SUPPLEMENT MATERIAL

METHODS

Cardiomyocyte isolation

The *Akap5* $^{-/-}$ allele was generated by gene targeting using standard techniques as previously described [1]. Mice were age matched littermates of 3 to 6 months of age maintained on a C57Bl/6 background. Animals were handled in accordance with the guidelines of the University of Washington Institutional Animal Care and Use Committee. Hearts were obtained from mice, 3 – 6 months of age, euthanized by an intraperitoneal injection of pentobarbitone (100 mg kg^{-1}). Ventricular myocytes were isolated following the procedure of Powell, Noma, Shioya & Kozlowski [2] for guinea-pig and modified as previously reported [3]. The cells were kept at room temperature (22-25 °C) in normal Tyrode (NT) solution composed of (mmol/L): 140 NaCl, 5 KCl, 10 Hepes, 10 glucose, 2 $CaCl_2$, and 1 $MgCl_2$; pH 7 4, until use (0.5-6 h after isolation).

Electrophysiology

Membrane Ca^{2+} currents were measured using the whole-cell configuration of the patch-clamp technique with an Axopatch-200A amplifier (Axon Instruments). Patch pipettes were pulled with a Flaming Brown-type puller (Sutter Instrument Co., Novato, CA, USA) to a nominal resistance of 1-3 MΩ and filled with an internal solution of the following composition (in mmol/L): 110 potassium aspartate, 30 KCl, 10 Hepes, 5 Mg^{2+}-ATP and 10 NaCl (pH 7.3). The 15 mV tip potential produced by this solution was corrected offline. During experiments cells were continuously superfused with normal Tyrode solution. Once a successful whole-cell patch-clamp was established, the external solution was changed to one containing (in mmol/L): 140 NaCl, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, 10 Hepes, and 0.01 TTX. Cells were held at -80 mV. To ensure that steady-state loading of the SR Ca^{2+} was attained, four depolarizing pulses (1 Hz) of 50 ms duration to 0 mV were applied before the cell was depolarized to a given test potential using the following protocol: Five hundred milliseconds after the fourth conditioning pulse, cells were depolarized to -40 mV slowly (1 mV every 12-5 ms) and held at this potential for 50 ms (to inactivate Na^{+} current) before rapid (step) depolarization to a test potential. All electrophysiological signals were analyzed using pCLAMP 6.01 software (Axon Instruments).

Field stimulation for calcium imaging

Cardiomyocytes were placed in a perfusion chamber and incubated with normal Tyrode at 22 – 25 ˚C. Field stimulation was performed with two platinum wires (0.5 cm separation) placed at the bottom of the perfusion chamber. An IonOptix Myopacer (IonOptix Corp, Milton, MA, USA) stimulator was used to deliver square voltage pulses (4 ms duration) with amplitude of 35 volts at a frequency of 1 Hz.

Measurements of $[Ca^{2+}]_i$

We used the fluorescent Ca^{2+} indicator Fluo-4 to measure changes in $[Ca^{2+}]_i$. Cells were loaded with the membrane-permeable acetoxymethyl-ester form of Fluo-4 (Fluo-4 AM, Invitrogen, Carlsbad, CA, USA) as previously described [4] for measurement of $[Ca^{2+}]_i$ that did not involved patch-clamping (i.e. Ca^{2+} sparks, SR Ca^{2+} load in paced and un-stimulated cells). For experiments that involved the simultaneous measurement of electrophysiological signals and $[Ca^{2+}]_i$, cells were loaded with the penta-potassium salt of Fluo-4 (50 µM) through the patch pipette. Confocal imaging of whole-cell $[Ca^{2+}]_i$ was performed using a Bio-Rad Radiance 2000 3confocal system (Cambridge, MA, USA) coupled to a Nikon TE300 inverted microscope equipped with a Nikon 60X oil immersion lens (NA = 1.4). This system was operated by Lasersharp 2000 (v. 4.0) software. Images were analyzed with custom software written in IDL

language (Research Systems, Boulder, CO, USA). Background-subtracted fluorescence signals were normalized by dividing the fluorescence (F) intensity at each time point by the resting fluorescence (F_0). Calibration of fluorescence signals was performed using the 'pseudo-ratio' equation [5]:

$$[Ca^{2+}]_i = K_d(F/F_0)/(K_d/[Ca^{2+}]_i,rest + 1 - (F/F_0)),$$

where F is the fluorescence intensity, F_0 is the resting fluorescence, K_d is the dissociation constant of Fluo4 (1100 nmol/L) and $[Ca^{2+}]_i$,rest is the resting Ca^{2+} concentration (150 nmol/L). The rate of decay of $[Ca^{2+}]_i$ transients was obtained by fitting the decaying phase of calibrated fluorescence signals with a standard single exponential function. F_{max} was determined at the end of each experiment by exposing cells to solution NT (see above) to which the Ca^{2+} ionophore ionomycin (10 μM), 2,3-butanedione monoxime (BDM, 20 mmol/L; to prevent contraction), and 20 mmol/L external Ca^{2+} had been added.

