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Article

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Tachycardia-induced metabolic rewiring as a driver of contractile dysfunction

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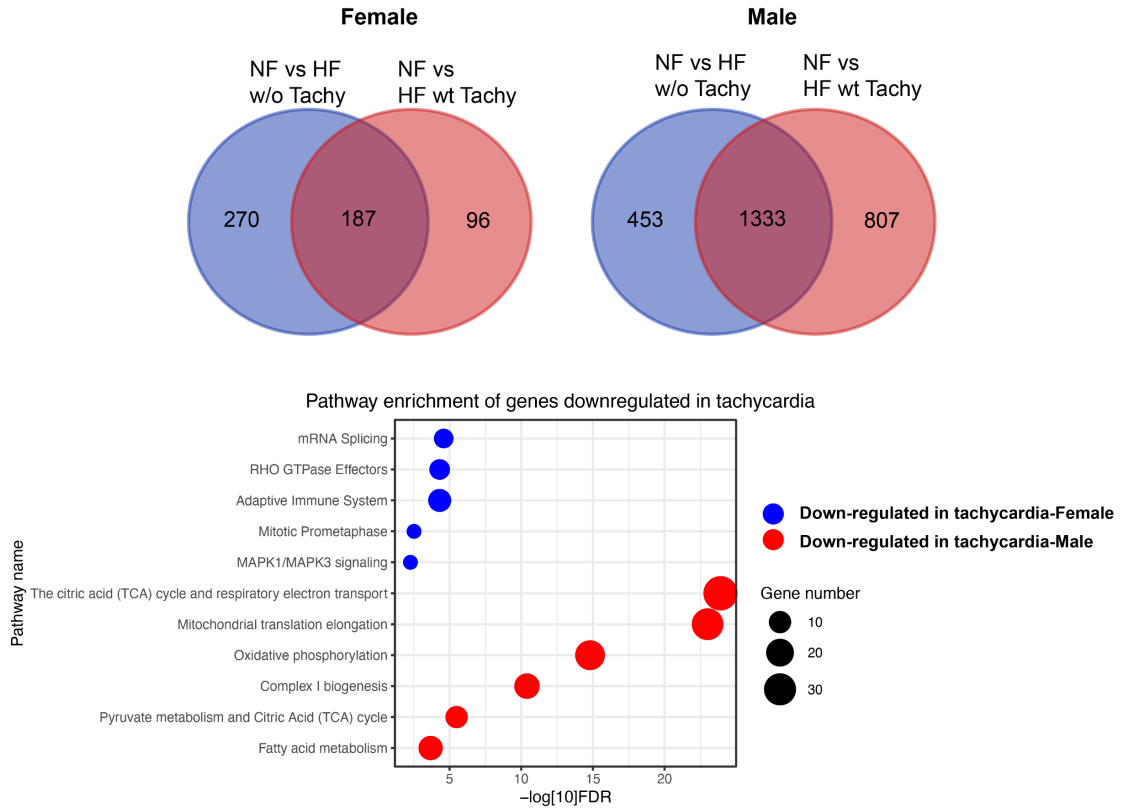
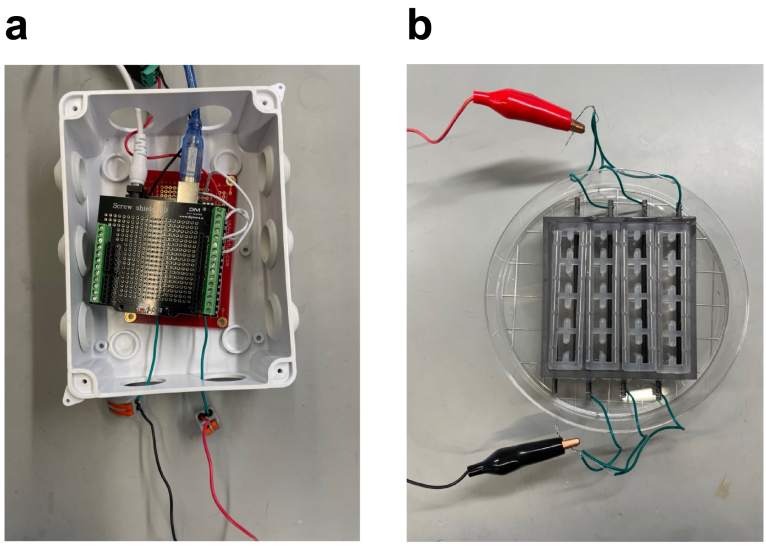
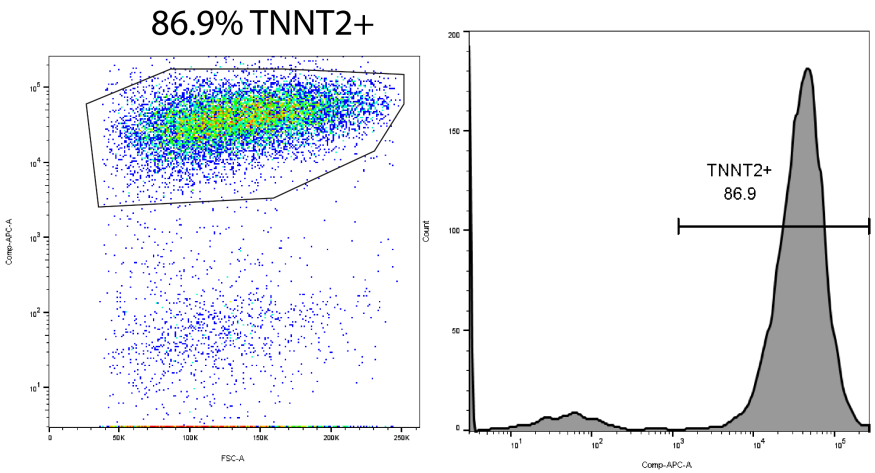
Contents



