Lysosomal signaling pathways influence heart rhythm, and regulate atrial function

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Online Methods

Animals Approvals

Mouse, rat and guinea pig animal models and tissue used in this investigation have local approval and all procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Specifically, all animal experiments were performed in accordance with the United Kingdom Home Office Guide on the Operation of Animal (Scientific Procedures) Act of 1986. All experimental protocols on mouse, rats and guinea pigs (in accordance to Schedule 1 killing; concussion followed by cervical dislocation) were approved by the University of Oxford and the University of Freiburg. Sprague Dawley rat litters, guinea pigs, C57BL/6NCrl mice and CD-1 mice were obtained from Envigo or Charles River. The $TPC2^{-/-}$ mice were created as described previously [2, 3].

Goat: The goat study was carried out in accordance with the principles of the Basel declaration and regulations of European directive 2010/63/EU. All animal procedures conformed to US National Institutes of Health guidelines and were approved by the local ethical committee of Maastricht University (DEC2014-025). Female Dutch milk goats were used in this study. Only female goats were used in AF model, as male goats are typically not retained in farming operations beyond a young age due to their limited use in reproduction and increased aggression seen in adult males. Anesthesia was induced with thiopental (10 mg/kg, Inresa Arzneimittel GmbH/Germany) and further maintained using Sufentanyl (6 µg/kg/h, Hameln Pharma/Germany), Midazolam (0.8 mg/kg/h, Actavis/Iceland) and Pancuronium (0.3 mg/kg/h, Organon/The Netherlands), anesthesia was followed by an open chest sacrifice experiment.

Drugs

Bafilomycin A1 (Bio-Techne Ltd, 1334/100U) and is an inhibitor of the vacuolar-type H^+-ATPase (V-ATPase), which is essential for acidifying intracellular compartments such as lysosomes. By inhibiting V-ATPase, bafilomycin A1 prevents lysosomal acidification. However, bafilomycin A1 has been identified to affect other cellular targets such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [26] and mitochondrial function [27]. In order to specifically target different components of the lysosomal signaling pathway, additional inhibitors were employed. The NAADP binding site antagonist *trans*-Ned-19 (Insight Biotechnology Ltd, sc-362814). TPC2-A1-N is a Ca^{2+}-permeable agonist of TPC2, which plays its role by mimicking the physiological actions of NAADP and SG-094, a specific TPC2 blocker to inhibits the Ca^{2+} release was kindly sent to us by Dr. Keller and Dr. Bracher. Ryanodine (559276, opens ryanodine receptors), thapsigargin (67526-95-8, SERCA pump inhibitor), isoprenaline (I6504), an agonist of the β-adrenergic receptors and DMSO (D5879) our solvent control vehicle used in the paper were purchased from Merck Life Science UK Limited. AC1 was inhibited using the AC1 selective inhibitor ST034307 (Tocris, United Kingdom), which has been shown to

demonstrate selectivity for AC1 over other AC isoforms at concentrations below 30 μM [28]. KN-93 is a potent, cell permeable inhibitor of CaM kinase II was also purchased by Tocris, United Kingdom.

Contraction setup

Experiments were performed according to previously published methods [38]. Hearts from male CD1 mice were rapidly dissected and place in warm, oxygenated physiological saline solution (PSS, in mM: NaCl 125, $NaHCO_3$ 25, KCl 5.4, NaH_2PO_4 1.2, $MgCl_2$ 1, glucose 5.5, $CaCl_2$ 1.8, pH to 7.4 with NaOH and oxygenated with 95 % O_2/5 % CO_2) containing heparin (10 U/ml). In a dissection chamber, left (data presented in Supplementary Fig. 1) and right atria were cleared, separated, and mounted to a force transducer in an organ bath as ventricular tissue was discarded. The organ bath was filled with PSS maintained at 37ºC oxygenated with 95 % O_2/5 % CO_2 and a resting tension between 0.2-0.3 g was applied to visualize contraction. Tension data were digitized using a PowerLabs bridge amplifier and recorded on LabChart5 software (all from AD Instruments, UK). Chronotropic and inotropic effects were measured in real-time using the Chart5 Ratemeter function. After left and right preparations had stabilized the effects of isoprenaline were investigated over a cumulatively increasing concentration range of 0.1 nM to 1000 nM, doses were administered every 3 minutes into the organ bath. Pharmacological agents, bafilomycin A1 and *trans*-Ned-19, both inhibitors of the lysosomal signaling pathway were added 45 minutes before first addition of isoprenaline to ensure effects. Preparations were excluded if stabilized beating rate under control conditions in the presence of PSS only was under 300 bpm or if preparations were arrhythmic.

Adult guinea SAN cell isolation

The methods were previously published by [8]. Guinea pig hearts were dissected and washed in heparin-containing PSS (20 IU per mL) to prevent blood clots forming and were mounted on a Langendorff setup for retrograde perfusion via the aorta. The heart was first perfused in a modified Tyrode's solution containing (in mM): NaCl 136, KCl 5.4, $NaHCO_3$ 12, sodium pyruvate 1, NaH_2PO_4 1, $MgCl_2$ 1, EGTA 0.04, glucose 5; gassed with 95 % O_2/5 % CO_2 to maintain a pH of 7.4 at 37°C for 3 minutes. Solution was switched to a digestion solution: the modified Tyrode's solution above containing 100 μM $CaCl_2$ and 0.04 mg/ml Liberase™ (Roche, Penzberg, Germany) and no EGTA. After 25 minutes of enzymatic digestion, the heart was removed. The right atrium was pinned in a dissection bath, opened by anterior incision, and the SAN was identified from anatomical features and tissue appearance. The SAN was dissected into thin strips (~2x5 mm) followed by gentle trituration and stored in a high potassium medium (KB, in mM: KCl 70, $MgCl_2$ 5, K^+ glutamine 5, taurine 20, EGTA 0.04, succinic acid 5, KH_2PO_4 20, HEPES 5, glucose 10; pH to 7.2 with KOH) at 4°C.

Calcium transient imaging of guinea pig SAN cells

Isolated atrial myocytes were incubated with 3 μM Fluo-5F-AM for 10 minutes at 37ºC in the dark in SAN KB containing (in mM): KCl 70, $MgCl_2$ 5, K^+ glutamine 5, taurine 20, EGTA 0.04, succinic acid

5, KH_2PO_4 20, HEPES 5, glucose 10; pH to 7.2 with KOH. Cells were then plated onto a glass coverslip for 10 minutes to adhere before imaging. All recordings were at 37°C under gravity-fed superfusion. Cells were visualized using a Zeiss Axiovert 200 with attached Nipkow spinning disk confocal unit (CSU-10, Yokogawa Electric Corporation, Japan). Excitation light, transmitted through the CSU-10, was provided by a 488 nm diode laser (Vortran Laser Technology Inc., Sacramento, CA). Emitted light was passed through the CSU-10 and collected by an iXON897 EM-CCD camera (Oxford Instruments, UK) at 60 frames per second with 2×2 binning (pixel size = 0.66667 μm^2). To avoid dye bleaching, video recordings were 10-20 seconds long with a frequency of 80 up to 100 frames per second. Ca^{2+} transients were measured using regions of interest (ROIs) in Andor iQ software (v 1.7) to record average whole cell fluorescence. Ca^{2+} transient measurements from spontaneously beating isolated SAN myocytes (n = 4), were obtained from a different guinea pig heart.

Neonatal rat atrial myocyte (NRAMs) cell isolation

NRAMs were isolated using a modified protocol from [70] and [71]. Hearts were dissected from 3-day-old Sprague Dawley rat pups, culled by cervical dislocation followed by exsanguination. Atria were separated from the ventricles and cut into 4 equal pieces. Atrial myocytes were digested enzymatically in trypsin (1mg/ml, Merck, UK) rocked at 4°C for 2 hours. Atrial myocytes were then enzymatically isolated a second time with collagenase (type IV, 1mg/ml, Merck, UK). Trypsin was replaced by 4ml collagenase solution and tissue was gently triturated using a plastic pipette for 1 minute to then be stirred gently in a water bath at 37°C for 2 minutes. The first supernatant was discarded. A second 4ml collagenase solution was added to the remaining tissue pellet and triturated for 1 minute using a wide bore pipette before also being stirred gently in a bath at 37°C for 2 minutes. The supernatant (4ml) was collected and added to 3ml cold Hank's buffered salt solution (HBSS) and stored on ice. This step was repeated 3 times to produce a total of four 7ml cell suspensions. Tubes were centrifuged at 2000 revolutions per minute (rpm) for 8 minutes. Supernatant was then removed, and the cells were resuspended in 2ml cardiomyocyte plating media (CPM: 67 % DMEM, 17 % M199, 10 % horse serum, 5 % FBS, 1 % Penicillin/Streptomycin) before being centrifuged at 1000 rpm for 10 minutes. All samples were then strained using a sterile cell strainer (70 µm). Isolated myocytes were pre-plated and incubated at 37°C (95 % O_2, 5 % CO_2) for 1 hour as a purification step to allow removal of fibroblasts. Myocytes were counted and seeded onto 24 mm laminin (66 $\mu g/cm^2$, Merck, UK) coated glass coverslips in 35 mm 6-well plates at a density of 30,000 cells per well.

hiPSC-CMs culture and cardiomyocyte differentiation (Winbo differentiation protocol)

The hiPSC-CMs were obtained from Dr Winbo [72], hiPSC-CMs stem cell study approved by institutional review committees in Umeå, Sweden (Regional Ethics Committee, Umeå University: Dnr 05-127M), and Auckland, New Zealand (Health and Disability Ethics Committees: 17/NTA/226), and all the participants have provided written informed consent. As previously described, reprogrammed from peripheral blood mononuclear cells from a healthy adult male using an integration-free kit

(CytoTune-iPS 2.0 Sendai Reprogramming Kit, Invitrogen, Life Technologies, now Thermo Fisher, Cat. Nos. A16517 and A16518) [72]. The hiPSC-CMs were differentiated into cardiomyocytes using previously published methods [72, 73]. Briefly, thawed hiPSC-CMs were plated on Geltrex™-covered 12-well plates ($0.3x10^6$ cells/well) and cultured in StemFlex™ medium. On day 0, at ~85 % confluency, the culture medium was changed to RPMI/B27- insulin medium with 6 µM GSK3-β inhibitor CHIR9902, followed by RPMI/B27-insulin medium with 5 µM Wnt inhibitor IWR1 on day 3. From day 7, the cells were grown in RPMI/B27+insulin medium, replaced every 72 hours. Spontaneously beating cardiomyocytes were further matured to a minimum of 21 days post hiPSC stage before experiments. For the purpose of Ca^{2+} transient imaging hiPSC-CMs were dissociated (Collagenase B 1 mg/ml in RPMI/B27+insulin medium) for 18 hours at 37°C and replated on 12 mm Geltrex™-covered glass coverslips before experiments.

hiPSC-CMs cardiac culture (Calamaio differentiation protocol)

Both hiPSC-aCMs lines used for all experiments were derived from healthy donors. In particular, one from a female donor with no diagnosed diseases (https://hpscreg.eu/cell-line/TMOi001-A, purchased from Thermo Fisher Scientific) [74] and the other, previously characterized and published from a 62-year-old male not affected by AF or other cardiac pathologies [75]. Approved protocols granted by the Ethical Committee of Brescia (protocol number 1737) and a written consent obtained from the patients, in agreement with the declaration of Helsinki. hiPSC-aCMs lines were maintained on human Biolaminin 521 LN-coated dishes in TeSR-E8™ medium (Thermo Fisher Scientific).