Measurement of SR Ca^{2+} content and Ca^{2+} Sparks

Caffeine induced $[Ca^{2+}]_i$ transients were induced as previously described [6]. The amplitude of the $[Ca^{2+}]_i$ transient evoked by the application of a Ca^{2+}- and Na^+-free (substituted with N-methyl-d-glucamine) solution containing 20 mmol/L caffeine (10 s; via a picospritzer) was used as an indicator of SR Ca^{2+} content. To ensure steady-state SR Ca^{2+} load, cells were subjected to a minimum of 10 preconditioning pulses (1 Hz) before caffeine was applied. Isoproterenol treatment was applied for 90 sec prior to measurement of SR Ca^{2+} content and Ca^{2+} sparks.

Ca^{2+} sparks were measured by loading cells with the potassium salt of Fluo-4-AM (50 μM, Invitrogen, Carlsbad, CA, USA). Fluorescence signals were then collected from unpaced cells using a Nikon swept field confocal microscope. Background-subtracted fluorescence signals were normalized by dividing the fluorescence (F) intensity at each time point by the resting fluorescence (F_0). Images were scanned using an argon ion laser beam for illumination at 488 nm (LiveScan Swept Field Confocal Microscope, Nikon, Melville, NY, USA). Emitted fluorescence was detected from 505 - 630 nm. XY confocal images were acquired every 45 to 55 ms. Automated analysis of images for Ca^{2+} sparks was performed using custom routines, written in Interactive Data Language (IDL version 6.2) as reported previously[7, 8].

NCX function was assessed as the rate constant of $[Ca^{2+}]_i$ decline (k_{Ca}) in the presence of caffeine[9]. To ensure steady-state SR Ca^{2+} load, cells were subjected to a minimum of 10 preconditioning pulses (1 Hz) before caffeine was applied. The time point used to measure NCX function with caffeine treatment was 90 sec after isoproterenol application.

Immunoprecipitation and Western blot analysis

Lysate buffer contained (in mmol/L): 10 Na_2HPO_4, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, with 1% TritonX-100 and 0.5% Na Deoxycholate. Before use protease and phosphatase inhibitor cocktail (P8340 and P2850, respectively, Sigma-Aldrich, St. Louis, MO, USA) was added. For isolate cardiomyocytes, 200 μL of lysate was added to each well then scraped and a hand held polytron PT-1200E (Kinematica, Bohemia, NY, USA) used to homogenize cells. For the in vivo experiments, 5 mg kg^{-1} of isoproterenol stabilized with 10 mg kg^{-1} ascorbic acid or 5 mg kg^{-1} propranolol was dosed intraperitoneal followed within 30 seconds with an intraperitoneal injection of pentobarbitone (100 mg kg^{-1}) to euthanize the mouse. The hearts were removed and washed once in ice cold PBS, pH 7.4. Hearts were homogenized at 5% (wt/vol) in lysate buffer with a polytron PT-10-35 (Kinematica, Bohemia, NY, USA) for 10 seconds. The homogenate was then spun at 10,000 x g for 10 min at 4 ^oC and the pellet discarded. The protein concentration of the supernatant was determined using the BCA assay (Therma Scientific, Rockford, IL, USA). For immunoprecipitation, one mg of protein was diluted into a 500 μL volume of IP buffer (in mmol/L): 10 Na_2HPO_4, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, 0.1%

tritonX-100 with fresh protease and phosphatase inhibitors. Two to four μg of capture antibody was added to the lysate and mixed at 4 – 6ºC overnight. The next day 100 μL of magnetic protein G beads were added to the lysate and washed per manufactures instruction (Millipore, Billerica, MA, USA). SDS-PAGE and Western blots used standard techniques[10]. The antibodies used for immunoprecipitation were: AKAP 150 (N-19): sc-6446, Adenylyl Cyclase V/VI (C-17) sc-590, and PKA IIα reg (C-20) sc-908 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Ryanodine Receptor, 559279 (Calbiochem, Merck KGaA, Darmstadt, Germany); Caveolin 3, 910420, Caveolin 1, 611338 (BD Transduction Laboratories, San Jose, CA, USA); Calcineurin (α-Subunit), C1956 (Sigma-Aldrich, St. Louis, MO, USA). Antibodies used for western blotting were are follows: AKAP150 C-20, sc-6445, Adenylyl Cyclase V/VI (C-17) sc-590, and PKA IIα reg (C-20) sc-908 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Ryanodine Receptor, 559279 (Calbiochem, Merck KGaA, Darmstadt, Germany); Caveolin 3, 910420, Caveolin 1, 611338 (BD Transduction Laboratories, San Jose, CA, USA); Calcineurin (α-Subunit), C1956 (Sigma-Aldrich, St. Louis, MO, USA); β_1-Adrenergic Receptor, ab3546-100 and β_1-Adrenergic Receptor, ab13989-50 (Abcam, Cambridge, MA, USA); CNC1 rabbit polyclonal for the α1c subunit of $Ca_v1.2$ and the phospho-Ser1928 $Ca_v1.2$ were generated as described previously [11]. The antibodies specific for phospho-Ser2808 on the RyR_2 [12] were either a gift from A.R.Marks (Columbia University) or purchased (A010-30, Badrilla, United Kingdom). The antibody specific for AC5 was a gift from S.F.Vatner, New Jersey Medical School [13].