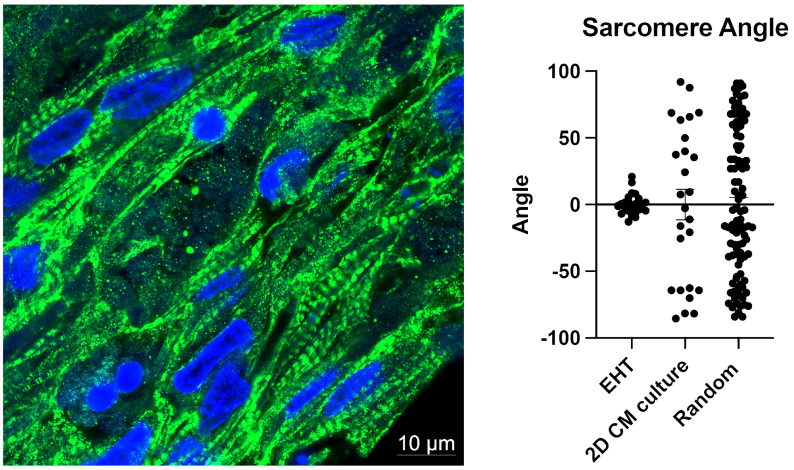
Supplementary Fig. 1 l Sub-analysis of genes and pathways specifically downregulated in patients with tachycardia by gender.
Genes specifically downregulated in patients with tachycardia were identified separately for female and male cohorts. 96 DEGs and 807 DEGs were identified respectively, followed by pathway enrichment analysis. Female and male cohorts were indicated by blue and red bubbles respectively. Fewer DEGs from the female cohort may be partially due to the smaller number of female participants.



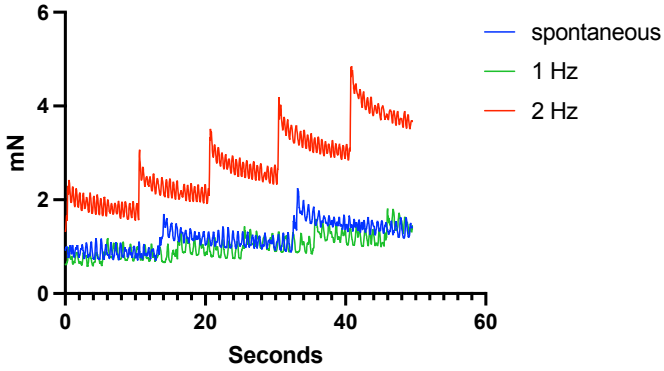
Supplementary Fig. 2 | Photos of the EHT tachypacing set-up.
a, Custom-built electrical stimulation device controlled by an Arduino micro-controller. **b**, Culture chamber for EHTs with carbon rods running across the two sides of each well.



Supplementary Fig. 3 | Purity of CMs generated from iPSCs.
iPSC-CMs were dissociated and stained with Alexa Fluor 647-conjugated antibody targeting cardiac troponin T, followed by flow cytometry analysis to quantify the percentage of cardiac cells in the differentiation.

