SUPPLEMENTAL MATERIAL

Supplemental Methods

Mice

Cardiomyocyte (CM)-specific *Prdm16* knockout mice were created by crossing a mouse line carrying conditional allele of *Prdm16* (*Prdm16*$^{fl/fl}$, The Jackson Laboratory, #024992)[34] and *Xmlc2-Cre* mice[31] or *cTnT-Cre* mice[30]. Mice carrying *Rosa26-tdTomato* reporter transgene[68] was crossed with *Prdm16*$^{fl/fl}$ mice to obtain *Prdm16*$^{fl/fl}$::*Rosa26-tdTomato*$^{+/+}$ mice, and then crossed with *Prdm16*$^{fl/+}$::*Xmlc2-Cre*$^{+/-}$ mice to label CMs with tdTomato fluorescence. *αMHC-MerCreMer* mice (The Jackson Laboratory, #005657)[40] were crossed with *Prdm16*$^{fl/fl}$ mice to generate inducible CM-specific *Prdm16* knockout mice (*Prdm16*^{icKO}). All mice were of C57BL/6NCrl or C57BL/6J background. Genotypes of mice were confirmed by polymerase chain reaction (PCR) analysis using embryonic yolk sac or tail extracts and *Prdm16*$^{fl/fl}$ primers (forward: 5'-TGCAGGGAGATTGACAAGTG-3', reverse: 5'-GTGGTTCCCTTGCACTGATT-3'), *Cre* primers (forward: 5'-GTTCGCAAGAACCTGATGGACA-3', reverse: 5'-CTAGAGCCTGTTTTGCACGTTC-3'), or Rosa26-tdTomato primers (WT-forward: 5'-AAGGGAGCTGCAGTGGAGTA-3', WT-reverse: 5'- CCGAAAATCTGTGGGAAGTC-3', tdTomato-forward: 5'- CTGTTCCTGTACGGCATGG-3', tdTomato-reverse: 5'-GGCATTAAAGCAGCGTATCC-3'). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of California San Diego with approved protocol # S01049.

Echocardiography

Neonatal mice were gently held in place on a heated platform. Echocardiography was then performed by using a VisualSonics Vevo 2100 ultrasound system (FUJIFILM) with a linear transducer 32-55MHz. Percentage fractional shortening (%FS) was used as an indicator of systolic cardiac function. Measurements of left ventricular (LV) internal diameter at end-diastole (LVIDd) and LV internal diameter at end-systole (LVIDs) were determined from the M-mode images.

Western blots

Mouse hearts were dissected and snap-frozen in liquid nitrogen. Total protein extracts were prepared by homogenization of hearts in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM Sodium Chloride, 1% NP-40, 0.1% SDS, 0.5% Sodium Deoxycholate, 1 mM EDTA) using a handheld pellet pestle (Sigma-Aldrich, for embryonic or postnatal mouse hearts) or a table-top homogenizer (Fisher Scientific, for adult hearts). Protein concentration was determined using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Tissue lysate was mixed with 4x LDS sample buffer and 10x Reducing Agent (Life Technologies) and incubated for 10 minutes at 70°C. Protein samples were separated on Bolt 4%-12% Bis-Tris gels or NuPAGE 3%-8% Tris-Acetate gels (Life Technologies) and transferred to PVDF membrane (Bio-Rad). Membranes were blocked in Tris Buffered Saline with 0.2% Tween-20 (TBST) supplemented with 5% BSA for 1 hour at room temperature (RT), and incubated with primary antibodies overnight at 4°C. Membranes were then washed with TBST and incubated with HRP-conjugated secondary antibodies for one hour before immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL) reagent (Bio-Rad) and captured by Bio-Rad ChemiDoc Imaging System. Catalogue numbers for antibodies used in western blots in this study: PRDM16, AF6295 (R&D systems); Tbx5, 42-6500 (Invitrogen); GAPDH, sc-32233 (Santa Cruz Biotechnology).

Histology, Immunofluorescence and EdU Labelling

Histology and immunofluorescence were performed as previously described[39]. Embryonic or postnatal mouse hearts were dissected at various developmental stages and fixed in ice-cold PBS with 4% PFA overnight at 4°C. Fixed hearts were then saturated in 5%, 10%, 15%, 20% sucrose in PBS, embedded in OCT Tissue-Tek (Thermo Fisher Scientific), and cut to 6 μm sections using a Leica CM 3050S cryostat (Leica Microsystems). For **histology**, sections were then stained with Hematoxylin and Eosin (H&E) using a standard protocol. Images were captured using a Hamamatsu NanoZoomer 2.0HT Slide Scanning System. For **immunofluorescence**, sections were blocked with PBST (1% BSA, 0.2% Tween-20 in PBS) for one hour, and then incubated with primary antibody solution (antibodies diluted in PBST supplemented with 5% donkey serum) overnight in a humidified chamber at 4°C. The next day, sections were washed three times with PBST and then incubated with secondary antibody solution (fluorescence-conjugated secondary antibodies diluted in PBST supplemented with 5% donkey serum) for 2 hours at RT. After washing with PBST three more times, sections were counterstained with DAPI and mounted in DAKO fluorescence mounting medium (Agilent). Images were captured using an Olympus FluoView FV1000 Confocal Microscope. For **EdU labelling** assay, pregnant female mice were given intraperitoneal injection of EdU (5-ethynyl-20-deoxyuridine, Invitrogen) two hours prior to embryo dissection. After cryosectioning of embryonic heart, EdU positive cells were detected with Click-iT assay (Invitrogen) using Alexa Fluor 647 azide according to the manufacturer's instructions, which was followed by primary and secondary antibody incubation per immunofluorescence procedures. Images were captured using an Olympus FluoView FV1000 Confocal Microscope. Catalogue numbers or sources for antibodies used in immunofluorescence

in this study: PRDM16 (Patrick Seale lab); Nkx2-5, sc-8697 (Santa Cruz Biotechnology); CD31, 550274 (BD Biosciences); Sox3, GTX129235 (GeneTex).

Fluorescence *In Situ* Hybridization (FISH)

Embryonic Day (E) 13.5 embryonic mouse heart sections were prepared using procedures described in '**Histology, Immunofluorescence and EdU Labelling'**. Procedures for FISH experiments were adapted from the protocol provided by Advanced Cell Diagnostics, Inc (ACD) using the RNAscope Multiplex Fluorescent Reagent Kit v2 (323100, ACD). Briefly, the slides were fixed in 4% PFA for 30 min and dehydrated in series of 50%, 70% and 100% ethanol gradients for 5 min each at RT, followed by Hydrogen Peroxide incubation for 10 min at RT and 1X Target Retrieval Reagent for 5 min at 98°C. Slides were then washed in distilled water, dehydrated in 100 % ethanol for 3 min, and air-dried. Slides were subsequently treated with Protease III solution for 30 min at 40°C in HybEZ Oven (321711, ACD). Following a washing step, slides were covered with probes and incubated for 2 hrs at 40°C. For signal amplification, slides were consecutively incubated in AMP 1, AMP 2 and AMP 3 reagents for 30 min at 40°C. The slides were then incubated with HRP-C1 for 15 min at 40°C, fluorophore for 15 min at 40°C and HRP blocker for 15 min at 40°C. The same steps were repeated twice for HRP-C2 and HRP-C3, respectively. Mounting media and cover slips were then added to slides for imaging. Images were captured using an ECHO Revolve microscope. Catalogue numbers for RNAscope probes used in this study: *Tbx5*, 519581-C2; *Mlc2a/Myl7*, 584271-C3; *Tbx20*, 511991-C2; *Hey2*, 404651-C2; *Mycn*, 477151; Nppa, 418691-C3; *Prdm16*, 584281-C3; *Cxcl12*, 422711-C3; *Hand1*, 429651-C2; *Tnnt1*, 466911-C3; *Thbs4*, 526821; *Tnni2*, 1065311-C1; *Cttnbp2*, 1065321-C2; *Acta1*, 808831-C3; *Nkx2-5*, 428241.

