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Atrial cardiomyopathy resulting from loss of Plakophilin-2 expression: response to adrenergic stimulation and implications for the exercise response

Kavya Phadke¹, Sergio D'Anna², Estefania Torres Vega², Junhua Xiao¹, Xianming Lin¹, Mingliang Zhang¹, Joseph Sall³, Feng-Xia Liang^{3,4}, David S. Park¹, Marina Cerrone¹, Alicia Lundby², Mario Delmar^{1,*}, Chantal J.M. van Opbergen^{1,*}

¹The Leon Charney Division of Cardiology, New York University Grossmann School of Medicine

²Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

³DART Microscopy Laboratory, New York University Grossmann School of Medicine

⁴Department of Cell Biology, New York University Grossmann School of Medicine

Abstract

Atrial arrhythmias occur in 20–40% of patients with Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) and associate with increased risk of sustained ventricular arrhythmias and inappropriate ICD shocks. The pathophysiology of atrial arrhythmias in ARVC remains unclear. Most cases of gene-positive ARVC are linked to pathogenic variants in the desmosomal gene *Plakophilin-2* (PKP2). Here, we test the hypothesis that loss of PKP2 expression leads to pro-arrhythmic changes in atrial cardiomyocytes. Atrial cells/tissue were obtained from a cardiac-specific, tamoxifen-activated model of PKP2 deficiency (PKP2cKO). In contrast to PKP2cKO ventricular myocytes, PKP2cKO atrial cardiomyocytes presented no significant differences in Ca^{2+}_i transient dynamics, SR load or action potential morphology. PKP2cKO atrial cardiomyocytes showed elevated ROS levels, increased frequency and amplitude of Ca^{2+} sparks, and increased diastolic $[\text{Ca}^{2+}]_i$ compared to control; the latter two parameters were further increased by isoproterenol (ISO) exposure and reversed by exposure to RyR blocker Dantrolene. We speculate that these ISO-dependent effects may impact on the exercise-related atrial arrhythmia risk in ARVC patients. Despite absence of changes in Ca^{2+}_i transient dynamics,

Correspondence: Mario Delmar, MD, PhD, Patricia and Robert Martinsen Professor of Cardiology, Leon H. Charney Division of Cardiology, New York University Grossmann School of Medicine, Science Building 435 E.30th Street 707, mario.delmar@nyulangone.org.

*Equally contributed. Co-corresponding authors.

Author Contributions

MD, CJMvO and AL performed the conception and design of the work. KP, SD, ETV, JX, XL, MZ, JS, AL, MD and CJMvO performed the acquisition, analysis and/or interpretation of data for the work. KP, MD and CJMvO drafted the work and KP, SD, ETV, JX, XL, MZ, JS, F-XL, DP, MC, AL, MD and CJMvO critically revised the work for important intellectual content. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

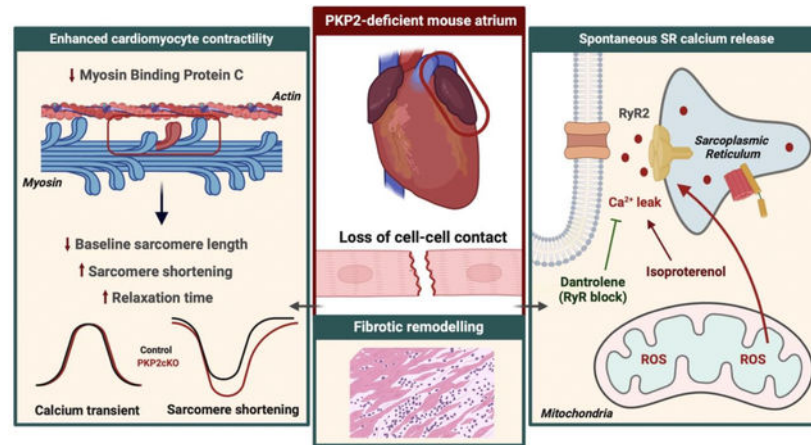
ADDITIONAL INFORMATION

Disclosures

None

PKP2cKO atrial cardiomyocytes showed enhanced sarcomere shortening and impaired sarcomere relaxation. Orthogonal transcriptomic analysis of human (GTEx) and PKP2cKO atrial tissue led to identification of 41 transcripts depending on PKP2 expression. Biochemical follow-up confirmed reduced abundance of sarcomeric protein Myosin Binding Protein C (MyBPC), potentially playing a role in cellular shortening and relaxation changes observed. Our findings provide novel insights into the role of PKP2 in atrial myocardium with potential implications to therapeutic management of AF in patients with PKP2-related ARVC.

GRAPHICAL ABSTRACT



Loss of plakophilin2 (PKP2) in the mouse atrium leads to impaired cell-cell contact and extensive fibrotic remodeling (middle panel). PKP2-deficient atrial cardiomyocytes exhibit enhanced sarcomere shortening and impaired relaxation, potentially related to reduced abundance of Myosin Binding Protein C (left panel). In addition, PKP2-deficient atrial cardiomyocytes present increased spontaneous sarcoplasmic reticulum (SR) calcium (Ca^{2+}) release events, further enhanced by Isoproterenol exposure and reversible by a RyR blocker (Dantrolene). SR Ca^{2+} leak is likely caused by elevated levels of oxidative stress (ROS) in the mitochondria (right panel). PKP2cKO: cardiac-specific, tamoxifen-activated knockout of PKP2, RyR2: ryanodine receptor, ROS: reactive oxygen species

Keywords

arrhythmogenic right ventricular cardiomyopathy; atrial fibrillation; plakophilin-2; ryanodine receptor; oxidative stress; adrenergic stimulation

INTRODUCTION

Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) is an inheritable condition characterized by a fibrofatty infiltration of the heart muscle (predominantly, but not exclusively, the right ventricular free wall), as well as increased risk of ventricular arrhythmias and sudden cardiac death (SCD)(Goff & Calkins, 2019; Jordà *et al.*, 2022). Most cases of gene-positive ARVC associate with pathogenic variants in the gene *PKP2*, coding for the desmosomal protein Plakophilin-2 (PKP2)(Bhonsale *et al.*, 2015). The

disease phenotype progresses over time, with lethal arrhythmias often observed in the early stages, before the onset of the cardiomyopathy (Groeneweg et al., 2014). While studies regarding the arrhythmogenic substrate in ARVC have focused on the risk of life-threatening ventricular arrhythmias, recent studies have drawn attention to the high prevalence of atrial fibrillation (AF) among ARVC patients. Recent studies show that the incidence of atrial arrhythmias ranges between 15–25% in ARVC, and that AF is the most prevalent supraventricular arrhythmia (59%) among patients (Camm et al., 2013; Cardona-Guarache et al., 2019; Baturova et al., 2020). Multiple biobank-driven genome-wide association studies recently revealed an association of AF with genetic variants in *PKP2*, further supporting the notion that PKP2 is important for atrial function (Nielsen et al., 2018; Zöller et al., 2024; Roberts et al., 2024). Atrial arrhythmias are clinically important for ARVC patients, as they associate with inappropriate implantable cardioverter-defibrillator shocks, increased risk of sustained ventricular arrhythmias and disease morbidity (Camm et al., 2013; Cardona-Guarache et al., 2019). However, the pathophysiology behind the development of atrial arrhythmias in ARVC and specifically, the association between PKP2 deficiency and atrial arrhythmogenic mechanisms, remains understudied.

It is generally accepted that both a substrate and a trigger are essential for the initiation and maintenance of arrhythmic events (Cluitmans et al., 2023). For ventricular arrhythmias in PKP2-deficient hearts, dysregulation of intracellular calcium (Ca^{2+}_i) homeostasis appears to be a critical trigger (van Opbergen et al., 2022), while a disrupted micro-structure may be sufficient substrate for arrhythmic events (Corrado et al., 2023). In the present study, we examined potential triggers and substrate elements that could affect the stability of the atrial rhythm in hearts deficient in PKP2. Our studies utilized a well-characterized model of a cardiac-specific, tamoxifen (TAM)-activated knockout of PKP2 (PKP2cKO) as well as an analysis of a database for genetic associations in humans (GTEx). Our experiments followed the path previously traced by our studies on PKP2-related ventricular dysfunction (Cerrone et al., 2017; Pérez-Hernández et al., 2022). Features such as fibrosis, excess ROS production and Ca^{2+}_i dysregulation were also observed in atrial myocardium, though important differences became apparent. An in-depth transcriptomics analysis revealed novel elements in the molecular framework supporting the atrial phenotype. Our observations suggest that recent therapeutic approaches to mitigate ventricular ARVC (van Opbergen et al., 2022; Wojciech Zareba et al. may be effective to treat the atrial disease.

METHODS

Ethical approval

Animal experiments were performed in 3- to 4-month-old mice expressing a cardiac-specific deletion of *Pkp2* (PKP2cKO). PKP2cKO mice were used at 21 days post injection (21 dpi) of TAM, a time point at which an arrhythmogenic cardiomyopathy of right ventricular predominance can be observed. Procedures conformed with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the New York University Institutional Animal Care and Use Committee (160726–03).

Animal care

Mice are housed within NYU Grossman School of Medicine in the 2nd and 3rd floors of the Science Building. All housing is provided in accordance with the Animal Welfare Act, the US Dept. of Agriculture Regulations and the NIH Guide for the Care and Use. NYU has a currently approved Animal Welfare Assurance Agreement with the NIH. The DLAR is staffed by an ACLAM board-certified attending veterinarian, six veterinary medicine staff members, and an additional 40 operational and care staff personnel. Animal care is provided by DCM seven days a week, and emergency veterinary care is available 24 hr/day. Food and water are continuously available to the animals and consumed ad libitum.

Data availability

The data that support the findings of this study are presented in the article and online supplementary files. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD052074. Additional information can be made available from the corresponding author upon reasonable request.

Reviewers can access the proteomics data with the following credentials:

Username: reviewer_pxd052074@ebi.ac.uk **Password:** 8Lo9drdN

Fibrosis quantification

Hearts were excised and rinsed in ice-cold PBS, then immediately perfusion-fixed in 4% paraformaldehyde for at least 72 hours at 4 °C. The hearts were then embedded in paraffin and sectioned at 5 µm thickness according to standard protocols at New York University Experimental Pathology Research Laboratory. Deparaffinized sections were stained with Masson's trichrome for fibrosis analysis. Images were acquired using a BZ-X800 microscope (Keyence, Osaka, Japan). For fibrosis quantification, the whole left atrial (LA) section was analyzed and trichrome positive fibrosis area was calculated as the percentage of the reference atrial area using Fiji ImageJ (NIH, Bethesda, MD).

Electron Microscopy

Sample Preparation: Mice were injected with 0.1 mL heparin (500 IU/mL i.p.) 20 min before heart excision and anaesthetized by inhalation of 100% CO₂. When deep anesthesia was confirmed, mice were sacrificed by cervical dislocation and hearts were extracted. The left atria was dissected and fixed at 0.1 M phosphate buffer (PB) containing 2% PFA and 2.5% glutaraldehyde at 4°C overnight. Fixed tissue was processed making use of the modified OTTO method(Vanslembrouck et al., 2018; Delgado et al., 2021). In brief, atrial tissue was washed with 0.1 M PB, post fixed in 2% OsO₄ /1.5% potassium ferrocyanide for 1.5 h at room temperature, then stained twice for 2 hours with freshly made 1% tannic acid (Electron Microscopy Sciences) at 4°C. The tissue was then washed in ddH₂O, placed in 2% aqueous OsO₄ for 40 min at room temperature, and en bloc stained in 1% aqueous uranyl acetate at 4°C overnight. The tissue was then washed with ddH₂O, dehydrated in a series of ethanol solutions (30, 50, 70, 85, 95, 100, 100%; 10 min each, on ice) and replaced

with ice-cold dry acetone for 10 min, followed by 10 min in acetone at room temperature. The sample was gradually equilibrated and embedded with EMbed812 resin (hard recipe, Electron Microscopy Sciences).

Serial Block-Face Scanning Electron Microscopy (SBF-SEM): The sample block was trimmed, and thin sections were cut to identify the area of interest. The sample block was then mounted on the 3View pin stab (Gatan, Pleasanton, CA, USA) using silver conductive epoxy (Ted Pella Inc., CA, USA) to electrically ground the tissue block. The specimen was trimmed again and coated with a thin layer of gold/palladium by Rave Safematic CCU-010 SEM coating system (Rave Scientific, Somerset, NJ). Serial block face imaging was performed using a Gatan OnPoint BSE detector in a Zeiss GEMINI 300 VP FESEM equipped with a Gatan 3View automatic microtome unit. The system was set to cut sections with 100 nm thickness, imaged with gas injection setting at 40% (2.0E-03mBar) with Focus Charge Compensation to reduce the charge, and images were recorded after each round of section from the block face using the SEM beam at 2.0 keV with a dwell time of 1.0 μ s/pixel and an X/Y pixel size of 6nm. Data acquisition settings and stage control were performed through the Gatan Digital Micrograph software (version 3.31). A stack of 500 .dm4 image files were converted to .tif files and assembled using ImageJ. Each image volume region obtained from the tissue block had a volume of $82\mu\text{m} \times 82\mu\text{m} \times 50\mu\text{m}$.

