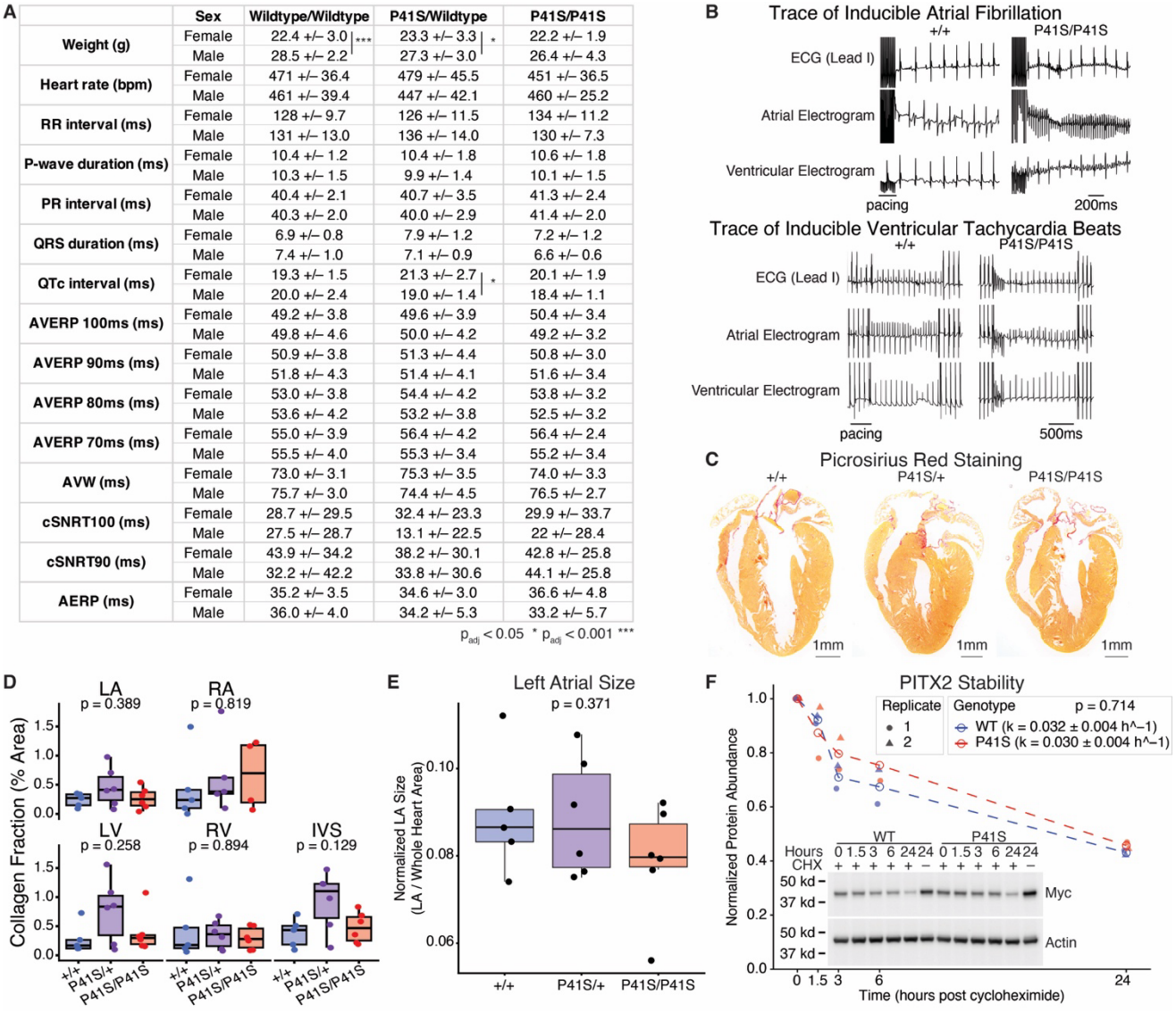
SUPPLEMENTAL FIGURES

A

	Sex	Wildtype/Wildtype	P41S/Wildtype	P41S/P41S
Weight (g)	Female	22.4 +/– 3.0 ***	23.3 +/– 3.3 *	22.2 +/– 1.9
	Male	28.5 +/– 2.2	27.3 +/– 3.0	26.4 +/– 4.3
Heart rate (bpm)	Female	471 +/– 36.4	479 +/– 45.5	451 +/– 36.5
	Male	461 +/– 39.4	447 +/– 42.1	460 +/– 25.2
RR interval (ms)	Female	128 +/– 9.7	126 +/– 11.5	134 +/– 11.2
	Male	131 +/– 13.0	136 +/– 14.0	130 +/– 7.3
P-wave duration (ms)	Female	10.4 +/– 1.2	10.4 +/– 1.8	10.6 +/– 1.8
	Male	10.3 +/– 1.5	9.9 +/– 1.4	10.1 +/– 1.5
PR interval (ms)	Female	40.4 +/– 2.1	40.7 +/– 3.5	41.3 +/– 2.4
	Male	40.3 +/– 2.0	40.0 +/– 2.9	41.4 +/– 2.0
QRS duration (ms)	Female	6.9 +/– 0.8	7.9 +/– 1.2	7.2 +/– 1.2
	Male	7.4 +/– 1.0	7.1 +/– 0.9	6.6 +/– 0.6
QTc interval (ms)	Female	19.3 +/– 1.5	21.3 +/– 2.7 *	20.1 +/– 1.9
	Male	20.0 +/– 2.4	19.0 +/– 1.4	18.4 +/– 1.1