Immunocytochemistry

Standard immunocytochemistry techniques were used are described previously [4]. Cells were incubated in M199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with pen/strep (Invitrogen, Carlsbad, CA, USA) at 37 ºC for 2 – 4 hr prior to treatment with drugs for the times indicated in Figure legends. Media was removed and the cells fixed with an ice cold solution of 2% paraformaldehyde in methanol for 10 min. Fixed cells were washed three times for 5 min each in phosphate buffered saline (PBS, pH 7.6). Blocking buffer, consisting of 20 % normal goat serum (Jackson ImmunoResearch Laboratories, Inc.) in an antibody dilution buffer (1 % IgG-free, protease-free BSA and 0.1 % Triton X-100 in PBS) was used for 30 min at room temperature to enhance cell permeability and to decrease non-specific binding of the antibodies. Cells were then washed three times in PBS. Myocytes were incubated overnight at 4 °C with the primary antibodies in antibody dilution buffer (1:500). The following day, cells were incubated for 2 h at room temperature with Alexa Fluor 488-conjugated (1:500) and Alexa Fluor 568-conjugated [1:500] secondary antibodies diluted in antibody dilution buffer. Following another wash with PBS, cells were mounted on slides using ProLong Antifade medium (Molecular Probes, Invitrogen, Carlsbad, CA, USA).

Slides were imaged using the Zeiss LSM 510 META at the Keck Microscopy Facility, University of Washington, using the Argon ion laser with lines at 488 nm, and Helium-neon laser with a 543 nm. Images were collected using a 63X Oil Immersion (PLAN APO N.A. 1.40). The fluorescence emitted by Alexa-488 and Alexa-568 was separated by the appropriate set of filters. Primary antibodies used were the same as above for western blots and we also used α-actinin A7811 (Sigma-Aldrich, St. Louis, MO, USA) as a marker for Z-lines.

Cyclic AMP Assay

All experiments were performed at 37ºC. The cardiomyocytes were plated in 6 well plates coated in mouse laminin, 10 μg per well, (23017-015, Invitrogen, Carlsbad, CA, UAS). Cells were incubated in M199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with pen/strep (Invitrogen, Carlsbad, CA, USA). One heart was divided between four wells. After 6 hours media was removed, and then replaced with media and either 100 nmol/L isoproterenol or vehicle

spited in at 1000X. After 90 sec the media was removed and to lyse the cells and stabilize the cAMP, 0.1 mol/L HCl in 100% ethanol was add to the cells. The cAMP concentration was measured using cAMP detection EAI as pre instruction (Assay Design, Ann Arbor, MI, USA). The protein concentration was measured for each well using 1% SDS to solubilize the protein and measured by BCA assay (Therma Scientific, Rockford, IL, USA), and used to normalize cAMP concentration between samples.

Statistical Analysis

Significant differences were determined by two-way ANOVA and post hoc Student's unpaired t test, as appropriate. Pearson's correlation coefficients [14] were calculated using Imaris software (Bitplane AG, Zurich, Switzerland).