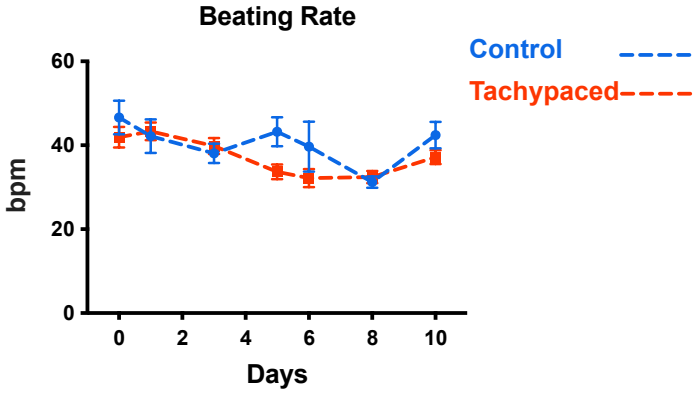


Supplementary Fig. 4 | Quantification of sarcomere alignment in 3D EHTs and 2D iPSC-CM culture. Angles of individual sarcomeres were measured in EHTs or iPSC-CMs cultured in 2D. A representative immunostaining image of EHTs were shown on the left. TNNT2 and nucleus were stained in green and blue (DAPI) respectively. A random distribution of 100 angles was computationally generated as a true negative control. n=26 technical replicates. 3D EHTs showed improved sarcomere alignment than 2D iPSC-CM culture.

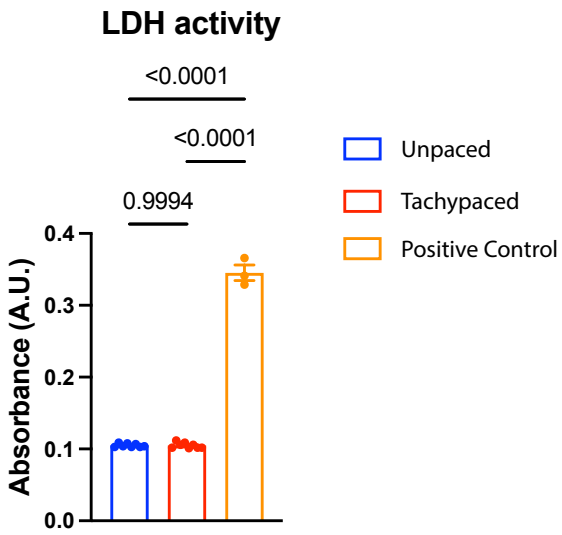


EHT ID	Electrical Stimulation	Max Active Stress (Pa = N/m2)
EHT #1	Spontaneous	0.55 kPa
	1 Hz	0.62 kPa
	2 Hz	0.63 kPa
EHT #2	Spontaneous	0.73 kPa
	1 Hz	0.51 kPa
	2 Hz	0.62 kPa
EHT #3	Spontaneous	0.39 kPa
	1Hz	0.40 kPa
	2Hz	0.44 kPa

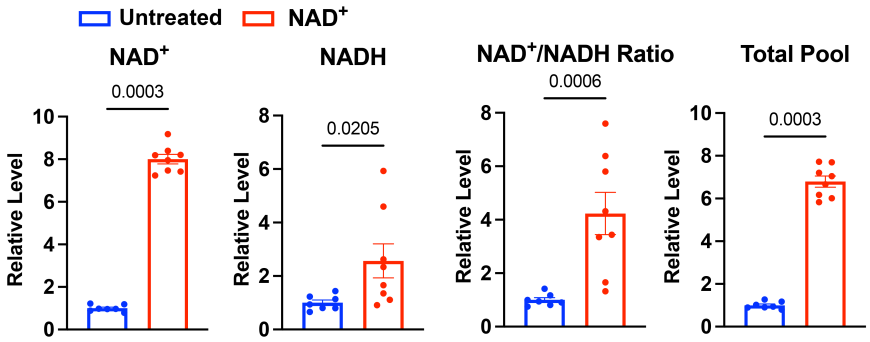
Supplementary Fig. 5 | Force measurements of EHTs under spontaneous beating, 1 Hz stimulation and 2 Hz stimulation.
Every 10 seconds, EHTs were stretched by 125 µm. Active force is calculated based on the amplitude of the small peaks. The large spikes are due to the increase in passive force by stretching. In all 3 tested EHTs, active contractile force increased from 1 Hz to 2 Hz. Representative traces from 1 EHT are shown on the left and the summary statistics are shown on the right.



