SUPPLEMENTAL MATERIAL

Supplementary Materials & Methods

Human tissue and DNA samples

Atrial myocardial tissue samples were obtained from patients undergoing cardiac surgery. Specimens were obtained from the right or left atria just prior to atrial cannulation for cardiopulmonary bypass. After excision, samples were rapidly frozen in liquid nitrogen and stored at -80°C until analyzed. The atrial samples were classified as patients with atrial fibrillation (AF) and without (No AF) a recorded history of AF. Although the atrial tissue samples consisted of tissue that would normally be discarded during surgery, permission to be used in this study was obtained from each patient. The study was approved by the Ethical Committee of the Hospital de la Santa Creu i Sant Pau (Barcelona) and the investigation conforms with the principles outlined in the *Declaration of Helsinki*.

Genomic DNA samples from 47 patients diagnosed of having atrial fibrillation and 100 healthy donors with no cardiac structural and/or functional diseases were obtained from the Spanish National DNA Bank (BNADN, Salamanca). This study was approved by the Ethical Committees of the Spanish National DNA Bank (BNADN, Salamanca) and of the University of Jaén and the investigation conforms with the principles outlined in the *Declaration of Helsinki*.

Transgenic mouse lines and breeding strategy

The *Pitx2*floxed, NppaCre and Mlc2vCre transgenic mouse line have been previously described (5, 11, 12). Generation of conditional atrial (NppaCre) and ventricular (Mlc2vCre) mutant mice was performed by intercrossing hemizygous Cre deletor mice with homozygous *Pitx2*floxed mice. Double heterozygous were selected by PCR and subsequently crossed with homozygous *Pitx2*floxed mice, respectively, yielding to controls (Cre-) (i.e. Mlc2vCre^{-}*Pitx2*$^{flox/flox}$ and NppaCre^{-}*Pitx2*$^{flox/flox}$, respectively), heterozygous Cre+/floxed (i.e. Mlc2vCre^{+}*Pitx2*$^{fl/-}$ and NppaCre^{+}*Pitx2*$^{fl/-}$, respectively) and homozygous Cre+/floxed (i.e. Mlc2vCre^{+}*Pitx2*$^{-/-}$ and NppaCre^{+}*Pitx2*$^{-/-}$,

respectively). Since homozygous mice were viable to adulthood in both conditional deletions, mice have been bred into a pure C57Bl/6J genetic background, and offspring from mouse lines matting were routinely screened for the presence of the *Pitx2* floxed allele and the Cre sequence as previously reported (15). This investigation conform the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Mouse genotyping

DNA for PCR screening was extracted from adult ear and/or tail samples and from the yolk sac in embryos. Screening of Cre and *Pitx2* floxed alleles was routinely done using used specific primers as detailed in Supplementary Table 1. Cycling conditions for Cre were as follows; 5 min at 95ºC, 35 cycles of 30s at 95ºC, 30s at 60ºC and 90s at 72ºC, and for *Pitx2* as follows; 5 min at 95ºC. 40 cycles of 30s at 95ºC, 30s at 60ºC and 90s at 72ºC, followed by a final extension step of 10 min at 72ºC, respectively. PCR products were separated in standard agarose electrophoresis and classified according to the expected band size.

Anatomical and histological analyses

Mice were sacrificed by cervical dislocation, after ECG recordings (see below). Adult hearts were carefully dissected and briefly rinse in Ringer´s solution and photographed. Samples processed for histochemistry and immunohistochemistry were fixed overnight in freshly made sterile 4% paraformaldehyde. Samples processed for RNA isolation were immediately snap-frozen in liquid nitrogen and stored at -80ºC until used. Staged wild-type control and chamber-specific *Pitx2* conditional embryos (E13.5, E15.5 and E17.5) were carefully dissected from uterus of time-controlled pregnant females, briefly rinse in sterile PBS and processed accordingly, Adult and embryonic samples processed for histochemistry and immunohistochemitry were dehydrated through graded ethanol steps and embedded in paraplast. Sections were cut at 10 µm and

processed for hematoxylin and eosin, Mallory´s thrichrome and/or picrosirius staining.

mRNA Isolation and Reverse Transcription

Embryonic hearts (E17.5) were dissected from pregnant tissue-specific conditional mutants. Neonatal (3 weeks) and adult Mlc2vCre*Pitx2* and Nppa-Cre*Pitx2* conditional mutants were also obtained. For NppaCre*Pitx2* (NppaCre^{-}*Pitx2*$^{flox/flox}$ and NppaCre^{+}*Pitx2*$^{-/-,}$ respectively) mouse mutants we carefully separately dissected the left atrial chambers, the right atrial chambers and the ventricular chambers, and stored in liquid nitrogen. For MlcvCre*Pitx2* (Mlc2vCre^{-} *Pitx2*$^{flox/flox}$ and Mlc2vCre^{+}*Pitx2*$^{-/-,}$ respectively), only the ventricular chambers were dissected and stored in liquid nitrogen.

RNA extraction was performed using six E17.5 pooled left, right atrial or ventricular samples of embryonic NppaCre*Pitx2* conditional mutants, respectively, corresponding on each case to either control (NppaCre^{-}*Pitx2*$^{flox/flox}$) or homozygous (NppaCre*Pitx2*$^{-/-}$) mutants. For Mlc2vCre*Pitx2* three pooled ventricular myocardium samples of Mlc2vCre^{+}*Pitx2*$^{-/-}$ and their corresponding controls (Mlc2vCre^{-}*Pitx2* $^{flox/flox}$) were used. RNA extraction of adult hearts was performed using three pooled left atrial samples NppaCre *Pitx2* conditional mutants (NppaCre^{-}*Pitx2*$^{flox/flox}$ and NppaCre^{+}*Pitx2*$^{-/-,}$ respectively) and a single ventricular myocardium sample of Mlc2vCre*Pitx2* conditional mutants (Mlc2vCre^{-}*Pitx2*$^{flox/flox}$ and Mlc2vCre^{+}*Pitx2*$^{-/-,}$ respectively). Total RNA was isolated using Trizol (Roche) according to manufacture's guidelines and DNase treated using RNase-Free DNase (Roche) for 1h at 30ºC. In all cases, at least three distinct pooled samples were used to perform the corresponding qRT-PCR experiments.