Quantitative Real-Time PCR

Left or right ventricles of E13.5 mouse hearts were isolated and snap-frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent per manufacturer's instructions (Life Technologies). cDNA was synthesized using SuperScript III reverse transcriptase (Life Technologies). Quantitative RT-PCR was performed on a CFX96 Real-Time System using iTaq Universal SYBR Green Supermix (Bio-Rad). *18S* rRNA was used as an internal control. Primer sequences for qRT-PCR are listed below:

Hey2-F: 5'- GCGTCGGGATCGAATAAATA-3'

Hey2-R: 5'- CCTGTAGCCTGGAGCATCTT-3'

Mycn-F: 5'- AGCACCTCCGGAGAGGATAC-3'

Mycn-R: 5'- ACGCACAGTGATCGTGAAAG-3'

18S-F: 5'- GGAAGGGCACCACCAGGAGT-3'

18S-R: 5'- TGCAGCCCCGGACATCTAAG-3'

RNA Sequencing

E13.5 embryonic hearts or isolated ventricles were homogenized in TRIzol (Invitrogen) and total RNA was isolated according to the manufacturer's instructions. The concentration and quality of purified RNA was assessed by TapeStation (Agilent). RNA integrity numbers (RIN) were in the range of 6.1 to 10. cDNA libraries were prepared using an Illumina TruSeq stranded mRNA kit according to manufacturer's instructions. Libraries were sequenced with an Illumina HiSeq 4000 sequencer to a sequencing depth of 20-70 million reads per sample.

Chromatin Immunoprecipitation and Sequencing

The procedures for PRDM16 ChIP-seq were modified from a previously reported protocol[25]. Separate left ventricles, right ventricles or whole ventricles (atria removed) of E13.5 mouse hearts were dissected and tear to smaller pieces by forceps. Tissues were then dual crosslinked by incubating in 1.5 mM EGS (ethylene glycol bis[succinimidyl succinate]) solution (Thermo Scientific) for 20 min at RT with rotation and then 1% formaldehyde plus 1.5 mM EGS for an additional 10 min at RT. Crosslinking was quenched by adding glycine to a final concentration of 125 mM and rotating for 5 min at RT. Tissues were then washed with 1X PBS and snap frozen in liquid nitrogen and stored at -80˚C. For chromatin shearing, tissues were resuspended in 300 µl lysis buffer (50 mM Tris-Cl pH 7.8, 10 mM EDTA, 1% SDS) and homogenized with a glass dounce homogenizer on ice. Chromatin shearing was performed in Bioruptor Pico (Diagenode) for 5-10 cycles (each cycle included 30 sec on and 30 sec off) at 4 ˚C. The sheared chromatin was cleared by spinning at 12, 000g for 10 min and transferred to a clean tube. For assessing chromatin shearing efficiency, 25 µl sheared chromatin was treated with RNase cocktail (Invitrogen) for 30 min at 37 ˚C and proteinase K (Thermo Scientific) at 65 ˚C for 3 hrs. DNA was purified by Qiagen QIAquick PCR purification kit and run on 1.5% agarose gel to determine the size of sheared DNA, which was kept within the range of 100-500 bp. After setting 5 µl aside for input control samples, the sheared chromatin was then diluted 10 times with dilution buffer (20 mM Tris-Cl pH 7.8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) and incubated with 5 µg sheep polyclonal anti-PRDM16 antibody (R&D Systems, #AF6295) overnight at 4 ˚C with rotation. Next day, 25 µl protein G dynabeads (Life Technologies) were washed twice with dilution buffer, added to the chromatin solution, and incubated 4 hrs at 4 ˚C with rotation. The beads were then washed once with TSE1 buffer (20 mM Tris-Cl pH 7.8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1%

SDS), three times with TSE2 buffer (20 mM Tris-Cl pH 7.8, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), once with LiCl buffer (10 mM Tris-Cl pH 7.8, 250 mM LiCl, 1 mM EDTA, 1% sodium deoxycholate, 1% IGEPAL) and twice with TE buffer (10 mM Tris-Cl pH 7.8, 1 mM EDTA). Each wash was 5 min at 4 ˚C with rotation. To elute protein/DNA complexes, beads were resuspended in pre-warmed 100 µl ChIP elution buffer (100 mM $NaHCO_3$, 1% SDS) and incubate at 65 ˚C for 30 min at a thermomixer (Eppendorf). The elution was repeated with another 100 µl ChIP elution buffer and combined. Input control samples were diluted with ChIP elution buffer to a final volume of 200 µl. Both ChIP eluate and input samples were treated with 1 µl RNase cocktail (Invitrogen) at 37 ˚C for 30 min and subsequently added with 8 µl 5 M NaCl to enhance the separation of DNA and Protein. 2 µl Proteinase K (20 mg/mL, Thermo Scientific) was then added to the solution and incubated at 50°C for 1 hr then 65°C overnight at a thermomixer. Next day, ChIP or input DNA were purified with a Qiagen QIAquick PCR Purification Kit and eluted twice with 30 µl pre-warmed buffer EB.

For library preparation, ChIP or input DNA were end repaired, ligated with barcoded adaptors, amplified and purified using Kapa HyperPrep PCR-free Kit and Kapa Real Time Amplification Kit. DNA fragments in the range of 200-500 bp were selected using a PippinHT Size Selection System. Libraries were sequenced in an Illumina HiSeq 4000 sequencer to a sequencing depth of ~40 million reads per sample.

Single Cell RNA sequencing

Two control and two *Prdm16*^{cKO} samples were used for scRNA-seq. Each sample consists of 3-4 E13.5 mouse hearts with atria removed. Tissue samples were digested with 0.25% trypsin (Gibco) at 37 ˚C for 30 min on a thermomixer and gently dispersed into single-cell suspension with

pipetting. The reaction was then quenched by adding DMEM containing 10% fetal bovine serum (FBS). Cells were filtered with a 40 μm cell strainer, spun down at 370 g and resuspended in DMEM containing 2% FBS. Subsequently, cells were subjected to fluorescence-activated cell sorting (FACS) in a BD Influx Cell Sorter or a BD FACSAria Fusion Cell Sorter, to select for live cardiomyocytes which had positive tdTomato fluorescence and negative DAPI staining. Sorted live cardiomyocytes were then captured with Chromium Next GEM Single Cell 3′ Reagent Kits v3.1 (10X Genomics) following manufacturer's instructions. Briefly, single cells were partitioned into nanoliter-scale Gel Beads-in-emulsion (GEMs) using Chromium Next GEM Chip G in the chromium controller. Immediately following GEM generation, the Gel Bead was dissolved, primers were released, and co-partitioned cell was lysed, and its mRNA were reverse transcribed into barcoded cDNA. After further cleanup and amplification, the cDNA was enzymatically fragmented and amplified via PCR to generate sufficient mass for library construction. After end repair, A-tailing, adaptor ligation and sample index PCR, the library comprised constructs containing the sample index, UMI sequences, barcode sequences, and Illumina standard sequencing primers P5 and P7 at both ends. The library was sequenced with Illumina NextSeq S4 platform to a sequencing depth of 140-270 million reads per sample.

Spatial Transcriptomics

Two pairs of *Prdm16*^{cKO}/control E13.5 embryonic mouse hearts and two pairs of *Prdm16*^{cKO}/control E15.5 embryonic mouse hearts were dissected and saturated in 5%, 10%, 15%, 20% sucrose in PBS, embedded in OCT Tissue-Tek (Thermo Fisher Scientific), and cut to 6 μm sections using a Leica CM 3050S cryostat. Sections were directly placed on capture areas of Visium Spatial Gene Expression Slide (10X Genomics) inside the cryostat chamber. Each of the

four capture areas was placed with one *Prdm16*^{cKO} section and one littermate control section, from one of the 2 pairs of E13.5 or the 2 pairs of E15.5 mouse hearts. After fixation, H&E staining and image capture, sections were incubated with permeabilization enzyme. Optimal tissue permeabilization time was pre-determined to be 8 min for both E13.5 and E15.5 heart sections using Visium Spatial Tissue Optimization Kit (10X Genomics). After permeabilization, mRNA was released from overlying cells and captured by primers on the gene expression spot (~5,000 per capture area). Reverse transcriptase mix was then added to the permeabilized tissue sections and produced spatially barcoded cDNA. Following second strand synthesis and denaturation, the barcoded cDNA was released from capture area and amplified to generate sufficient mass for library construction. After end repair, A-tailing, adaptor ligation and sample index PCR, the library comprised constructs containing the sample index, UMI sequences, spatial barcode sequences, and Illumina standard sequencing primers P5 and P7 at each end. The library was sequenced with Illumina NextSeq S4 platform to a sequencing depth of 80-100 million reads per sample.

