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***Pitx2*, an Atrial Fibrillation Predisposition Gene, Directly Regulates Ion Transport and Intercalated Disc Genes**

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Abstract

Background—*Pitx2* is the homeobox gene located in proximity to the human 4q25 familial atrial fibrillation locus. When deleted in the mouse germline, *Pitx2* haploinsufficiency predisposes to pacing induced atrial fibrillation indicating that reduced *Pitx2* promotes an arrhythmogenic substrate. Previous work focused on *Pitx2* developmental functions that predispose to atrial fibrillation. Although *Pitx2* is expressed in postnatal left atrium, it is unknown whether *Pitx2* has distinct postnatal and developmental functions.

Methods and Results—To investigate *Pitx2* postnatal function, we conditionally inactivated *Pitx2* in the postnatal atrium while leaving its developmental function intact. Unstressed adult *Pitx2* homozygous mutant mice display variable R-R interval with diminished P-wave amplitude characteristic of sinus node dysfunction, an atrial fibrillation risk factor in human patients. An integrated genomics approach in the adult heart revealed *Pitx2* target genes encoding cell junction proteins, ion channels, and critical transcriptional regulators. Importantly, many *Pitx2* target genes have been implicated in human atrial fibrillation by genome wide association studies.

Immunofluorescence and transmission electron microscopy studies in adult *Pitx2* mutant mice revealed structural remodeling of the intercalated disc characteristic of human atrial fibrillation patients.

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Conclusions—Our findings, revealing that *Pitx2* has genetically separable postnatal and developmental functions, unveil direct *Pitx2* target genes that include channel and calcium handling genes as well as genes that stabilize the intercalated disc in postnatal atrium.

Keywords

atrial fibrillation; sinus node dysfunction; genome-wide analysis; genetics; knockout models; *Pitx2*

Atrial fibrillation (AF), the most prevalent adult arrhythmia in humans, results in an increased risk of stroke, dementia and heart failure¹. Electrical impulses that are critical for a coordinated, physiologic heartbeat originate in the sinoatrial node (SAN) and are transduced from the pacemaker region into both atria. In AF, fibrillatory atrial impulses override normal conduction pathways with resulting irregular ventricular conduction.

Because of its clinical significance, great efforts have been expended to investigate the genetic underpinnings of AF using unbiased genome wide approaches such as genome wide association study (GWAS). Multiple studies have revealed a single nucleotide variant (SNV) on human 4q25 that was associated with familial AF (reviewed in²). Patients with the 4q25 variant exhibited early onset AF that was independent from known AF risk factors. The 4q25 SNV also had prognostic value as patients with this variant are prone to AF recurrence after ablation therapy³. The gene poor 4q25 region harbors the *Pitx2* homeobox gene which has been implicated in AF predisposition using mouse models⁴⁻⁷.

Atrial fibrillation and associated arrhythmias

AF may result from new, pathologic sources of electrical impulses. For example, many cases of ectopic electrical activity originate in the pulmonary vein⁸. Other sites of ectopy include the left atrial posterior wall, superior vena cava, interatrial septum, crista terminalis, and coronary sinus myocardium^{9,10}. In addition to ectopy, other causes of AF involve atrial myopathy that disrupts normal atrial conduction and promotes re-entrant circuits. One common example of AF secondary to myopathy is fibrosis that in some cases may be due to elevated Tgf β signaling¹¹.

Work from the Framingham study has shown that patients with PR interval prolongation, also called first degree atrioventricular block (AVB), often develop AF¹². In addition to AVB, A sinus node dysfunction (SND) is also an AF risk factor in human patients¹³. Notably, progression to higher grade arrhythmias with time is also common reflecting the importance of aging in arrhythmogenesis. Although the mechanistic connection between SND, PR interval prolongation, and AF are poorly understood, all three conditions may involve an atrial myopathy with defective atrial impulse conduction¹⁴.

Predisposition to AF may result from a developmental defect that results in an adult heart with subclinical abnormalities that subsequently manifest as overt disease after environmental insults or aging. Alternatively, postnatal homeostatic genes may be required to maintain normal tissue structure and physiology. Small changes in homeostatic gene level may result in subclinical disease until an AF-inducing stress is encountered.

Pitx2 and predisposition to atrial fibrillation

Pitx2 encodes three isoforms, *Pitx2a*, *Pitx2b*, and *Pitx2c* that are generated by alternative splicing and dual promoter usage⁴. *Pitx2c* is generated via an intergenic promoter while the *Pitx2a* and *Pitx2b* isoforms, generated by alternative splicing, use an upstream 5' flanking promoter. The *Pitx2c* isoform is expressed on the left side of the embryo while the *Pitx2a* and *Pitx2b* isoforms are expressed symmetrically in the head within eyes and craniofacial structures⁴. In the mouse, *Pitx2c* expression continues in the postnatal atrium while human PITX2C is also the predominant isoform in left atrium^{4, 6, 15}. Studies in isoform-specific knock out mice in our lab also revealed that the *Pitx2c* isoform is the dominant isoform in determining left right asymmetry (LRA) during development¹⁶. *Pitx2* haploinsufficient (*Pitx2*^{null +/-}) adult mice and *Pitx2c*^{+/-} adult mice were prone to AF when challenged by programmed stimulation indicating that reduced *Pitx2c* levels during development creates an arrhythmogenic substrate^{4, 6}. Notably, *Pitx2* levels are also decreased in the atria of human AF patients⁵.