First strand cDNA was synthesized at 50ºC for 1h using 1 µg of RNA, oligo-dT primers and Superscript III Reverse Transcriptase (Invitrogen) according to manufacture's guidelines Negative controls to assess genomic contamination were performed for each sample, without reverse transcriptase, which resulted in all cases in no detectable amplification product.

qRT-PCR (mRNA)

RT-PCR was performed in Mx3005Tm QPCR System with a MxPro QPCR Software 3.00 (Stratagene) and SYBR Green detection system. Reactions were performed in 96-well plates with optical sealing tape (Cultek) in 20 μL total volume containing SYBR Green Mix (Finnzymes) and the corresponding cDNA. Two internal controls, mouse βactin and GAPDH, were used in parallel for each run. Amplification conditions were as follows: denaturisation step of 95ºC for 10 min, followed by 40 cycles of 95ºC for 30s, 60ºC for 30s, 72ºC for 30s; with final elongation step of 72ºC for 10 min. All primers were designed to span exon-exon boundaries using online Primer3 software Primer3input (primer3 www. cgi v 0.2) as provided in **Table 1**. No amplifications were observed in PCR control reactions containing only water as the template. Each PCR reaction was performed at least three times to obtain representative averages. The Livak method was used to analyze the relative quantification RT-PCR data (37) and normalized in all cases taking as 100% the wild-type (control) value, as previously described (38).

qRT-PCR (microRNA)

miR-1 microRNA qRT-PCR was performed using Exiqon LNA microRNA qRT-PCR primers and detection kit according to manufacturer's guidelines. All reactions were always run in triplicates using 5S as normalizing control, as recommended by the manufacturer. SyBR Green was used as quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Relative measurements were calculated as described by Livak & Schmittgen (37) and control measurements were normalized to represent 100%, as previously described (38).

Electrophysiological measurements

Transmembrane action potentials were recorded in isolated left and right atria of male NppaCre^{-}*Pitx2*$^{flox/flox}$ and NppaCre^{+}*Pitx2*$^{-/-}$ mice

(n=5, per group), and in thin papillary muscles from male Mlcv2Cre^{-} *Pitx2*$^{flox/flox}$ and Mlc2vCre^{+}*Pitx2*$^{-/-}$ mice through glass microelectrodes filled with 3 M KCl (tip resistance, 8-15 MΩ) using procedures described previously (13,14). Multicellular preparations were perfused with a modified Tyrode´s solution of the following composition: NaCl 125, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 24, NaH_2PO_4 0.42 and glucose 11. The solution was bubbled with 95% O_2 and 5% CO_2 (pH=7.4) and maintained at a temperature of 35°C. The microelectrode was connected via Ag-AgCl wire to high-input impedance, capacity-neutralizing amplifiers (model 701; WPI, New Haven, CT, USA). Driving stimuli were rectangular pulses (1-2 ms in duration) delivered from a multipurpose programmable stimulator (CS-220; Cibertec SA, Madrid, Spain). Action potentials were stored in a computer by use of Acknowledge software. The following parameters of the transmembrane action potential were measured: resting membrane potential (RMP), amplitude (APA) and action potential duration (ADP) measured at the 20% (APD_{20}), 50% (APD_{50}), and 90% (APD_{90}) level of repolarization. The preparations were driven at 3 Hz and a period of 1 h was allowed for equilibration, during which a stable impalement was obtained.

ECG recordings

Mice were anesthetized with 2mg/Kg Ketamine (PARKE-DAVIS, S.L.) intraperitoneally. Electrocardiogram (ECG) recordings were registered and analyzed using a digital acquisition and analysis system (Power Lab/4SP; www.adinstrument.com). Dual Bio Amplifier was connected to the ECG Lead Switch Box to enable recording of standard lead configurations. For routine screening, surface ECG (lead II) were recorded from needle electrodes that were inserted subcutaneously in the limbs and tape secured. The signal is acquired for about 10 minutes using Chart 4.2.3 software. When recordings were finished, the limb electrodes are removed and mice were allowed to recover and returned to their cage. The signal averaged ECG waveform and

the 1st derivate were analyzed using SAECG (signal-averaged electrocardiogram) extension for Chart 4 software (AD Instruments).

Cell culture and microRNA-1 transfection assays

HL-1 mouse immortalized atrial myocardial (39) cells were used to assay microRNA-1 gain-of-function experiments. HL-1 cells ($6*10^5$ cells per dish) were culture under appropriate cell culture condition (39) and plated 30mm culture dishes. Pre-miR-1 were transfected with lipofectamine 2000 (Invitrogen) into HL-1 cells at 5 mmol according to manufacturer's guidelines, respectively. Negative controls included non transfected cells as well as FAM-labeled pre-miR negative control transfected cells, which also allow evaluation of the transfection efficiency. In all cases, transfection efficiencies were greater than 50%, as revealed by observation of FAM-labeled pre-miR transfection. After 4 hours transfection, HL-1 cells were culture in appropriate cell culture media as reported by Claycomb et al. 1998. Cells were collected 24h (pre-miR treatment) after transfection. Negative control and transfected cells were collected and processed for RNA isolation using Trizol-base standard protocols. RNA quality and integrity was evaluated using a Nanodrop spectrophotometer and cDNAs were retro-transcribed accordingly. qRT-PCR measurement of several mRNA transcripts was evaluated as described above. Control measurements levels were normalization represent 100%, as previously described (38).

Cell culture, Pitx2c overexpression and siRNA transfection assays

HL-1 cells ($6*10^5$ cells per well) were transfected with CMV-*Pitx2*c construct at two distinct plasmid concentrations (2 and 4 μg/well) using lipofectamine 2000 (Invitrogen), according to manufacturer's guidelines. Cells were harvested for 48 hours and processed for RNA isolation as previously described. Transfection efficiency was evaluated by assessment of CMV-EGFP transfected cells, which resulted in all cases in more that 60% transfected cells. In addition, in

all cases, *Pitx2*c quantitation was evaluated by qRT-PCR, which resulted in 5 to 8-fold increase.

