SUPPLEMENTAL MATERIAL

Supplemental Methods

Ankyrin-B βII spectrin conditional knock-out (cKO) mice. Ankyrin-B cKO mice were generated by the introduction of LoxP sites flanking exon 24 of *Ank2*. The strategy results in the deletion of 73 bp of coding sequence: the splicing of exon 23-exon 25 leads to a frame shift resulting in a premature stop codon in exon 25. Mice were crossed to generate pure lines of floxed mice devoid of the neomycin cassette. Mice were screened by PCR and Southern analyses (genOway). Animals were crossed with mice expressing Cre under the cardiac promoter α-myosin heavy chain (αMHC-Cre) resulting in specific loss of ankyrin-B in adult cardiac myocytes. βII spectrin cKO mice were generated by the introduction of LoxP sites flanking exon 3 of the gene.[1] These animals were then crossed with αMHC-Cre mice.

Immunoblots and immunostaining. Tissue was harvested and immediately placed into ice cold homogenization buffer (in mM: 50 Tris-HCl, 10 NaCl, 320 sucrose, 5 EDTA, 2.5 EGTA; supplemented with 1:1000 protease inhibitor cocktail and 1:1000 PMSF). Following quantification, tissue lysates were analyzed on Mini-PROTEAN tetra cell (BioRad) on a 4-15% precast TGX gel (BioRad). Gels were transferred to a nitrocellulose membrane using the Mini-PROTEAN tetra cell (BioRad). Membranes were blocked for 1 hour at room temperature using a 3% BSA solution or 5% milk solution and incubated with primary antibody overnight at 4°C. Densitometry analysis was done using ImageLab software (BioRad). For all experiments, protein values were normalized against an internal loading control (actin, GAPDH, calsequestrin).

Super-resolution image acquisition, reconstruction, and sample preparation. Our custom built STORM system is based on an inverted microscope (IX71, Olympus America Inc.) with 1.49 NA 100x oil

immersion total internal reflection fluorescence (TIRF) objective. A 647 nm diode laser (Vortran Laser Technology Inc.) is used to both excite and activate Alexa Fluor 647 (Life Technologies, CA). An EMCCD camera (iXon Ultra 897, Andor Technologies, CT) is used for image acquisition. The sample holder is mounted on a 3D piezo stage (Nano- LPS, Mad City Lab). An infrared 980 nm laser is used in combination with the piezo stage for the axial Zero Drift Correction (ZDC).[2] The super-resolution image is reconstructed using a tracklet-based method as described.[3] The effective resolution is approximately 35 nm. Control or βII spectrin cKO cardiomyocytes were labeled using RyR_2 or βII-spectrin primary antibody as described [4] for 24 hours. Cells were then washed and incubated with an Alexa Fluor 647 conjugated secondary goat-anti-mouse (Life Technologies, CA) or goat-anti-rabbit secondary antibody (Life Technologies, CA) for 3 hours at room temperature. Glass bottom culture dishes (MatTek, MA) were coated with Matrigel (Corning Inc, MA, 1:6 dilution) for 45 minutes. 50 μl labeled cell suspension was pipetted into the dish and settled for 1 hour before washing with the imaging buffer.[5] Cells were imaged in the imaging buffer and typically 60,000 to 80,000 frames were acquired with a frame rate of 56 fps.

Site Directed Mutagenesis. Primers were designed to insert the p.R990Q mutations into the spectrin-binding domain of ankyrin-B in pcDNA3.1+. Primers were used in concert with the Stratagene QuikChange Site-Direct Mutagenesis kit and manufacturer's instructions. Sequences were verified before experiments.

Production and purification of fusion proteins. cDNAs for the WT ankyrin-B, and p.R990Q spectrin-binding domains were PCR-amplified, cloned into pGEX6P-1 (Amersham), and sequenced to confirm correct sequences. To facilitate cloning, all constructs were engineered to contain 5′ *Eco*RI and 3′ *Xho*I restriction sites. BL21(DE3)pLysS cells were transformed with the ankyrin-B pGEX6P-1 constructs and grown overnight at 37 °C in LB supplemented with 0.05 g/L ampicillin. The overnight cultures were

subcultured for large-scale expression. Cells were grown to an optical density of 0.6 and induced with 1 mM isopropyl 1-thio-α-D-galactopyranoside (IPTG) for 4 h at 37 °C. Cells were centrifuged for 10 min at 8,000 x *g*, re-suspended in PBS, and frozen at -80 °C following re-suspension. Cells were lysed by thawing. The crude extract was suspended in a solution of PBS, 1 mM DTT, 1 mM EDTA, 40 g/mL AEBSF, 10 g/mL leupeptin, 40 g/mL benzamidine, 10 g/mL peptstatin (Lysis buffer). Lysates were homogenized by sonication, centrifuged to remove cellular debris, and the supernatant incubated with glutathione-sepharose overnight at 4 °C. The overnight incubation was centrifuged and washed in PBS. A small aliquot was separated by SDS-PAGE and Coomassie Blue stained to quantitate immobilized protein.

