Expanded Materials and Methods

Animal

Male and female age matched C57BL/6J WT, OCT3KO mice (2-4 months) and neonatal, β_1ARKO, β_2ARKO and $\beta_1\beta_2$ARKO were used in this study. Male New Zealand rabbits (3-6 months old) and CD® IGS rats (3-5 months) were also used. All the experiments including cardiomyocyte isolation, mouse echocardiogram, *in vivo* injection, and tissue harvesting were approved by the Institutional Animal Care and Use Committees (IACUC, Protocol 20234 and 20597) of University of California, Davis. OCT3/Slc22a3-KO mice were gifted by Dr. Paul J Gasser from the Department of Biomedical Sciences, Marquette University. Mice genotypes were confirmed by using PCR as previously described. [19]

Reagents

(-)-isoproterenol hydrochloride (I6540), epinephrine bitartrate salt (E4375), dobutamine hydrochloride (D0676), and L-(-)-norepinephrine (+)-bitartrate salt monohydrate (A9512) from Sigma-Aldrich (I6504, St. Louis, MO) were freshly prepared each day in water at 10 mmol/L and further diluted to working concentration before use. (±)-sotalol hydrochloride (S0278), (±)-propranolol hydrochloride (P0884), carvedilol (C3993), atenolol (A7655), ICI118551(I127), CGP20712A(C231) and corticosterone (27840) were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescence dye BODIPY-tagged β antagonist (±)-propranolol (FL-prop) was purchased from Hello Bio (HB7820, Princeton, NJ).

Echocardiography

Male and female WT and OCT3KO mice were subjected to echocardiography recording using a Vevo 2100 Imaging System with a 22-55 MHz MS550D linear probe (Visual Sonic, Canada). [33, 35] Mice were anaesthetized with isoflurane while maintaining stable body temperature and respiratory rate, and electrocardiogram (ECG) was monitored. Cardiac function was recorded with echocardiogram at baselines, 3 minutes after intraperitoneal injection of epinephrine (EPI, 10μg/kg, i.p) or isoproterenol (ISO, 10μg/kg, i.p.) in a blind fashion. Additionally, administration of norepinephrine (NE, 1-1000μg/kg, i.p.) minimally promoted increases in cardiac contractility, most likely due to systemic baroreflex responses induced vessel constriction. [34]

Heart Harvesting and Plasma Collection

Body weight of each mouse was recorded before being euthanized. Mice were anesthetized with 4% isoflurane in oxygen. After thoracotomy, the heart was quickly excised and collected for biochemistry and cAMP assay. Whole blood was collected in tubes containing heparin as an anti-coagulant, and centrifuged at 1000 x g, 4ºC for 30 minutes. The supernatant was collected and stored at -80ºC.

NE Measurement Assay

NE in plasma and heart tissues was determined using norepinephrine ELISA kit (KA1891, Abnova) according to the manufacturer's instructions. Heart tissues were homogenized by a glass homogenizer in cold PBS (9 mL PBS for 1g of tissue. Protease inhibitor PMSF was added into the PBS). Heart tissues, plasma and standards were added to the extraction plate. The samples were mixed with 250 µL of water, 50 µL of assay buffer, and 50 µL of extraction buffer, and incubated by shaking for 30 minutes at room temperature. After removing the solution, the plates were washed with wash buffer. The plates were then incubated with 150 µL of acylation buffer and 25 µL of acylation reagent for 15 minutes at RT. The plates were washed before incubation with 150 µL of hydrochloric acid for 10 minutes at RT to extract NE. After extraction, 20 µL of the extracted samples were incubated in a sample plate with 25 µL of the enzyme solution for 30 minutes at RT. The samples were then mixed with 50 µL of the noradrenaline antiserum and incubated at room temperature for 2 hours at RT. The solutions were discarded before the plates were rinsed for 3 times with wash buffer. The samples were then mixed with 100 µL of the conjugated antibody enzyme and incubated for 30 minutes at RT before rinsing 3 times with wash buffer. An additional 100 µL of the substrate was added into each well and incubated for 25 minutes at RT. The reaction was stopped with addition of 100 µL of the stop solution. The absorbance of each samples and standards were read at 450nm with a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). The standard curve was plotted according to the manufacturer's protocol. The NE concentrations of the samples were interpolated from the standard curve.

cAMP Assay

Cyclic AMP concentration in mice hearts was measured using the cAMP-Glo™ Assay kit (V1501, Promega, Madison, WI) as previously reported according to manufacturer's instruction. [35] Briefly,

100 mg heart tissues were homogenized in 100 µL sample preparation buffer (50 mmol/L Tris-HCl, 30 mmol/L $MgCl_2$, IBMX 500 µmol/L, Ro20-1724 100 µmol/L, pH to 7.5). 40 µL of sample were added to a 96-well plate together with 10 µL of 5x cAMP detection solution, mixed and incubated for 20 minutes at room temperature. An additional 50 µL of 2x Kinase Glo solution was added to the mix and incubated 10 minutes at room temperature ºC. The color intensity was detected with a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Isolation of PM and Internal Membrane Compartment Fractions (non-PM) of Mouse Hearts

Mouse heart tissues were homogenized in osmotic lysis buffer (25 mmol/L Tris-HCl, pH 7.4; 5 mmol/L EDTA, pH 8.0; 1 mmol/L PMSF; 2 µg/mL aprotinin; and 2 µg/mL leupeptin). Cell debris and nuclei were removed by centrifugation at 3000 rpm for 5 minutes at 4ºC. The supernatant was then centrifuged at 18,300 rpm for 30 minutes at 4ºC using a Fiberlite™ F50L-24 fixed-angle rotor (Thermo Fisher Scientific, Waltham, MA). Pellets representing the PM fraction were resuspended in Triton lysis buffer (25 mmol/L Hepes, pH 7.4; 5 mmol/L EDTA; 150 mmol/L NaCl; 0.5 % Triton X-100; and protease inhibitors containing 2 mmol/L Na_3VO_4, 1 mmol/L PMSF, 10 mmol/L NaF, 10 µg/mL Aprotinin, 5 mmol/L Bestatin, 10 µg/mL Leupeptin, and 2 µg/mL Pepstain A) for Western blotting. The supernatant was further centrifuged at 50,000 rpm for 1 hour at 4ºC; the pellets obtained in this step contained internal membrane compartment fractions and were resuspended in the Triton lysis buffer mentioned above for Western blotting. The protein concentration was determined using the BCA Assay kit (23225, Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (50 µg) were resolved on an SDS-PAGE gel and detected with anti-β_1AR antibody (V-19, rabbit polyclonal IgG, SC-568, Santa Cruz Biotechnology, Dallas, TX), anti-SERCA2 Antibody (clone IID8, mouse monoclonal IgG, MAB2636, Millipore Sigma, Burlington, MA), or anti-insulin receptor β antibody (C-19, rabbit polyclonal IgG, SC-711, Santa Cruz Biotechnology, Dallas, TX). All primary antibodies were revealed with IRDye 800 CW Goat anti-Rabbit IgG secondary antibody (1:5000, #926-32211, Licor, NE) or IRDye 800 CW Goat anti-Mouse IgG secondary antibody (1:5000, #926-32210, Licor, NE) using the Odyssey detection system (Li-cor Biosciences, Lincoln, NE). The optical density of the bands was analyzed with NIH Image J software (https://imagej.nih.gov/ij/).

Quantitative Radioligand Binding Assay

Quantitative radioligand binding assays were conducted as previously described. [17, 35]. 20 μg of protein from each PM and non-PM fraction above was resuspended in 500 μL ligand binding buffer (75 mmol/L Tris-HCl, pH 7.4; 2 mmol/L EDTA, pH 8.0; and 12.5 mmol/L $MgCl_2$). Tubes containing 150 pmol/L of the nonselective βAR antagonist (^{125}I)-cyanopindolol (^{125}I-CYP, Perkin Elmer, MA, USA) were used to determine the total βAR binding; tubes containing 150 pmol/L ^{125}I-CYP and 10 μmol/L alprenolol were used to determine non-specific binding. Assays were performed in triplicate. After vigorously shaking for 90 minutes at room temperature, the reaction was terminated by harvesting onto Whatman GF/B glass fiber filters (Brandel Inc., MD). The filters were then washed 3 times with ligand binding buffer. Bound radioactivity was measured using a liquid scintillation counter (Tri Carb 2500 TR, Perkin Elmer, Boston, MA). Specific binding was calculated as the difference between total and non-specific binding and expressed as fmol/mg protein.

Cardiomyocyte Isolation, and Contractility and Calcium Transient Assay

Rabbits were sacrificed under general anesthesia (induction with propofol 2 mg/kg followed by 2-5 % isoflurane in 100 % oxygen). After thoracotomy, the heart was quickly excised and rinsed in cold Ca^{2+}-free minimum essential medium (MEM). The left coronary ostium was cannulated using a 4F catheter. After perfusion was established, the right atrium and non-perfused right ventricle free wall were removed, and the catheter was secured with a purse-string suture. [38] The remainder of the isolation procedure was executed as previously described. [18]

Adult ventricular cardiomyocytes (AVMs) from mice and rats were isolated, and myocyte contractility was measured as previously described. [17, 36] Briefly, mice or rats were anesthetized with 3-5% isoflurane, and hearts were quickly excised and mounted on a Langendorff perfusion apparatus. Hearts were then perfused with a collagenase and protease solution (0.5 mg/mL collagenase and 0.1 mg/mL protease for mouse; 1.0 mg/mL collagenase and 0.2 mg/mL protease for rat). Freshly isolated AVMs were loaded with Fluo-4 AM (5 μM, Molecular Probes, Eugene, OR) before being placed in beating buffer (NaCl 120 mmol/L, KCl 5.4 mmol/L, NaH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, HEPES 20 mmol/L, Glucose 5.5 mmol/L, $CaCl_2$ 1mmol/L, pH 7.1). AVMs were pretreated with or without β-blocker/inhibitors for 5 minutes. AVMs were paced at 1 Hz with voltage of 50 V using the SD9 stimulator (Grass Technology, Warwick, RI). Calcium

transients and cell length at basal condition and after stimulation of βAR-agonist were recorded on an inverted microscope (Zeiss AX10) at 20x magnification using MetaMorph® software (Molecular Devices, Sunnyvale, CA). Percent sarcomere shortening (% $\Delta L/L_0$) was calculated using MetaMorph® software. The calcium transient analysis was performed using Image J software and custom-written routines in IDL (Interactive Data Language, ITT) as previously described. [33, 35] We tested multiple cells for each animal. The average value of cells from each animal was plotted as one data point per animal in the figures.

