SUPPLEMENTAL MATERIAL

Structural and functional characterization of a $Na_V1.5$-mitochondrial couplon.

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Methods

Focused Ion Beam milling combined with Scanning Electron Microscopy (FIB-SEM)

FIB-SEM sample preparation

The protocol was modified from Wilke *et al*.[24] Mice C57BL/6 were anaesthetized by inhalation of 100% CO_2. After deep anesthesia was confirmed by lack of response to otherwise painful stimuli, mice were euthanized by cervical dislocation. All procedures conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the NYU IACUC committee under protocol number 160726. The heart was surgically excised, perfused with 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer saline (PBS) and kept in the same fixative solution at 4°C overnight. After washing with 0.1 M PBS for 30 min, the heart was placed in 2% OsO_4/1.5% potassium ferrocyanide in PBS for 1 h at room temperature, washed 3x in ddH_2O and then placed in a filtered solution of 1% thiocarbohydrazide (EMS) in ddH_2O for 20 min at room temperature to allow for additional osmium-staining. Subsequently, the tissue was then washed 3x in ddH_2O and placed in 2% aqueous OsO_4 for 30 min at room temperature. Finally, the tissue was washed 3 in ddH_2O and placed in 1% aqueous uranyl acetate at 4°C overnight. The next day, tissue was washed 3x in ddH_2O.

En bloc lead staining was performed to enhance membrane contrast.[25] A lead aspartate solution was made by dissolving 0.066 g of lead nitrate in 10 ml of 0.003 m aspartic acid pH 5.5 and kept at 60°C for 30 min. The lead aspartate solution was filtered, and the tissue was stained at 60°C for 30 min. It was determined that this enhanced osmium-staining protocol, combined with *en bloc* lead staining, was critical for enhancing membrane contrast. The tissue was then washed 3x in ddH_2O, dehydrated in a series of ethanol solutions (30, 50, 70, 85, 95 and 100%; 10 min each, on ice) and then placed in ice-cold dry acetone for 10 min, followed by 10 min in acetone at room temperature. The tissue was then gradually equilibrated with Durcupan ACM Araldite embedding resin (Electron Microscopy Sciences, EMS, PA) by placing in 25% Durcupan/acetone for 2 h, 50% Durcupan/acetone 2 h, 75% Durcupan/acetone for 2 h, and 100% Durcupan

overnight. Finally, it was embedded in fresh 100% Durcupan and placed at 60°C for 48 h to allow Durcupan polymerization and complete the embedding procedure.

FIB-SEM data acquisition

As previously described,[26] the sample was trimmed and thin sections were cut on slot grids to identify the area of interest. The sample block was then mounted on the s.e.m. sample holder using double-sided carbon tape (EMS). Colloidal silver paint (EMS) was used to electrically ground the exposed edges of the tissue block. The entire surface of the specimen was then sputter coated with a thin layer of gold/palladium and the tissue imaged using back scattered electron (BSE) mode in a FEI Helios Nanolab650 dual beam s.e.m. Images were recorded after each round of ion beam milling using the s.e.m. beam at 2 keV and 100 pA with a working distance of 2.5 mm. Data acquisition occurred in an automated way using the Auto Slice and View G3 software.

FIB-SEM data analysis

Upon inspection of the virtual slices, some mitochondria and lateral membrane were segmented to obtain their 3D-rendered models. The 3D model allows the visualization of the spatial interrelations between mitochondria and lateral membrane, the tubular extensions of mitochondria and their different orientations. Segmentation and visualization were performed in Amira (Visage Imaging, San Diego, CA).

Inmunostaining

For immunolocalization, HL-1 cells were incubated with 1 µmol/L Mitotracker Deep Red FM (Invitrogen, catalog number #M22426) for 5 min, washed 3x with PBS and subsequently fixed in 4% PFA (Electron Microscopy Sciences) for 10 min. Cells were then permeabilized in 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. Polyclonal rabbit NCLX (Biorbyt, catalog number #orb317904, Lot: BS9098) primary antibody diluted in blocking buffer (1:50) was incubated for 1h. After 3x washes with PBS, Alexa Fluor 568 goat anti-rabbit (1:300, Invitrogen, catalog number #A11011,

Lot: 1778925) was applied for 1h at room temperature and 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.

Cardiomyocyte dissociation

Adult mouse ventricular myocytes were obtained by enzymatic dissociation. Mice C57BL/6N were injected with 0.1 mL heparin (500 IU/mL i.p.) 20 min before heart excision and anaesthetized by inhalation of 100% CO_2. Deep anesthesia was confirmed by lack of response to otherwise painful stimuli. The mouse was then euthanized by cervical dislocation and the heart 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 Ca^{2+}-free solution containing (in mmol/L): 113 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 0.6 Na_2HPO_4, 0.6 KH_2PO_4, 12 $NaHCO_3$, 10 $KHCO_3$, 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 tissue was 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 (Ca^{2+}-free perfusion buffer with 5% bovine calf serum) and the Ca^{2+} concentration was increased gradually to 1.0 mmol/L. Cardiomyocytes were kept in Tyrode's solution containing (in mmol/L): 148 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 0.4 NaH_2PO_4, 15 HEPES and 5.5 Glucose, pH 7.40. Cells were used within 8 h after isolation.

Single molecule localization microscopy (SMLM) by stochastic optical reconstruction microscopy (STORM)

Freshly isolated ventricular cardiomyocytes from wild-type mice were plated on laminin-coated coverslips and left to adhere for at least 30 minutes before fixation (4% PFA-PBS). Previous to fixation, some coverslips were incubated with 1 µmol/L Mitotracker Deep Red FM (Invitrogen, catalog number #M22426) for 5 min. Cells were then permeabilized with

0.1% Triton X-100 in PBS for 10 minutes. Blocking was done in PBS containing 2% bovine serum albumin (Sigma), 2% glycine (Sigma) and 0.2% gelatin (Sigma) for 30 min. Primary antibodies diluted in blocking buffer were incubated for 1 h at room temperature, followed by 3 washes in PBS, and secondary antibodies incubation for 10 min. Antibodies used were: rabbit polyclonal NCLX (1:50, Biorbyt, catalog number #orb317904, Lot: BS9098), rabbit polyclonal $Na_V1.5$ (1:50, Sigma catalog number #S0819, specific for $Na_V1.5$ isoform), mouse monoclonal α-actinin (1:200, Sigma, catalog number #A7811), rabbit polyclonal $Na_V1.5$ conjugated to Alexa Fluor goat anti-rabbit 568 (1:100, Invitrogen, catalog number #A10494), Alexa Fluor goat anti-rabbit 568 (1:10000, Invitrogen, catalog number #A11011, Lot: 1778925) and Alexa Fluor goat anti-rabbit 488 (1:10000, Invitrogen, catalog number #A11008, Lot: 2179202). Negative controls were performed under the same conditions but without primary antibodies.