AVERP 100ms (ms)	Female	49.2 +/– 3.8	49.6 +/– 3.9	50.4 +/– 3.4
	Male	49.8 +/– 4.6	50.0 +/– 4.2	49.2 +/– 3.2
AVERP 90ms (ms)	Female	50.9 +/– 3.8	51.3 +/– 4.4	50.8 +/– 3.0
	Male	51.8 +/– 4.3	51.4 +/– 4.1	51.6 +/– 3.4
AVERP 80ms (ms)	Female	53.0 +/– 3.8	54.4 +/– 4.2	53.8 +/– 3.2
	Male	53.6 +/– 4.2	53.2 +/– 3.8	52.5 +/– 3.2
AVERP 70ms (ms)	Female	55.0 +/– 3.9	56.4 +/– 4.2	56.4 +/– 2.4
	Male	55.5 +/– 4.0	55.3 +/– 3.4	55.2 +/– 3.4
AVW (ms)	Female	73.0 +/– 3.1	75.3 +/– 3.5	74.0 +/– 3.3
	Male	75.7 +/– 3.0	74.4 +/– 4.5	76.5 +/– 2.7
cSNRT100 (ms)	Female	28.7 +/– 29.5	32.4 +/– 23.3	29.9 +/– 33.7
	Male	27.5 +/– 28.7	13.1 +/– 22.5	22 +/– 28.4
cSNRT90 (ms)	Female	43.9 +/– 34.2	38.2 +/– 30.1	42.8 +/– 25.8
	Male	32.2 +/– 42.2	33.8 +/– 30.6	44.1 +/– 25.8
AERP (ms)	Female	35.2 +/– 3.5	34.6 +/– 3.0	36.6 +/– 4.8
	Male	36.0 +/– 4.0	34.2 +/– 5.3	33.2 +/– 5.7