Localization of βAR in Mouse AVMs by Fluorescent Ligand Binding and Confocal Microscopy

Freshly isolated mouse AVMs were cultured on laminin-coated coverslips (24 hours) before100 nmol/L fluorescence-labelled propranolol (FL-Prop) was added to the culture medium. In the competition experiments, different beta-adrenergic receptor blockers/inhibitors or agonists was added 15 minutes prior to the addition of FL-Prop. After incubation with FL-Prop for 15 minutes, cells were washed with culture medium for 10 minutes twice at room temperature and then fixed with 4% paraformaldehyde at room temperature for 15 minutes. The coverslips were then mounted on slides with mounting medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA). Confocal imaging was carried out on Zeiss LSM 700 Axio Observer confocal microscope (Oberkochen, Germany) using a Zeiss Plan-Apochromat 63×/1.4 oil objective.

Cell Culture and Adenovirus Infection

To culture and infect AVMs, freshly isolated cells were plated on mouse laminin (Life Technologies, Grand Island, NY)-coated dishes with different culture media for different species. For rabbit AVM culture, PC-1 (Lonza Walkersville, MD, USA) supplemented with 1% Penicillin-Streptomycin-Glutamine (PSG, pH 7.4) was used. For rat AVMs, minimum essential medium (MEM, M1018, Sigma-Aldrich, St. Louis, MO) with 10 % 2,3-Butanedione monoxime (BDM, B0753, Sigma-Aldrich, St. Louis, MO) was used. For mouse AVMs, MEM (MEM, M1018, Sigma-Aldrich, St. Louis, MO) supplemented with 1% PSG, 4 mmol/L $NaHCO_3$, 0.2% BSA, 10 mmol/L HEPES, 6.25 μmol/L blebbistatin, and 10 % FBS was used as previously reported. [36] For mouse neonatal myocytes, DMEM (MEM, M1018, Sigma-Aldrich, St. Louis, MO) supplemented with 1% PSG, 4 mmol/L $NaHCO_3$, 0.2% BSA, 10 mmol/L HEPES, and 10 % FBS was used as

previously reported [37]. Recombinant adenoviruses were generated with pAdeasy system (Qbiogene, Carlsbad, CA) as previously described [32]. AVMs and neonatal myocytes were infected with adenovirus at MOI of 100 for 18 (for Imaging experiments) or 36 (for Fret experiments) hours.

Super-resolution imaging by multicolor SP8 Falcon LIGHTNING

Isolated mouse cardiomyocytes were cultured on laminin-coated coverslips (2 hours) before infection with β_1AR-overexpressing adenovirus or FLAG-tagged β_2AR-overexpressing adenovirus respectively. 18 hours after infection, cells were fixed in 4% paraformaldehyde (15 minutes) followed by washes with PBS (3 times, 15 minutes). Cells were then permeabilized/blocked with 0.3% Triton-X, 2% goat serum in PBS (30 minutes). β_1AR-overexpressed cells were incubated with primary antibodies [Rabbit anti-β_1AR (1:200 dilution, SC-568, SCBT, CA), mouse anti-SERCA2 (1:200, MAB2636, Millipore, CA) or mouse anti-RyR2 (1:200, MA3-925, Fisher Thermo, CA). PBS washing (3 times, 15 minutes) was applied before incubation of diluted secondary antibody, Goat-anti-Rabbit Alexa Fluor 594 (1:1000, A-11012, Invitrogen, USA) and Goat-anti-Mouse Alexa Fluor 488 (1:1000, A-11001, Invitrogen, USA). β_2AR-overexpressed cells were incubated with primary antibodies [mouse anti-SERCA2 (1:200) or mouse anti-RyR2 (1:200)]. PBS washing was applied before incubation of secondary antibody, Goat-anti-Mouse IgG2b Alexa Flour 546 (1:1000, A-21143, Invitrogen, USA) and Goat-anti-Mouse IgG1 Alexa Fluor 488 (1:1000, A-21121, Invitrogen, USA). For imaging, coverslips were washed 3 times with PBS before mounting with VECTASHIELD Antifade Mounting Medium (1:1000, H-1000, Vector Labs). Super-resolution images were obtained using the LIGHTNING mode in TCS SP8 Falcon confocal microscope (Leica). 63x/1.3 oil immersion lens, and a zoom of 5.0 (pixel size of 40 nm) were used here. The SP8 LIGHTNING system enables acquisition with a minimum of averaging and enhanced signal-to-noise ratio. Randomized images of 10-20 different cells from 5 independent experiments were used to calculate the Pearson's correlation coefficient by JACoP plug-in in the ImageJ software.

Fluorescent Resonance Energy Transfer (FRET) Assay

In the current study, we applied a series of FRET-based AKAR biosensors to detect localized PKA activities. A-kinase activity reporter (AKAR) is a recombinant protein composed of a

phosphoamino acid-binding domain and a PKA-specific substrate which are in between a cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). When phosphorylated by PKA, conformational changes of AKAR lead to an increase in FRET between CFP and YFP. The PM-AKAR3, MF-AKAR3 and SR-AKAR3 used in the current study have been reported previously. [17, 36] Briefly, the regular PKA activity biosensor, AKAR3, was fused with the sequence KKKKKKSKTKCVIM derived from K-Ras kinase to generate general PM-targeted AKAR3, termed PM-AKAR3.[12] AKAR3 was fused with the helical transmembrane domain PQQARQKLQNLFINFCLILICLLLICIIVMLL of PLB to generate an SR membrane-anchored AKAR3, termed SR-AKAR3.[9] AKAR3 was fused with TnT to anchor onto troponin complex on the myofilament (MF-AKAR3, [18]). The localized AKAR3 cDNA was subcloned into a shuttle vector, pAdTrack-CMV, to generate a recombinant adenovirus according to the manufacturer's instructions (Quantum Biotechnologies, Republic of South Africa). The recombinant adenovirus was then generated with the pAdEasy system (Qbiogene, Carlsbad, CA). FRET recording on AVMs infected with PM-AKAR3, MF-AKAR3 or SR-AKAR3 biosensors was performed as previously described. [17, 36] Images were acquired on a Leica DMI 3000B microscope with a 40×/1.3 NA oil-immersion objective lens (Leica Biosystems, Buffalo Grove, IL). The charge-coupled device camera was controlled by Metafluor software (Molecular Devices, Sunnyvale, CA). FRET image acquisition settings and FRET intensity measurements were carried out as previously described.[5] Briefly, donor CFP was excited at 430-455 nm and emission fluorescence was collected with two filters (475DF40 for cyan and 535DF25 for yellow) every 30 seconds, with an exposure time of 200 ms. Fluorescence emission intensity at 545nM (YFP) and 480 nM (CFP) were subjected to background subtraction, YFP/CFP ratio was analyzed as F/F0, in which F is the ratio at time t and F0 is the baseline ratio. We tested multiple cells for each animal. The average value of cells from each animal was plotted as one data point per animal in the figures. In dose curves, the YFP/CFP ratios were normalized against the maximal changes induced by ISO in the absence of β-blocker or by IBMX plus forskolin.

Western Blotting

Left ventricular tissue and isolated AVMs were used in this study. Left ventricular extracts were prepared as previously described. [33] Left ventricular tissues were lysed using FastPrep-24 lysing matrix D beads (MP Biomedicals) in lysis buffer (25 mmol/L Hepes, pH 7.4; 5 mmol/L EDTA;

150 mmol/L NaCl; 0.5 % Triton X-100; and protease inhibitors containing 2 mmol/L Na_3VO_4, 1 mmol/L PMSF, 10 mmol/L NaF, 10 μg/mL Aprotinin, 5 mmol/L Bestatin, 10 μg/mL Leupeptin, and 2 μg/mL Pepstain A). Freshly isolated AVMs were allowed to adhere to laminin-coated 6-well plate (2 hours) before treating with or without different β-blockers for 5 minutes and incubated in a 37°C incubator, followed by stimulation with ISO (100 nmol/L) or NE (100 nmol/L) for 5 minutes. AVMs were lysed using lysis buffer (25 mmol/L Hepes, pH 7.4; 5 mmol/L EDTA; 150 mmol/L NaCl; 0.5 % Triton X-100; and protease inhibitors containing 2 mmol/L Na_3VO_4, 1 mmol/L PMSF, 10 mmol/L NaF, 10 μg/mL Aprotinin, 5 mmol/L Bestatin, 10 μg/mL Leupeptin, and 2 μg/mL Pepstain A), then centrifuged at 15,000 x g (15 minutes). Equal amounts of protein (15 μg for PLB detection and 50 μg for other detections) were resolved on an SDS-PAGE gel and detected with anti-phospho-PLB serine 16 (1:1000; A010-12, Badrilla, Leeds, United Kingdom), anti-PLB (1:1000; A010-14, Badrilla, Leeds, United Kingdom), anti-SERCA2 (1:500; MAB2636, Millipore), anti-RyR2 (1:1000; MA3-925, Thermo Fisher Scientific), anti-phosphor-RyR2808 (1:500, ab59225, Abcam), anti-troponin (1:500, #4002, CST), anti-phosphor-troponin23/24 (1:1000, #4004, CST), anti-β_1AR (1:500, sc-568, Santa Cruz), anti-phospho-PLM63 (1:500, gifted from Dr. Donald Bers, University of California, Davis), anti-PLM (1:500, gifted from Dr. Donald Bers, University of California, Davis), anti-MyBPC, anti-phospho-MyBPC serine273, anti-phospho-MyBPC serine 282, anti-phospho-MyBPC serine 302 (1:1000, gifted from Dr. Sakthivel Sadayappan, University of Cincinnati), anti-LTCC (1:1000, custom antibody from Abmart, NJ, USA), anti-phospho-LTCC serine 1928 (1:1000, custom antibody Abmart, NJ, USA). All primary antibodies were revealed with IRDye 800 CW Goat anti-Rabbit IgG secondary antibody (1:5000, #926-32211, Licor, NE) or IRDye 800 CW Goat anti-Mouse IgG secondary antibody (1:5000, #926-32210, Licor, NE) using the Bio-Rad ChemiDoc MP Imagers (Bio-Rad Laboratories, Hercules, CA). The optical density of the bands was analyzed with NIH Image J software (https://imagej.nih.gov/ij/). The arbitrary units (A.U.) of WB was defined as the ratio of intensity of protein of interest over the intensity of a reference protein or as the intensity of phosphorylated proteins over intensity of total proteins as indicated.