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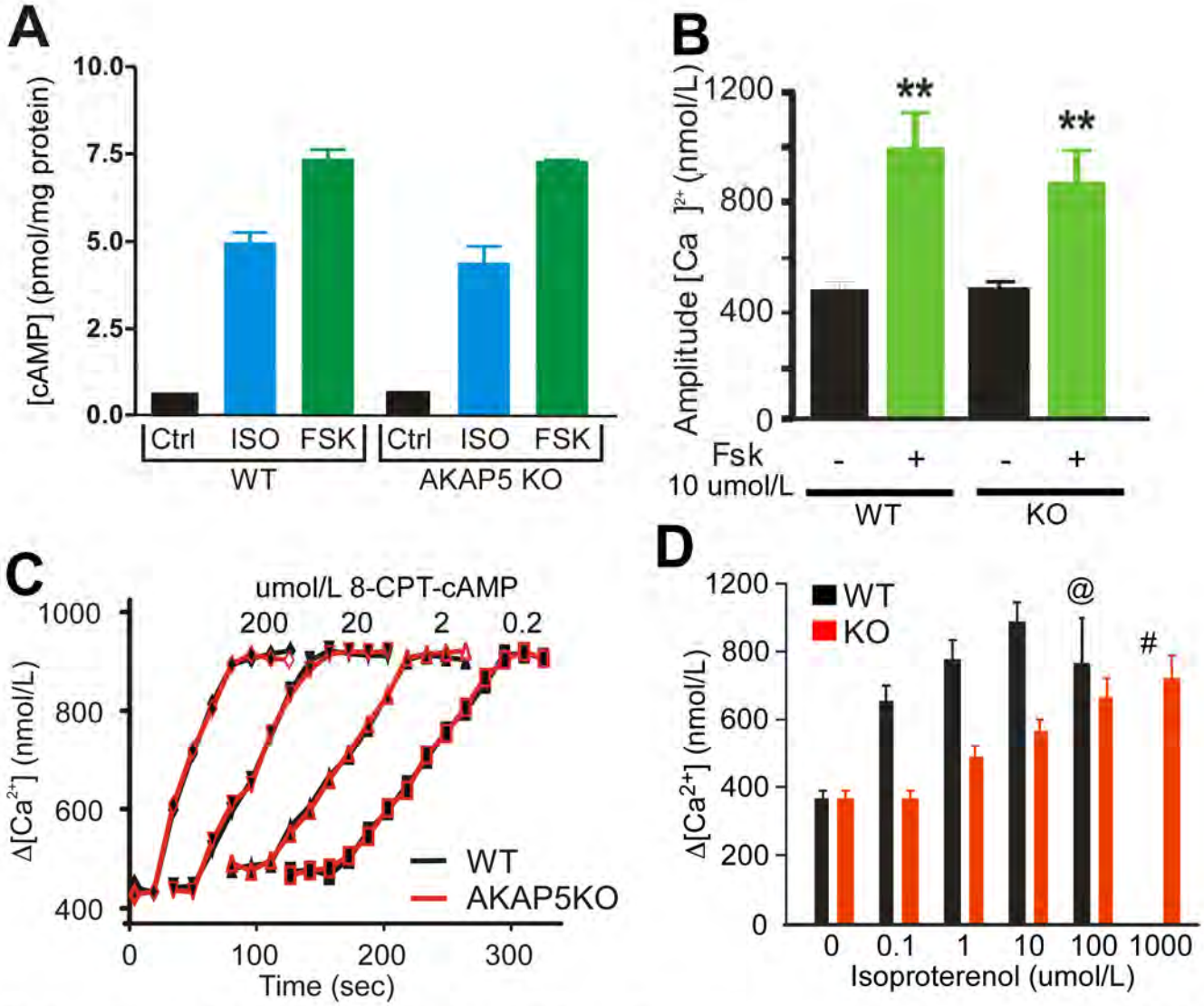
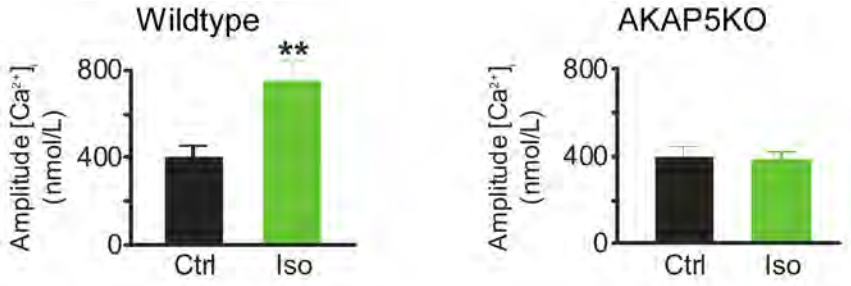
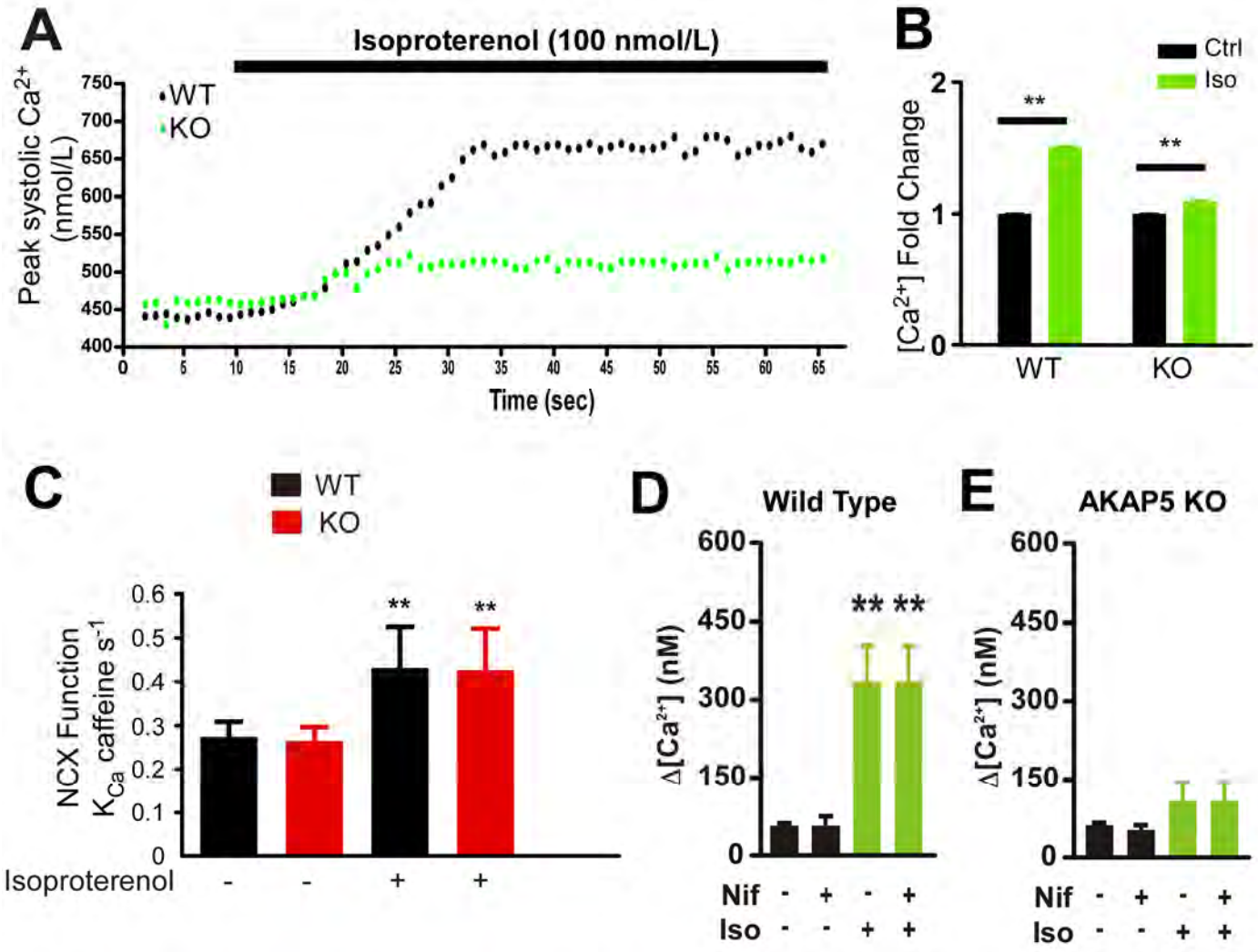