Previous experiments indicated that SAN genes were expanded in left superior caval vein and left atrium of *Pitx2*^{null/+} and *Pitx2*^{null/null} mutant embryos indicating a developmental defect⁴. Optical mapping experiments showed *Pitx2* conditional mutant embryos had a functional left-sided SAN that could override normal atrial cardiomyocyte depolarization⁷. Furthermore, *Pitx2c* heterozygous adult mice had shortened action potential duration without fibrosis or structural defects suggesting an electrophysiologic mechanism for arrhythmogenesis in *Pitx2c* germline mutants⁶.

It is unknown whether *Pitx2* has a postnatal homeostatic function. To study *Pitx2* postnatal function, we generated a *Pitx2* conditional knock out (CKO) mouse line that deletes *Pitx2* in postnatal atrium. The adult *Pitx2* CKO mice had abnormal electrocardiography with irregular R-R interval and low voltage P waves indicating SND with impaired atrial conduction. A genome-wide search for *Pitx2* targets by ChIP-Seq and microarray assays revealed genes encoding cell adhesion/cell junction proteins, ion channels, and transcription factors. Many *Pitx2* target genes are AF risk genes identified in human GWAS². Using immunofluorescence and transmission electron microscopy (TEM) we obtained evidence for structural remodeling of the intercalated disc (ID) – the structure that mediates cardiomyocyte electro-mechanical coupling¹⁷. Our data indicate a postnatal role for *Pitx2* in AF predisposition and uncover novel *Pitx2* target genes that provide new mechanistic etiologies for AF.

Materials and Methods

Mouse alleles and transgenic lines

The *MCK-Cre* transgenic line, *R26R* transgenic line, *Pitx2* conditional null or floxed allele and Flag knock in allele have been described^{4, 18}. The *Pitx2* conditional null allele contains *LoxP* sites flanking the exon encoding the N terminus of homeodomain and conditionally removes function of all *Pitx2* isoforms.

Telemetry ECG

Implantation of the telemetry transmitter ETA-F10 (Data Sciences International) was done following the protocol of the manufacturer. Transmitters were placed intraperitoneally and ECG leads were at a lead II configuration. ECG data was collected by DSI Telemetric Physiological Monitor System and processed by Dataquest A.R.T. 3.1 software (Data Sciences International) without stress from restraint or anesthesia.

Microarray assay

Pitx2 control and mutant hearts were collected from 3-, 6- and 12-week-old mice. At each time point, three controls and three mutants were collected as biological replicates. cDNA microarray analysis was performed using Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA).

ChIP-Sequencing

Chromatin immunoprecipitation was performed as described previously using EZ ChIP™ kit (Millipore) and Anti-FLAG M2 Affinity Gel (Sigma)⁴. For library construction, 10-50 ng of purified ChIP DNA was end-repaired using Ion Plus Fragment Library Kit (Part no. 4471252, Life Technologies) and purified with two rounds of AMPure® XP beads (Beckman Coulter, Brea, CA) capture to size select fragments 100–250 bp in length. End-repaired DNA was ligated to Ion Torrent compatible adapters. Followed by 15 cycles of PCR amplification before downstream template preparation. DNA fragments of completed library ranged from approximately 170–220 bp in length. Sequencing was performed on Ion Torrent PGM system (Life Technologies).

Statistical analysis

Microarray raw data were generated using Affymetrix MAS5.0, global scaling was performed to set mean target intensity to 100. The normality of the microarray data was checked using quantile-quantile plot and Shapiro-Wilk test on R (3.0.1). Differential expressed genes were detected using R package limma (3.16.8)¹⁹ with threshold p-value 0.05 and fold change ≥ 1.5 . Benjamini-Hochberg correction was used to calculate false discovery rate. All the differential expressed genes have FDR < 0.35 . Clusters were automatically detected using R package HOPACH (2.20.0)²⁰. The distance matrix was calculated using cosine angle method. Over-represented gene ontology terms were identified using GO-Elite²¹ with Z score > 3 , FDR < 0.10 . FDR was calculated using Benjamini-Hochberg correction after permutation ($n = 5000$). qRT-PCR and luciferase reporter assay results were tested using two-sample Wilcoxon test in R. Difference of R-R interval between wild type and mutant mice was tested using two-tailed, unpaired Student's t-test using R ($n > 400$). ChIP-Seq peaks were identified using Homer²² under the assumption that the local density of the reads follow an Poisson distribution. The peaks were called using cutoff: read number enrichment ≥ 4 folds, FDR < 0.001 , FDR effective Poisson p-value $< 3.7e-8$, and minimum read number ≥ 5 .

Results

Generation of *Pitx2* conditional knock out mice

To generate a *Pitx2* conditional loss of function (*Pitx2* CKO) allele that deletes all *Pitx2* isoforms in postnatal atrium, mice with the *muscle creatine kinase (MCK)-Cre* driver were crossed to mice bearing the *Pitx2* conditional null (Flox) allele (Fig. S1 A). *MCK-Cre* directs Cre activity in skeletal and cardiac muscle with a perinatal onset around birth^{18, 23}. We examined Cre activity using the *R26R LacZ* reporter. MCK-Cre activity initiates in atria after birth and within ventricles in a mosaic pattern after E15.5 (Fig. S1B). Since *Pitx2* is predominantly expressed in left atrium at these fetal stages and in adult, *MCK-Cre* is a valuable tool to address *Pitx2* function in postnatal left atrium^{4, 16}. Both immunostaining and realtime RT-PCR revealed that *Pitx2* was efficiently deleted in neonatal left atrium (Fig. S1 C, D). Although *Pitx2* is also inactivated in skeletal muscle, we did not detect skeletal muscle phenotypes in *Pitx2* CKO mice perhaps due to overlapping function with *Pitx3*²⁴.