Cardiomyocyte dissociation

Isolated murine atrial myocytes were obtained by enzymatic dissociation. Mice were injected with 0.1 mL heparin (500 IU/mL i.p.) 20 min before heart excision and anaesthetized by inhalation of 100% CO₂. When deep anesthesia was confirmed, mice were sacrificed by cervical dislocation and the heart was surgically removed via thoracotomy and placed in a Langendorff column. The isolated hearts were perfused sequentially at a constant flow rate of 3 mL/min with Ca²⁺-free solution containing (in mM): 113 NaCl, 4.7 KCl, 1.2 MgSO₄, 0.6 Na₂HPO₄, 0.6 KH₂PO₄, 12 NaHCO₃, 10 KHCO₃, 10 HEPES, 5.5 Glucose and 30 Taurine, pH 7.40 with NaOH and then an enzyme (collagenase type II; Worthington, Lakewood, NJ, USA) solution for 10 min. Perfusate temperature was maintained at 37°C. After digestion, the left atrial was dissected, cut into small pieces and minced by gentle mechanical agitation with a Pasteur pipette. The isolated cardiomyocytes were suspended in 10 mL of stop buffer (Ca²⁺- free perfusion buffer with 5% bovine calf serum) and the Ca²⁺ concentration was increased gradually to 1.0 mM. Cardiomyocytes were kept in Tyrode's solution containing (in mM): 148 NaCl, 5.4 KCl, 1.0 MgCl₂, 1.0 CaCl₂, 0.4 NaH₂PO₄, 15 HEPES and 5.5 Glucose, pH 7.40. Cells were used within 5 h after isolation.

Electrophysiological measurements

Action potential: Current clamp was used to measure the adult left atrial myocyte action potential as in (Lübke et al., 2013). Briefly, the recording pipette solution contained (in mmol/l): 135 KCl, 1 MgCl₂, 10 EGTA, 10 HEPES, and 5 Glucose, pH 7.2 with KOH. The bath solution contained (in mmol/l): 136 NaCl, 4 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES and 10 Glucose, pH 7.4 with NaOH. Resting membrane potentials, action potential amplitudes, and action potential duration were measured.

Sodium current: All electrophysiological recordings were conducted in the whole-cell configuration at room temperature, as in (Lübckemeier et al., 2013). Pipette resistance was maintained within the range of 1.5 to 1.8 MΩ. For sodium current recordings, recording pipettes were filled with a solution containing (in mmol/l): NaCl 5, CsF 135, EGTA 10, MgATP 5 and HEPES 5, pH 7.2 with CsOH. Cells were maintained in a solution containing (in mM): NaCl 5, CsCl 132.5, CaCl₂ 1, MgCl₂ 1, CdCl₂ 0.1, HEPES 20 and Glucose 11, pH 7.35 with CsOH. Voltage clamp protocols were as follows: for determination of peak current voltage relation, 200 msec voltage pulses were applied to V_m -90 mV to +30 mV in 5 mV voltage steps, from a holding potential of V_m = -120 mV. Interval between voltage steps was 3 sec. Steady state inactivation was determined by stepping V_m from -130 mV to -40 mV, followed by a 30 msec test pulse to V_m = -40mV to elicit I_{Na}.

Calcium imaging

Ca²⁺ transients and Ca²⁺ sparks: Isolated left atrial myocytes were loaded for 10 min with Fluo-8 AM (AAT Bioquest) in Tyrode's solution containing (in mM): 140 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES and 5.6 glucose followed by additional 10 min wash in Tyrode's solution. The Ca²⁺ transients were induced by applying depolarizing pulses at 1 Hz using a pair of electrodes coupled to a stimulator (MyoPacer, IonOptix). Ca²⁺ fluorescence was imaged using a laser scanning confocal microscopy system (Leica TCS SP5) fitted with a 63× oil immersion objective lens. Dyes were excited at 488 nm using an Ar laser and fluorescence emission at wavelengths >510 nm was detected. To measure Ca²⁺ transients or Ca²⁺ sparks, line scan images were captured at 1.43 ms/line with LAS AF (Leica) software. Recording of Ca²⁺ sparks was preceded by a train of electrical pulses at 1 Hz for 10 sec. Under this condition, the frequency of Ca²⁺ sparks was stable during the experimental period. To quantify Ca²⁺ transients, local averaged Ca²⁺ signals were measured using ImageJ (NIH) software. Traces of local Ca²⁺ transients were expressed as the mean fluorescence of each line normalized to the mean resting fluorescence (F/F₀). For automated detection and analysis of Ca²⁺ sparks in line scan images, we used a thresholding algorithm, the SparkMaster plugin, implemented within ImageJ software.

SR Ca²⁺ load: Experiments for detection of SR load in isolated left atrial myocytes relied on the use of a ratiometric method as described in (Lohr, 2003). Briefly, myocytes were loaded with the Ca²⁺- sensitive dyes Fluo-3 AM (1.67 μM) and Fura Red AM (5 μM; Molecular Probes) for 30 min. The excitation wavelength was 488 nm. Fluo-3 (emission at 505–550 nm) and Fura Red (emission at >615 nm) were imaged simultaneously. SR Ca²⁺ load was measured in line- scanning mode at the sampling rate of 1.43 ms/line. When the cells were exposed to 10 mM caffeine, the fluorescence from Fluo-3 was increased, while the fluorescence of Fura Red was reduced. In both experiments, images were recorded after 10 s of rest following a train of electrical pulses at 1 Hz, using Leica TCS SP5 confocal microscope with a 63x oil immersion objective lens. The ratio of the intensity of the two images (F_{Fluo-3}/F_{Fura Red}) was calculated by using LAS X software and the F_{Fluo-3}/F_{Fura Red} images were obtained with ImageJ plugin Ratio Plus.

Determination of [Ca²⁺]_i: For ratiometric Ca²⁺ imaging to determine quantitatively [Ca²⁺]_i, isolated left atrial myocytes were loaded with 3 μM Fura Red AM for 30 min and

the dye was alternately excited with the 458 and 488 nm argon laser. Fluorescence emission at wavelengths >585 nm was detected using a laser scanning confocal microscopy system (Leica TCS SP5). The paired images were analyzed by the method described by Lohr et al (Lohr, 2003). Briefly, a Fura Red fluorescence ratio (F_{458}/F_{488}) was calculated from the fluorescence intensity emitted under each excitation wavelength, making use of the ImageJ plugin Ratio Plus.

Dantrolene treatment in mice

PKP2cKO mice at 21dpi were manually restrained, injected intraperitoneally with 20 mg/kg (compound/body weight) Dantrolene sodium suspension (Ryanodex) dissolved in 0.9% normal saline, or saline (of equivalent volume), and returned to their cage. After 30 minutes, mice were sacrificed and left atrial myocytes isolated as explained in the previous sections.

***In vitro* experiments with beta-adrenergic agonist**

Isolated left atrial myocytes were loaded with the required fluorescent dyes and incubated for 10 minutes with 100 nM isoproterenol (ISO) (isoprenaline hydrochloride; Sigma Aldrich, USA) in 1.8mM Ca^{2+} Tyrode solution, to test the response of control and PKP2cKO myocytes towards beta-adrenergic stimulation. Ca^{2+} transients, Ca^{2+} sparks, SR load and intracellular Ca^{2+} levels were examined accordingly, as explained in the previous sections.

Sarcomere shortening measurements

Sarcomere length was recorded using a video-based edge-detecting IonOptix system. Briefly, isolated left atrial myocytes were bathed in 1.8mM calcium Tyrode's solution for 10 minutes at room temperature (RT) before starting the experiment. Myocytes were incubated in a glass bottom round dish on top of an inverted Leiss microscope (40x magnification) in combination with a transmitted light camera (IonOptix MyoCam-S). Experiments were conducted at room temperature, and cells were paced at 1Hz (60 beats/min) by field stimulation (MyoPacer, IonOptix, Westwood, MA, USA). If a myocyte fulfilled the selection criteria (good morphology, well-followed pacing, no spontaneous beating, $<10\%$ decrease in contractility during the 10-minute pre-baseline recording), myocytes were paced for 20 seconds to achieve a steady state condition and then recorded for 20–30 seconds. Sarcomere shortening data analysis was performed using IonWizard (IonOptix, Westwood, MA, USA) software. Sarcomere shortening was measured from 15 averaged sarcomere waveforms.

Mass spectrometry measurements of Ryr2 phosphorylation

The left atria were dissected from six Control and PKP2cKO mice at 21 dpi. Immediately following dissection, atrial tissue was snap-frozen in liquid nitrogen. Subsequently, atrial samples from each animal were homogenized using a Precellys 24 homogenizer (Bertin Instruments) and solubilized for 30 minutes at 4°C in ice-cold lysis buffer consisting of 50 mM Tris (pH 8.5), 150 mM NaCl, 10 mM KCl, 1% sodium deoxycholate, 1% NP-40, 5 mM EDTA, 5 mM sodium fluoride, 5 mM β -glycerolphosphate, 1 mM sodium orthovanadate, and 1 tablet of complete inhibitor cocktail (Roche). Following solubilization, atrial lysates

were centrifuged to remove insoluble debris, and the protein concentrations were quantified using the PierceTM Rapid Gold BCA Protein Assay Kit (Thermo Scientific).

300 µg of solubilized lysate was precleared using 60 µg of Dynabeads Protein A (Invitrogen) for 20 minutes at 4°C. The precleared lysate was then incubated with 0.6 µg of anti-Ryr2 antibody (ARR002, Alomone) for 45 minutes at 4°C. Next, the antibody-protein complex was captured using 0.6 µg of Dynabeads Protein A pre-blocked with bovine serum albumin (BSA). The beads were thoroughly washed with lysis buffer followed by phosphate-buffered saline (PBS) and subsequently stored at -20°C until mass spectrometry analysis. Washed beads were incubated for 30 min with elution buffer 1 (2 M Urea, 50 mM Tris-HCl pH 7.5, 2 mM DTT, 20 µg/ml trypsin) followed by a second elution for 5 min with elution buffer 2 (2 M Urea, 50 mM Tris-HCl pH 7.5, 10 mM Chloroacetamide). Both eluates were combined and further incubated at room temperature overnight. Tryptic peptide mixtures were acidified to 1% TFA and loaded on Evotips (Evosep). Peptides were separated on 15 cm, 150 µM ID columns packed with C18 beads (1.9 µm) (Pepsep) on an Evosep ONE HPLC applying the '30 samples per day' method and injected via a CaptiveSpray source and 10 µm emitter into a timsTOF pro mass spectrometer (Bruker) operated in PASEF mode (Meier et al., 2018).

Raw MS data were processed using Fragpipe (version 21.1) (<https://fragpipe.nesvilab.org/>) with the LFQ-phospho built-in workflow. All MS and MS/MS spectra were matched against an in-silico tryptic digest of a database comprising all reviewed SwissProt mouse protein entries, supplemented with reversed copies of all sequences, and commonly observed contaminants. Maximal MS/MS tolerance was set to 20 p.p.m., and a maximum of two missed cleavages were allowed. False discovery rates were set at 0.01 for both peptides and proteins. Carbamidomethylated cysteines were designated as fixed modifications, whereas oxidation of methionine, N-terminal acetylation, and STY phosphorylation were considered variable modifications. The minimum peptide length was set at seven amino acids. Due to the similarity of the samples, the match-between-runs option was enabled with default parameters. Normalized intensities in the 'combined_site_STY_79.9663.tsv' file were used for statistical analyses using the Perseus software (version 2.0.9.0) (Max-Planck-Institute of Biochemistry). Identification of phosphosites was refined by filtering to retain only those quantified in at least 4 out of 6 replicates from either group. Differential phosphorylation analysis between the two groups was conducted using Student's t-test comparisons. To account for multiple testing, the false discovery threshold was set at 0.05 after Benjamini-Hochberg correction.

Mitochondrial superoxide radical anion (O₂⁻) detection

For atrial cardiomyocytes, superoxide production was measured using the pro-fluorescent dye MitoSOX Red (Invitrogen)(Pérez-Hernández et al., 2021). Freshly isolated non-permeabilized left atrial myocytes were loaded with 5 µmol/L MitoSOX Red in Hanks' Balanced Salt Solution (HBSS) for 10 min at 37°C. For mitochondrial loading validation, cells were further incubated with 200 nmol/L Mitotracker Deep Red FM (Thermo Fisher) for 10 min at 37°C. After washing with HBSS, cells were placed on the stage of a Leica SP5 confocal microscope (63× oil immersion lens) where MitoSOX fluorescence was

recorded using at 514 λ_{ex} and λ_{em} 530–580 nm. MitoTracker was excited at 633 nm and fluorescence emission at 650–800 nm was detected. MitoSOX colocalized with MitoTracker suggesting that it was selectively loaded into mitochondria. MitoTracker was used to draw the ROIs. In both cases, image analysis and quantification were done using LAS X and ImageJ software.

RNA Sequencing

RNA from 6 control and 6 PKP2cKO left atria from mice at 21 dpi were extracted using RNA-easy Mini kit (Qiagen). RNA-Seq library preps were made using the Illumina TruSeq RNA Library Preparation Kit v2 using 500 ng of total RNA as input, amplified by 12 cycles of PCR, and run on an Illumina 2500 (v4 chemistry), as single read 50 at the Genome Technology Center at NYUMC. Approximately 200 million reads per sample were generated. Sequencing results were demultiplexed and converted to FASTQ format using Illumina Bcl2FastQ software. Quality Control (QC) for the RNA-Seq reads was assessed using FastQC software. Next, reads were aligned to the mouse genome (build mm10/GRCm38) with Spliced Transcripts Alignment to a Reference (STAR). PCR duplicates were removed using the Picard toolkit (open-source, MIT license). HTSeq package was utilized to generate counts for each gene. The read counts of each transcript were normalized to the length of the individual transcript and to the total mapped read counts in each sample and expressed in counts per millions. In order to validate the intra-group homogeneity, we first performed a principal component analysis (PCA). PCA is based on two principal components PC1 and PC2. The first principal component (PC1) is the direction along which the samples show the largest variation. The second principal component (PC2) is the direction uncorrelated to the first component along which the samples show the largest variation. Next, we visualized the genes with a largest variance using a hierarchical clustering heatmap. For each gene, we compared the expression levels between Control and PKP2cKO RNAs. Gene expression differences were evaluated using Fisher's exact test after normalizing by the total number of mapped reads in each lane. The resulting p-values were corrected via the Benjamini and Hochberg method. Differentially expressed genes were defined as those with log2 changes of at least 0.25-fold between a pair of samples at FDR of 0.05 for genes with a count above 10. Dataset was analyzed using R software version 4.03 and ad hoc packages.

