Supplementary Materials

Supplementary Materials and Methods

1. Genes Analyzed in AnkB-p.E1485G Proband with ARVC on Autopsy

Clinical genetic testing was performed with a commercial combined arrhythmia and cardiomyopathy panel (Ambry Genetics, Aliso Viejo, CA, USA) containing 79 genes:

ABCC9, ACTC1, ACTN2, AKAP9, ANKRD1, BAG3, CACNA1C, CACNA2D1, CACNB2, CALR3, CASQ2, CAV3, CRYAB, CSRP3, DES, DMD, DSC2, DSG2, DSP, EMD, EYA4, FXN, GATA4, GLA, GPD1L, ILK, JAG1, JPH2, JUP, KCNE1, KCNE2, KCNE3, KCNH2, KCNJ2, KCNJ8, KCNQ1, LAMP2, LDB3/ZASP, LMNA, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYOM1, MYOZ2, MYPN, NEBL, NEXN, NKX2.5, PDLIM3, PKP2, PLN, PRKAG2, PTPN11, RAF1, RBM20, RYR2, SCN1B, SCN3B, SCN4B, SCN5A, SGCD, SNTA1, TAZ, TBX1, TBX5, TCAP, TMEM43, TMPO TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, TXNRD2, VCL

2. Whole-Exome Sequencing in a Multi-Generational ARVC Kindred

Whole exome capture was performed using the Agilent SureSelect Human Exome Library Preparation kit (Agilent Technologies, Santa Clara, CA) and paired end sequencing was subsequently completed on an Illumina HiSeq 2500 platform. Reads were trimmed for adaptor read-through and low quality using Trimomatic and then aligned to the GRCh37/hg19 build human reference genome using BWA-MEM *(1)*. Polymerase chain reaction (PCR) duplicates were marked using Picard tools and local re-alignment, base recalibration, and variant calling were performed using GATK *(2)*.

Sequencing data was analyzed on SNP and Variation Suite version 8 (SVS, Golden Helix, Inc.) and aimed to identify shared heterozygous variants between the 2 affected individuals evaluated. All shared variants were filtered against gnomAD and those with an allele frequency > 0.005% were discarded. A standard coding variant (frameshift, nonsense, non-synonymous and synonymous) classification list was generated to identify candidates.

Based on biological plausibility, the novel ANK2-Met1988Thr mutation was selected as the top candidate. Sanger sequencing was utilized to confirm its presence in the probands and affected family members and absence in an unaffected family member.

3. ANK2 Sequencing and Bioinformatic Analysis at Study Sites and ARVC Registries

Amsterdam Academic Medical Center (University of Amsterdam)

Exome sequencing and analysis

Whole exome sequencing was carried out on 19 Dutch probands with genotype negative Task Force Criteria Positive ARVC. Captured fragments were sequenced on the Hiseq2000 sequencer (Illumina, San Diego, CA, USA) to an average depth of >50 reads per target base; mean fold coverage of the *ANK2* exons and flanking intron boundaries was 282.2. The sequence reads were aligned to the human reference genome (UCSC NCBI37.1/hg19) using SOAPaligner (version 2.21, Beijing Genomics Institute, Shenzhen, China), and functional annotation of high-quality variants was performed using SOAPsnp software (Beijing Genomics Institute) for the single-nucleotide variation *(3)*. For insertion/deletion detection, sequence reads were aligned by BWA and annotated by GATK for break-point identification *(2, 4)*.

Data analysis was performed in the statistical programming environment R version 3.2.1 (Vienna, Austria). Synonymous variants not located at splice sites and variants with a frequency >0.1% in the following publically available databases were excluded from further analysis: (1) the Genome Aggregation Database (gnomAD) Browser, (2) the National Heart, Lung, and Blood Institute Exome Sequencing Project, (3) Phase 1 version 3 of the 1000 Genomes project (data release August 2015), (4) Genome of the Netherlands and an in-house exome database *(5, 6)*.

Median coverage depth of the 53 *ANK2* exons that underwent sequencing was 241-fold. Two *ANK2* variants, Thr466Met and Thr3744Asn, meeting pre-specified criteria were identified among 19 genotype negative Task Force Criteria positive ARVC study participants. Both variants were subsequently confirmed with Sanger sequencing.

Johns Hopkins Medical Center

Exome sequencing and analysis

26 samples underwent whole-exome sequencing following capture of the CCDS exonic and flanking intronic regions totaling ~51Mb using the Agilent SureSelect Human All Exon V4 51Mb Kit (Agilent Technologies, Santa Clara, CA, USA). An additional 84 samples were captured using a custom Agilent 500kb panel that contained the *ANK2* gene. 100 base pair paired-end sequencing was performed using the Illumina HiSeq2000 platform (Illumina, San Diego, CA). Reads were aligned to the reference genome (NCBI human genome assembly build 37; Ensembl core database release 50_361) using the Burrows-Wheeler Alignment (BWA) tool and single nucleotide variants (SNVs) and small insertion-deletions (indels) were identified using SAMtools *(4, 7)*. Local realignment and base call quality recalibration was performed using GATK *(2, 8)*. Potential causal variants were identified using standard filtering criteria: SNV and indel minimal depth of 8X, root mean square mapping quality of 25, strand bias p-value below 10^{-4}, end distance bias below 10^{-4}, and filtering out SNVs within 3bp of an indel and indels within 10bp of each other; followed by the use of the PhenoDB *(9)* Variant Analysis to design the prioritization strategy *(10)*. Rare heterozygous or homozygous functional variants (missense, nonsense, splice site variants and indels) were prioritized, excluding variants with a minor allele frequency >0.1% in gnomAD or 1000 Genomes Project, and/or those that were present in dbSNP 126, 129, 131, 135 and/or 137 *(6)*. Variants found within in-house controls (CIDRVar 51Mb) were also excluded.

The median depth of coverage of the 53 *ANK2* exons that underwent sequencing was 136-fold. A total of 9 *ANK2* variants meeting our pre-specific criteria were identified among 110 genotype negative Task Force Criteria positive ARVC study participants.

Clinical sequencing

Five genotype negative Task Force Criteria positive ARVC study participants underwent *ANK2* sequencing as part of clinical testing with commercially available panels. The panels, along with their commercial vendor, are provided below:

Invitae Corporation (San Francisco, CA, USA) - Performed in 2 patients

ABCC9, ACTC1, ACTN2, AKAP9, ALMS1, ANK2, ANKRD1, BAG3, CACNA1C, CACNA2D1, CACNB2, CALM1, CALM2, CALM3, CALR3, CASQ2, CAV3, CRYAB, CSRP3, CTF1, CTNNA3, DES, DMD, DSC2, DSG2, DSP, DTNA, ELAC2, EMD, EYA4, FHL1, FHL2, FKRP, FKTN, GATA4, GATA6, GATAD1, GLA, GPD1L, HCN4, ILK, JPH2, JUP, KCND3, KCNE1, KCNE2, KCNE3, KCNE5, KCNH2, KCNJ2, KCNJ5, KCNJ8, KCNQ1, LAMA4, LAMP2, LDB3, LMNA, MTO1, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOM1, MYOZ2, MYPN, NEBL, NEXN, NKX2-5, NPPA, PDLIM3, PKP2, PLN, PRDM16, PRKAG2, RAF1, RANGRF, RBM20, RYR2, SCN10A, SCN1B, SCN2B, SCN3B, SCN4B, SCN5A, SDHA, SGCD, SLMAP, SNTA1, TAZ, TCAP, TGFB3, TMEM43, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TRDN, TRPM4, TTN, TTR, TXNRD2, VCL.

GeneDx (Gaithersburg, MD, USA)

ABCC9, ACTC1, ACTN2, AKAP9, ANK2, ANKRD1, BAG3, BRAF, CACNA1C, CACNB2, CASQ2, CAV3, CRYAB, CSRP3, DES, DMD, DSC2, DSG2, DSP, DTNA, EMD, FKTN, GATAD1, GLA, GPD1L, HCN4, HRAS, ILK, JPH2, JUP, KCNE1, KCNE2, KCNE3, KCNH2, KCNJ2, KCNJ5, KCNJ8, KCNQ1, KRAS, LAMA4, LAMP2, LDB3, LMNA, MAP2K1, MAP2K2, MTND1, MTND5, MTND6, MTTD, MTTG, MTTH, MTTI, MTTK, MTTL1, MTTL2, MTTM, MTTQ, MTTS1, MTTS2, MYBPC3, MYH7, MYL2, MYL3, MYLK2, MYOZ2, MYPN, NEBL, NEXN, NKX2-5, NRAS, PDLIM3, PKP2, PLN, PRKAG2, PTPN11, RAF1, RBM20, RANGRF, RYR2, SCN1B, SCN3B, SCN4B, SCN5A, SGCD, SNTA1, SOS1, TAZ, TCAP, TMEM43, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, VCL.

Ambry Genetics (Aliso Viejo, CA, USA)

ABCC9, ACTC1, ACTN2, ANKRD1, BAG3, CALR3, CAV3, CRYAB, CSRP3, DES, DMD, DSC2, DSG2, DSP, EMD, EYA4, FXN, GLA, ILK, JPH2, JUP, LAMP2, LDB3/ZASP, LMNA, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYOM1, MYOZ2, MYPN, NEBL, NEXN, PDLIM3, PKP2, PLN, PRKAG2, PTPN11, RAF1, RBM20, RYR2, SCN5A, SGCD, TAZ, TCAP, TMEM43, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, TXNRD2, VCL.

Ambry Genetics (Aliso Viejo, CA, USA)

ABCC9, ACTC1, ACTN2, AKAP9, ANK2, ANKRD1, BAG3, CACNA1C, CACNA2D1, CACNB2, CALR3, CASQ2, CAV3, CRYAB, CSRP3, DES, DMD, DSC2, DSG2, DSP, EMD, EYA4, FXN, GATA4, GLA, GPD1L, ILK, JAG1, JPH2, JUP, KCNE1, KCNE2, KCNE3, KCNH2, KCNJ2, KCNJ8, KCNQ1, LAMP2, LDB3/ZASP, LMNA, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYOM1, MYOZ2, MYPN, NEBL, NEXN, NKX2.5, PDLIM3, PKP2, PLN, PRKAG2, PTPN11, RAF1, RBM20, RYR2, SCN1B, SCN3B, SCN4B, SCN5A, SGCD, SNTA1, TAZ, TBX1, TBX5, TCAP, TMEM43, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, TXNRD2, VCL.

The ANK2-p.Gly290Ser variant, which met pre-specified criteria, was identified in a single patient that underwent clinical screening.

Familial Cardiomyopathy Registry, University of Colorado

Sequencing of *ANK2* was performed using the TruSight One Sequencing Panel (Illumina, San Diego, CA, USA), a next generation sequencing panel containing 4,813 genes. Paired-end sequencing was performed on an Illumina HiSeq 2500 sequencer and then mapped using GSNAP *(11)*. Variants were called with GATK and classified with Annovar *(2, 12)*. Variants that changed an amino acid or altered splice sites were selected and dbNSFP was subsequently used to provide predictions for all non-synonymous single-nucleotide polymorphisms *(13)*. Variants predicted to be damaging in at least one of the prediction algorithms were retained, while missense and truncation variants present in greater than 1.0% in the 1000 Genomes Project were discarded.