Pitx2 CKO mice have abnormal cardiac conduction

We implanted telemetry transmitters into control and *Pitx2* CKO adult mice and collected resting electrocardiography (ECG) data in awake mice. Compared to normal sinus rhythm in controls (Fig. 1 A), ECG tracing for *Pitx2* CKO mice show abnormal heart rate with irregular R-R intervals and low voltage P waves (Fig. 1 B, Table S1). This phenotype was observed in all *Pitx2* CKO mice studied by resting telemetry ECG but none of the controls (Table S2). As sinus node sets heart rhythm and P wave represents atrial depolarization, these phenotypes indicate sinus node dysfunction (SND) with impaired atrial conduction. Importantly, SND is closely associated with AF in human patients¹².

Unbiased discovery of *Pitx2* regulated target genes

We performed microarray transcriptional profiling on 3-, 6-, and 12-week-old whole hearts from *Pitx2* CKO and control mice (Fig. S2). Most genes were upregulated in *Pitx2* CKO mutant hearts suggesting that *Pitx2* is a transcriptional repressor in the adult heart (Fig. 2 A, S3). We performed clustering analysis on all differentially expressed genes and identified five gene expression categories in *Pitx2* CKO hearts compared to controls. Groups I, IV, and V genes were significantly upregulated in the *Pitx2* CKO at the 12 week stage. In contrast, group III genes were upregulated at the 3 and 6 week stages in *Pitx2* CKO hearts. Group II genes, the only gene class that was downregulated, were reduced at 12 weeks in *Pitx2* CKO hearts (Fig. 2 B). Genes in groups I, IV, and V were involved in cell junction assembly, as well as, proliferation and migration (Fig. 2 C).

To investigate *Pitx2* direct target genes, a ChIP-Seq assay was performed at the 12 week stage on heart tissue. Overlay of *Pitx2* ChIP-Seq data and the 12-week expression profiling revealed genes that are likely direct *Pitx2* target genes. Gene ontology (GO) analysis of the ChIP-Seq/microarray overlay gene-set indicated that most *Pitx2* target genes are involved in cell-cell junction organization and ion channel physiology (Fig. 3 A, S4). Another important GO term providing insight into *Pitx2* mediated arrhythmias was arrhythmogenic right ventricular cardiomyopathy (ARVC) thought to be a desmosome defect. We categorized *Pitx2* direct target genes into four main groups based on gene function including cell

junction assembly (Group A), ion transport (Group B), ARVC (Group C), and transcriptional regulation (Group D, Fig. 3 B). Enriched ChIP sequencing tags (peaks) were identified in potential regulatory chromosomal regions for these targets (Fig. 4 A, S5).

Motif analysis identified the Pitx2 binding element as the most highly enriched in the ChIP-Seq dataset validating our experiment²⁵ (Fig. 3 C). Other transcription factor binding sites were also enriched in the ChIP-Seq data set providing further insight into potential Pitx2 co-factors in the adult heart (Fig. 3 C). Among potential Pitx2 co-factors, Nkx2.5 is of great interest because it has been implicated in human AF in GWAS²⁶.

Our data sets indicated that other genes that have been implicated in human AF through GWAS or other genetic analysis are potential Pitx2 target genes such as *Kcnq1*, *Cav1*, and *Zfx3*²⁷⁻³³. Other Pitx2 target genes such as *Cacna1d* and *Tbx20*, have also been directly implicated in AF in mouse models or other human arrhythmias such as prolonged QRS (Table S3)^{26, 34-36}.

Pitx2 directly regulates genes involved in cell junction assembly, ion transport, and transcriptional regulation

We validated gene expression changes for genes in ChIP-Seq/microarray overlay by realtime RT-PCR. We also evaluated a number of candidate genes, previously implicated in human arrhythmias, that were unchanged on the microarray. Among these were *Kcnq1*, *Cav1*, *Tbx20*, and *Zfx3*. In *Pitx2* CKO hearts, we found increases in gene expression levels for ion channel and calcium handling genes such as *Cacna1d*, *Cacna2d2*, *Kcnq1*, *Kcnj11*, *Ryr2*, *Jph2*, and *Atp2a2*. We saw upregulated expression of genes encoding transcriptional regulators Hdac7, *Tbx20*, and *Zfx3* as well as *Cav1*, a component of calveolae.

Significantly, elevated Hdac activity has been implicated in AF vulnerability, structural remodeling, and fibrosis³⁷. *Tbx20* is a central component of a gene regulatory network that includes channel genes and other transcriptional regulators³⁸. *Zfx3*, a homeodomain zinc finger transcription factor that was implicated in AF via GWAS, is also required for normal pituitary development as is *Pitx2*³⁹. Caveolins are known to associate with channel proteins and are thought to modify channel function⁴⁰. Our results also revealed higher mRNA expression levels for the genes *Gja1*, *Emd*, *Dsp*, and *Plec*, encoding ID components that are required for structural homeostasis and signaling between cardiomyocytes (Fig. 4B). Genes that were unchanged or reduced in RT-PCR assays are listed in Table S4.