HL-1 cells ($6*10^5$ cells per well) were transfected with siRNA-*Pitx2* (Sigma), at different concentrations, 25nM and 50nM, using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following the supplier's protocol. 10^5 cells per well were seeded and transfected in serum free conditions for 5 hr, after that cells were collected at 24h. siRNA efficiency was measured as percentage of *Pitx2* expression levels as compared to non-transfected controls. In all cases, silencing of *Pitx2* was higher than 70-80%.

Immunofluorescence staining and confocal analysis

Embryonic hearts (E16.5) were extracted from chamber-specific conditional and control pregnant females, respectively. Adult hearts from chamber-specific *Pitx2* conditional and controls were also collected. For immunofluorescent experiments, embryos and isolated adult hearts were fixed overnight in 4% paraformaldehyde in PBS, dehydrated in increasing ethanol steps and embedded in paraplast. Tissues samples were sectioned at 10 μm and mounted in 3-aminopropyl-triethoxy-silane (AAS) coated slides.

Tissue slides were deparaffinised at 65ºC during 30 min, hydrated through decreasing graded ethanol steps, and briefly rinsed in bidest water. Unspecific bindings were blocked for 30 min in TBSA_BSAT (10 mM Tris, 0,9% NaCl, 2% bovine serum albumin, 0,1% Triton X-100, 0,02% sodium azide) at room temperature. Subsequently tissue sections were incubated overnight with the corresponding primary antibody (1:100) diluted in TBSA-BSAT. The antibodies used were: rabbit anti desmin (D8281; Sigma), anti-hcn4 (APC-052, Alomone) and anti-Nav1.5 (ASC-005, Alomone). The excess of primary antibody was removed by a brief rinse in TBSA-BSAT. Thereafter, the sections were incubated in darkness with the corresponding anti-rabbit Cy2-conjugated (Jackson Lab, USA) or anti-mouse TRITC-conjugated (DAKO) secondary antibodies (1:100) respectively, during 5 hours. Sections were washed in TBSA-BSAT,

rinsed in PBS and incubated in DRAQ-5™ (Red Fluorescent Cell-Permeable DNA probe, from Biostatus Limited, UK) diluted (1:1000) in PBS for 10 min. The excess of DRAQ-5™ was removed by a wash in PBS, briefly rinsed in water, dehydrated and mounted in DPX. The specificity of the primary antibody was assessed by lack of primary antibody incubation which resulted in all cases in no detectable signal. The samples were conserved in total darkness until analysed. Images were obtained using a Leica Laser Scanning Confocal Microscope and further edited using Adobe Photoshop software (version 7.0).

Western blotting

Adult hearts from either wild type (NppaCre^{-}*Pitx2*$^{flox/flox}$) or homozygous (NppaCre^{+}*Pitx2*$^{-/-}$) mutants, were collected, processed accordingly and stored in liquid nitrogen. Total protein extraction of was done using single hearts. These samples were lysated in a small volume of 1 ml RIPA buffer (50mM Tris pH 8,2, 1mM EDTA, 0,1% p/v Triton X-100, 1mM PMSF, cocktail protease) using sonication. Protein quantitation was performed using standard Commassie Protein Assay (Pierce). 10mg of total protein was loaded in homogeneous 12,5% SDS-PAGE gels. Gels were blotted onto nitrocellulose and probed against Kir2.1 (ab-80969-500, Abcam) or Nav1.5 (ASC-005, Alomone) while α-tubuline was used as internal loading control (T-5168, Sigma). Primary antibody incubation was performed at 1:200, 1:100 and 1:14000, respectively. Corresponding secondary anti-rabbit or anti-mouse antibodies (1:10000 dilution) were used to reveal Kir2.1, Nav1.5 and α-tubuline, respectively. Signal detection was performed using ECL Plus (GE).

Statistical analyses

qRT-PCR data statistical analyses were performed using unpaired Student t- test. p values <0.05 were considered statistically significant and are stated on each corresponding figure legend. Deviation from the Hardy–Weinberg equilibrium was tested by a Fisher's exact test for testing the null of independence of the group and genotype in a (2 by 2) contingency table with fixed marginals (alternative hypothesis: true odds ratio is not equal to

1; p-values were obtained directly using the hypergeometric distribution. Allele frequencies were estimated from genotype frequencies by gene counting. The study was analyzed as a case/ control study comparing the allele frequency of both SNPs in the atrial fibrillation (47) and control (100) groups. The odds ratio (OR) [95% CI] for both SNPs associated with genotype was estimated from logistic regression analysis adjusted for AF type (isolated *vs* associated) and atrial fibrillation/control. A p-value <0.05 was considered statistically significant. All computations of p-value, OR and confidence interval were carried out with the two ways contingency table analysis software (www.statpages.org/ctab2x2.html). General linear models (GLMs) were carried out for testing the genotype dependence on the independent age and group variables. The outcome was generated using a binomial distribution (for testing the presence or absence of mutation) under a logistic model as the link function.To analyze simultaneously both polymorphisms, haplotype analyses were performed by use of Haploview software. In these analyses, the haplotype combining the risk alleles at each locus was used as the reference.