GTEX

Gene read counts from the heart atrial appendage were acquired from the Genotype-Tissue Expression V8 (GTEx) consortium. Differential expression was conducted using a negative binomial distribution with the Empirical Bayes approach via the DeSeq2 package. Genes with no reads in more than 50% of samples and genes with counts <10 were not included. The gene counts were then normalized using variance stabilization transformation from the R package DeSeq2 (version 3.8) to ensure homoscedasticity. P-value significance was set to 0.05. A Pearson's correlation and linear regression test were used to detect co-expression with *Pkp2*. P-values were corrected via the Benjamini and Hochberg method. Combination of RNA-Seq dataset from PKP2cKO mice with GTEx dataset was performed in R version 4.0.3.

Real Time PCR

Total RNA was extracted and purified using RNeasy Mini Kit (QIAGEN) from the left atria of 3 PKP2cKO and 3 control mice (PKP2flox/flox/Cre negative) at 21 dpi. Complementary DNA was generated using High Capacity Reverse Transcript Kit (ThermoFisher) with equal amount of total RNA from PKP2KO and control atria tissues. Real time PCR was performed with PowerUp SYBR Green reagents and applied on StepOnePlus Real Time PCR System. Relative Quantification was calculated as $RQ = 2^{-\Delta\Delta CT}$ and RQs of each reaction were normalized to control mice.

Mice primers: *GAPDH* was used as reference gene with forward primer TGACGTGCCGCTGGAGAAA, and reverse primer AGTGTAGCCCAAGATGCCCTTCAG. *PKP2* forward primer GCAGCCGAAGTAGGAAACTAA and reverse AATGCCACAGCCACTCAA. *MYBPC3* forward primer AGGCTGTGTTTAAGTGTGAGG and reverse CATCGTCAATGGTCAGTTTGTG.

Western blot analysis

To analyze protein expression levels of PKP2 and MYBPC3, we used a standard protocol. Briefly PKP2cKO and control mice were euthanized at 21 dpi and left atrial samples were cryopreserved immediately in liquid nitrogen. The samples were then homogenized in extraction buffer containing protease and phosphatase inhibitors (50 mM Tris pH 8.0, 150 mM NaCl, 0.02% Sodium azide, 1% Triton X-100, 1 mM PMSF, 1 mM Na₃VO₄, 50 mM NaF and Complete Protease Inhibitor (Roche)). Protein concentration was determined using the BCA kit (Thermo Fisher). Samples were run on 4–12% precast polyacrylamide SDS gradient gels (BioRad) and transferred onto nitrocellulose membranes (BioRad), subsequently incubated in blocking buffer consisting of PBS with Tween-20 (0.1%) and 1% nonfat dry milk. Membranes were then incubated with specific primary antibody (PKP2 polyclonal Boster Biological, MYBPC3 monoclonal Santa Cruz) diluted in 1% nonfat dry milk overnight at 4°C followed by wash steps and secondary antibodies (Odyssey goat anti-mouse[®]680RD or antirabbit[®]800). Antigen complexes were visualized with the Odyssey Infrared Imaging System (LICOR). The band intensity was quantified with ImageJ software.

Immunofluorescent labelling

Freshly isolated left atrial cardiomyocytes plated on laminin-coated coverslips were fixed in 4% PFA (Electron Microscopy Sciences) for 10 min, and kept in PBS at 4°C until further use. For immunolocalization of Myosin Binding Protein C3 (MYBPC3), cells were permeabilized with 0.1% Triton X-100 in PBS (Sigma) for 10 min and incubated in blocking buffer: 2% bovine serum albumin (Sigma), 2% glycine (Sigma) and 0.2% gelatin (Sigma) for 30 min. The primary antibody Mouse monoclonal anti- MYBPC3 (1:100, Santa Cruz Biotechnology #137180) was incubated for 1 h at room temperature. After 3x washes with PBS, secondary antibody Alexa Fluor 568 goat anti- mouse (1:300, Invitrogen, #A1100) was incubated for 1 h at room temperature. After 3x washes with PBS, cells were stained for DAPI 500 nmol/L for 5 min. Coverslips were mounted with ProLong Gold

antifade reagent (Invitrogen, Ref P36930, Lot: 2204416) and images were obtained using a laser scanning confocal microscope Leica SP5.

Statistical analysis

Numerical results are given as mean and standard deviation (SD). All pooled data originating from separate animals were tested using the workflow designed by Sikkel et al (Sikkel et al., 2017)(1) to determine the validity of the assumption of independence vis a vis data clustering. When clustering was identified, hierarchical analysis was employed. All data sets were tested for normal distribution by the Shapiro-Wilk and Kolmogorov-Smirnov tests and considered normally distributed when passing one of these two tests. Significance was determined by parametric or non-parametric methods, as appropriate. The specific methods used to determine significance were therefore tailored to the individual datasets, and they are specified in the figure legends describing the corresponding datasets. Data were analyzed using GraphPad Prism v8.0. Sarcomere shortening data were analyzed using IonWizard (IonOptix, Westwood, MA, USA).

RESULTS

We examined the electrophysiological profile, and the basic parameters reflecting Ca^{2+}_i homeostasis, in PKP2cKO atrial myocytes harvested 21 days post injection (21 dpi) of TAM, a time point at which an arrhythmogenic cardiomyopathy of right ventricular predominance can be observed (Cerrone et al., 2017). As shown by the data in Figure 1 we detected no significant changes in the morphology of the action potential, nor did we identify an effect on peak density or steady-state inactivation of the sodium current. Moreover, and in sharp contrast to what we have observed in ventricular myocytes harvested at the same time point after TAM (Cerrone et al., 2017), we detected no differences in the amplitude of elicited Ca^{2+}_i transients or in the time course of Ca^{2+}_i decay after activation when comparing control to PKP2cKO cells (Figure 2A&B; bars labelled “control” and “PKP2cKO”). In addition, acute exposure to isoproterenol (ISO) elicited an increase in the amplitude of the transient and a reduced time constant of transient decay of similar magnitude in both cell populations (Figure 2A&B; bars labelled “Control + ISO and PKP2cKO + ISO”). Yet, we observed a large increase in the frequency and amplitude of Ca^{2+} sparks in PKP2-deficient cells (comparing “control” versus “PKP2cKO”). PKP2cKO cells were also more susceptible to modulation by beta-adrenergic stimulation (Figure 3A&B; other parameters in Fig 4A&B; comparison effect of ISO in Figure 5A&B). This pattern was consistent with the observed measurements of diastolic Ca^{2+} levels, presenting a significant increase consequent to ISO stimulation in the PKP2cKO cells (Figure 6A; comparison effect of ISO in Figure 5C). SR load increased by ISO exposure in both control and PKP2cKO cells; the magnitude of the SR load after ISO was significantly larger in control than in PKP2cKO cells, likely because of excessive leak of Ca^{2+} out of the SR in the latter group (Figure 5DE & 6B).

The results above suggest an “eagerness” for the RyR2 channels in PKP2cKO ACMs to flicker and release Ca^{2+} into the cytoplasmic space during diastole. Further evidence was obtained through acute *in vivo* treatment of PKP2cKO mice with Dantrolene, a well-

characterized ryanodine receptor blocker(Kobayashi et al., 2009). Data are shown in Figure 3&6. PKP2cKO mice were injected with either DMSO or Dantrolene (30mg/kg) 30 minutes prior to euthanasia (see also Methods). The results show that Dantrolene mitigated the ISO-dependent increased frequency of Ca^{2+} sparks and their amplitude in PKP2cKO cells (Figure 3A–C). A corresponding mitigation of diastolic Ca^{2+} elevation and increase in SR load was observed (Figure 6A&B).

Previous studies have indicated that the open probability of RyR2 channels can be modulated by its phosphorylation state, particularly in Ser-2808 and Ser-2814(Wehrens et al., 2006; Uchinoumi et al., 2016). We therefore utilized mass spectrometry analysis to evaluate for abundance differences of RyR2 phosphorylation sites in control and PKP2cKO atria. We obtained excellent coverage of RyR2 phosphorylation sites; our data revealed no difference between groups (Figure 7A), thus indicating that the increased frequency and amplitude of the Ca^{2+} sparks could not be accounted for by changes in RyR2 phosphorylation in those sites. On the other hand, reactive oxygen species (ROS), known to post-translationally modify RyR2 oxidation state and increase open probability, were more abundant in PKP2cKO myocytes when compared to control (Figure 7B–C).

Loss of PKP2 leads to enhanced sarcomere shortening in adult ventricular myocytes(Cerrone et al., 2022). Those results were interpreted as consequent to the increased amplitude and duration of Ca^{2+}_i transients. Interestingly, a similar analysis of sarcomere shortening in atrial PKP2cKO myocytes, which show no difference in Ca^{2+}_i transient amplitude or duration, also revealed that loss of PKP2 expression associates with increased shortening, reduced sarcomere length at rest and a prolonged relaxation time when compared to control (Figure 8A–E). Yet, while isolated myocytes were able to maintain contractility, we observed a drastic loss of muscle integrity at the expense of a large increase in collagen abundance in the PKP2cKO atria when compared to control (Figure 9A&B). These results were similar to those previously reported for ventricular tissue, where loss of PKP2 expression associated with a loss of muscle mass (and in the case of the left ventricle, reduced ejection fraction) while the fractional shortening of individual myocytes was in fact, enhanced(Cerrone et al., 2022). Finally, as suspected, the structural remodeling in the macro- scale was also apparent at the micro-scale, as PKP2cKO in atrial myocytes led to the expected loss of intercalated disc integrity, as shown by the electron microscopy images presented in Figure 9C and in Supplemental Movies 1&2.

To further characterize the effects that loss of PKP2 exert on the molecular profile of the atria, we conducted a differential transcriptome analysis comparing 6 atria from PKP2cKO hearts versus 6 atria from controls. The differential transcriptome is presented in the form of a volcano plot in Figure 10A. Positive and negative numbers in the x-axis reflect the magnitude of the differential, expressed as Log_2Fc . Only 215 transcripts were significantly down-regulated and 136 were up-regulated in PKP2cKO samples ($|\log_2\text{FC}| > 0.25$, $\text{FDR} < 0.05$). As expected, given the nature of the experimental model, the largest change was found in the transcript abundance of *Pkp2* (downregulated) and the estrogen receptor *Esr1* (upregulated). The complete dataset is available as Supplemental Table 1. As a next step, we sought to discover functionally relevant co-regulated transcripts in the human transcriptome. For this purpose, we took advantage of the GTEx repository.

We implemented a previously described protocol (Montnach et al., 2018) to explore the relation between the abundance of the *Pkp2* transcript and the abundance of each of the 27,856 transcripts stored in the GTEx v8 databank under the human atria category. The pipeline for data generation is shown in Figure 11. The resulting volcano plot (Figure 10B) presents two arms: one, to the right of the ordinate, indicating that the abundance of *Pkp2* correlated directly with the abundance of a given transcript (a positive regression coefficient in the linear relation; see Figure 11) and the one to the left of the ordinate, where the abundance of *Pkp2* is inversely correlated with the specific transcript (negative regression coefficient). Overall, the plot shows a high degree of association between the abundance of *Pkp2* and that of 12,611 genes with a slope of the linear regression fit of more than 0.5 and an adjusted p-value (padj) value of <0.001, of which 5,419 transcripts were positively correlated and 7,192 transcripts were negatively correlated. Pathway analysis of those genes is shown in Supplemental Table 2. We found consistency with pathways previously identified from bulk transcriptomics of PKP2cKO tissue (Cerrone et al., 2017) and of ARVC human samples (Pérez-Hernández et al., 2022) and that have been linked to the pathophysiology of ARVC, such as oxidative phosphorylation, citrate cycle (TCA cycle), and nucleotide excision repair (Figure 12). We also found that, consistent with previous studies, there is an over-representation of pathways linked to neurodegenerative diseases (Figure 12B).