Based on phylogenetic conservation and DNAase hypersensitivity, we cloned 1-3 kb DNA sequences flanking multiple Pitx2 ChIP-Seq peaks (Fig. 4A) into luciferase reporters and performed transactivation experiments in cultured cells. Luciferase activity for the ChIP-Seq regions that are in close proximity to *Tbx20*, *Cacna1d*, *Kcnq1*, *Cav1*, and *Emd* were significantly repressed upon Pitx2 co-transfection indicating Pitx2 directly represses gene expression through binding to these chromosomal regions (Fig. 4C).

Pitx2 mutants have an atrial cardiomyopathy with disrupted junctional complexes

Among the top Pitx2 target gene function categories are cell junction assembly and ARVC. To investigate ID status in *Pitx2* CKO hearts, we used immunostaining with antibodies

against β -catenin to label adherens junctions in the ID. β -catenin mediates cell adhesion between cardiomyocytes and also functions as a cell signaling molecule once activated by Wnt signaling. In controls, β -catenin clearly marked IDs while in *Pitx2* CKO there were fewer distinct IDs and more β -catenin expression on lateral aspects of cardiomyocytes (Fig. 5 A-D).

We used transmission electron microscopy (TEM) to further investigate ID structure in *Pitx2* mutants. In contrast to controls (Fig. 5 E-G), *Pitx2* mutant IDs had widened spaces between junctions indicating ID deterioration (Fig. 5 H-J). We also found evidence for mitochondrial dysfunction with swollen and vacuolated mitochondria in *Pitx2* CKO hearts (Fig. 5 H-J). *Pitx2* CKO cardiomyocytes also had indistinct Z-lines indicating that *Pitx2* is required for maintaining normal sarcomere cytoarchitecture (Fig. 5 H-J).

Discussion

Pitx2 is the gene in proximity to the 4q25 familial AF locus. Previous work indicated that reduced *Pitx2* levels result in AF predisposition. Prior to our experiments, it was unknown whether *Pitx2* was important in postnatal heart since previously reported adult phenotypes could result from developmental defects. In addition to uncovering a *Pitx2* postnatal function, our findings reveal novel *Pitx2* target genes and that *Pitx2* is required for ID homeostasis in postnatal atrium (Fig. 6).

***Pitx2* postnatal deletion results in sinus node dysfunction**

Pitx2 deletion in postnatal atrium caused resting atrial arrhythmias indicating that in addition to its developmental function, *Pitx2* has a genetically separable postnatal function in left atrial homeostasis. The predominant arrhythmia we observe with resting telemetry is SND that is known to be clinically linked to AF in human patients. SND, can result from defective impulse generation from the sinus node. In addition, SND similar to AF, is associated with diffuse atrial remodeling resulting in an arrhythmogenic substrate. Consistent with this, we observe anatomic disruption of cardiomyocyte junctions in *Pitx2* CKO hearts¹⁴.

The *Pitx2c*^{+/-} mice that have a 37% reduction in *Pitx2c* levels have no structural abnormalities or obvious histologic defects but are still prone to AF⁶. Molecular analysis indicates that *Pitx2c*^{+/-} mice have electrical remodeling with changes in channel gene expression. The authors also noted changes in desmosomal genes, such as *Dsg2*, raising the possibility that *Pitx2c*^{+/-} mice may also be prone to structural remodeling. Taken together with previous studies, our findings reveal that *Pitx2* has both developmental and postnatal functions that predispose the heart to atrial arrhythmias.

***Pitx2* regulates intercalated disc maturation and homeostasis**

Our array data reveal that major changes in gene expression were detected at 12 weeks of age. Moreover, ChIP-Seq data support the model that *Pitx2* directly regulates genes involved in ID homeostasis. Structural remodeling of intercellular connections and an increase in fibrosis represent known anatomic substrates for AF. Notably, *Pitx2* mutants have upregulated expression of ID genes suggesting that the correct stoichiometry of ID

component genes is important to maintain ID structure. The immunofluorescence showing β -catenin mislocalization and TEM revealing breakdown of intercellular junctions also strongly support the notion that *Pitx2* is required for ID maturation and homeostasis.

In the developing and postnatal heart, ID components including desmosomes, gap junctions, and adherens junctions are initially expressed in a dispersed pattern. As the heart matures, ID components become localized into the ID at the cardiomyocyte termini⁴¹. At the transcriptional level, expression of genes such as *Emd* and *Gjal* is reduced as the heart matures^{42, 43}. Intriguingly, *Emd* is known to function as a β -catenin interacting protein that also modulates β -catenin intracellular localization⁴⁴. It is conceivable that lateralized β -catenin that we observe in the *Pitx2* mutant left atrium may be due to elevated *Emd* levels. Together, our data indicate that *Pitx2* is required for this transcriptional downregulation of ID component genes in left atrium myocardium.

Our findings have implications for AF progression to chronic AF. The severe anatomic abnormalities observed in *Pitx2* CKO hearts suggest that these are irreversible structural defects. Moreover, the TEM data suggest that the *Pitx2* CKO hearts have deteriorating mitochondria suggesting cellular compromise further supporting the notion that *Pitx2* CKO arrhythmias are irreversible. The TEM data also revealed that *Pitx2* CKO atrial cardiomyocytes had Z-line defects suggesting that *Pitx2* also regulates sarcomere homeostasis. Interestingly, we previously observed formation of M-lines and more mature sarcomeric structure in *Pitx2* CKO extraocular muscle, that normally lacks M-lines, suggesting context dependent functions for *Pitx2* in sarcomere regulation⁴⁵.