No *ANK2* variants meeting pre-specified criteria were identified among 32 genotype negative Task Force Criteria positive ARVC study participants.

University Hospital Linköping

ANK2 sequencing was performed as part of a custom next-generation sequencing panel containing 84 genes that was created using SureDesign (Agilent Technologies, Santa Clara, CA, USA). Standard settings were used for DNA obtained from blood samples, while High Sensitivity and formalin fixed paraffin embedded (FFPE) settings, including molecular barcoding, were used for FFPE samples. Library preparation was performed using the HaloPlex Target Enrichment System (Agilent

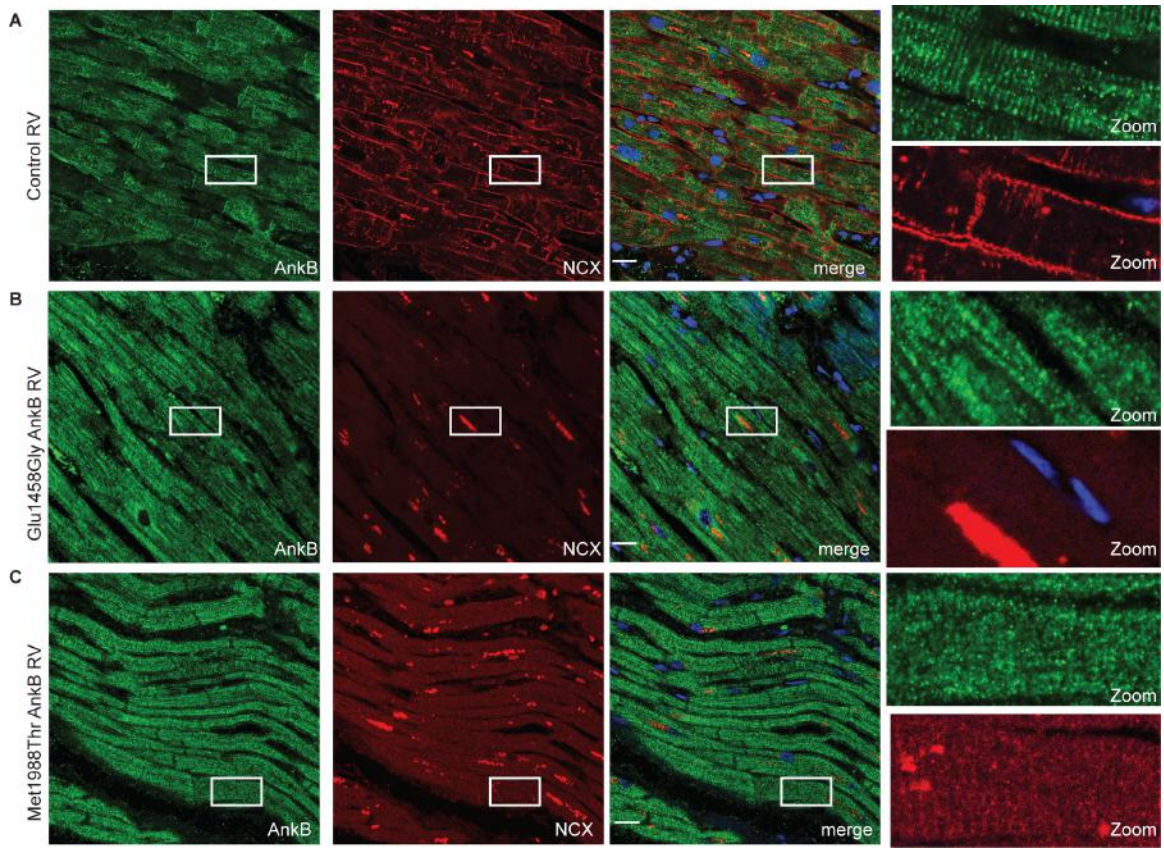
Technologies) in accordance with manufacturer recommendations. Paired-end sequencing was performed using MiSeq (Illumina, San Diego, CA, USA). FASTQ files from FFPE samples were analyzed using SureCall (Agilent Technologies), while FASTQ files from blood samples were analyzed using an in-house bioinformatics pipeline and non-synonymous and splice sites variants with allele frequencies < 0.1% in gnomAD were retained for further analysis *(14)*.

Two *ANK2* variants, Ile964Val and Arg2069His, meeting our pre-specified criteria were identified among 19 genotype negative Task Force Criteria positive ARVC study participants. Both variants were subsequently confirmed with Sanger sequencing.

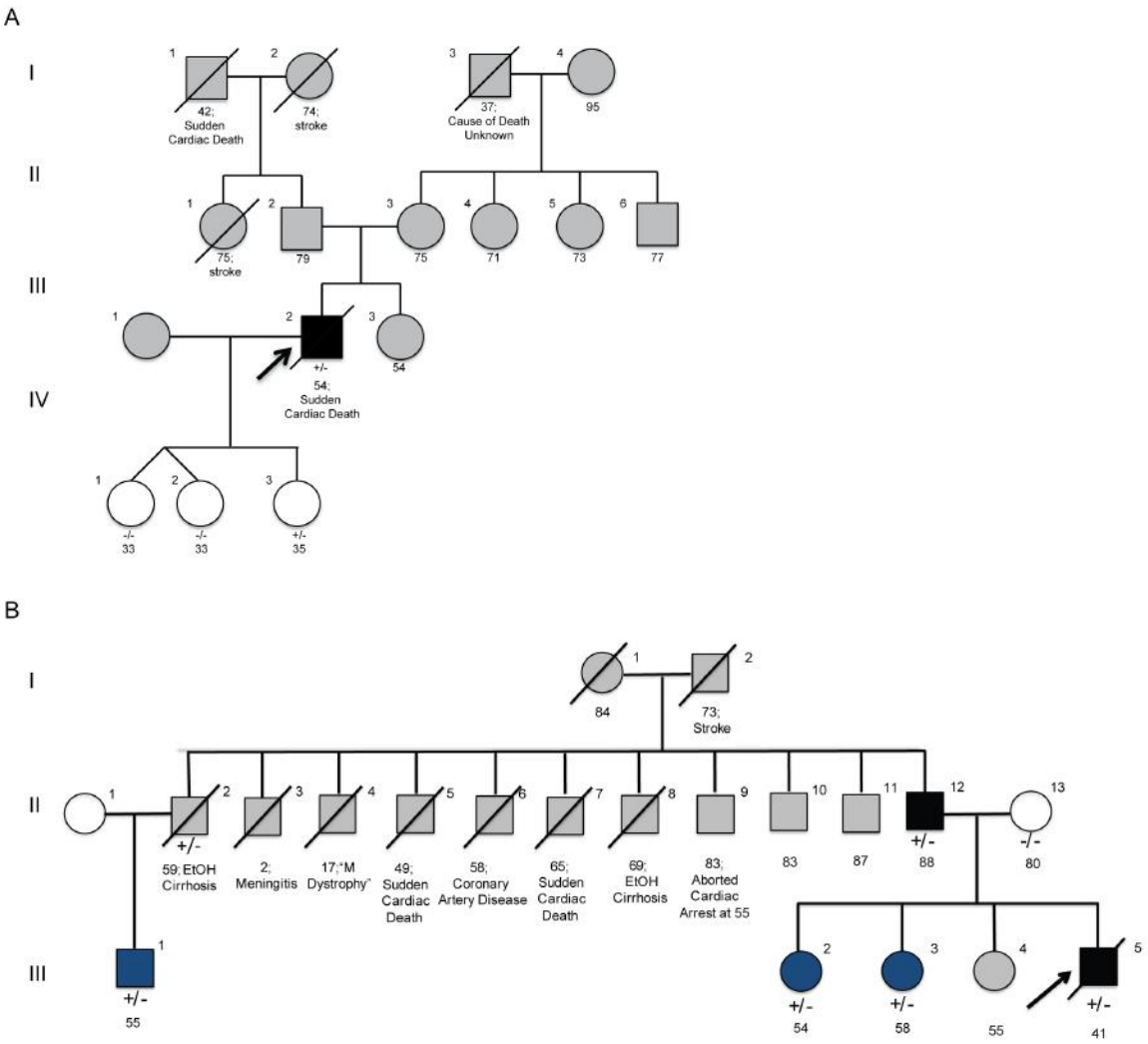
Canadian ARVC Registry and Toronto General Hospital

The Illumina Design Studio tool was used to generate TruSeq Custom Amplicon sequencing targets for *ANK2*. Samples were prepared according to standard library preparation protocols (Illumina Inc., San Diego, CA, USA) and then sequenced on the Illumina MiSeq sequencer using the v2 300 cycle sequencing kit in 2 x 150 paired end sequencing mode. The sequencer was configured to generate .bam and .vcf files using on-board MiSeq Reporter software invoking GATK based variant calling, using default parameters *(8)*. The .vcf files were then annotated using a custom-scripted instance of ANNOVAR *(12)*. Variants that were within 15 base pairs of an exon boundary and had a prevalence of < 0.1% in either 1000 Genomes, Exome Sequencing Project or gnomAD population databases were retained for consideration. The median coverage depth of the 53 *ANK2* exons sequenced was 255-fold.