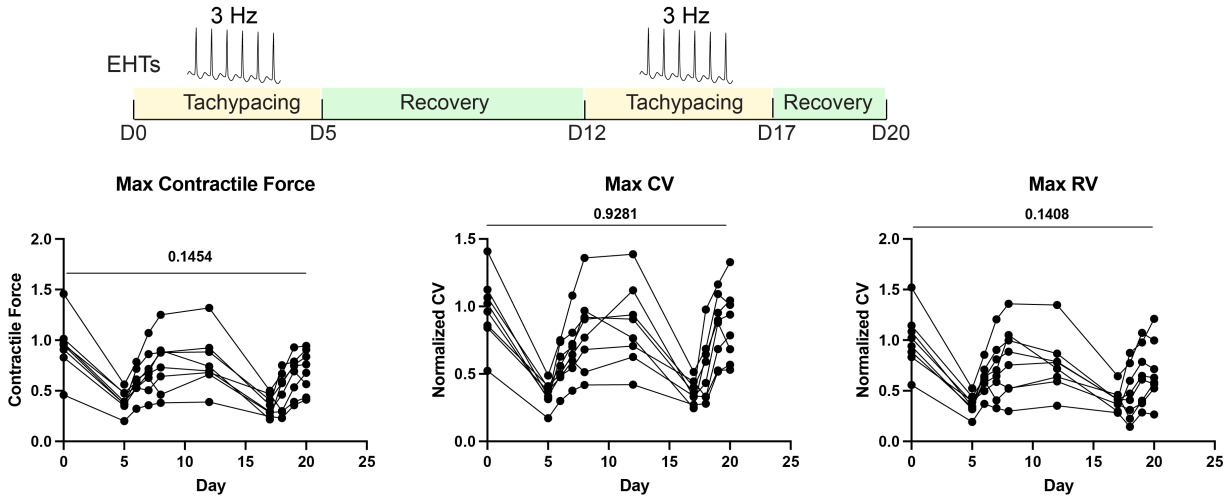
Supplementary Fig. 6 | Spontaneous beating rate of tachypaced or unpaced EHTs.
EHTs were tachypaced or unpaced for 5 days, then allowed for recovery for 5 days. Beating rate was measured without pacing. No significant difference in beating rate was observed between unpaced and tachypaced EHTs. n=7 EHTs for each group. Two-way ANOVA with Bonferroni's multiple comparisons test. Data are displayed as mean ± s.e.m.



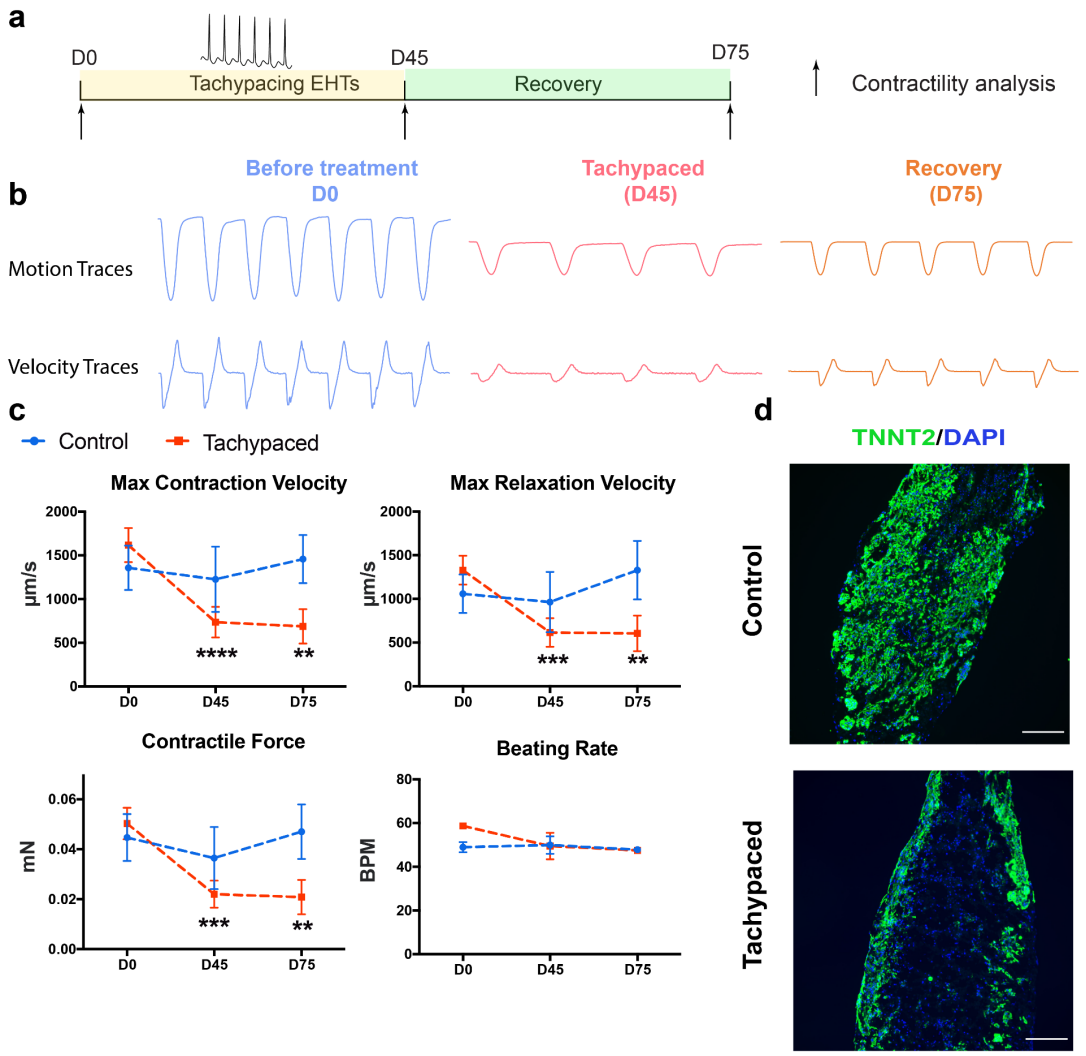
Supplementary Fig. 7 | Effect of tachypacing on cell viability in EHTs.
Lactate dehydrogenase activity assay of conditioned medium from unpaced or tachypaced EHTs was performed. Positive control (high LDH activity) was provided with the assay kit. n=8 technical replicates. One-way ANOVA with Tukey's multiple comparisons test. Data are displayed as mean ± s.e.m.