The nature of the GTEx plot is only correlative. The differential transcriptomic data (via RNA-seq of PKP2cKO vs control atria) on the other hand, directly reflects changes in the abundance of a given gene consequent to reduced PKP2 expression. We therefore cross-referenced both datasets to identify transcripts that show a significant correlation with PKP2 abundance in the human heart and that depend on PKP2 expression, as determined by the RNA-seq experiments. We found that 33 of the 215 downregulated transcripts in the PKP2cKO mouse had a significantly positive correlation with *Pkp2* (blue circles in Figure 10B) and 8 of the 136 upregulated transcripts had a significantly negative correlation with *Pkp2* (red circles in Figure 10B). The complete datasets are provided in Supplemental Table 3. Our results suggest that genes involved in the pro-fibrotic remodeling of the heart (e.g. *COL4A4*, *COL8A1*, *COL18A1* and *P3H3*), in an intercalated disc-transcription axis (*JUP*) and in mitochondrial function (e.g. *NDUFB4*, *COX7A2*, *ATP5MD*, *DNAJC15* and *NDUFB1*) are part of a common gene network that is subject to modulation by the levels of *Pkp2* expression. Interestingly, this network also includes genes coding for proteins that regulate sarcomere function (e.g. *MYBPC3*, *ITGA7*, *SGCA* and *LRRC39*). As a final step, we tested whether the prediction obtained through the discovery process outlined in Figure 10, was confirmed by direct experimental methods. We focused on MYBPC3, a transcript coding for a protein critical for the regulation of sarcomere function in the adult heart and related to several cardiomyopathies, as well as atrial fibrillation (Hershberger et al., 2010; Owais et al., 2024). As shown in Figure 13, RT-PCR (A), Western blot (B) and immunolocalization (C) experiments revealed a reduced abundance of the MyBPC3 protein in PKP2cKO atrial myocardium and isolated PKP2cKO atrial myocytes when compared to control. These results were consistent with those obtained through the transcriptomic pipeline and supported the notion of a link between PKP2 and MyBPC3 that can have

important consequences in the generation of the atrial cardiomyopathy observed in PKP2 deficient hearts.

DISCUSSION

We carried out a comprehensive characterization of the role of PKP2 in the atrial myocardium, using a model of PKP2 deficiency (PKP2cKO mice) with a well-characterized ventricular phenotype. In contrast to the previously-reported observations in PKP2cKO ventricular myocytes (Cerrone et al., 2017), atrial PKP2cKO myocytes presented no significant differences in Ca^{2+} transient dynamics, SR load and action potential morphology. However, PKP2cKO atrial myocytes showed an increased frequency and amplitude of Ca^{2+} sparks, in combination with increased diastolic intracellular Ca^{2+} and cellular ROS levels. ISO exposure further increased the frequency of Ca^{2+} sparks and the levels of diastolic Ca^{2+} , and those effects were reversed by acute *in vivo* treatment with Dantrolene, a known RyR blocker. PKP2cKO atrial myocytes also presented enhanced sarcomere shortening and impaired sarcomere relaxation. Transcriptomic analysis followed by direct biochemical studies identified a reduced abundance of the sarcomeric protein MyBPC. The latter, we speculate, may have a mechanistic role in the cellular shortening and relaxation changes observed. A comparison between the changes in PKP2cKO ventricular versus atrial myocytes is presented in Figure 14.

Fibrotic infiltration of the ventricular wall is well-documented in PKP2cKO mice. As reported by Cerrone et al (Cerrone et al., 2017), a significant increase in collagen content is first noted in the RV free wall 21 days after TAM injection (21 dpi), eventually being also manifest in the LV free wall (at 28 dpi; see (Cerrone et al., 2017)). For the present study, mice were euthanized and studied at 21 days post-TAM. This time point has been chosen in previous reports from our lab ((Cerrone et al., 2017; van Opbergen et al., 2022, 2024; Pérez-Hernández et al., 2022)) because of a consistent characteristic: RV dilation, RV fibrosis, and yet, minor LV fibrosis with preservation of ejection fraction; in other words, a cardiomyopathy of right ventricular predominance with only minor involvement of the left ventricle. The data presented here show abundant collagen infiltrates in the left atria at 21 dpi, thereby preceding LV fibrosis and also preceding a reduction of left ventricular ejection fraction below 45% (Cerrone et al., 2017). Left atrial fibrosis seems to appear even at an earlier stage, prior to significant increase in collagen abundance in the ventricular walls, as hinted by a close analysis of previously published results (see Figure 1 of Cerrone et al; heart at 14 dpi (Cerrone et al., 2017)). These results suggest that atrial remodeling is not consequent to a failure of LV contractile function, but rather, a process intrinsic to the loss of PKP2 in the atria itself. Whether the fibrotic process contributes, through direct or paracrine regulation, to the observed changes in Ca^{2+}_i homeostasis, remains unclear. On the other hand, it is possible that myocyte-intrinsic changes in contractility, ROS production and Ca^{2+}_i dysregulation (e.g, Fig 2,3,6,7&8) favor myocyte damage and myocyte death, which would be followed by a reparative fibrosis throughout the structure. Both processes in combination (a cell-intrinsic pro-fibrotic process consequent to PKP2-dependent fluctuations in the transcriptional network on one hand, and myocyte damage produced by the proliferative fibroblasts, on the other) would generate a positive feedback loop that would facilitate the damage of the atria as a whole.

Exercise negatively impacts ARVC progression and is considered an independent risk factor for arrhythmias consequent to PKP2 deficiency.(Sawant et al., 2014) In ARVC patients, sympathetic input can be particularly arrhythmogenic and sympathectomy is an effective method of treatment in some patients.(Assis et al., 2019) However, the effect of catecholaminergic input on the PKP2 deficient atrial myocardium remains understudied. Here, we show that beta-adrenergic stimulation (by ISO) of PKP2-deficient atrial myocytes elicited an enhanced increase in the frequency and amplitude of Ca^{2+} sparks, compared to control cells. PKP2cKO cells were also more susceptible to beta-adrenergic stimulation in regard to diastolic Ca^{2+} levels, presenting a larger increase consequent to ISO stimulation, while the SR load upon ISO exposure tended to be lower in PKP2cKO compared to control cells. These data suggest that adrenergic stimulation of PKP2-deficient atrial myocytes causes an excessive leak of Ca^{2+} out of the SR, a well-known pro-arrhythmic trigger in cardiac myocytes. We thereby speculate that exercise, besides increasing the susceptibility for ventricular arrhythmias, as well enhances the risk for atrial arrhythmias in patients with PKP2-related ARVC.

Our previous studies show that in PKP2 deficient ventricular tissue, dysregulation of intracellular Ca^{2+} homeostasis is based on transcriptional changes of Ca^{2+} handling related genes. Yet, a similar transcriptional remodeling was not observed in PKP2 deficient atrial myocytes. Moreover, substantial structural and functional differences exist between atrial and ventricular myocytes regarding Ca^{2+} handling. Atrial myocytes lack substantial T-tubules, and possess two populations of RyRs: one minor group (junctional RyRs) that sits just beneath the sarcolemma and another group (non-junctional RyRs) located deep inside the cell and not forming a dyadic structure with sarcolemmal calcium channels(Mackenzie et al., 2004; Bootman et al., 2006). Importantly, previous studies showed that, in PKP2cKO ventricular myocytes, there is increased proximity between $\text{Ca}_v1.2$ and RyR2 and this proximity can play a role in the observed increase in the Ca^{2+} transient amplitude(Cerrone et al., 2017). Thus, the structural differences between atrial and ventricular myocytes may explain, at least in part, the differences in terms of Ca^{2+} transient dynamics.

Ca^{2+} reuptake into the SR is more robust in atrial myocytes, yielding a relatively greater content of Ca^{2+} in the SR when compared to ventricular myocytes(Hove-Madsen et al., 2004; Mackenzie et al., 2004; Bootman et al., 2006; Neef et al., 2010; Voigt et al., 2012). At first glance, an increase in SR Ca^{2+} leak (as observed in our studies) would be expected to reduce the SR Ca^{2+} pool; yet, no changes in SR Ca^{2+} load were detected. This seemingly paradoxical result is reminiscent of previous results showing that the CASQ2-K180R mutation provokes spontaneous RyR2 Ca^{2+} release, without affecting SR load or the expression of Ca^{2+} -handling related proteins(Wleklinski et al., 2022). In that study, CASQ2-K180R was specifically found to impair the dynamic buffering of Ca^{2+} within the SR, without affecting total SR Ca^{2+} content. It would be interesting to speculate that PKP2 deficiency as well impacts on the intra-SR Ca^{2+} buffering in atrial myocytes. In addition, it is important to highlight that mitochondria influence excitation-contraction coupling by means of Ca^{2+} uptake, buffering and release(Dedkova & Blatter, 2013). Changes in mitochondrial Ca^{2+} levels due to mitochondrial dysfunction can potentially influence diastolic Ca^{2+} , RyR2 function and SR load in PKP2cKO atrial myocytes by means other than (or in addition to) elevated ROS levels. Dissecting the contribution

of individual components to the overall change in RyR2 activity will be a complex and interesting endeavor in future studies. Our data show that Dantrolene was very effective in reducing the extent of spontaneous SR Ca^{2+} release events and diastolic Ca^{2+} levels, while improving SR Ca^{2+} load, upon ISO exposure. However, Dantrolene was not as effective under control (non ISO-stimulated) conditions. When β -adrenergic agonists are applied to atrial myocytes, there is a rapid change in spatial properties of the intracellular Ca^{2+} signals, whereby Ca^{2+} signaling acutely changes from being a peripheral-only elevation of Ca^{2+} to a global response in which the non-junctional RyRs participate (Mackenzie et al., 2004). The involvement of a larger pool of RyR2 channels possibly explains the more prominent effect of Dantrolene upon ISO exposure. The beneficial effect of Dantrolene on atrial Ca^{2+} dysregulation has been confirmed in human heart failure and AF cardiomyocytes (Hartmann et al., 2017) and, combined with our data, suggest that Dantrolene could be a potential antiarrhythmic for both atrial and ventricular arrhythmias in ARVC patients carrying PKP2 mutations. Dantrolene is a clinically approved drug, currently used for the treatment of malignant hyperthermia (Krause et al., 2004), and therefore amenable for repurposing to treat the ARVC patient population. It is important to note that our studies characterize the development of potential substrates and triggers in the setting of PKP2 deficiency in atrial tissue, but the anatomical differences between mice and human preclude us from making an assessment of atrial fibrillation in mice as a relevant parameter in the context of the human disease.

Our GTEx dataset suggested that PKP2 expression is coordinated with genes of importance for cardiomyocyte sarcomere function, mitochondrial energy production and pro-fibrotic remodeling of the heart. We then complemented GTEx with transcriptomic data from PKP2cKO mice, allowing us to identify not only transcripts whose abundance seem to concur, but also that their abundance depends on the expression of PKP2. Through this process, we showed that PKP2 expression is coordinated with (a) the expression of a transcript involved in cell-cell contact and the signal transduction from the intercalated disc to the nucleus (*JUP*), (b) transcripts involved in sarcomere function (*MYBPC3*, *SGCA* and *LRRC39*), (c) oxidative phosphorylation (e.g. *NDUFB4*, *COX7A2*, *ATP5MD*, *DNAJC15* and *NDUFB1*) and (d) collagen syntheses (e.g. *COL4A4*, *COL8A1*, *COL18A1* and *P3H3*). The broad discovery (omics) approach thus pointed out to individual candidates to be tested by direct biochemical methods. Follow-up studies confirmed the reduction in MyBPC3 transcript and protein and as such, bases for the hypothesis that loss of PKP2 leads to mechanical dysfunction of the atrial myocytes, and that the latter results, at least in part, from changes in the transcript abundance of an important sarcomere protein.

Mutations in *MYBPC3* can lead to various forms of cardiomyopathies, such as dilated cardiomyopathy, hypertrophic cardiomyopathy and left ventricular noncompaction cardiomyopathy (Hershberger et al., 2010; Yuan et al., 2017; Tudurachi et al., 2023). In addition, a recent genome-wide association study showed that genetic variants in *MYBPC* significantly relate to atrial fibrillation, confirming the importance of MYBPC for atrial function (Owais et al., 2024). The protein MyBPC associates with both actin and myosin and as such, serves as a brake that limits cross-bridge interactions during contraction. Our data shows that PKP2cKO atrial myocytes present impaired relaxation and enhanced

sarcomere shortening, consistent with down-regulation of MyBPC3. It is worth mentioning that previous studies have reported seemingly contrasting findings regarding the regulatory effect of MyBPC3. On one hand, Fraysse et al. showed that cardiomyocytes of mice carrying a *MYBPC3* point mutation, frequently associated with HCM, exhibited higher myofilament Ca^{2+} sensitivity, shorter diastolic sarcomere length, faster Ca^{2+} transient decay and reduced *in vivo* fractional shortening (Fraysse et al., 2012). On the other hand, Toepfer et al. showed that stepwise loss of MyBPC3 resulted in augmentation of myosin contractility and prolongation of the sarcomere shortening relaxation time (Toepfer et al., 2019). Of note, these studies were performed in ventricular myocytes; yet, the results seem to concur with the changes in sarcomere shortening dynamics observed in PKP2 deficient atrial cardiac myocytes.

We acknowledge that the genetic deficit in PKP2 created for the purpose of this study does not reproduce that of humans with ARVC, where PKP2 deficiency is very rarely recessive and it does not affect only one cell type or developmental stage. In fact, no experimental model, be it murine or cell-based, properly recapitulates ARVC. In this study, we identify the atrial endophenotype of a protein that, when mutated in a human heart, causes ARVC. It is important to note that existing evidence suggests that PKP2-related ARVC is consequent to a loss of function of the PKP2 protein (Dries et al., 2021). The PKP2cKO model has been used to generate pre-clinical data toward actionable treatment in humans (e.g., a pilot clinical trial on use of flecainide in ARVC patients; [NCT03685149](#) (Wojciech Zareba et al., 2023), and PKP2-related gene therapy; RP-A601 (van Opbergen et al., 2024) and (Wu et al., 2024)). Future studies will allow us to discern the extent of the similarities between the murine and the human atrial phenotype, and whether the murine model is also a useful tool to advance atrial-related therapy in humans.