RNA-Seq Analysis

Sequencing reads were mapped to GENCODE mouse transcripts reference (release M22, GRCm38.p6) and transcription levels were quantified using salmon (version: 1.2.1, parameters: -l A --validateMappings --seqBias --gcBias). Subsequently, gene expression levels were generated using tximport (version: 1.10.1) and differential expression analysis was carried out using DEseq2 (version: 1.22.2) in R. Benjamini-Hochberg correction for multiple testing was applied to correct p-value of each gene as FDR, and used FDR < 0.05 as a threshold for differentially-expressed genes (DEGs). Lists of downregulated DEGs and upregulated DEGs were separately examined for statistical enrichment of gene ontology (GO) terms and biological pathways in Toppgene

(https://toppgene.cchmc.org). Redundant GO terms were removed by REVIGO (http://revigo.irb.hr).

ChIP-seq Analysis

Sequencing reads were aligned to the mouse genome (GRCm38, mm10) using bwa (version: 0.7.12-r1039) and peaks were identified using MACS2 (version: 2.1.1.20160309, default parameters). Subsequently, motif analysis was carried out using Homer and differential binding analysis was calculated using DESeq2.

scRNA-seq and Spatial Transcriptomics Analysis

Single cell transcriptomic reads and spatial transcriptomic reads were processed using Cell Ranger (version: 4.0.0, 10x Genomics) and Space Ranger (version: 1.2.0, 10x Genomics) respectively. Further analysis was performed using Seurat (version: 3.2.0) on high quality cells with less than twenty percent of mitochondrial contaminations and larger than 2500 measured genes. To sufficiently separate cell populations, high resolution graph-based clustering was carried out on 30 principal components followed by UMAP dimension reduction, generating 53 subclusters. Clusters were further assembled by expression pattern of marker genes, which were identified from spatial transcriptomics data using Loupe Browser (version 5.0.0, 10x Genomics). For compact or trabecular myocardium marker genes, most enriched genes of spatial clusters mapped to compact or trabecular myocardium were calculated using 'Up-Regulated Genes Per Cluster' functionality of Loupe Browser; For left ventricle (LV) or right ventricle (RV) marker genes, ST spots belong to LV or RV were manually assigned to LV or RV clusters and generated most enriched genes with 'Up-Regulated Genes Per Cluster' function. Seurat and Loupe Browser software applied

Benjamini-Hochberg correction for multiple testing to correct p-values (FDR). The FDR of top enriched genes are usually several orders of magnitude below the FDR < 0.01 threshold for statistical significance. Next, regulators of expression network were inferred using SCENIC (version: 1.1.2.1).

Quantification and Statistical Analysis

Left ventricular, right ventricular and IVS thickness were measured with Nanozoomer Digital Pathology (NDP) view software (Hamamatsu). LV or RV thickness was measured from the base of trabeculae to epicardium. For an individual heart, all measurements were averaged values of 10-20 different positions on its sections.

For cardiomyocyte (CM) proliferation and apoptosis measurement, CM number was either counted with ImageJ/Fiji's cell count function (Nkx2-5$^+$ nuclei) or manually (overlay of α-actinin and DAPI). EdU$^+$/Nkx2-5$^+$ nuclei (proliferative CMs) were identified by merging EdU images (green) with Nkx2-5 images (red), and using ImageJ/Fiji's color threshold function to select for the yellow signal generated by overlaying red Nkx2-5 signal and green EdU signal. Double positive nuclei were then counted with ImageJ/Fiji's cell count function. Same settings were applied to all images for measurement. cCSP3$^+$ CMs were counted manually. Anatomical parts of the heart (LV compact layer, LV trabeculation, IVS, RV compact layer, RV trabeculation) were manually defined with ImageJ/Fiji's ROI (region of interest) function.

Data are presented as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using GraphPad Prism 9 software, with two-tailed unpaired Student's t test or Mixed-

effects two-way ANOVA. *P*-values less than 0.05 were considered significant and reported as *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.

Supplemental Figures

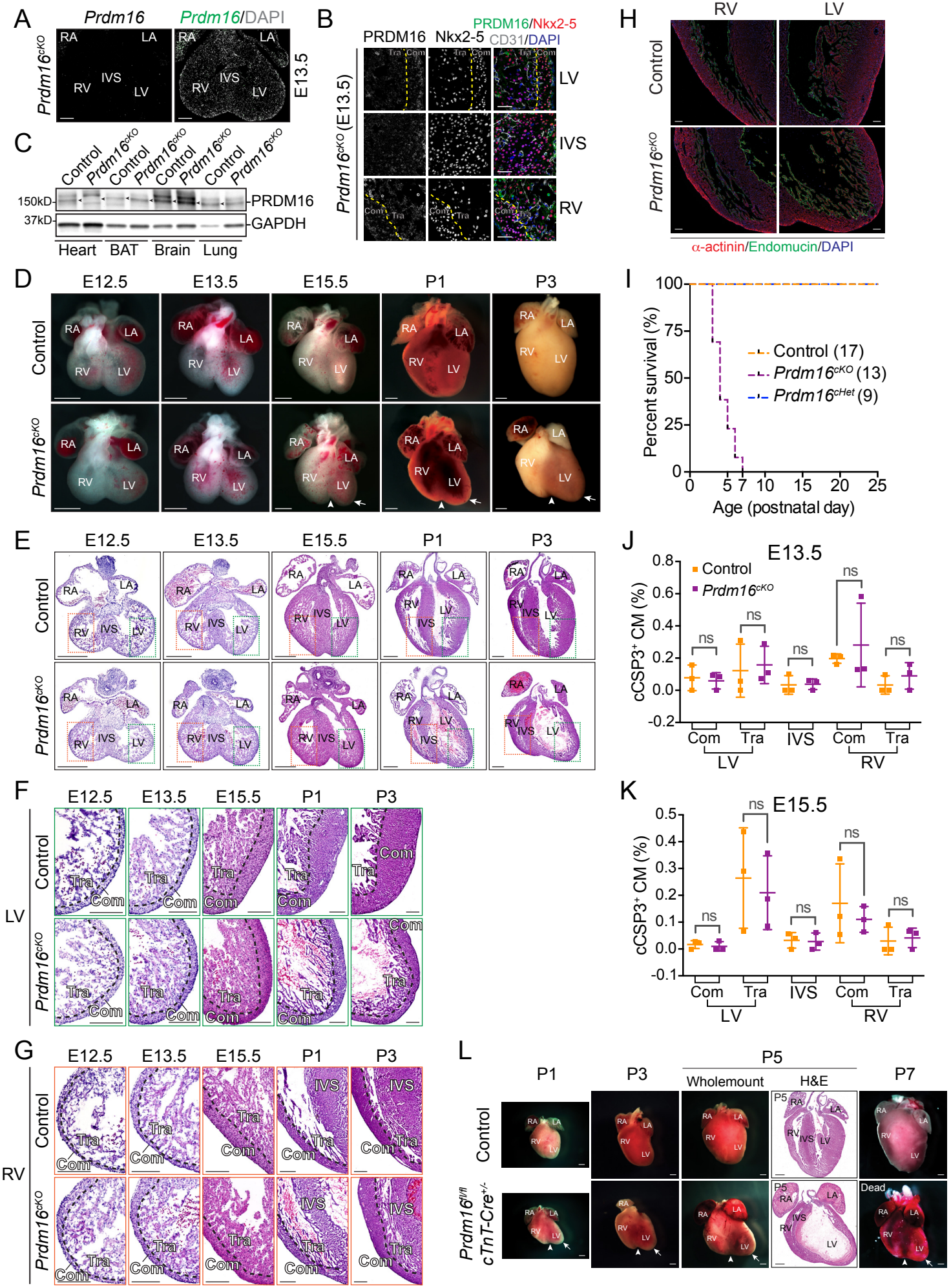


Figure S1. Cardiomyocyte-specific deletion of PRDM16 recapitulates left ventricular noncompaction. Related to Figure 1.

(A) Representative images of *Prdm16 in situ* hybridization (ISH) performed on E13.5 *Prdm16*^{cKO} mouse embryonic heart cryosections. Images were captured with the same parameters as Figure 1A. DNA was stained with DAPI. LV, left ventricle. RV, right ventricle. IVS, interventricular septum. LA, left atrium. RA, right atrium. Scale bars, 0.2 mm.