Cardiac differentiation was carried out by monolayer culture on Matrigel® hESC-qualified Matrix (Corning, Corning, NY, USA) coated dishes using the PSC Cardiomyocytes Differentiation Kit (Thermo Fisher Scientific), following manufacturer instructions. To induce atrial differentiation, an adaptation of the method previously described [76] was used. Briefly, cells were treated with 1 µmol/L atRA (Sigma) starting from day 3 of differentiation, and the medium was changed every day until day 7. Then, cells were maintained in Cardiomyocytes Maintenance Medium (Thermo Fisher), changing the medium every other day. On the 21st day of differentiation, hiPSC-aCMs culture was enriched using the PSC-Derived Cardiomyocyte Isolation Kit (Miltenyi Biotech) following manufacturer instructions, which allows magnetic separation with highly specific CM surface markers. Immediately after magnetic separation, iPSC-CMs were collected and cryopreserved in liquid nitrogen. For the experiments, atrial differentiated hiPSC-aCMs were thawed on Matrigel-coated dishes and maintained at 37°C (95 % O_2, 5 % CO_2) with Cardiomyocyte Maintenance Medium with B-27 Supplement 50X and penicillin/streptomycin 100X (Thermo Fisher Scientific). Cells were culture until 28 days before running FRET or live imaging.

hiPSC differentiation protocol (Sharma protocol) and super-resolution microscopy

(a) The iPSC differentiation protocol is based on previously published protocols [77, 78]. Briefly undifferentiated healthy human stem cells (ATCC) were seeded onto Geltrex coated dishes at a density of 1×10^5 cells/cm^2 and allowed to expand for 48 hours until 80 % confluent. At this stage, (day 1 of differentiation) cells were treated with StemPro-34 medium (Life Technologies), containing recombinant human Activin A (Life Technologies) and recombinant human BMP4 (R&D Systems) for 48 hours. Media exchange was performed on day 3 into medium consisting of RPMI (Life Technologies) supplemented with 1× B-27 (Life Technologies) and a small molecule inhibitor, KY02111 (R&D Systems) and XAV939 (R&D Systems). From day 5 onward, cells were maintained in RPMI medium supplemented with B-27 only, and media changes were performed every 2 days. For Elyra experiments, cells were re-plated on Ibidi µ-slide 8 well glass bottom (#1.5H (170 µm +/- 5 µm) D 263 M Schott glass, sterilized) that were coated with geltrex. LysoTracker Red DND-99 (L7528) staining was performed on the cells for 20 minutes, washed twice in PBS, fixed in 4 % formaldehyde, washed with PBS and surface covered in 100 µL PBS, slide protected with tin foil, stored at 4°C until imaging was performed.

(b) Super-resolution microscopy: Imaging of hiPSC-cardiomyocytes was performed on an Elyra7 microscope (Zeiss). Images were captured using the Apotome modality (5 phases) and a 63x 1.4 NA Plan-Apochromat objective (Zeiss, catalog # 420782-9900-799). Samples were excited with a 561 nm (output set to 200 mW) HR DPSS laser at 6 % power for LysoTracker Red imaging and a 405 nm (50 mW) HR diode laser at 4 % power for DAPI imaging, while emission was detected using two PCO Edge 4.2 sCMOS cameras, with an exposure time of 600 ms, and a 560 nm long pass beam splitter. Images were captured as z-stacks with a z-interval of 0.273 µm. Captured images were processed using the Zen Black (ZEN 3.0 SR, version 16) SIM^2 module in LEAP mode using an iteration of 20, regularization weight of 0.04 and processing/output sampling of 3x. The final z-interval for LEAP processed images was 0.091 µm.

Microscope component	Zeiss Elyra
Light source	Zeiss Laser module for HR systems 230V (000000-2269-457) Zeiss Laser HR Diode 405 nm (000000-2005-027) 50 mW at 4 % power Zeiss Laser HR DPSS 561 nm (000000-2266-094) 500 mW – set to 200 mW output - at 6 %
Excitation/Emission optics	Laser Blocking Filter: 405/488/561/642 Grating period: 27.5 µm Grating: 5 Phases Filter module: DuoLink SR QUAD (000000-2290-250) with secondary beam splitter (SBS) and emission filters (EF): SBS LP 560 with EF BP420-480/BP495-550 & EF BP570-620 & LP655
Objective lens	Zeiss 63×/1.40 Oil DIC Plan-Apochromat (420782-9900-799)
Detector	PCO.edge 4.2 CLHS, 2x PCO.edge sCMOS cameras (000000-2263-137) Exposure time: 600 ms 1280 × 1280 pixels, 16 bit depth
Special settings	Post-processing SIM2 module (ZEN 3.0 SR, version 16) in LEAP Mode processing Iterations: 20 Regularisation Weight: 0.04 Processing/Output sampling: 3× Mean Best fit filter

(c) Image analysis: Three-dimensional image analysis was performed using Imaris (version 10.2, Oxford Instruments, Belfast, UK). Segmentation was carried out separately for cells, nuclei, and lysosomes using the Surfaces and Spots modules with the following parameters.

Cell segmentation was performed on the widefield lysosome channel using the surface module, taking advantage of unspecific background/autofluorescence staining, with surface smoothing enabled (surface grain size: 1.5 µm) and without background subtraction. A manual intensity threshold was adjusted for each image, and objects smaller than 10 voxels were excluded.

Nuclear segmentation was performed on the super-resolved DAPI channel using the surface module with smoothing enabled (surface grain size: 0.25 µm) and background subtraction applied (diameter of largest sphere: 5 µm). A manual intensity threshold was adjusted for each image, and objects smaller than ~10^5 voxels were excluded.

Lysosome detection was performed on the super-resolved lysosome channel using the Spots module with estimated XY/Z diameters of 0.209 µm / 0.600 µm, background subtraction enabled, and a quality filter threshold of 858. Region growing was based on local contrast with a manual threshold of 12.316 and diameters calculated from volume. Following segmentation, lysosomes located inside nuclei (shortest distance to nuclei < 0 µm) or outside cells (shortest distance to cells > 0 µm) were excluded from analysis. Lysosome counts were normalized to a standard unit cell volume to enable comparison between images and experimental conditions.

Imaris Object creation parameters:

Cells:
[Algorithm]
  Enable Region Of Interest = false
  Enable Region Growing = false
  Enable Tracking = false
  Enable Classify = false
  Enable Shortest Distance = true
[Segmentation Setup]
  Source Channel Index = 2
  Enable Smooth = true
  Surface Grain Size = 1.50 µm
  Enable Eliminate Background = false
[Threshold]
  Active Threshold = true
  Enable Automatic Threshold = false
  Manual Threshold Value = 7622.89
  Active Threshold B = false
[Filter Surfaces]
  "Number of Voxels Img=1" above 10.0

Nuclei:
[Algorithm]
  Enable Region Of Interest = false
  Enable Region Growing = false
  Enable Tracking = false
  Enable Classify = false
  Enable Shortest Distance = true

[Segmentation Setup]
Source Channel Index = 3
Enable Smooth = true
Surface Grain Size = 0.250 µm
Enable Eliminate Background = true
Diameter Of Largest Sphere = 5.00 µm
[Threshold]
Active Threshold = true
Enable Automatic Threshold = false
Manual Threshold Value = 457.479
Active Threshold B = false
[Filter Surfaces]
"Number of Voxels Img=1" above 6.04e5

Lysosomes:
[Algorithm]
Enable Region Of Interest = false
Enable Region Growing = true
Enable Tracking = false
Enable Classify = false
Enable Region Growing = true
Enable Shortest Distance = true
[Source Channel]
Source Channel Index = 1
Estimated XY Diameter = 0.209 µm
Estimated Z Diameter = 0.600 µm
Background Subtraction = true
[Filter Spots]
"Quality" above 858
[Spot Region Type]
Region Growing Type = Local Contrast
[Spot Regions]
Region Growing Automatic Treshold = false
Region Growing Manual Threshold = 12.316
Region Growing Diameter = Diameter From Volume
Create Region Channel = false

Calcium transient imaging of hiPSC-CMs

Spontaneously beating hiPSC-CMs grown on 12 mm coverslips were transferred to the Ca^{2+} imaging rig and incubated with 3 µM Fluo-5F-AM in extracellular solution adjusted for use in hiPSC-CMs (in mM: NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, D-Glucose 10) [79] for 15 minutes at 37°C in the dark. Ca^{2+} transient imaging experiments were performed during continuous perfusion with 37°C extracellular solution via an in-line heater. A control recording of spontaneous cardiomyocyte activity was obtained for 10 minutes before addition of DMSO (0.1 %, Merck Life Science UK Limited) or bafilomycin A1 (100 nM, Bio-Techne Ltd) for 10 minutes. Thereafter 3 nM isoprenaline (Merck Life Science UK Limited) was added to the extracellular solution and Ca^{2+} transients and 10-12 second recordings were taken every minute for 4 minutes. Recordings were done at 40 frames per second (exposure 25ms per image) using an ORCA-Fusion Digital CMOS camera C14440-20UP (Hamamatsu) and HCImageLive software (Hamamatsu). Cells were imaged using an Olympus BX51WI microscope.

Patch-clamp on purified hiPSC-aCMs

Voltage-clamp experiments in whole-cell configuration were performed on hiPSC-aCMs (day 30 of differentiation) at 37°C using a Multiclamp 700B patch-clamp amplifier and a Digidata 1440A (Axon Instruments, Molecular Devices, USA). The ultrarapid delayed rectifier potassium current (I_{Kur}) was recorded as the 4-aminopyridine-sensitive component. To evaluate the current-voltage (IV) relationship, voltage steps of the duration of 300 ms were applied in the range of -50 mV to +50 mV (in -10mV increments) from a holding potential of -50 mV. The extracellular solution composition was (in mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$. 5 HEPES, 10 Glucose (pH 7.4 with NaOH, 308 mOsm). Borosilicate glass pipettes with a 3-5 MΩ resistance were filled with an intracellular solution containing (in mM): 120 KCl, 20 HEPES, 10 Mg-ATP, 2 $MgCl_2$, 0.2 EGTA-KOH (pH 7.1 with KOH, 290 mOsm). 10 µM nifedipine was added to the extracellular solution to block Ca^{2+} currents; 50 µM 4-aminopyridine added to the extracellular solution and delivered to the cells through a gravity-driven perfusion system. Signals were sampled at 10 kHz and low-pass filtered at 800 Hz. Data are represented as mean±SEM.

Immunofluorescence staining on purified hiPSC-aCMs

hiPSC-CMs (day 30 of differentiation) were plated on glass coverslip coated with Matrigel®. After 48h Cells were fixed using PFA 4 % for 15 minutes RT. Three washes of 5 minutes each at RT in PBS high salt buffer (PBS-HS) were performed. Before antibody staining, the cells were incubated with the permeabilization solution (gelatin 0.4 % Triton X-100 0.6 %, phosphate buffer 40 mM, NaCl 0.9 M, and saponin 0.1 %) for 15 minutes at RT. Two washes were then conducted with the PBS-HS solution. The non-specific binding sites of the primary antibody were blocked with PBS + 0.5 % bovine serum albumin (BSA) + 1 % Donkey Serum for 1 hour at RT. The excess was removed with two 5 minutes washes in PBS-HS. The primary (Anti-Pro-Atrial Natriuretic Peptide Antibody, clone 11E3.9 mouse

monoclonal, Sigma-Aldrich, Catalogue MABC1032; Anti-K_v1.5 Antibody, rabbit polyclonal, Alomone labs, Catalogue #APC-004) antibodies were incubated overnight (o/n) at 4 °C and secondary (Donkey Anti-mouse Alexa Fluor 594, Catalogue # A-11005, diluted 1:600; Donkey Anti-rabbit Alexa Fluor 488, Catalogue # A-21206, diluted 1:800, Thermo Fisher Scientific) were incubated for 1 hour at RT in the dark, in permeabilization solution. The excess antibodies were then removed with two washes (5 min, RT) in PBS-HS and two washes in PBS low salt (LS). Nuclear Staining was performed with DAPI (10 minutes RT) and washed in PBS LS. The slides were coverslipped with DAKO fluorescence mounting medium. The Zeiss LSM710 confocal microscope, equipped with a 63× oil immersion objective, was used for image acquisition as a single optical section. Zeiss ZEN Microscope Lite software version and ImageJ 1.48V were used for image processing.