In conclusion, we describe that loss of PKP2 expression affects cellular Ca^{2+} handling and electrophysiology differently in the atrial versus ventricular myocardium. We speculate that an increased probability of opening of Ryr2 channels, supported by increased ROS production and hence, likely RyR2 oxidation, serve as a pro-arrhythmic trigger that, combined with a highly fibrotic substrate, can facilitate atrial arrhythmias. We also found that the dysregulation of Ca^{2+}_i can be amplified by catecholaminergic input and that RyR blockers can dampen these pro-arrhythmic effects, and we describe a PKP2-related gene network that includes various components of the ARVC molecular phenotype. Taken together, our findings provide novel insights into the role of PKP2 in the atrial myocardium with potential implications to the therapeutic management of AF in patients with PKP2-related ARVC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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KEY POINTS

- Atrial arrhythmias occur in a large group of patients with Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC), a cardiac disease mostly caused by pathogenic variants in the desmosomal gene Plakophilin-2 (PKP2). Exercise is considered an independent risk factor for arrhythmias consequent to PKP2 deficiency.
- We show that loss of PKP2 expression affects cellular calcium handling and electrophysiology differently in left atrial versus ventricular myocardium and causes extensive atrial fibrosis.
- PKP2-deficient atrial cardiomyocytes present increased spontaneous SR calcium release events, further enhanced by isoproterenol exposure and reversible by a RyR blocker (Dantrolene). In addition, PKP2-deficient atrial myocytes exhibit impaired relaxation and enhanced sarcomere shortening, likely related to reduced abundance of Myosin Binding Protein C.
- We speculate that cellular effects reported upon isoproterenol impact on the exercise-related atrial arrhythmia risk in ARVC patients.
- We further propose that therapeutic approaches aimed at mitigating ventricular damage may be effective to treat the atrial disease in ARVC.

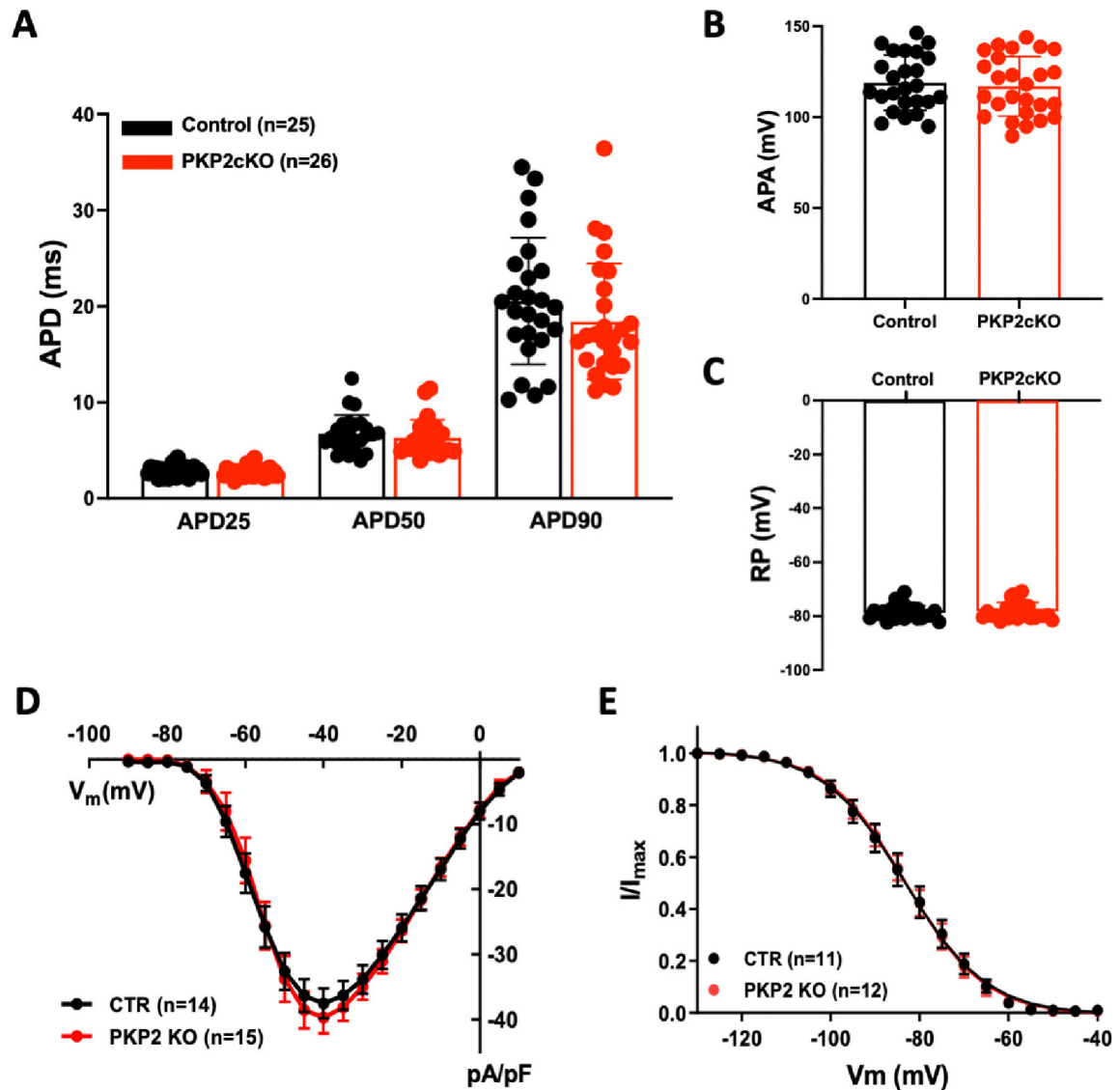


Figure 1: Action potential duration and sodium current properties recorded from control and plakophilin-2 conditional knockout (PKP2cKO) atrial myocytes.
A) Bar graphs depicting action potential duration at 25% (APD25), 50% (APD50) and 90% (APD90) of repolarization, **B)** action potential amplitude (APA) and **C)** resting membrane potential (RMP) in control (black bar) and PKP2cKO (red bar). **D)** Average peak sodium current density as a function of voltage command. Peak current amplitude at -40 mV. **E)** Voltage-dependence of I_{Na} steady-state inactivation curves. Voltage for half-maximal inactivation ($V_{1/2}$). Data is presented as mean $\pm$ SD. Number of cells noted in the corresponding plots; 5 mice per group. Test for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkell et al (Sikkell et al., 2017). Hierarchical statistics were used for all parameters. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests.

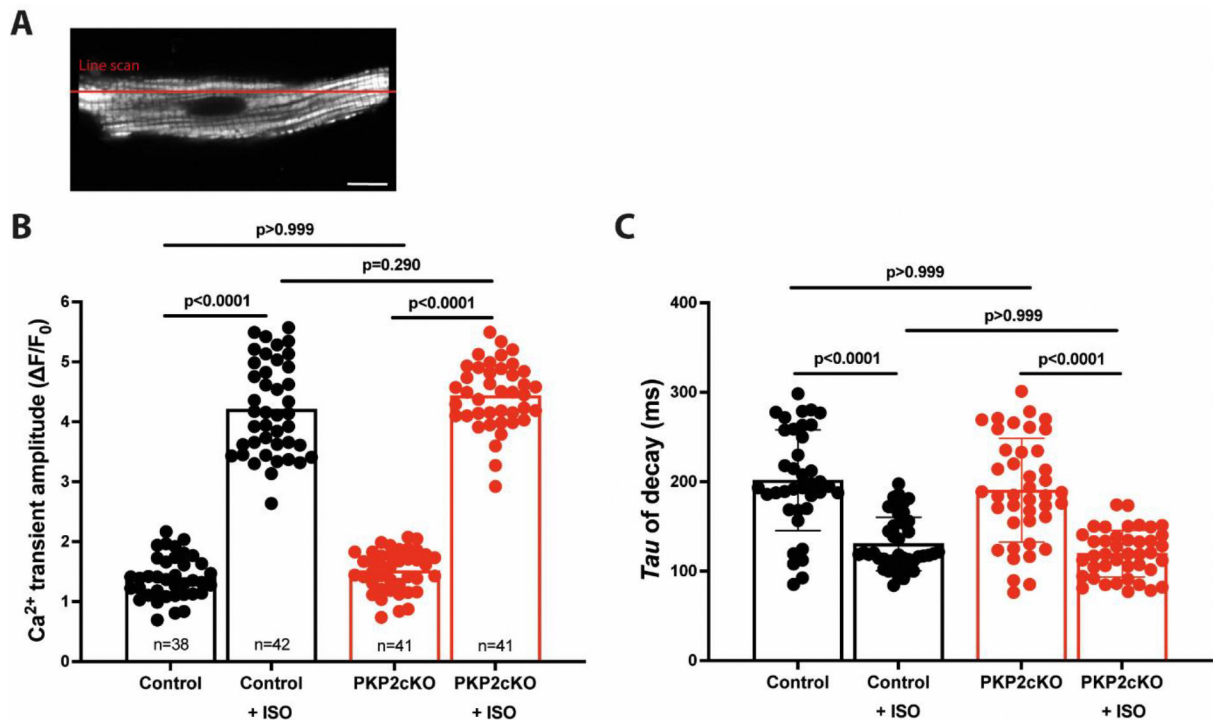


Figure 2: Calcium transient dynamics and the extent of calcium sparks in control and plakophilin-2 conditional knockout (PKP2cKO) atrial cardiac myocytes; response to beta adrenergic stimulation.

A) Representative example of the line scan positioning during calcium handling recordings by confocal microscopy in atrial myocytes. Scale bar: 5 μm . **B**) Calcium transient amplitude and **C**) Tau of decay in atrial control and PKP2cKO cardiac myocytes, at baseline or exposed to 100 nmol/L isoproterenol (ISO). Data is presented as mean $\pm$ SD. Number of cells noted in the corresponding bars; 4 mice per group. Tested for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkel et al (Sikkel et al., 2017). Hierarchical statistics were used for both calcium transient amplitude and tau decay. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests.

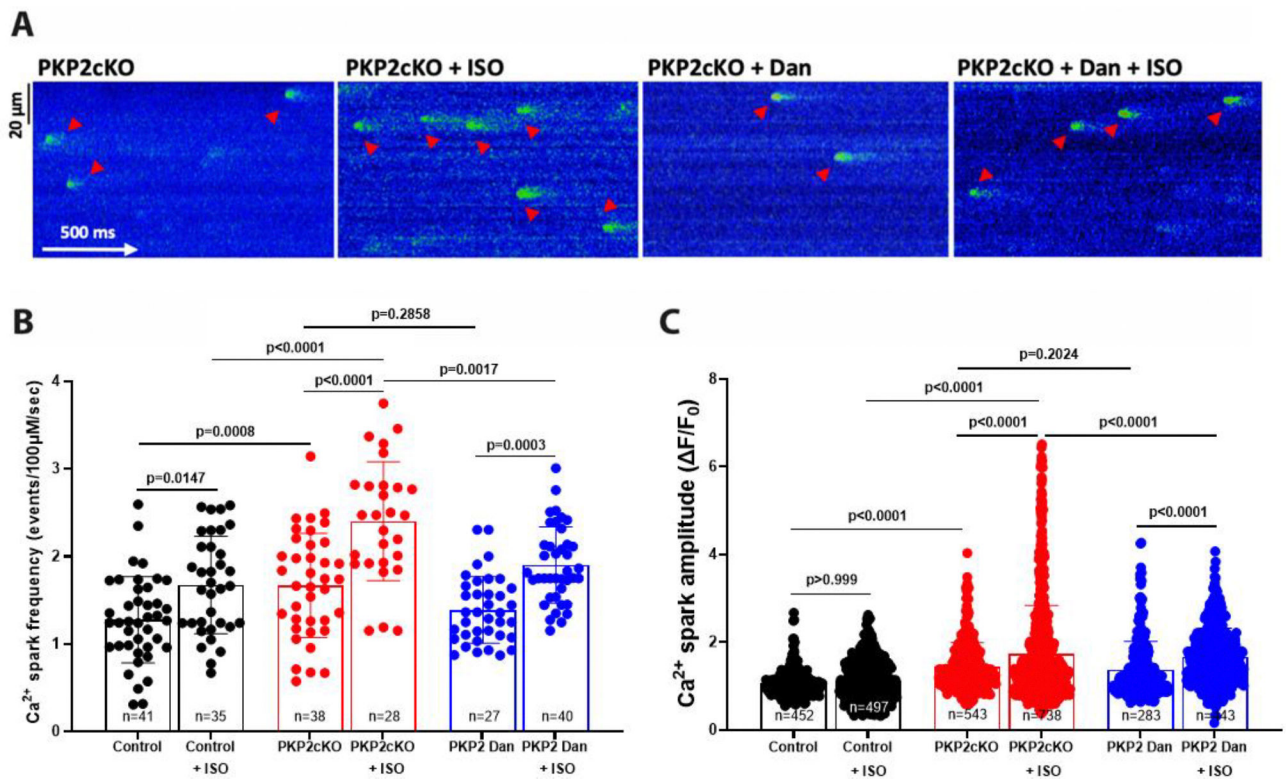


Figure 3: Extent of calcium sparks and calcium spark amplitude in atrial myocytes from control, plakophilin-2 conditional knockout (PKP2cKO) and Dantrolene-treated PKP2cKO mice; response to beta adrenergic stimulation.

A) Confocal line-scan images of calcium sparks in atrial cardiac myocytes from PKP2cKO mice treated Dantrolene, at baseline or exposed to 100 nmol/L ISO. **B-C)** Quantitative analysis of calcium spark frequency (B) and amplitude (C) in atrial cardiac myocytes from control, PKP2cKO and Dantrolene-treated PKP2cKO mice, at baseline or exposed to 100 nmol/L ISO. Number of cells noted in the corresponding bars; 4 mice per group. Tested for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkel et al (Sikkel et al., 2017). Hierarchical statistics were used for both parameters. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests.

