A Distinct Cellular Basis for Early Cardiac Arrhythmias, the Cardinal Manifestation of Arrhythmogenic Cardiomyopathy, and the Skin Phenotype of Cardiocutaneous Syndromes

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Expanded Material and Methods

Studies in the animal models conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee. Human tissue use was approved by the Institutional Review Board.

Generation of custom-made rabbit anti CSPG4 antibodies: Three antibodies against the mouse CSPG4 protein (UniProt ID#: Q8VHY) were generated by immunization of rabbits with the antigen peptides, KELKLQTPADTVLSDSAPH-Cys, SSGSLDLPYLKGISRPLRGC, and RLSDGESFSQSDLQAGRVTYRAT-Cys.

Detection of expression of CSPG4 in the ventricular myocardium: To detect expression of CSPG4 in the heart, fresh frozen thin myocardial sections were co-immunostained with a custom-made rabbit anti CSPG4 antibody and an antibody against either α-actinin (ACTN1) to mark the myocytes, connexin 40 (GJA5 or CX40) to mark the CCS, and contactin 2 (CNTN2), also a marker for the CCS. Details of antibodies are provided in Online Table I.

Isolation and culture of myocyte-depleted cells from the human hearts: Cardiac myocyte depleted cell fraction was obtained by collagenase type 2 digestion of ~100 mg of fresh tissue. [1] The tissue samples were minced and incubated in a 0.1% collagenase 2 solution (Worthington Biochemical Corp; Lakewood, NJ08701; cat# LS004176) for 45 min at 37 °C under gentle agitation. The collagenase activity was stopped by adding 10 mL of α Modification media (αMEM, Hyclone; cat# SH30265.01) supplemented with 10% stem cell certified Fetal Bovine Serum (ES-FBS, Hyclone; cat# SH30070.03-E) and 1% Antibiotic-Antimycotic solution (Gibco; cat # 15240). The solution was filtered through a 40 μm cell strainer (BD Bioscience cat# 352340) and centrifuged for 5 min at 300g. The cell pellet was washed one time with PBS and re-suspended in a complete medium, composed by a α MEM supplemented with 20% ES-FBS, and 1% Antibiotic-Antimycotic solution, plated in 60-mm plates coated with 0.1% gelatin and placed in a 5% CO2 humidified incubator at 37 °C.

Isolation of mouse cardiac CSPG4^{pos} cells: To isolate CSPG4^{pos} cells, myocyte-depleted cardiac cells were subjected to flow cytometry and FACS using a custom-made rabbit anti CSPG4 antibody, as

described before with modifications. [1] In brief, 1-3 months old mice were anesthetized by intraperitoneal (IP) injection of pentobarbital (62 mg/Kg) and anti-coagulated upon IP injection of 200 U of heparin. The heart was explanted and washed with cold sterile PBS. The ascending aorta was cannulated with a 22G blunt needle under a magnifying dissection microscope (Leica, S6D). The cannula was positioned above the aortic valve cusps and connected to a retrograde perfusion system. To remove excess blood, the heart was perfused with PBS at a constant rate of 4 mL/min at 42 °C and then the perfusate was switched to 275U/mL type 2 collagenase in α-MEM medium (Worthington, Lakewood, NJ). The digestion with collagenase was continued until the heart became pale and spongy. The heart was then disconnected from the cannula and minced into small pieces using fine scissors in a 60-mm dish containing 2 ml of the digestion buffer (collagenase). The mixture was pipetted up and down gently several times with a sterile plastic transfer pipet (2 mm opening). The digestion was stopped by adding 8 ml of 10% calf serum in the α-MEM medium. The cell suspension was filtered through a 100 μm nylon mesh, transferred to a 15-ml polypropylene conical tube, and centrifuged at 300 g for 5 min at 4 °C. The supernatant was removed, the cell pellet was washed twice in a MACS buffer (Miltenyi Biotec), and resuspended in 500 mL of MACS buffer. Cell were then incubated with the primary antibody for 1 hr at 4 °C in the dark, followed by two washes in the MACS buffer and incubation with the secondary antibody for 1 h. Cells suspensions were washed in the MACS buffer, filtered through a 35 μm nylon mesh and analyzed by FACS.

FACS isolated mouse CSPG4^{pos} cells were plated onto 0.1% gelatin coated plates in a growth medium [α MEM supplemented with 10% ES-FBS, 10 ng/mL mouse basic Fibroblast Growth Factor (bFGF, R&D, Minneapolis, MN; cat #3139-FB), 1000 U/mL of mouse Leukemia Inhibitory Factor (mLIF, Millipore; cat # ESG1106) and 1% Antibiotic-Antimycotic solution] and incubated at 37 °C in a 5% CO2 humidified incubator overnight.