$p_{adj} < 0.05$ * $p_{adj} < 0.001$ ***



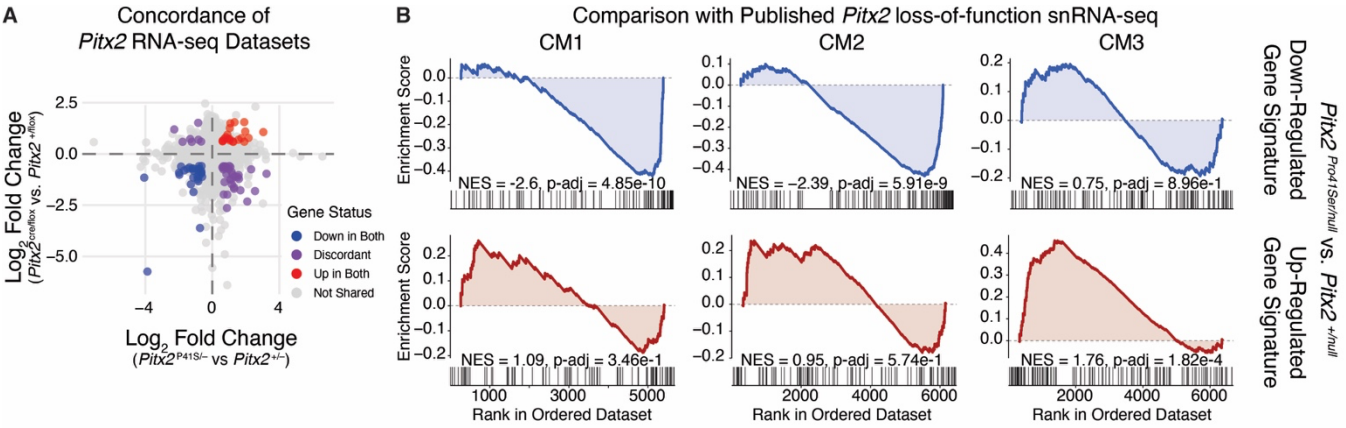
Supplemental Figure 1.

A. Table of body weight, electrocardiogram measurements, and programmed electrical stimulation of atrioventricular and sinoatrial nodes for female and male mice for each genotype is listed. Statistical differences identified using multi-factor ANOVA.

B. Representative traces for inducible atrial fibrillation (top) and ventricular tachycardia (bottom) followed programmed electrical stimulation.

C. Representative Picrosirius red staining for wildtype (+/+), *Pitx2*^{Pro41Ser} heterozygous (P41S/+), and *Pitx2*^{Pro41Ser} homozygous (P41S/P41S) mice.

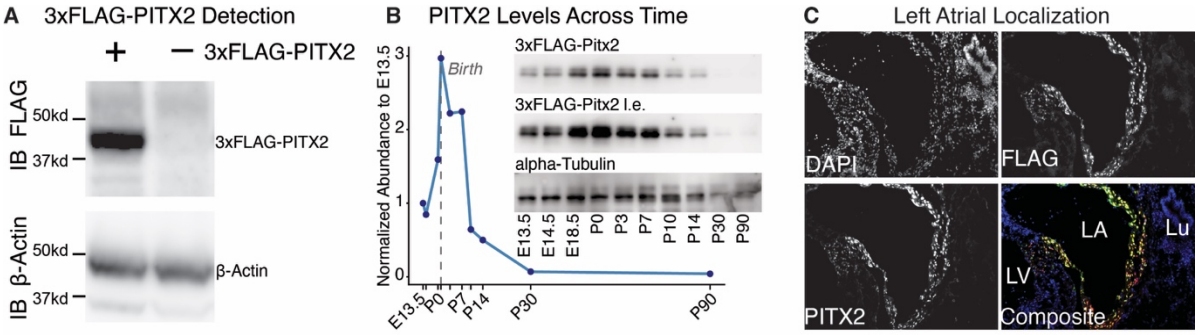
D. Quantification of collagen fraction (percent, %, area) for each chamber of the heart by boxplot. P-value was calculated by ANOVA.
E. Box plots showing left atrial area normalized to whole heart area. P-value was calculated by ANOVA.
F. Western blot for Myc-tagged PITX2 wildtype or Pro41Ser in HEK293T 0, 1.5, 3, 6, and 24 hours after cycloheximide (CHX) treatment.



Supplemental Figure 2.

A. Scatterplot showing the fold change for every gene in both the *Pitx2*$^{Pro41Ser/–}$ to *Pitx2*$^{+/–}$ adult LA RNA-seq (**Figure 1E**) and *Pitx2*$^{creneo/flox}$ to *Pitx2*$^{+/flox}$ E13.5 LA RNA-seq (**Figure 1G**).

B. Gene set enrichment analysis (GSEA) comparing the genes found in *Pitx2*$^{Pro41Ser/–}$ to *Pitx2*$^{+/–}$ adult LA RNA-seq (**Figure 1E**) with previously published snRNA-seq dataset (3).