Samples were imaged using a custom-built platform based on an inverse microscopy setup Leica DMI3000 as described before.[27] Imaging conditions were achieved by addition of 200 mmol/L mercaptoethylamine and an oxygen scavenging system (0.4 mg/ml glucose oxidase, 0.8 µg/mL catalase and 10% (wt/wt) glucose) to the fluorophore-containing sample.

Movies containing at least 2000 frames were submitted to a home-built software in Matlab for precise single molecule localization. Reconstructed super-resolved images were processed with a smoothing filter (“Gaussian blur” function in ImageJ), adjusted for brightness and contrast and filtered to a threshold to obtain a binary image. Cluster detection and parameters were obtained using the function “Analyze particles” in ImageJ.

As described previously,[27] in order to standardize measurements, distances between $Na_V1.5$ and NCLX, either at the lateral membrane or at the cell interior, were measured with an automated script written in Python. The images from the super-resolution fluorescence microscopy and their corresponding ROI files provided the input to the script. The script utilized the image processing packages scikit-image, and “Mahotas,” an open source software for scriptable computer vision (http://dx.doi.org/10.5334/jors.ac). Two clusters were considered separate if one was at least 20 nm/1 pixel apart from another in any direction.

Interaction factor analysis

The algorithm provides a measure of the degree to which the distance/orientation of the mitochondria (red) and $Na_V1.5$ (green) in manually drawn regions of interest (ROIs) are different from random distributions. First, the bounding box of the ROI is cropped from the larger image. Then, a reference line along the chain of subsarcolemmal mitochondria, parallel to the cell membrane, is determined and an angle of inclination of an object (a $Na_V1.5$ cluster) measured in relation to the reference line. The position of the reference line is determined by finding the line over which the sum of intensities of the red channel is maximum. $Na_V1.5$ clusters were considered ellipse objects; the centroid was used to measure the shortest distance to the reference line and the long axis of the ellipse was used to determine the angle of inclination. These parameters were compared to those obtained by placing a random arrangement of (green) clusters over the same ROI. The randomized images were created using the python package "IFSimPy".[78] The python source code is available on github at https://github.com/FenyoLab/IFSimPy. Briefly, randomized images were created by segmenting the clusters of interest (i.e. $Na_V1.5$ or α-actinin) and then, for each cluster, randomizing its position and angle of rotation within the ROI, and placing it on the new image. Please note that, for simulating proximities, the ROIs were limited by the edge of the cell.

Mitochondrial Ca^{2+} dynamics

As previously described,[27] mitochondrial Ca^{2+} was measured with Rhod-2 AM dye. Briefly, non-permeabilized left ventricular myocytes from wild-type mice were loaded with 2 μmol/L Rhod-2 AM (Thermo Fisher) and 10% FBS at 4°C for 70 min; followed by a 37°C incubation (FBS free) for 3-5 h. 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, the cells were placed on the stage of a Leica SP5 confocal microscope (63× oil immersion lens) where Rhod-2 fluorescence was recorded by excitation at 543 nm and measurement of the emitted light at 560-600 nm. MitoTracker was excited at 633 nm and fluorescence emission at 650-800 nm was detected. Image analysis and quantification was made using LAS X and ImageJ software. After this protocol, we

observed colocalization of Rhod-2 with MitoTracker, suggesting that Rhod-2 was selectively loaded into mitochondria.

The bath solution contained (in mmol/L): 148 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 0.4 NaH_2PO_4, 15 HEPES and 5.5 Glucose, pH 7.40. To evaluate the mitochondrial Ca^{2+} dynamics in response to different drugs, we monitored Rhod-2 fluorescence signal before (F_0) and after (F) adding in the bath: TTX 10 µmol/L or 100 nmol/L (Na-channel blocker, Tocris), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, an ionopore of protons, Tocris) 1 µmol/L + oligomycin A 2 µmol/L (an inhibitor of F0/F1-ATPase, Sigma), and CGP-37157 (NCLX antagonist, Tocris) 1 µmol/L when combined with other drugs and 2 µmol/L when alone. After TTX addition, cells were paced at 0.5 Hz in order to open Na-channels. FCCP and CGP-37157 were dissolved in DMSO, oligomycin A in ethanol, and TTX in water, as stock solutions. The final concentration of DMSO and ethanol in the bath solution were 0.01–0.02% and 0.15%, respectively. Negative controls (no drug) were performed by adding 10 µL solution with respective DMSO and ethanol percentages to the bath, and Rhod-2 fluorescence was measured after 5-10 min.

To check for t-tubule preservation, live isolated myocytes were labelled with 2 µg/ml wheat germ agglutinin (WGA) for 10 minutes after we completed the protocol used for the measurements of mitochondrial Ca^{2+}.

Statistics

Numerical results are given as means ± standard error of the mean (S.E.M.). Data on mitochondrial Ca^{2+} dynamics were tested for normality using the Shapiro-Wilk test and the Kolmogorov-Smirnov test. For each drug treatment, Student's t-test was used to compare SSM *vs* IFM or treatment *vs* negative control Rhod2 fluorescence changes (ratio F_{drug}/F_0). Analysis was done using GraphPad Prism 8 software. Differences were considered to be significant at $p<0.05$.

Reported values are unadjusted p-values. The adjusted p-values do not change the outcome of the significance but they do slightly change the p-values in some comparisons, as seen in the table below (marked in orange):

Pair compared	Reported P Value	Adjusted P Value
1st-2nd bar	n.s.	n.s.
3th-4th	*	**
5th-6th	**	**
7th-8th	n.s.	n.s.
9th-10th	***	*
1st-3th	###	###
1st-5th	###	#
1st-7th	n.s.	n.s
1st-9th	#	##
2nd-4th	n.s.	n.s.
2nd-6th	n.s.	n.s.
2nd-8th	n.s.	n.s.
2nd-10th	n.s.	n.s.

n.s. non significant; *p<0.05; **p<0.01 (SSM *vs* IFM); #p<0.05; ###p<0.001 (treatment *vs* negative control).

Mitochondrial superoxide detection

Superoxide production was measured using the indicator MitoSOX Red (Invitrogen), according to the manufacturer's instructions. MitoSOX is a fluorescent dye dihydroethidium-derivative bound to a lipophilic cation. Briefly, freshly isolated non-permeabilized left ventricular myocytes from wild-type mice were loaded with 5 µmol/L MitoSOX Red in Hanks' Balanced Salt Solution (HBSS) for 10 min at 37°C. For mitochondrial loading validation, some 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 by excitation at 514 nm and measurement of the emitted light at 530-580 nm. MitoTracker was excited at 633 nm and fluorescence emission at 650-800 nm was detected. Image analysis and quantification were done using LAS X and ImageJ software. After this protocol, we observed colocalization of MitoSOX with MitoTracker, suggesting that MitoSOX was selectively loaded into Mitochondria. To evaluate mitochondrial superoxide changes in response to TTX, we measured MitoSOX fluorescence signal before (F_0) and after (F) adding TTX 10 µmol/L to the bath followed by 0.5Hz pacing to open $Na_V1.5$ channels.