Supplementary Fig. 8 | Effect of NAD+ supplementation on NAD+ and NADH levels in EHTs. Tachypaced EHTs were treated with 1 mM NAD+ or vehicle control for 24 hours and subjected to NAD+ and NADH measurements. Data were normalized against untreated control. n=7 untreated EHTs and n=8 NAD+-treated EHTs. Two-tailed Mann–Whitney test. Data are displayed as mean ± s.e.m.



Supplementary Fig. 9 | Reversibility of TIC in EHTs.
EHTs were subjected two rounds of tachypacing (5 days of pacing for each round) and allowed to recover after each round of pacing. Each data point represents one EHT. Contractile force, max contraction velocity, max relaxation velocity were quantified. Data were normalized against the baseline value on day 0. n= 6 EHTs. One-way ANOVA with Tukey's multiple comparisons test. Data are displayed as repeated measure of each EHT over time.



Supplementary Fig. 10 | Long-term tachypacing resulted in irreversible damages to EHTs. **a**, Experimental outline: EHTs (SCVI-273) were tachypaced at 3 Hz for 45 days and then allowed to recover for 30 days. Contractility was measured on day 0, day 45 and day 75. **b**, Representative motion traces and velocity traces of tachypaced EHTs on day 0, day 45 and day 75. **c**, Maximum contraction velocity, relaxation velocity, contractile force and beating rate of tachypaced EHTs (indicated by red) and unpaced control EHTs (indicated by blue); for control EHTs; n=6 for day 0 and day 45, and n=3 for day 75; for tachypaced EHTs, n=8 for day 0 and day 45, and n=7 for day 75. Two-way ANOVA with Bonferroni's multiple comparisons test. Data from D45 and D75 were compared with D0 within each group (tachypaced or control). **: P<0.01; ***: P<0.001; ****: P<0.0001. **d**, Representative immunofluorescence images of control and tachypaced EHTs (day 75). EHTs were cryosectioned and stained for TNNT2 (indicated by green) and DAPI (indicated by blue). scale bar, 200 µm. Data are displayed as mean±s.e.m.

Supplementary methods

Electrical pacing of EHTs. The customized stimulation chamber was designed using Fusion 360 and manufactured by computer numerical control (CNC) milling at the Stanford Physics Department machine shop. The chamber was made of polycarbonate, a material compatible with autoclaving. Carbon rods of 3 mm in diameter (Ladd Research) were inserted into the chambers so that two carbon rods were situated on the two sides of the EHTs. For the electrical output, an H-bridge circuit was designed using MultisimLive and assembled by manual soldering, then connected to an Arduino microcontroller. This design allows for reversing the direction of the electrical current after each pulse and hence avoids the oxidation of the culture medium. The electrical pacing intensity was 4 volts/cm with a pulse duration of 6 ms. After each use, the carbon rods were replaced, and the stimulation chamber was autoclaved for the next experiment.