(B) Representative images of PRDM16, Nkx2-5 and CD31 immunofluorescences (IF) performed on E13.5 *Prdm16*^{cKO} mouse embryonic heart cryosections. Images were captured with the same parameters as Figure 1B. The approximal boundaries between compact myocardium (Com) and trabecular myocardium (Tra) are indicated by yellow dashed lines. Scale bars, 50 mm.

(C) PRDM16 western blot using protein samples extracted from heart, brown adipose tissue (BAT), brain and lung of P3 control and *Prdm16*^{cKO} mice. Black arrowheads indicate PRDM16 band which was specifically diminished in heart sample while remained unchanged in other tissues of *Prdm16*^{cKO} mouse. GAPDH serves as a loading control.

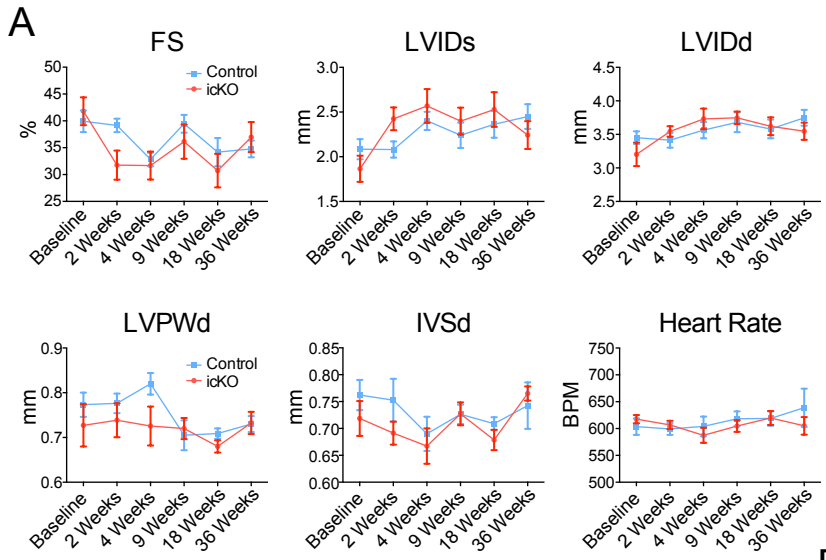
(D-G) Representative wholemount (D) and H&E stained whole heart (E), LV (F) and RV (G) cryosection images of E12.5-P3 control and *Prdm16*^{cKO} mouse hearts. Clefts at the apexes and enlarged left ventricles of *Prdm16*^{cKO} heart are indicated by white arrowheads and white arrows, respectively (D). The boundaries between compact myocardium (Com) and trabecular myocardium (Tra) are depicted by black dashed lines (F-G). Scale bars: 0.5 mm (D-E), 0.2 mm (F-G).

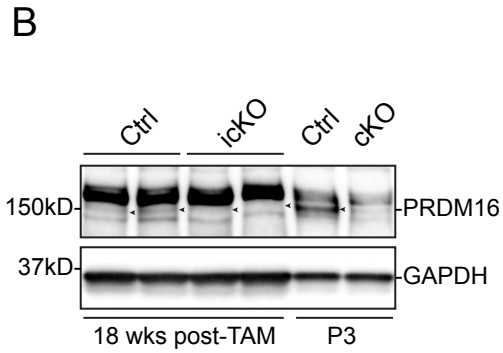
(H) Representative images of α-actinin and Endomucin immunofluorescences (IF) performed on P1 control and *Prdm16*^{cKO} postnatal heart cryosections. Scale bars, 100 μm.

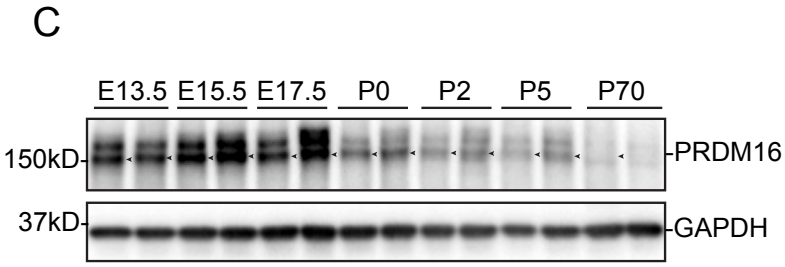
(I) Survival curves of control (*Prdm16*$^{fl/fl}$ or *Prdm16*$^{fl/+}$, n = 17), *Prdm16*^{cKO} (*Prdm16*$^{fl/fl}$::*Xmlc2-Cre*$^{+/-}$, n = 13) and *Prdm16*^{cHet} (*Prdm16*$^{fl/+}$::*Xmlc2-Cre*$^{+/-}$, n = 9) mice up to 25 days after birth.

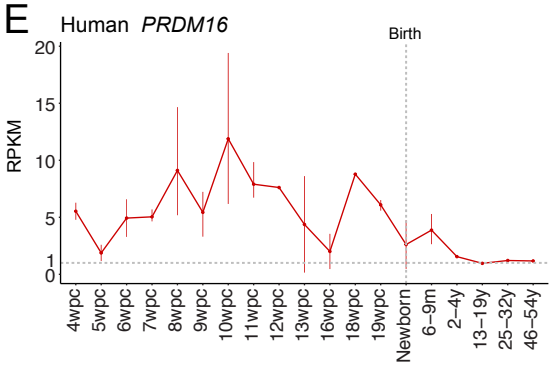
(J-K) Quantitative analyses of cleaved caspase 3 positive (cCSP3^{+}) CMs from cCSP3 and Nkx2-5 IF performed on E13.5 (I) and E15.5 (J) control and *Prdm16*^{cKO} hearts (E13.5, n = 3 hearts per group, n = 3 sections per heart; E15.5, n = 3 hearts per group, n = 2-3 sections per heart). Data are represented as mean ± SEM. Statistical significance was determined with two-tailed Student's t test (ns, not significant).

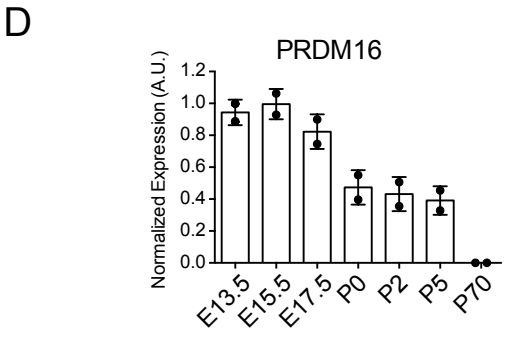
(L) Wholemount (P1, P3, P5 and P7) and H&E stained cryosection (P5) images of control and *Prdm16*$^{fl/fl}$::*cTnT-Cre*$^{+/-}$ mouse hearts. A cleft at the apex and the enlarged left ventricle of *Prdm16*$^{fl/fl}$::*cTnT-Cre*$^{+/-}$ heart are indicated by a white arrowhead and a white arrow, respectively. Scale bars, 0.5 mm.