Co-immunoprecipitation (Co-IP)

Heart tissues were homogenized by 2 mL of lysis buffer above and centrifuged at 13,200 rpm for 30 minutes at 4ºC. The supernatant was transferred to a fresh centrifuge tube and precleared by

adding 10 μL of Protein A-Sepharose beads (GE17-0780-01, Millipore, Burlington, MA) together with 1.0 μg of control IgG antibody (sc-66931, SCBT, CA) for 1 hour at 4 ºC. The precleared supernatant (1 mL) was incubated with 30 μL of Protein A-Sepharose beads and 2 μg of anti-β_1AR (sc-568, SCBT, CA) or anti-IgG antibody (sc-66931, SCBT, CA) at 4 ºC overnight. The beads were then rinsed with lysis buffer for three time. The bead-bound proteins were solubilized with 30 μL 2x SDS sample loading buffer (#161-0747, Bio-Rad Laboratories, Hercules, CA) and subject to electrophoresis to probe the target proteins (ADRB1, RyR2, SERCA2, PLB).

Single molecule Pulldown (SiMPull) assay

Heart lysates from above were diluted and used for single-molecule pulldown (SiMPull) analysis.[20] β_1AR proteins from heart lysate were pulled down on SiMPull slides by 10 nmol/L of biotinylated goat anti-rabbit IgG together with 5 nmol/L anti-β_1AR antibodies as indicated. Immobilized proteins were visualized by a prism-type total internal reflection fluorescence (TIRF) microscope. For the β_1AR and SERCA2 association, the bound SERCA2 was visualized with Alexa-488-conjugated mouse anti-SERCA2 antibody (1 nmol/L; sc-376235 AF488, SCBT, CA). Mean spot count per image and standard deviation were calculated from images taken from 10–20 different regions using scripts written in MATLAB as previously described. [20]

Proximity Ligation Assay (PLA)

In situ PLAs were performed according to the manufacturer's procedure to detect endogenous protein-protein interactions in WT mouse AVMs using the Duolink® kit (DUO92101, Sigma-Aldrich, St. Louis, MO). This protocol allows for the detection of two target proteins that are in close proximity (< 40 nm, https://www.sigmaaldrich.com/technical-documents/protocols/biology/duolink-troubleshooting-guide.html). Briefly, freshly isolated WT mouse AVMs were fixed, permeabilized, and blocked. Cells were then incubated with primary antibodies against β_1AR (1:500, rabbit IgG, SC-568, SCBT, Dallas, TX) and SERCA2 (1:500, mouse IgG, MAB2636, Millipore, Burlington, MA), or normal mouse IgG (1:500, mouse IgG, SC-2025, SCBT, Dallas, TX), or Junctophilin-2 (1: 500, mouse IgG, SC-377086, SCBT, Dallas, TX) followed by the appropriate secondary antibodies containing PLA probes. After polymerase reactions, cells were visualized by the Zeiss LSM 700 Axio Observer confocal microscope (Oberkochen, Germany) using a Zeiss Plan-Apochromat 20 x objective. Confocal images were

collected using 405 nm excitation for DAPI and 555 nm excitation for red PLA positive signals. Z-stack imaging was processed and analyzed with Image J (https://imagej.nih.gov/ij/) and Zen software from Zeiss (Oberkochen, Germany).

Data Analysis

Pooled data are represented as the mean ± SEM. Group sizes were determined by an a priori power analysis for a two-tailed, two-sample t-test with an α of 0.05 and power of 0.8, in order to detect a 10% difference signal at the endpoint. Animal were grouped with no blinding but randomized during the experiments. Fully blinded analysis was performed by different persons carrying out the experiments and analysis, respectively. Both male and female animals were used in experiments. No samples or animals were excluded from analysis. Representative figures/images reflected the average level of each experiment. Normality of the data was assessed using Shapiro-Wilk test in GraphPad Prism 8 with significance at alpha = 0.05 (GraphPad Inc., San Diego, CA)., If N<=5, the data were assumed normality due to the central limit theorem. Comparisons between two groups were performed by paired (echocardiograph) and unpair (for all others) two tailed t-test, between more than two groups by one-way ANOVA or two-way ANOVA followed by Tukey's post-hoc using Prism 8.0 software (GraphPad). For data without normal distribution in Online Figure XIIIB, we used non-parametric Mann-Whitney test to compare two unpaired groups. All bar graphs are shown as mean ± standard error of the mean (SEM). Statistical significance analysis was performed using GraphPad Prism 8 (GraphPad Inc., San Diego, CA). A value of two-tailed P <0.05 was considered statistically significant. We did not perform multiple testing across the entire body of the work.

Wang et al. Online Figure Legends

Online Figure I. Characterization of endogenous β_1AR in isolated AVMs. (A) The specificity of anti-β_1AR antibody was verified in WT and β_1AR-KO mouse heart tissue lysates by western blot. (B) WT AVMs were stained with FL-Prop (100 nmol/L) in the presence of different concentration of Prop (0.1-10 μmol/L). Scale bar, 10 mm. (C, D) WT, β_1AR-KO, β_2AR-KO, $\beta_1\beta_2$AR-KO AVMs were stained with FL-Prop (100 nmol/L). Red: FL-Prop; blue: DAPI. Scale bar = 10 μm.

Online Figure II. Colocalization of β_1AR and SERCA2, RyR2 in mouse AVMs. (A) Representative confocal images of individual mouse AVMs displaying staining against β_1AR, SERCA2, RyR2 as indicated. β_1AR were expressed by infection with recombinant adenovirus. The white boxes highlight the areas showing in Figure 1C and D. Scale bar = 10 μm. (B) Exemplary fluorescence images of PLA labeled with antibodies against β_1AR and IgG, β_1AR and SERCA2, and β_1AR and RyR2 in AVMs. Positive PLA signal (red), DAPI (blue). The white boxes highlight the areas showing in Figure 1F. Scale bar = 10 μm. (C) Exemplary fluorescence images and quantification of PLA signal after labeling with antibodies against β_1AR and IgG, β_1AR and Junctophilin 2 (JP2) in AVMs. Positive PLA signal (red), DAPI (blue). Scale bar = 5 μm. Data are shown as mean ± S.E.M; AVMs (in the parenthesis) and animal numbers are indicated in the panels. P value was obtained by student *t*-test.

Online Figure III. Co-staining of β_2AR and SERCA2, RyR2 in mouse AVMs. (A and B) Representative confocal images of individual mouse AVMs displaying staining against β_2AR, SERCA2, RyR2 as indicated. β_2AR were expressed by infection with recombinant adenovirus. The white boxes highlight the areas showing in the low rows. Scale bar = 10 μm.

Online Figure IV. Subcellular PKA activity in WT and OCT3KO AVMs after adrenergic stimulation at different doses. (A-D) Time courses of FRET responses in isolated AVMs from WT mice expressing PM-AKAR3 and SR-AKAR3 before and after stimulation with ISO (100 nmol/L), NE (1000 nmol/L), EPI (1 μmol/L) or Dob (10 μmol/L). Data are shown as mean ± S.E.M from 5 mice; p values were obtained by two-way ANOVA analysis with Tukey's multiple comparison test (E) The time to peak responses were plotted after stimulation with different agonists in panel A-D. Data are shown as mean ± S.E.M; AVMs (in the parenthesis) and animal numbers are indicated in the panels. p values were obtained by one-way ANOVA analysis with Tukey's multiple comparison test. (F-I) Quantification of FRET responses in isolated AVMs from WT (black) and OCT3KO (red) mice expressing PM-AKAR3 and SR-AKAR3 after stimulation with NE (100 nmol/L) or ISO (100 nmol/L) after pretreatment with either β_1AR antagonist CGP20712a (300 nmol/L) or β_2AR antagonist ICI118551 (100 nmol/L). FRET was analyzed with F/F0 of YFP/CFP ratio; the maximal increases in YFP/CFP ratios were obtained. Data are shown as mean ± S.E.M; AVMs (in the parenthesis) and animal numbers are indicated in the panels. p values were obtained by two-way ANOVA analysis with Tukey's multiple comparison test.

Online Figure V. Subcellular PKA activity and PKA phosphorylation of substrates in WT and OCT3KO AVMs after adrenergic stimulation at different doses. (A) Quantification of FRET responses in isolated AVMs from WT (black) and OCT3KO (red) mice expressing MF-AKAR3 after stimulation with ISO (100 nmol/L) or NE (100 nmol/L). FRET was analyzed as F/F0 of YFP/CFP ratio; the maximal increases in YFP/CFP ratios were obtained. AVMs (in the parenthesis) and animal numbers are indicated

in the panels. Data are shown as mean ± S.E.M. P values were obtained by two-way ANOVA analysis with Tukey's multiple comparison test. (B) Western blot to show phosphorylation and total protein levels of TnI, MyBPC, RyR, and LTCC in WT and OCT3-KO AVMs after stimulation with ISO (100 nmol/L) or NE (100 nmol/L). (C-H) The western blots were quantified. A.U. is defined as the ratio of intensity of phosphorylated proteins over intensity of total proteins; and p values were obtained by two-way ANOVA analysis with Tukey's multiple comparison test. (I and J) The expression of β_1AR were detected in WT and OCT3-KO hearts. The western blots were quantified. A.U. is defined as the ratio of intensity of β_1AR over intensity of GAPDH; and p value was obtained by student *t*-test.

Online Figure VI. Sarcomere shortening, calcium transient and rate of calcium decay in WT and OCT3KO AVMs after beta-adrenergic stimulation. (A-C) Time courses of sarcomere shortening (SS), Ca^{2+} transient amplitudes, and Ca^{2+} transient decay tau in WT AVMS after stimulation with ISO (100 nmol/L) or NE (100 nmol/L) and Dob (10 μmol/L). Data are shown as mean ± S.E.M from 5 mice. (D-G) Ca^{2+} transients of AVMs were recorded with Fluo-4 dye and at 1Hz pacing. Representative traces of Ca^{2+} transient before (close line) and after (dash line) application of 100 nmol/L ISO or NE in WT and OCT3KO mouse AVMs. (H, I) Quantification of Ca^{2+} transient amplitude in WT and OCT3KO AVMs in response to 100 nmol/L ISO or NE. (J, K) Calculation of rate of Ca^{2+} decay (Tau) in WT and OCT3KO AVMs in response to 100 nmol/L ISO or NE. From panel H to K, data are shown as mean ± S.E.M; AVMs (in the parenthesis) and animal numbers are indicated in the panels. p values were obtained by two-way ANOVA with Tukey's multiple comparison test.