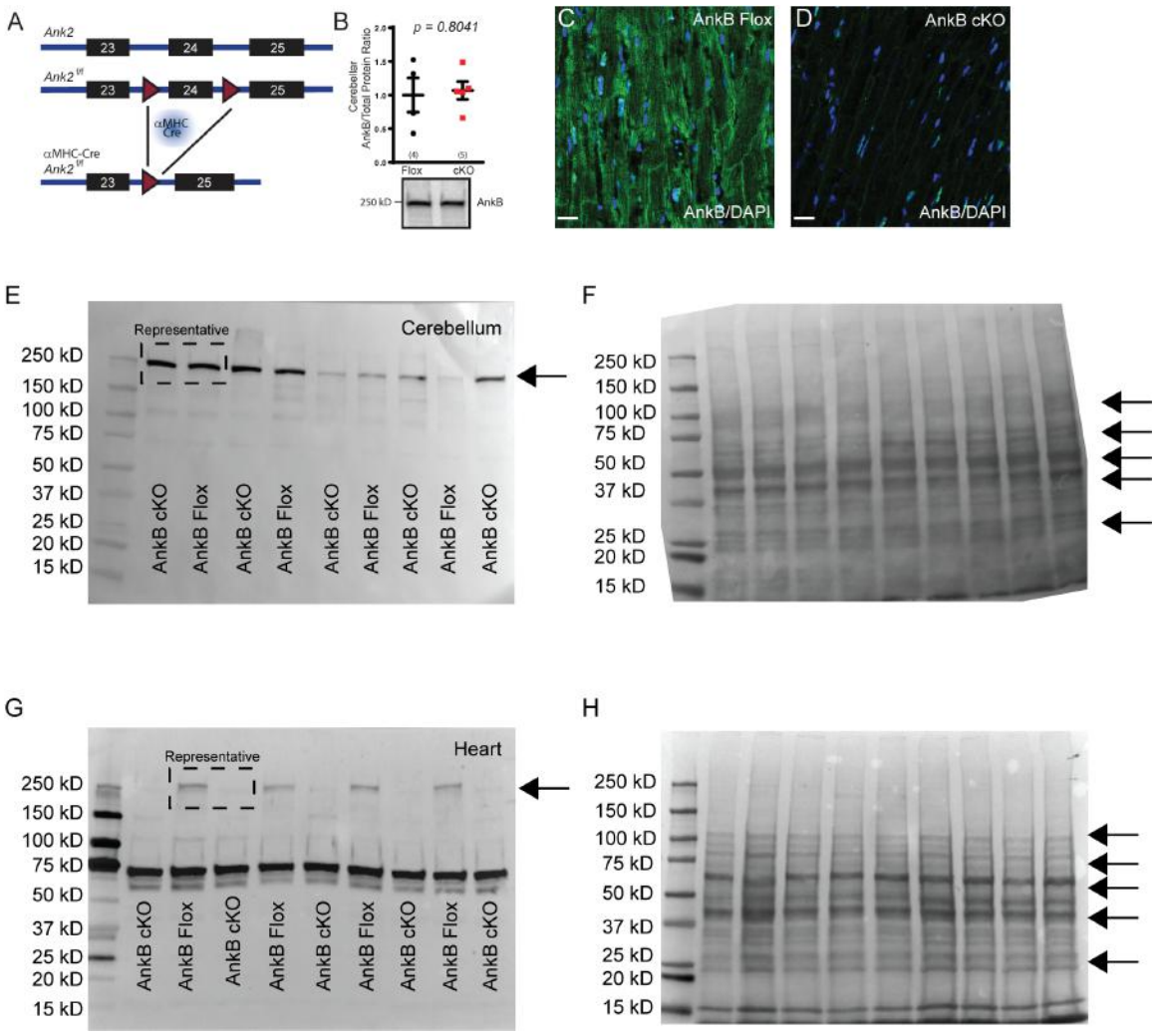
No *ANK2* variants meeting our pre-specific criteria were identified among 26 genotype negative Task Force Criteria positive ARVC study participants.



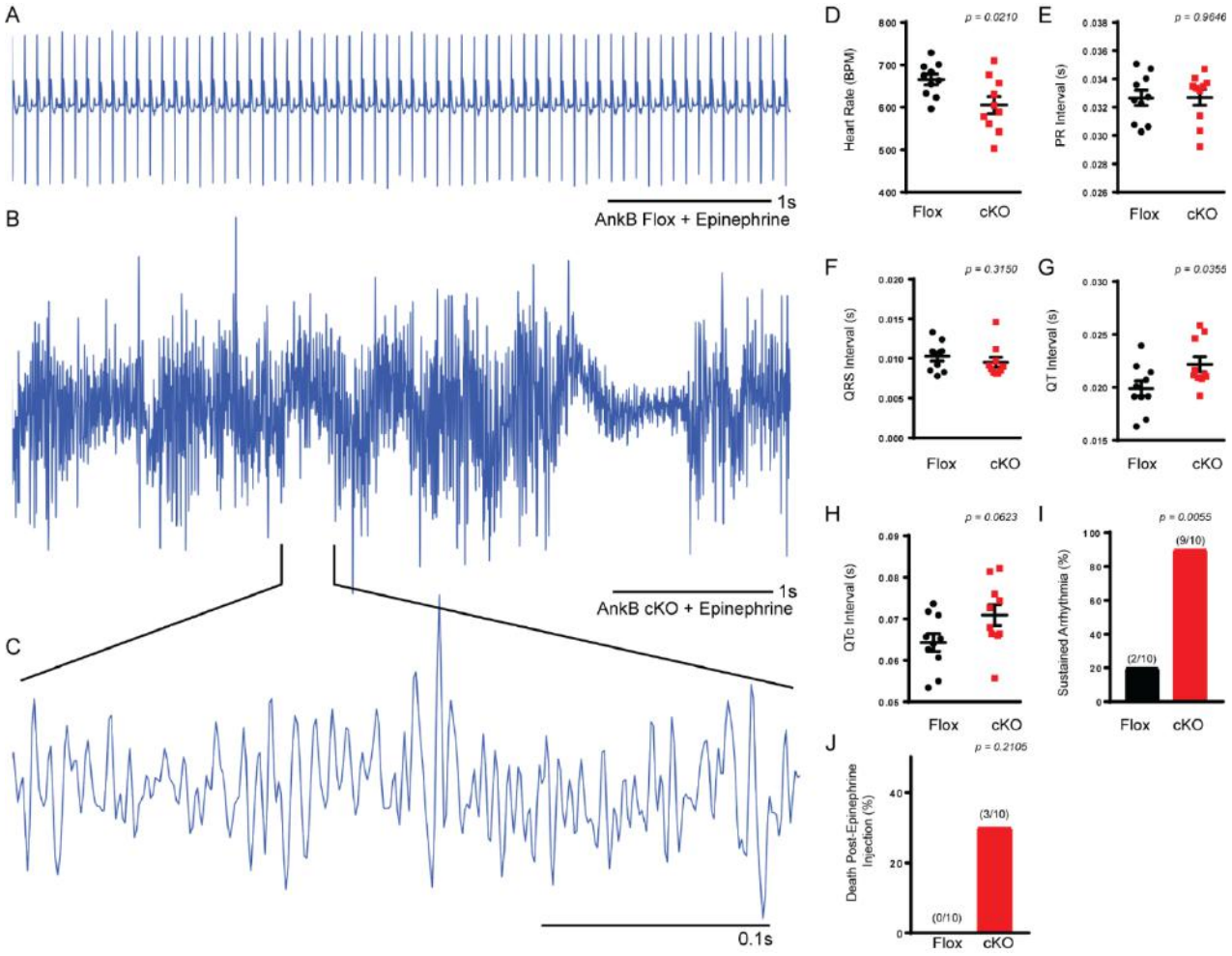
Supplemental Figure 1 | Patients with AnkB variants display abnormal localization of AnkB and NCX. a-c, Confocal microscopy images detailing localization of AnkB and NCX in control right ventricle (**a**), AnkB-p.Glu1458Gly right ventricle (**b**), and AnkB-p.Met1988Thr right ventricle (**c**). Scale bars represent 20 μm.



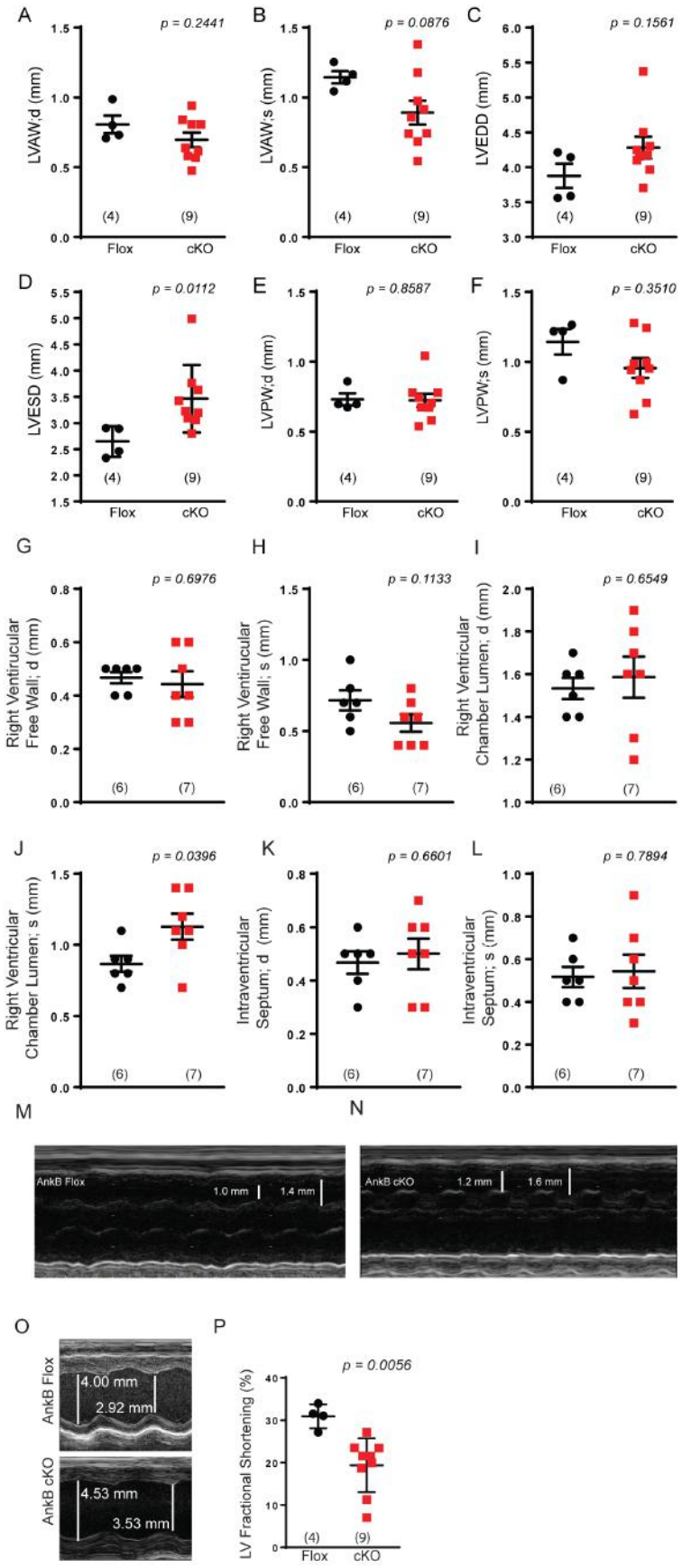
Supplemental Figure 2 | AnkB-p.Glu1458Gly and -p.Met1988Thr family pedigrees. Black and navy blue filled circles denote affected family members with Definite and Borderline Task Force Criteria positive diagnoses, respectively. Family members that were inaccessible or declined evaluation are shaded in grey. Genotype is denoted as +/- and age at evaluation or at time of death, along with presumed cause of death, is provided.