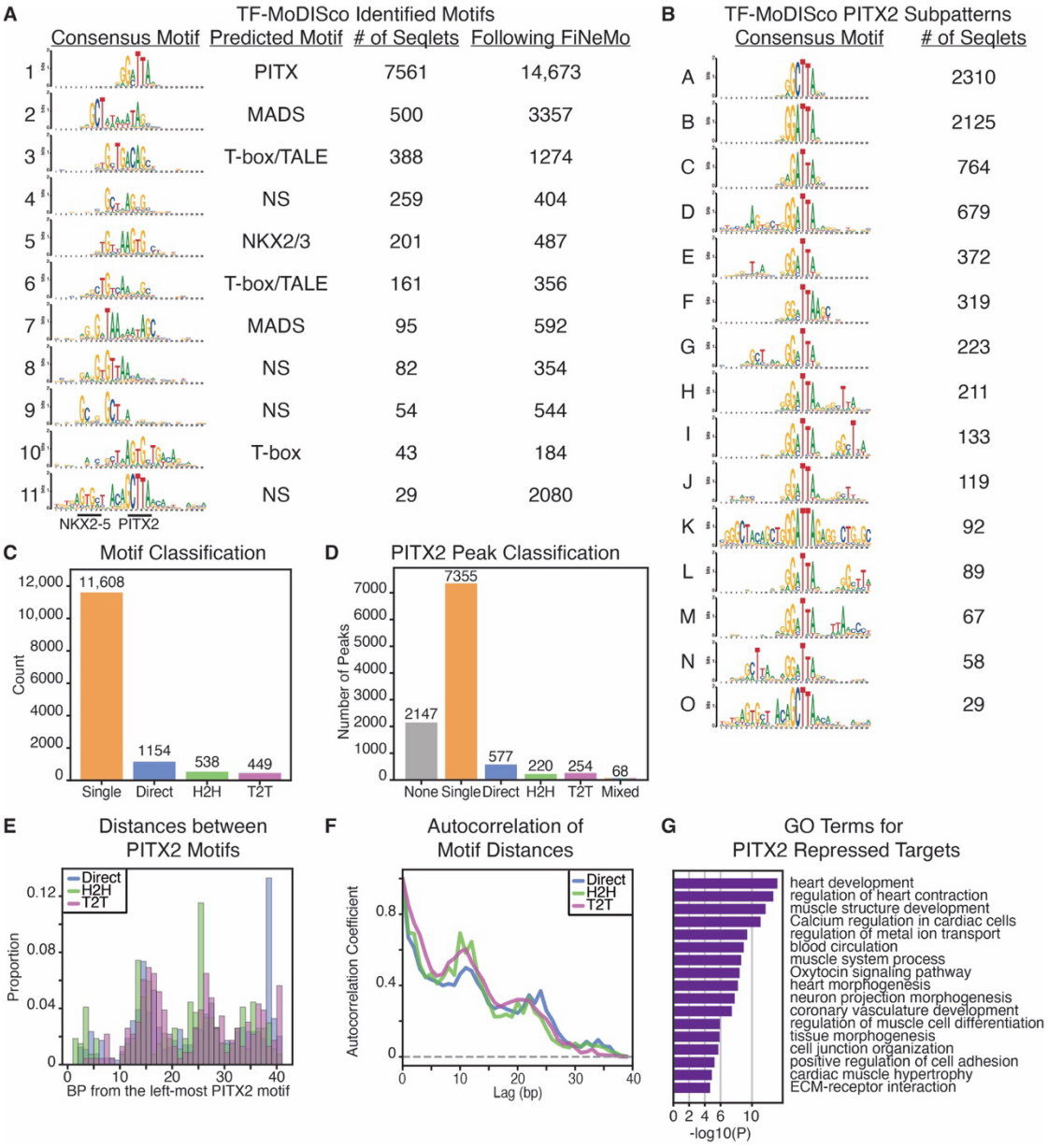


Supplemental Figure 3.

A. Western blot of heterozygous Pitx23x-FLAG-Pitx2c mouse whole embryo E14.5 lysates and littermate controls using an anti-FLAG antibody.