A
FS
LVIDs
LVIDd
LVPWd
IVSd
Heart Rate
Control
icKO
%
mm
BPM
Baseline
2 Weeks
4 Weeks
9 Weeks
18 Weeks
36 Weeks


B
Ctrl
icKO
Ctrl
cKO
150kD
37kD
PRDM16
GAPDH
18 wks post-TAM
P3


C
E13.5
E15.5
E17.5
P0
P2
P5
P70
150kD
37kD
PRDM16
GAPDH


E
Human PRDM16
Birth
RPKM
4wpc
5wpc
6wpc
7wpc
8wpc
9wpc
10wpc
11wpc
12wpc
13wpc
16wpc
18wpc
19wpc
Newborn
6–9m
2–4y
13–19y
25–32y
46–54y


D
PRDM16
Normalized Expression (A.U.)
E13.5
E15.5
E17.5
P0
P2
P5
P70

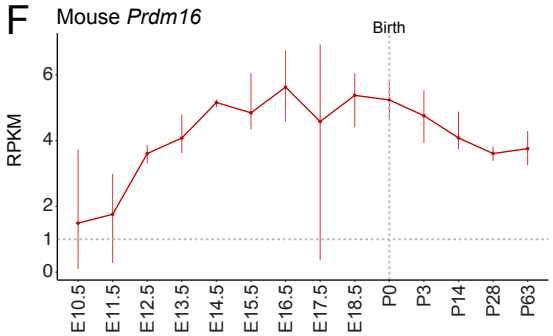

F
Mouse Prdm16
Birth
RPKM
E10.5
E11.5
E12.5
E13.5
E14.5
E15.5
E16.5
E17.5
E18.5
P0
P3
P14
P28
P63

Figure S2. PRDM16 is dispensable in adult cardiomyocyte. Related to Figure 1.
(A) Echocardiographic parameters fraction shortening (FS), left ventricle internal dimension, systolic (LVIDs), left ventricle internal dimension, diastolic (LVIDd), left ventricle posterior wall thickness, diastolic (LVPWd), interventricular septum thickness, diastolic (IVSd) and heart rate (M-mode) of 8-week-old control and *Prdm16*^{icKO} adult mice before tamoxifen injection (baseline) and 2, 4, 9, 18, 36 weeks post tamoxifen injection (baseline: *n* = 7-9 per group; 2, 4, 9, 18 weeks: *n* = 7-8 per group; 36 weeks: *n* = 4 per group). Tamoxifen was dissolved in peanut oil and injected intraperitoneally at 40mg/kg/day for 3 consecutive days. Data are represented as mean ± SEM. Mixed-effects two-way ANOVA was conducted to examine if there were significant differences in cardiac function between control and *Prdm16*^{icKO} mice over time. For all the echocardiography parameters tested, neither the interaction effects between genotype (control/*Prdm16*^{icKO}) and time (from baseline to 36 weeks post-induction) nor the main effects for genotype (control/*Prdm16*^{icKO}) were statistically significant.
(B) PRDM16 western blot using protein samples extracted from control and *Prdm16*^{icKO} ventricles 18 weeks post tamoxifen (TAM) injection. Black arrowheads indicate PRDM16 band. Protein samples from P3 control and *Prdm16*^{cKO} neonatal mouse ventricles were used as positive and negative controls, respectively. GAPDH serves as a loading control.
(C-D) PRDM16 western blot (C) and its densitometry quantification result (D) normalized to GAPDH using protein samples extracted from E13.5-P70 wild type mouse ventricles (*n* = 2 per group). Data are represented as mean ± SEM. Black arrowheads indicate PRDM16 band.
(E-F) Expression profiles of human *PRDM16* (E) and mouse *Prdm16* (F) from Cardoso-Moreira et al., 2019. RPKM, reads per kilobase of exon per million mapped reads. wpc, weeks post-conception. m, months. y, years.

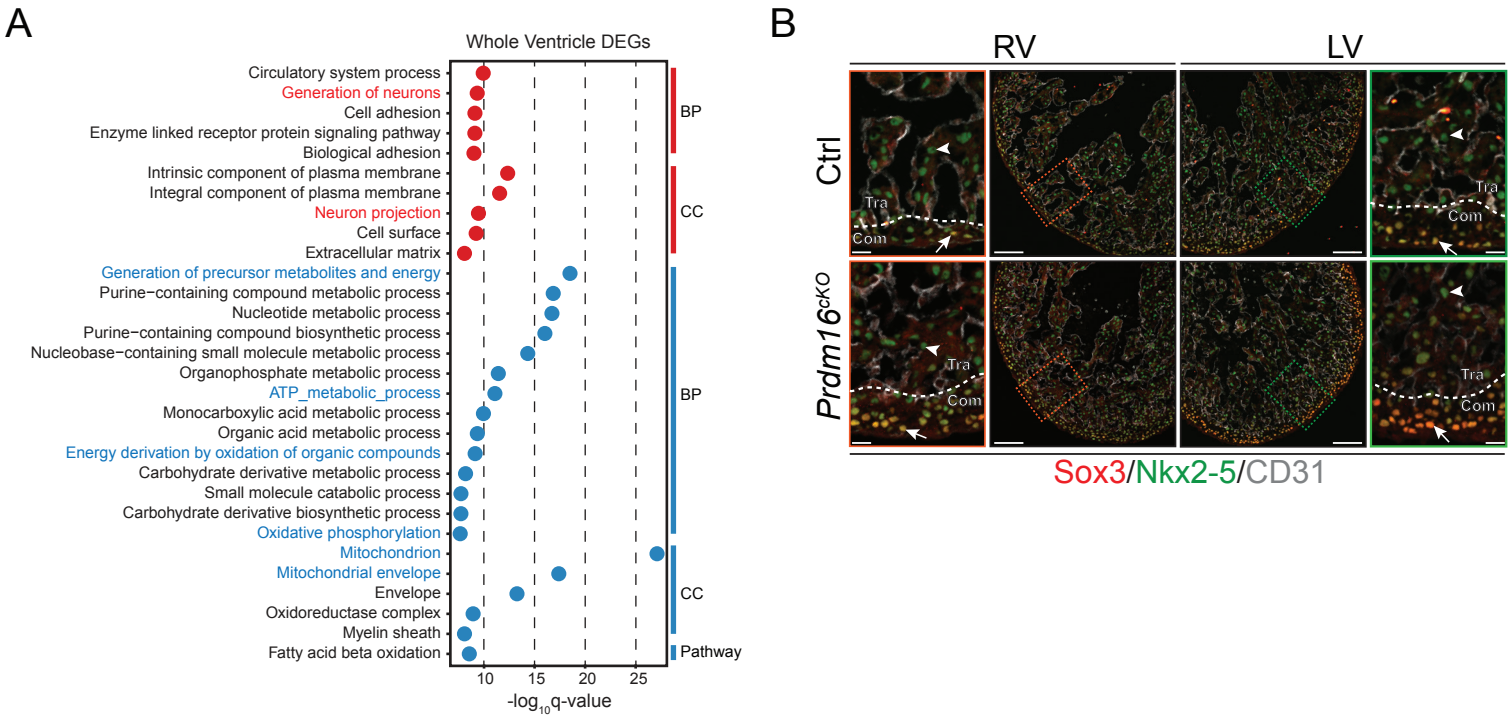


Figure S3. Loss of PRDM16 leads to widespread gene misregulation in developing heart. Related to Figure 2.

(A) Gene ontology analysis of whole ventricle DEGs of E13.5 *Prdm16*^{cKO} mice compared with littermate controls. BP: biological process. CC: cellular component. Notable GO terms are highlighted in red (upregulated) or blue (downregulated).

(B) Representative images of Sox3, Nkx2-5 and CD31 immunofluorescences (IF) performed on E13.5 mouse embryonic heart cryosections. Compact myocardial (Com) CMs (Nkx2-5-positive) with high expression of Sox3 were indicated by white arrows. Trabecular myocardial (Tra) CMs with low expression of Sox3 were indicated by white arrowheads. The approximal boundaries between compact myocardium and trabecular myocardium are depicted by white dashed lines. Scale bars: 100 mm (whole ventricle view), 20 mm (magnified view).