Online Figure VII. Sarcomere shortening, calcium transient and rate of calcium decay in WT and OCT3KO AVMs after subtype specific beta-adrenergic stimulation. (A-F) Sarcomere shortening (SS), Ca^{2+} transient amplitudes, and Ca^{2+} transient decay tau in WT and OCT3-KO AVMS after stimulation with ISO (100 nmol/L) or in the presence of β_1AR antagonist CGP20712a (300 nmol/L) or β_2AR antagonist ICI118551 (100 nmol/L). The maximal responses were plotted. Data are shown as mean ± S.E.M; AVMs (in the parenthesis) and animal numbers are indicated in the panels. P values are obtained by one-way ANOVA analysis with Tukey's multiple comparison test. (G-L) Sarcomere shortening (SS), Ca^{2+} transient amplitudes, and Ca^{2+} transient decay tau in WT and OCT3-KO AVMS after stimulation with NE (100 nmol/L) or in the presence of β_1AR antagonist CGP20712a (300 nmol/L) or β_2AR antagonist ICI118551 (100 nmol/L). The maximal responses were plotted. Data are shown as mean ± S.E.M; AVMs (in the parenthesis) and animal numbers are indicated in the panels. P values are obtained by one-way ANOVA analysis with Tukey's multiple comparison test.

Online Figure VIII. Effects of β-blockers and β-agonists on staining of endogenous β_1AR with fluorescence-labeled propranolol in mouse AVMs. (A) Schematics showing competitive detection of endogenous β_1AR by fluorescence-labeled propranolol **(**FL-Prop) in the absence and presence of β-blocker (non-permeant β-blocker SOTA and membrane permeant β-blocker PROP or β-agonist ISO or NE. (C and D) WT AVMs were stained with FL-Prop (100 nmol/L) after pretreatment with vehicle solution (CON), SOTA (25 μmol/L), PROP (10 μmol/L), ISO (10 μmol/L) or NE (10 μmol/L).

Online Figure IX. Activation of β_1ARs at the SR promotes PKA activity at the local domain in mouse, rat, and rabbit AVMs. (A-D) Mouse, rat, and rabbit AVMs expressing PM-AKAR3 or SR-AKAR3 FRET biosensors were stimulated with 100 nmol/L ISO with or without 5-minute pretreatment of SOTA (25

μmol/L) and PROP (10 μmol/L). FRET was analyzed with F/F0 of YFP/CFP ratio; the maximal increases in YFP/CFP ratios were obtained. Data are shown as mean ± S.E.M, AVMs (in the parenthesis) and animal numbers are indicated in the panels. P values were obtained by One-way ANOVA with Tukey's multiple comparison test. (E-F) Rat AVMs expressing PM-AKAR3 and SR-AKAR3 FRET biosensors were stimulated with 100 nmol/L ISO with or without 5-mintue pretreatment with Atenolol (ATEN, 25 μmol/L) and carvedilol (CARV, 10 μmol/L). Data are shown as mean ± S.E.M, AVMs (in the parenthesis) and animal numbers are indicated in the panels. P values were obtained by one-way ANOVA analysis with Tukey's multiple comparison test.

Online Figure X. Activation of β_1ARs at the SR promotes PKA activity at the local domain in mouse WT, β_1AR-KO, and β_2AR-KO neonatal myocytes. (A-F) WT, β_1AR-KO, and β_2AR-KO neonatal myocytes expressing PM-AKAR3 or SR-AKAR3 FRET biosensors were stimulated with 100 nmol/L ISO with or without 5-minute pretreatment of SOTA (25 μmol/L) and PROP (10 μmol/L). FRET was analyzed with F/F0 of YFP/CFP ratio; the maximal increases in YFP/CFP ratios were obtained. Data are shown as mean ± S.E.M; AVMs (in the parenthesis) and animal numbers are indicated in the panels. P value were obtained by One-way ANOVA with Tukey's multiple comparison test.

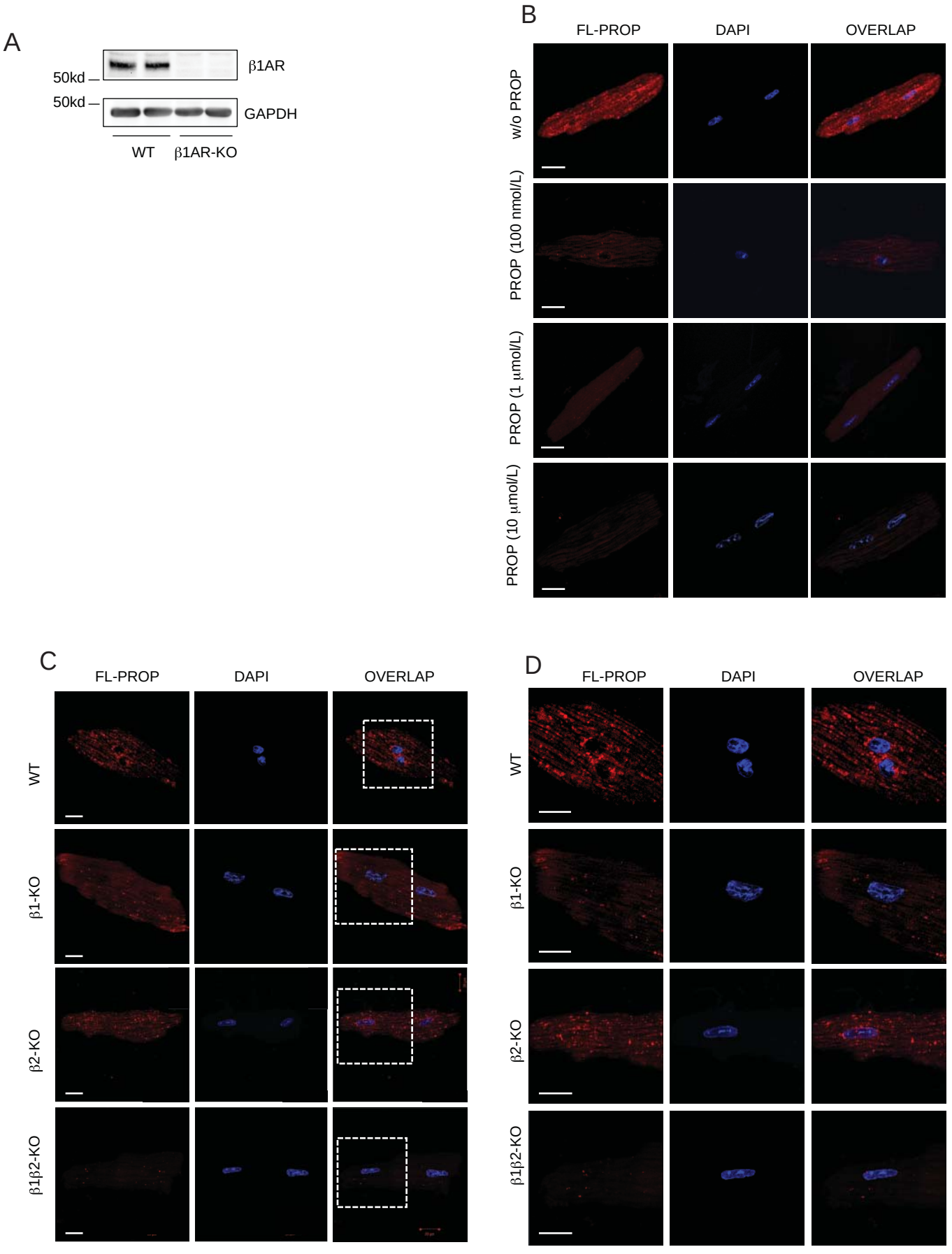
Online Figure XI. Activation of β_1AR in the SR promotes PLB phosphorylation in mouse, rat and rabbit AVMs. (A-D) mouse, rat, and rabbit AVMs were stimulated 100 nmol/L ISO for 5 minutes with or without pretreatment of membrane non-permeant β-blocker sotalol (SOTA, 25 μmol/L) or membrane permeant β-blocker (PROP, 1 μmol/L). Phosphorylation of PLB at serine 16 and of LTCC at serine 1928 was detected by western blot and quantified. Data are shown as mean ± S.E.M; animal numbers are indicated in the panels. A.U. (arbitrary unit) is defined as the ratio of intensity of phosphorylated proteins over intensity of total proteins. P values were obtained by one-way ANOVA analysis with Tukey's multiple comparison test.

Online Figure XII. Differential regulation of local β_1AR activation by membrane non-permeant β-blocker sotalol in WT and OCT3KO AVMs. (A, B) Schematics showing detection of PKA activity at the PM and SR with localized FRET biosensors after application of ISO or NE in the presence of sotalol (SOTA) pretreatment in WT and OCT3KO AVMs. In all experiments, AVMs were pretreated with 25 μmol/L SOTA. (C, D) WT and OCT3KO AVMs expressing with targeted PM-AKAR3 and SR-AKAR3 biosensors, representative curves show compartmentalized PKA FRET responses to 100 nmol/L ISO stimulation followed by addition of forskolin (FSK,10 μmol/L) and IBMX (100 μmol/L). FRET was analyzed with F/F0 of YFP/CFP ratio. The time courses of YFP/CFP ratios were normalized to the maximal increases induced by FSK and IBMX. (E, F) WT and OCT3KO AVMs expressing with targeted PM-AKAR3 and SR-AKAR3 biosensors, representative time courses show compartmentalized PKA FRET responses to 100 nmol/L NE stimulation followed by addition of FSK (10 μmol/L) and IBMX (100 μmol/L). The time courses of YFP/CFP ratios were normalized to the maximal increases induced by FSK and IBMX.