***Pitx2* and ion current regulation**

Our data support previous observations that multiple genes involved in calcium handling and potassium channels are changed in *Pitx2* mutants⁶. The ChIP-Seq data indicate that multiple genes that compose the calcium release unit such as *Ryr2*, *Jph2*, and *Atp2a2* are directly regulated by *Pitx2*⁴⁶. Genes involved in calcium homeostasis have been implicated in both AF initiation and progression to chronic AF⁴⁷. For example, Ca²⁺/calmodulin dependent protein kinase II delta (CaMKII delta) has been shown to phosphorylate the Ryanodine Receptor 2 (RyR2) at S2814 resulting in incomplete RyR2 closure with calcium leak. Increased phosphorylation of RyR2 has been demonstrated in human patients with chronic AF, as well as, other AF models^{48, 49}. Our ChIP-Seq/microarray datasets indicate that *RyR2* is upregulated in *Pitx2* mutant adult hearts suggesting that in addition to phosphorylation transcriptional regulation may be a regulatory node for modulating calcium handling in atrial cardiomyocytes.

In *Pitx2* CKO postnatal hearts the inward rectifier *Kcnj11* was both directly bound by *Pitx2* and upregulated at the mRNA level, a likely AF risk factor⁵⁰. This upregulation is consistent with the shortened action potential duration that has been previously described in *Pitx2* mutants^{5, 6}.

Pitx2 and AF in the developing and postnatal heart

Our findings suggest that postnatal atrial cardiomyocytes require persistent *Pitx2* expression for ID homeostasis and maturation. *Pitx2* regulates separate mechanisms, a developmental mechanism and a postnatal homeostatic mechanism, that result in an arrhythmogenic substrate. This is also reflected in the expression dynamics of *Pitx2* target genes in the developmental and postnatal contexts. *Scn5a* and *Kcnj10* are downregulated in *Pitx2* mutant atrium during development but upregulated in postnatal mutants⁵. One explanation for this is that *Pitx2* may have separate cofactors at developmental and postnatal stages that result in distinct transcriptional readouts.

Our findings also have implications for human AF. Previous GWAS implicated multiple genes as potential contributors to the pathogenesis of human AF and prolonged PR interval². Our *Pitx2* ChIP-Seq data identified several of these genes as potential *Pitx2* target genes in postnatal hearts (Table S2)^{2, 26}. Since *Pitx2* regulates many of these genes in the postnatal and adult heart, it is conceivable that drugs can be developed to modulate the molecular interaction between *Pitx2* and its target genes. Since fetal therapies currently lack feasibility, our findings that many important *Pitx2* regulated events occur postnatally strengthens the likelihood that *Pitx2* mediated AF may be treatable in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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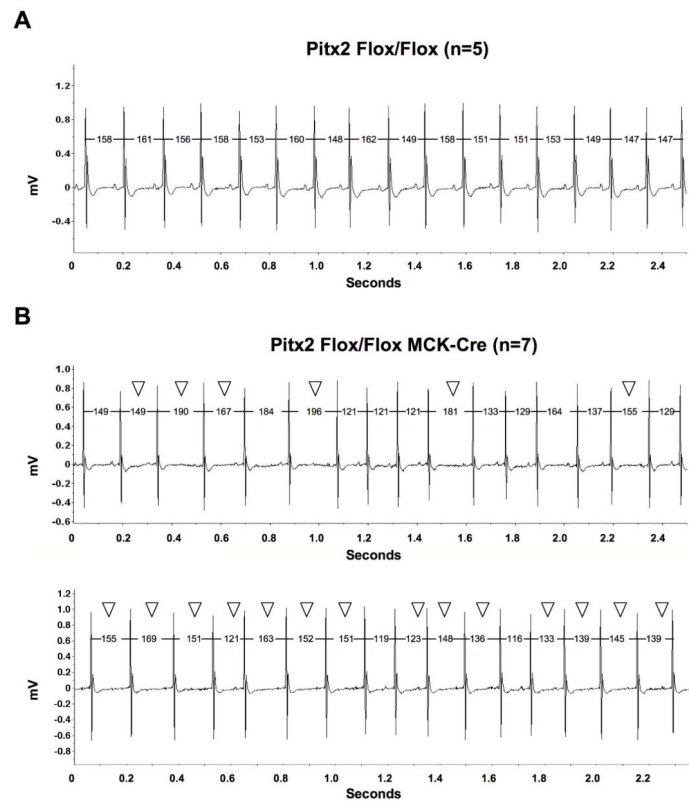


Figure 1.

Pitx2 conditional knock out mice have abnormal heart function – sinus node dysfunction with impaired atrial conduction. By telemetry ECG tracing in awake mice, heart function of the adult control (*Pitx2* Flox/Flox) and *Pitx2* CKO (*Pitx2* Flox/Flox *MCK-Cre*) mice were tested. (A) In control mice, normal sinus rhythm was observed. (B) In the *Pitx2* CKO mice, mutant heart function including irregular R-R intervals and low voltage of P waves (open arrowheads) were observed. Tracings from one control and two *Pitx2* CKO mice are shown. All mice shown were 3-month-old. The unit of R-R interval is millisecond (ms).