Statistics

Numerical results are given as means ± S.E.M. Mitochondrial superoxide data were tested for normality using the D'Agostino & Pearson test, Shapiro-Wilk test and Kolmogorov-Smirnov test. All data in all tests passed the normality test (alpha=0.05). Student's t-test was used to compare SSM *vs* IFM MitoSOX fluorescence changes (ratio F_{TTX}/F_0). Analysis was done using GraphPad Prism 8 software. Differences were considered to be significant at $p<0.05$.

SCN5A correlation analysis in human left ventricle

RNAseq raw data from human left ventricular tissue were downloaded through the Genotype-Tissue Expression v8 (GTEx) consortium (https://www.gtexportal.org/home/datasets). Genes were filtered by removing the ones with no transcripts per million (TPM) in <50% of samples and genes TPM <1 in >5% of samples. The variance stabilizing transformation was performed to normalize gene counts, as implemented in the R package DESeq2 (version 1.16.1). Co-variants such as gender, age, and RNA quality (rin= RNA integrity number) were used in the linear regression analysis.[79]

hiPSC-CM model of SCN5A knockout

hiPSC maintenance

The hiPSC line 19c3 was previously derived from peripheral blood mononuclear cells of a healthy male using Sendai virus (Invitrogen) and expressing an exogenous TNNT2 promoter-derived Zeocin resistance cassette. The hiPSCs were passaged at a ratio of ~1:15 every 4 days using 0.5 mmol/L EDTA (for 6 min at RT), achieving ~75-80% confluence. The cells were routinely maintained in B8 medium on 1:800 diluted growth factor reduced Matrigel (Corning), except for the first 24 h after passage when B8 was supplemented with 2 µmol/L thiazovivin (LC Labs, T-9753), hereby referred to as B8T medium.[28] All cultures (pluripotent and differentiation) were maintained in 2 mL medium per 9.6 cm^2 of surface area or equivalent.

Genome editing

To generate *SCN5A* knockout gRNA expression vectors, two gRNAs targeting all splicing variants of *SCN5A* were designed using an online CRISPR design tool (Benchling) with high predicted on–target score and minimal predicted off–target effect. DNA oligos (IDT) encoding each gRNA with BbsI ligation overhangs were annealed and inserted into the BbsI restriction site of a pSpCas9(BB)–2A–Puro (PX459, Addgene 62988) plasmid. The constructed gRNA expression plasmids were confirmed by Sanger sequencing (Eurofins) with the LKO1_5_primer (5'–GACTATCATATGCTTACCG–3'). CRISPR/Cas9–mediated knockout of each gene independently was induced after cell passage by electroporation of 5×106 hiPSC with 5 µg of each gRNA expression vector. Subsequently, cells were maintained for 48 h in B8T medium supplemented with 0.5 µg/ml of puromycin (Gibco). Puromycin resistant individual colonies were picked and expanded ~10 days after electroporation. Clones with indels were identified by genomic sequencing with primers outside of the targeting region. Genomic DNA was extracted from the cell pellets using a Quick–DNA Miniprep Plus kit (Zymo).

hiPSC-CMs differentiation

Differentiation into hiPSC-CMs was performed according to previously described protocol with slight modifications.[29,30] Briefly, at the start of differentiation (day 0), B8 medium was changed to R6C, consisting of RPMI 1640 (Corning, 10-040-CM), supplemented with 6 µmol/L of glycogen synthase kinase 3-β inhibitor CHIR99021 (LC Labs, C-6556). On day 1, medium was changed to RPMI, and on day 2 medium was changed to RBA-C59, consisting of RPMI supplemented with 2 mg/ml fatty acid-free bovine serum albumin (GenDEPOT, A0100), 200 µg/mL L-ascorbic acid 2-phosphate (Wako, 321-44823) and 0.5 µmol/L Wnt-C59 (Biorbyt, orb181132). Medium was then changed on day 4 and then every other day with RBAI consisting of RPMI supplemented with 0.5 mg/mL fatty acid-free bovine serum albumin, 200 µg/ml L-ascorbic acid 2-phosphate, and 1 µg/mL E. coli-derived recombinant human insulin (Gibco, A11382IJ). Contracting cells were noted from day 7, differentiated cardiomyocytes were treated with 25 µg/mL of Zeocin from day 10 to day 14. On day 20 of differentiation, cardiomyocytes were dissociated using DPBS for 20 min at 37°C followed by 1:200 Liberase TH (Roche, 5401151001) diluted in DPBS for

20 min at 37°C, centrifuged at 300×g for 5 min, and filtered through a 100 µm cell strainer (Falcon). hiPSC-CMs were then plated on a Matrigel-coated 18 mm cover glass (Warner Instruments) for action potential measurements and into 30 mm culture dishes for membrane current recordings.