Cryosectioning and immunostaining of EHTs. EHTs were fixed in 4% paraformaldehyde (PFA) overnight, washed with PBS buffer, and soaked in 30% sucrose (Sigma-Aldrich) overnight. The tissues were then snap-frozen in Tissue-Tek O.C.T. Compound (Sakuraus). Frozen tissues were cryosectioned using a Cryostat (Thermo Fischer Scientific). Tissue slices were attached to Superfrost Plus microscope slides (Fisher Scientific). Tissue slides were stored at -80 °C. For immunostaining, the tissue sections were permeabilized with 0.1% Triton X-100 (Thermo Fisher Scientific) in PBS and incubated at room temperature for 15 min, washed 3 times with 0.1 % Tween-20 (Sigma-Aldrich) in PBS (PBST), and then blocked with 3% BSA in PBST for 30 min at room temperature. The samples were incubated with the primary antibodies overnight at 4 °C, washed with PBST, and then incubated with the goat anti-rabbit IgG or goat anti-mouse IgG antibodies (Thermo Fisher Scientific) for 1 hour at room temperature. After washing with PBST 3 times, fluorescence imaging was performed on a Leica DMi8 fluorescence microscope and processed with Fiji Image J. For the primary antibodies: anti-cardiac troponin T antibody (Abcam) was used to detect the cardiac sarcomeric structure, and an anti-vimentin antibody (Abcam) was used to detect the non-myocytes (e.g., fibroblasts).

Canine microarray data. Microarray data of tachypacing-induced HF was retrieved from NCBI GEO at GSE9794. Differentially expressed genes (DEG) between different groups were identified using the DESeq2 R package. Genes with a Benjamin-Hochberg corrected $p< 0.05$ were considered significant. The functions and pathways of the DEGs were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Reactome pathway database. To identify potential common pathways, DEGs were analyzed for biological process and pathway enrichment using KOBAS and PANTHER within canis familiaris species. Pathway enrichment analysis was performed to identify significantly enriched metabolic pathways or signal transduction pathways using the FDR < 0.05 as a threshold of significance.

Western blot analysis. After 5 days of tachypacing, EHTs were removed from the silicone racks. Two EHTs were pooled together as one sample and lysed using RIPA buffer supplemented with proteinase inhibitor (Thermo Fisher Scientific). Water bath sonication (Diagenode) was applied with 20 cycles (30 seconds on/30 seconds off) to break down the EHTs, followed by centrifugation and collection of the supernatant. Protein concentration was quantified using BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were kept cool during the extraction. For Western blotting, 20 µg of protein was separated by NuPAGE 4-12% Bis-Tris Gel (Invitrogen) and transferred onto PVDF membranes using Trans-Blot Turbo System (BioRad), then blocked with 3% BSA in TBS tween buffer (Thermo Fisher Scientific) for 30 min at room temperature. Membranes were incubated with primary antibodies at 4 °C overnight and then with secondary antibodies for 1 hour at room temperature. Membranes were washed with TBS tween between each step. Images were acquired on a BioRad Gel Doc XR Imaging System. Primary antibodies used were: Total human OXPHOS antibody cocktail (Abcam), GLUT1 antibody (Proteintech), ACSL1 antibody (Proteintech), Phospho CaMKII Thr286 (Cell Signaling Technology), CaMKII antibody delta (GeneTex), Phospho Troponin I Ser23/24 (Cell Signaling Technology), Cardiac troponin I (R&D Systems), phospholamban pSer16 (Badrilla), phospholamban pThr17 (Badrilla), phospholamban monoclonal antibody (Badrilla), Serca2 antibody (Cell Signaling Technology), HIF-1 alpha antibody (Proteintech). Secondary antibodies used were HRP-linked anti-rabbit IgG antibody (Cell Signaling Technology) and HRP-linked anti-mouse IgG antibody (Cell Signaling Technology). Dilutions of all antibodies were carried out as instructed by the manufacturers. Protein was stained using Pierce™ Reversible Protein Stain Kit (Thermo Fisher Scientific) as the loading control. Protein band intensity was quantified using the volume tool in Image Lab (BioRad).