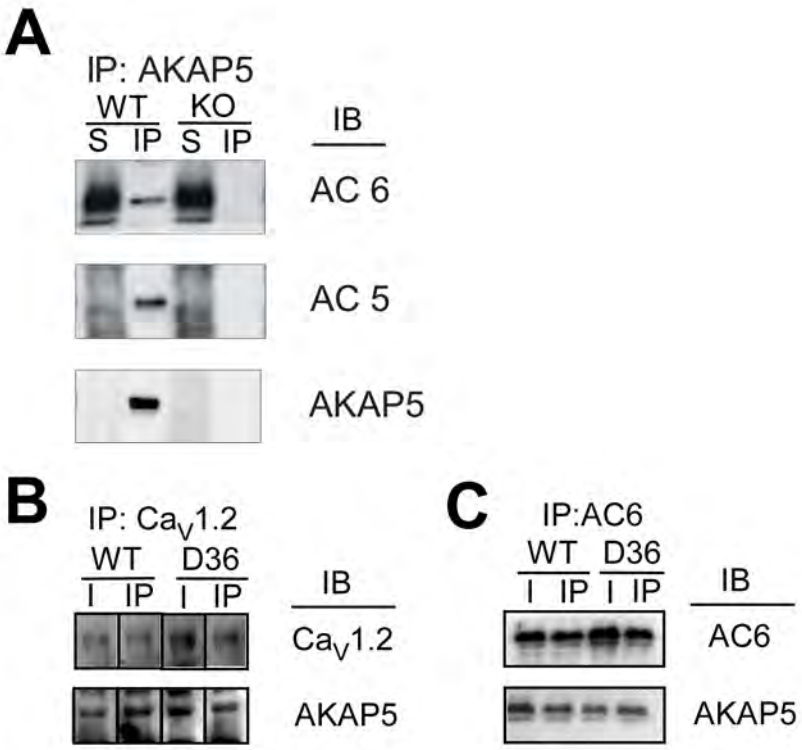
Online Figure I. Response of WT and KO cardiomyocytes to stimulation of the cAMP/PKA pathway. A, Stimulation of cAMP production in isolated ventricular myocytes from WT and KO animals after a 90 sec treatment with 100 nmol/L isoproterenol (Iso) or 10 umol/L forskolin (Fsk). **B,** Forskolin (Fsk, 10 umol/L) stimulation of $[Ca^{2+}]_i$ transients in isolated cardiac myocytes from WT and KO animals. Calcium transients were measured with Fluo-4 and converted to calcium concentrations as described in methods. (mean ± s.e.m., n=9 animals, 3-4 cells analyzed per animal; **P<0.001). **C,** Time course of calcium transient response in field stimulated cardiomyocytes treated with various concentrations of exogenous cAMP analog, 8-CPT-cAMP, measured with Fluo-4 and converted to calcium concentrations as described in Methods (mean, N=3). **D,** Isoproterenol dose response of $[Ca^{2+}]_i$ transients in isolated cardiac myocytes from WT and KO animals. Calcium transients were measured with Fluo-4 and converted to calcium concentrations as described in methods. # = WT myocytes responded to 1000 umol/L isoproterenol by rounding up preventing measurement. @ = Six of 12 WT cells treated with 100 umol/L isoproterenol responded with spontaneous transients that decreased the field stimulation $[Ca^{2+}]$ transients amplitude. (mean ± s.e.m., n=3 animals, 3-4 cells analyzed per animal)