B. Western blot of homozygous *Pitx2*$^{3x\text{-}FLAG\text{-}Pitx2c}$ mouse left atrium across developmental time from E13.5 to postnatal day (P) 90 using anti-FLAG antibody. Values were normalized to alpha-tubulin loading control.

C. Immunofluorescence imaging of frozen tissue showing the left atrium (LA), left ventricle (LV), and lungs (Lu) stained with anti-FLAG (red) and anti-PITX2 (green) antibodies.

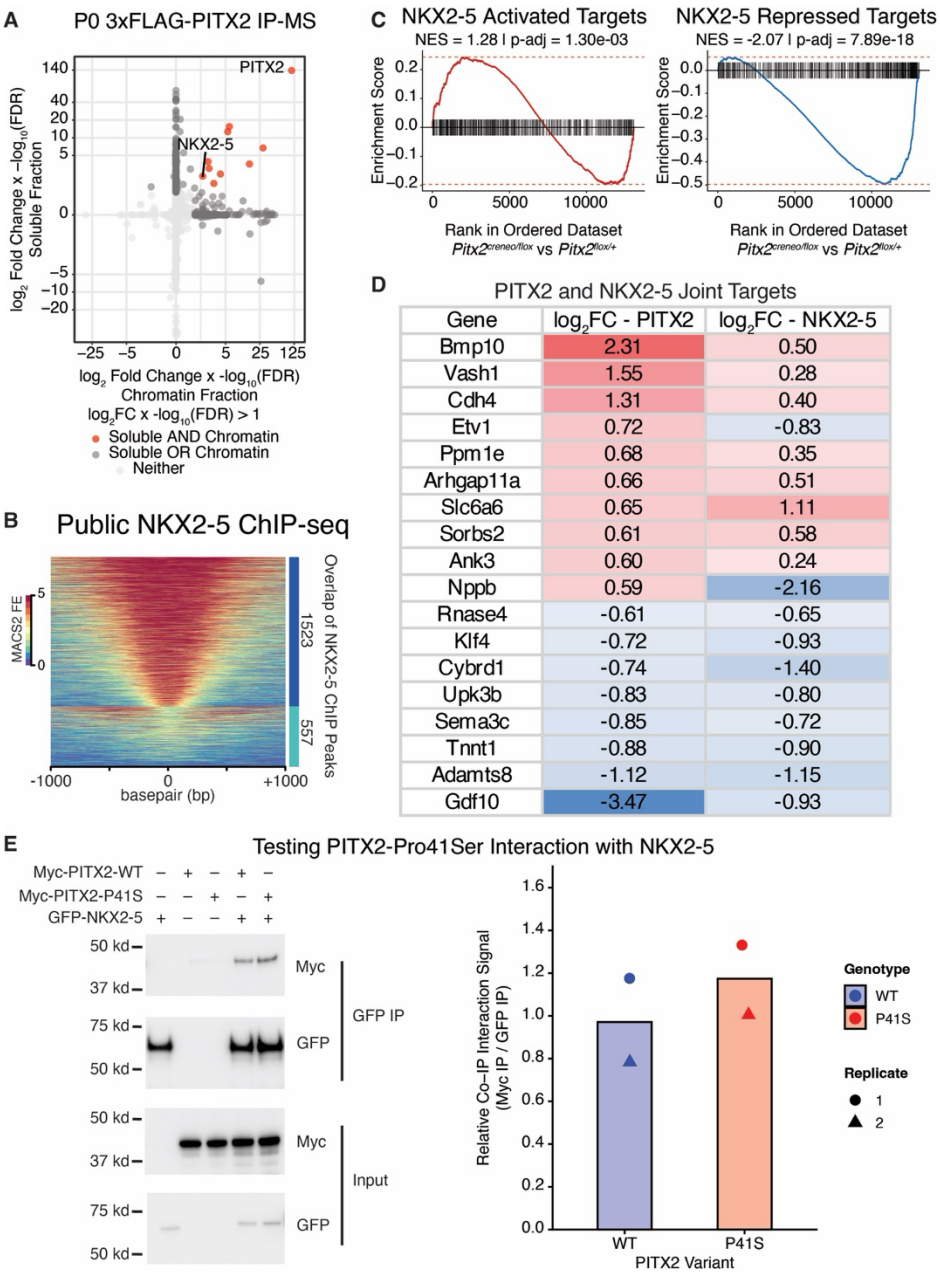


Supplemental Figure 4.