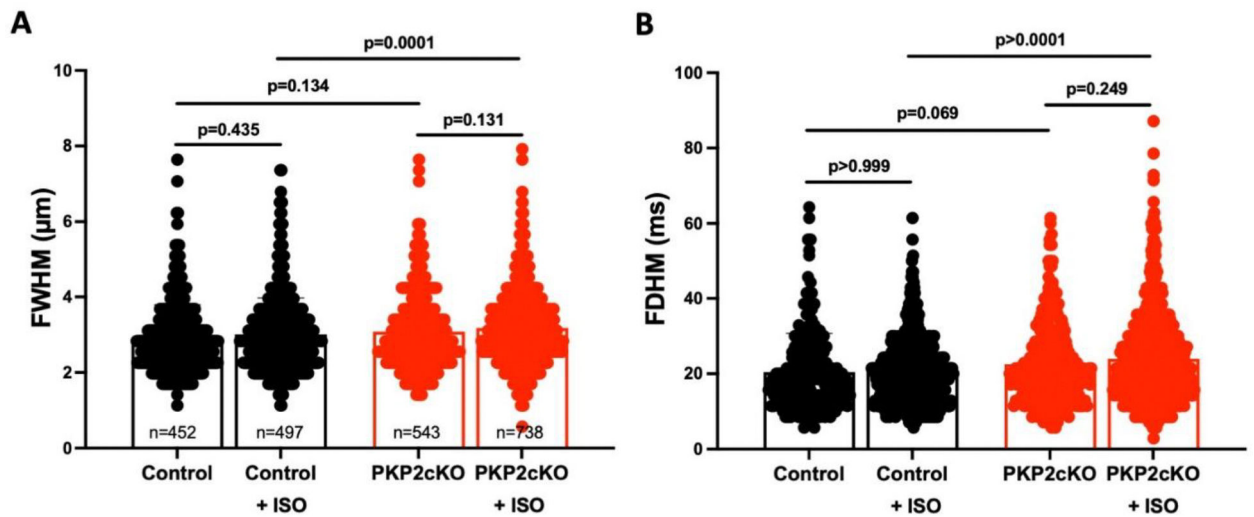


Figure 4: Width and duration of Ca^{2+} sparks from control and plakophilin-2 conditional knockout (PKP2cKO) atrial myocytes.

A) Full-width at half maximum (FWHM; μm) and **B)** Full-duration at half maximum (FDHM; ms) of Ca^{2+} sparks recorded from atrial myocytes isolated from control (black bars) and PKP2cKO mice (red bars) 21 days post-TAM, at baseline and upon 100 nM isoproterenol (ISO) exposure. Data are presented as mean $\pm$ SD. N=4 mice per group. Test for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkel et al (Sikkel et al., 2017). Hierarchical statistics on animal -and cell level were used for both FWHM and FDHM analysis. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests.

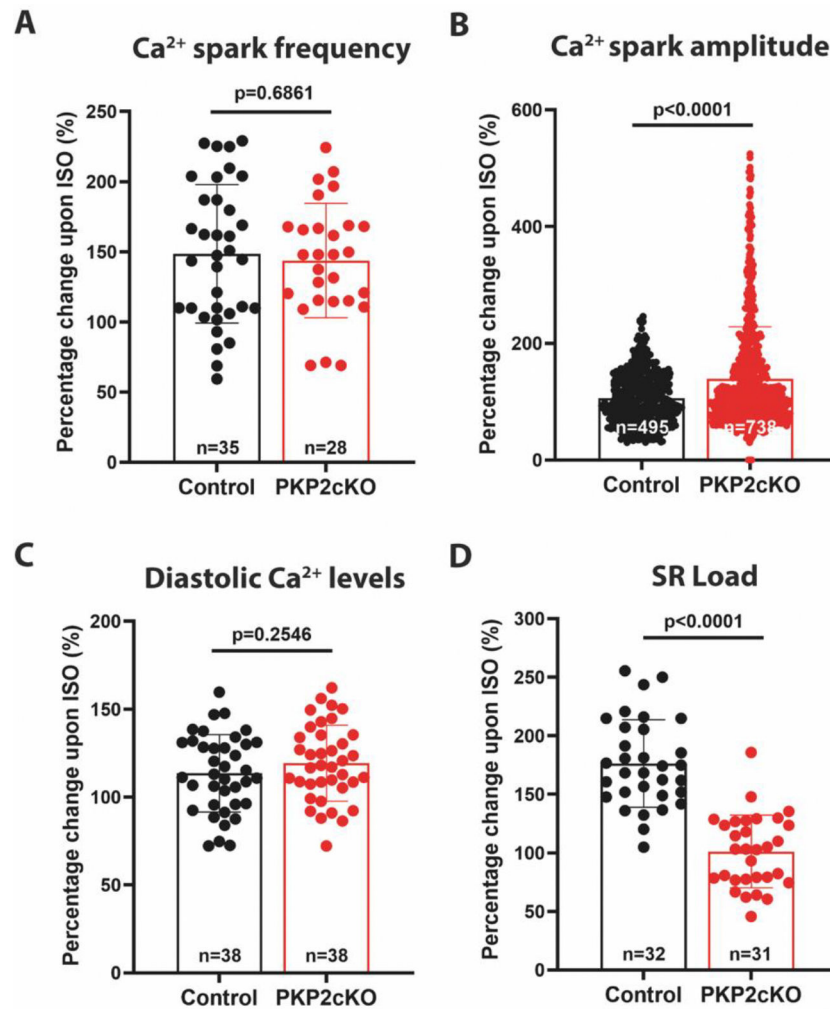


Figure 5: Effect of isoproterenol (ISO) on calcium handling parameters in PKP2cKO atrial myocytes.

Percentage of change in Ca²⁺ spark frequency, Ca²⁺ spark amplitude, SR load and diastolic Ca²⁺ levels. Normalized to values obtained in the absence of ISO (100%). Data is presented as mean $\pm$ SD. Number of cells noted in the corresponding bars; 4 mice per group, unpaired Students T-test.

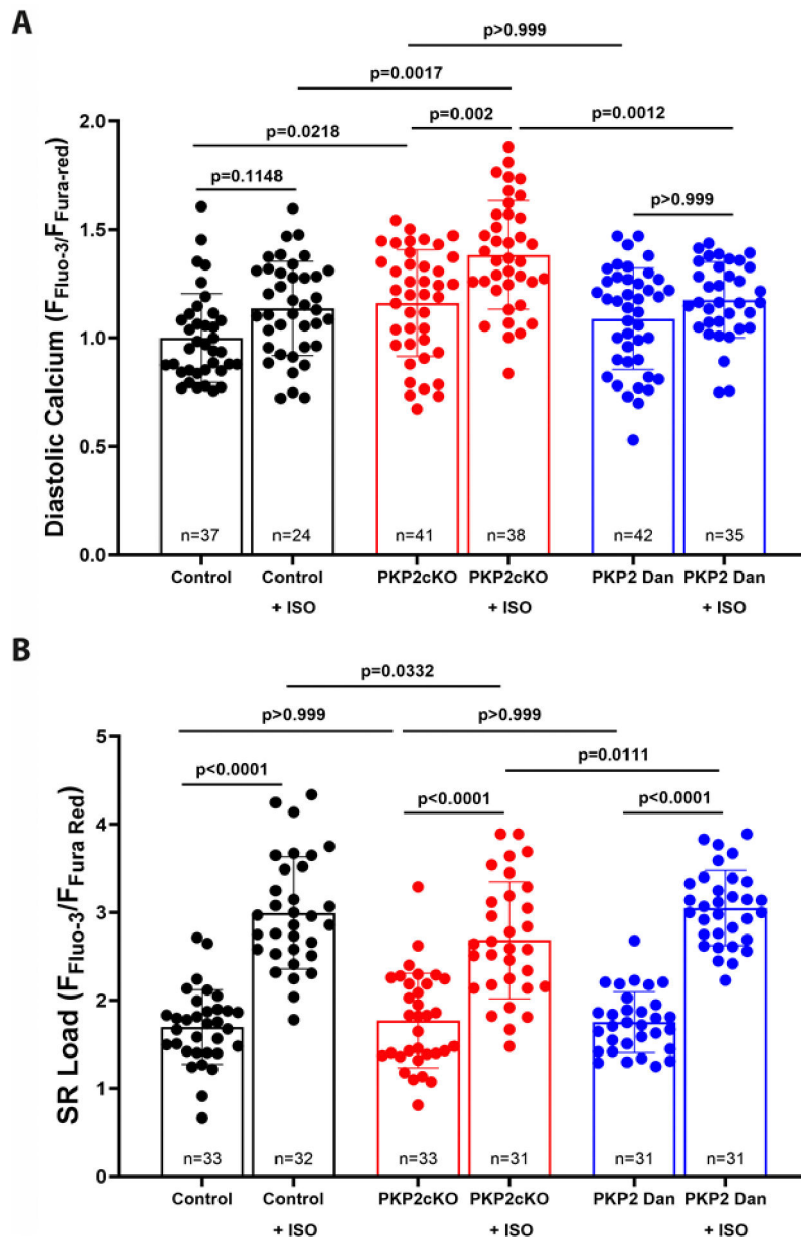


Figure 6: Intracellular calcium content and SR load in atrial cardiac myocytes from control, plakophilin-2 conditional knockout (PKP2cKO) and PKP2cKO mice treated with Dantrolene; response to beta adrenergic stimulation.

A) Diastolic calcium levels and **B)** SR calcium load in atrial cardiac myocytes from control, PKP2cKO Dantrolene-treated PKP2cKO mice, at baseline or exposed to 100 nmol/L isoproterenol (ISO). SR load is recorded upon a pulse of caffeine (10 mmol/L). Intracellular calcium levels and SR load were detected by a ratiometric method ($F_{\text{Fluo-3}}/F_{\text{Fura Red}}$). Data is presented as mean $\pm$ SD. Number of cells noted in the corresponding bars; 4 mice per group. Tested for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkel et al (Sikkel et al., 2017). Hierarchical statistics were used for both parameters. Normality of distribution was tested by Shapiro-Wilk and Kolmogorov-Smirnov tests.

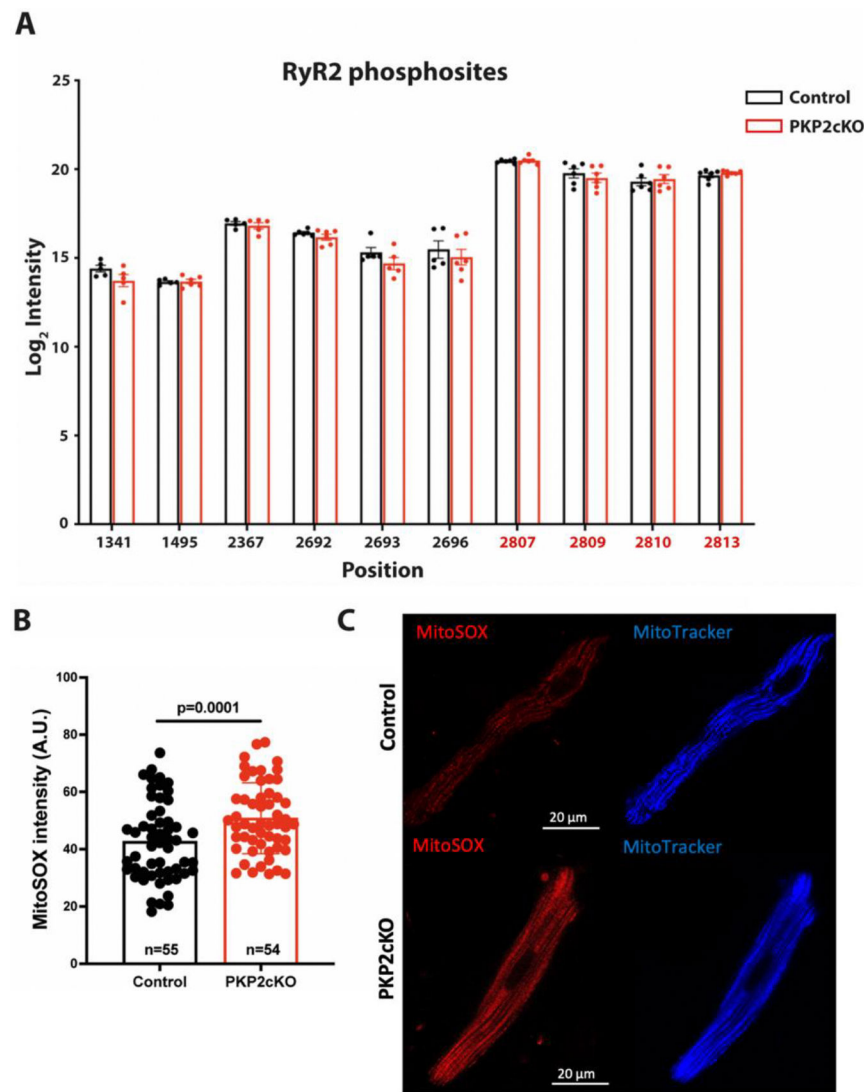


Figure 7: Post-translational modifications in the atrial myocardium from plakophilin-2 conditional knockout (PKP2cKO) mice.