ECG experiments. ECG recordings of ambulatory mice were obtained using subcutaneously implanted radiotelemeters (DSI, St. Paul, MN). For baseline HR analysis, continuous ECG data was collected for 1 hour on seven separate days. Only ECG complexes with clearly defined onset and termination signals were sampled. ECG parameter measurement was performed by one individual and confirmed by two or more individuals. For stress tests, mice were initially injected intraperitoneally with both low dose (0.2 mg/kg) and high dose (2 mg/kg) epinephrine. Baseline recordings were performed prior to each injection for at least 5 minutes and for at least 15 minutes after the injection. Non-sustained and sustained arrhythmias were identified using standard ECG analysis guidelines. Variability was assessed over a 10 min period and expressed as the average heart rate plus standard deviation. In a separate group of mice, surface ECG recordings were obtained under anesthesia with 2% isoflurane. Three needle electrodes were placed subcutaneously in the standard limb configuration. For each mouse, 15 min of continuous data were sampled at 4 kHz with a PowerLab 4/30 interface (AD Instruments). Analysis was performed offline using LabChart 7 Pro (AD Instruments).

Electrophysiology. Membrane currents were assessed by use of an Axopatch-200B amplifier and a CV-203BU head stage (Axon Instruments). Experimental control, data acquisition, and data analysis were accomplished with the use of software package PClamp 10 with the Digidata 1440A acquisition system (Axon Instruments).

Calcium wave studies. Isolated and fluo-4 loaded ventricular myocytes were analyzed for calcium waves. Myocytes were field stimulated at 0.5 Hz and upon cessation of stimulation were continuously monitored for spontaneous calcium wave formation for 30 seconds. Data in Fig. 5K represents total spontaneous waves for each genotype over the 30 second interval. Fig. 5L represents waves/myocyte over the first 15 seconds of observation.

Action Potentials. Action potentials (APs) were performed using multiple pacing frequencies ± superfusion with 1μM isoproterenol. In parallel experiments, myocytes were pre-treated with 100 nM ryanodine.

Antibodies. The following antibodies were used in this study: mouse monoclonal anti-NCX1 (Swant), rabbit polyclonal anti-βII spectrin, ankyrin-B, ankyrin-R, and ankyrin-G, CaMKIIδ/CaMKIIδ pS287 (Badrilla), mouse monoclonal anti-$Ca_v1.2$ (Affinity Bioreagents), βI spectrin (neuromab), rabbit polyclonal anti-$Na_v1.5$[4], Na/K ATPase (Millipore), actin (Santa Cruz Biotechnology), α-actinin (Sigma), αII spectrin (Sigma), βII spectrin, connexin43 (Invitrogen), α-tubulin (Sigma), N-cadherin (Invitrogen), desmin (Sigma), GAPDH (Fitzgerald), RyR_2 and SERCA2 (Affinity Bioreagents).

Human variant rescue studies. Assays to evaluate human ankyrin-B mutations were performed in primary control and ankyrin-B cKO myocytes. Ankyrin-B R990Q, as well as two ankyrin-B mutations previously shown to lack βII spectrin-binding[6] (DAR976AAA, A1000P) were evaluated in parallel experiments.

In vitro binding assays. In vitro binding assays were performed as previously described using GST-fusion proteins and ^{35}S-labelled *in vitro* translation products. Reactions were performed at 4°C for 3 hours in a high stringency binding buffer (50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 500 mM NaCl, 0.1% Triton X-100), washed 5 times in a high stringency wash buffer (1 M NaCl binding buffer), separated by SDS-PAGE, and visualized by phosphorimaging. All binding experiments were replicated at least three times.

Structural modeling. Analysis of ankyrin-B/βII spectrin interactions were performed using the high resolution structure of the ankyrin-B ZZUD tandem. All structural figures were prepared using PyMOL (www.pymol.org).

Echocardiography. Transthoracic echocardiogram was performed using the Vevo 2100 (Visualsonics). The mice were anesthetized using 2.0 % isoflurane in 95% O2 / 5% CO_2 at a rate of ~ 0.8 L/min. Anesthesia was maintained by administration of oxygen and ~1% isoflurane. Electrode gel was placed on the ECG sensors of the heated platform and the mouse was placed supine on the platform to monitor electrical activity of heart. A temperature probe was inserted into the rectum of the mouse to monitor core temperature of ~ 37°C. The MS-400 transducer was used to collect the contractile parameters of the heart in the short axis M-mode. Transverse aortic constriction was performed as described.[7]

Neonatal cardiomyocyte experiments. Experiments were performed as described, however for transfections, unlike previous studies in global ankyrin-B$^{-/-}$ myocytes in post-natal day 1 myocytes[6] (done at P1 as global ankyrin-B mice die immediately after birth[8]), experiments in this manuscript were performed to evaluate expression in post-natal 7 day myocytes to coincide with expression and striation of βII spectrin in the myocyte. Based on our new findings, while βII spectrin requires ankyrin-B

expression in immature myocytes (<post-natal day 3), this relationship reverses by post-natal day 7 where βII spectrin expression is required for ankyrin-B expression.