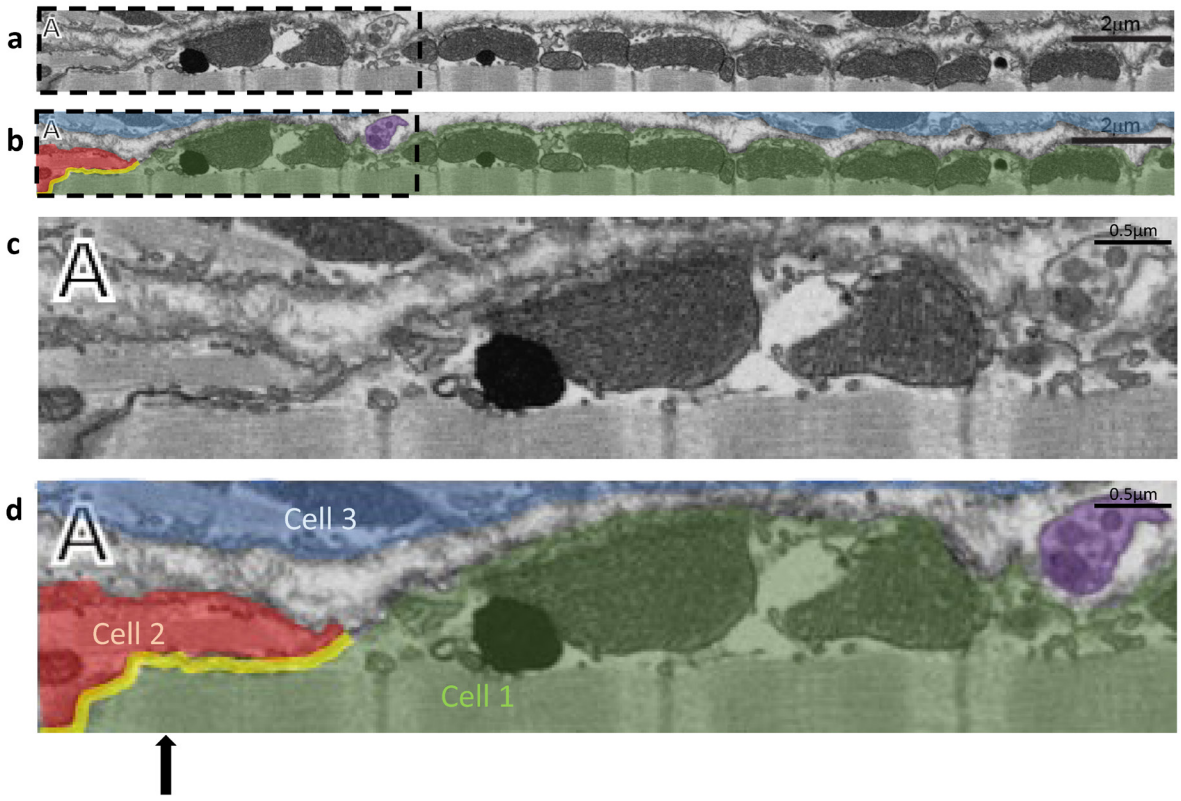
Quantitative Real–time PCR

RNA was isolated using TRI reagent (Zymo) and Direct–zol RNA microprep kit (Zymo) including on-column DNase digestion to remove genomic DNA. cDNA was produced from 1 µg of total RNA using a High Capacity RNA–to–cDNA kit (Applied Biosystems). All PCR reactions were performed in triplicate in a 384–well plate format using TaqMan Gene Expression Master Mix in a QuantStudio 5 Real–Time PCR System (both Applied Biosystems) with following TaqMan Gene Expression Assays (Applied Biosystems): 18S (Hs99999901_s1), SCN5A (Hs00165693_m1), TNNT2 (Hs00943911_m1) SLC8A1 (Hs01062258_m1), and SLC8B1 (Hs00227951_m1), ATP2A2 (Hs00544877_m1), MYL7 (Hs01085598_g1), MYH7 (Hs01110632_m1), KCNQ1 (Hs00923522_m1), TNNI3 (Hs00165957_m1), KCNJ2 (Hs01876357_s1). Relative quantification of gene expression was calculated using 2-ΔΔCt method.

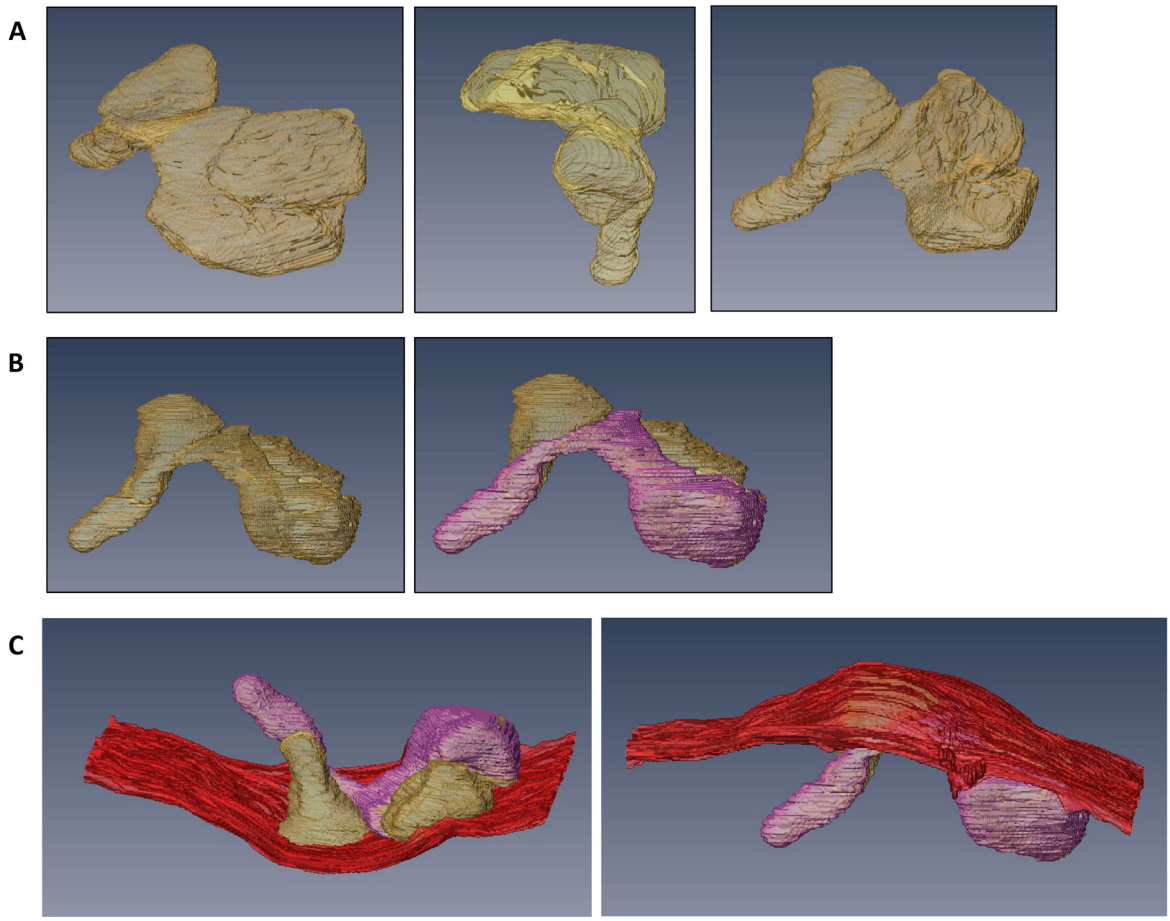
Statistics

Numerical results are given as means ± S.E.M. For the hiPSC-CM analysis, Mann Whitney U-test was used to compare gene expression in SCN5A-KO and WT. Data was analyzed in GraphPad Prism 8 software. Differences were considered to be significant at $p<0.05$.

Online Figures and Legends:

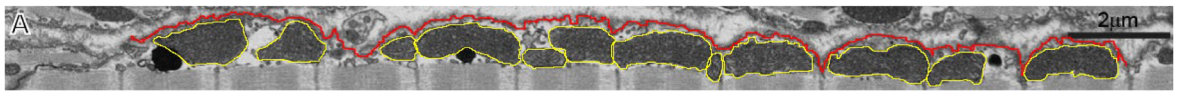


Supplemental Figure I. FIB-SEM single plane of a complete 3D-volumetric image of the cell membrane and the subjacent mitochondria. **a** and **c**. Same FIB-SEM single plane as Figure 1 panel B.A. The black dotted box is enlarged in panel **c**. **b**. Colored diagram to distinguish the different structures. The black dotted box is enlarged in panel **d**. From the bottom edge of the image in the left corner (black arrow), moving up we see the following structures in order of appearance: the last sarcomere of cell 1 (green); a gap junction (yellow); myofibrils of cell 2 (red); the membrane of cell 2 as it wraps its own cell; the intercellular space; the membrane of cell 3 (blue) and its the interior; the upper edge of the image. Of note, the intercellular space includes a non-myocyte cell between cells 1 and 3 (in magenta in the upper right corner of panel **d**).