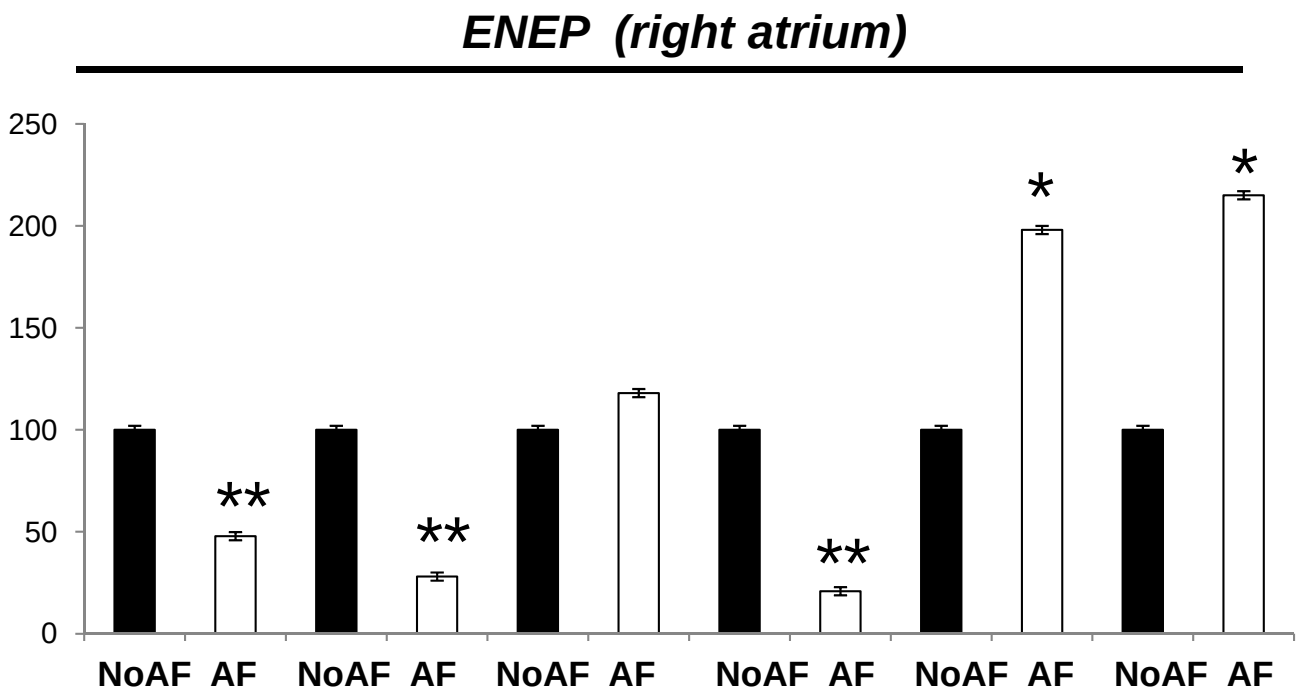
References

37. Livak KJ. Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-408.

38. Domínguez JN. Navarro F, Franco D, Thompson RP, Aránega AE.Temporal and spatial expression pattern of beta1 sodium channel subunit during heart development. Cardiovasc Res. 2005; 65:842-850.