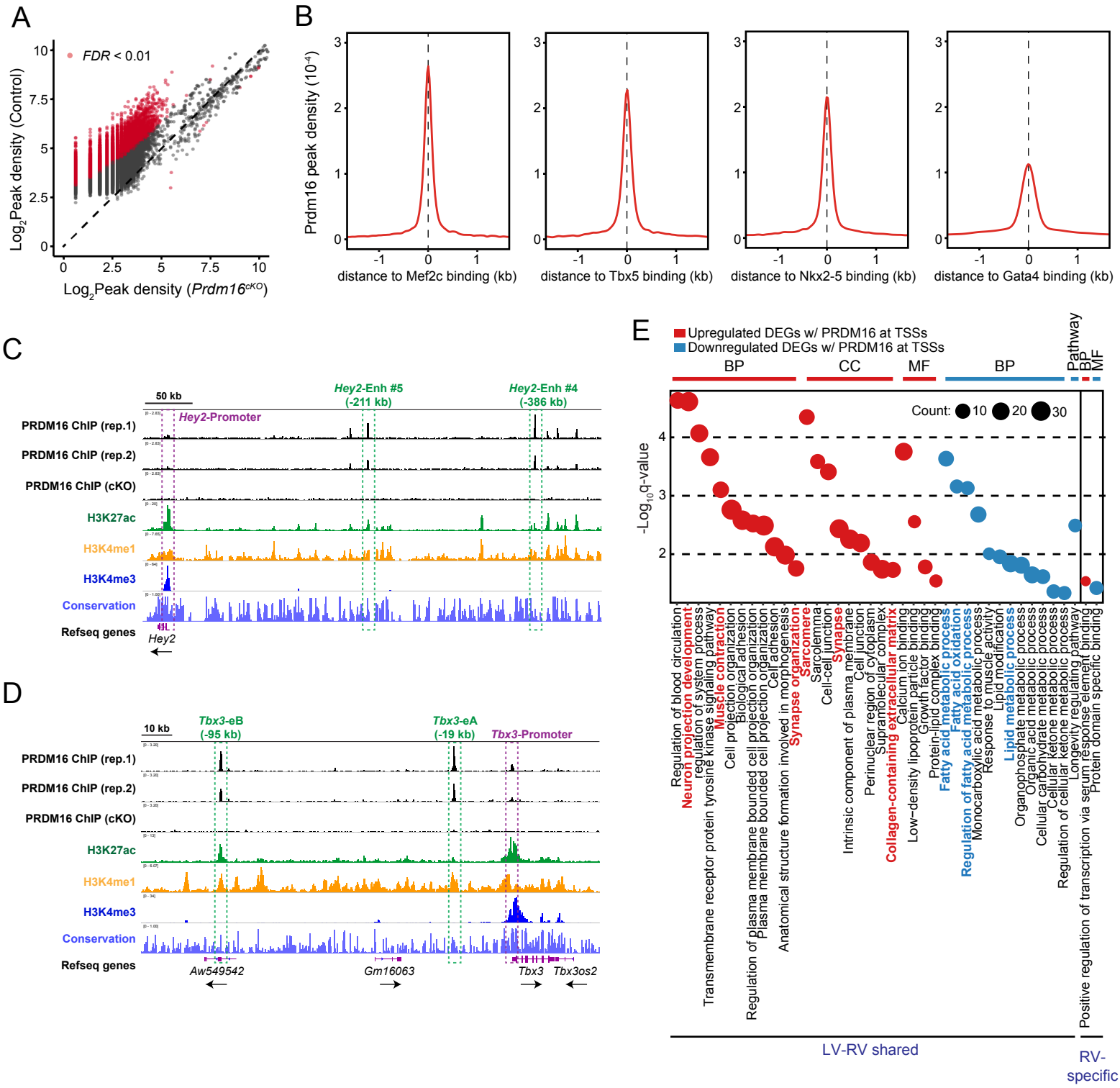

A
FDR < 0.01
Log2Peak density (Control)
Log2Peak density (Prdm16cKO)
B
Prdm16 peak density (10-4)
distance to Mef2c binding (kb)
distance to Tbx5 binding (kb)
distance to Nkx2-5 binding (kb)
distance to Gata4 binding (kb)
C
50 kb
Hey2-Enh #5 (-211 kb)
Hey2-Enh #4 (-386 kb)
Hey2-Promoter
PRDM16 ChIP (rep.1)
PRDM16 ChIP (rep.2)
PRDM16 ChIP (cKO)
H3K27ac
H3K4me1
H3K4me3
Conservation
Refseq genes
Hey2
D
10 kb
Tbx3-eB (-95 kb)
Tbx3-eA (-19 kb)
Tbx3-Promoter
PRDM16 ChIP (rep.1)
PRDM16 ChIP (rep.2)
PRDM16 ChIP (cKO)
H3K27ac
H3K4me1
H3K4me3
Conservation
Refseq genes
Aw549542
Gm16063
Tbx3
Tbx3os2
E
Upregulated DEGs w/ PRDM16 at TSSs
Downregulated DEGs w/ PRDM16 at TSSs
BP
CC
MF
BP
Pathway
BP
MF
Count: 10 20 30
-Log10q-value
Regulation of blood circulation
Neuron projection development
regulation of system process
Transmembrane receptor protein tyrosine kinase signaling pathway
Muscle contraction
Cell projection organization
Biological adhesion
Regulation of plasma membrane bounded cell projection organization
Plasma membrane bounded cell projection organization
Cell adhesion
Anatomical structure formation involved in morphogenesis
Synapse organization
Sarcomere
Sarcolemma
Cell-cell junction
Synapse
Intrinsic component of plasma membrane
Cell junction
Perinuclear region of cytoplasm
Supramolecular complex
Collagen-containing extracellular matrix
Calcium ion binding
Low-density lipoprotein particle binding
Growth factor binding
Protein-lipid complex binding
Fatty acid metabolic process
Fatty acid oxidation
Regulation of fatty acid metabolic process
Monocarboxylic acid metabolic process
Response to muscle activity
Lipid modification
Lipid metabolic process
Organophosphate metabolic process
Organic acid metabolic process
Cellular carbohydrate metabolic process
Cellular ketone metabolic process
Regulation of cellular ketone metabolic process
Longevity regulating pathway
Positive regulation of transcription via serum response element binding
Protein domain specific binding
LV-RV shared
RV-specific

Figure S4. PRDM16 functions as a bifunctional transcription regulator in the genome. Related to Figure 3.

(A) Comparison of the mean read density between PRDM16 ChIP-seq peaks in wild type and *Prdm16*^{cKO} ventricles. Red dots represent differentially enriched binding sites (*FDR* < 0.01).

(B) PRDM16 binding profiles within a 10 kb window centered on Mef2c, Tbx5, Nkx2-5 and Gata4 binding sites in embryonic mouse heart.

(C-D) IGV view showing histone modifications and PRDM16 binding at *Hey2* (C) and *Tbx3* (D) loci. Promoters or *in vivo* validated enhancers of *Hey2* and *Tbx3* are indicated by purple or green boxes, respectively. Direction of transcription is indicated by black arrows.

(E) Gene ontology analysis of LV-RV shared or RV-specific DEGs with promoter PRDM16 binding. Each analysis is divided into four categories: biological process (BP), cellular component (CC), molecular function (MF) and KEGG pathway. Notable GO terms are highlighted in red (upregulated) or blue (downregulated).

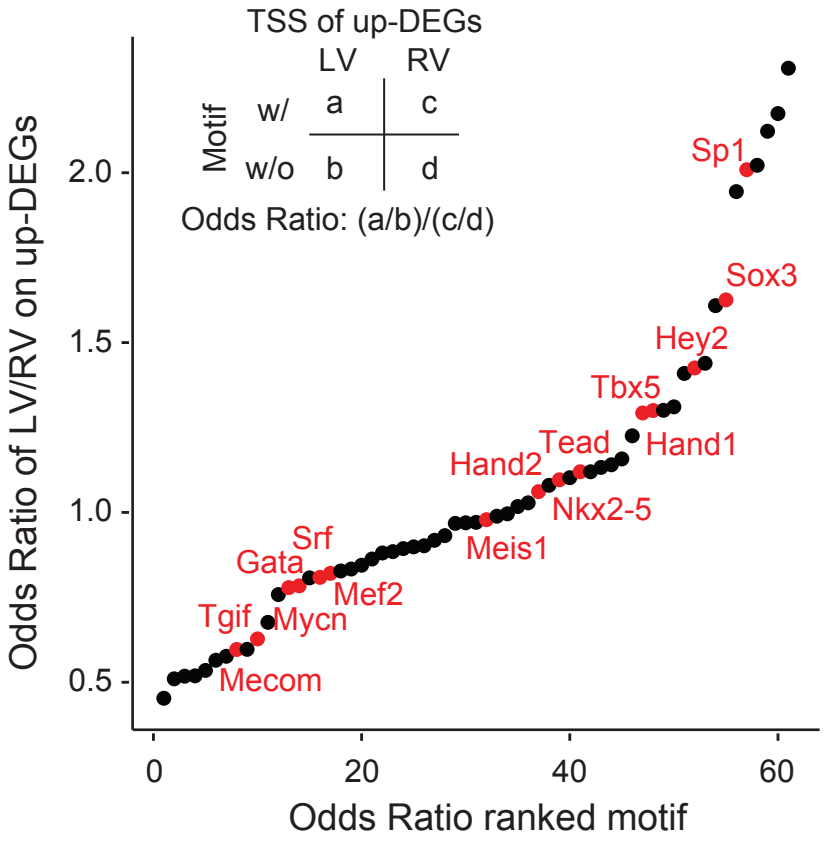


Figure S5. Cooperation of PRDM16 with LV-enriched TFs. Related to Figure 4.