Immunostaining and live cell imaging

NRAMs were fixed with 2 % paraformaldehyde for 15 minutes. The cells were washed 3 times with PBS. Cells were then blocked with 10 % Donkey serum, 0.3 % BSA and 0.1 % triton X100 in PBS for 1 hour. Following the removal of the blocking buffer, cells were incubated overnight in 4°C with primary antibody (1:100, LAMP2 (PA1-655)). Cells were then washed 3 times with PBS and incubated in the dark at room temperature (RT) with the secondary antibody goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular probes, Eugene, Ore). Cells were washed 3 times in PBS and coverslips were mounted on slides with Vectashield™ with DAPI to stain the nuclei.

Lysosomes of NRAMs and hiPSC-CMs were labelled with Lysotracker Red (Thermo Fisher Scientific Inc). Cells were washed twice and incubated at RT in the dark for 30 minutes in Tyrode (in mM: NaCl 119, $NaHCO_3$ 25, NaH_2PO_4 1.0, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 1.8, and glucose 10) with 50 nM Lysotracker. Cells were washed twice and left in Tyrode for live imaging using Nikon Eclipse Ti Inverted Fluorescence Microscope with a 40x oil-immersion objective with illumination light at 546 nm.

Cells were viewed using a Nikon eclipse Ti inverted confocal microscope (Nikon) with a 63x/1.2 water objective Plan Apo VC 60xA WI DIC N2 lens. NIS-Element viewer (Nikon) was used to acquire multichannel fluorescence images. For detection of DAPI, fluorescence excitation. Excitation at 561 nm was collected at 595 nm for detection of AlexaFluor 568 nm and imaged sequentially at 2048 × 2048 (12 bits).

Förster resonance energy transfer (FRET)

FRET imaging experiments were performed on day 3 of culture and 24 hours after infection of the NRAMs with adenovirus carrying EPAC-S^{H187} cytosolic biosensor [34, 80] at a multiplicity of infection of 1000 virus particles per cell. During experiments, cells were maintained at RT in extracellular (E1) buffer (in mM: NaCl 140, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, glucose 15, HEPES 10, pH 7.2 with NaOH). Live dynamic measurements were done using an inverted microscope attached to a cool SNAP HQ2 camera

and an optical beam splitter (Photometrics) for simultaneous recording of YFP and CFP emissions. FRET changes were measured as changes in the background-subtracted 480 nm/545 nm fluorescence emission intensity on excitation at 430 nm and normalized to the maximum FRET change induced by saturation. For recording, cells were left to stabilize for 4 minutes, drugs, or controls were then added, bafilomycin A1 (100 nM), *trans*-Ned-19 (3 μM) or 0.1 % DMSO as a solvent control. 1 nM of isoprenaline or 10 μM of TPC2-A1-N [35] was then added between 3-6 minutes and left until effect showed a peak and plateau. To induce saturation response of cAMP, FSK (10 μM, EMD millipore) to activate ACs and 3-isobutyl-1-methylxanthine (IBMX, 100 μM, Sigma-Aldrich) a competitive non-selective phosphodiesterase inhibitor was added.

4pi-SMS imaging

Single molecule localization microscopy data was acquired from a custom built 4Pi Single Molecule Switching (SMS) microscope [81]. Localizations were accumulated for the LAMP2 structural lysosomal marker throughout the cytosol of a single neonatal rat cardiomyocyte. Fixing and LAMP2 antibody staining of samples were prepared similarly as for immunolabelling (above) and the imaging buffer used was standard GLOX dSTORM imaging buffer. Fixed sample was mounted between two #1.5H x Ø 25 mm coverslips and sealed with two-part silicone adhesive. The raw images were acquired with 10 ms exposure time at 100 frame per second for 10 minutes with standard GLOX dSTORM imaging buffer. The power density of 642 nm excitation laser was 7.5 Kw/cm2. The localizations were summed within voxels measuring 16 nm in X and Y and 87.5 nm in Z. The number of localizations within each voxel were resampled to fall within an 8-bit range. Two large dark circular regions visible in the image of the cell (Supplementary Fig. 2a) are due to fluorescent beads that are laid on top of the cells as fiducial markers for drift correction. These regions have obscured part of the cell volume and thus were cropped out from the reconstructed 3D data. Imaris v10 was used to analyze the image data.

Goat model

The goat model in this study (female C. hircus) is described in [25], where AF was induced and maintained in female goats (C. hircus) for 6 months (AF goat model was created as methodologically described in [24]. Goat were anesthetized and followed by an open chest sacrifice experiment (N=4 AF and N=4 sham controls) in order to collect tissue and were euthanized [24]. Atrial goat biopsies were either snap frozen or chemically fixed with Karnovsky fixative for electron microscopy studies.

Human tissue permissions

The study protocol conformed to the principles outlined in the declaration of Helsinki and was approved by local and national ethics committees. Tissue used in the research study was obtained from three centers, UK and Germany.

The Royal Papworth Hospital Research Tissue Bank, Cambridge, UK (all male patients, left atrial tissue). Written consent was obtained for all tissue samples using Royal Papworth Hospital Research

Tissue Bank's ethical approval (East of England - Cambridge East Research ethics committee – 18/EE/0269).

Human right atrial tissue from male and female patients in sinus rhythm and AF were obtained from the University of Göttingen. Experimental protocols were approved by the ethics committee of the University Medical Center Göttingen (No. 4/11/18).

Human biopsies of the atrial appendage used for proteomics (Supplementary Figures 8-11) were obtained from patients (male and female) undergoing on-pump cardiac surgery at the John Radcliffe Hospital (Oxford, United Kingdom). The study was approved by the Research Ethics Committee (reference no. 07/Q1607/38), and all patients gave written, informed consent."

Transmission electron microscopy (TEM)

Cardiac tissue was chemically fixed and sectioned using methods described in [23]. Lysosomes were identified by their documented appearance and single lipid bilayer [53]. Analysis and measurements were made using Gatan and ImageJ software.

Transmission electron microscopy and tomography of murine and goat tissue

3D electron tomography

All animal (murine (mixed gender), goat (female)) tissue was fixed using iso-osmotic Karnovsky's fixative (2.4 % sodium cacodylate, 0.75 % paraformaldehyde, 0.75 % glutaraldehyde) as described previously [82]. Semi-thick (300 nm) sections were prepared and imaged at the Electron Microscopy Core Facility, European Molecular Biology Laboratory (EMBL) Heidelberg using 300 kV Tecnai TF30 (FEI Company, now Thermo-Fisher Scientific, Eindhoven, The Netherlands) as described previously [83]. Dual-axis tilt series were aligned, reconstructed, and combined using IMOD. Lysosomes were identified by their documented appearance and single lipid bilayer [53]. Analysis and measurements were made using IMOD and ImageJ software.

Transmission electron microscopy of human tissue (Oxford)

Human tissue was fixed in 4 % formaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 1 hour at RT ahead of transfer to storage buffer (0.25 % glutaraldehyde in buffer). Samples were then prepared with microwave assistance through the following procedure. Following a rinse step in buffer, samples were treated with 50 mM glycine in buffer, rinsed again in buffer and osmicated in 1 % osmium tetroxide and 1.5 % potassium ferrocyanide in buffer. Samples were then rinsed with MilliQ water followed by incubation in 2 % uranyl acetate (aq.). Samples were then rinsed with MilliQ and dehydrated through an ethanol series and infiltrated with TAAB Hard Plus resin. An extended infiltration was carried out over 3 days at room temperature ahead of embedding and polymerisation. Sections of 90 nm were cut from the resin blocks using a Leica UC7 Ultramicrotome and collected onto 3 mm copper grids. The sections were then incubated with 10 nm gold fiducial

markers and post-stained with lead citrate. Images were collected using a JEOL 1400 Flash 120kV TEM equipped with a Gatan Rio camera. Lysosomes were identified by their documented appearance and single lipid bilayer [53]. Analysis and measurements were made using Gatan and ImageJ software.

Transmission electron microscopy of human tissue (Göttingen)

Human tissue was fixed in 4 % formaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 1 hour at RT ahead of transfer to storage buffer (0.25 % glutaraldehyde in buffer). Samples were then rinsed in buffer, treated with 50 mM glycine in buffer, rinsed again in buffer and osmicated in 2 % osmium tetroxide and 1.5 % potassium ferrocyanide in buffer for 1 hour at 4°C with rotation. Samples were then rinsed with MilliQ water and incubated overnight in 0.5 % uranyl acetate (aq.). The following day samples were rinsed with MilliQ then dehydrated through an ethanol series and infiltrated with and embedded in TAAB Hard Plus resin over 3 days. Sections of 250 nm were cut from the resin blocks using a Leica UC7 Ultramicrotome and collected onto 3 mm copper grids. The sections were then incubated with 10 nm gold fiducial markers and post-stained with lead citrate. Tilt series were collected using a JEOL 2100 Plus 200kV TEM equipped with a Gatan OneView camera and reconstructed using IMOD (Etomo).

Rodent isolated cardiomyocyte cytosolic pH measurements

All experiments were performed in accordance with the Home Office Guidance on the operation of The Animals (Scientific Procedures) Act 1986 (H.M.S.O.). Rat ventricular myocytes were isolated according to methods described in [84] and loaded with cSNARF1 in control conditions and in the presence of 1 μM bafilomycin A1. Superfusion experiments have been described in detail in [84] and drug was delivered using a rapid switcher device which allowed us to superfuse the cells and alternate between control and drug rapidly, with minimal use of solution.

Tissue sample preparation for proteomics

Human right atrial appendage tissue from SR and AF patients were individually homogenized in a bead homogenizer in RIPA buffer and were reduced using 4X Lithium Dodecyl Sulfate (LDS) buffer and β-mercaptoethanol. Tissue samples were analyzed by LC-MS/MS using a Dionex Ultimate 3000 (Thermo Scientific) coupled to a timsTOF Pro (Bruker, [85]) using a 75 μm x 150 mm C18 column with 1.6 μm particles (IonOpticks) at a flow rate of 400 nL/minute. A 17- minute linear gradient from 2 % buffer B to 30 % buffer B (A: 0.1 % formic acid in water. B: 0.1 % formic acid in acetonitrile) was used. These data are available on PRIDE (Project accession: PXD042391). Atrial tissue contains a range of different cell types from atrial myocytes to vascular smooth muscle cells, endothelial cells, neurons, immune cells and fibroblasts meaning changes in the expression of lysosomal components in specific cell types within the atria in AF cannot be ruled out.

Mass Spectrometry

The TimsTOF Flex (Bruker) was operated in PASEF mode using Compass Hystar 5.0.36.0. Settings for the 11 samples per day method were as follows: Mass Range 100 to 1700 m/z, 1/K0 Start 0.6 $V \cdot s/cm^2$ End 1.6 $V \cdot s/cm^2$, Ramp time 110.1 ms, Lock Duty Cycle to 100 %, Capillary Voltage 1600 V, Dry Gas 3 l/min, Dry Temp 180°C, PASEF settings: 10 MS/MS scans (total cycle time 1.27sec), charge range 0-5, active exclusion for 0.4 min, Scheduling Target intensity 10000, Intensity threshold 2500, CID collision energy 42eV. Settings for the 50 and 180 samples per day method were as follows: Mass Range 100 to 1700m/z, 1/K0 Start 0.85 $V \cdot s/cm^2$ End 1.3 $V \cdot s/cm^2$, Ramp time 100 ms, Lock Duty Cycle to 100 %, Capillary Voltage 1600V, Dry Gas 3 l/min, Dry Temp 180°C, PASEF settings: 4 MS/MS scans (total cycle time 0.53 sec), charge range 0-5, active exclusion for 0.4 min, Scheduling Target intensity 24000, Intensity threshold 2000, CID collision energy 42 eV.