References

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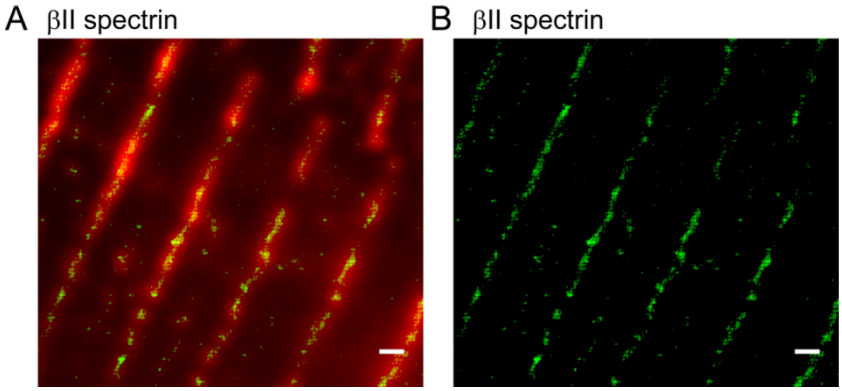
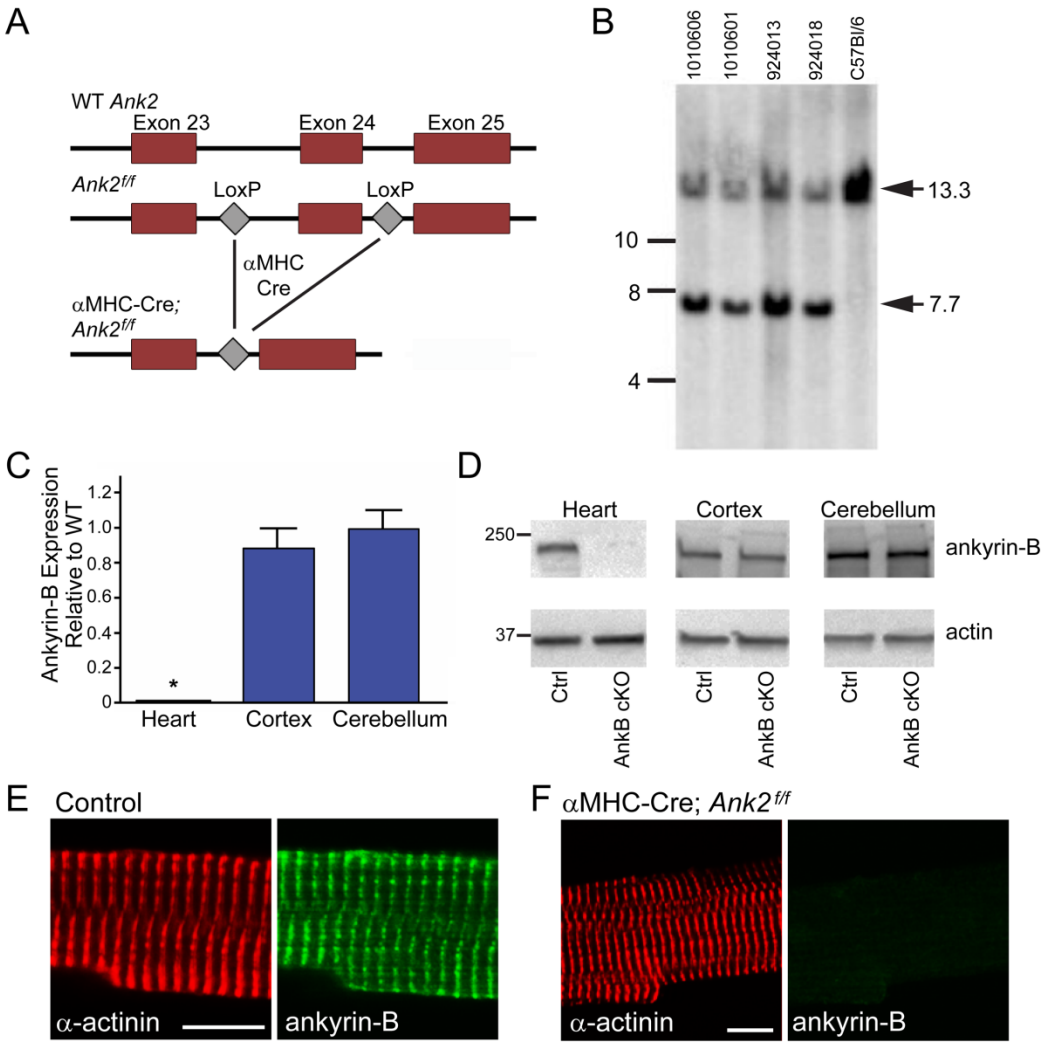
Supplemental Figure 1