A. All patterns identified by TF-MoDISco following ChromBPNet analysis of E13.5 PITX2 CUT&Tag data, with additional motif identification by Fi-NeMo. For Motif #11, both the NKX2-5 and PITX2 motifs are labeled.

B. All PITX2 sub-patterns identified by TF-MoDISco following ChromBPNet analysis of E13.5 PITX2 CUT&Tag data, including PITX2 motifs from both Motif #1 and Motif #11 shown in A.

C. PITX2 motifs were categorized as single (not within 40 bp of another PITX2 motif) or as occurring in direct tandem, head-to-head (H2H; e.g., TAATC-GATTA), or tail-to-tail (T2T; e.g., GATTA-TAATC) orientations.
D. The union set of 10,621 PITX2 peaks was classified into groups containing single PITX2 motifs, direct tandem repeats, H2H repeats, T2T repeats, a combination of several types of PITX2 motifs, or those containing no identified PITX2 footprints.
E. For each set of paired PITX2 motifs (*e.g.*, Direct, H2H, and T2T), a proportional histogram shows the distance between motifs.
F. For each set of paired PITX2 motifs (*e.g.*, Direct, H2H, and T2T), the autocorrelation coefficient is plotted for each lag distance between adjacent pairs of motifs, revealing 10bp periodicity of H2H motifs and 6bp periodicity of direct tandem motifs.
G. Select GO terms associated with the 146 down-regulated genes associated with PITX2 footprint-containing peaks.



Gene	log_2FC - PITX2	log_2FC - NKX2-5
Bmp10	2.31	0.50
Vash1	1.55	0.28
Cdh4	1.31	0.40
Etv1	0.72	-0.83
Ppm1e	0.68	0.35
Arhgap11a	0.66	0.51
Slc6a6	0.65	1.11
Sorbs2	0.61	0.58
Ank3	0.60	0.24
Nppb	0.59	-2.16
Rnase4	-0.61	-0.65
Klf4	-0.72	-0.93
Cybrd1	-0.74	-1.40
Upk3b	-0.83	-0.80
Sema3c	-0.85	-0.72
Tnnt1	-0.88	-0.90
Adamts8	-1.12	-1.15
Gdf10	-3.47	-0.93

Supplemental Figure 5.

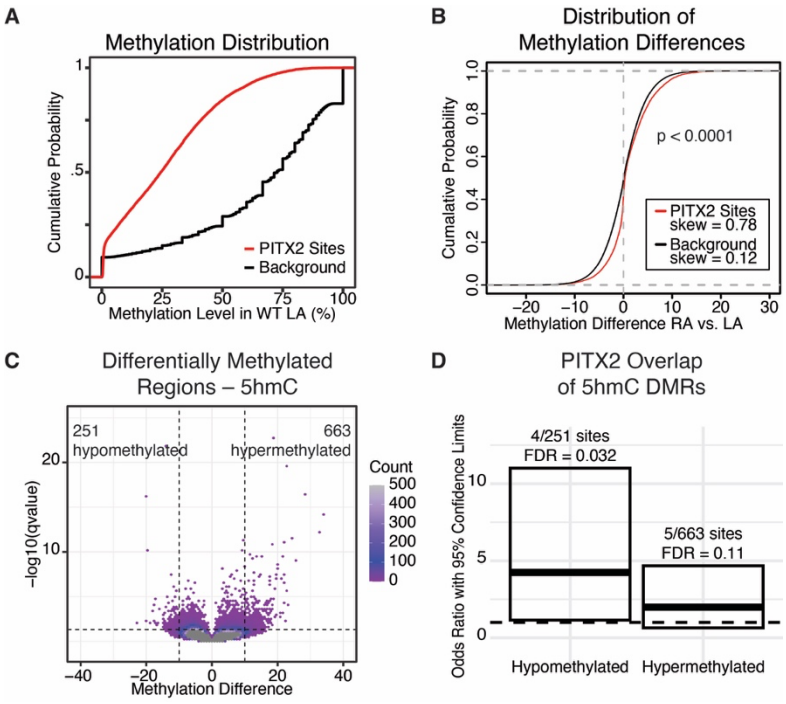
A. Immunoprecipitation-Mass Spectrometry (IP-MS) of 3xFLAG-PITX2. The x-axis shows the log_2 fold change * $-log_{10}$(FDR) for the chromatin fraction, while the y-axis shows the same for the soluble fraction. Identified proteins that showed a value greater than 1 in only one comparison are highlighted in dark grey, while proteins with a value greater than 1 in both comparisons are highlighted in red, including PITX2 and NKX2-5.