To detect and quantify expression of the genes of interest in cardiac CSPG4^{pos} cells, these cells were collected directly into the TRIzol LS Reagent (Invitrogen; cat # 10296010 and 10296028) during FACS and used for RNA extraction. In brief, 20ug of glycogen was added to each TRIzol sample and RNA

was first extracted with a phenol/chloroform/isopropanol mixture, precipitated with 100% ethanol and used in the qPCR reactions.

Detection of expression of desmosome proteins in isolated cardiac CSPG4^{pos} cells: To determine whether cardiac CSPG4^{pos} cells expressed desmosome proteins, isolated cardiac CSPG4^{pos} were stained with antibodies against desmosome proteins DSP, PKP2, and JUP (Online Table I).

Isolation of mouse adult cardiac myocytes: To verify specificity of *Dsp* deletion in CSPG4^{pos} cells but not in cardiac myocytes, the latter cells were isolated, and co-stained with antibodies against CSPG4, ACTN2, and DSP. To isolate cardiac myocytes, the heart was harvested, as described above, and placed in a perfusion buffer [120 mM NaCl, 15 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, $7H_2O$, 30 mM Taurine, 4.6 mM $NaHCO_3$, 10 mM HEPES, 10 mM 2,3-Butanedione monoxime (BDM), and 5.5 mM Glucose; pH 7.0], as published. [1] The ascending aorta was cannulated and the heart was perfused in a retrograde perfusion system, as described above, with the perfusion buffer at a constant rate of 4 mL/min at 42 °C. Upon washing out the blood, the perfusate was switched to a collagenase 2 digestion buffer (Worthington, Lakewood, NJ). Approximately 2-3 min after perfusion with the collagenase, 7.5 ul of 100 mM $CaCl_2$ was added to the buffer and the digestion was continued until the heart became pale and felt spongy upon gentle pinching. The heart was removed from the cannula, minced into small pieces, and cells were dissociated, as described above. The digestion was stopped by adding stop buffer (10% calf serum and 12.5 μM $CaCl_2$ in the perfusion buffer) and the cell suspension was filtered through a 100 μm nylon mesh into a polypropylene conical tube. An aliquot of 100 μl of 200 mM ATP was added to the tube and the myocytes were allowed to sediment by gravity for a few minutes at 4 °C, which was then followed by centrifugation at 20g for 3 min. Following removal of the supernatant, cells were subjected to a three-step calcium reintroduction by resuspending the cells in aliquots of 10 mL of stop buffer containing 2 mM ATP and increasing concentration of $CaCl_2$ to 100 μM, 400 μM, and 900 μM for 3 minutes each. After each calcium reintroduction step, the suspension was centrifuged at 20g for 3 min and the pellet was resuspended in the buffer containing the corresponding incremental concentration of $CaCl_2$. The final pellet was resuspended in a cardiac myocyte plating media (MEM media, 1% penicillin-streptomyocin,

10% Calf serum, 10 mM BDM, and 2 mM ATP). Cardiac myocytes were plated on laminin coated cover slips for immunofluorescence or 6 well plates for RNA and protein extraction, and incubated immediately in a 2% CO2 incubator at 37°C. After 2 hours of incubation to allow the cells to attach to the substrate, the cells were collected for RNA extraction or fixed in 4% paraformaldehyde for immunostaining.

***Cspg4*-DsRed.T1 reporter mice:** *Cspg4*-DsRed.T1 is a BAC transgenic reporter mouse line that expresses a red fluorescence protein variant under the transcriptional regulation of the *Cspg4* locus (Stock No: 008241, Jackson Laboratory). [2] In this mouse model, DsRed.T1 protein serves as a surrogate marker representing expression pattern of the CSPG4 protein.

***Cspg4-Cre/Esr1*:Dsp*$^{W/F}$ and *Cspg4-Cre/Esr1*:Dsp*$^{F/F}$ mice:** To determine biological and functional significance of expression of DSP in CSPG4^{pos} cells, *Dsp* gene was conditionally deleted using the inducible cre deleter BAC transgenic mice *Cspg4-Cre/Esr1** (Stock No: 008538; Jackson Laboratory). [3] In brief, *Cspg4-Cre/Esr1** mice were crossed to *Dsp*$^{F/F}$ [4] to generate *Cspg4-Cre/Esr1*:Dsp*$^{W/F}$ mice and subsequently *Cspg4-Cre/Esr1*:Dsp*$^{F/F}$ mice. Twenty-one day old mice carrying one or two floxed *Dsp* and the cre recombinase alleles were treated with I.P. injection of tamoxifen at 100 μg/g/d for 5 days. Activation of the cre recombinase by tamoxifen is expected to delete the floxed exon 2 in the *Dsp* gene specifically in cells that are transcriptionally regulated by the *Cspg4* locus. Mice were genotyped by PCR of tail DNA. Oligonucleotide primers used in PCR reactions are listed in Online Table I. Age and sex-matched wild type (WT) were used as controls in all experiments.