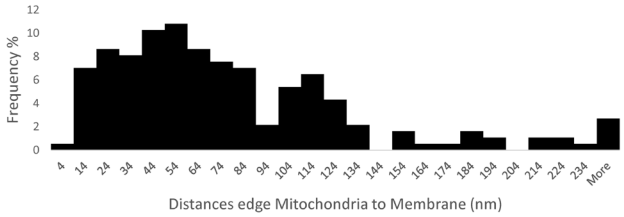


Supplemental Figure II. 3D-rendered models of structures visible after image segmentation: mitochondria cluster and tubular extension (brown and purple) and lateral membrane (red). **A**. Three-dimensional view of the mitochondria in different orientations. **B**. Mitochondrial tubular extension connecting two mitochondria columns. **C**. Mitochondria cluster lateral membrane interaction.

A



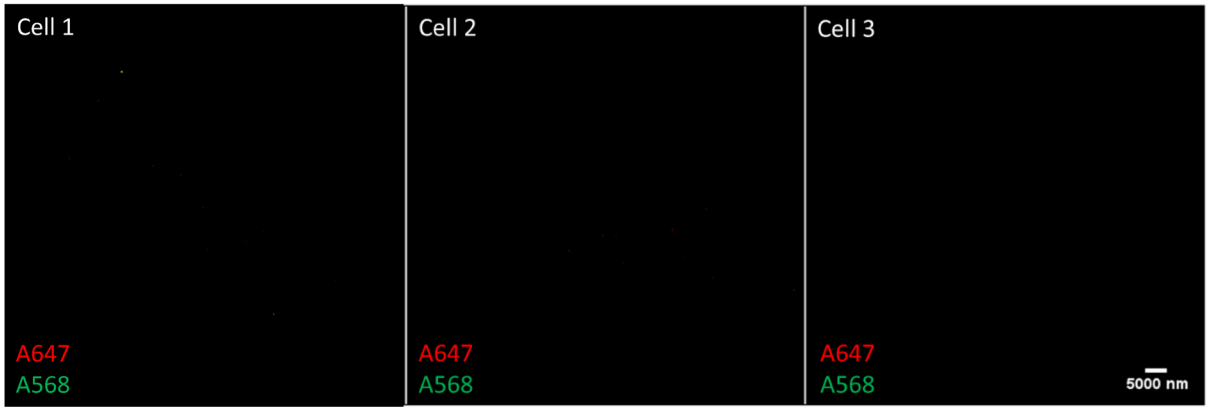
B



C

Mean distance ± S.E.M. (nm)	Median distance (nm)
77.2 ± 4.97	60

Supplemental Figure III. Distances SSM to the membrane. A. Same FIB-SEM single plane as Figure 1 panel B.A, with colored contours (yellow for the mitochondria; red for the membrane). **B**. Distribution histogram representing the distances from the edge of SSM to the membrane. Approximately 13% of the mitochondrial surface in the immediate subsarcolemmal space is less than 20 nm apart from the membrane. **C.** Mean and median distance values (nm). n=185 observations from 1 mouse.

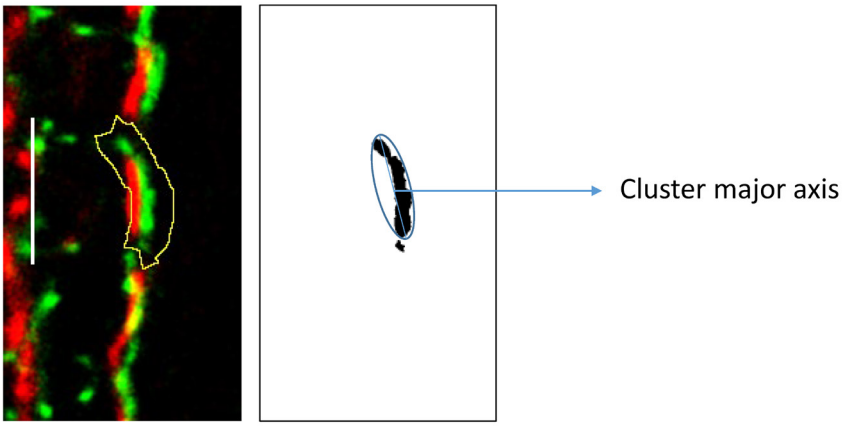


Supplemental Figure IV. Negative control immunostainings of STORM-acquired images. Three representative images from three different cells from 1 mouse are shown. Fixed isolated myocytes were incubated with secondary antibodies (A647 + A568) in the absence of primary antibodies. Scale bar 5 μm.

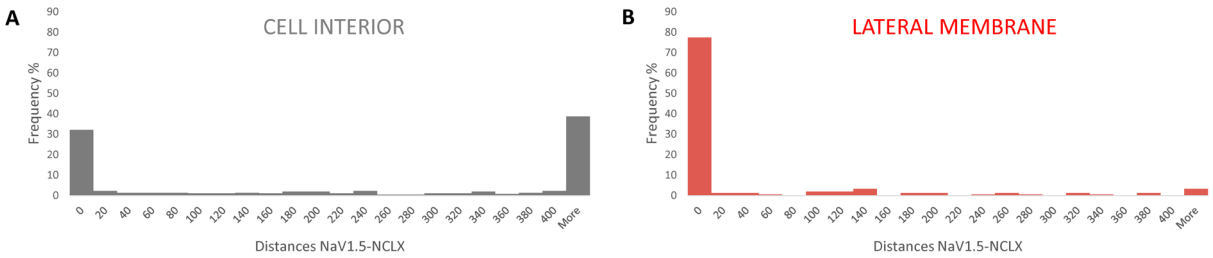
A

Cluster size	Circularity index	Cluster major axis
156.46 ± 18.7 µm^2	0.506 ± 0.02	1.36 ± 0.135 µm

B



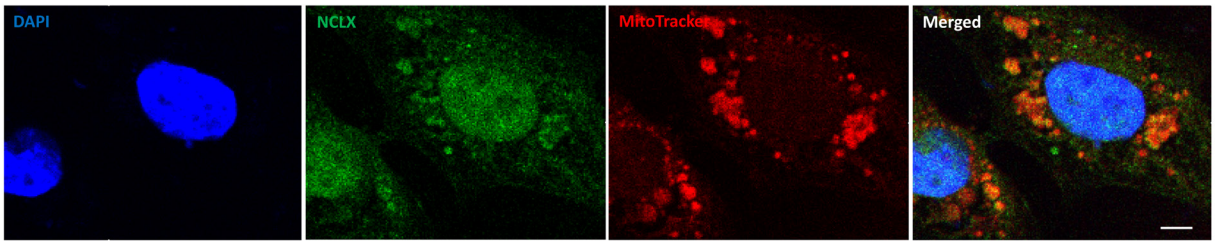
Supplemental Figure V. Quantitative analysis of the lateral membrane Na$_V$1.5 subpopulation clusters. A. Cluster properties: values are Mean ± standard error of the mean. We analyzed a total of 297 clusters from 15 cells, harvested from 3 mice. **B**. Left: Representative STORM-acquired image at the lateral membrane of Na$_V$1.5 (green) and Mitotracker (red). The contour of the ROI is marked in yellow. Scale bar 2 µm. Right: Mask showing the Na$_V$1.5 cluster from the ROI. Na$_V$1.5 clusters were defined by an ellipse (in blue) and the major axis was measured.