B. Overlap of the 2080 PITX2/NKX2-5 joint footprints with published P0 atrial NKX2-5 ChIP-seq (32). Signal represents from the MACS2 fold enrichment around the footprint located at 0 base pairs (bp).

C. Gene set enrichment analysis (GSEA) for NKX2-5 targets from previously published E18.5 conditional *Nkx2-5* mutant bilateral atrial RNA-seq (33).

D. Table of DEGs in both the *Pitx2*$^{creneo/flox}$ vs. *Pitx2*$^{+/flox}$ E13.5 LA transcriptional profiling and publicly available E18.5 atrial conditional knockout of *Nkx2-5* (33) that are also associated with a joint PITX2/NKX2-5 footprint (Motif #11 from **Supplemental Figure 4A**).

E. Immunoprecipitation (IP) Western blot analysis looking for enrichment of Myc-tagged wildtype PITX2 (Myc-PITX2-WT) or Pro41Ser (Myc-PITX2-P41S) by GFP-NKX2-5 in HEK293T cells. Normalized values on the right.



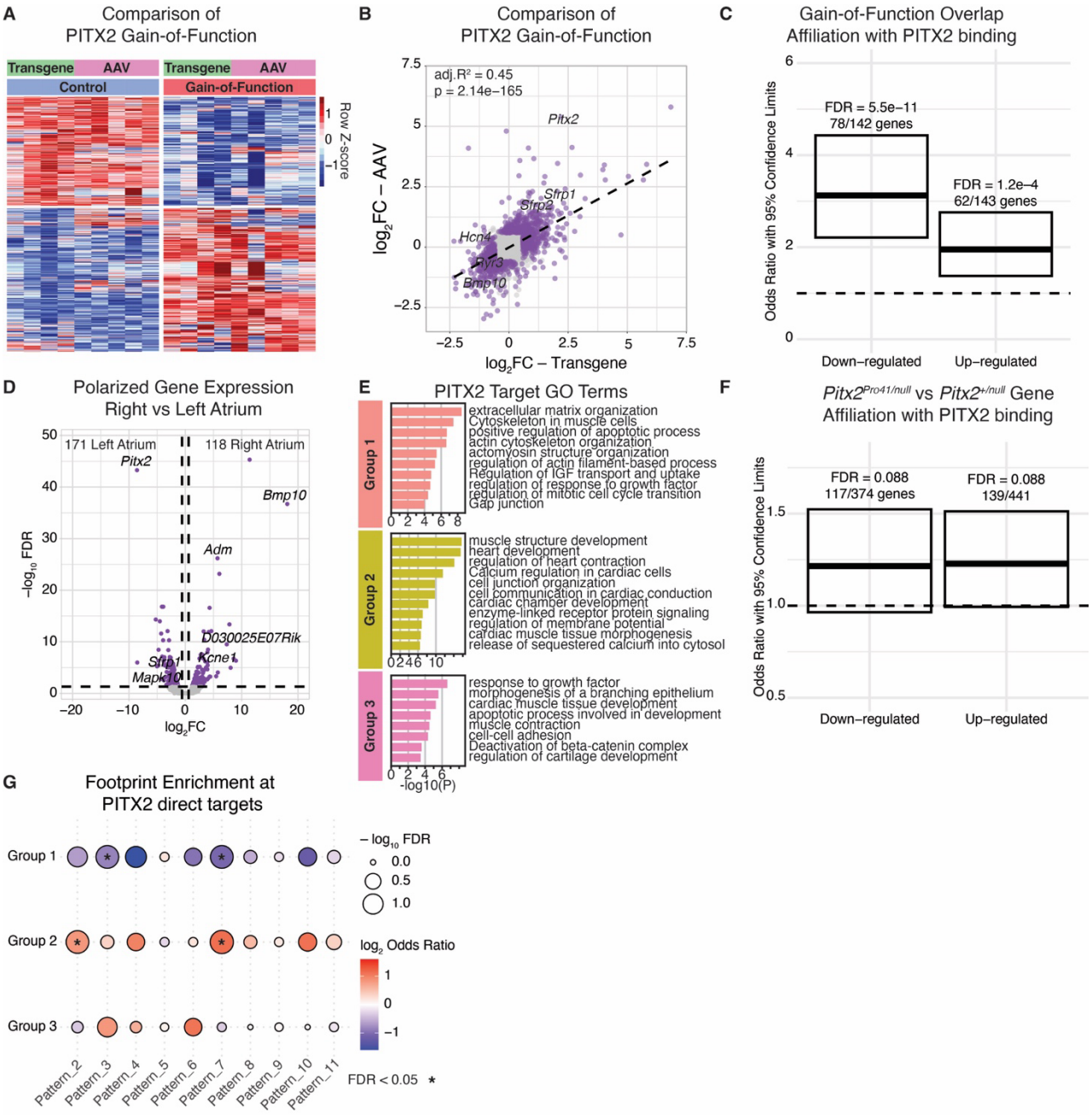
Supplemental Figure 6.