Quantitative polymerase chain reaction (qPCR): qPCR was performed as published and the relative normalized values (2^-ΔΔ method, shown as relative to the WT mice) were used to compared the transcript levels. [5, 6] Oligonucleotide primers and probes are listed in online Table I.

Immunoblotting: Immunoblotting was performed as published. [5, 6] In brief, total proteins were extracted by homogenizing heart tissues or the collected cells in a RIPA lysis buffer [1X formulation: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% Sodium deoxycholate and 0.1% Sodium Dodecyl Sulphate (SDS); Pierce Biotechnology, Rockford, 8 IL; cat #89901] in the presence of protease inhibitors (complete protease inhibitor cocktail; Roche Diagnostics, GmbH, Mannheim, Germany;

cat # 11-697-498-001;). Protein concentration was measured using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories; cat # 5000111). Aliquots of 30-50 μg of total protein were denatured in a Laemmli loading buffer at 95 °C for 5 min, loaded onto SDS-polyacrylamide gels (PAGE), subjected to electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 hr in 5% non-fat milk and incubated with the primary antibody of interest overnight at 4 °C. After 3 washes in TBS, the membranes were incubated with the corresponding horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour and the signal was detected by chemiluminescence. The membranes were stripped by incubation in the Restore PLUS Western Blot stripping Buffer (Thermo Scientific, Hudson, New Hampshire, cat #46430) for 10 min at room temperature, washed in TBS for 3 times, and reprobed with another antibody of interest. The primary and secondary antibodies and their titers are listed in Online Table I.

Immunofluorescence: Immunofluorescence staining to detect expression and localization of the proteins of interest was performed as published. [5, 6] In brief, the whole heart was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc. Torrance, CA 90501; cat#4583) and frozen in isopentane (2-Methyl Butane, Sigma-Aldrich St Louis, MO, cat #320404). Thin myocardial sections (5 μm) were cut from the OCT blocks and fixed in 4% formaldehyde for 2.5 min. FACS isolated cells were cultured overnight and fixed in 4% formaldehyde for 10 min. The samples were washed 3 times in PBS and blocked in a 5% donkey serum diluted in PBS for 1 h at room temperature. After blocking, samples were incubated with antibodies against the target protein (list of antibodies is available in Online Table I) in 1% BSA in PBS overnight at 4 °C. After 3 washes, samples were incubated with fluorescence-labeled secondary antibodies in 1% BSA for 1 hour at room temperature. Samples were washed and stained with a 0.1 mg/mL of 4′, 6 Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich St Louis, MO; cat# D8417) or DRAQ5 (Abcam, ab108410) for 2 min at room temperature, the latter two to identify the nuclei. After 3 washes in PBS wash, samples were mounted in fluorescent mounting medium (Dako North America Inc.6392 Via Real, Carpinteria CA 93013, cat# S3023), and examined under fluorescence microscopy (Zeiss, Axioplan Fluorescence Microscope).

Histology and immunohistochemistry: Whole heart histology was examined by H&E, Masson Trichrome, and Oil Red O staining of thin myocardial sections. For Oil Red O staining, thin myocardial sections prepared from OCT embedded hearts were washed one time with PBS and then fixed in 10% formalin for 15 min at room temperature. After 10 min washing under running water, samples were stained in a modified Mayer`s hematoxylin solution (Richard-Allan Scientific, Kalamazoo, MI; cat # 72804) for 2 min. Samples were then washed for 10 min in water, placed in 100% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264) for 2 min and then placed in Oil Red O 0.5% solution in propylene glycol (Poly Scientific, Bay Shore, NY; cat #s1848) overnight at room temperature. The Oil Red O solution was removed and the samples were dipped 2 times in 85% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264A). After 20 min washing the slides were mounted with aqueous mounting medium.

For H&E staining, the heart was formalin fixed by perfusion and incubated overnight in formalin at 4 °C. Tissue was dehydrated by ethanol xylene gradient and embedded in paraffin. Paraffin embedded hearts were sectioned into 5 μm horizontal or coronal sections, and dried overnight at 37 °C. Sections were deparaffinized by incubating twice in Xylene for 5 min each, 2 min each in 100%, 95%, and 70% ethanol for 2 min each, followed by washing with water for 1 min. Tissue was stained with Hematoxylin for 30 min at room temperature followed by 10 dips in water. Tissue was incubated in 70% ethanol for 1 min and immediately stained with Eosin Y solution for 30 seconds. Tissue was then dehydrated by ethanol xylene gradient. Slides were covered with coverslip using Cytoseal^{TM} XYL (Thermo Scientific, 8312-4).