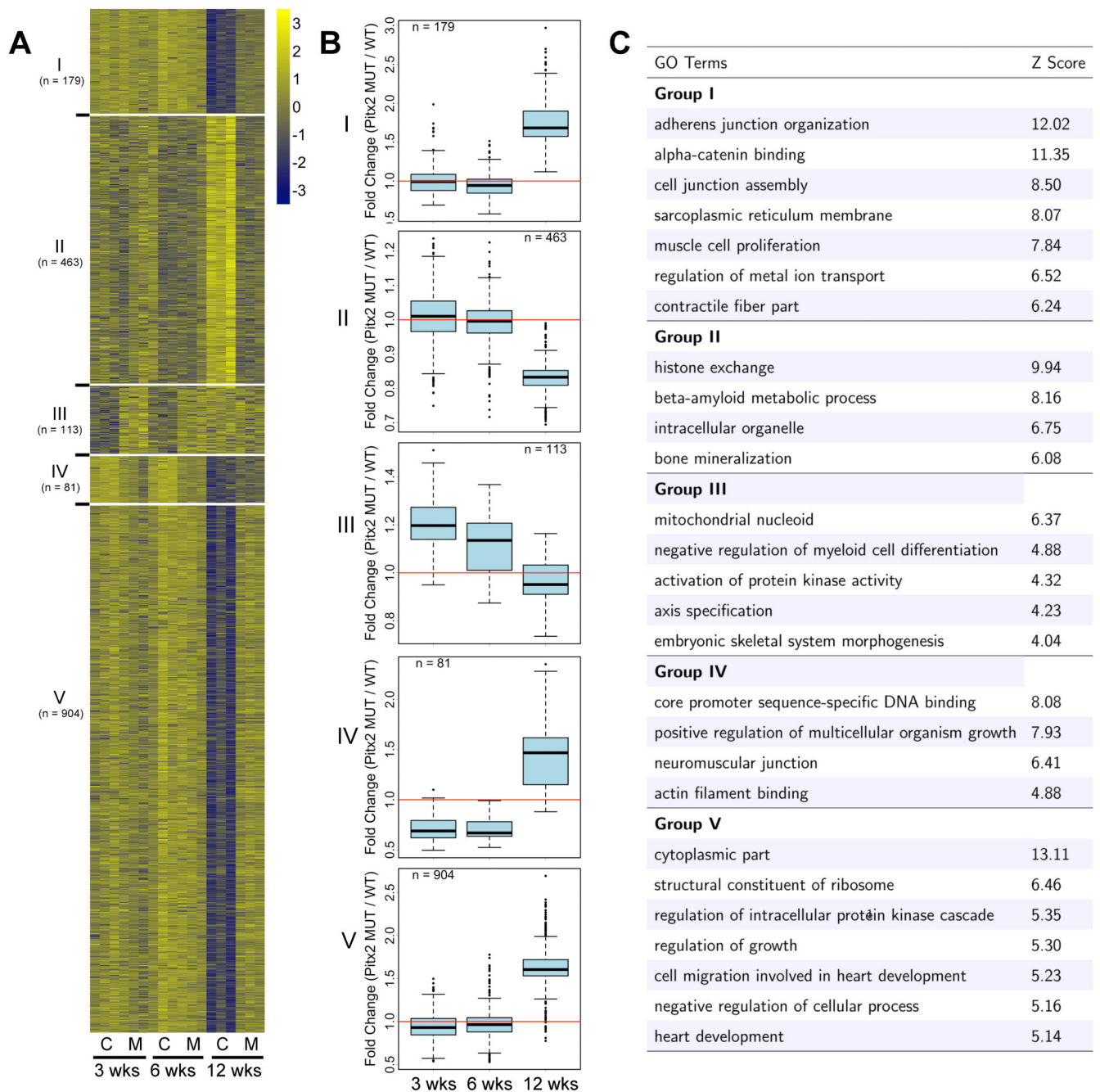


Figure 2.

Age-related gene expression profiling in *Pitx2* mutants. (A) Clustering analysis of gene expression profile across three time points in control and *Pitx2* mutant hearts. Expression patterns of five representative groups are shown in heat map. The color bar represents relative expression level for each gene across different samples. (B) Differential expression between *Pitx2* mutant and control across three time points. Mean value for each group was used to calculate fold change between mutants and controls. Box plots for selected clusters show median (middle line), third and first quartile (top and bottom hinges of the boxes), whiskers and outliers (black dots). (C) Gene ontology analysis of genes in each group. GO terms related to biological process and molecular function categories.