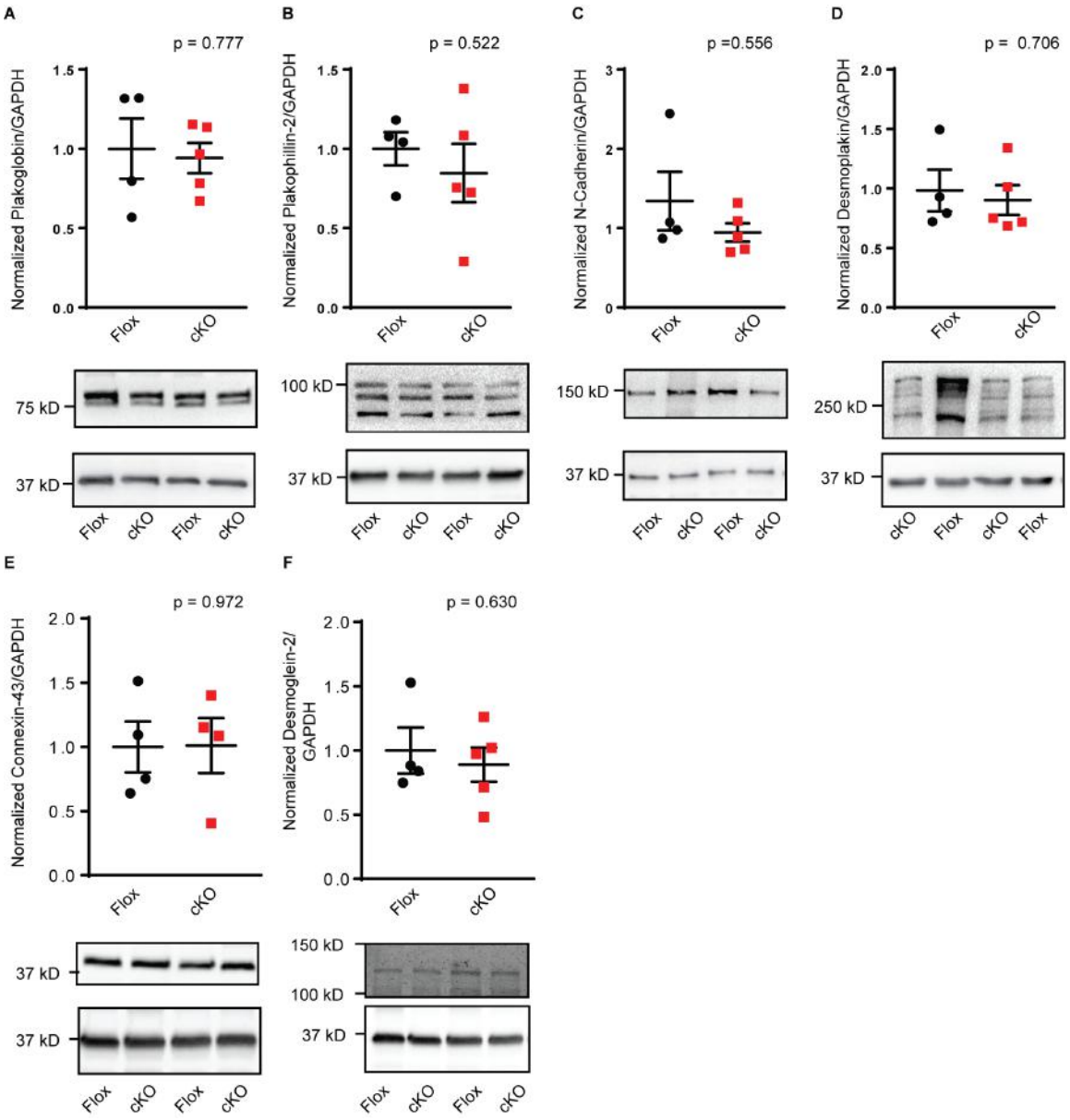
Supplemental Figure 3 | AnkB cKO mice exhibit cardiac specific deletion of AnkB. **a-d,** Strategy for deletion of *Ank2* in cardiomyocytes (**a**), immunoblotting for AnkB in cerebellum of AnkB Flox and cKO mice, n =4,5, (**b**), and immunofluorescence images of AnkB in AnkB Flox (**c**) and AnkB cKO (**d**) cardiac cryoslices. Scale bars represent 20 µm. Images representative of n = 3 from each group. **g-h**,AnkB immunoblot (arrow represents AnkB) (**e**) and corresponding total protein Ponceau stain(arrows indicate bands quantified for total protein) (**f**) in cerebellar tissue from AnkB Flox andAnkB cKO mice. **g-h**, AnkB immunoblot (arrow represents AnkB) (**g**) and corresponding totalprotein Ponceau stain (arrows indicate bands quantified for total protein) (**h**) in cardiac tissuefrom AnkB Flox and AnkB cKO mice. The immunoblots shown in this figure are the full immunoblots from Figure 3.



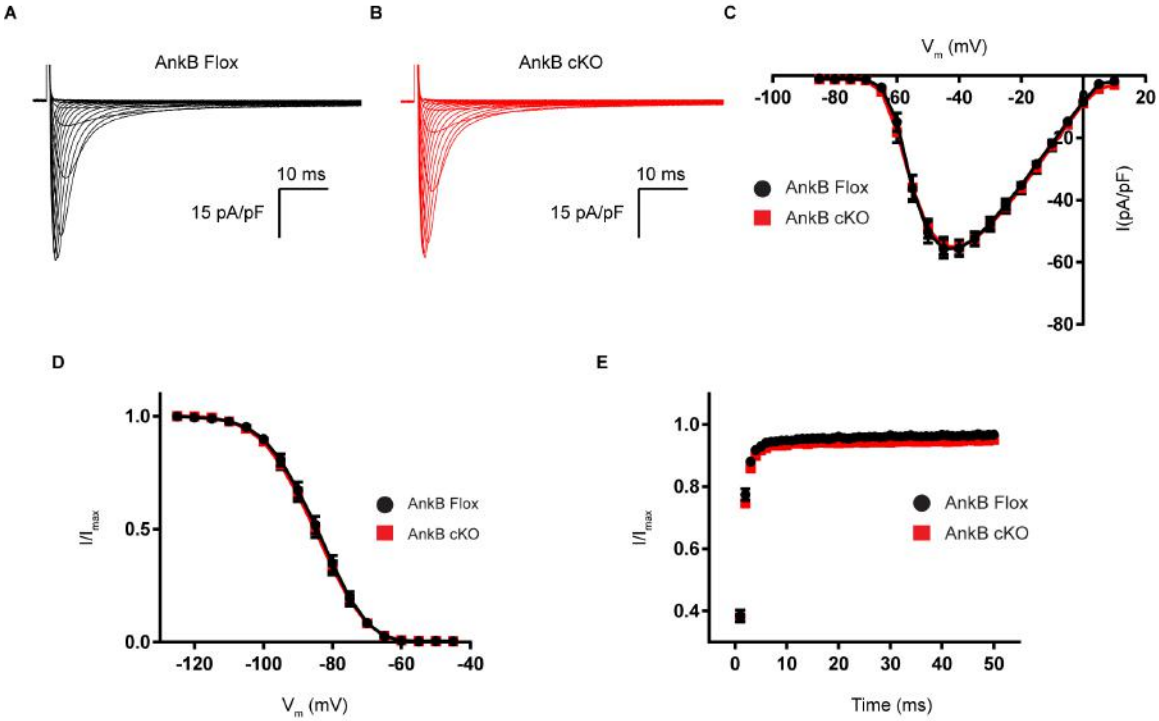
Supplemental Figure 4 | Representative electrocardiograms and complete electrocardiogram parameters of AnkB cKO mice. **a-c**, Representative electrocardiograms from AnkB Flox mice (**a**) and AnkB cKO mice demonstrating fatal ventricular fibrillation (**b,c**) following 2.0 mg/kg injection of epinephrine. **d-h** Electrocardiogram parameters: heart rate (**d**), PR interval (**e**), QRS duration (**f**), QT interval (**g**) QTc interval (**h**) in telemetry-implanted AnkB Flox and AnkB cKO mice. **i-j**, Percentage of mice with sustained arrhythmia (**i**) and death post (**j**) epinephrine injection in AnkB Flox and AnkB cKO mice, n = 10,10. Shown are mean ± SEM; statistical analysis is two tailed parametric t-test at 95% CI in **d,e,f,g,h**. Statistical analysis is Fisher's exact test in **i,j**.


A
p = 0.2441
LVAW;d (mm)
(4)
(9)
Flox
cKO
B
p = 0.0876
LVAW;s (mm)
C
p = 0.1561
LVEDD (mm)
D
p = 0.0112
LVESD (mm)
E
p = 0.8587
LVPW;d (mm)
F
p = 0.3510
LVPW;s (mm)
G
p = 0.6976
Right Ventricular Free Wall; d (mm)
(6)
(7)
H
p = 0.1133
Right Ventricular Free Wall; s (mm)
I
p = 0.6549
Right Ventricular Chamber Lumen; d (mm)
J
p = 0.0396
Right Ventricular Chamber Lumen; s (mm)
K
p = 0.6601
Intraventricular Septum; d (mm)
L
p = 0.7894
Intraventricular Septum; s (mm)
M
N
AnkB Flox
1.0 mm
1.4 mm
AnkB cKO
1.2 mm
1.6 mm
O
AnkB Flox
4.00 mm
2.92 mm
AnkB cKO
4.53 mm
3.53 mm
P
p = 0.0056
LV Fractional Shortening (%)

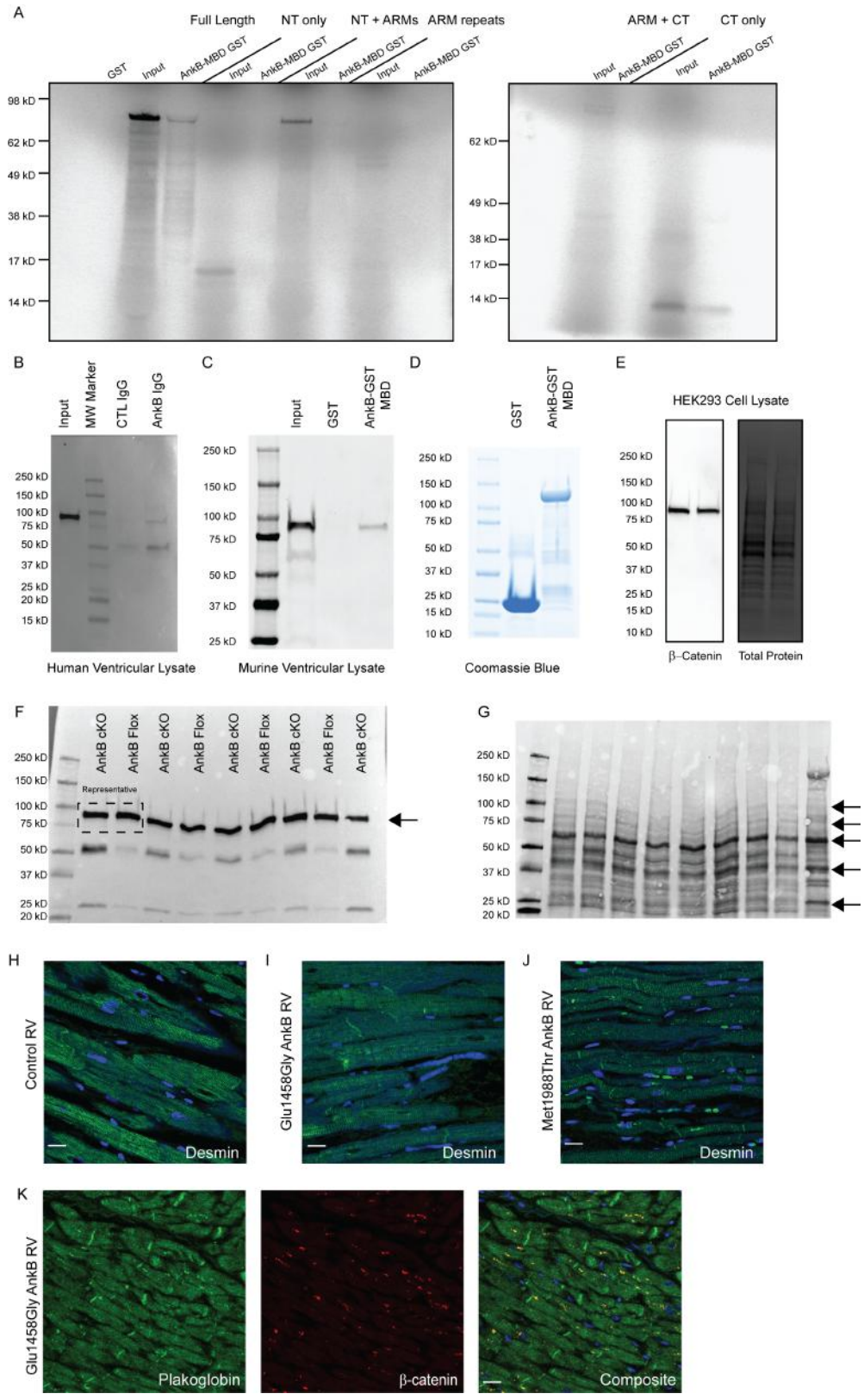
Supplemental Figure 5 | Complete echocardiographic parameters of AnkB cKO mice. a-f, Echocardiogram parameters: left ventricular anterior wall at diastole (LVAW;d, **a**), left ventricular anterior wall at systole (LVAW;s, **b**), left ventricular end diastolic diameter (LVEDD, **c**), left ventricular end systolic diameter (LVESD, **d**), left ventricular posterior wall at diastole (LVPW;d, **e**), left ventricular posterior wall at systole (LVPW;s, **f**), n =4,9, right ventricular free wall at diastole (**g**), right ventricular free wall at systole (**h**), right ventricular chamber lumen at diastole (**i**),right ventricular chamber lumen at systole (**j**), intraventricular septum at diastole (**k**), intraventricular septum at systole (**l**), n =6,7. Representative AnkB Flox and AnkB cKO right ventricular m-mode images (**m,n**) **o-p**. Representative m mode left ventricular echocardiogram images (**o**) and quantification of left ventricular fractional shortening, n = 4,9. (**p**). Shown are mean ±SEM;statistical analysis is two tailed parametric t-test at 95% CI.