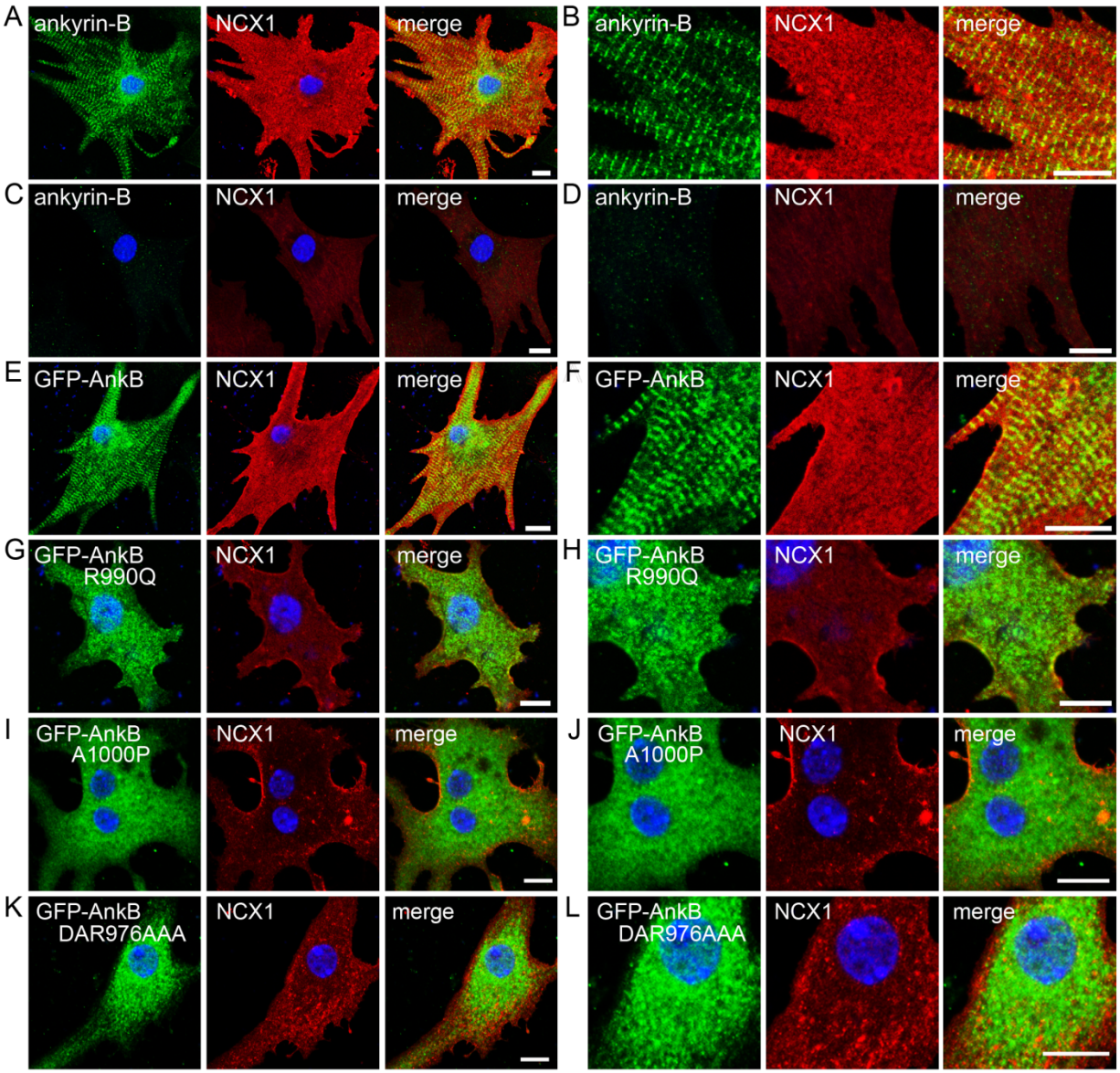
Supplemental Figure 1. Localization of βII spectrin in myocytes. (**A**) Overlay of a TIRF image (red) and the super-resolution image (green) of βII spectrin in control myocytes. (B) Super-resolution image of βII spectrin. Scale bar: 500 nm.

Supplemental Figure 2



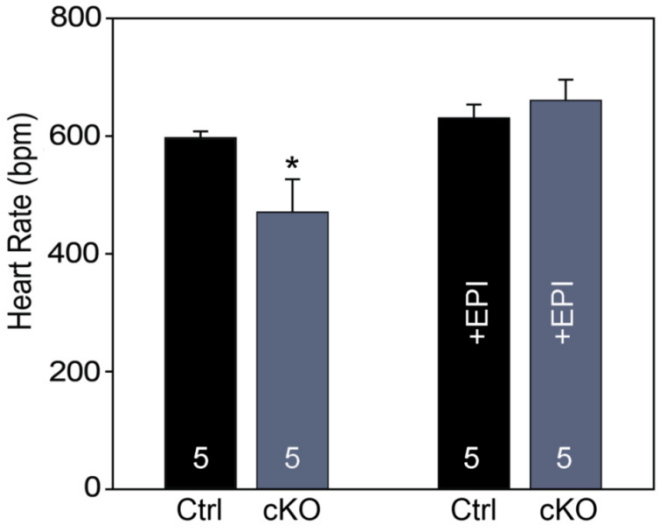
Supplemental Figure 2. Generation and validation of mice lacking ankyrin-B in ventricular cardiomyocytes. (**A**) Targeting strategy to generate Cre-dependent loss of cardiac ankyrin-B. (**B**) Southern blot analysis of heterozygous Neo-excised conditional knockout (αMHC-Cre; *Ank2*$^{f/f}$, cKO). Genomic DNA of tested animals was compared with WT DNA from C57/Bl6 mouse. Spe I digested DNAs were blotted on nylon membrane and hybridized with external 3' probe. (**C-D**) Expression of ankyrin-B in tissues from ankyrin-B cKO mouse relative to control mice ($p<0.05$ for ankyrin-B cKO heart compared with control heart). (**E-F**) Ankyrin-B immunostaining in control and ankyrin-B cKO mouse myocytes. Bar= 10 μm.