Analysis and statistics on mass spectrometry data

The raw protein intensities of individual SR vs AF group samples were Log transformed and normalized by median subtraction to identify the significantly regulated proteins of interest in AF. The violin plots were created with the InstantClue omics tool version, win-0.12.1 [86]. The protein intensities of the AF vs SR groups were log-transformed and normalized using the Z score. The kernel density estimation of protein intensities for the distribution between groups was represented using a gradient of color code, red to blue. To study the biological significance, the differentially expressed proteins were subjected to protein network analysis using the Ingenuity Pathway Analysis (IPA) software (Qiagen Inc.) based on curated databases from the literature. IPA is a tool used in omics studies to suggest/predict the effects of specific conditions on biological outcomes. Datasets containing protein identifiers (UniProt) and corresponding expression values (Log2 [Fold change]) of AF vs. sinus rhythm controls were uploaded, and predicted networks were analyzed. Top analysis-ready molecules are found in Supplementary File 3. For visual interpretation, these peptides were input with their FC values into Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA) to generate top canonical pathways with color-coded measures of relative FC values (Supplementary Fig. 6c). Figures for canonical pathways were generated in IPA run on 2024-03-21 content version 111725566 with analysis set on Ingenuity Knowledge Base (Genes Only) to include Direct and Indirect relationships.

Statistics

Dose-response curves (log[agonist] vs. response [three parameters]) were used to calculate EC_{50} values and maximum responses in murine atrial preparations and are reported as mean and with 95 % confidence interval (CI) of best-fit value. Guinea pig and human Ca^{2+} transient experiments were background-subtracted and normalized using $\Delta F/F_0$ and expressed as % change from baseline (after stabilization of the beating rate and prior addition of isoprenaline). Ca^{2+} transient data was analyzed by a non-parametric approach (Mann–Whitney U test) to all datasets to ensure consistent analysis, as not all data were normally distributed and data are presented in the Results section as median (interquartile

range: 25th–75th percentile). FRET datasets were collected from individual cells (2-9 cells per experiment) and were averaged to give n=1 and data in graphs are represented as n=number of averaged cells per experiments and N=number of litters used for neonatal isolations (10-14 pups per litter) or differentiations. For FRET analysis, data are given as FRET change percentage of the saturating FSK/IBMX response. FRET datasets and measurements obtained from EM are presented as median (interquartile range: 25th–75th percentile). Statistical analysis was run using Kruskal Wallis followed by Dunn's multiple comparison or non-parametric Mann- Whitney U test. Differences were considered statistically significant at values of $P < 0.05$. Statistical significance, when achieved, is indicated as $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. Statistical analysis was performed using Prism v10 software (GraphPad, CA, USA).

Supplementary material online

Supplementary Figure 1 (related to figure 1): Left atrial force transducer experiments. a: Dose-response curves to show change in tension generated on cumulative addition of isoprenaline to stimulated (5Hz) murine left atria preparations under control conditions (circles, n=7) and in the presence of either 100 nM bafilomycin A1 (diamonds, n=9) or 3 µM *trans*-Ned-19 (triangles, n=6). **b**: Comparison of tension force in beating murine left atrial preparations in PSS prior to addition of drug or stimulation with isoprenaline. **c**: Comparison of change in beating force (in %) in beating murine left atrial preparations in PSS on addition of DMSO, 100 nM bafilomycin A1 or 3 µM *trans*-Ned-19, prior to stimulation with isoprenaline. **d**: Dose-response curves to show change in tension generated on cumulative addition of isoprenaline to stimulated (5Hz) murine left atria preparations in WT mice (circles, n=11) and in $TPC2^{-/-}$ mice (squares, n=11). Dose-response curves in panel a and d were fitted using log(agonist) vs. response (three-parameter model) and best-fit values for top (maximum response) and EC_{50} are reported with 95 % confidence intervals (CI, calculated using profile likelihood method). Data in panel b and c are presented as median (interquartile range: 25th–75th percentile). Statistical significance was determined using the Kruskal–Wallis test followed by Dunn's multiple comparisons test, maximal response in panel e was analyzed using unpaired t-test. Asterisks indicate significance level for effect of bafilomycin A1 and *trans*-Ned-19, non-significant (ns)=P>0.05; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Supplementary Figure 2 (related to figure 4e): 4Pi-SMS super-resolution microscopy analysis. a: 4Pi-SMS super-resolution microscopy analysis; scale bar represents 2 µm. Representative Z-projection of the 4Pi SMS imaged data indicating lysosomes (spheres) which are less than (yellow) or more than (green) 500 nm of their nearest neighbour in cultured and fixed NRAMs. Scale bar 2 µm. **b:** Distribution of nearest neighbor distances between lysosomes in cultured and fixed NRAMs. **c:** Distribution of measured lysosome volumes in cultured and fixed NRAMs.

Supplementary Figure 3 (related to figure 4): characterization of hiPSC-aCMs. a: Representative 3D reconstruction of high-resolution confocal image showing lysosomes (magenta) and nucleus (cyan) in hiPSC-CMs; scale bar represents 4 µm. **b:** Quantification of lysosomal density (N=6 field of views), presented as lysosomes/μm^3. **c:** Summary table of lysosomal measurements in hiPSC-CMs. Values represent the mean distance in µm of the three closest lysosomes to each other per cell, with corresponding minimum (Min), maximum (Max) and standard deviation (Std. Dev., N=6 field of views). Data presented as mean±Std. Dev. Analyses in b and c were performed across N=6 field of views, which corresponds to 2137 lysosomes identified within a cellular volume of 64408 μm^3.

Supplementary Figure 4 (related to figure 4): characterization of hiPSC-aCMs. a: Representative sample traces of potassium currents elicited at +50 mV in control (black) or in the presence of 50 µM

4-AP in hiPSC-aCMs (i) and in hiPSC-CMss (ii). **b**: Current-voltage relationships of 4-AP-sensitive currents in hiPSC-aCMs and in hiPSC-CMss (n= number of cells, N=number of independent differentiations) **c:** Image showing representative staining in hiPSC-aCMs; the plasma membrane localization of $K_v1.5$ (i, green), nuclear Staining DAPI (ii, blue), and merge image (iii, using ImageJ)**. d:** Image showing representative staining in hiPSC-aCMs of pro-ANP localization in perinuclear region e cytosolic granuli (i, red), nuclear Staining DAPI (ii, blue), and merge image (iii, using ImageJ)**.** Scale bar 20μm. Data are represented as mean±SEM and was analyzed using ANOVA followed by Šídák's multiple comparisons test.; not significant (ns); *P< 0.05, **P< 0.01, ***P< 0.001.

Supplementary Figure 5 (related to figure 6): Goat left atrial electron microscopy. Snap shots from goat left atrial sinus rhythm control (column **a**) and atrial fibrillation (column **b**) from 3D tomograms to show proximity of Lysosomes (Ly) to Mitochondria [67] and Sarcoplasmic Reticulum [68]. Scale bars represent 200 nm.

Supplementary Figure 6 (related to figure 2): Rodent isolated cardiomyocyte cytosolic pH measurements. a: Rat ventricular myocytes, loaded with cSNARF1 in control condition and in the presence of 1 μM bafilomycin A1; scale bar represents 100 μm. **b**: Rat ventricular myocytes, loaded with cSNARF1 and measured in HEPES buffer extracellular solution (in mM: NaCl 130, KCl 4.5, HEPES 20, $CaCl_2$ 1, $MgCl_2$ 1, Glucose 11) exposed to 1 μM bafilomycin A1 by dual microperfusion (n=8). Data are represented as mean±SEM.

Supplementary Figure 7: Spatial interaction between lysosome-sarcoplasmic reticulum and lysosome-mitochondria. a-b: Representative 3D electron tomography images of murine SAN including raw images (e and f top panels) and reconstructed organelles in 3D (e and f bottom panels) of lysosomes (red), sarcoplasmic reticulum (blue) and mitochondria (yellow)**.** Model: lysosomes (Ly, red), sarcoplasmic reticulum (SR, blue), mitochondria (Mito, yellow); scale bars represent 200 μm.

Supplementary Figure 8 (related to discussion): IPA analysis of human tissue samples. a: Violin Plot of protein intensity preparations in right atrial appendage human samples from atrial fibrillation patient (N=6) and patient with a normal sinus rhythm (N=7). **B:** Volcano Plot of differential gene expression of human samples from atrial fibrillation patient (N=6) and patient with a normal sinus rhythm (N=7), MaxLFQ is a generic method for label-free quantification, proteins with fold changes greater than 2 are highlighted, Instant Clue v0.12.1. See Supplementary File 1 for protein list. **c:** The highest ranked canonical IPA pathways of the differentially expressed proteins (using a score cut off -log (p values) are greater than 3.8). The horizontal axis shows the pathway names, and the vertical axis is the p-value (-log) of each pathway. Blue bars: negative z-score; orange bars: positive z-score and clear bars are indicative of a z-score of 0, and thus have no difference in activity. Grey shaded bars indicate that there is no activity pattern available identified by IPA, despite highly significant association of the proteins within the pathway. See Supplementary File 2 for list.

Supplementary Figure 9 (related to discussion): Pathway analysis of human tissue samples. a: Pathways analysis in IPA; Bubble plot showing top canonical pathways when comparing AF with sinus rhythm control samples. The intensity of the color depicts the relative increase (red) or decrease [65] in protein counts with the size of the bubble representing the number of proteins found in the pathway. **b:** Integrin Signaling Pathway showing in color all proteins found within the dataset. Red represents upregulation in AF. Whereas Green depicts down regulation **c,** microautophagy top canonical pathway showing in color all proteins found within the dataset. Red represents upregulation in AF. **d:** Plot of differentially expressed proteins (p= 1.98E-05) from the microautophagy regulation pathway showing how each protein changes with AF.

Supplementary Figure 10 (related to discussion): Top 10 networks analyzed in IPA. **a**: Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance; **b**: Cell Cycle, Infectious Diseases, Organismal Injury and Abnormalities; **c**: Developmental Disorder, Hematological Disease, Hereditary Disorder; **d**: Cell Morphology, Neurological Disease, Organismal Injury and Abnormalities; **e**: Connective Tissue Disorders, Dermatological Diseases and Conditions, Developmental Disorder; **f**: Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry; **g**: Cardiac Arrythmia, Cardiovascular Disease, Hereditary Disorder; **h:** Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance; **i**: Developmental Disorder, Embryonic Development, Organismal Injury and Abnormalities; **j:** Cellular Assembly and Organization, Cellular Function and Maintenance, Infectious Diseases. List of regulated proteins used in IPA can be found in Supplementary File 2 and Supplementary File 4 contains Ingenuity Canonical Pathways Analysis which includes molecules.

Supplementary Figure 11 (related to discussion): The top regulator effects and interaction networks mapped by IPA. a: CALR, DNMT3A, ESRRA (consistency score of 1.152). **b**: ESRRG (consistency score of 0.577). **c**: MMP9 (consistency score of -6.0).

Supplementary Files:

Supplementary File 1 (related to discussion, student T-test): Volcano Plot excel output file. Used to determine significant data points in a data set with a permutation-based FDR, X- axis column displays: log2 fold changes and Y- axis column displays: -log10 p values. Column headers are gene/protein description and columns labelled with the sample IDs are showing the mass spectrometry signal intensities in log2, normalized by MaxLFQ [69].

Supplementary File 2 (related to discussion): List of significant up and down regulated proteins taken from supplementary file 1 for analysis in IPA.

Supplementary File 3 (related to discussion): Canonical Pathways Analysis in IPA.

Supplementary File 4 (related to discussion): List of molecules identified through IPA. The table summarizes molecules with associated scores and top disease and functions linked to each network. Higher scores indicate stronger relevance.

Supplementary Videos:

Supplemental Video 1 (related to Figure 7): Left atrial goat tissue (sham control). 3D animation of tomographic reconstruction of cell in Fig. 7d. Red: Lysosome, Blue: Sarcoplasmic reticulum, Yellow: Mitochondria.

Supplemental Video 2 (related to Figure 7): Left atrial AF goat tissue (atrial fibrillation). 3D animation of tomographic reconstruction of cell in Fig. 7e. Red: Lysosome, Blue: Sarcoplasmic reticulum, Yellow: Mitochondria.