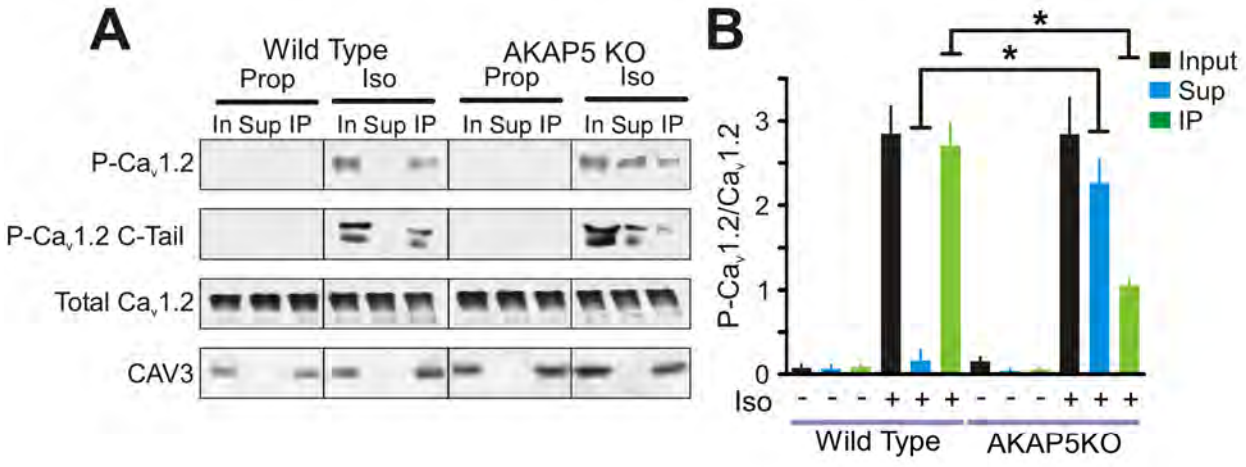
Online Figure II. AKAP5 KO whole cell $[Ca^{2+}]_i$ transients fail to respond to isoproterenol even though the I_{Ca} increases. Simultaneous recording of the whole cell $[Ca^{2+}]_i$ transients from the same cells shown in Figure 3A for LTCC current measurements. Transients were measured at +10 mV using Fluo-4.