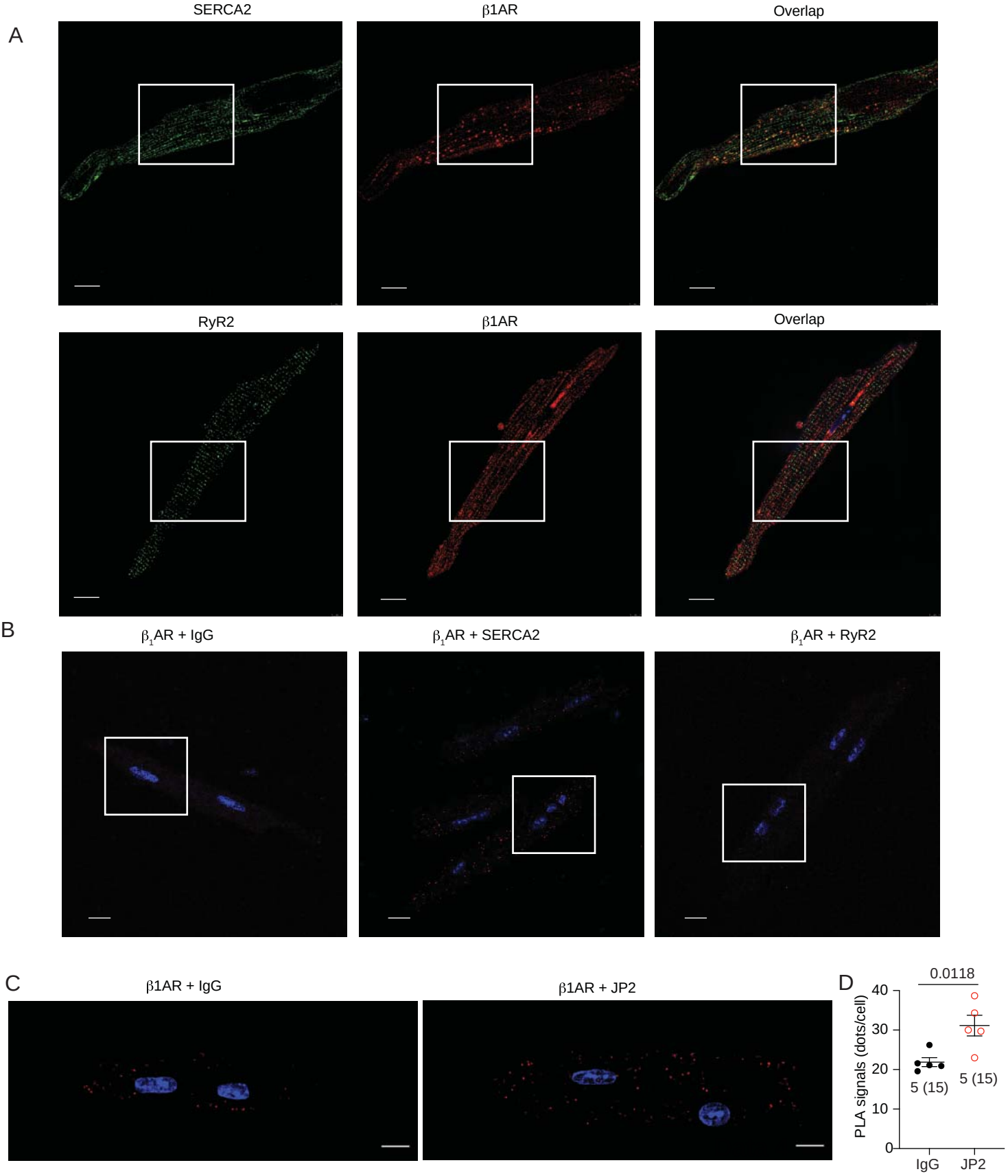
Online Figure XIII. Protein expression in WT and OCT3KO hearts. (A) Representative western blots show gene expression in WT and OCT3KO hearts. (B) Western blots were quantified in bar graphs. N = 6 mice. A.U. (arbitrary unit) is defined as the ratio of intensity of proteins over intensity of GAPDH or total

proteins as indicated. P values were obtained by student's *t*-test; for PLB/GAPDH and RyR2/GAPDH, p values were obtained by Mann Whitney test.