Supplemental Video 3 (related to Figure 6): Mouse Sino-Atrial Node tissue. 3D animation of tomographic reconstruction of cell in Fig. 6e-f. Red: Lysosome, Blue: Sarcoplasmic reticulum, Yellow: Mitochondria.

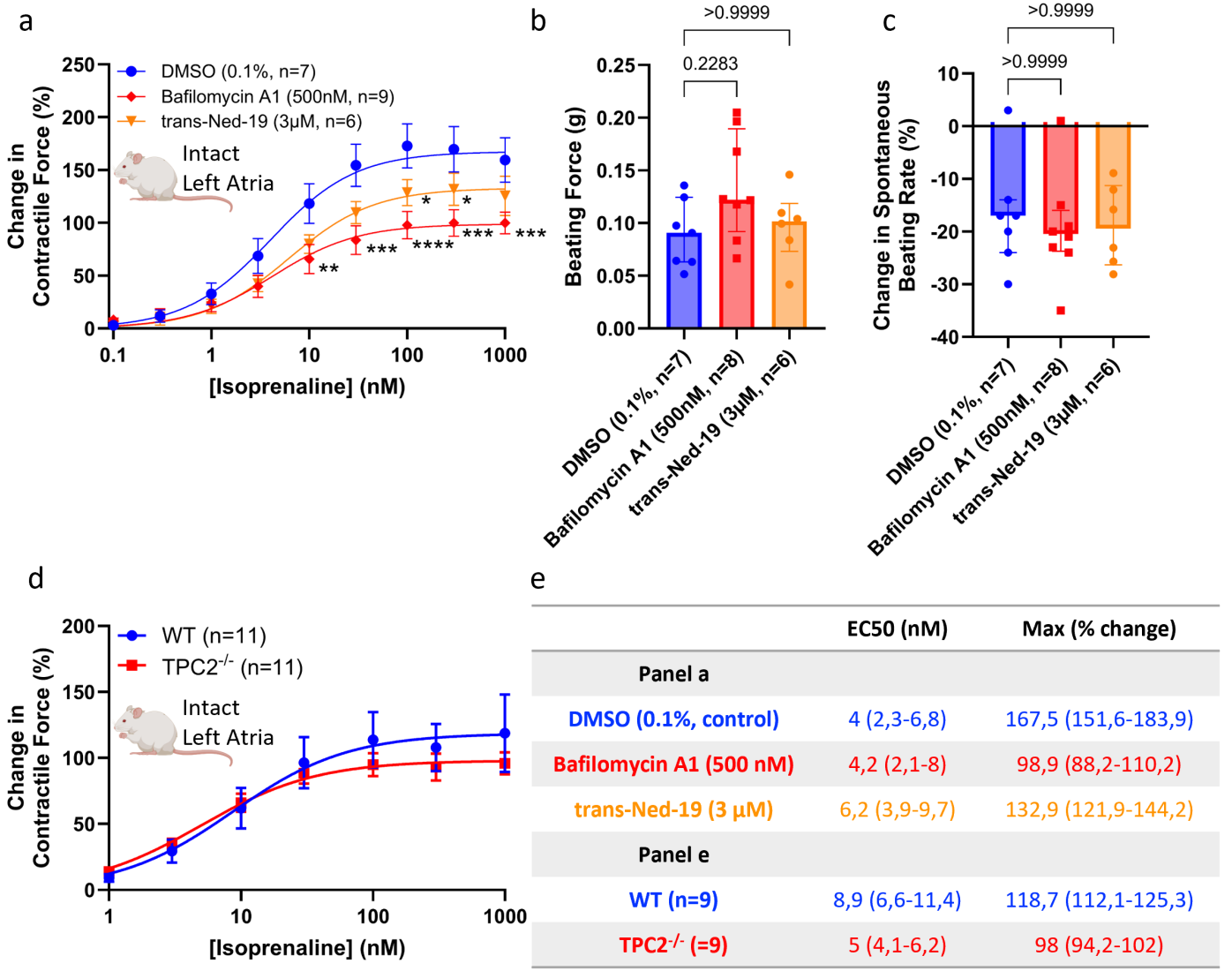
Supplementary Table:

Supplementary Table 1 (related to Figure 7): Summary table of surgeries underwent by SinusR and ChronicAF patient went biopsies were collected from both University Medical Center Göttingen and Royal Papworth Hospital Research Tissue Bank.

Royal Papworth Hospital Research Tissue Bank					
Surgery information undergone by the patients:					
SinusR					
TB Code	**Age**	**Sex**	**Samples**	**Surgery**	
TB23.0052	43	male	LA	MV replacement	
TB23.0066	59	male	LA	MV repair	PFO closure
ChronicAF					
TB Code	**Age**	**Sex**	**Samples**	**Surgery**	
TB23.0202	59	male	LA	MV repair	
TB23.0196	75	male	LA	MV repair	
TB23.0065	78	male	LA	MV repair	TV repair

Germany				
Surgery information undergone by the patients:				
SinusR				
Pat. Nr.	**Age**	**Sex**	**Samples**	**Surgery**
1234	58	female	RA	AKE
1239	49	male	RA	AKE
1341	76	female	RA	ACB
1377	57	male	RA	ACB
1380	79	female	RA	ACB
1448	63	male	RA	ACB and MI
ChronicAF				
Pat. Nr.	**Age**	**Sex**	**Samples**	**Surgery**
1338	75	male	RA, LA	ACB
2265	81	male	RA	AKE and TKE/R
2292	66	female	RA, LA	ACB and MI
2302	75	male	RA, LA	ACB, TIA and MI
2304	68	male	RA, LA	ACB

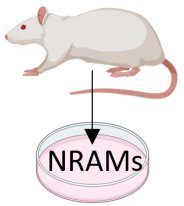
LA=left atrial tissue
RA=right atrial tissue
ACB=aortocoronary bypass surgery
AKE=aortic valve replacement
TKE/R=tricuspid valve replacement/reconstruction
TV=tricuspid valve
MV=mitral valve
TIA=transient ischemic attack
MI=myocardial infarction
PFO closure = Patent Foramen Ovale closure

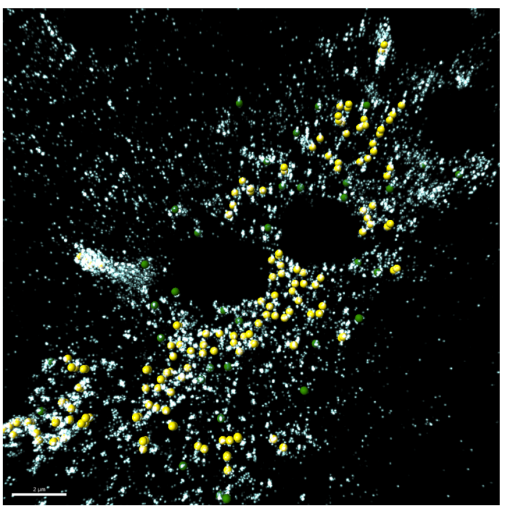


e

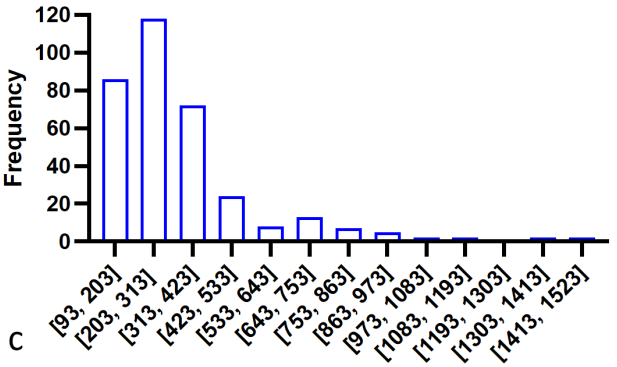
	EC50 (nM)	Max (% change)
Panel a		
DMSO (0.1%, control)	4 (2,3-6,8)	167,5 (151,6-183,9)
Bafilomycin A1 (500 nM)	4,2 (2,1-8)	98,9 (88,2-110,2)
trans-Ned-19 (3 µM)	6,2 (3,9-9,7)	132,9 (121,9-144,2)
Panel e		
WT (n=9)	8,9 (6,6-11,4)	118,7 (112,1-125,3)
TPC2$^{-/-}$ (=9)	5 (4,1-6,2)	98 (94,2-102)

a


NRAMs



b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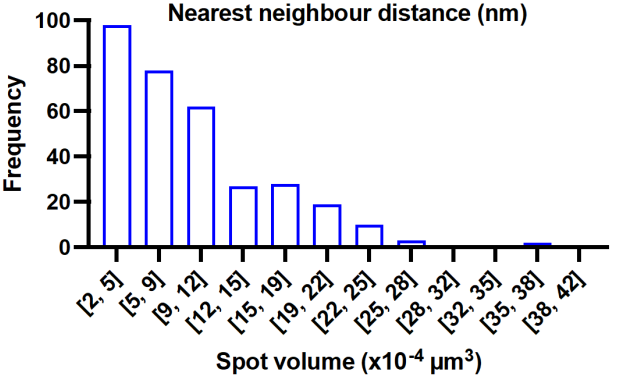
Nearest neighbour distance (nm)

c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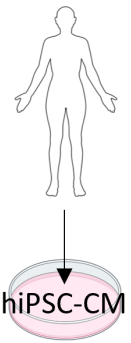
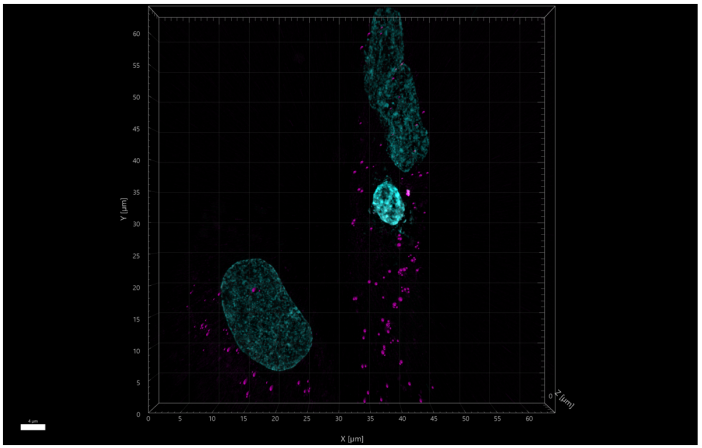
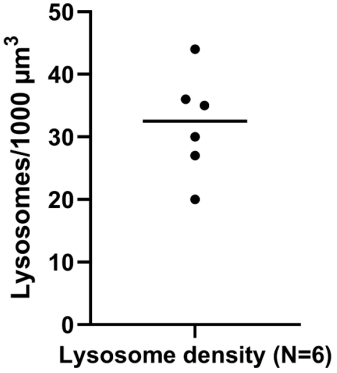
Spot volume (x10-4 µm3)