Relative transcription factor motif enrichment at TSS of LV-specific or RV-specific upregulated DEGs. Notable TF motifs are highlighted in red.

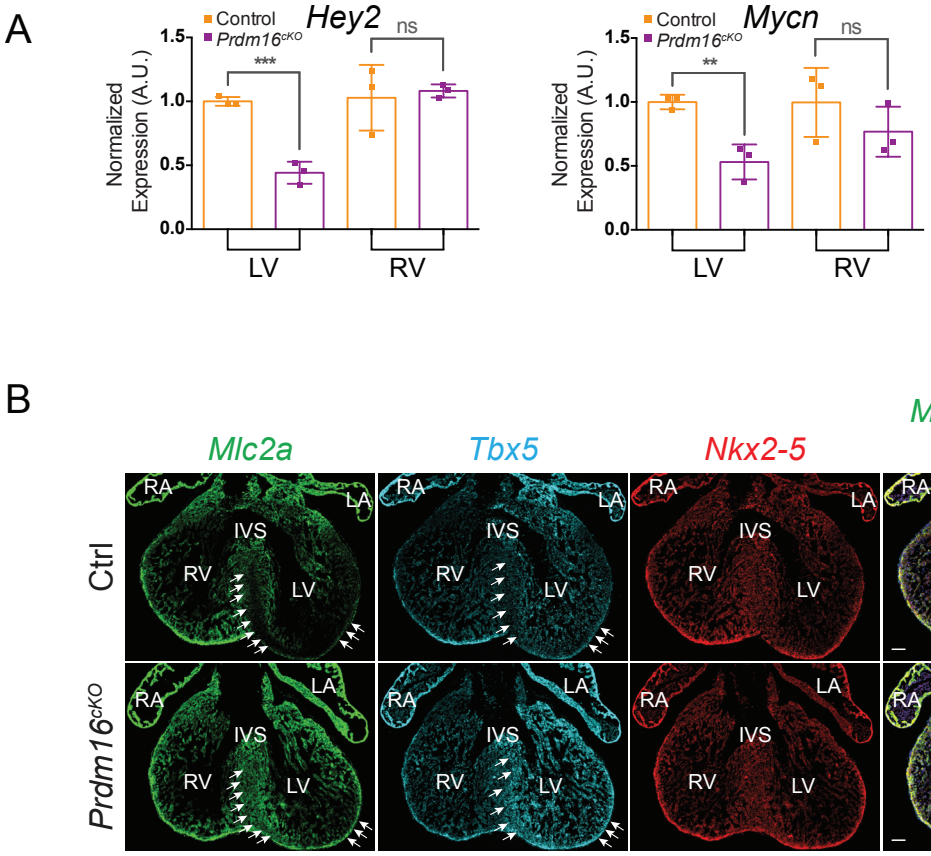


Figure S6. PRDM16 activates compact genes while repressing trabecular genes in LV compact myocardium and IVS. Related to Figure 5.

(A) qRT-PCR analysis of *Hey2* and *Mycn* normalized to *18S* rRNA (artificial units, A.U.) using RNA extracted from LV or RV of E13.5 *Prdm16*^{cKO} and littermate control mice (n = 3 per group). Data are represented as mean ± SEM. Statistical significance was determined with two-tailed Student's *t* test (ns, not significant; ***p* < 0.01, ****p* < 0.001).

(B) Representative images of *Mlc2a/Myl7* and *Tbx5 in situ* hybridization (ISH) performed on E13.5 mouse embryonic heart cryosections. The regions in which *Mlc2a* and *Tbx5* expression ectopically expanded in *Prdm16*^{cKO} are indicated by white arrows. Scale bars, 0.1 mm.

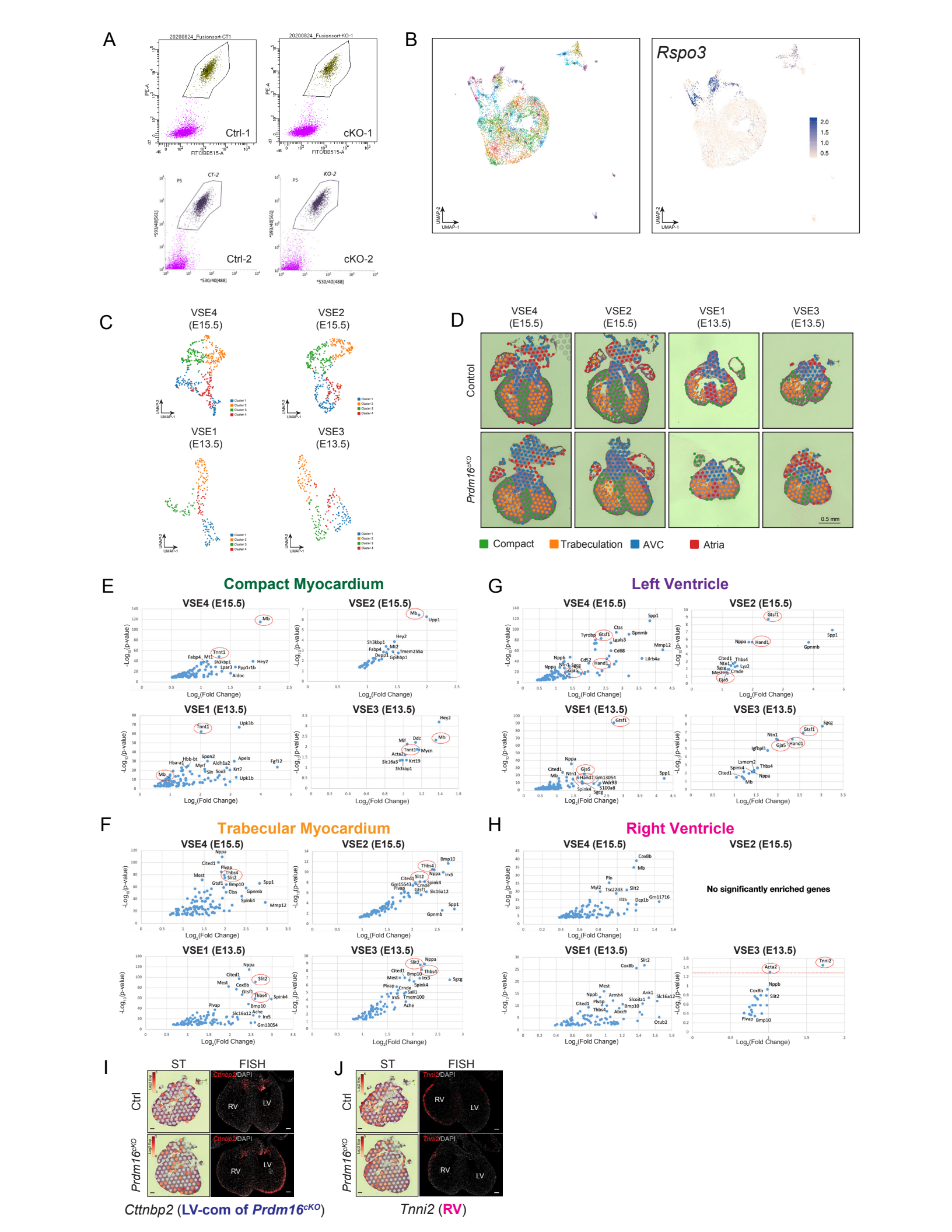

A
20200824_Fusionsort-CT1
20200824_Fusionsort-KO-1
Ctrl-1
cKO-1
Ctrl-2
cKO-2
B
Rspo3
C
VSE4 (E15.5)
VSE2 (E15.5)
VSE1 (E13.5)
VSE3 (E13.5)
D
Control
Prdm16cKO
0.5 mm
Compact
Trabeculation
AVC
Atria
E
Compact Myocardium
F
Trabecular Myocardium
G
Left Ventricle
H
Right Ventricle
No significantly enriched genes
I
ST
FISH
Cttnbp2/DAPI
RV
LV
J
Tnni2/DAPI
Cttnbp2 (LV-com of Prdm16cKO)
Tnni2 (RV)

Figure S7. Single-cell RNA-seq uncovers a PRDM16-anchored transcription network in LV. Related to Figure 6.