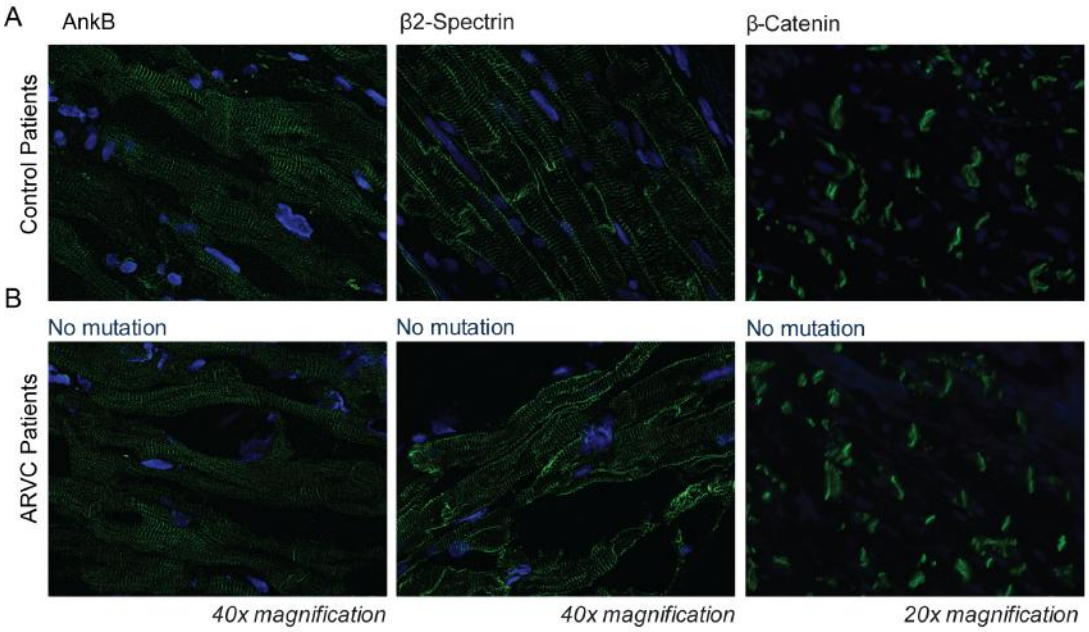
Supplemental Figure 6 | Immunoblots of intercalated disc proteins. a-f, Quantitative immunoblots from AnkB Flox and AnkB cKO cardiac lysates for plakoglobin (**a, n =4,5**), plakophillin-2 (**b, n =4,5**), N-cadherin (**c, n =4,5**), desmoplakin (**d, n =4,5**), connexin-43 (**e, n =4,4**), and desmoglein-2 (**f, n =4,5**) and GAPDH loading controls. Shown are mean ± SEM; statistical analysis is parametric t-test at 95% CI.



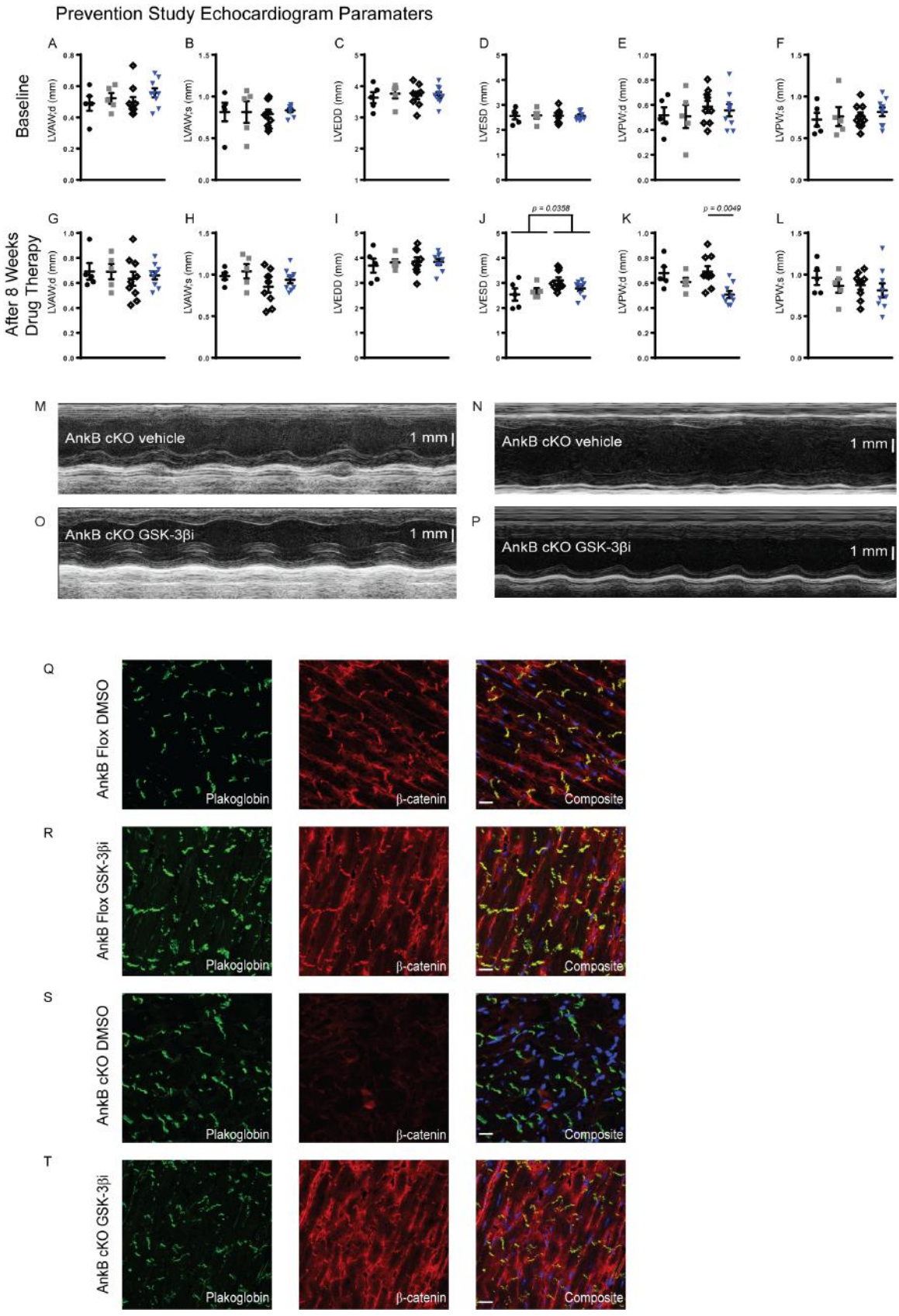
Supplemental Figure 7 | AnkB cKO mice fail to exhibit cardiac sodium channel dysfunction. **a-b**, Representative recordings of whole cell I_{Na} from AnkB Flox and AnkB cKO ventricular myocytes. (**c**) Current-voltage relationship, (**d**) voltage-dependent activation and voltage-dependent inactivation curves, and (**e**) time-dependent recovery of I_{Na} in AnkB Flox and AnkB cKO ventricular myocytes. No significant difference was observed in peak I_{Na} at experimental voltages ranging from -60 to -15mV (p=N.S.), in $V_{½}$ as determined by Boltzmann fits of the steady-state voltage-dependent inactivation (p=N.S.) and time-dependent recovery (p=N.S.; N=3,3, n=12,14) Shown are mean ± SEM; statistical analysis is two tailed parametric t-test at 95% CI.


A
Full Length
NT only
NT + ARMs
ARM repeats
ARM + CT
CT only
GST
Input
AnkB-MBD GST
98 kD
62 kD
49 kD
38 kD
17 kD
14 kD
B
Input
MW Marker
CTL IgG
AnkB IgG
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
Human Ventricular Lysate
C
Input
GST
AnkB-GST MBD
Murine Ventricular Lysate
D
GST
AnkB-GST MBD
Coomassie Blue
E
HEK293 Cell Lysate
β–Catenin
Total Protein
F
AnkB cKO
AnkB Flox
Representative
G
H
Control RV
Desmin
I
Glu1458Gly AnkB RV
J
Met1988Thr AnkB RV
K
Plakoglobin
β-catenin
Composite

Supplemental Figure 8 | Complete Gels for AnkB -β-catenin binding experiments and β-catenin immunoblots. a-e, Complete gels for direct binding experiment between AnkB-MBD GST and 35[S]-β-catenin (**a**), co-immunoprecipitation assay between AnkB IgG and β-catenin (**b**), pulldown assay between AnkB-MBD GST and β-catenin (**c**), Coomassie Blue gel of *in vitro* translated products (d), HEK293 cell lysate β-catenin immunoblot (e), and complete AnkB Flox and AnkB cKO β-catenin immunoblot (arrow represents β-catenin, **f**) and corresponding total protein Ponceau stain (arrows represent bands quantified for total protein), **g**) in AnkB Flox and AnkB cKO cardiac lysates. **h-j**,Immunofluorescence images of desmin in control (**h**), AnkB-p.Glu1458Gly (**i**), and AnkB-p.Met1988Thr (**j**) right ventricular tissue. Immunofluorescence images of plakoglobin and β-catenin in AnkB-p.Glu1458Gly proband (**k**).Scale bars represent 20 μm. The immunoblots shown in this figure are the full immunoblots from Figure 5.