Masson Trichrome staining was performed per the manufacturer's protocol (Sigma, HT15-1KT) using paraffin embedded heart sections. First, whole heart sections were deparaffinized as described above. Slides were incubated for 15 min in Bouin's solution preheated to 56°C (Sigma, HT10-1). Slides were washed in running tap water for 10 min, followed by staining in Working Weigert's Iron Hematoxylin Solution for 5 min. Slides were rinsed in running distilled water for 5 min and stained with Biebrich Scarlet-Acid Fuchsin (Sigma, HT15-1). Slides were dipped one time in distilled water and placed in Phosphotungstic/Phosphomolybdic Acid solution for 5 min, followed by analine blue solution (Sigma

HT15-4) for 5 min and 1% acetic acid for 2 min. Finally, slides were rinsed in water, dehydrated, and mounted, as described above.

Five μm-sections were prepared from Paraffin wax-embedded back skin tissue and dewaxed using standard methods. Dewaxed sections were either stained with Hematoxylin and Eosin (H&E) or further processed for antibody staining. Antigen retrieval was performed using pepsin (1 mg/mL, Sigma-Aldrich) at pH 2, citrate buffer (10 mM, trisodium citrate dihydrate**;** 2.94 g/L) at pH 6 or Tris-EDTA (10 mM Tris base and 1 mM EDTA) at pH 9. Section were stained with primary antibody (see Table I) overnight at 4 °C then incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies. To reveal the signal, sections were incubated with 3,3'-diaminobenzidine (DAB). Sections were imaged using a Leica DM4000 optical microscope.

Detection and quantification of apoptosis: Apoptosis was analyzed in the heart by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, using the fragEL ^{TM} DNA Fragmentation Detection Kit (Millipore; cat # QIA-39). In brief, formalin fixed paraffin embedded thin myocardial sections were stained for fluorescein-labeled deoxynucleotides and counterstained with DAPI. The number of TUNEL positive cells was counted in 11 high magnification fields per section, 10 thin sections per heart, and in 5 mice per group (a total of ~20,000 cells per heart) and presented as percentage of the total cells, the latter identified by DAPI staining.

Echocardiography: 2D, M mode, and Doppler echocardiography was performed as published using a HP 5500 Sonos echocardiography unit equipped with a 15-MHz linear transducer. [1, 5-7] Echocardiography was performed in 3-6 months old *Cspg4-Cre/Esr1*:Dsp*$^{W/F}$ and *Cspg4-Cre/Esr1*:Dsp*$^{F/F}$ mice and their corresponding WT controls. In brief, mice were anesthetized by IP injection of sodium pentobarbital (60 mg/Kg body weight) and chest hair were removed with hair removal cream Wall thicknesses and left ventricular end diastolic and end systolic diameters were measured from M-mode images from the parasternal two-dimensional short-axis view at the tip of the mitral leaflet, using the leading-edge method. Left ventricular fractional shortening and mass were calculated as previously

described. [1, 5-7] All echocardiographic data represent the mean of 3 measurements on different cardiac cycles.

Electrocardiography: Mice were anesthetized as described above and kept on a heating plate set at 37°. 29-gauge needle electrodes were inserted subcutaneously into the forelimbs and ECG and heart rate were recorded and analyzed using the Animal Bio Amp, PowerLab, and the LabChart7 software (all from AD Instrument).

Electrophysiology (EP): Mice were anesthetized with isoflurane (average 1.75% in 100% oxygen), sufficient to maintain an adequate level of anesthesia, and 100% oxygen. Temperature was monitored continuously and maintained between 36-37 °C degrees. Surface electrocardiograms were monitored throughout. Intracardiac bipolar atrial and ventricular electrograms were obtained using a 1.1F octapolar catheter (EPR-800; Millar Instruments), as described. [8, 9] EP pacing protocols, including single, double, and burst ventricular pacing protocols were used for ventricular tachycardia (VT) induction at baseline and after injection of isoproterenol (3 mg/kg i.p.). Two drive trains were used at the baseline (90 msec and 70 msec) and after isoproterenol injection (80 msec and 70 msec). Ventricular doubles at baseline were performed at cycle lengths of 90/50/40 msec and 80/50/40 msec and on isoproterenol at 80/50/40 msec and 70/50/40 msec. Ventricular burst pacing was performed at a drive train of 60 msec. The atrial stimulation protocol to evaluate inducibility of atrial fibrillation was performed with incremental atrial pacing, as described. [9] All pacing protocols were performed a maximum of three times. Non-sustained and sustained VT were defined as reproducible (at least twice) ectopic ventricular rhythms lasting 4 to 9 beats and ≥10 beats, respectively. Atrial fibrillation was defined as reproducible rapid and fragmented atrial electrograms with irregular ventricular response for ≥1 second.