a



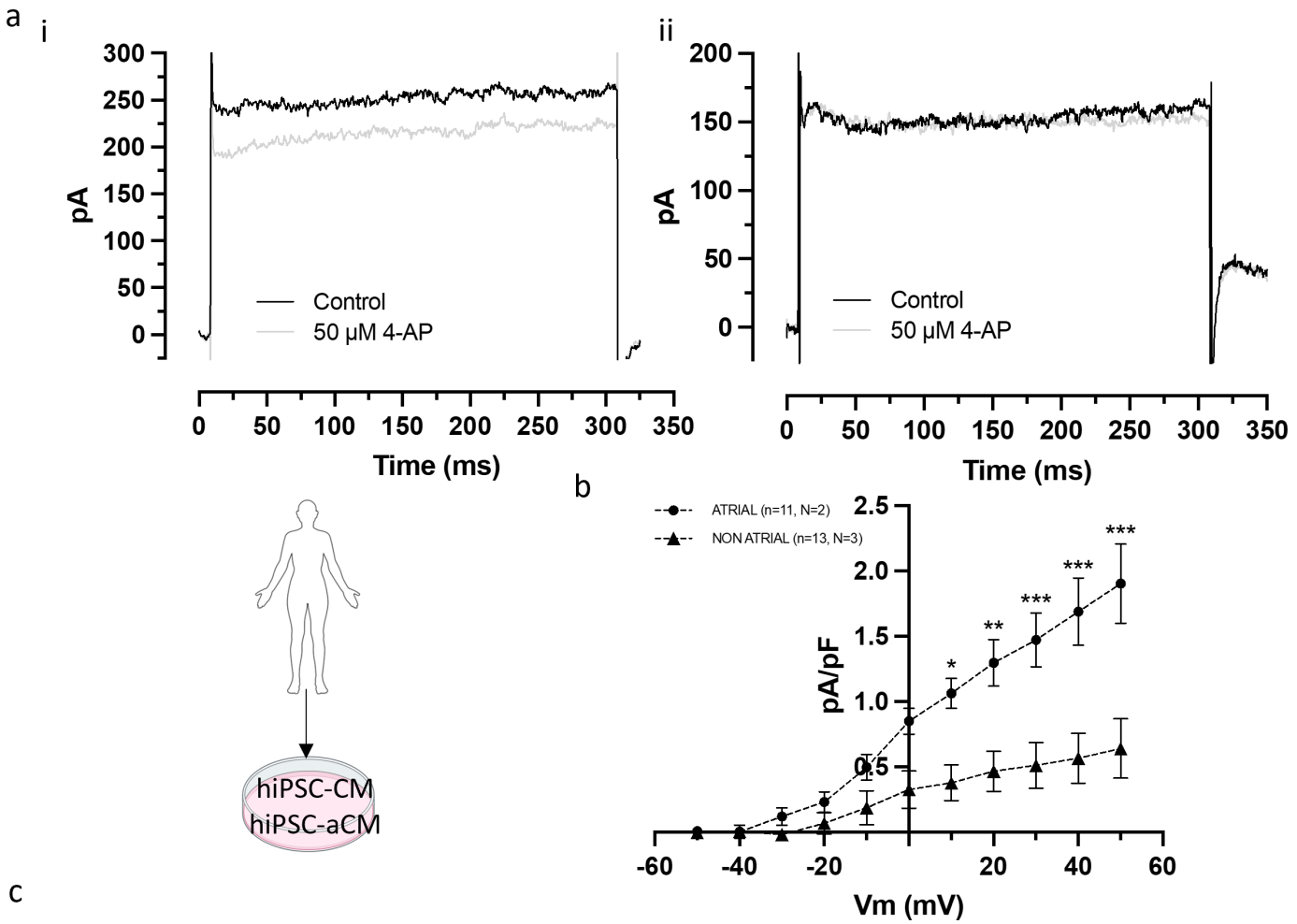


b



c

	Measurements in µm			
hiPSCs-CM	Min	Max	Mean	Std. Dev.
1	0.39	5.69	1.93	1.13
2	0.48	19.62	1.99	1.62
3	0.4	8.4	1.94	1.17
4	0.37	10.79	1.67	1.06
5	0.33	4.41	1.56	0.87
6	0.40	11.31	1.96	1.4
Mean	0.4	10.03	1.84	1.21


a
i
ii
pA
Control
50 µM 4-AP
Time (ms)
hiPSC-CM
hiPSC-aCM
b
ATRIAL (n=11, N=2)
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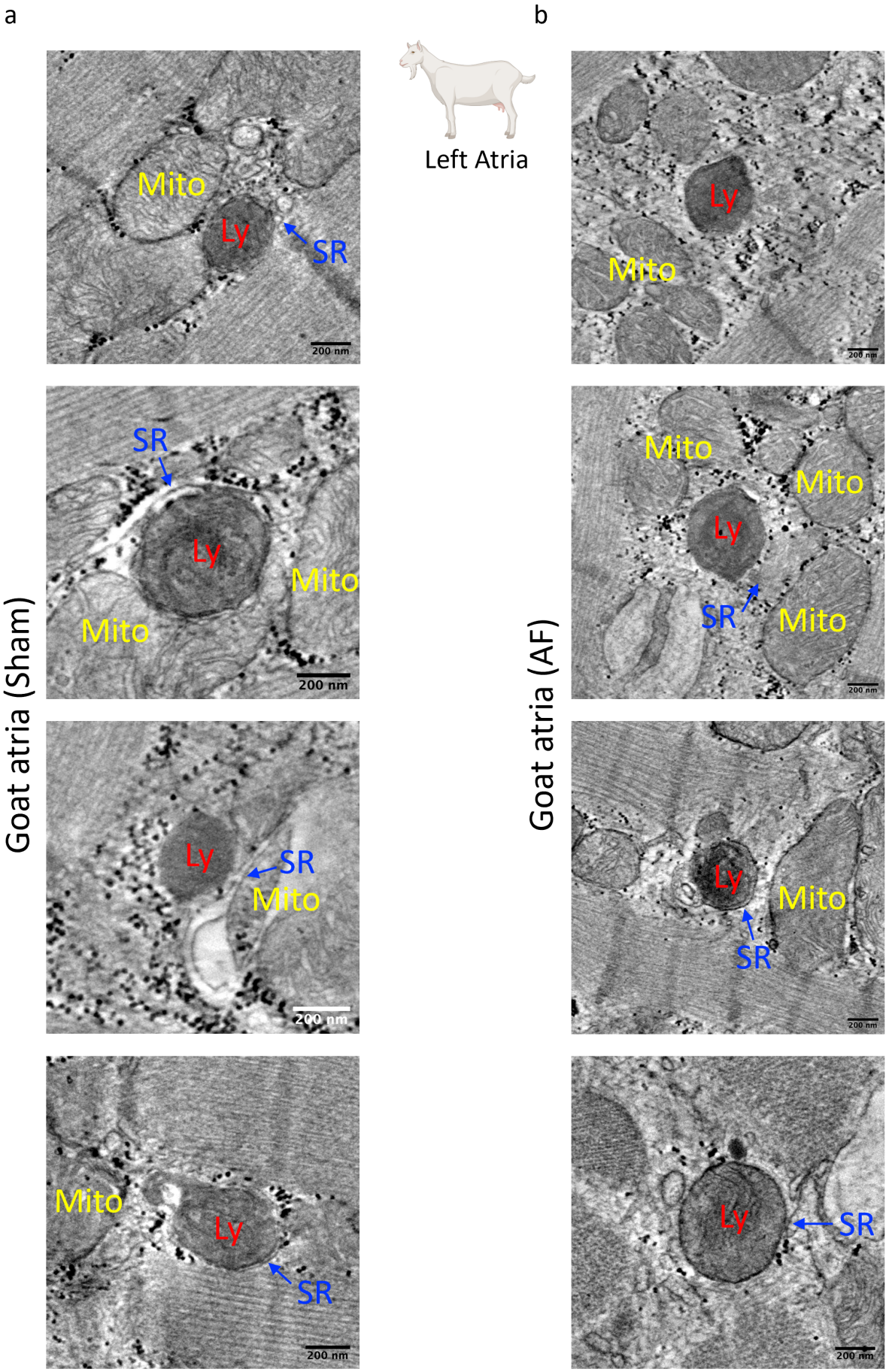
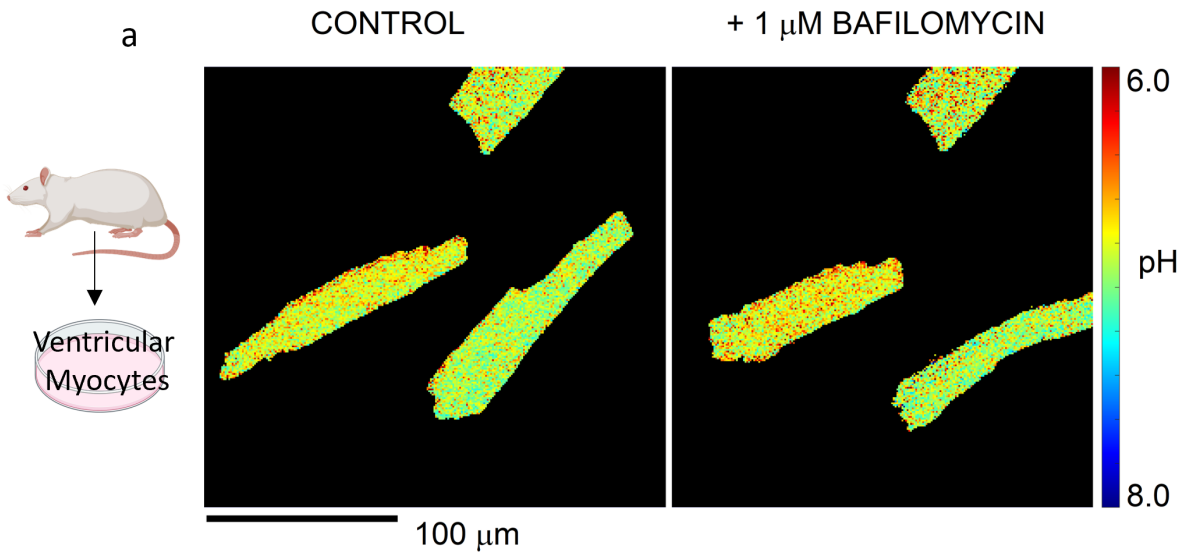
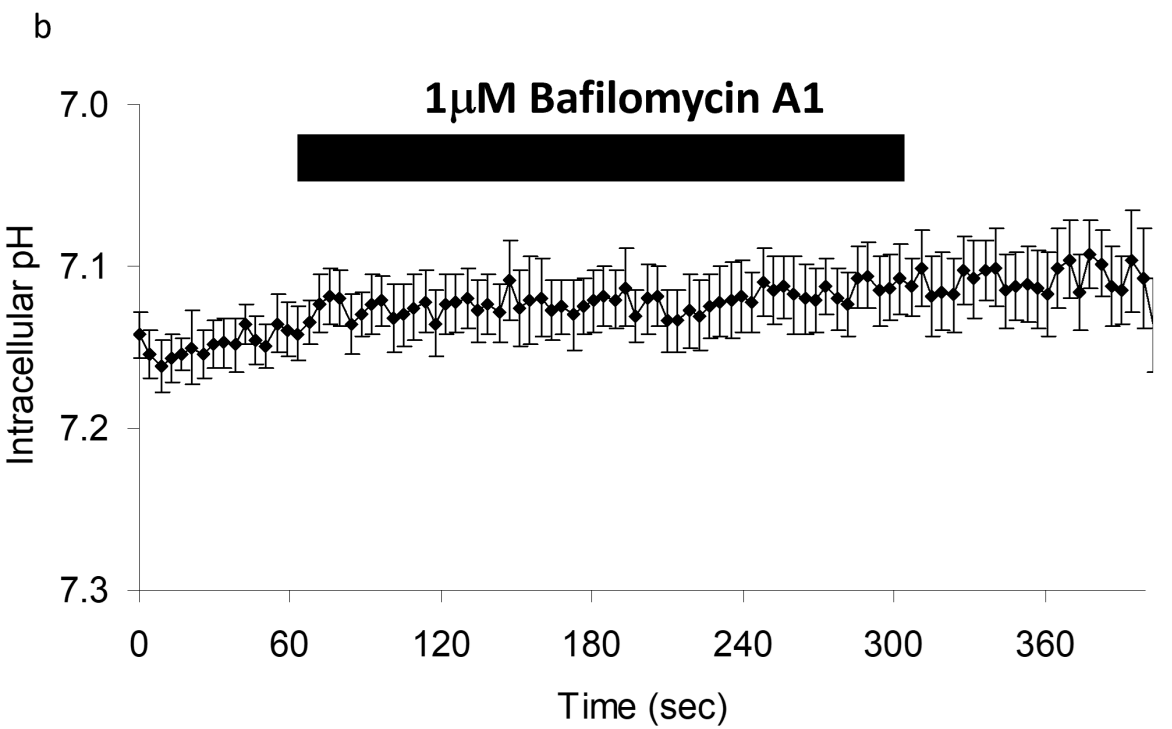
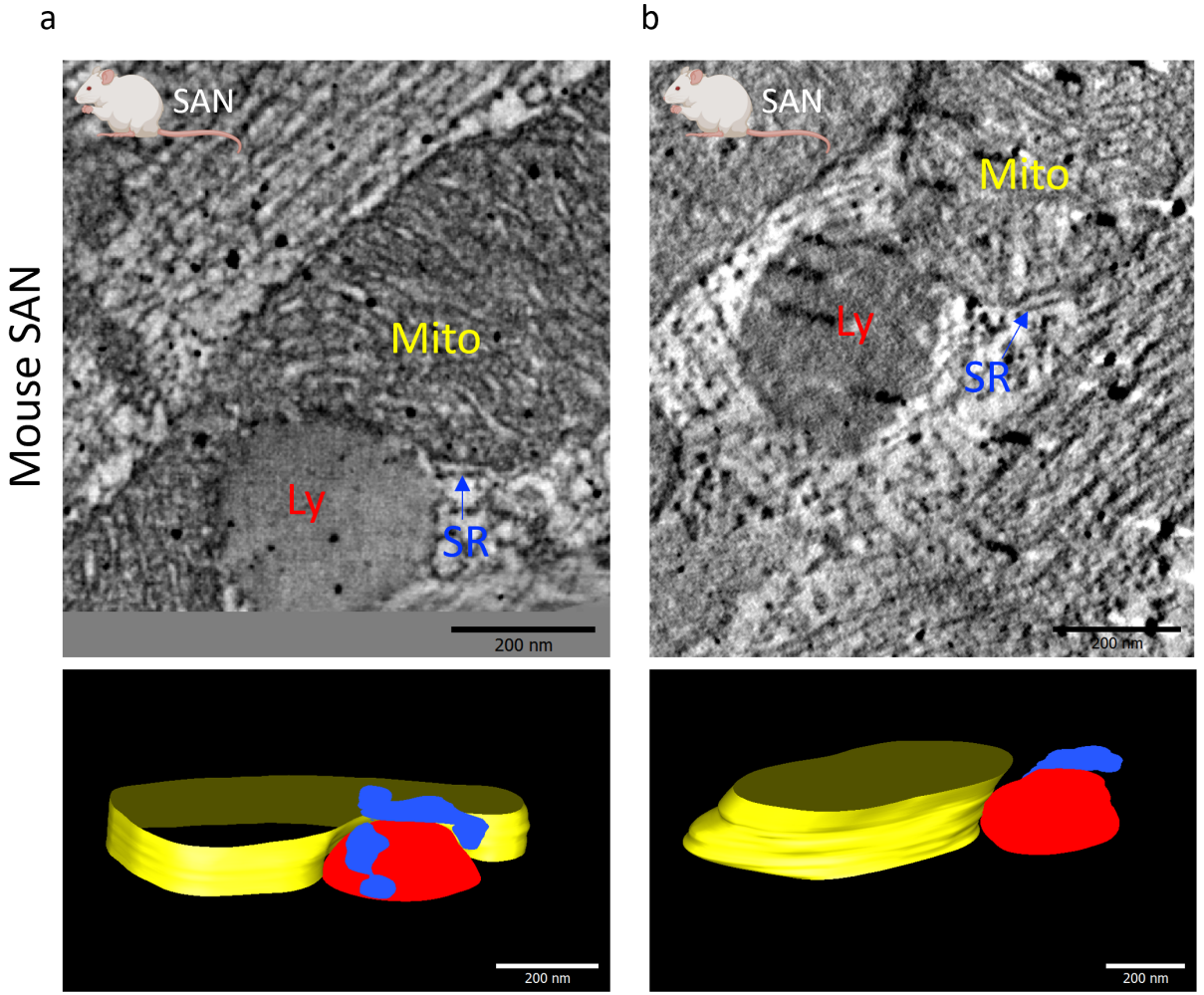
pA/pF
Vm (mV)
c
d
20 µm