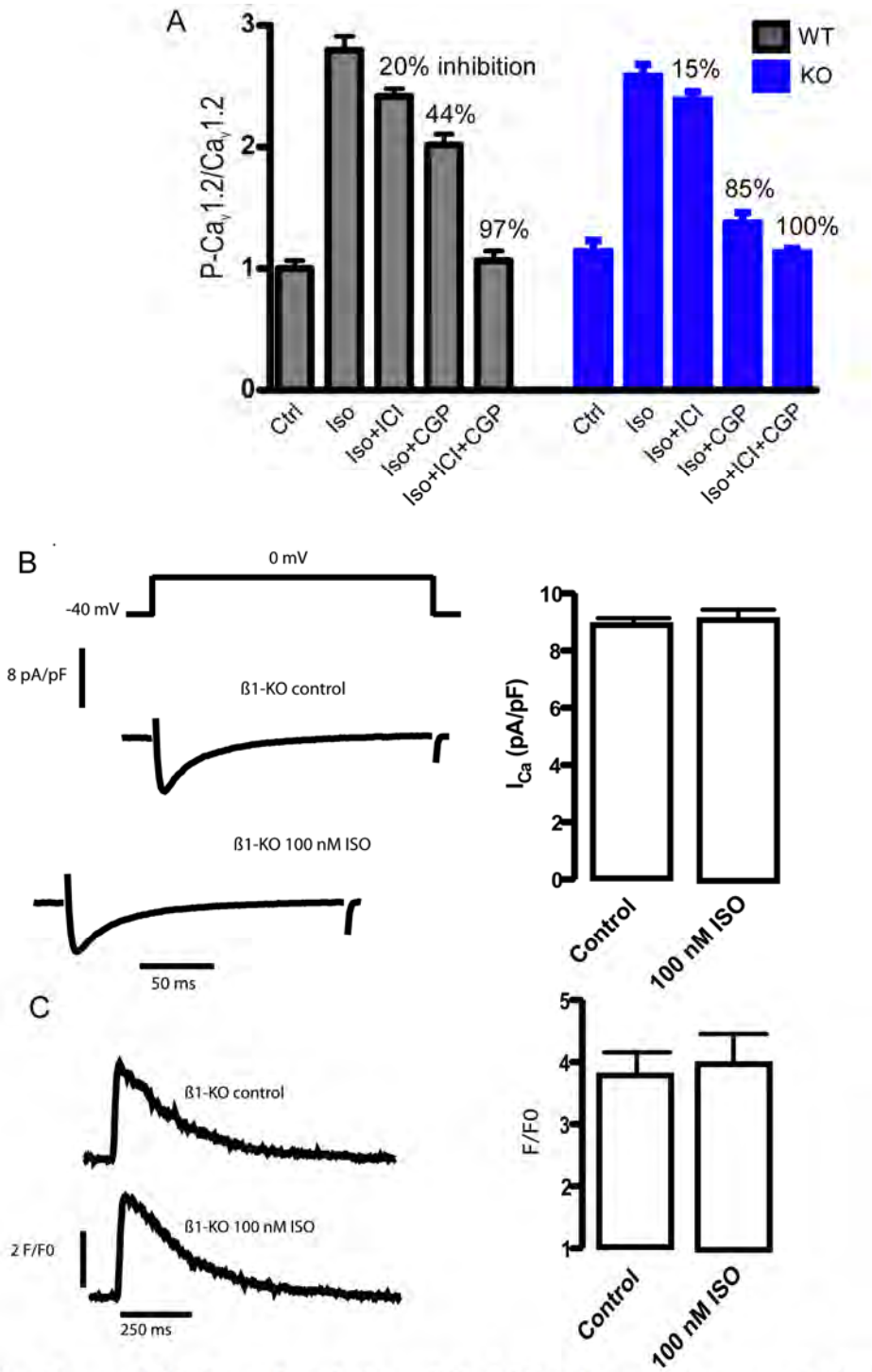
Online Figure III. Ca^{2+} dynamics in the cardiomyocyte. **A,** The peak amplitude of the $[Ca^{2+}]_i$ transient is shown for a representative WT and KO myocyte. During isoproterenol stimulation there is a robust increase in the peak amplitude of the $[Ca^{2+}]_i$ transient in WT as expected and a very small increase in KO myocytes. **B,** The $[Ca^{2+}]$ was normalized for each cell by comparing the peak amplitude of the $[Ca^{2+}]_i$ transient after stimulation with Iso to that before stimulation. A small 1.09 fold difference is seen in KO myocytes after isoproterenol (Iso, 100 nmol/L) stimulation whereas the WT cells demonstrate a 1.51 fold change. (mean ± s.e.m, $P<0.001$, N=9). **C,** Average rate constant k_{Ca} for $[Ca^{2+}]_i$ decay during caffeine exposure used as an indirect indicator of NCX function. Ten pulses were used to fill the SR of the myocyte before caffeine (10 mmol/L) treatment. (mean ± s.e.m, 4 animals, 8 cells/animal, **$P<0.001$ vs. WT and KO). Details are described in Methods. **D-E,** Isoproterenol (Iso, 100 nmol/L) stimulation of $[Ca^{2+}]_i$ spark amplitude in WT (D) and KO (E) cardiomyocytes. Nifedipine (Nif, 25 nmol/L) was used to block L-type Ca^{2+} channels (mean ± s.e.m, N=3).