39. Claycomb WC. Lanson NA Jr, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A. 1998; 95:2979-2984.

A



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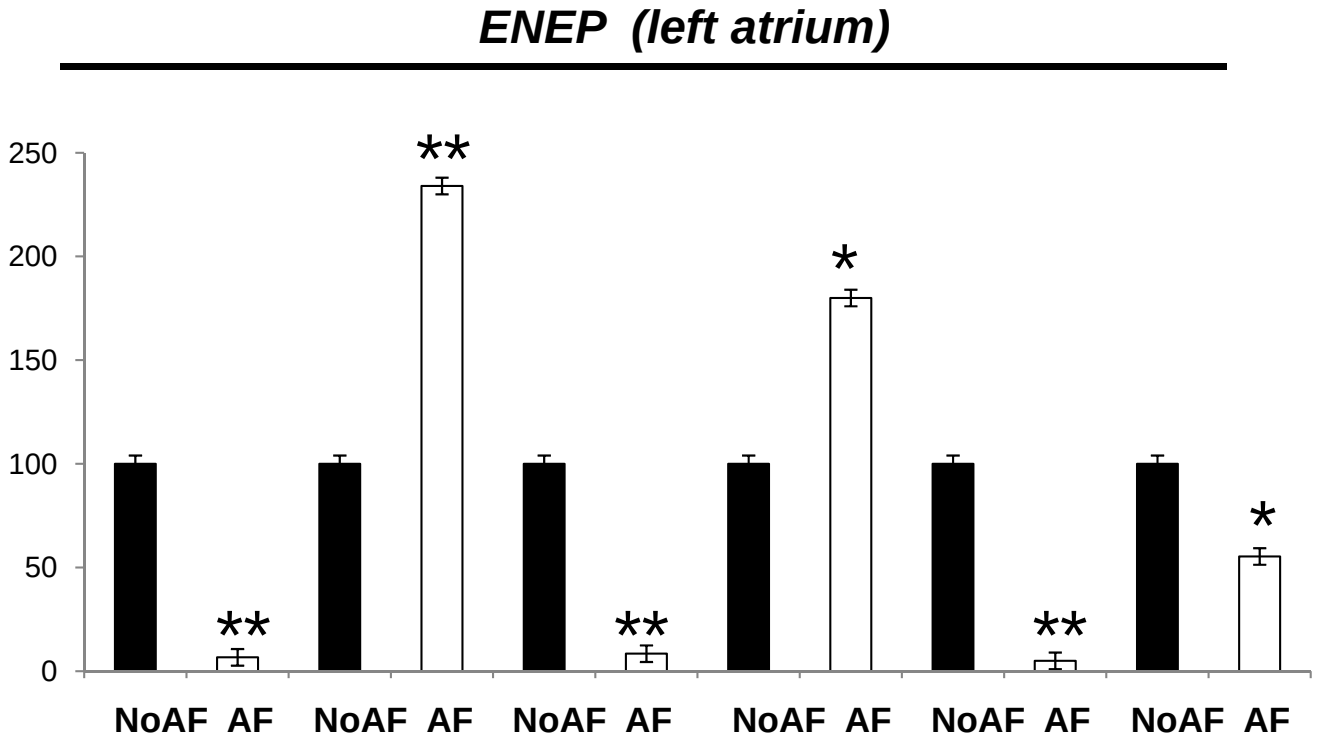
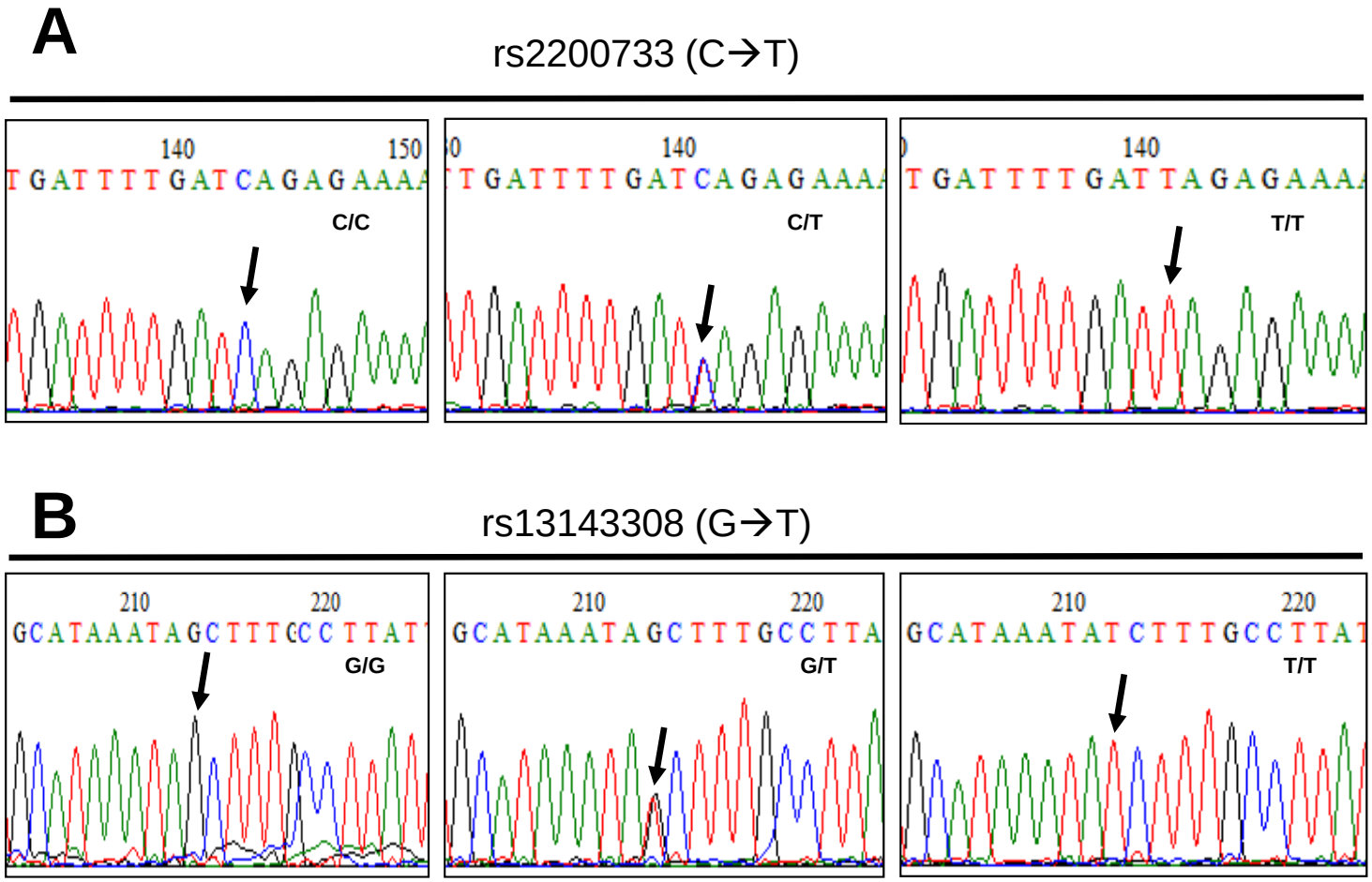
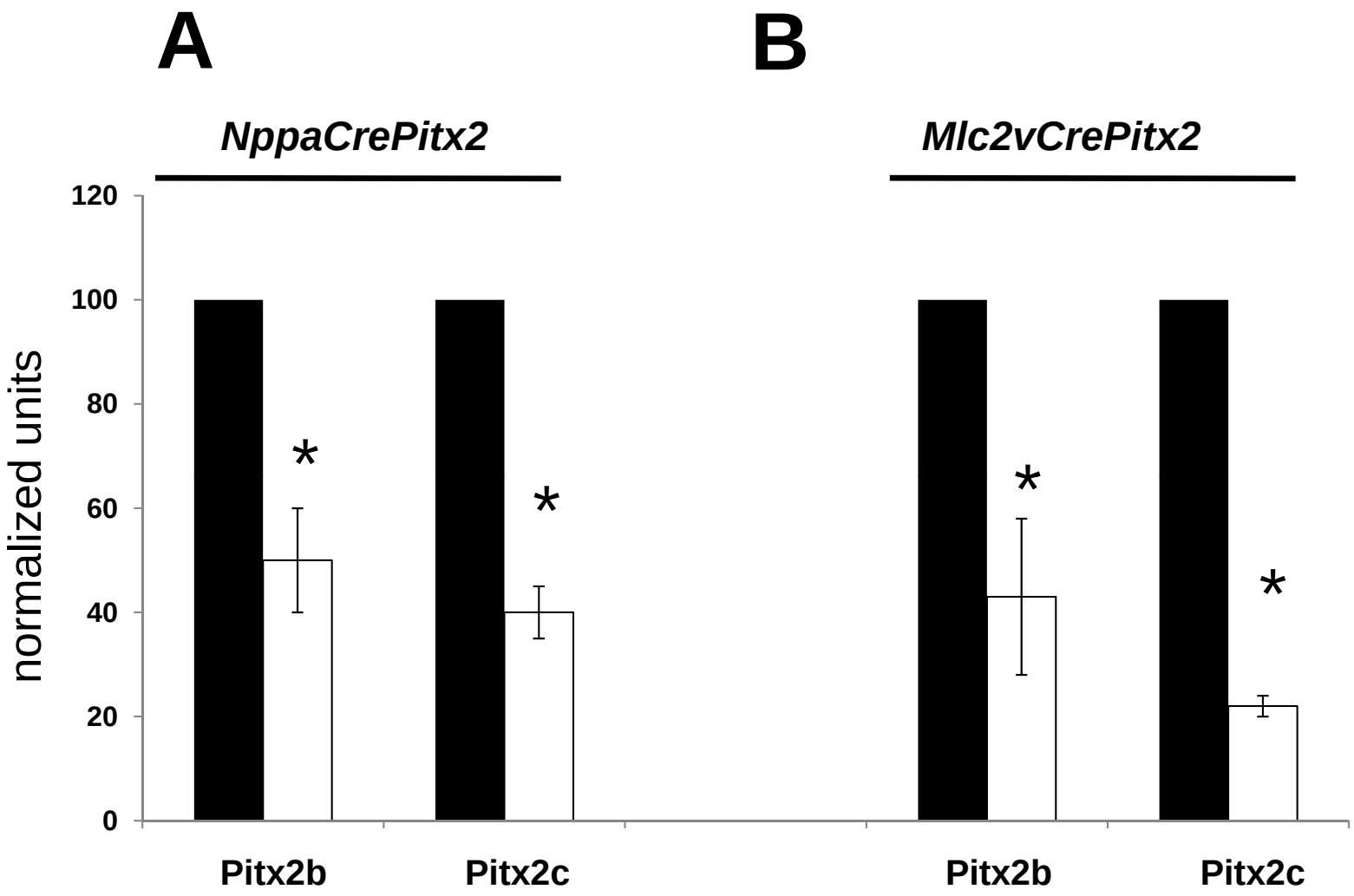