Supplemental Figure 3



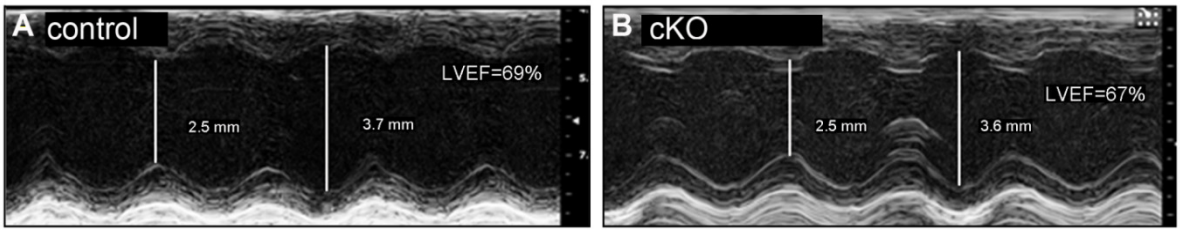
Supplemental Figure 3. Ankyrin-B R990Q in primary cardiomyocytes. Compared with control myocytes (**A-B**), ankyrin-B cKO myocytes (**C-D**) at post-natal day 7 display loss of ankyrin-B expression and membrane targeting of the Na/Ca exchanger (NCX1). (**E-F**) Expression of GFP-ankyrin-B is sufficient to rescue the striated pattern of ankyrin-B and NCX1 expression in ankyrin-B cKO myocytes. (**G-H**) While expressed in ankyrin-B cKO myocytes (green), GFP-ankyrin-B p.R990Q is abnormally localized and lacks ability to rescue abnormal Na/Ca exchanger localization. As a control for ankyrin-B R990Q, two mutant ankyrin-B polypeptides that lack βII spectrin-binding activity (**I-L**; A1000P, DAR976AAA) were analysed in parallel. Bar=10 μm.

Supplemental Figure 4



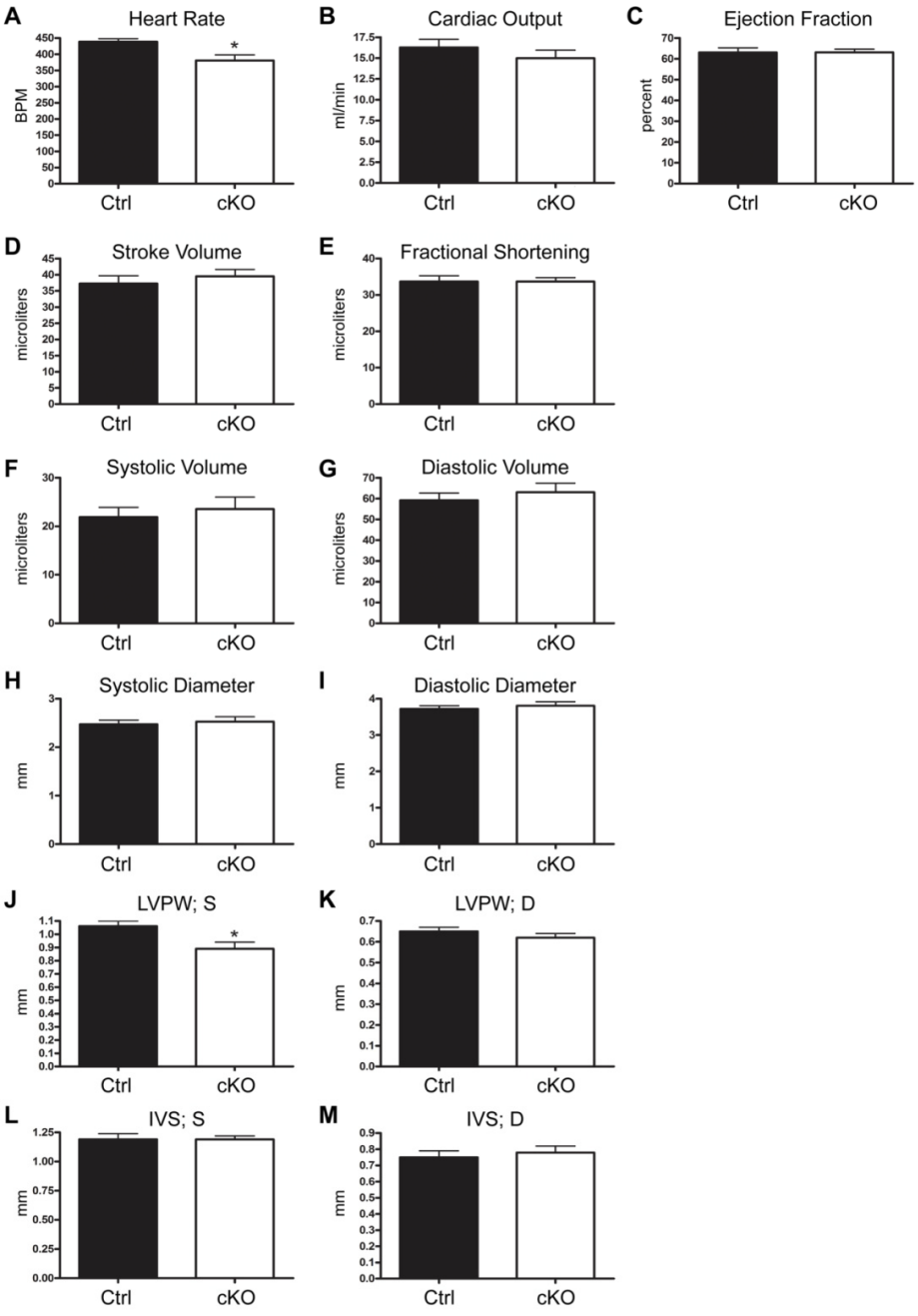
Supplemental Figure 4. βII spectrin cKO mice display bradycardia but normal peak heart rate response. Heart rate of conscious control mouse and βII spectrin cKO mice at baseline and following 2 mg/kg epinephrine I.P. Data represent mean heart rates of five mice/genotype recorded by telemetry ($p<0.05$ for cKO mice versus control mice at baseline).