(A) Fluorescence-activated cell sorting (FACS) for CMs based on tdTomato fluorescence.

(B) UMAP plots of FACS-sorted E13.5 CMs showing the expression of outflow tract CM marker gene *Rspo3*.

(C-D) Unsupervised clustering of Spatial Transcriptomics (ST) spots (C) and mapping to their spatial positions (D).

(E-H) Diagrams showing top enriched genes in compact myocardium (E), trabecular myocardium (F), left ventricle (G) or right ventricle (H) calculated from corresponding ST spots.

(I-J) ST expression profiles and representative FISH images of *Cttnbp2* (I) and *Tnni2* (J) in E13.5 control and *Prdm16*^{cKO} hearts. Scale bar, 0.1 mm.

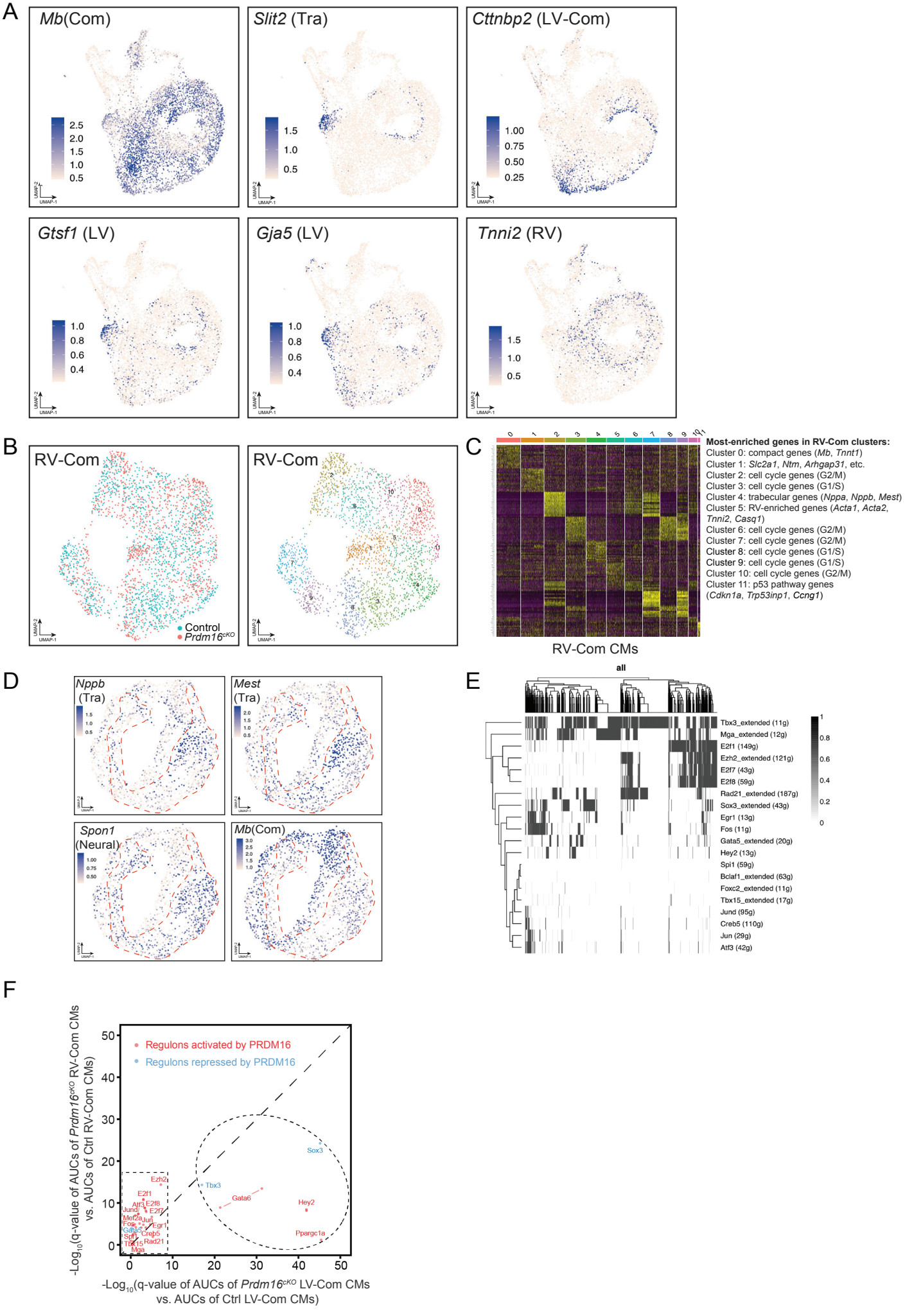

A
Mb(Com)
Slit2 (Tra)
Cttnbp2 (LV-Com)
Gtsf1 (LV)
Gja5 (LV)
Tnni2 (RV)
B
RV-Com
RV-Com
Control
Prdm16cKO
C
Most-enriched genes in RV-Com clusters:
Cluster 0: compact genes (Mb, Tnnt1)
Cluster 1: Slc2a1, Ntm, Arhgap31, etc.
Cluster 2: cell cycle genes (G2/M)
Cluster 3: cell cycle genes (G1/S)
Cluster 4: trabecular genes (Nppa, Nppb, Mest)
Cluster 5: RV-enriched genes (Acta1, Acta2, Tnni2, Casq1)
Cluster 6: cell cycle genes (G2/M)
Cluster 7: cell cycle genes (G2/M)
Cluster 8: cell cycle genes (G1/S)
Cluster 9: cell cycle genes (G1/S)
Cluster 10: cell cycle genes (G2/M)
Cluster 11: p53 pathway genes (Cdkn1a, Trp53inp1, Ccng1)
RV-Com CMs
D
Nppb (Tra)
Mest (Tra)
Spon1 (Neural)
Mb(Com)
E
all
Tbx3_extended (11g)
Mga_extended (12g)
E2f1 (149g)
Ezh2_extended (121g)
E2f7 (43g)
E2f8 (59g)
Rad21_extended (187g)
Sox3_extended (43g)
Egr1 (13g)
Fos (11g)
Gata5_extended (20g)
Hey2 (13g)
Spi1 (59g)
Bclaf1_extended (63g)
Foxc2_extended (11g)
Tbx15_extended (17g)
Jund (95g)
Creb5 (110g)
Jun (29g)
Atf3 (42g)
F
Regulons activated by PRDM16
Regulons repressed by PRDM16
-Log10(q-value of AUCs of Prdm16cKO RV-Com CMs vs. AUCs of Ctrl RV-Com CMs)
-Log10(q-value of AUCs of Prdm16cKO LV-Com CMs vs. AUCs of Ctrl LV-Com CMs)
Sox3
Tbx3
Gata6
Hey2
Ppargc1a
Ezh2
E2f1
Atf3
E2f8
Jund
E2f7
Mef2a
Jun
Fos
Egr1
Gata5
Creb5
Spi1
Tbx15
Rad21
Mga

Figure S8. Single-cell RNA-seq uncovers a PRDM16-anchored transcription network in LV. Continued from Figure S7.

(A) UMAP plots showing the expression of additional anatomical marker genes in FACS-sorted CMs.

(B) UMAP plots of RV compact myocardial CMs assigned to genotypes (left panel) or subclusters (right panel).

(C) Heatmap showing most-enriched genes in subclusters of RV compact myocardial CMs.

(D) UMAP plots of LV compact myocardial CMs showing the expression of additional subcluster marker genes.

(E) SCENIC (single-cell regulatory network inference and clustering) results on LV and RV compact myocardial CMs.

(F) Correlation analysis of TF regulon activity between LV and RV compact myocardial CMs.

Supplemental Excel Files

Supplemental Excel File 1 (Table S1): Differentially expressed genes between left ventricle and right ventricle of E13.5 control mice or *Prdm16*^{cKO} mice, related to Figure 2.

Supplemental Excel File 2 (Table S2): Differentially expressed genes in whole ventricle, left ventricle or right ventricle of E13.5 *Prdm16*^{cKO} mice compared with littermate controls, related to Figure 2.

Supplemental Excel File 3 (Table S3): PRDM16 direct target genes (with PRDM16 binding at promoters) in whole ventricle, left ventricle or right ventricle of E13.5 mice, related to Figure 3.