Supplemental Figure 9 | AnkB, β-catenin, and βII-spectrin remain normally localized in non-AnkB related arrhythmogenic cardiomyopathy. a-b, Immunofluorescence images of AnkB, βII -spectrin, and β-catenin in control (**a**) and ARVC (**b**) human cardiac tissue. We observed similar findings (normal AnkB, βII spectrin, β-catenin) in all samples from diseased (n=4, no ANK2 variant) versus non-diseased (n=5) sections.


Prevention Study Echocardiogram Paramaters
Baseline
After 8 Weeks Drug Therapy
A
B
C
D
E
F
G
H
I
J
K
L
LVAW;d (mm)
LVAW;s (mm)
LVEDD (mm)
LVESD (mm)
LVPW;d (mm)
LVPW;s (mm)
p = 0.0358
p = 0.0049
M
AnkB cKO vehicle
1 mm
N
AnkB cKO vehicle
1 mm
O
AnkB cKO GSK-3βi
1 mm
P
AnkB cKO GSK-3βi
1 mm
Q
AnkB Flox DMSO
R
AnkB Flox GSK-3βi
S
AnkB cKO DMSO
T
AnkB cKO GSK-3βi
Plakoglobin
β-catenin
Composite

Supplemental Figure 10 | Complete echocardiographic parameters of GSK3-βi prevention Study and β-catenin immunofluorescence a-f, Echocardiogram parameters: LVAW;d, (**a**), left ventricular LVAW;s, (**b**), LVEDD,(**c**), LVESD, (**d**), LVPW;d, (**e**), LVPW;s, (**f**) of vehicle and GSK3-βi treated mice at baseline. **g-l**, Echocardiogram parameters: LVAW;d,(**g**), LVAW;s, (**h**), LVEDD, (**i**), LVESD, (**j**), LVPW;d,(**k**), LVPW;s, (**l**) of vehicle and GSK3-βi treated mice after 8 weeks of drug therapy, n =5,5,9,9. **m-p,** Representative m-mode echocardiograms in AnkB cKO mice at 4 weeks of age (**m,n**) and after 8 weeks of drug therapy in vehicle and GSK3-βi therapy AnkB cKO mice (**o,p**).**q-t**, Immunofluorescence images of plakoglobin and β-catenin in vehicle and GSK3-βi treated AnkB Flox and AnkB cKO mice (**m-p)**. Images representative of n=3 from each genotype and treatment condition. Shown are mean ± SEM; statistical analysis is two way ANOVA with Tukey post-hoc comparisons.

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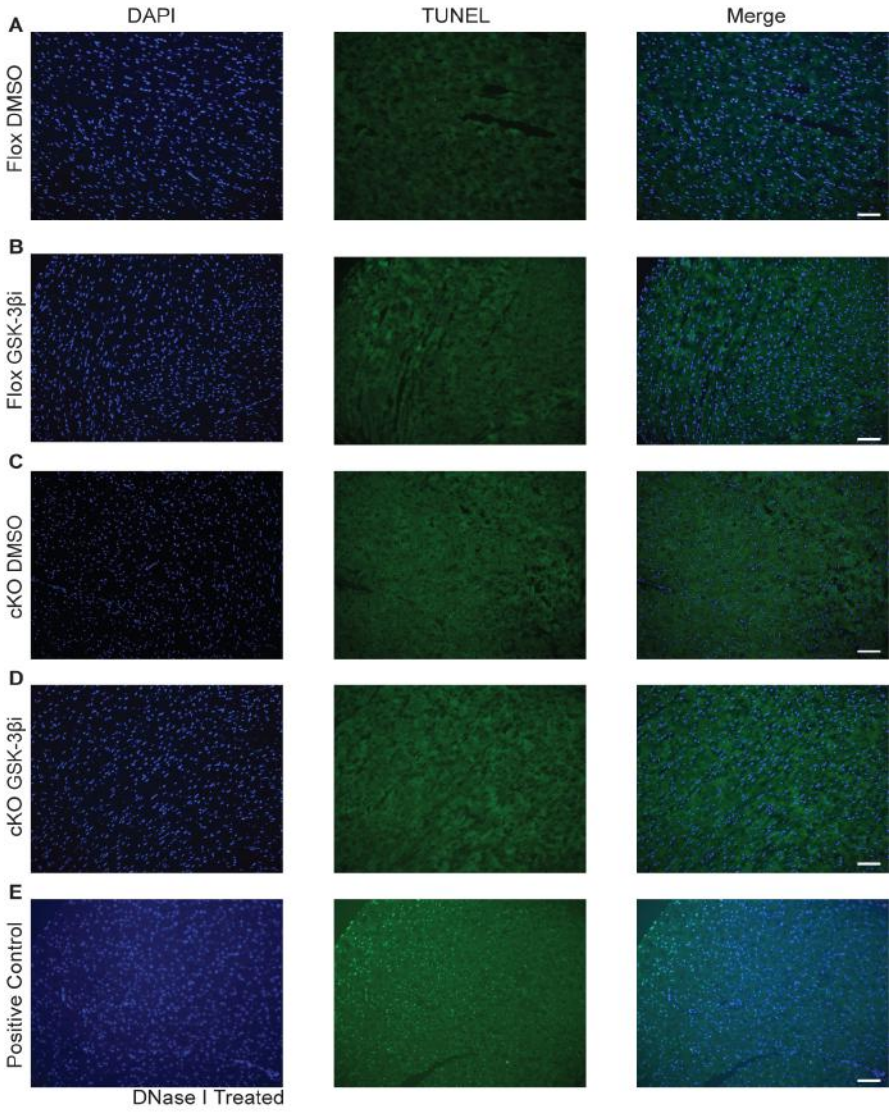
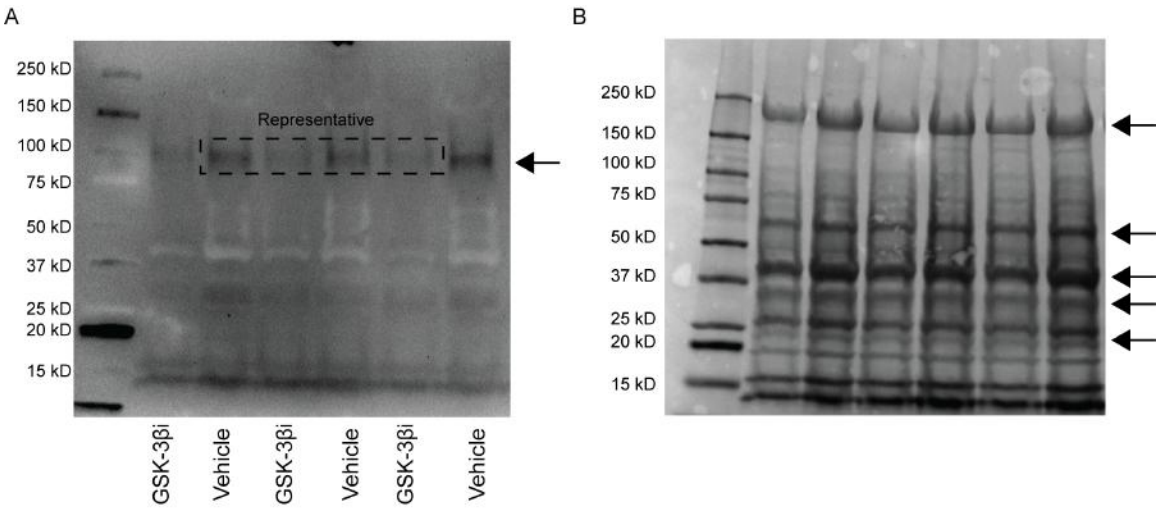
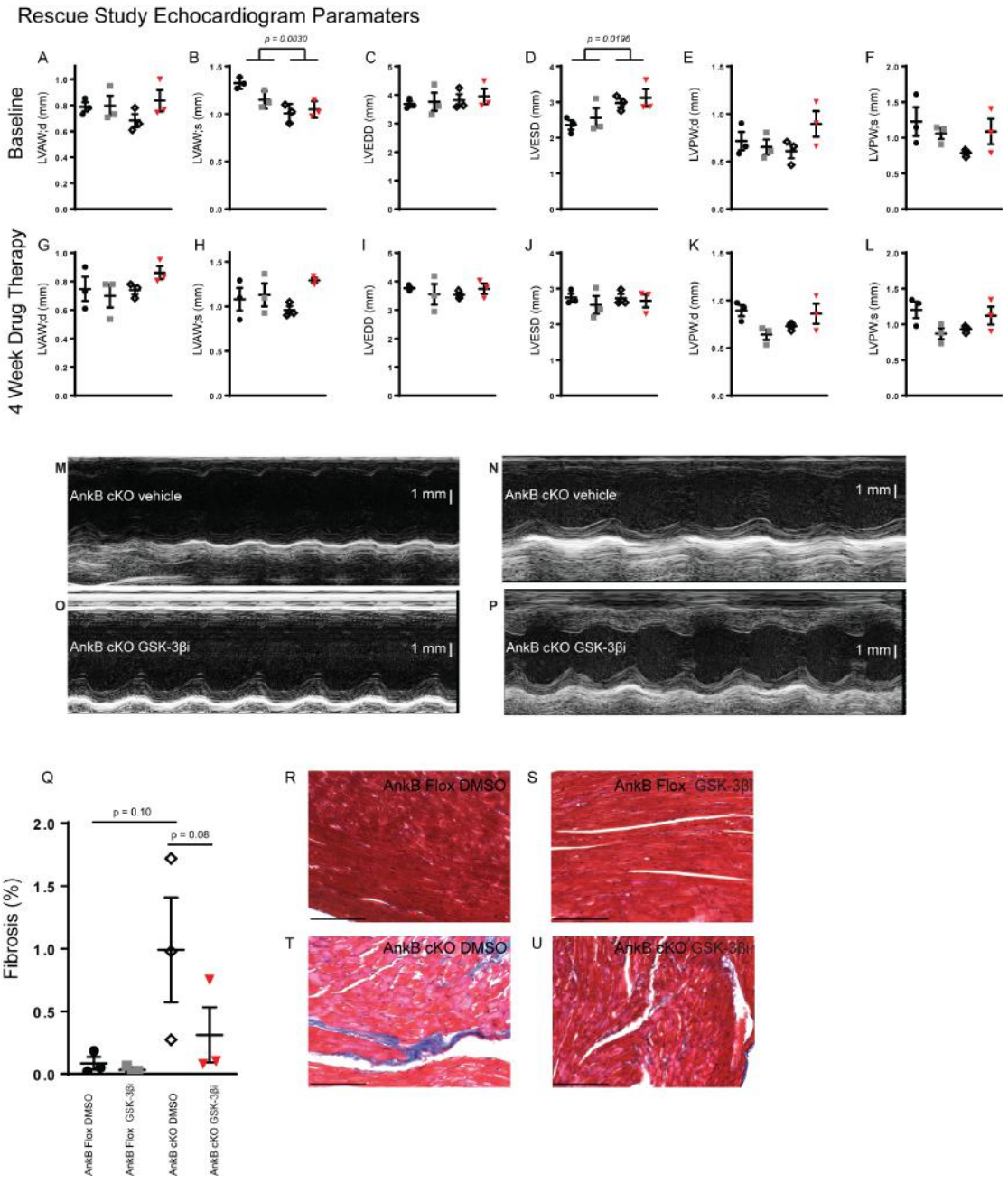