Supplemental Figure 5



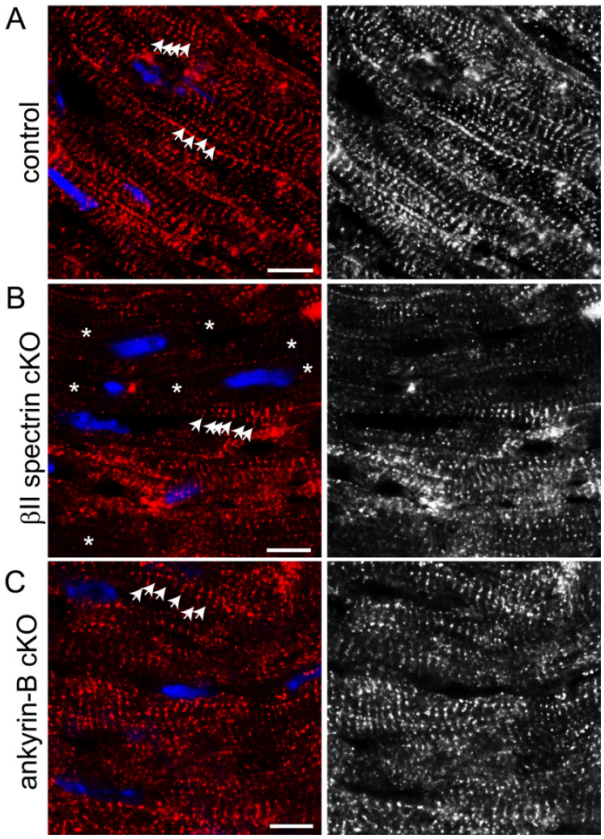
Supplemental Figure 5. βII spectrin cKO mice display normal cardiac function. (**A**) Normal baseline LVEF (69%) for 8 week old control mouse using M-mode end-systolic and -diastolic measurements. (**B**) Eight week βII spectrin cKO mouse with baseline LVEF of 67%.

Supplemental Figure 6



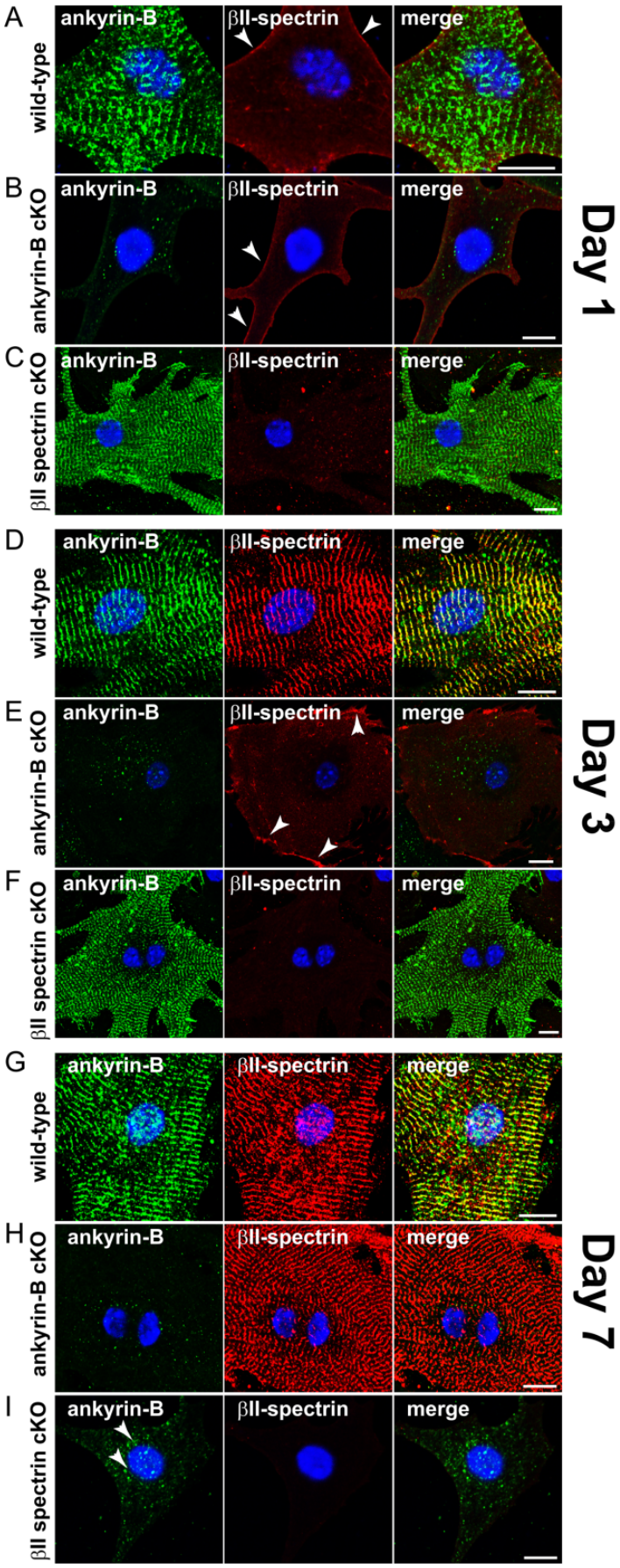
Supplemental Figure 6. βII spectrin cKO mouse cardiac measurements. (**A**) βII spectrin cKO mice displayed decreased heart rates when compared to control mice (n=5/genotype; $p<0.05$). There was no significant difference between control and βII spectrin cKO mice with respect to (**B**) cardiac output, (**C**) ejection fraction, (**D**) stoke volume, (**F**) systolic volume, (**G**) diastolic volume, (**E**) fractional shortening and (**H**) systolic and (**I**) diastolic diameter (n=5/genotype; N.S.). (**J**) Control mice displayed significantly more left ventricular posterior wall thickness (LVPW) during systole than βII spectrin cKO mice (n=5 mice/genotype, $p<0.05$). (**K**) LVPW thickness during diastole was similar between control and βII spectrin cKO mice (n=5 mice/genotype; N.S.). Intraventricular septal (IVS) thickness did not differ between control and βII spectrin cKO mice during systole (**L**) or diastole (**M**); (n=5 mice/genotype; N.S.).