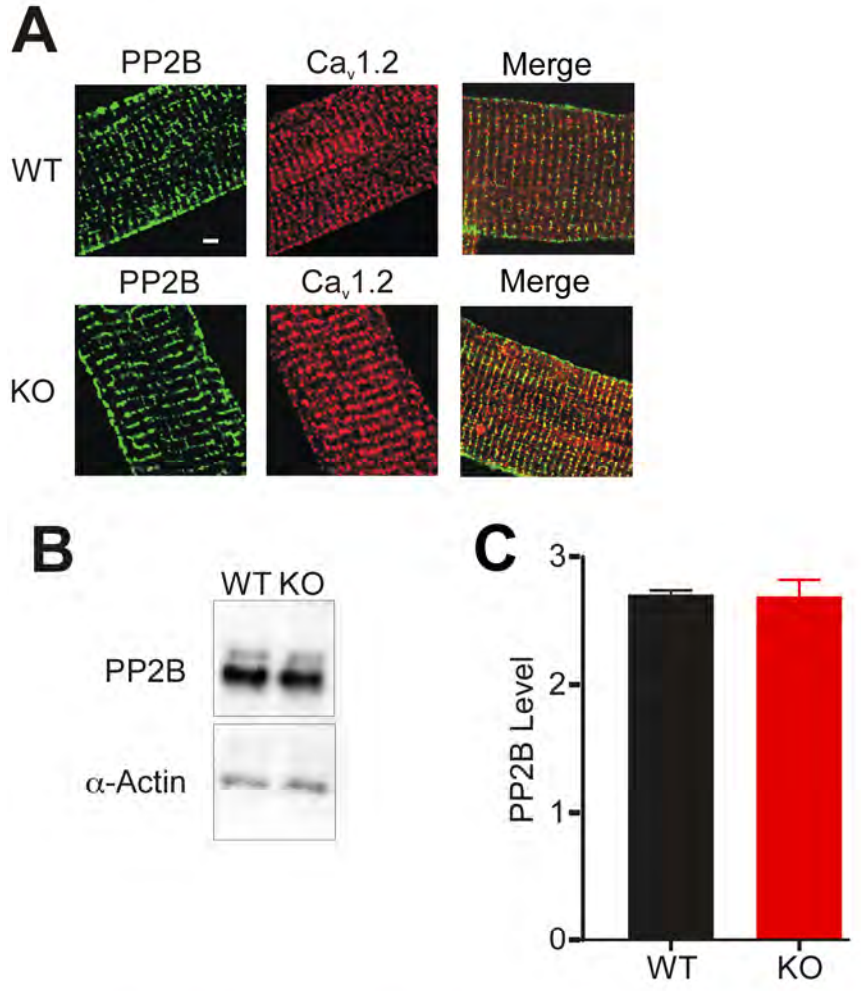
Online Figure IV. Both AC5 and AC6 interact with AKAP5 and the D36 mutant still interacts with both AC6 and $Ca_v1.2$. Immunoprecipitations were done from heart extracts of WT and KO animals. Tissue was homogenized in buffer containing 1% TX-100 and 0.5% sodium deoxycholate and antibody-bound proteins were recovered using protein G magnetic beads as described in Online Methods. **A,** Immunoprecipitations with antibodies against AKAP5, AC6, and AC5 were run on western blots and probed with the antibodies listed under immunoblot (IB). **B,** Immunoprecipitation with antibodies for $Ca_V1.2$ were run on western blots and probed with the antibodies listed under immunoblot (IB). **C,** Immunoprecipitation with antibodies against AC6, were run on western blots and probed with the antibodies listed under immunoblot (IB).



Online Figure V. In vivo phosphorylation of $Ca_v1.2$ in response to isoproterenol or propranolol. A, WT and KO mice were administered Iso (5mg/kg i.p.) or propranolol (5mg/kg i.p.). After 5 min, hearts were isolated and extracts were immunoprecipitated with an antibody against CAV3. An equal fraction of the input (In), the supernatant from the CAV3 immunoprecipitation (Sup) and the immunoprecipitated fraction (IP) was loaded for western blotting and probed with antibodies against $Ca_v1.2$, CAV3, and P-$Ca_v1.2$ (Ser1928). Both the intact P-$Ca_v1.2$ at ~240 kDa and the C-tail at ~54 kDa are shown. **B,** The P-$Ca_v1.2$/$Ca_v1.2$ ratio based on densitometry and averaged from 4 separate experiments is shown in the right panel (mean ± s.e.m, *P<0.05).



Online Figure VI. Phosphorylation of $Ca_v1.2$ by β1 or β2 Adrenergic Receptor stimulation in WT myocytes and effects of Iso on LTCC current and calcium transient in β1-AR KO myocytes. **A,** Isolated cardiomyocytes were treated with isoproterenol (Iso, 100 nmol/L), isoproterenol plus β2-AR selective blocker ICI118551 (1 umol/L), isoproterenol plus β1-AR specific blocker CGP20712A (1 umol/L), or isoproterenol plus both ICI and CGP inhibitors (mean ± s.e.m, N=3). Extracts were subjected to western blot with antibodies against P-Cav1.2 and Cav1.2 as described in Online Figure V and the ratio of phospho to total Cav1.2 was measured by densitometry and normalized to the WT control value. The percent inhibition by antagonist is shown above the bar. **B,** ICa records from a representative β1 KO (Adrb1 KO) ventricular myocyte before and after the application of 100 nM Iso. ICa was evoked by a step depolarization to 0 mV for 300 ms from the holding potential of -40 mV. Bar plot shows the mean ± SEM of the amplitude of ICa at 0 mV before and after Iso ($n = 7$, $p > 0.05$) **C,** Action potential-evoked [Ca2+]i transients in a representative β1-KO myocyte before and after Iso. Bar plot of the mean ± SEM of the amplitude of an action potential-evoked [Ca2+]i transient before and after Iso.



Online Figure VII. Localization and quantification of PP2B in WT and AKAP5 KO myocytes. **A.** Immunocytochemistry of PP2B (left panel), $Ca_v1.2$ (center panel), and merged image (left panel) in WT and KO ventricular cardiomyocytes. Bar = 5 um. **B,** PP2B protein expression in WT and KO heart compared to α-actin on western blots. **C,** Level of PP2B expression normalized to α-actin as a loading control. (mean ± s.e.m, N=3).