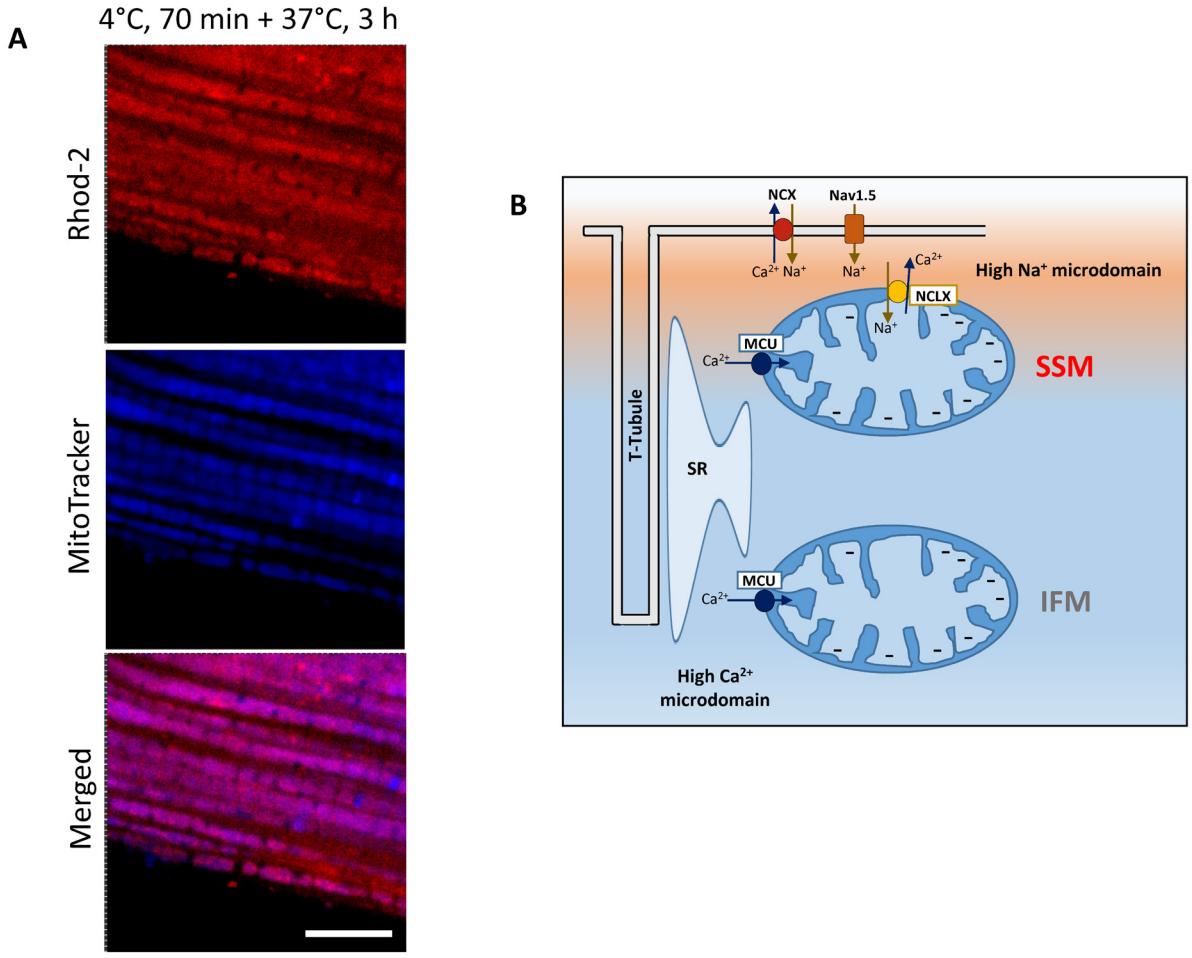
C

	Mean distance ± S.E.M. Nav1.5-NCLX (nm)	Median distance Nav1.5-NCLX (nm)	% colocalization (<20 nm)
LATERAL MEMBRANE	52.3 ± 11.1	0	77.5 %
CELL INTERIOR	388 ± 24.3 ***	231.3	32.2 %

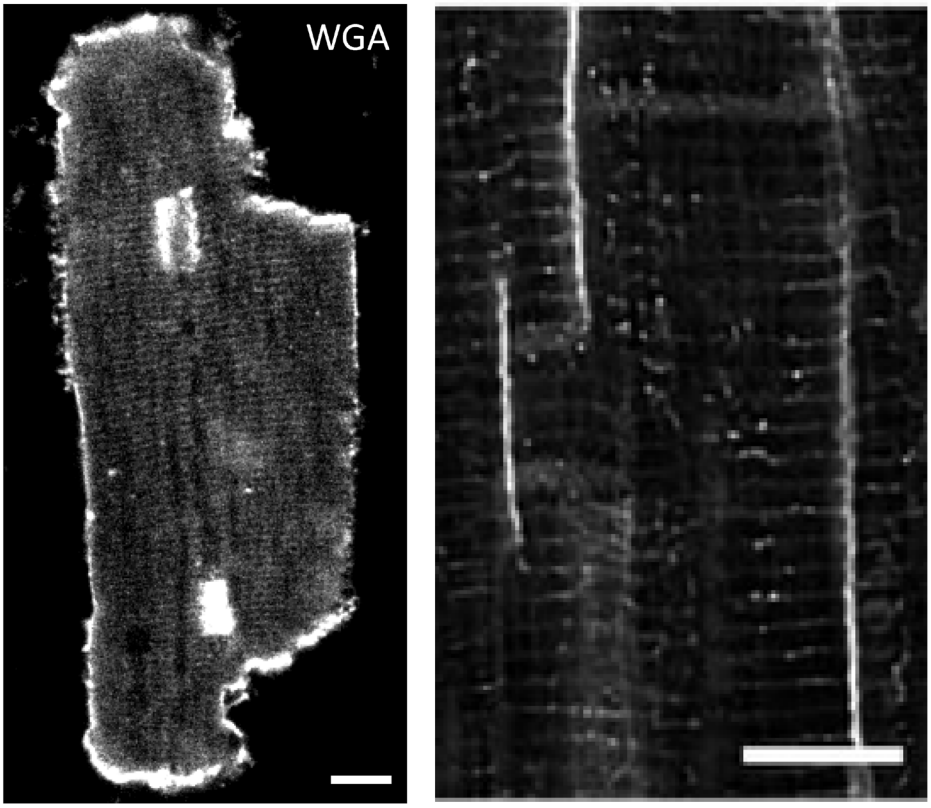
Supplemental Figure VI. Distances between Nav1.5 and NCLX at the lateral membrane *vs* the cell interior. **A-B.** Distribution histogram representing Nav1.5-NCLX distances at the cell interior (A) and lateral membrane (B). **C**. Mean and median distance, and percentage of colocalization (<20 nm) were measured. Statistical test: Mann Whitney; ****p*<0.0001. Cell interior n=348 clusters from N= 3 mice; Lateral membrane n=151 clusters from N= 3 mice.