Supplemental Figure 7



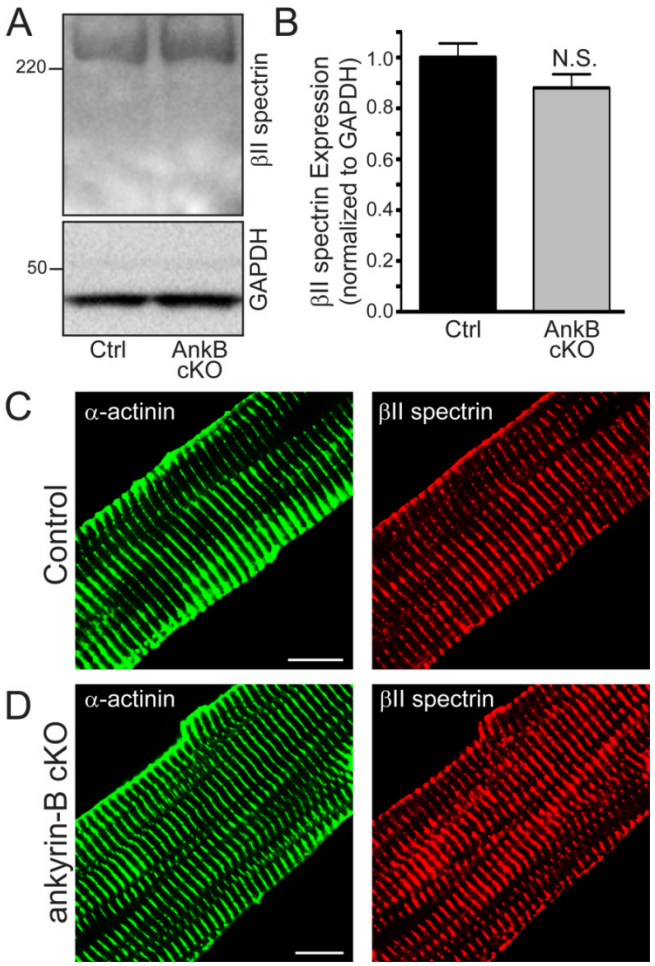
Supplemental Figure 7. βII spectrin cKO hearts display heterogeneity in RyR2 expression. RyR2 expression in (**A**) control, (**B**) βII spectrin cKO, and (**C**) ankyrin-B cKO left ventricle heart sections (multiple sections from n=3 hearts/genotype examined). RyR2 localization was generally homogeneous along the Z-line of control and ankyrin-B cKO hearts (white arrowheads), we observed heterogeneity in RyR2 expression both across myocyte sections as well as within single βII spectrin cKO myocytes (note * in panel B). Bar=10 μM.

Supplemental Figure 8



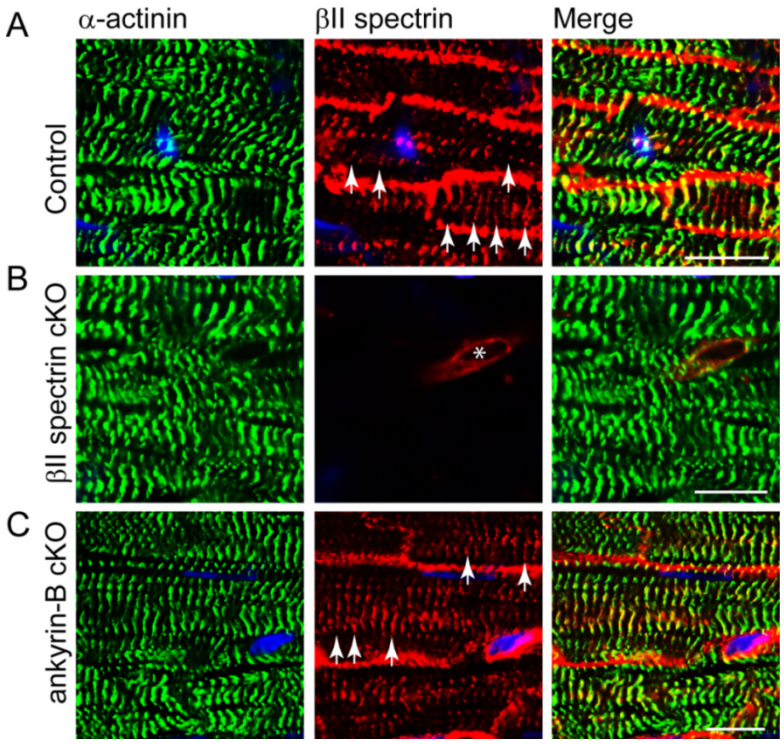
Supplemental Figure 8. Ankyrin-B targets βII spectrin in immature neonatal myocytes, whereas βII spectrin targets ankyrin-B in mature and adult myocytes. (**A-C**) Localization of ankyrin-B and βII spectrin in post-natal day 1 (P1) neonatal cardiomyocytes from control (**A**), ankyrin-B cKO (**B**), and βII spectrin cKO myocytes (**C**). Note that βII spectrin expression is minimal in the P1 myocyte whereas ankyrin-B is expressed and striated in control myocytes. Also note that ankyrin-B is expressed and striated in βII spectrin cKO myocytes. (**D-F**) At P3, βII spectrin is expressed in control myocytes and requires ankyrin-B for expression and localization. (**G-I**) In contrast to post-natal days 1-3, ankyrin-B is not required for βII spectrin expression or targeting. In fact, in these maturing myocytes, similar to adult myocytes, βII spectrin is required for ankyrin-B expression. Scale equals 10 microns in all panels.