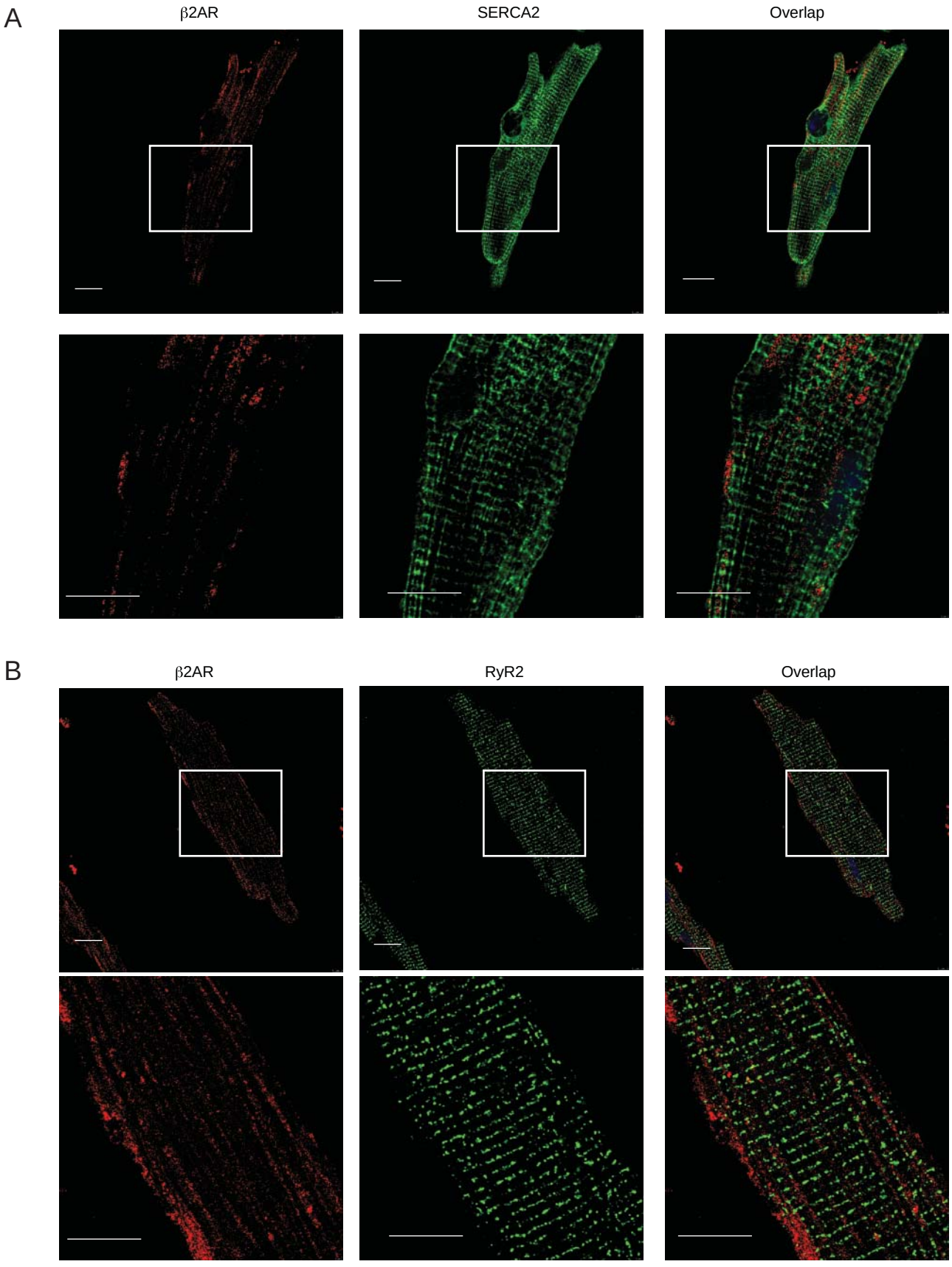
Online Figure I



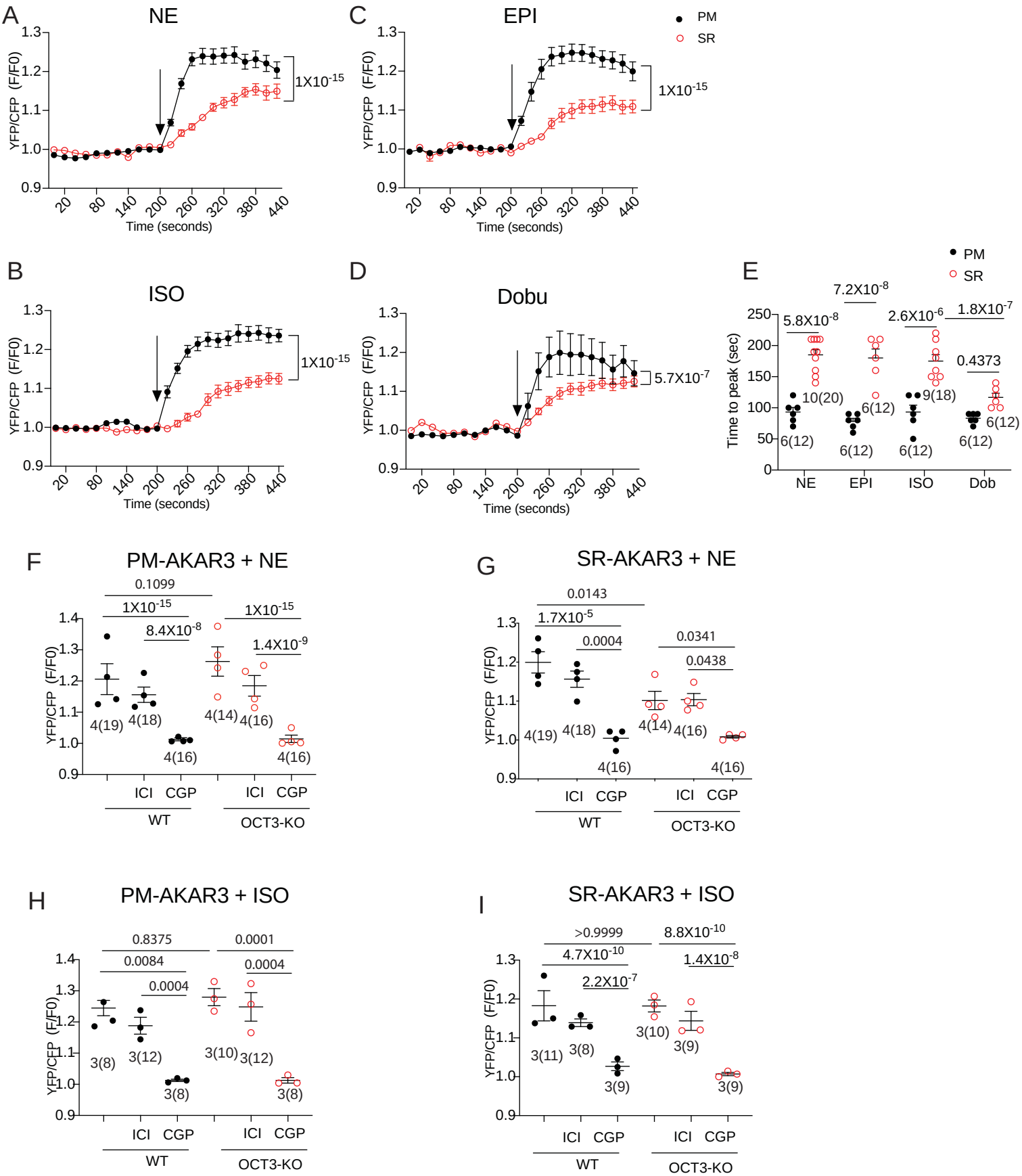
Online Figure II



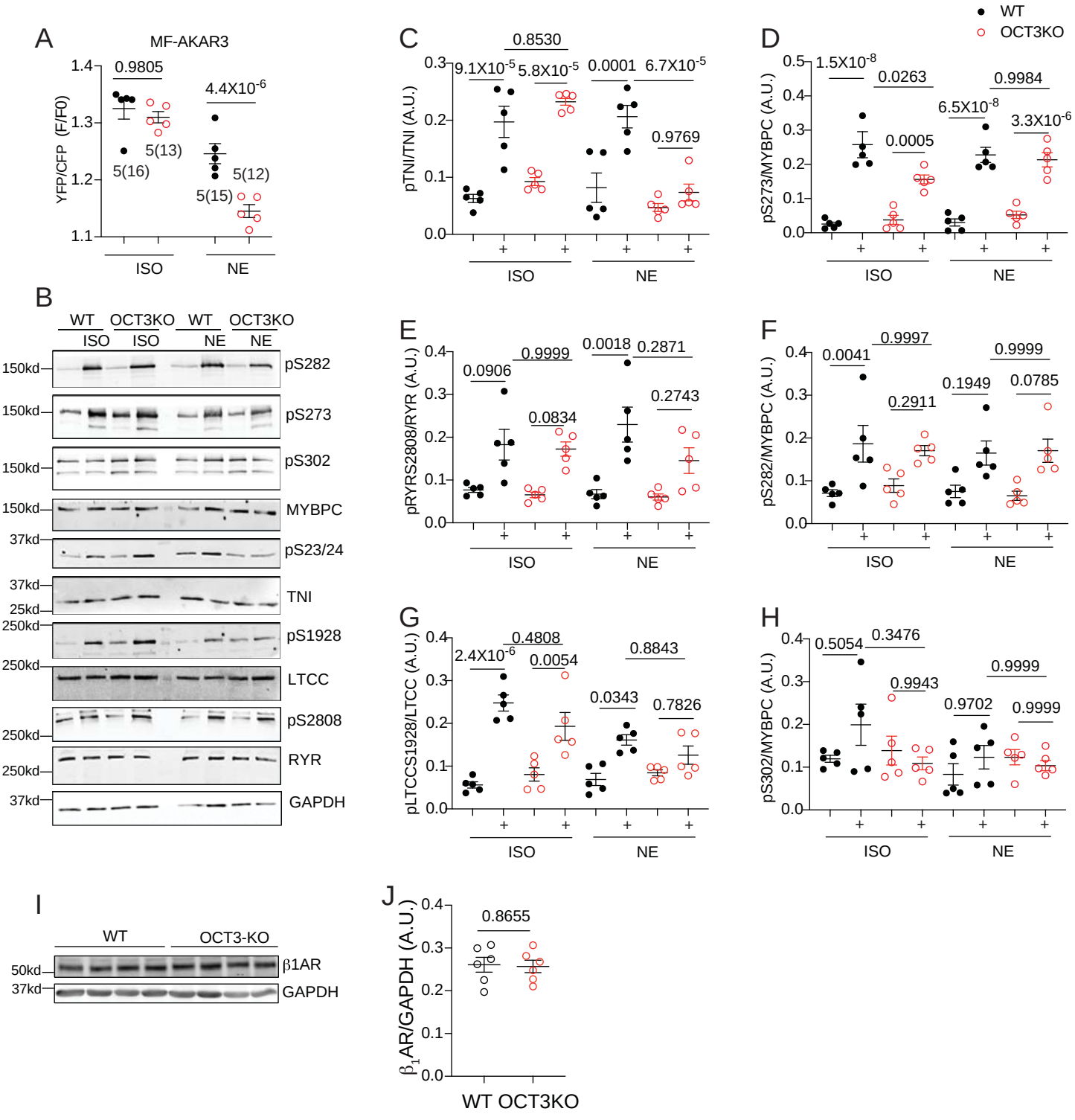
Online Figure III



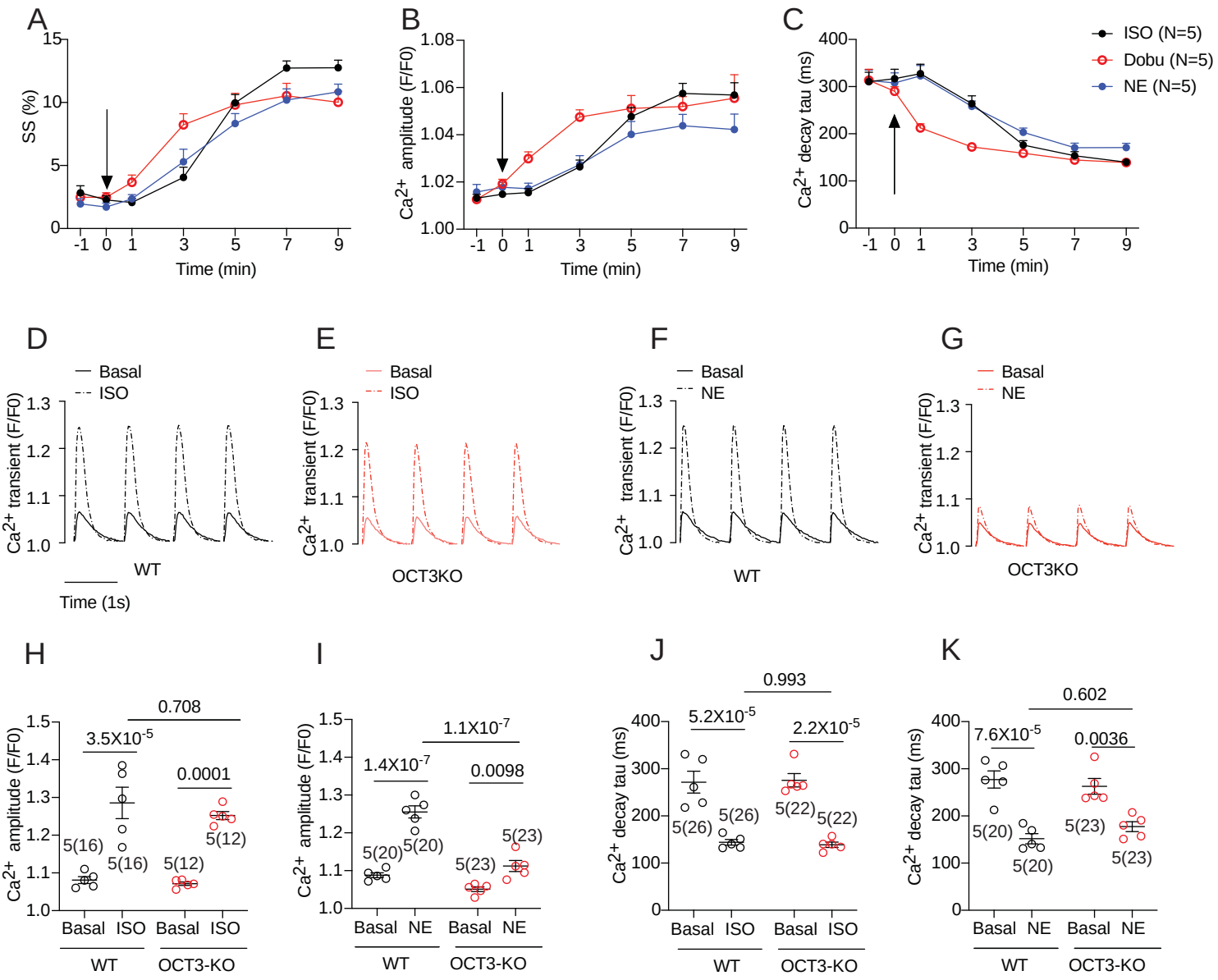
Online Figure IV