To evaluate heart rate variability, heart rates were measured for an average of 3 beats every 30 seconds during the electrophysiology study. Heart rates were not measured if within 10 seconds of a pacing drive train. The average change in heart rate during the EP study were compared between the WT and *Cspg4-Cre/Esr1*:Dsp*$^{F/F}$ mice, who completed the EP studies.

Statistical analysis: Data that followed a Gaussian distribution pattern were presented as mean ± SD and were compared between two groups by t test and among multiple groups by ANOVA followed by post-hoc pairwise comparisons. Otherwise, data were presented as the median values and compared by Kruskall-Wallis test, as were the categorical data.

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Online Supplementary Material

Online Table I

A. Information on antibodies used in the experiments

Antibody	Company	Catalogue#	Concentration
DSP1/2	Progen	65146	1:1,000 (IF, IB)
PKP2	Progen	651101	1:1,000 (IF)
DSG2	Progen	61002	1:1,000 (IF)
DSC2/3	Santa Cruz	SC-70994	1:1,000 (IF)
JUP	Santa Cruz	SC-1497	1:100 (IF in skin, citrate antigen retrieval)
	Invitrogen	13-8500	1:1,000 (IF for $CSG4^{pos}$ cells)
ACTN2	Sigma	A7811	1:1,000 (IF)
CSPG4	Millipore	AB530B, AB530	1:1,000 (IF, IB)
CSPG4-clone 45	Custom-made		1 μg per 10^6 cells (FACS)
CSPG4-clone 47	Custom-made		Not used
CSPG4-clone 49	Custom-made		1:1,000 (IF)
CNTN2	R&D systems	364900	1:200
Cx40	Life technology	AF4439	1:1,000
KRT1	Covance	PRB-165P	1:6,000 (DAB, pepsin)
KRT6	Covance	PRB-169P	1:2,000 (DAB, pepsin)
KRT14	Covance	PRB-155P	1:2,000 (DAB, pepsin)
MKI67 (Ki67)	Abcam	ab16667	1:100 (DAB, citrate)
TP53	Santa Cruz	sc-6243	1:500 (DAB, citrate)
TP63	Abcam	ab735	1:800 (DAB, citrate)
BLIMP1 (PRDM1)	eBioscience	14-5963-82	1:100 (DAB, citrate)
CASP3	Cell Signaling	9661	1:400 (DAB, citrate)
BCL2	BD Transduction Labs	D46620	1:200 (DAB, citrate)
FAS	Santa Cruz	sc-48357	1:250 (DAB, citrate)
TNFα	Antigenix America	RMPF326B	1:100 (DAB, citrate)
F4/80	Abcam	sb111101	1:100 (DAB, Tris-EDTA)
Anti-Mouse Powervision HRP	Immunologic	DPVM110HRP	DAB
Anti-Mouse Powervision HRP	Immunologic	DPVR110HRP	DAB
Anti-Rabbit IgG, Alexa Fluor 594	Life technologies	21207	1:1,000 (IF)

Anti-Rabbit IgG, Alexa Fluor 488	Life technologies	21206	1:1,000 (IF)
Anti-Mouse IgG, Alexa Fluor 594	Life technologies	21203	1:1,000 (IF)
Anti-Mouse IgG, Alexa Fluor 488	Life technologies	21202	1:1,000 (IF)
Anti-goat IgG, Alexa Fluor 488	Life technologies	11055	1:1,000 (IF)
Anti-goat IgG, Alexa Fluor 594	Life technologies	11058	1:1,000 (IF)
PE Rat Anti-Mouse CD140a (PDGFRA)	BD Pharmingen	562776	1μg:10^6 cells (FACS)
PE Rat Anti-Mouse CD140b(PDGFRB)	eBioscience	12-1402-81	1 μg:10^6 cells (FACS)
α-Tubulin	Cell Signaling	2125	1:1,000 (IB)
Anti-Rabbit IgG, HRP linked	Cell Signaling	7074	1:3,000 (IB)
Anti-Mouse IgG, HRP linked	Cell Signaling	7076	1:4,000 (IB)