Supplemental Figure 9



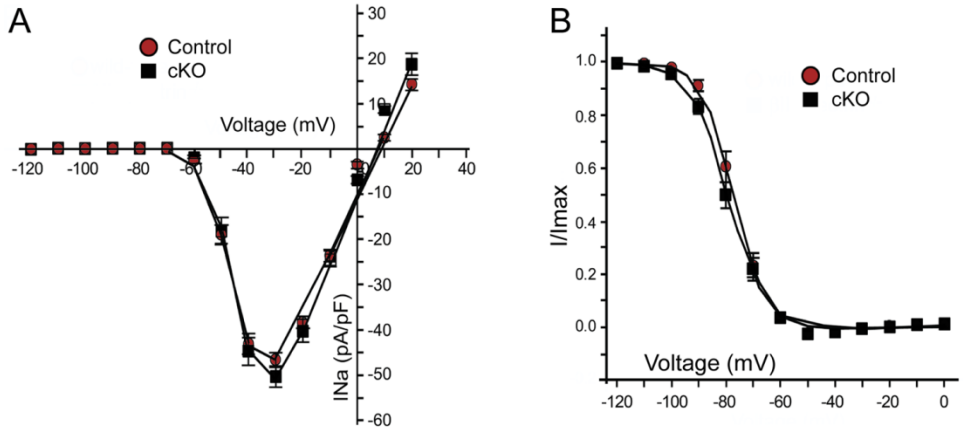
Supplemental Figure 9. βII spectrin levels are unchanged in hearts of ankyrin-B cKO mice. Expression levels of βII spectrin is not significantly different in protein lysates (A-B) from control (Ctrl, n=4) versus ankyrin-B cKO hearts (n=4; p=N.S.) or by immunostaining (C-D) of adult cardiomyocytes (red). Scale bar equals ten microns.

Supplemental Figure 10



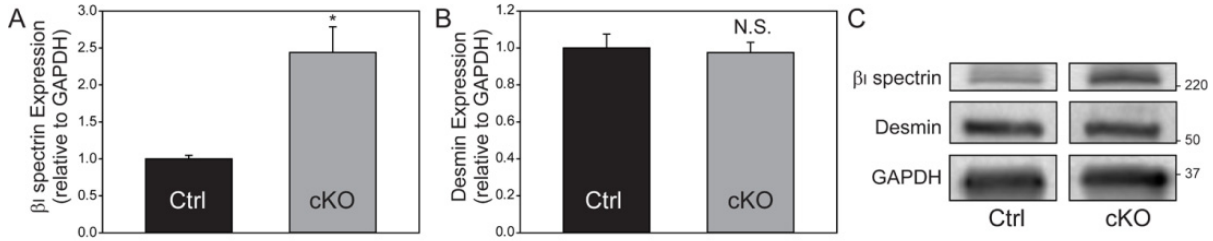
Supplemental Figure 10. Ankyrin-B is not required for βII spectrin expression or localization. βII spectrin localization (red) in control, βII spectrin cKO, and ankyrin-B cKO left ventricle. Sections are co-labeled with α-actinin. Note that in C, βII spectrin is normally expressed and striated in the absence of ankyrin-B. Also note that minor background of βII spectrin staining in βII spectrin cKO is small vessel (*), not ventricular myocytes. Bar=10 μM.

Supplemental Figure 11



Supplemental Figure 11. βII spectrin deficiency does not alter cardiomyocyte INa. **(A-B)** Control (n=10) and βII spectrin cKO mouse myocytes (n=11) display no difference in I_{Na} phenotypes (N.S.).

Supplemental Figure 12



Supplemental Figure 12. βII spectrin deficiency results in increased expression of βI spectrin. **(A, C)** βI spectrin levels are increased nearly two-fold in βII spectrin cKO hearts (n=5) compared to control hearts (n=5 hearts; $p<0.05$). **(B,C)** Desmin expression levels are equivalent between control (n=5) and βII spectrin cKO hearts (n=5; p=N.S.).