Supplemental Figure VII. NCLX antibody validation. Inmunofluorescence localization of NCLX (green), Mitotracker (red) and DAPI (blue) in HL-1 cells. Scale bar 5 μm. Notice the colocalization between Mitotracker and NCLX.

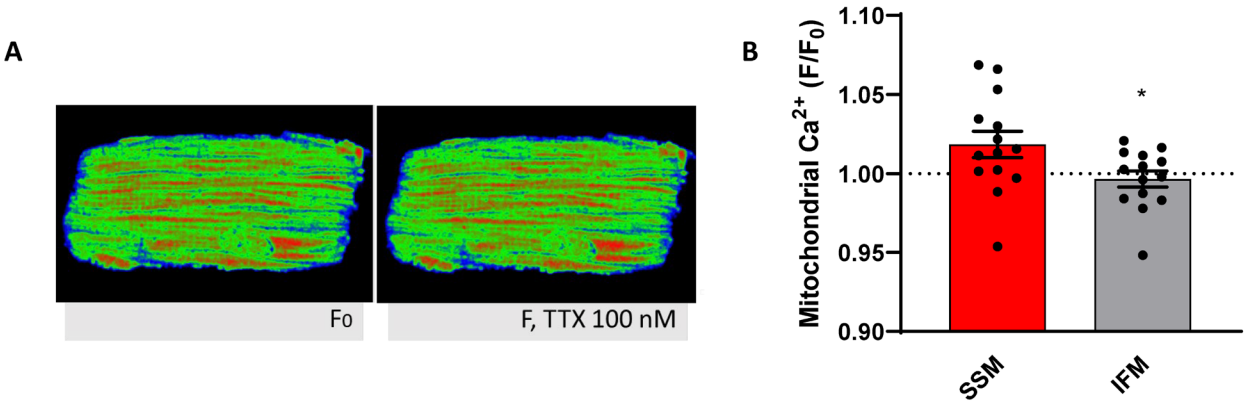


Supplemental Figure VIII. Rhod-2 colocalization with Mitotracker in isolated adult cardiomyocytes. **A.** Rhod 2 loading into mitochondria is temperature-dependent. Ventricular myocytes were incubated with 2 µmol/L Rhod-2 AM at 4°C for 1 h followed by 37°C for 3-5 h and then labelled with 200 nmol/L MitoTracker Deep Red FM for another 5 min. Rhod-2 and MitoTracker were visualized with confocal fluorescence imaging. Merged image shows that Rhod-2 was selectively loaded into the mitochondria. Scale bar 10 µm. **B**. Schematic diagram of the subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) localization. SSM are just beneath the surface sarcolemma whereas IFM localize between the myofibrils. We found NCLX primarily located in SSM, where its proximity to $Na_V1.5$ channels creates a high Na^+ microdomain (dark orange) similar to the Ca^{2+} microdomain (blue) created between the sarcoplasmic reticulum (SR) and the mitochondrial calcium uniporter (MCU).

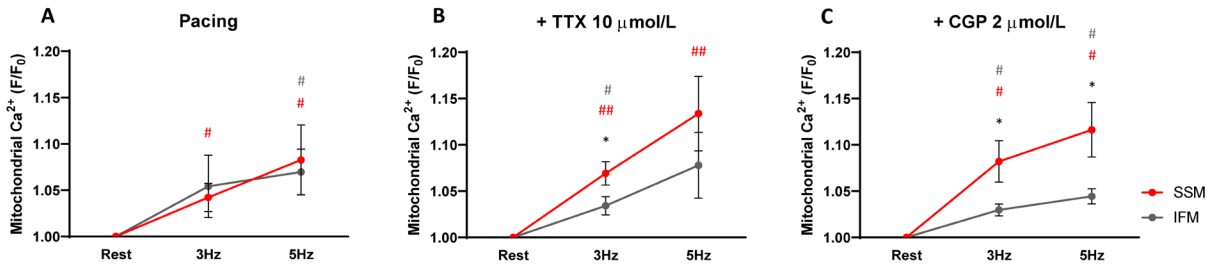


Adapted from Crossman et al, *PLoS ONE* 2011; 6(3):e17901.

Supplemental Figure IX. Preserved t-tubules after mitochondrial Ca^{2+} experiments. Live isolated myocytes were labelled with 2 µg/ml wheat germ agglutinin (WGA) for 10 minutes after we completed the protocol used for our measurements of mitochondrial Ca^{2+} (as in Fig. 5 and described in the Methods section of the Ms). WGA is a plant lectin that binds to sialic acid residues. Note the preserved t-tubular structure, comparable to the one presented in previous publications on the right panel (Adapted from Crossman et al, *PLoS ONE* 2011; 6(3):e17901). Scale bar 10 µm.

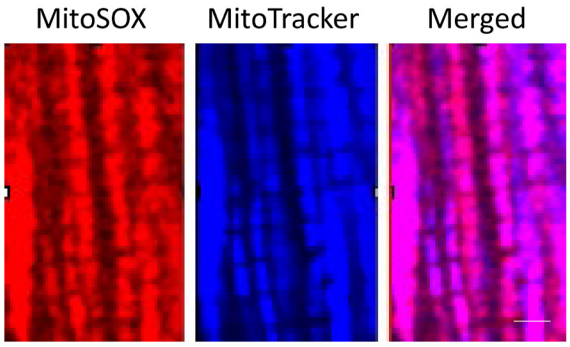


Supplemental Figure X. Mitochondrial Ca^{2+} dynamics in relation to availability of TTX-sensitive Nav-channels. **A**. Confocal 2D images of cardiomyocytes loaded with Rhod 2-AM at 4°C and then incubated at 37°C for 3-5 hours (see Methods). Two images from the same cell are shown: at rest (F_0; left), and after adding TTX 100 nmol/L (F; right). Two regions were defined, SSM (as the first string of mitochondria immediately below the cell surface) and IFM (in the interior of the cell). **B**. Quantitative analysis of F/F_0 ratio in SSM (red bar) and IFM (gray bar). We observed a tendency for an increase in mitochondrial Ca^{2+} in SSM after adding TTX (p=0.078), and no changes in IFM Ca^{2+} retention, while the ratio F/F_0 SSM *vs* IFM was indeed statistically significant (p<0.05; (n=14 cells, N=3 mice), suggesting a possible minor contribution of TTX-sensitive isoforms at the lateral membrane. Interestingly, it has been demonstrated that these TTX-sensitive channels can be upregulated by exogenous factors (Lin et al, *Heart Rhythm*, 2011; 8(12):1923-30), which could lead to changes in mitochondrial calcium homeostasis. Statistical test: Student's t-test between SSM and IFM. *p<0.05 *vs* SSM.