B. TaqMan gene expression probes (from life technologies)

Cspg4 Mm00507257_m1

Dsp Mm01351876_m1

Gapdh Mm99999915_g1

C. SYBR green oligonucleotide primers:

- ***Gapdh*:**

Forward: AAC TTT GGC ATT GTG GAA GG
Reverse: GGA TGC AGG GAT GAT GTT CT

- ***Actc1:***

Forward: TGT CAC CAC TGC TGA ACG TG
Reverse: CTG GCC GTC AGG AAG TTC ATA

- ***Myh6*:**

Forward: CTG TTC CTC TCT CCG TCC AG
Reverse: ATT CTG TCA CTC AAA CTC TGGT

D. PCR oligonucleotide primers for mouse genotyping:

- **Floxed *Dsp*:**

Forward: TAAGCTCCCCTCACTTCTCCAG
Reverse: TTCTCTTTGTCTGTTGCCATGT

- ***Cspg4-Cre:***

Forward: GCG GTC TGG CAG TAA AAA CTA TC
Reverse: GTG AAA CAG CAT TGC TGT CAC TT

Internal Positive Control Forward: CTA GGC CAC AGA ATT GAA AGA TCT
Internal Positive Control Reverse: GTA GGT GGA AAT TCT AGC ATC ATC C

- **Ds-Red.T.1:**

Forward: TTC CTT CGC CTT ACA AGT CC
Reverse: GAG CCG TAC TGG AAC TGG
Internal Positive Control Forward: CTA GGC CAC AGA ATT GAA AGA TCT
Internal Positive Control Reverse: GTA GGT GGA AAT TCT AGC ATC ATC C

TABLE II

Electrophysiological Measurements in 2-3 Months Old Wild Type and *Cspg4Cre/Esr1*:Dsp*F/F *Mice*

	Baseline			Isoproterenol		
	WT n = 7	***Cspg4-Cre/Esr1*:Dsp*$^{F/F}$ n = 10**	**p**	**WT n = 7**	***Cspg4-Cre/Esr1*:Dsp*$^{F/F}$ n = 10***	**p**
Heart Rate (bpm)	618 ± 49	545 ± 101	0.10	708 ± 40	645 ± 42	<0.01
PR interval (ms)	30.0 ± 1.1	28.1 ± 2.9	0.20	26.9 ± 2.9	27.4 ± 2.4	0.66
QRS duration (ms)	9.4 ± 1.3	9.8 ± 1.0	0.52	9.9 ± 1.4	10.1 ± 0.9	0.66
QTc (ms)	42.3 ± 3.4	46.1 ± 5.1	0.10	44.7 ± 3.1	48.0 ± 4.8	0.15
AVERP at 88msec	38.0 ± 10.9	46.9 ± 8.4	0.08	38.3 ± 6.9	38.7 ± 6.1	0.93
AVW (ms)	59.1 ± 8.5	66.0 ± 8.0	0.11	56.7 ± 6.1	62.0 ± 6.0	0.11
VAW (ms)	73.3 ± 11.5	88.6 ± 13.7	0.04	69.8 ± 8.3	77.3 ± 10.0	0.17
VBCL (ms)	39.4 ± 0.5	44.5 ± 7.2	0.09	40.1 ± 1.8	43.1 ± 5.6	0.20
VERP at 88/78 ms	20.7 ± 6.6	29.1 ± 10.6	0.08	26.1 ± 11.6	25.3 ± 10.9	0.88

Abbreviations: AVERP – atrioventricular effective refractory period when pacing at a drive train cycle length of 88msec; AVW – atrioventricular wenckebach cycle length; VAW – ventriculo-atrial wenckebach cycle length; VBCL – ventricular block cycle length; VERP – ventricular effective refractory period when pacing at a drive train of 88msec (baseline) and 78msec (isoproterenol). *Two mice did not complete the isoproterenol pacing protocols due to bradycardic death.

TABLE III

Echocardiographic Data in 3 Months Old Wild Type and *Cspg4Cre/Esr1*:Dsp*$^{F/F}$ *Mice*

	WT	***Cspg4Cre/Esr1*:Dsp*$^{F/F}$**	**p**
N	14	14	N/A
M/F	8/6	7/7	
Age (months)	2.95 ± 1.17	2.96 ± 1.1	0.98
Body weight (g)	20.4 ± 4.0	14.6 ± 2.4	0.000067
HR (bpm)	562 ± 60	532 ± 108	0.372
IVST (mm)	0.83 ± 0.12	0.62 ± 0.07	7.08E-6
PWT (mm)	0.78 ± 0.18	0.60 ± 0.08	0.002
LVEDD (mm)	2.83 ± 0.39	2.87 ± 0.3	0.76
LVEDDi (mm/g)	0.11 ± 0.02	0.19 ± 0.028	2.21E-08
LVESD (mm)	0.90 ± 0.22	0.99 ± 0.21	0.3
FS (%)	68.14 ± 6.20	65.44 ± 6.0	0.25
LVM (mg)	64.86 ± 13.24	45.63 ± 11.86	0.0004
LVMi (mg/g)	2.57 ± 0.52	2.93 ± 0.65	0.123