RNA extraction and quantitative real-time PCR (qPCR) analysis. EHTs were rinsed with PBS buffer and then manually cut into fine pieces in PBS, spun down at 100 x g for 5 min, and then resuspended in QIAzol lysis reagent (Qiagen). After 20 min of shaking, the resulting solution was stored at -80 °C or directly used for RNA extraction using the Qiagen miRNeasy Mini Kit (Qiagen). High-Capacity RNA-to-cDNA kit (Thermo

Fisher Scientific) was used for the reverse transcription of 1 µg RNA. Each qPCR reaction contained 12.5 ng of template cDNA, 250 nM of the forward and reverse primers, 1x iTaq Universal SYBR Green Supermix (BioRad), and various volumes of DI water so that the final volume was 20 µL. PCR reaction was monitored with CFX Real-Time PCR System (Bio-Rad). Relative expression of each gene was normalized against the expression of beta-2-microglobulin (B2M) or GAPDH. Designs of primers were acquired from PrimerBank (https://pga.mgh.harvard.edu/primerbank) and synthesized by the Stanford Protein and Nucleic Acid Facility.

Lactate assay. Extracellular lactate from tachypaced EHTs or unpaced EHTs were quantified using Lactate-Glo Assay (Promega). Briefly, the culture medium was taken from tachypaced or unpaced EHTs on day 3 and day 5 of pacing. The medium was serially diluted with DPBS. Diluted samples were mixed at 1:1 ratio with the detection reagent prepared according the manufacturer's instructions. Luminescence was recorded in a Cytation 5 Plate Reader (Biotek). The intensity of the light signal is proportional to the lactate level.

NAD+ and NADH measurements. NAD+ and NADH were quantified using the NAD/NADH-Glo Assay Kit (Promega). EHTs were homogenized in 0.2 N NaOH with 1 % dodecyltrimethylammonium bromide (DTAB) using the Next Advance Bullet Blender. After centrifugation, the resulting supernatant (100 µL) was split equally into two tubes for the subsequent quantification of NAD+ and NADH, respectively. For NAD+ measurement, 25 µL of 0.4 N HCl was added to facilitate NADH degradation at 60 °C for 10 min. Samples were then neutralized with 25 µL of 0.5 M Trizma base, resulting in a final volume of 100 uL. To prepare for NADH measurement, samples were heated at 60 °C for 10 min to remove NAD+. 25 µL of 0.4 N HCl and 25 µL of 0.5 M Trizma base were added to the samples, bringing the total volume to 100 µL. The detection reagent was prepared and mixed with the processed samples at a 1:1 ratio in a white-bottom 96-well plate. After 30-60 min of incubation at room temperature, luminescence was measured in a Cytation 5 Plate Reader (Biotek). The concentration of NAD+ or NADH is proportional to the luminescence intensity.

Metabolomics. EHTs were homogenized by the Next Advance Bullet Blender, and the metabolites were extracted using methanol/water (80/20, v/v). The samples (50 µL) were transferred to HPLC vials for direct injection into the LC/MS system within 24 hours of extraction. LC/MS analysis was performed using the negative ion mode on an Agilent 1290 Infinity LC coupled to an Agilent 6545 Q-TOF with an Agilent Jet Stream Source. The LC chromatographic separation was optimized to achieve the best selectivity on an Agilent InfinityLab Poroshell 120 HILIC-Z column (2.1 mm × 150 mm, 2.7 µm, PEEK-lined). In brief, mobile phase A was 10 mM ammonium acetate in water with 2.5 µM medronic acid (pH=9), and mobile phase B was 10 mM ammonium acetate acetonitrile/water (85/15, v/v) with 2.5 µM medronic acid (pH=9). A non-linear mobile phase gradient from 96% to 65% B in 24 min was used with a re-equilibration time of 5 minutes. Other key LC parameters were as follows: LC flow rate was 0.25 mL/min; autosampler temperature was 4 °C; injection volume was 3 µL, and the column temperature was 50 °C. The 6545 Q-TOF mass spectrometer parameters were optimized to achieve the best sensitivity and broad coverage of metabolites. The mass spectrometer parameters were as follows: acquisition mass range is 60-1200 m/z, Vcap was 3500 V, the gas temperature was 225 °C at the flow of 13 L/min, and the sheath gas temperature was 350 °C at a flow of 12 L/min and nebulizer was set at 35 psi. Dynamic mass axis correction was achieved by continuous infusion of a reference mass solution.