Chinchilla et al., Supplementary Figure 2



Chinchilla et al., Supplementary Figure 1



Chinchilla et al., Supplementary Figure 3

Supplementary Table 1

Mouse qRT-PCR	**Oligonucleotide sequence**
Cre Forward	ATCTTCCAGGCGCACCATTGCCCCTGT
Cre Reverse	TGACGGTGGGAGAATGTTAATCCATATTGG
Pitx2 Forward	TCGTGTCTTAAAAGGATGTGTTTCTTC
Pitx2 Reverse	TTCTGGAGGGTTTTCTTGTTCTAG
Gapdh Forward	TCCTGGTATGACAATGAATACGGC
Gapdh Reverse	TCTTGCTCAGTGTCCTTGCTGG
Gusb Forward	ACGCATCAGAAGCCGATTAT
Gusb Reverse	ACTCTCAGCGGTGACTGGTT
Nkx2.5 Forward	AGGTACCGCTGTTGCTTGAA
Nkx2.5 Reverse	CAAGTGCTCTCCTGCTTTCC
Pitx2b Forward	GATAACCGGGAATGGAGACC
Pitx2b Reverse	GTCTTTCTGGGGCAGAGTTG
Pitx2c Forward	GCCCACATCCTCATTCTTTC
Pitx2c Reverse	CCTCACCCTTCTGTCACCAT
Nppa Forward	TCT CAG AGG TGG GTT GAC CT
Nppa Reverse	CCT GTG TAC AGT GCG GTG TC
Bmp10 Forward	TCAAGACGCTGAACTTGTCG
Bmp10 Reverse	GTTCAGCCATGACGACCTCT
Kcnj2 Forward	GGTGTCAGCGCAAACAGTTGC
Kcnj2 Reverse	AGAGATGGATGCTTCCGAGA
Kcnj12 Forward	GACAGAAACAGCATCCACCA
Kcnj12 Reverse	GTGTATGCACCTTGCCATTG
Kcnj4 Forward	AGACCCTCCTCGGACCTTAC
Kcnj4 Reverse	AGACGTTACACTGGCCGTTC
Gja1 Forward	ACAGCGAAAGACTGTT
Gja1 Reverse	TTTGACTTCACCAAGG
Scn1b Forward	TGC TCA TTG TGG TGT TGA CC
Scn1b Reverse	CCT GGA CGC CTG TAC AGT TT
Scn5a Forward	GGA GTA CGC CGA CAA GAT GT
Scn5a Reverse	ATC TCG GCA AAG CCT AAG GT
Mhl7 Forward	TGCTTTATTCCCACCT
Mhl7 Reverse	AGTCCCAGGTAAGCTG
Mef2c Forward	GGGGTGAGTGCATAAGAGGAG
Mef2c Reverse	AGAAGAAACACGGGGACTATGGG
Mlc2a Forward	AAGCCATCCTGAGTGCCTTCCG
Mlc2a Reverse	GGTGTCAGCGCAAACAGTTGC
Mlc2v Forward	CCT CTC TGC TTG TGT GGT CA
Mlc2v Reverse	AAA GAG GCT CCA GGT CCA AT
Col1a1 Forward	CACCTGGTCCACAAGGTTTC
Col1a1 Reverse	ACCATCCAAACCACTGAAGC
Col3a1 Forward	AATGGCTCACACAAAG
Col3a1 Reverse	CACCTGAAGGCGTGTT

Gata4 Forward	GCAGCAGCAGTGAAGAGATG
Gata4 Reverse	GCGATGTCTGAGTGACAGGA
Gata6 Forward	CTACACAAGCGACCACCTCA
Gata6 Reverse	CCAGAGCACACCAAGAATCC
islet-1 Forward	TCCCATCCCTAAGCAC
islet-1 Reverse	ACCAATTGTCCACCAT
siRNA Pitx2 sense	GUC CAU ACA AUC UCC GAU AdTdT
siRNA Pitx2 antisense	UAU CGG AGA UUG UAU GCA CdTdT
Human SNP genotyping	**Oligonucleotide sequence**
rs2200733 Forward	ACTAGCAAGCCCTCCAGGTT
rs2200733 Reverse	GCAAACCACTGCCCTAAGAG
rs13143308 Forward	TGGGGGATGGACCAGTATAA
rs13143308 Reverse	TTGCCAGAAGAGCTTCAGTATG
Human qRT-PCR	**Oligonucleotide sequence**
PITX2A Forward	GGCGTGTGTGCAATTAGAGA
PITX2A Reverse	GGTCCACACAGCGATTTCTT
PITX2B Forward	TCGAGTTCACGGACTCTCCT
PITX2B Reverse	GAGCTGCTGGCTGGTAAAGT
PITX2C Forward	CTTTCCGTCTCCGGACTTTT
PITX2C Reverse	CGCGACGCTCTACTAGTCCT
GAPDH Forward	AGCCACATCGCTCAGACAC
GAPDH Reverse	AACCATGTAGTTGAGGTCAATGAA
PPIA Forward	TCGAGTTGTCCACAGTCAGC
PPIA Reverse	TTCATCTGCACTGCCAAGAC
ENEP Forward	TTTCTCCTGCTCCAGCTTGT
ENEP Reverse	AGAAACCTTGGCCGAATTG