DAPI
TUNEL
Merge
A
Flox DMSO
B
Flox GSK-3βi
C
cKO DMSO
D
cKO GSK-3βi
E
Positive Control
DNase I Treated
F
p= N.S.
% TUNEL Positive Cells
2.5
2.0
1.5
1.0
0.5
0.0
Flox DMSO
Flox GSK-3βi
cKO DMSO
cKO GSK-3βi

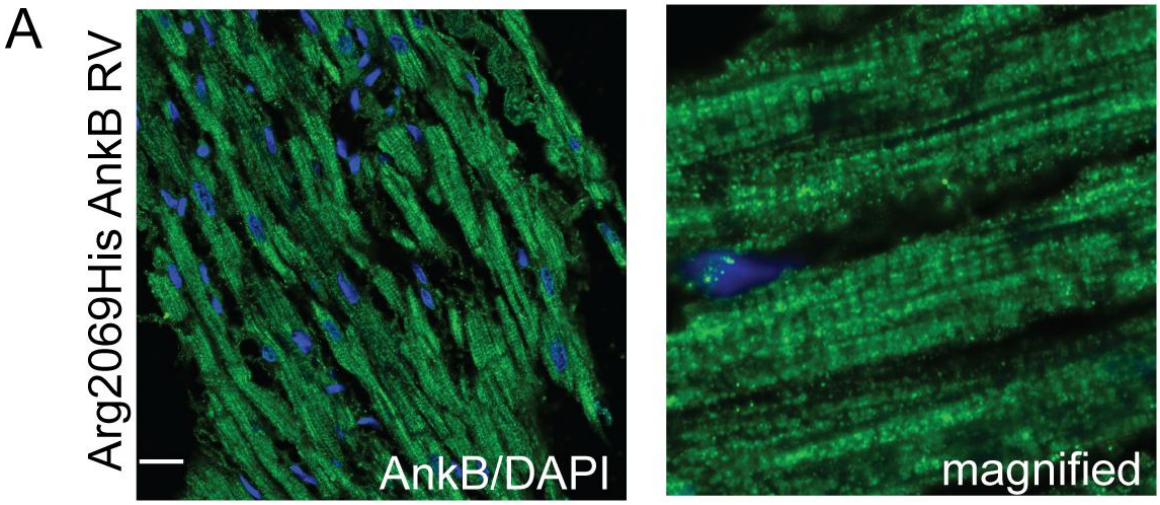
Supplemental Figure 11 | AnkB cKO mice treated with SB-216763 fail to demonstrate differences in TUNEL positive nuclei compared to vehicle treated controls. a-f, Representative TUNEL staining images from AnkB Flox Vehicle (**a**) AnkB Flox SB-216763 (**b**) AnkB cKO Vehicle (**c**), AnkB cKO SB-216763 (**d**) and DNase I treated (**e**) cardiac cryoslices, and quantification of TUNEL positive nuclei (**f**). Images represent n = 3 from each group and statistics performed in **f** were completed using a two-way ANOVA. Scale bars represent 100 μm.



Supplemental Figure 12 | Complete immunoblot of Phospho-β-catenin. **a-b**, Immunoblot of phospho-β-catenin (**a**) and corresponding total protein Ponceau stain (**b**), n =3,3. The immunoblots shown in this figure are the full immunoblots from Figure 6.


Rescue Study Echocardiogram Paramaters
Baseline
4 Week Drug Therapy
A
B
C
D
E
F
G
H
I
J
K
L
LVAW;d (mm)
LVAW;s (mm)
LVEDD (mm)
LVESD (mm)
LVPW;d (mm)
LVPW;s (mm)
p = 0.0030
p = 0.0196
M
N
O
P
AnkB cKO vehicle
AnkB cKO GSK-3βi
1 mm
Q
R
S
T
U
Fibrosis (%)
p = 0.10
p = 0.08
AnkB Flox DMSO
AnkB Flox GSK-3βi
AnkB cKO DMSO
AnkB cKO GSK-3βi

Supplemental Figure 13 | Complete echocardiographic parameters of GSK3-βi rescue study and Masson's trichrome analysis. a-f, Echocardiogram parameters: LVAW;d, (**a**), LVAW;s, (**b**), LVEDD, (**c**), LVESD (**d**),LVPW;d, (**e**), LVPW;s, (**f**) of vehicle and GSK3-βi treated mice at baseline. **g-l**, Echocardiogram parameters: LVAW;d,(**g**), LVAW;s, (**h**), LVEDD, (**i**), LVESD, (**j**), LVPW;d, (**k**), LVPW;s, (**l**) of vehicle and GSK3-βi treated mice after 4 weeks of drug therapy, n =3,3,3,3. **m-p,** Representative m-mode echocardiograms in AnkB cKO mice at 12 weeks of age (**m,n**) and after 4 weeks of drug therapy in vehicle and GSK3-βi therapy AnkB cKO mice **q-u,** Percent fibrosis by area quantification in vehicle and GSK3-βi treated AnkB Flox and AnkB cKO mice (**q**). Representative Masson's trichrome images from vehicle and GSK3-βi treated AnkB Flox and AnkB cKO mice. (**r-u**). Images representative of n=3 from each genotype and treatment condition. Scale bars represent 100 um. Shown are mean ± SEM; statistical analysis is two way ANOVA with Tukey post-hoc comparisons.



Supplemental Figure 14 | AnkB displays abnormal localization in RV of human AnkBp.Arg2069His proband. a. Immunofluorescence images for AnkB in right ventricular free wall tissue from the deceased AC proband possessing the p.Arg2069His mutation (**a**). Scale bar represents 20 microns.

Supplemental Table S1: Protein Altering Variants Identified by Exome Sequencing with Allele Frequencies <0.005% and Shared by Affected Family Members (Grey Shading Reveals Additional Variants Present when Sisters of Proband with Late Potentials on SAECG were Excluded from Analysis) in the AnkB-p.Met1988Thr Kindred

Gene	Chr:position GRCh 37	Allele	Amino Acid	gnomAD	*In Silico* Analysis			
				AF (%)	PolyPhen-2	SIFT	Mutation Taster	CADD
ANK2	4:114275737	T>C	p.Met1988Thr	Novel	PrD	D	DC	24.4
ASPN	9:95237025	insTGA	p.Asp51_Glu52insAsp	Novel	-	-	P	-
C4orf21	4:113461250	C>T	p.His1981Tyr	Novel	B	D	P	12.25
CHD8	14:21899513	G>C	p.Thr97Ser	Novel	B	T	P	0.918
FAM104B	X:55172596	G>C	p.Gly89Ala	Novel	PrD	D	DC	21.6
FAM104B	X:55172659	T>C	p.Ile70Thr	Novel	B	T	P	7.275
FAM104B	X:55172716	G>A	p.Ser51Asn	Novel	B	T	P	9.342
HERC3	4:89527065	A>G	p.Ile31Val	0.00082	B	T	P	1.825
HYDIN	16:70894087	T>C	p.Thr4005Ala	Novel	PoD	D	DC	25.7
IRF2BPL	14:77493973	C>T	p.Arg55Cys	Novel	PrD	D	DC	28.9
KIAA0556	16:27692798	A>G	p.Asn296Ser	Novel	B	T	P	0.001
KRT24	17:38859683	G>T	p.Gly88Val	Novel	B	T	P	6.575
LLGL2	17:73566278	G>A	p.Gly606Ser	Novel	PoD	D	DC	29.6
MESP2	15:90320144	C>G	p.Gln186Glu	Novel	B	T	P	0.457
MYH14	19:50789814	C>T	p.Arg1506Cys	Novel	PrD	D	DC	35
MYO18A	17:27425163	C>A	p.Ala1317Glu	Novel	PoD	D	DC	25.4

NCAN	19:19339028	A>G	p.Thr867Ala	Novel	B	T	P	0.071
OR51A4	11:4967541	C>T	p.Arg264Cys	0.001625	B	D	P	16.42
PTDSS2	11:490009	G>T	p.Gln414His	Novel	PoD	T	DC	22.9
RP1L1	8:10467589	A>G	p.Glu1340Gly	Novel	PoD	T	P	10.26
SLC26A9	1:205904934	G>T	p.Arg55Ser	Novel	B	T	DC	21.8
TMEM54	1:33360973	C>T	p.Arg176His	0.001695	PrD	T	DC	27.1
ZGRF1	4:113461250	G>A	p.His1981Tyr	Novel	B	T	P	1.717
ZNF439	19:11979204	insT	p.Lys441fs	Novel	-	-	DC	-
ZNF717	3:75787476	G>A	p.Ser433Asn	Novel	B	T	P	0.433
ZNF717	3:75788230	G>A	p.Val182Ile	Novel	B	T	P	4.466
ZNF717	3:75788273	C>A	p.Phe167Leu	Novel	B	T	P	2.9242
ZNF717	3:75788411	C>G	p.Asn121Lys	Novel	B	T	P	0.051

Chr = chromosome, gnomAD = genome aggregation database, AF = Allele Frequency, PrD = probably damaging, PoD = possibly damaging, B= benign, T = tolerated, D = damaging, DC = disease causing, P = polymorphism

Supplemental Table S2: Protein Altering Variants Identified by Exome Sequencing in the Proband Possessing the AnkB-Met1988Thr mutation in Known Cardiomyopathy Genes with Allele Frequencies < 5%.

Gene	Chr:position GRCh 37	Allele	Amino Acid	gnomAD	*In Silico* Analysis			
				AF (%)	PolyPhen-2	SIFT	Mutation Taster	CADD
ALMS1	2:73613032	insGGA	p.Glu28_Ala29insGlu	Novel	-	-	P	-
CACNA1C	12:2794921	G>A	p.Glu1865Lys	Novel	B	T	DC	22.8
DMD	X:31986607	G>A	p.Arg814Trp	2.65	PoD	D	P	24.8
DSC2	18:28650748	A>C	p.Leu732Val	0.1209	B	T	P	16.75
DSG2	18:29111109	G>A	p.Val392Ile	0.2205	B	D	P	10.96
JUP	17:39925713	C>T	p.Arg142His	4.40	PrD	D	P	29.6
TTN	2:179605725	T>C	p.Ile3762Val	1.79	B	T	P	4.836

Chr = chromosome, gnomAD = Genome Aggregation Database, AF = Allele Frequency, PrD = probably damaging, PoD = possibly damaging, B= benign, T = tolerated, D = damaging.