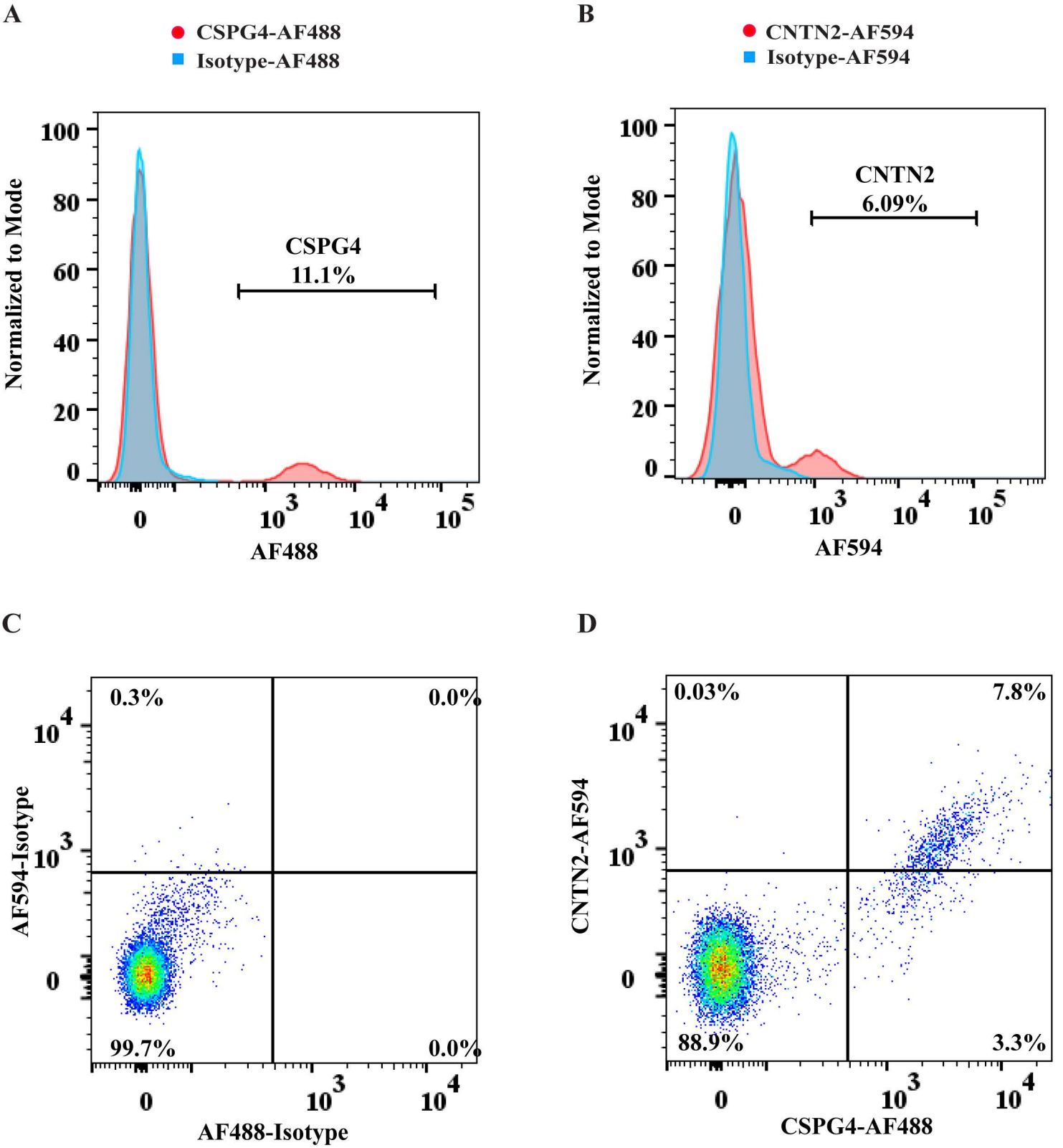
Abbreviations: HR, heart rate; bpm, beats per minutes; IVST, interventricular septal thickness; PWT, posterior wall thickness; LVEDD, left ventricular end diastolic diameter; LVEDDi, LVEDD divided by the body weight; LVESD, left ventricular end systolic diameter; LVESDi , LVESD divided for the body weight; FS, fractional shortening; LVM, left ventricular mass; LVMi, LVM divided by the body weight.