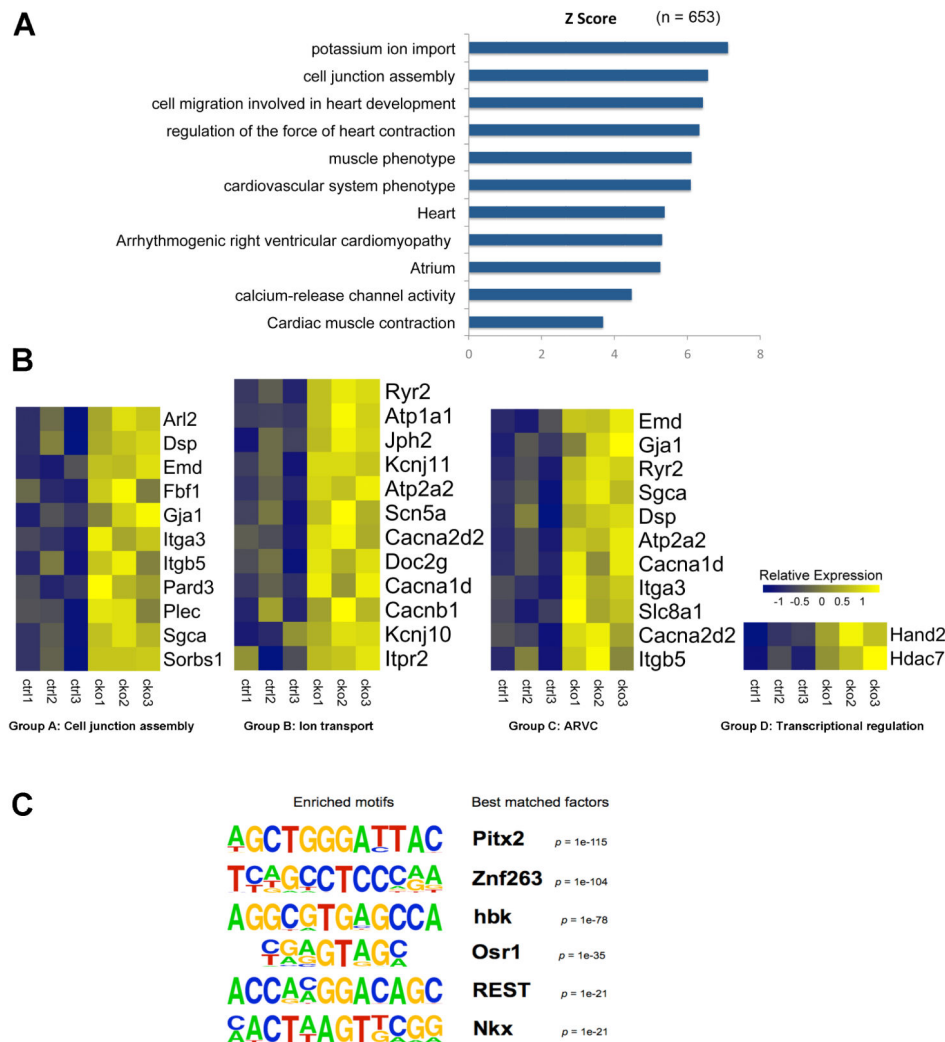


Figure 3.

Overlay of Pitx2 ChIP-Seq and gene expression profiling assays for *Pitx2* CKO heart. (A) Gene ontology (GO) analysis for genes (n=653) from overlay of Pitx2 ChIP-Seq and *Pitx2* CKO microarray assays (genes in close proximity to Pitx2 binding chromosomal regions identified by Pitx2 ChIP-Seq assay that also show significant change in microarray assay for *Pitx2* CKO heart). Both assays performed on 3-month-old adult mouse hearts. (B) Heat map of gene expression profiling obtained by *Pitx2* CKO microarray assay. The color bar represents relative expression level of log-transformed value for each gene across different samples. Ctrl1-Ctrl3: control mouse heart (*Pitx2* Flox/Flox); CKO1-CKO3: *Pitx2* CKO mouse heart (*Pitx2* Flox/Flox *MCK-Cre*). (C) Motif analysis for peaks from Pitx2 ChIP-Seq (n=11,280). Enriched DNA binding motifs and best matched factors are shown.

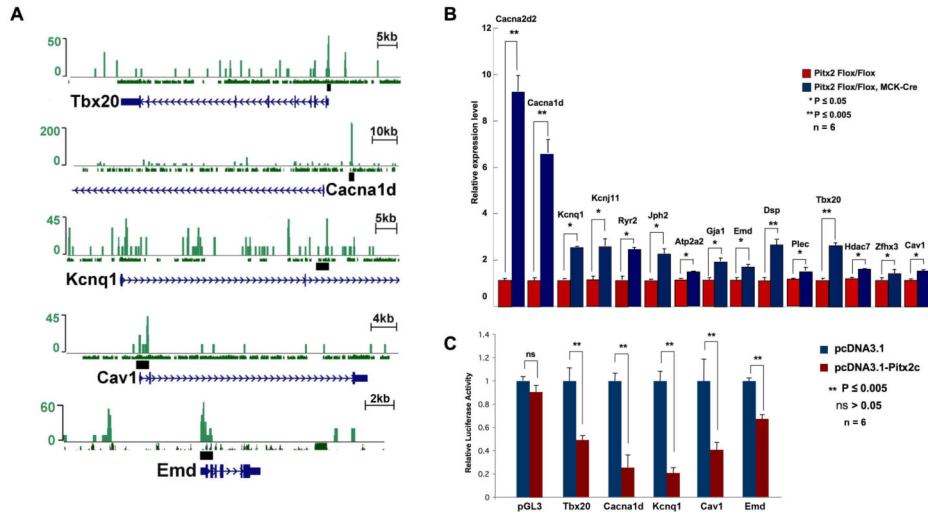


Figure 4.

Validation of potential Pitx2 targets from ChIP-Seq and microarray assays. (A) Genome browser tracks for potential targets of Pitx2 in adult heart identified by ChIP-Seq. Peaks from ChIP-Seq, conservation with human genome and the chromosomal regions used in reporter assay are shown. Normalized ChIP-Seq tag numbers are shown on the Y-axis. (B) Alteration in expression levels for the potential Pitx2 targets were detected by realtime RT-PCR in the left atrium of 3 to 4-month-old control and *Pitx2* conditional knock out mice. (n=6) (C) Reporter assay for ChIP-Seq identified potential targets of Pitx2. Chromosomal regions were named after the genes in closest proximity. Values and error bars represent mean and standard deviation (n=6).