A) Mass spectrometry–based investigation of ryanodine receptor 2 (Ryr2) phosphorylation state in control (black bars) and PKP2cKO (red bars) atria. Measured peptide intensities are shown for phosphorylated peptides covering the indicated Ryr2 phosphorylation sites. Amino acid positions for phosphorylation sites falling within the identified hot spot of Ryr2 are highlighted in red. Mean $\pm$ SD, atria from 6 mice per group. Evaluation of abundance differences between the two groups was conducted using Student's t-test and corrected for multiple hypothesis testing by Benjamini-Hochberg. There were no significant differences. **B)** Quantitative analysis of MitoSOX intensity (AU) in control and PKP2cKO atrial cardiac myocytes. **C)** Representative images of atrial cardiac myocytes labelled with MitoSOX and MitoTracker. Data is presented as mean $\pm$ SD. Number of cells noted in the corresponding bars; 3 mice per group. Tested for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkel et al (Sikkel

et al., 2017) and hierarchical statistics were used. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests.

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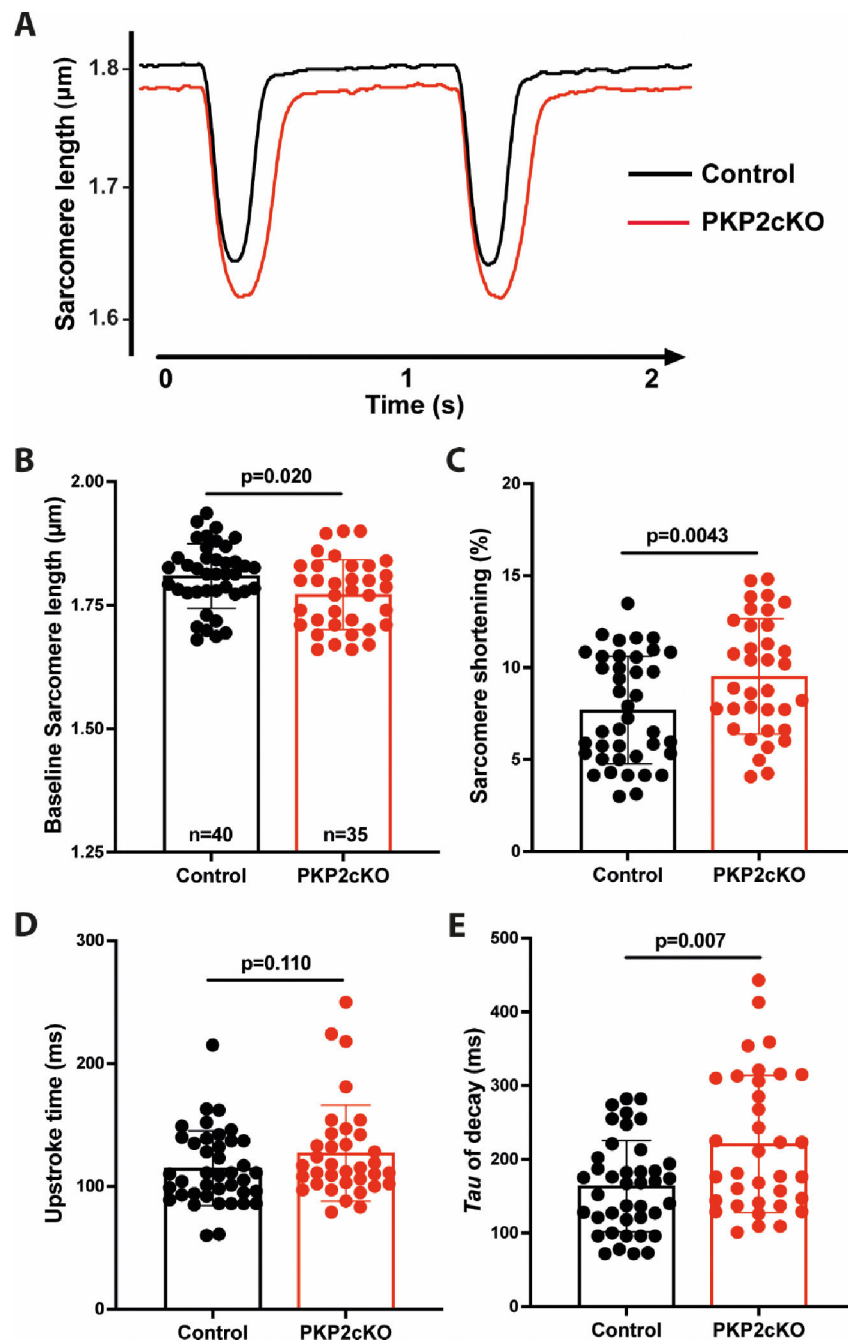


Figure 8: Sarcomere shortening dynamics in control and plakophilin-2 conditional knockout (PKP2cKO) atrial myocytes.

A) Representative examples of time course of sarcomere shortening (in control and PKP2cKO atrial cardiac myocytes during 1 Hz field stimulation. **B-E)** Quantitative analysis of **(B)** Baseline sarcomere length, **(C)** percentage of sarcomere shortening, **(D)** upstroke time and **(E)** τ of decay in control (black bars) and PKP2cKO (red bars) atrial cardiac myocytes. Data is presented as mean $\pm$ SD. Number of cells noted in the corresponding bars; 4 mice per group. Tested for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkel et al

(Sikkel et al., 2017). Hierarchical statistics were used for all four parameters. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests.

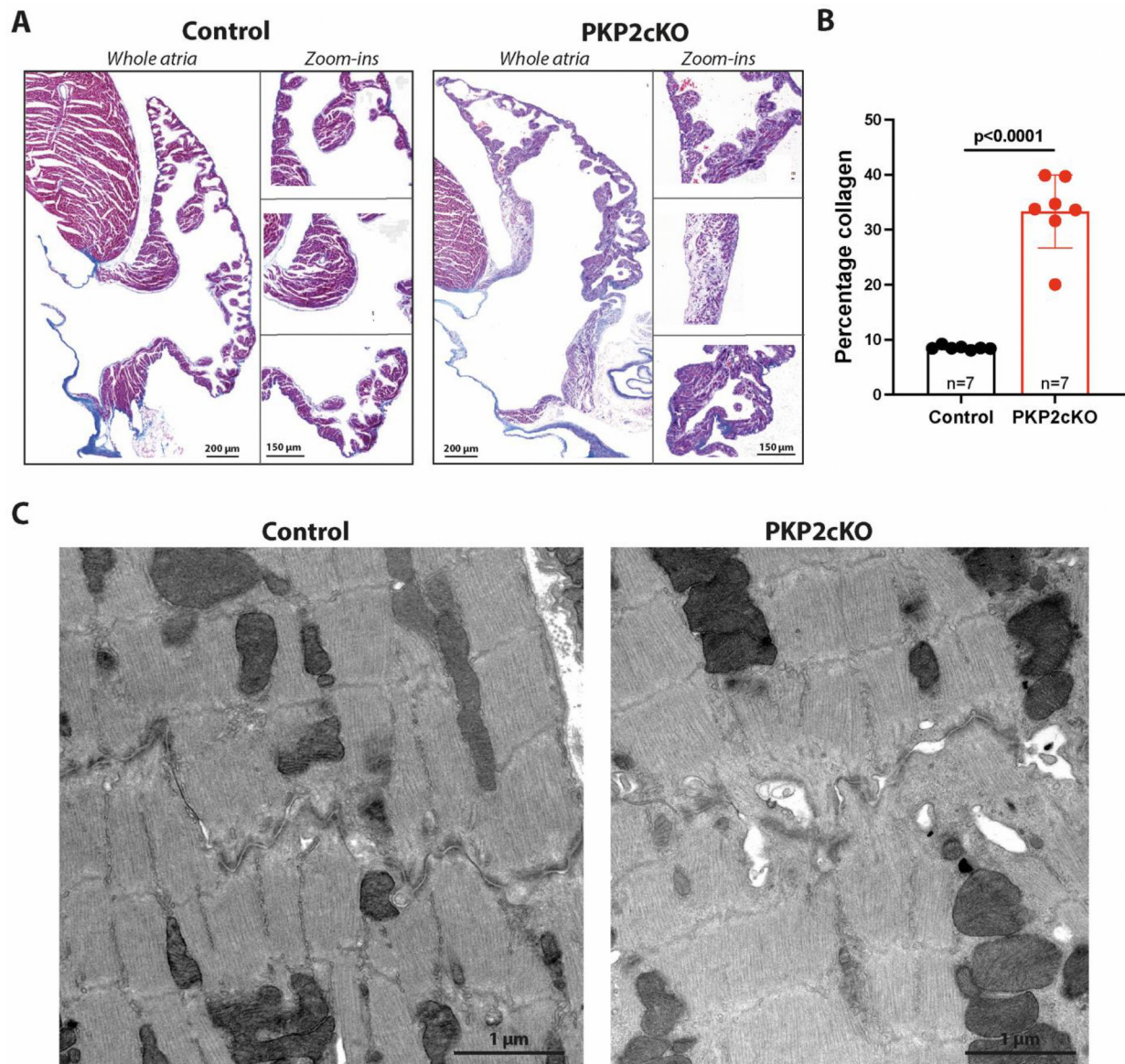


Figure 9: Structural remodeling on the macro and micro scale in atria from plakophilin-2 conditional knockout (PKP2cKO) mice.

A) Representative examples of Masson's Trichrome staining in left atria from control and PKP2cKO mice. Per genotype, left panels represent the entire atria and panels on the right zoom-ins throughout the atrium. **B)** Quantitative analysis of percentage of collagen in the left atrium from control and PKP2cKO mice. Data is presented as mean $\pm$ SD; 7 mice per group; Student's Test. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests. **C)** Single frame of a serial block face-scanning electron microscopy (SBF-SEM) - acquired set showing the ultrastructure of the intercalated disc from control (left panel) and PKP2cKO (right panel) left atrial tissue. Scale bar: 1 μ m. A complete 3D volume is presented in supplemental movies 1 and 2.

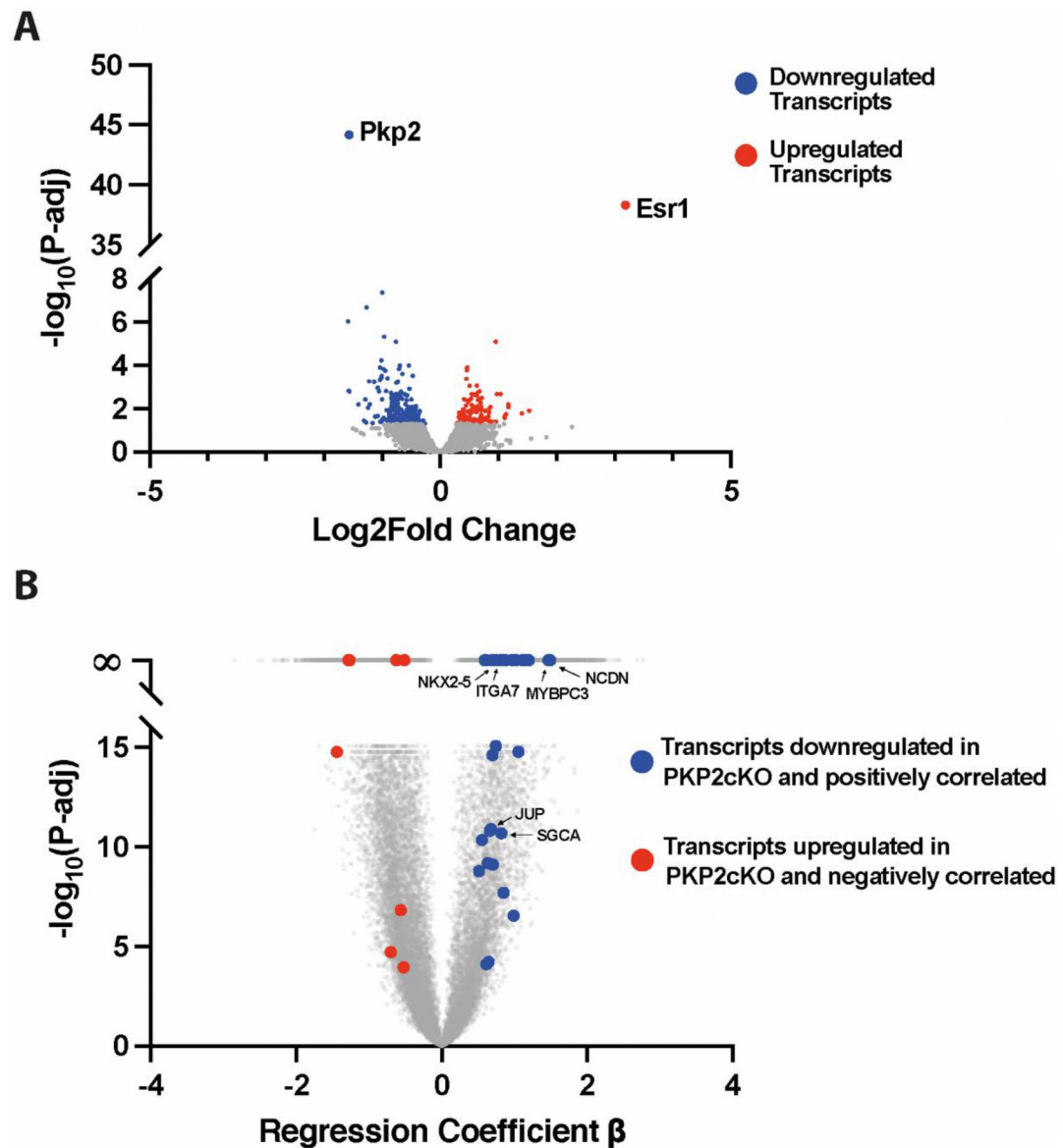


Figure 10: Transcripts correlated with plakophilin 2 (PKP2) expression in the human and murine atrial myocardium.