A. Genome-wide distribution of DNA methylation by WGBS at all PITX2 sites in control E13.5 LA tissue, with a median methylation of approximately 24%.

B. Genome-wide distribution of differential DNA methylation by WGBS at all PITX2 sites having a moderate skew ($p < 0.0001$) towards increased methylation in control RA compared to LA, independent of DMR status.

C. Hexagonal heatmap displaying the distribution of DMRs from enzymatic 5hmC-seq comparing *Pitx2*$^{creneo/flox}$ versus *Pitx2*$^{+/flox}$ E13.5 LA.

D. Fisher's Exact Test enrichment of PITX2 footprint-containing peaks at hypomethylated and hypermethylated sites identified by enzymatic 5hmC-seq in E13.5 left atria from *Pitx2*$^{creneo/flox}$ versus *Pitx2*$^{+/flox}$ E13.5 mice.



Supplemental Figure 7.

A. Heatmap displaying the differentially expressed genes in both *Pitx2* gain-of-function models in the RA. Row and column clusters were determined by hierarchical clustering.

B. Scatterplot displaying the log_2FC of the transgene gain-of-function model on the x-axis and the log_2FC of the AAV gain-of-function model on the y-axis. Genes in purple are differentially expressed in both datasets and show a significant correlation with an adjusted $R^2 = 0.45$; p = 2.14e-165.

C. Enrichment by Fisher's Exact Test of PITX2 footprint-containing peaks affiliation to up- and downregulated genes identified by both gain-of-function models, as shown in B.

D. Volcano plot comparing P7 wildtype RA and LA via RNA-seq. Differentially expressed genes (purple), FDR < 0.05, absolute $\log_2$ fold-change ($\log_2$FC) > $\log_2$(1.5).

E. GO terms related to the three groups of genes identified in (**Figure 3I**).

F. Enrichment by Fisher's Exact Test of PITX2 footprint-containing peaks affiliation to up- and downregulated genes identified in the *Pitx2*$^{Pro41Ser/-}$ to *Pitx2*$^{+/-}$ adult LA by RNA-seq (**Figure 1E**).

G. Enrichment by Fisher's Exact Test of non-PITX2 footprints in PITX2 peaks (**Supplemental Figure 4A**) at the different groups of PITX2 targets (**Figure 3I**). Asterisk * indicate comparisons with an FDR < 0.05.