Supplementary Table 2

	Sex	Age	Diabetes	Systole BP	Dyastole BP	HTA	FA type	Isolated/CM	rs2200733	rs13143308
1	Male	72	NO	100	65	NO	Paroxysmal	CM	T/T	G/G
2	Male	64	NO	160	100	NO	Paroxysmal	Isolated	C/T	T/G
3	Female	58	NO	146	80	YES	Paroxysmal	Isolated	C/C	T/T
4	Female	75	NO	128	78	NO	Paroxysmal	Isolated	C/T	T/G
5	Female	64	NO	147	74	YES	Paroxysmal	Isolated	C/T	T/G
6	Male	81	NO	128	95	YES	Paroxysmal	CM	C/C	T/G
7	Male	75	NO	155	65	YES	Paroxysmal	Isolated	C/C	T/T
8	Female	73	YES	162	95	YES	Paroxysmal	Isolated	T/T	G/G
9	Female	70	NO	90	58	YES	Paroxysmal	Isolated	C/C	G/T
10	Female	60	YES	160	88	YES	Paroxysmal	Isolated	C/T	G/T
11	Female	72	NO	130	80	YES	Paroxysmal	Isolated	C/C	G/G
12	Male	46	NO	138	96	NO	Paroxysmal	Isolated	C/C	G/G
13	Male	58	NO	130	84	NO	Paroxysmal	Isolated	C/T	G/T
14	Male	68	YES	135	66	YES	Paroxysmal	CM	C/C	G/G
15	Male	74	NO	129	80	YES	Permanent	Valvulopathy	C/T	G/T
16	Female	65	YES	110	77	NO	Permanent	Isolated	C/C	T/T
17	Male	73	NO	136	102	YES	Permanent	CM	C/C	G/T
18	Female	78	YES	125	72	YES	Paroxysmal	Isolated	C/C	G/G
19	Male	52	NO	132	75	YES	Paroxysmal	Isolated	C/C	G/T
20	Female	58	NO	123	80	NO	Paroxysmal	Isolated	C/T	G/G
21	Female	81	NO	144	93	YES	Paroxysmal	Isolated	C/T	G/T
22	Male	59	YES	131	79	YES	Paroxysmal	Isolated	C/T	G/T
23	Male	65	NO	130	80	NO	Paroxysmal	Isolated	C/T	G/T
24	Male	78	NO	134	74	YES	Paroxysmal	CM	C/C	G/G
25	Male	60	NO	98	62	NO	Paroxysmal	Isolated	C/C	G/G
26	Male	65	NO	152	89	YES	Paroxysmal	Isolated	C/T	G/T
27	Female	78	YES	167	97	YES	Paroxysmal	CM	C/C	G/G
28	Female	74	NO	140	70	YES	Paroxysmal	CM	C/T	G/T
29	Male	85	NO	131	79	NO	Permanent	CM	C/C	G/G
30	Male	77	NO	110	85	YES	Permanent	CM	C/C	G/G
31	Male	75	YES	127	68	YES	Permanent	Isolated	C/T	G/G
32	Male	57	NO	98	71	YES	Paroxysmal	CM	C/C	T/T
33	Male	76	NO	118	69	YES	Permanent	CM	C/C	G/G
34	Male	57	NO	136	81	YES	Paroxysmal	Isolated	C/T	T/T
35	Female	86	NO	181	86	YES	Permanent	CM	C/C	G/T
36	Female	59	YES	136	78	YES	Paroxysmal	Isolated	C/T	G/G
37	Female	86	NO	120	70	YES	Paroxysmal	Isolated	C/C	G/G
38	Female	77	NO	123	100	YES	Paroxysmal	CM	C/C	T/T
39	Male	38	NO	119	82	YES	Paroxysmal	Isolated	C/C	T/T
40	Female	90	NO	153	81	NO	Paroxysmal	Isolated	T/T	G/G
41	Male	63	NO	137	89	YES	Paroxysmal	Isolated	C/T	G/T
42	Male	43	NO	119	88	YES	Paroxysmal	Isolated	C/C	G/G
43	Female	83	YES	115	62	YES	Permanent	CM	C/T	G/G
44	Female	77	NO	118	97	NO	Paroxysmal	Isolated	C/C	G/T
45	Female	78	NO	147	86	YES	Paroxysmal	Isolated	C/C	G/G
46	Male	77	NO	116	61	NO	Permanent	CM	C/C	G/T
47	Male	81	NO	139	67	NO	Permanent	CM	C/C	G/G