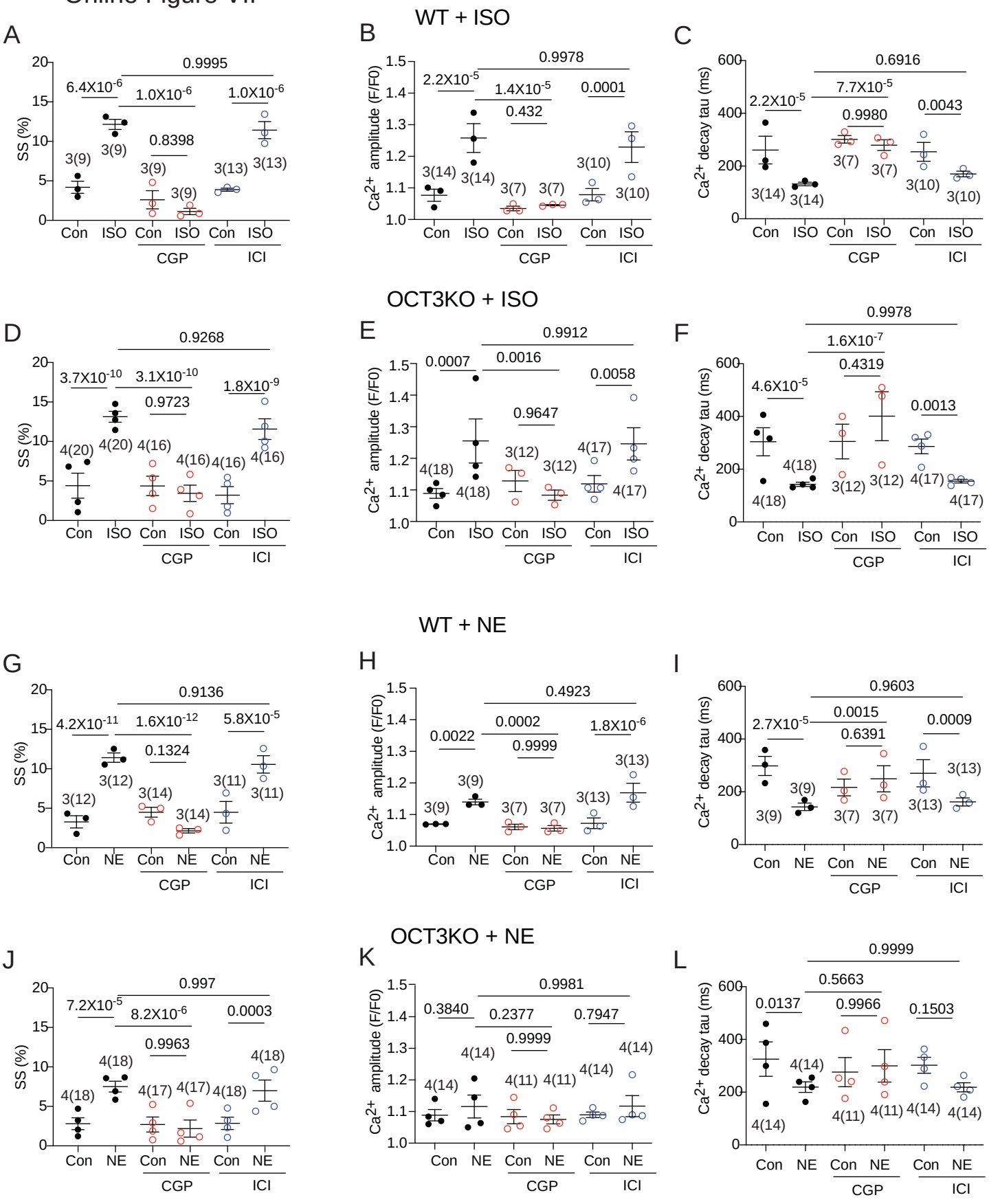
Online Figure V



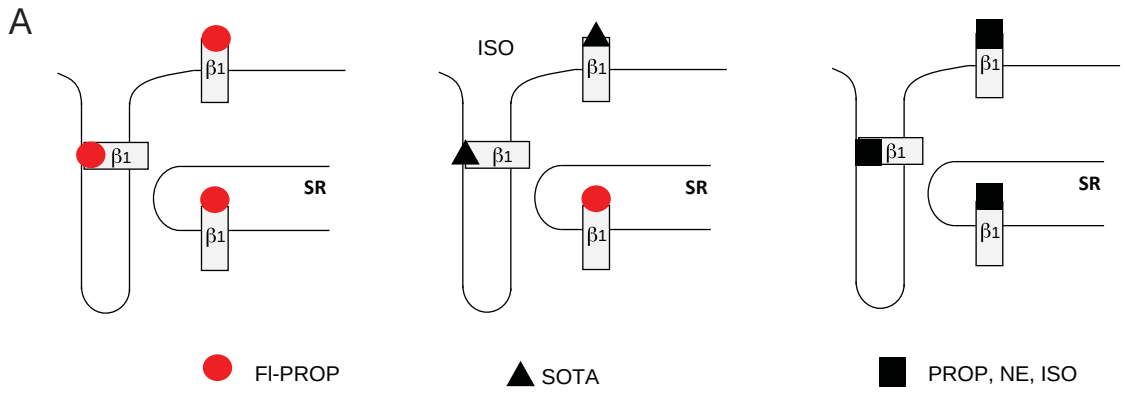
Online Figure VI

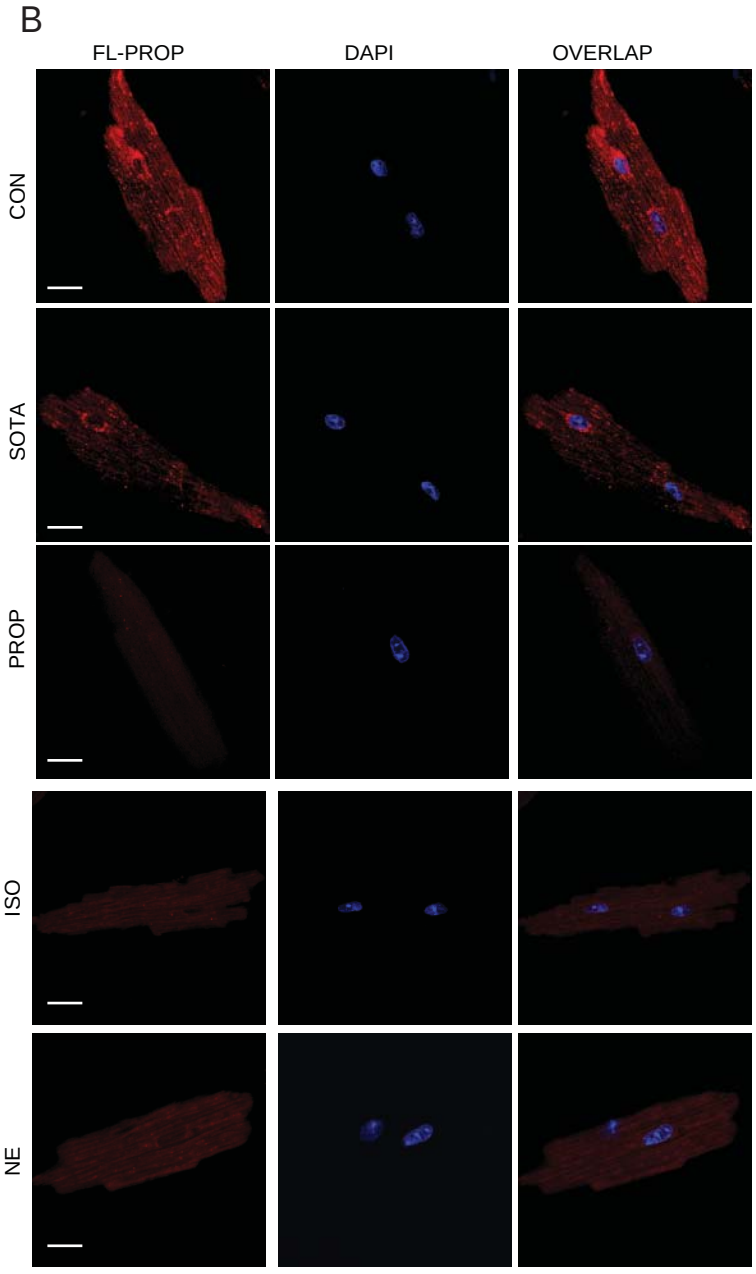


Online Figure VII

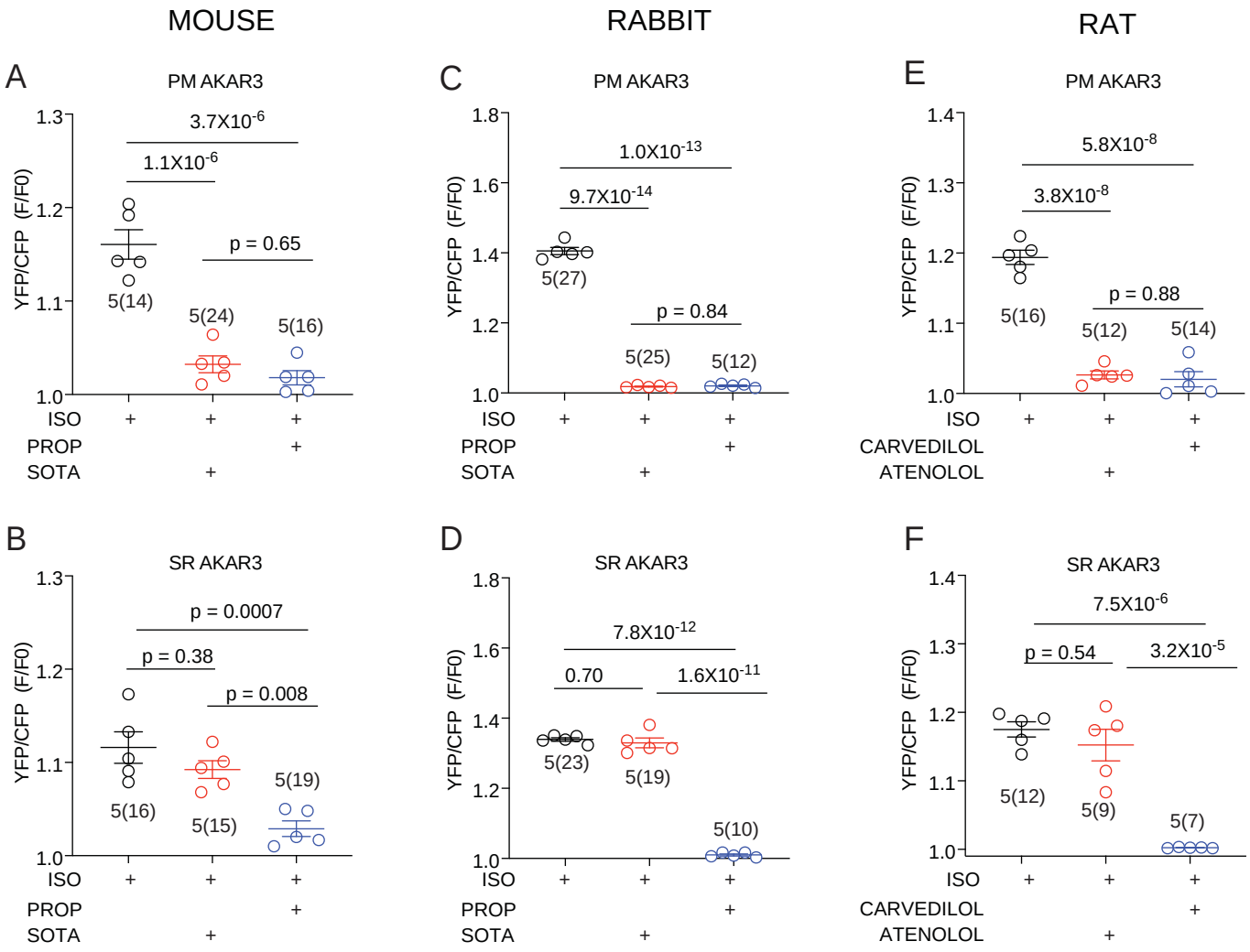


