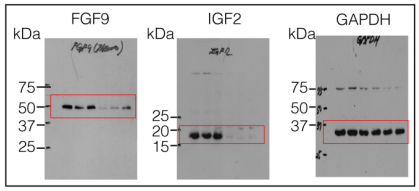


Figure 3A

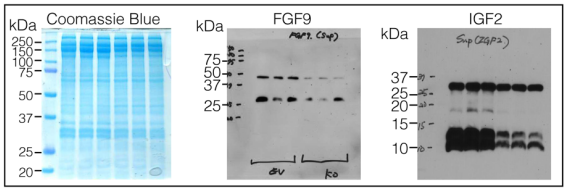


Figure 4B

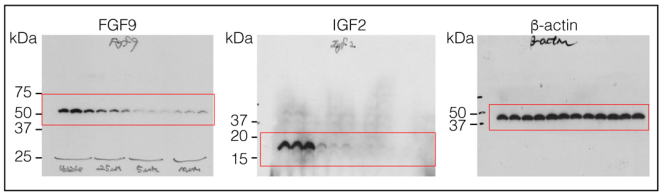


Figure 4C

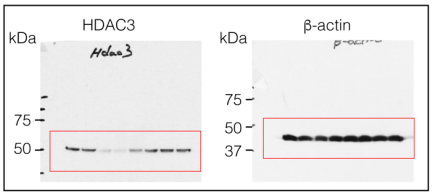


Figure 5D (Left)

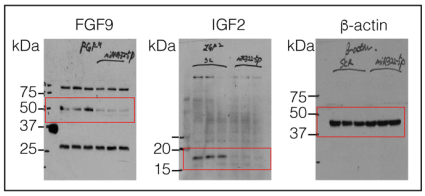


Figure 5D (Right)

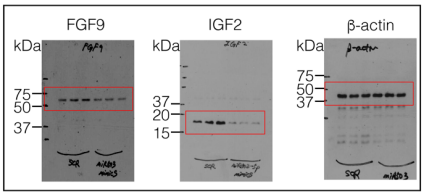


Figure 6B

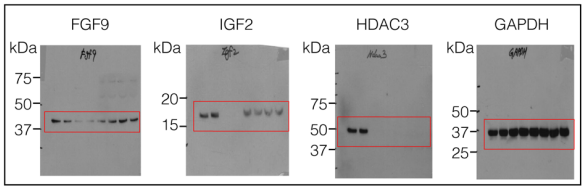
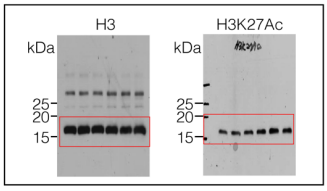
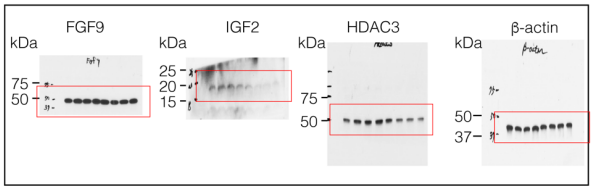


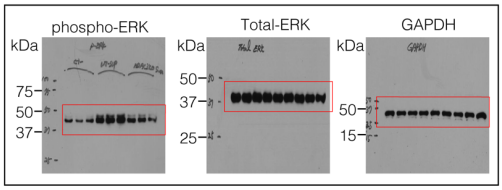
Figure 7A



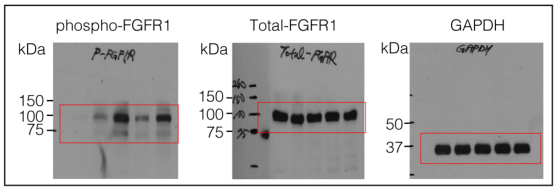
Supplemental Figure 6



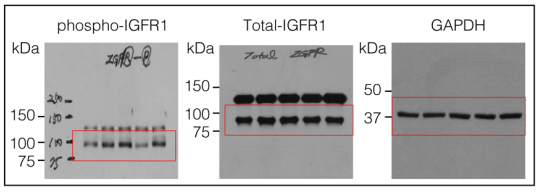
Supplemental Figure 7A



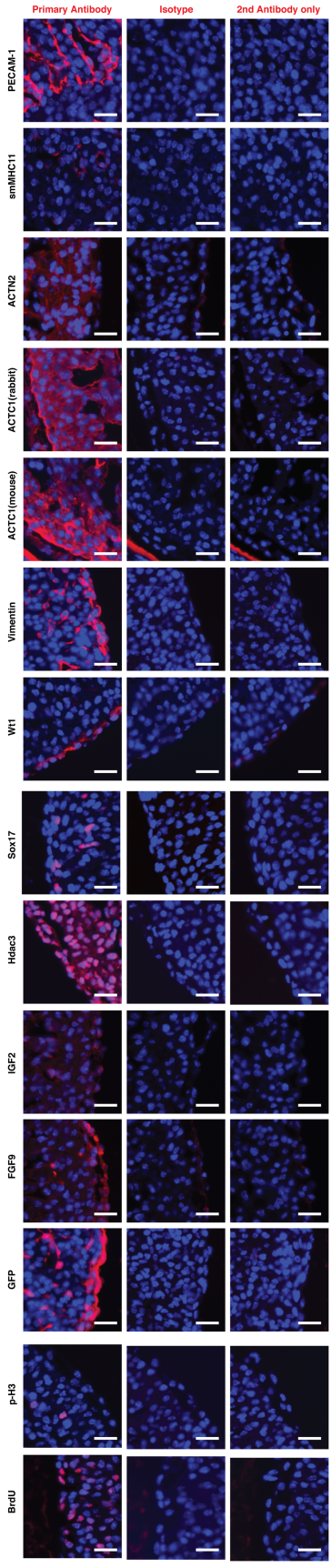
Supplemental Figure 7B (Left)



Supplemental Figure 7B (Right)



Online Figure S8. Documentation of full scans of Western blots.


Primary Antibody
Isotype
2nd Antibody only
PECAM-1
smMHC11
ACTN2
ACTC1(rabbit)
ACTC1(mouse)
Vimentin
Wt1
Sox17
Hdac3
IGF2
FGF9
GFP
p-H3
BrdU

Online Figure S9. Immunofluorescence staining with various antibodies along with their corresponding isotype and secondary antibody only controls. Scale bars: 25 um.