Supplementary Table 3

	Sex	Age	rs2200733	rs13143308
1	Male	61	C/C	G/G
2	Male	53	C/C	G/G
3	Male	59	C/C	G/G
4	Female	49	C/C	G/T
5	Male	53	C/C	G/G
6	Female	55	C/C	G/G
7	Male	59	C/T	G/G
8	Female	69	C/T	G/T
9	Male	57	C/C	G/T
10	Male	53	C/C	G/G
11	Male	45	C/T	G/T
12	Female	61	C/C	G/T
13	Female	49	C/C	G/T
14	Female	47	C/C	G/T
15	Male	63	C/C	G/T
16	Female	55	C/T	G/T
17	Male	64	C/T	G/G
18	Female	56	C/T	G/G
19	Female	43	C/C	G/G
20	Female	43	C/C	G/G
21	Female	56	C/T	G/T
22	Female	46	C/C	G/G
23	Male	50	C/C	G/G
24	Female	51	C/C	G/G
25	Female	49	C/C	G/G
26	Male	65	C/C	G/G
27	Female	50	C/C	G/G
28	Female	57	C/C	G/G
29	Female	43	C/C	G/G
30	Female	45	C/C	G/G
31	Female	45	C/T	G/G
32	Female	56	C/T	G/G
33	Female	52	C/C	G/G
34	Female	47	C/C	G/G
35	Male	65	C/T	G/G
36	Male	45	C/T	G/G
37	Female	57	C/C	G/G
38	Male	45	C/C	G/G
39	Male	56	C/C	G/G
40	Female	57	C/C	G/G
41	Female	62	C/C	G/G
42	Female	57	C/T	G/G
43	Female	44	C/T	G/G
44	Male	60	C/T	G/G
45	Female	52	C/C	G/G
46	Male	66	C/C	G/G
47	Female	45	C/T	G/G
48	Male	52	C/C	G/G
49	Female	56	C/C	G/G
50	Female	50	C/C	G/G
51	Female	44	C/C	G/G
52	Female	59	C/C	G/G
53	Female	51	C/C	G/G
54	Male	57	C/C	G/G
55	Male	51	C/C	G/G
56	Female	45	C/C	G/G
57	Female	49	C/C	G/G
58	Male	53	C/C	G/G
59	Female	54	C/C	G/G
60	Female	44	C/T	G/G
61	Male	52	C/T	G/G
62	Female	53	C/C	G/G
63	Male	51	C/C	G/G
64	Male	54	C/T	G/G
65	Male	52	C/T	G/G
66	Female	56	C/T	G/G
67	Male	54	C/C	G/G
68	Female	44	C/T	G/G
69	Male	58	C/C	G/G
70	Male	53	C/T	G/G
71	Female	52	C/C	G/G
72	Male	48	C/C	G/G
73	Male	58	C/C	G/G
74	Female	44	C/C	G/G
75	Male	51	C/C	G/G
76	Female	54	C/C	G/G
77	Female	55	C/C	G/G
78	Male	53	C/C	G/G
79	Male	63	C/C	G/G
80	Female	51	C/C	G/G
81	Female	48	C/C	G/G
82	Female	43	C/C	G/G
83	Male	56	C/C	G/G
84	Male	52	C/C	G/G
85	Male	52	C/C	G/G
86	Female	45	C/C	G/G
87	Female	54	C/C	G/G
88	Male	58	C/C	G/G
89	Female	62	C/C	G/G
90	Female	45	C/C	G/G
91	Male	58	C/C	G/G
92	Male	51	C/C	G/G
93	Male	64	C/C	G/G
94	Female	58	C/C	G/G
95	Male	49	C/C	G/G
96	Female	44	C/C	G/G
97	Male	53	C/C	G/G
98	Male	64	C/C	G/G
99	Male	55	C/C	G/G
100	Female	53	C/C	G/G

Supplementary Table 4

	Sex	Age	Diabetes	Hypertension	FA/ No AF	Surgery	Biopsies
1	Male	51	NO	YES	sinus rythmn	Valve replacement	LA
2	Male	58	NO	YES	sinus rythmn	Heart transplatation	LA
3	Female	79	YES	YES	sinus rythmn	Valve replacement	LA
4	Female	78	NO	NO	sinus rythmn	Valve replacement	LA
5	Female	61	NO	NO	permanent AF	Valve replacement	LA
6	Male	75	NO	YES	permanent AF	Valve replacement	LA
7	Female	74	YES	YES	permanent AF	Bypass	LA
8	Male	75	YES	NO	permanent AF	Valve replacement	LA
9	Male	15	NO	NO	sinus rythmn	Valve replacement	RA
10	Male	67	NO	NO	sinus rythmn	Valve replacement	RA
11	Male	18	NO	NO	sinus rythmn	Valve replacement	RA
12	Male	54	NO	NO	sinus rythmn	Valve replacement	RA
13	Female	74	NO	NO	sinus rythmn	Valve replacement	RA
14	Female	67	NO	YES	permanent AF	Valve replacement	RA
15	Male	71	NO	YES	permanent AF	Valve replacement	RA
16	Male	58	YES	NO	paroxysmal AF	Valve replacement	RA
17	Female	58	NO	NO	permanent AF	Valve replacement	RA
18	Female	65	NO	NO	permanent AF	Valve replacement	RA

Supplementary Table 5

	delta CT (PITX2C/PPIA)	
	right atrium	
	mean	SD
NoAF #1 RA	10,84	1,27
AF #1 RA	14,72	2,43
NoAF #2 RA	8,74	0,54
AF #2 RA	14,26	0,89
NoAF #3 RA	6,4	1,61
AF #3 RA	13,35	1,48
NoAF #4 RA	10,83	1,49
AF #4 RA	15,52	1,49
NoAF #5 RA	20,45	1,12
AF #45 RA	12,75	0,65
	left atrium	
	mean	SD
NoAF #1 LA	6,73	0,81
AF #1 LA	9,25	0,44
NoAF #2 LA	8,51	1,13
AF #2 LA	16,35	0,49
NoAF #3 LA	7,28	0,27
AF #3 LA	20,83	0,01
NoAF #4 LA	17,58	0,92
AF #4 LA	7,92	0,86

Supplementary Figure 1 Chromatograms of SNPs (rs2200733 and rs13143308) sequencing.

Supplementary Figure 2 qRT-PCR analyses of ENPEP in right (panel A) and left (panel B) atrial samples of NoAF and AF patients. Observe that expression of ENPEP displays in approximately 50% of cases a significant decrease of expression in AF patients as compared to NoAF, whereas in the remaining 50% displays no significant changes or increased expression in AF patients as compared to AF. Thus, ENPEP expression in right and left atrial samples seems to be independent of AF.

Supplementary Figure 3 qRT-PCR analyses of *Pitx2*b and *Pitx2*c expression in atria (NppaCre*Pitx2*) and ventricular (Mlc2vCre*Pitx2*) chamber-specific conditional *Pitx2* mouse mutants corresponding to E16.5 atrial and ventricular chambers, respectively. Relative expression of *Pitx2*b and *Pitx2*c, in age-matched control negative littermates (black bars), as compared to conditional mutants (white bars). Control levels are normalized to 100%. Observe that *Pitx2*b and *Pitx2*c are decreased between 60 to 80% in both atrial and ventricular chamber-specific *Pitx2* conditional mutants.

Supplementary Table 1. Oligonucleotide sequences used for qRT-PCR expression analyses, SNPs (rs2200733 and rs13143308) genotyping and *Pitx2* siRNA silencing.

Supplementary Table 2. Clinical data and rs2200733 and rs13143308 genotype corresponding to the AF cohort of patients

Supplementary Table 3. Clinical data and rs2200733 and rs13143308 genotype corresponding to the control cohort of patients.

Supplementary Table 4. Clinical data corresponding to the AF and No AF cohorts used for PITX qRT-PCR analyses in the atrial biopsies.

Supplementary Table 5. Mean values of the delta Ct value between PITX2C levels and PPIA levels in NoAF and AF right and left atrial qRT-PCR analyses. SD, standard deviation.