Immunoprecipitation (IP) and western blot. Immunoprecipitation of acetylated lysine from EHTs was conducted using the Pierce™ Classic Magnetic IP/Co-IP Kit as specified by the manufacturer's instructions. Briefly, EHTs were sonicated in IP buffer containing 1X deacetylase, protease, and phosphatase inhibitors. Lysates were collected after centrifugation, and total protein was quantified by BCA assay. Input protein was collected from each sample and incubated in 1X sample buffer for 10 min at RT and stored for subsequent western blot analysis. Lysate (350 µg protein/EHT in 500 µL reaction volume) was incubated with 5 µL Acetyl lysine antibody (Cell Signaling Technology) or species-matched control immunoglobulin (Cell Signaling Technology) at 4°C overnight with mild agitation. Magnetic beads were pre-swollen in IP buffer, then used to immunocapture antibodies from lysates for one hour at RT. Antibody-bead conjugates were pelleted from each sample using the DynaMag™-2 Magnet. Pellets were gently washed three times in IP buffer. To elute protein, pellets were incubated in 1X reducing sample buffer for 10 min at RT. Samples were pelleted, and the supernatant was collected from each sample and stored. For western blot analysis, samples were loaded onto a 4%-12% gradient gel for 10% SDS-PAGE, transferred to PVDF membrane, and blocked with 5% BSA in TSBT. Proteins were detected by specific antibodies and corresponding secondary antibodies (listed below). Signals were visualized by HRP-derived chemiluminescence using a Bio-Rad imager. Protein levels were quantified using Image Lab software from Bio-Rad Technologies.

Mitochondrial respiration using Seahorse XF96 Analyzer. The oxygen consumption rate (OCR) of iPSC-CMs and EHTs was measured with an XF96 Flux Analyzer (Seahorse Bioscience, Agilent). The Seahorse utility plate was hydrated with calibration buffer and incubated in a non-CO_2 incubator overnight at 37 °C prior to experiments. For iPSC-CMs, cells were seeded on Matrigel-coated microplates 2 days prior to the experiment. On the day of the experiment, culture media was replaced with assay media (DMEM-based media supplemented with 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate) and incubated for 30 mins in a non-CO_2 incubator at 37 °C. The microplate was calibrated in the Seahorse Analyzer for 20 mins then a baseline OCR was measured. Sequential injections of inhibitors (final concentration 2.5 µM oligomycin, 1 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone and 1 µM antimycin A + 1 µM rotenone) were added into each well, and changes in OCR were measured. For EHTs, tissues were directly loaded onto uncoated microplates containing assay media (same as above). The microplate was centrifuged at RT for 5 min at 300 x g to orient EHTs onto the bottom of each well, then incubated for 30 mins in a non-CO_2 incubator at 37 °C. The microplate was calibrated in the Seahorse Analyzer for 20 mins then a baseline OCR was measured. Sequential injections of inhibitors (final concentration 10 µM dobutamine, and 2 µM antimycin A + 2 µM rotenone) were added into each well, and changes in OCR were measured.

EHT force measurement. The isometric force test apparatus consists of an isometric force transducer (Model 724480, Harvard Apparatus, Holliston, MA) with two selectable ranges, 0–0.005 N and 0–0.05 N, and accuracy of ±1%; a micrometer positioning system (Model 47700, World Precision Instruments (WPI), Sarasota, FL); and a tissue holder with two 5 mm flexible platinum stimulation electrodes (Model 47050, WPI, Sarasota, FL) positioned next to two L-shaped stainless-steel holders. During tissue stimulation, an EHT was immersed in Tyrode's solution (Millipore-Sigma, T2397). The media was constantly circulated via a variable flow mini-pump (Model 57951-016, VWR, West Chester, PA). The media bath was continuously bubbled with 95% O_2 and 5% CO_2 and maintained at 37 °C. An electrical field stimulator (Model SIU-102, Warner Instruments, Hamden, CT) was used to pace tissue by applying biphasic pulses (1.0-2.0 Hz, 10 ms pulse width, 20 V peak-peak) across the titanium electrodes spaced approximately 1-2 cm apart across the EHT. Analog voltage signals from the force transducer were transferred to a USB data acquisition device at a 25 Hz sampling rate (USB-6009, National Instruments (NI), Austin, TX) and displayed, analyzed, and recorded with LabView 8.2 software (NI, Austin, TX). Each EHT (n = 3) was mounted to the force transducer using vascular clamps at either end of the EHT and were allowed to equilibrate with oxygenated Tyrode's for 30 min at 37 °C. The EHT was stretched 125 µm every 10 seconds with no electrical stimulation (spontaneous contractions) or 1 Hz or 2 Hz field stimulation. In response to stretch, the EHT displays an immediate increase in force (also known as systolic force, with a parallel increase in diastolic force) then relaxes until it reaches a new steady state. The time interval is long enough to allow for a new steady state to develop. The linear relationship between stress and strain is the Young's modulus. Maximum active force (also known as twitch force, which is the difference between systolic force and diastolic force) is calculated by the average of the 10 highest active forces. Similarly, the maximum active stress is calculated by computing the ratio of the maximum active force to starting EHT cross-sectional area.