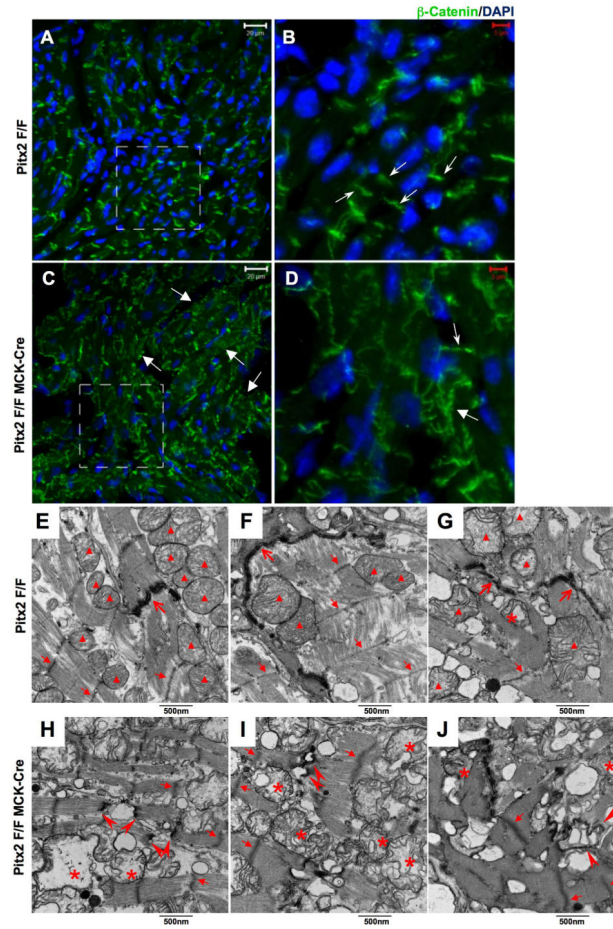


Figure 5.

Pitx2 CKO mouse heart has altered expression pattern for β -catenin and disrupted intercalated discs (IDs). (A-D) β -catenin immunostaining on left atrium sections of 3-month-old control (*Pitx2* Flox/Flox) (A-B) and *Pitx2* CKO (C-D) mice shows enhanced expression of β -catenin on cardiomyocytes lateral aspect (big arrows in D) and in cytoplasm (big arrows in C). Small arrows point to intercalated discs. (E-J) Electron microscopy shows disrupted IDs in 1-year-old *Pitx2* CKO heart. (E-G) in control left atrium, IDs (big arrows) and Mitochondria were intact (triangles). (H-J) in *Pitx2* CKO left atrium, some IDs were disrupted (arrowheads). Large number of mitochondria were deteriorated (asterisks). Indistinct Z-lines were observed in *Pitx2* CKO left atrium (small arrows).

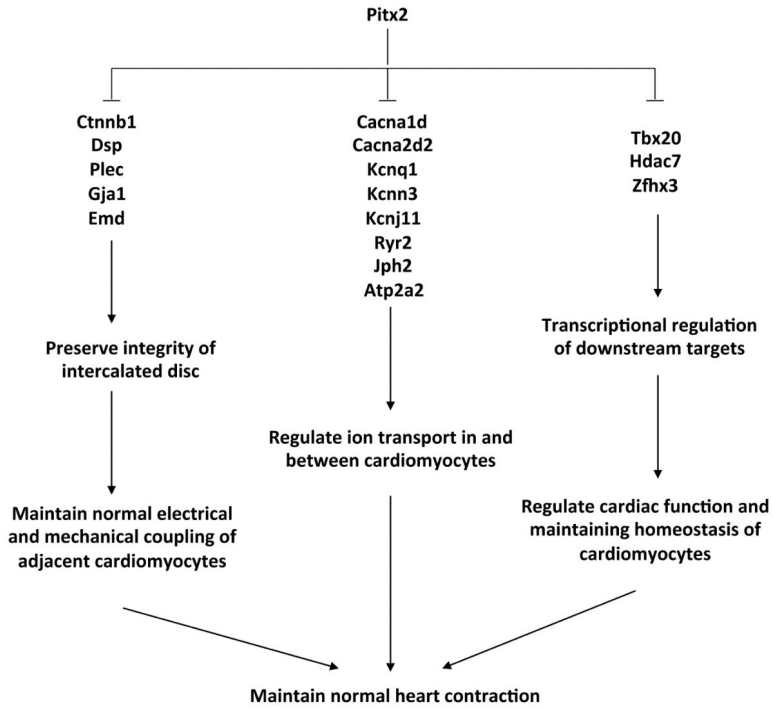


Figure 6.

Model of Pitx2’s function in postnatal heart. In postnatal heart, Pitx2 binds to its targets through DNA binding domain and regulates expression of targets. Three groups of genes were identified to be targets of Pitx2. The first group contains genes regulating cell adhesion/cell junction assembly, which is critical for preserving normal structure of intercalated disc. The second group contains genes that regulate ion transportation in and between cardiomyocytes, which are important for normal conduction of electrical signal that induces heart contraction. The third group contains transcription regulators that regulate a large number of downstream targets and involved in regulating cardiac function and maintaining homeostasis of cardiomyocytes.