Online Figure VIII

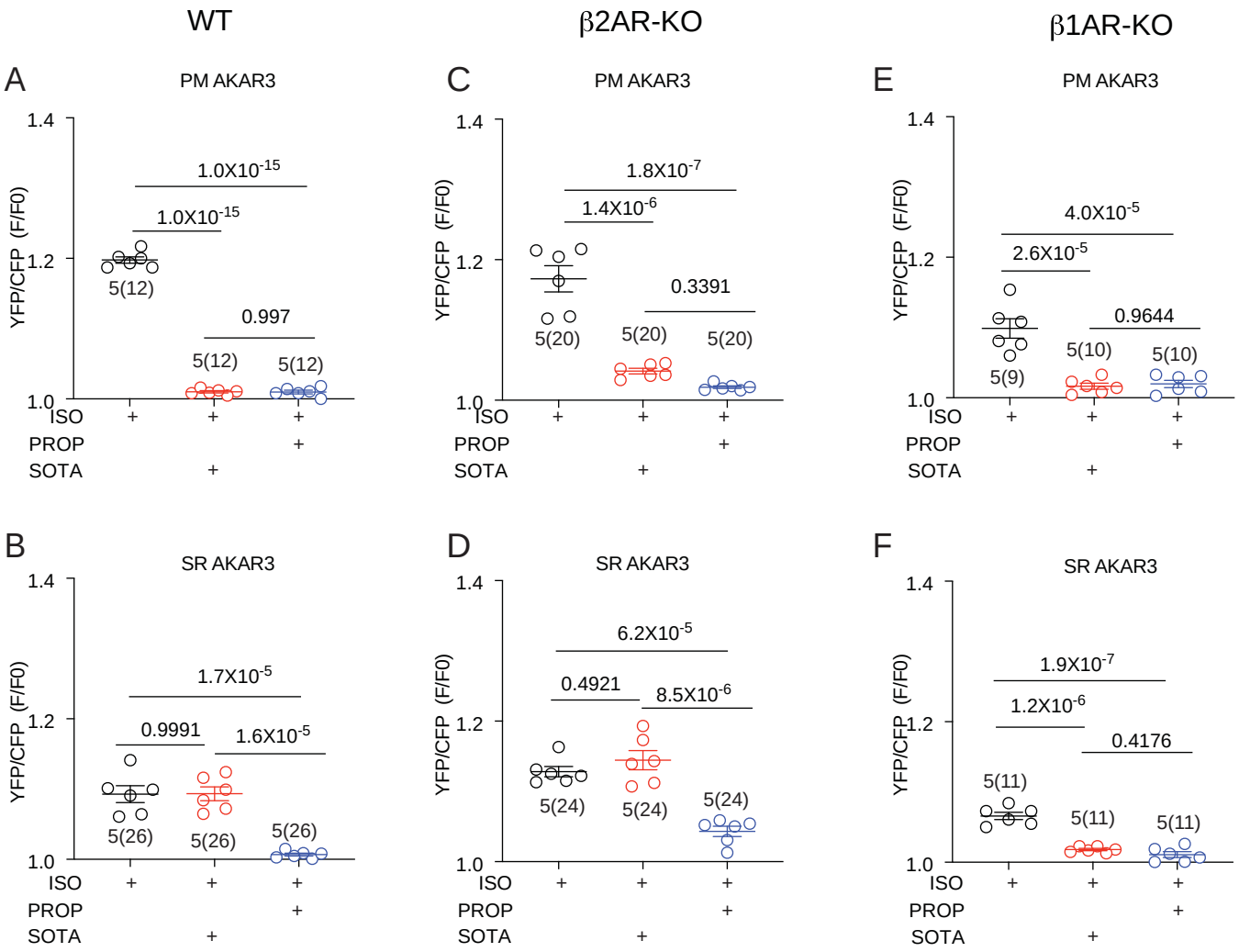




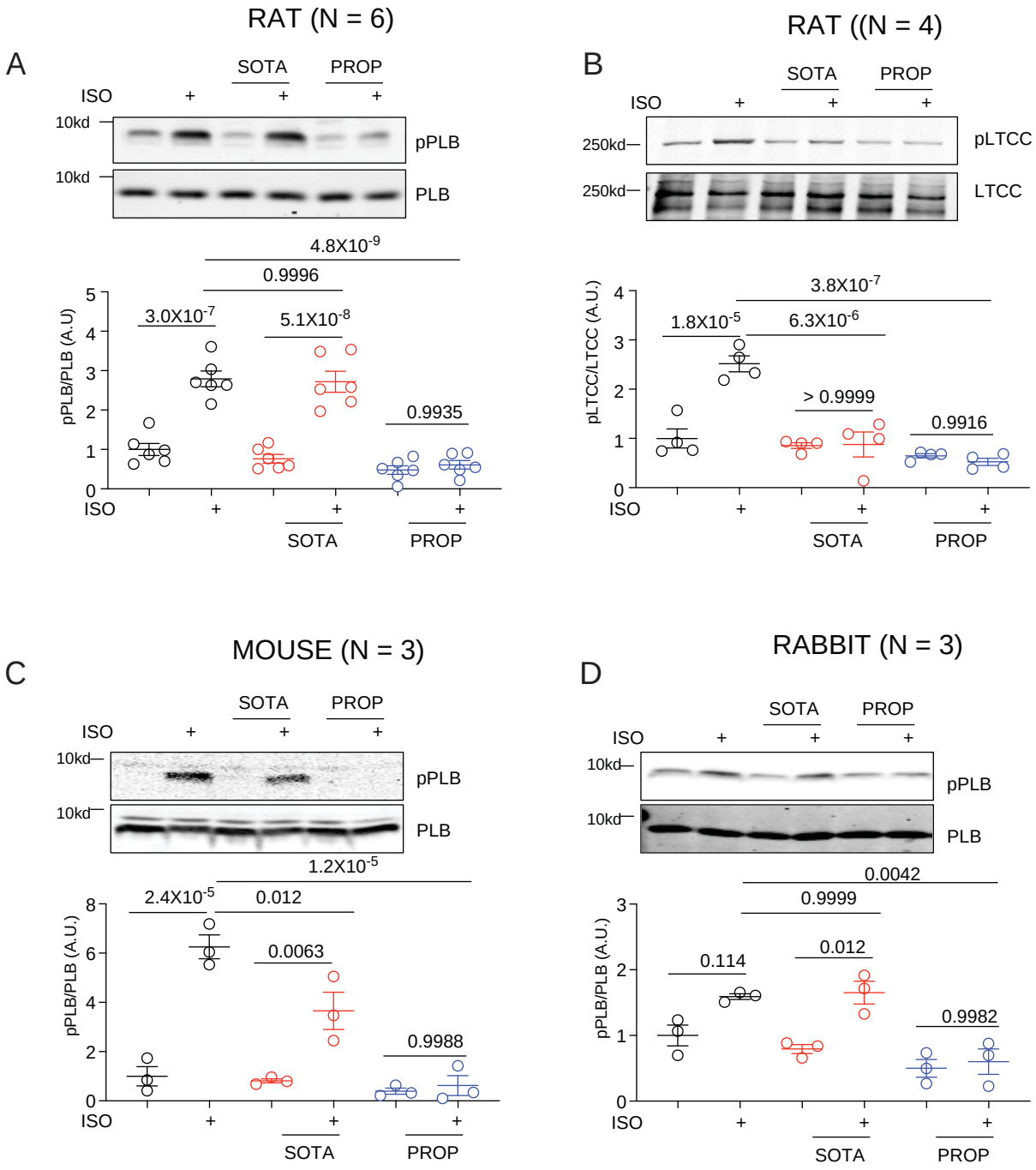
Online Figure IX



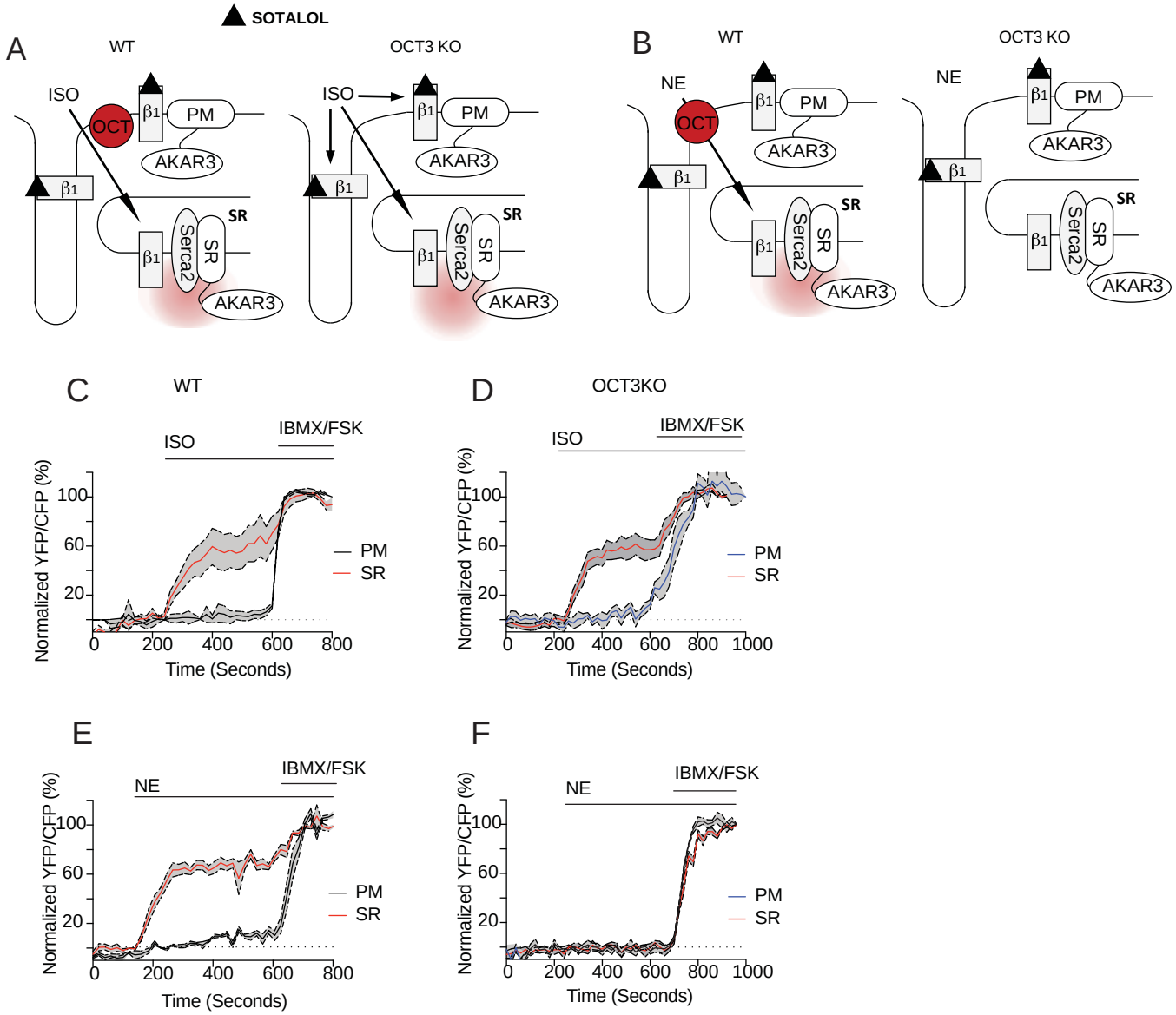
Online Figure X



Online Figure XI



Online Figure XII



Online Figure XIII