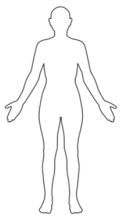

a
b
Left Atria
Goat atria (Sham)
Goat atria (AF)
Mito
Ly
SR
200 nm


a
CONTROL
+ 1 μM BAFILOMYCIN
Ventricular Myocytes
6.0
pH
8.0
100 μm

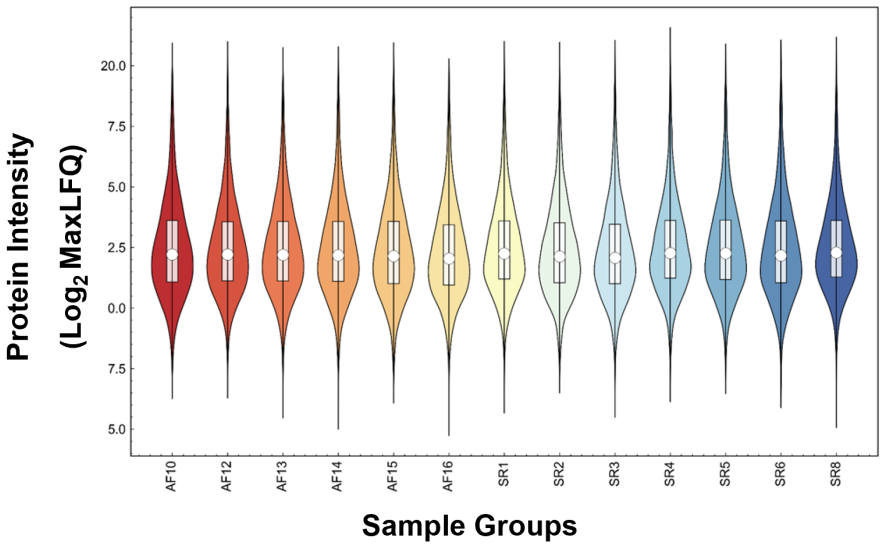

b
1μM Bafilomycin A1
Intracellular pH
7.0
7.1
7.2
7.3
0
60
120
180
240
300
360
Time (sec)


a
b
Mouse SAN
SAN
Mito
Ly
SR
200 nm
SAN
Mito
Ly
SR
200 nm
200 nm
200 nm

a



Right Atrial Appendage



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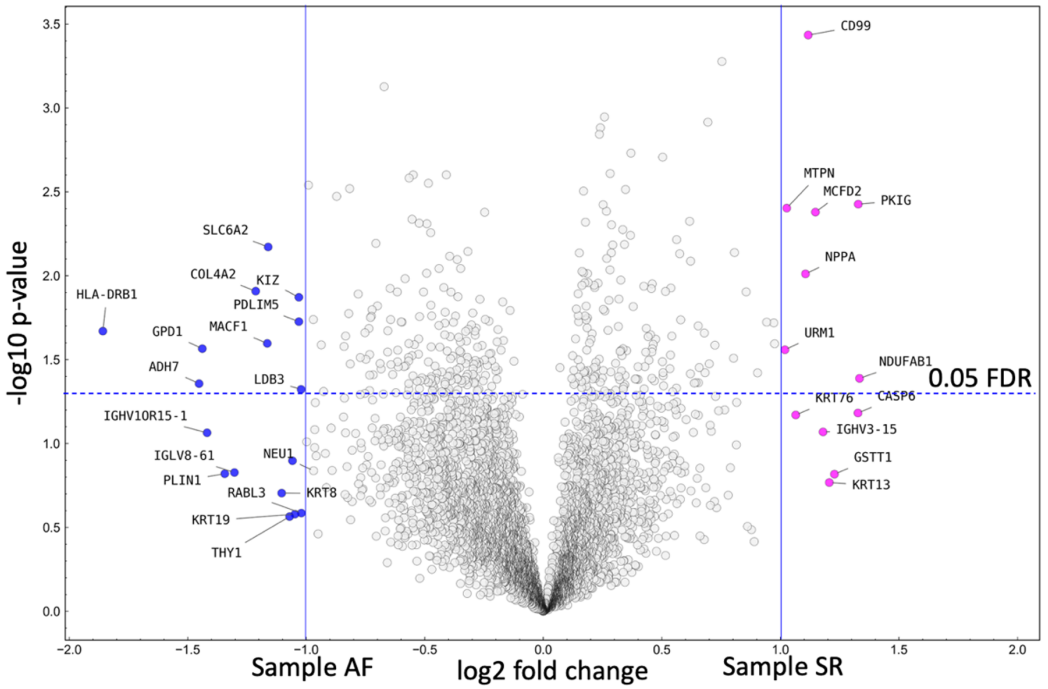

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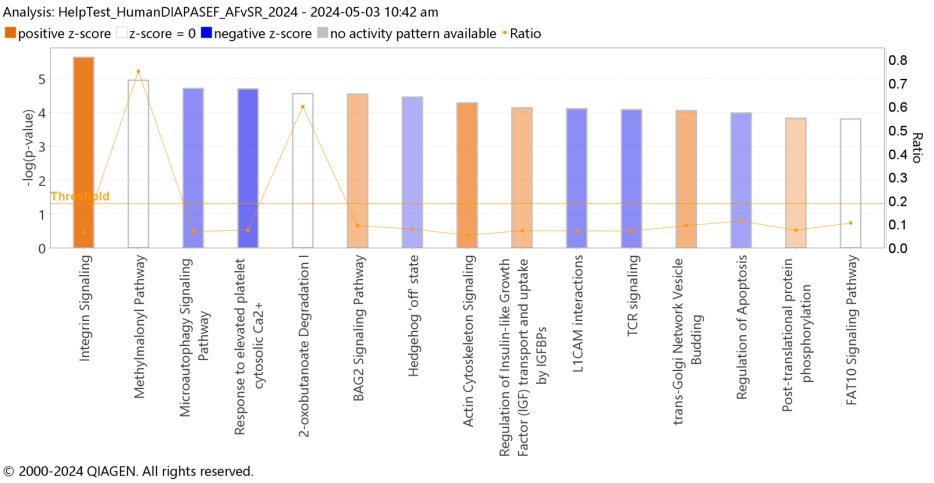

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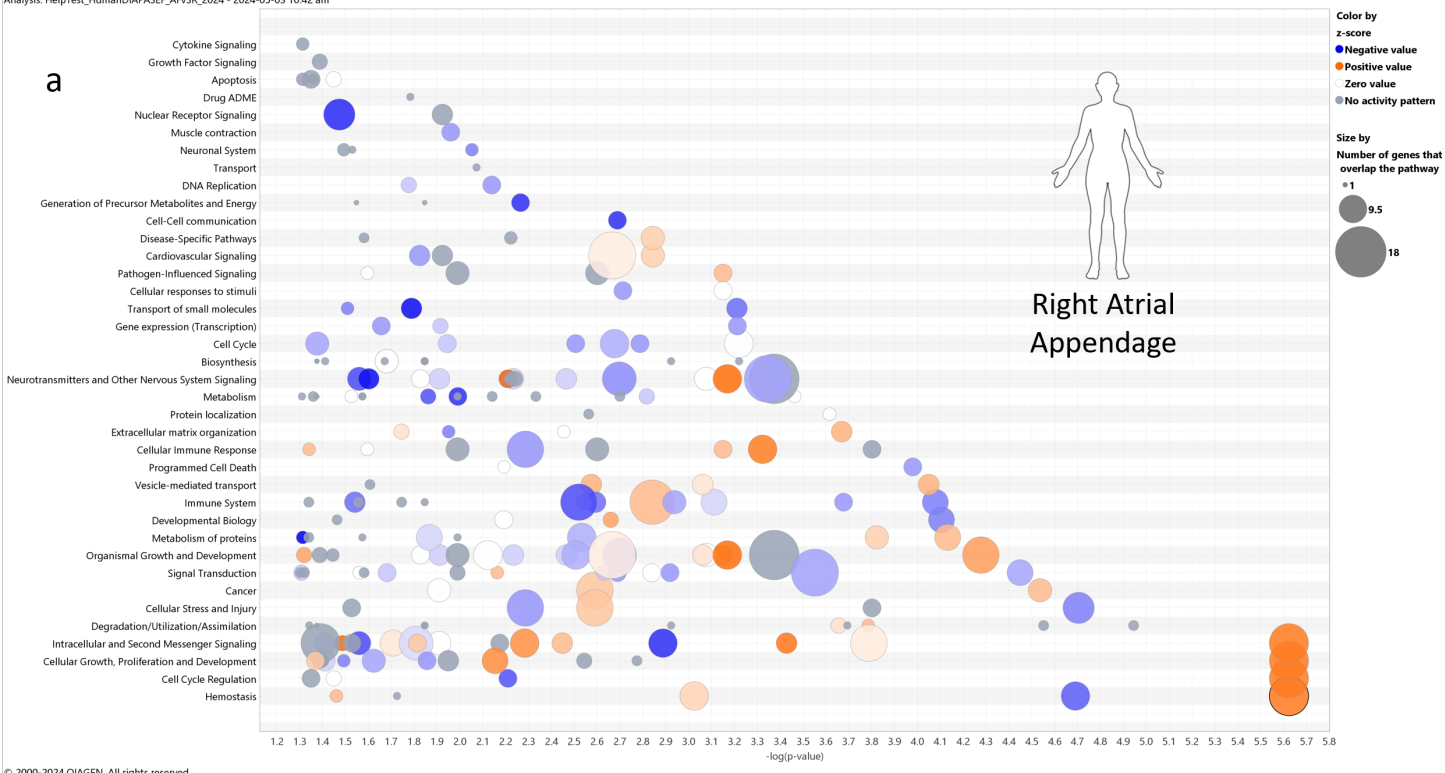
a