List of Cardiomyopathy Genes Screened: *ABCC9, ACTC1, ACTN2, AGL, ALMS1, ALPK3, ANKRD1, BAG3, BRAF, CACNA1C, CALR3, CAV3, CHRM2, CRYAB, CSRP3, CTF1, CTNNA3, DES, DMD, DOLK, DSC2, DSG2, DSP, DTNA, EMD, EYA4, FHL1, FHL2, FKRP, FKTN, FLNC, GAA, GATA4, GATA6, GATAD1, GLA, HCN4, HRAS, ILK, JPH2, JUP, KRAS, LAMA4, LAMP2, LDB3, LMNA, LRCC10, MAP2K1, MAP2K2, MED12, MIB1, MTND1, MTND5, MTND6, MTTD, MTTG, MTTH, MTTI, MTTK, MTTL1, MTTL2, MTTM, MTTQ, MTTS1, MTTS2, MURC, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOM1, MYOZ2, MYPN, NEBL, NEXN, NKX2-5, NPPA, NRAS, PDLIM3, PLEKHM2, PKP2, PLN, PRDM16, PRKAG2, PTPN11, RAF1, RBM20, RIT1, RYR2, SCN5A, SGCD, SLC22A5, SOS1, TAZ, TCAP, TGFB3, TMEM43, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, TXNRD2, VCL*

Supplemental Table S3: Clinical Characteristics of Genotype Negative Arrhythmogenic Right Ventricular Cardiomyopathy Probands from Multi-Center Cohort

Characteristics	ARVC Cases n = 207
Age at Presentation, years	39.2 (14.3)
Male Sex	123 (59.4)
White Race	204 (98.6)
Family History of ARVC*	48 (23.2)
TF Major Criteria	1.9 (0.8)
TF Minor Criteria	1.6 (1.1)
RV systolic dysfunction	159 (76.8)
LV systolic dysfunction	48 (23.2)
Arrhythmia History	
Cardiac Syncope	86 (41.5)
Sustained VT	131 (63.3)
Aborted Cardiac Arrest	10 (4.8)
Implanted ICD	170 (82.1)

Data are n (%) or mean (SD). ARVC = arrhythmogenic right ventricular cardiomyopathy, *First- or second-degree relative, TF = Task Force, RV = right ventricular, LV = left ventricular, VT = ventricular tachycardia, ICD = implantable cardioverter defibrillator

Supplemental Table S4: Clinical Features of Arrhythmogenic Right Ventricular Cardiomyopathy Probands Possessing a Rare *ANK2* Variant

	G290S	T466M	T790I, N3554Y	G859R	I964V	E1458G **	E1458G	R1582Q	M1988T **	T2059M	R2069H	R2069H	K2337E	D3177H	T3744N
Age*	49	26	30	50	16	52	43	59	41	32	12	53	20	41	30
Sex	F	M	F	M	M	M	F	M	M	M	M	M	M	F	M
Race	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
Clin Hx															
Syncope	Y	-	-	Y	Y	Y	Y	-	-	Y	-	Y	Y	-	-
VT	Y	Y	Y	Y	-	-	Y	-	-	Y	-	Y	Y	Y	Y
ACA	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-
CHF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SCD	N	-	-	-	-	Y	-	-	Y	-	Y	-	-	-	-
AF	N	-	-	-	AFl	-	-	-	-	-	-	-	-	-	AFl
SND	N	-	-	-	-	Y	-	-	-	-	-	-	-	-	N
TFC															
Tissue	-	-	-	-	-	++	-	-	++	-	++	-	-	-	-
Structural	-	-	++	++	++	++	++	-	NE	++	NE	++	++	-	-
Repolarization	++	+	++	++	-	-	++	-	-	++	NE	++	++	++	-
Depolarization	-	-	-	-	-	-	-	+	-	-	NE	-	+	+	+

Arrhythmia	+	-	+	+	+	-	+	+	-	+	NE	++	+	+	+
FHx	-	-	-	-	+	-	-	++	++	-	-	-	-	-	-
LV dysfn	N	N	N	N	N	Y	N	N	N	Y	NE	N	N	N	No
ECG (ms)															
PR-interval	166	140	144	238	188	172	158	162	147	172	NE	280	184	156	240
QRS duration	82	110	72	104	102	88	92	98	98	90	NE	101	90	80	130
QTc	399	456	413	476	409	540	432	416	420	470	NE	428	399	422	407
Variants in other CM genes	MYH6 p.G1826N	-	-	-	DSP p.N593S	-	-	-	DSG2 p.V392I, DSC2 p.L732V	-	-	-	-	-	SCN5A-p.F861Wfs*90

*Age at first presentation (years), **Initially identified probands separate from multi-centre cohort. M = male, F = female, W = white, Clin Hx = Clinical History, Y = yes, N = no, VT = ventricular tachycardia, ACA = aborted cardiac arrest, CHF = congestive heart failure, SCD = sudden cardiac death, AF = atrial fibrillation, AFl = atrial flutter, SND = sinus node dysfunction, TFC = Task Force Criteria, + = Minor Task Force criterion, ++ = Major Task Force criterion, NE = not evaluated, FHx = family history, LV dysfn = left ventricular dysfunction, ms = milliseconds, QTc = Bazett corrected QT-interval, CM = cardiomyopathy

Supplemental Table S5: Allele Frequencies and *In Silico* Analyses of *ANK2* mutations Identified in Arrhythmogenic Right Ventricular Cardiomyopathy Probands

***ANK2* Mutation**			**ANK2**	**gnomAD**	***In Silico* Analysis**			
Chr:position GRCh 37	**Allele**	**Amino Acid**	**Domain**	**Allele Freq (%)**	**PolyPhen-2**	**SIFT**	**MT**	**CADD**
4:114163342	G>A	Gly290Ser	MBD	0.00248	PrD	T	DC	26.2
4:114186063	C>T	Thr466Met	MBD	Novel	PrD	D	DC	34.0
4:114213663	C>T	Thr790Ile	MBD	0.00257	B	T	P	21.2
4:114232437	G>A	Gly859Arg	SBD	Novel	PrD	D	DC	33.0
4:114239766	A>G	Ile964Val	SBD	0.00308	PoD	T	P	13.73
4:114269433	A>G	Glu1458Gly	SBD	0.04222	PoD	T	DC	23.7
4:114274519	G>A	Arg1582Gln	SBD	0.06375	B	T	P	0.004
4:114275737	T>C	Met1988Thr	CTD	Novel	PrD	D	DC	24.4
4:114275950	C>T	Thr2059Met	CTD	0.07120	PrD	T	P	13.92
4:114275980	G>A	Arg2069His	CTD	0.08589	PrD	D	P	23.1

4:114276783	A>G	Lys2337Glu	CTD	Novel	B	T	P	0.082
4:114279303	G>C	Asp3177His	CTD	Novel	B	T	P	14.08
4:114288920	C>A	Thr3744Asn	CTD	0.06180	B	D	DC	14.49

Chr = chromosome, gnomAD = Genome Aggregation Database, Allele Freq = Allele Frequency, MT = Mutation Taster, MBD = membrane minding Domain, SBD = spectrin binding domain, CTD = C-terminal domain, PrD = probably damaging, PoD = possibly damaging, B= benign, T = tolerated, D = damaging, DC = disease causing, P = polymorphism

Supplemental Table S6: Allele Frequencies and *In Silico* Analyses of Variants of Unknown Significance Identified in Arrhythmogenic Right Ventricular Cardiomyopathy Probands Possessing *ANK2* variants

ARVC Case	Gene	Chr:position GRCh37	Allele	Amino Acid	gnomAD AF (%)	*In Silico* Analysis			
						PolyPhen-2	SIFT	Mutation Taster	CADD
1	*MYH6*	14:23853740	delGGinsAA	Gly1826Asn	Novel	-	T	P	-
3	*DSP*	6:7571692	A>G	Asn593Ser	0.0511	B	T	DC	16.4
9	*DSC2*	18:28650748	A>C	Leu732Val	0.1209	B	D	P	9.099
9	*DSG2*	18:29111109	G>A	Val392Ile	0.2205	B	D	P	12.99

ARVC Case number refers to Supplemental Table S4, Chr = chromosome, gnomAD = genome aggregation databse, AF = Allele Frequency, B= benign, T = tolerated, D = damaging, DC = disease causing, P = polymorphism

Supplemental Table S7: Delineation of Animal Use by Sex, Age, and Genotype.

Experiment	Number of Male Mice (AnkB Flox, AnkB cKO)	Number of Female Mice (AnkB Flox, AnkB cKO)	Ages of Mice (Weeks)
Left ventricular assessment at baseline	2,5	2, 4	10-12
Right ventricular assessment at baseline	3,3	3,4	10-12
Sodium channel recordings	3,3	0,0	6-8
Telemeter ECG Implantation	6,7	4,3	6-8
Heart Weight/ Tibia Length	4,4	4,5	12-14
SB-216763 Prevention Study	6,10	4,8	3-4
SB-216763 Rescue Study	1,3	5,3	11-13

References

1. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30(15):2114–2120.

2. McKenna A et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 2010;20(9):1297–1303.

3. Gu S, Fang L, Xu X. Using SOAPaligner for Short Reads Alignment. *Curr. Protoc. Bioinforma.* 2013;44:11.11.1-17.

4. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinforma. Oxf. Engl.* 2010;26(5):589–595.

5. Lek M et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 2016;536(7616):285–291.

6. 1000 Genomes Project Consortium et al. An integrated map of genetic variation from 1,092 human genomes. *Nature* 2012;491(7422):56–65.

7. Li H et al. The Sequence Alignment/Map format and SAMtools. *Bioinforma. Oxf. Engl.* 2009;25(16):2078–2079.

8. DePristo MA et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* 2011;43(5):491–498.

9. Hamosh A et al. PhenoDB: a new web-based tool for the collection, storage, and analysis of phenotypic features. *Hum. Mutat.* 2013;34(4):566–571.

10. Sobreira N, Schiettecatte F, Boehm C, Valle D, Hamosh A. New tools for Mendelian disease gene identification: PhenoDB variant analysis module; and GeneMatcher, a web-based tool for linking investigators with an interest in the same gene. *Hum. Mutat.* 2015;36(4):425–431.

11. Wu TD, Nacu S. Fast and SNP-tolerant detection of complex variants and splicing in short reads. *Bioinforma. Oxf. Engl.* 2010;26(7):873–881.

12. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* 2010;38(16):e164–e164.

13. Liu X, Jian X, Boerwinkle E. dbNSFP v2.0: a database of human non-synonymous SNVs and their functional predictions and annotations. *Hum. Mutat.* 2013;34(9):E2393-2402.

14. Gréen A et al. Assessment of HaloPlex amplification for sequence capture and massively parallel sequencing of arrhythmogenic right ventricular cardiomyopathy-associated genes. *J. Mol. Diagn. JMD* 2015;17(1):31–42.