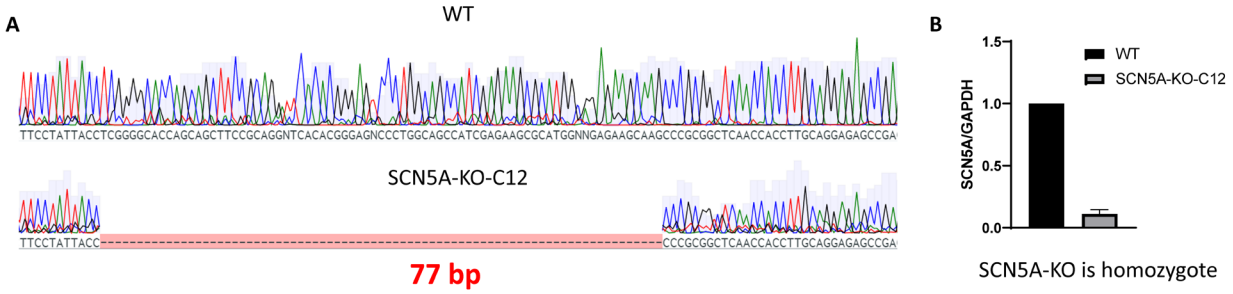


Supplemental Figure XI. Mitochondrial Ca^{2+} dynamics as a function of pacing rate. Rhod-2 was recorded in live myocytes at rest and during stimulation at either 3Hz and 5Hz. Data were collected in control solution (panel **A**), and in the presence of either 10 μmol/L of TTX (panel **B**), or 2 μmol/L of the NCLX inhibitor CGP-37157 (panel **C**). Rapid pacing at 3Hz and 5Hz in control solution (**A**) led to mitochondrial Ca^{2+} accumulation in both SSM and IFM (N=3, *vs* rest #p<0.05), and no difference was observed between the mitochondrial subpopulations. These results suggest that mitochondrial Ca^{2+} is dynamically regulated to keep apace with increased cellular demand. When TTX was added (10 μmol/L, **B**), there was a tendency for SSM to accumulate more Ca^{2+} in response to pacing (3Hz *vs* 3Hz+ TTX p=0.06; 5Hz *vs* 5Hz+ TTX p=0.07) and when comparing the two mitochondrial subpopulations, mitochondrial Ca^{2+} increased significantly more in SSM than in IFM, *p<0.05). Similar results were obtained in the presence of CGP-37157 (**C**).

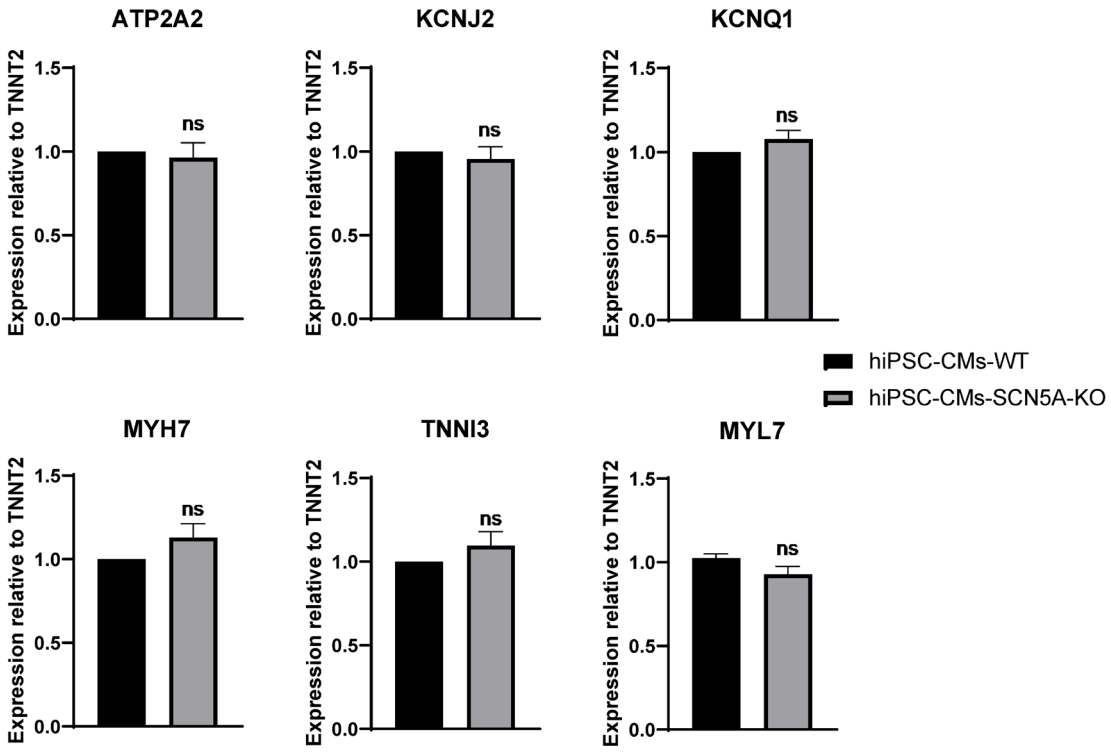
Pacing: n=8 cells from 3 mice; +TTX: n= 7 from 3 mice; +CGP: n=9 from 3 mice. Statistical test: Student's t-test; *p<0.05 IFM *vs* SSM; #p<0.05 *vs* rest (in red for SSM rest and gray for IFM rest), ##p<0.01 *vs* rest; No significant changes by adding TTX or CGP *vs* only pacing.



Supplemental Figure XII. MitoSOX colocalization with Mitotracker in isolated adult cardiomyocytes. Ventricular myocytes were loaded with 5 μmol/L MitoSOX for 10 min at 37°C and then 200 nmol/L MitoTracker Deep Red FM for another 10 min. MitoSOX and MitoTracker were visualized with confocal fluorescence imaging. Merged image shows that MitoSOX was selectively loaded into the mitochondria. Scale bar 2 μm.



Supplemental Figure XIII. DNA sequencing showing SCN5A KO in human-induced pluripotent stem cells. A. Sequencing of SCN5A KO clone 12 (SCN5A-KO-C12) evidenced a 77-base pair deletion in the CRISPR target region (underlined). **B**. Relative SCN5A expression normalized to GAPDH in wild-type (WT) and SCN5A-KO-C12 lines. N=3 for WT and KO.



Supplemental Figure XIV. Cardiomyocytes derived from hiPSC WT and SCN5A KO lines express cardiovascular markers. Quantitative real-time RT-PCR of hiPSC-CM from both cells lines. Relative mRNA expression normalized to TNNT2. Statistical test: Mann Whitney showed no significant (ns) differences between the WT and the KO lines. N=4 for WT and KO.