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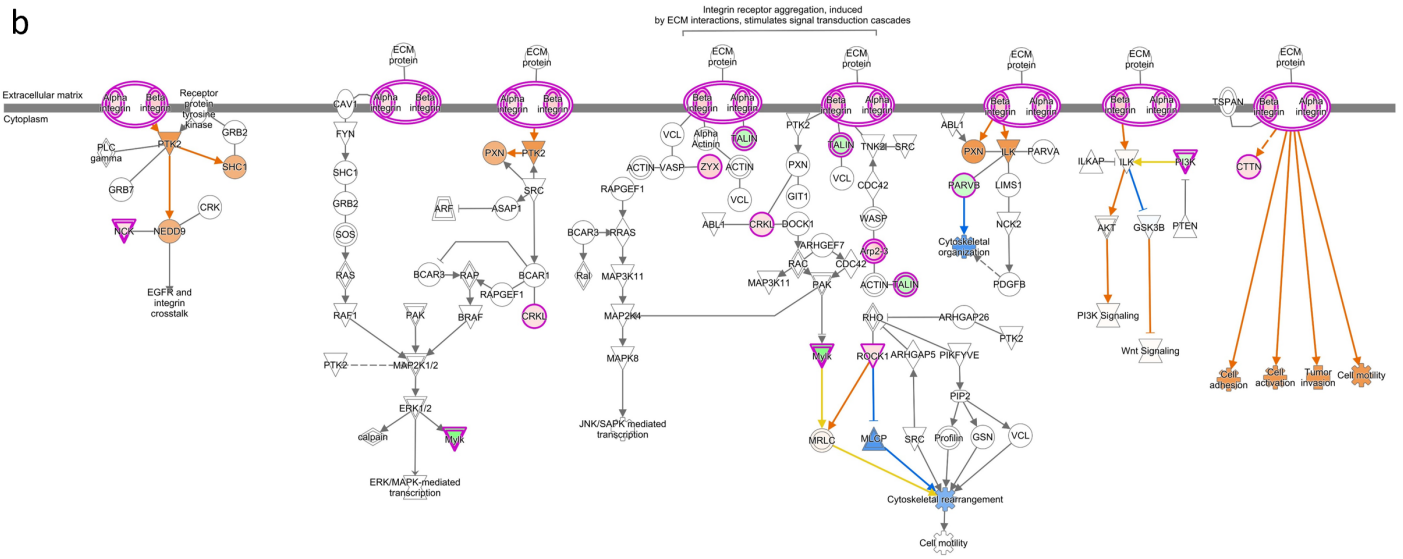

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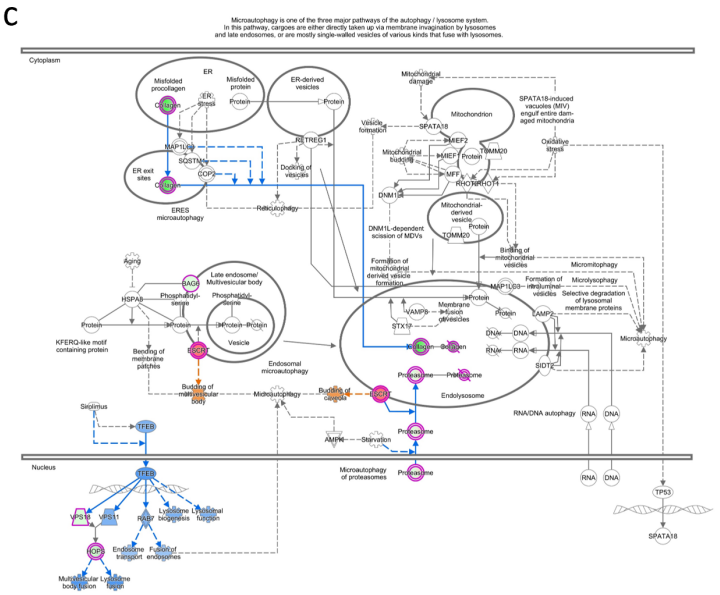

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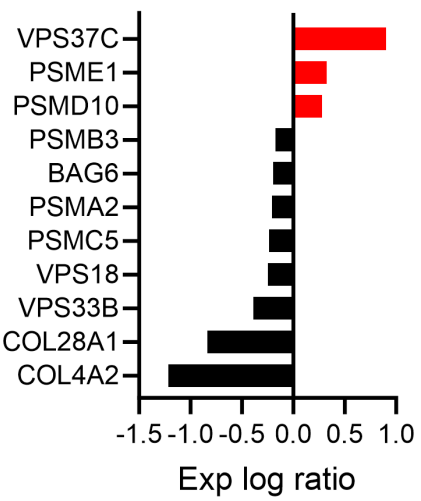

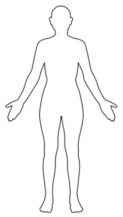

Right Atrial Appendage

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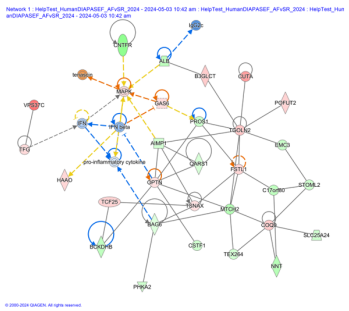

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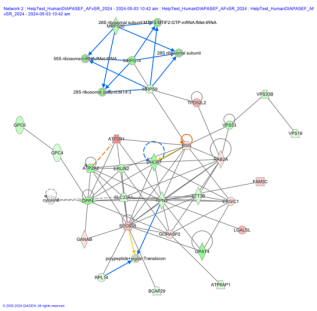

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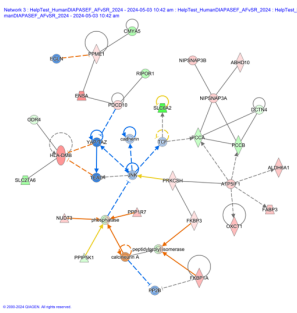

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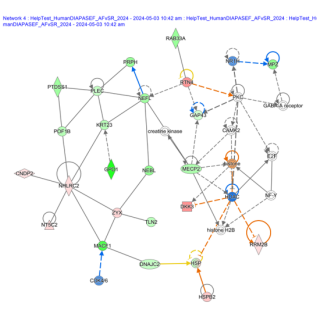

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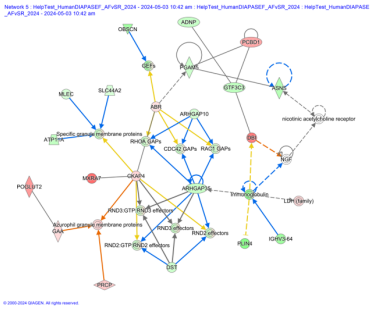

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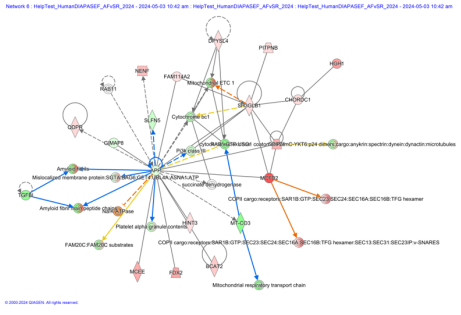

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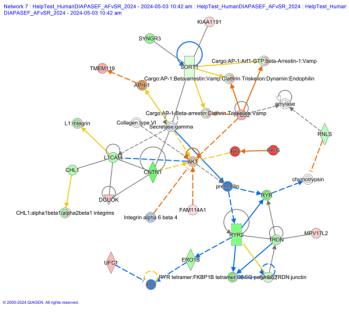

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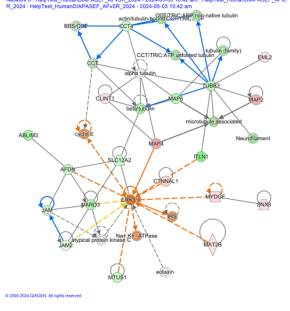

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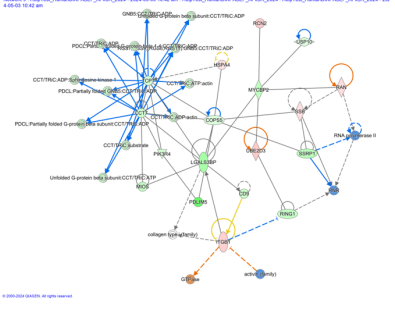

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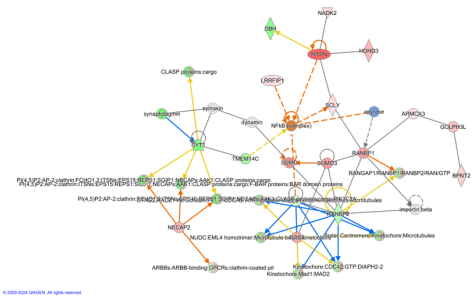
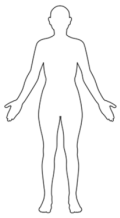
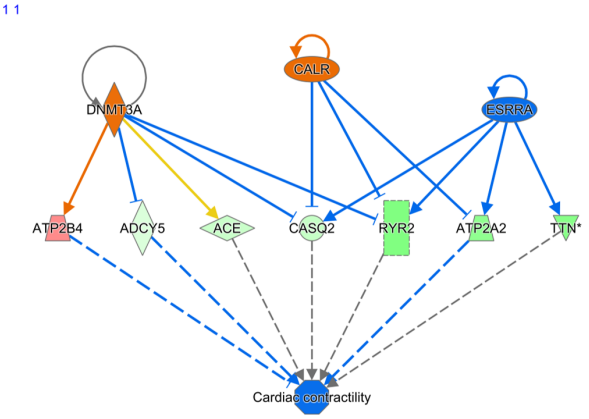


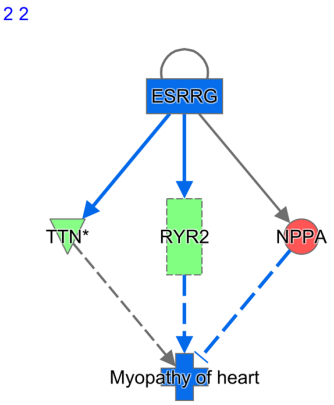


Right Atrial Appendage

a



b



c