A) Volcano plot showing the differential transcriptome analysis in left atria from control versus plakophilin-2 conditional knockout (PKP2cKO) mice. Thresholds of $|\log_2\text{FC}| > 0.25$ and 5% false discovery rate ($\text{FDR} < 0.05$). Notice the interruption in the ordinate axis to include the data corresponding to *Pkp2* and to *Esr1*. **B)** Volcano plot showing the association analysis of the human atrial transcriptome in relation to PKP2 expression. Blue dots and red dots: transcripts significantly downregulated and upregulated, respectively, in atrial PKP2cKO RNAseq dataset. Selected gene names are indicated in the figure. Dots on top, and marked with an infinity sign in the ordinates, represent transcripts with a $-\log_{10} \text{p adj}$ values larger than 30.

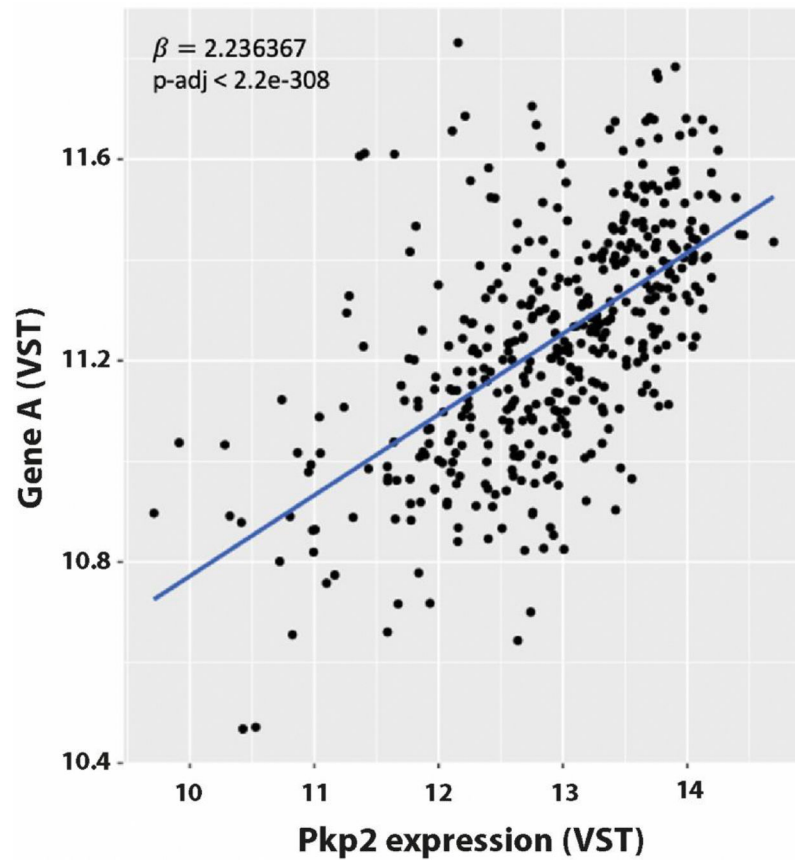


Figure 11: Linear regression of normalized Gene A expression vs normalized Pkp2 expression. Linear regression between each transcript and Pkp2 normalized expression was used to obtain the regression coefficient (β). Regression coefficient and p-value were obtained from line of best fit. P-value was corrected by Benjamini-Hochberg method.

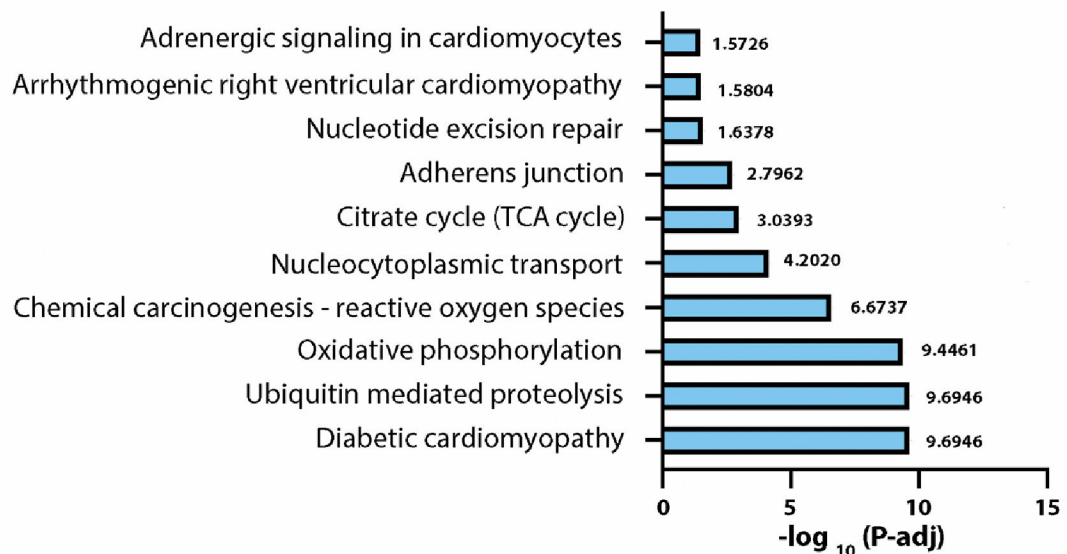
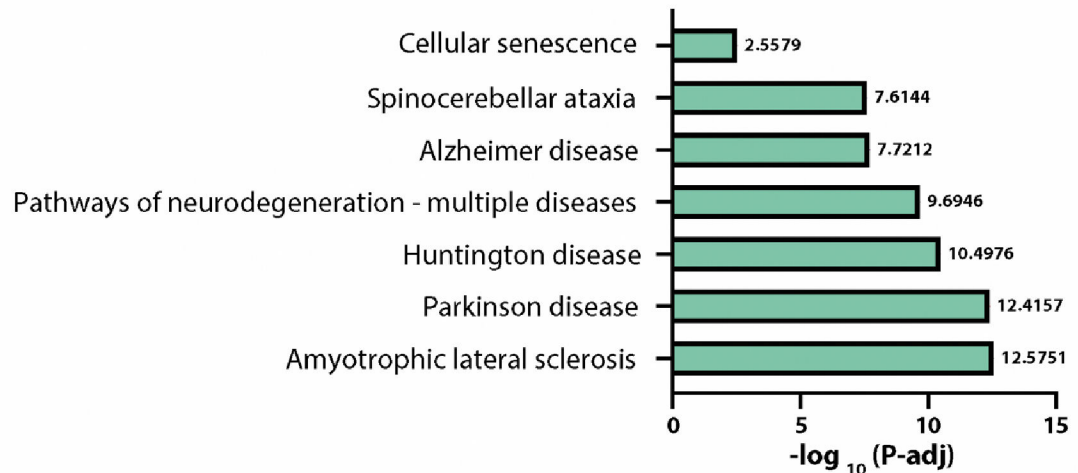
A**Pathways linked to ARVC pathophysiology****B****Pathways linked to neurodegenerative diseases**

Figure 12: KEGG pathway analysis on human atrial GTEx dataset, of genes significantly correlated to *Pkp2* expression.

Highlighted are pathways linked to **A**) the pathophysiology of Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) and **B**) neurodegenerative diseases. Thresholds of | regression coefficient | >0.5 and 5% P-adjusted ($P\text{-adj} < 0.001$) for significant correlation.

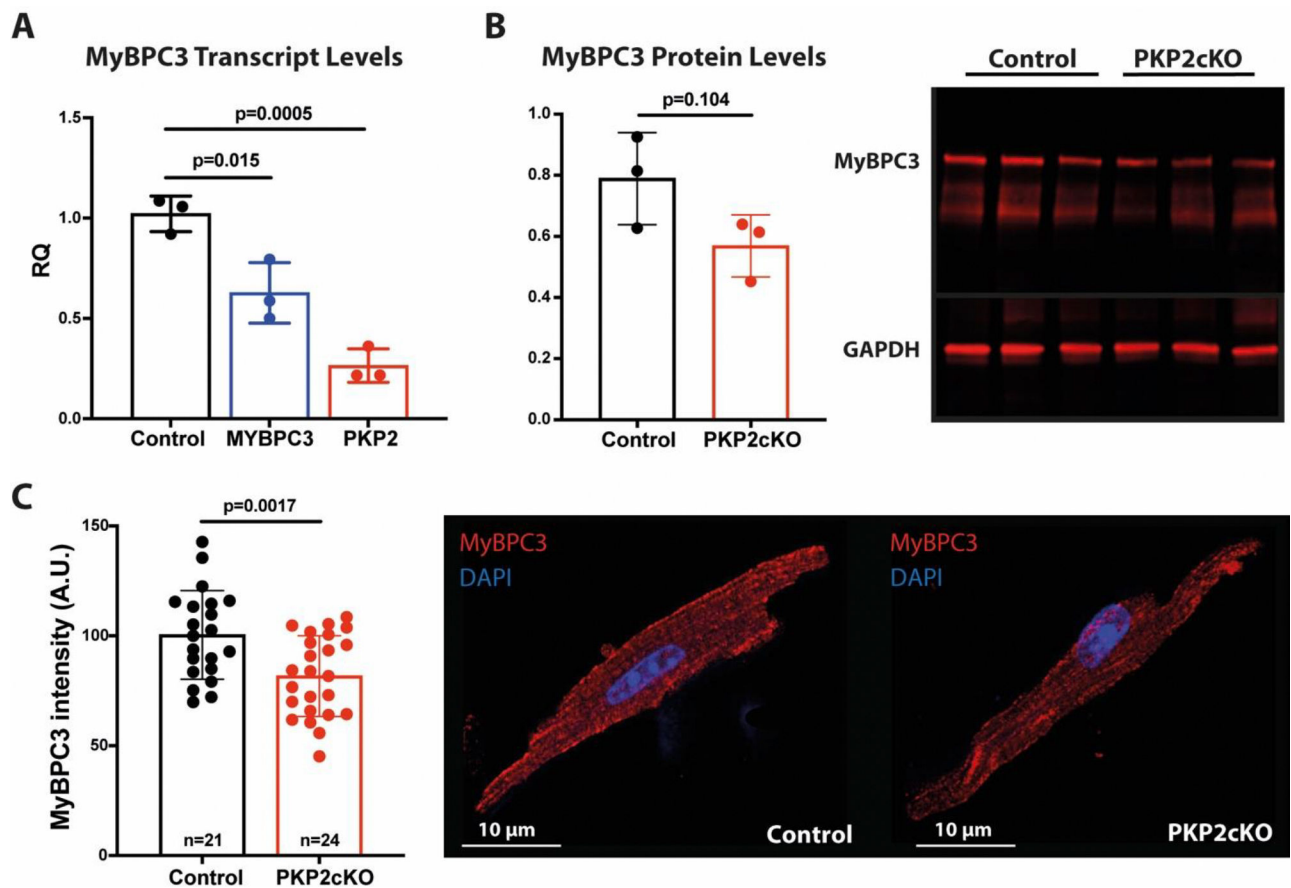


Figure 13: Down-regulation of myosin binding protein C in atrial myocardium and atrial myocytes from plakophilin-2 conditional knockout (PKP2cKO) mice.

A) RT-PCR analysis to examine transcript levels of MyBPC3 and PKP2 in control and PKP2cKO left atria. Values are normalized to transcript levels in control mice. Data is presented as mean $\pm$ SD; 3 mice per group. **B)** Quantitative analysis (left panel) and gel images (right panel) of MyBPC3 protein detected in left atria from control and PKP2cKO mice. Values are normalized to GAPDH levels in corresponding samples. Molecular weight of MyBPC3 is 140 kDa. Data is presented as mean $\pm$ SD; 3 mice per group. **C) Left panel:** Quantitative analysis of MyBPC3 intensity (AU) in control and PKP2cKO atrial cardiac myocytes. **Right panel:** Representative images of atrial cardiac myocytes immunostained for MyBPC3. Data is presented as mean $\pm$ SD. Number of cells noted in the corresponding bars; 3 mice per group. Tested for clustering versus validity of assumption of independence between data obtained from more than one mouse within a group, as in Sikkell et al (Sikkell et al., 2017); hierarchical statistics were used. Normality of distribution was tested by Shapiro-Wilk and Kolgomorov-Smirnov tests.

PKP2cKO vs. Control	Ventricle	Atria
Baseline sarcomere length	=	-
Sarcomere shortening (SS)	+	+
SS upstroke time	+	=
SS <i>tau</i> of decay	+	+
Ca ²⁺ transient amplitude	+	=
Ca ²⁺ transient upstroke time	+	=
Ca ²⁺ transient <i>tau</i> of decay	+	=
Ca ²⁺ spark frequency	+	+
Ca ²⁺ Spark amplitude	+	+
SR Ca ²⁺ load	+	=
Diastolic Ca ²⁺ levels	+	+
Action potential duration	+	=
Sodium current	=	=

Figure 14:

Overview of outcomes on electrophysiological parameters measured in plakophilin-2 conditional knockout (PKP2cKO) ventricular and atrial myocytes, versus control. Based on data described in this manuscript, as well as in (Cerrone et al., 2017, Pérez-Hernández et al. 2021, Cerrone et al., 2022). SS: sarcomere shortening, Ca²⁺: calcium, = : no change, - : reduction, + : increase.