TABLE IV

Echocardiographic Data in 8 Months Old Wild Type and *Cspg4Cre/Esr1*:Dsp$^{W/F}$ Mice*

	WT	***Cspg4Cre/Esr1*:Dsp$^{W/F}$***	**p**
n	19	12	N/A
M/F	13/6	7/5	
Age (months)	8.5 ±2.95	8.80 ±3.64	0.57
Body weight (g)	30.90 ± 3.36	31.83 ± 5.51	0.83
HR (bpm)	567.4 ± 68.35	538.33 ± 70.57	0.28
IVST (mm)	0.9 ± 0.062	0.87 ± 0.06	0.09
PWT (mm)	0.9 ±0.07	0.85 ± 0.05	0.07
LVEDD (mm)	3.0 ± 0.22	3.1 ± 0.23	0.18
LVEDDi (mm/g)	0.098 ±0.010	0.100 ± 0.013	0.64
LVESD (mm)	1.08 ± 0.15	1.22 ± 0.21	0.04
FS (%)	63.8 ± 5.20	60.63 ± 7.34	0.19
LVM (mg)	78.9 ± 18.31	83.93 ± 11.4	0.41
LVMi (mg/g)	2.6 ± 0.63	2.68 ±0.40	0.64

Abbreviations are as in Table III



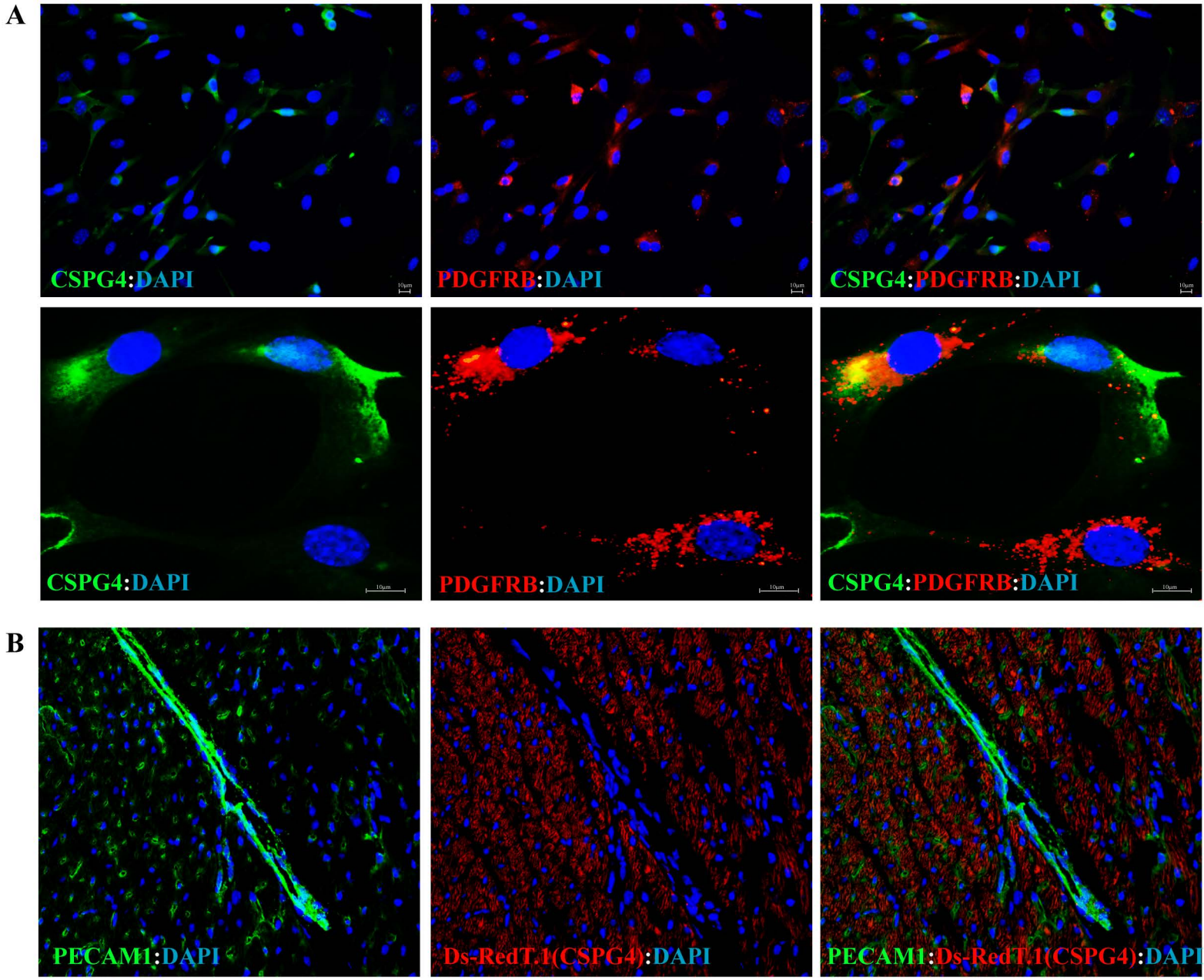
Online Figure I: FACS analysis of cardiac CSPG4^{pos} cells

A. Distribution plot of non-myocytes cardiac cells sorted against a custom-made anti CSPG4 antibody

B. Distribution plot of non-myocytes cardiac cells sorted against an established CNTN2 antibody

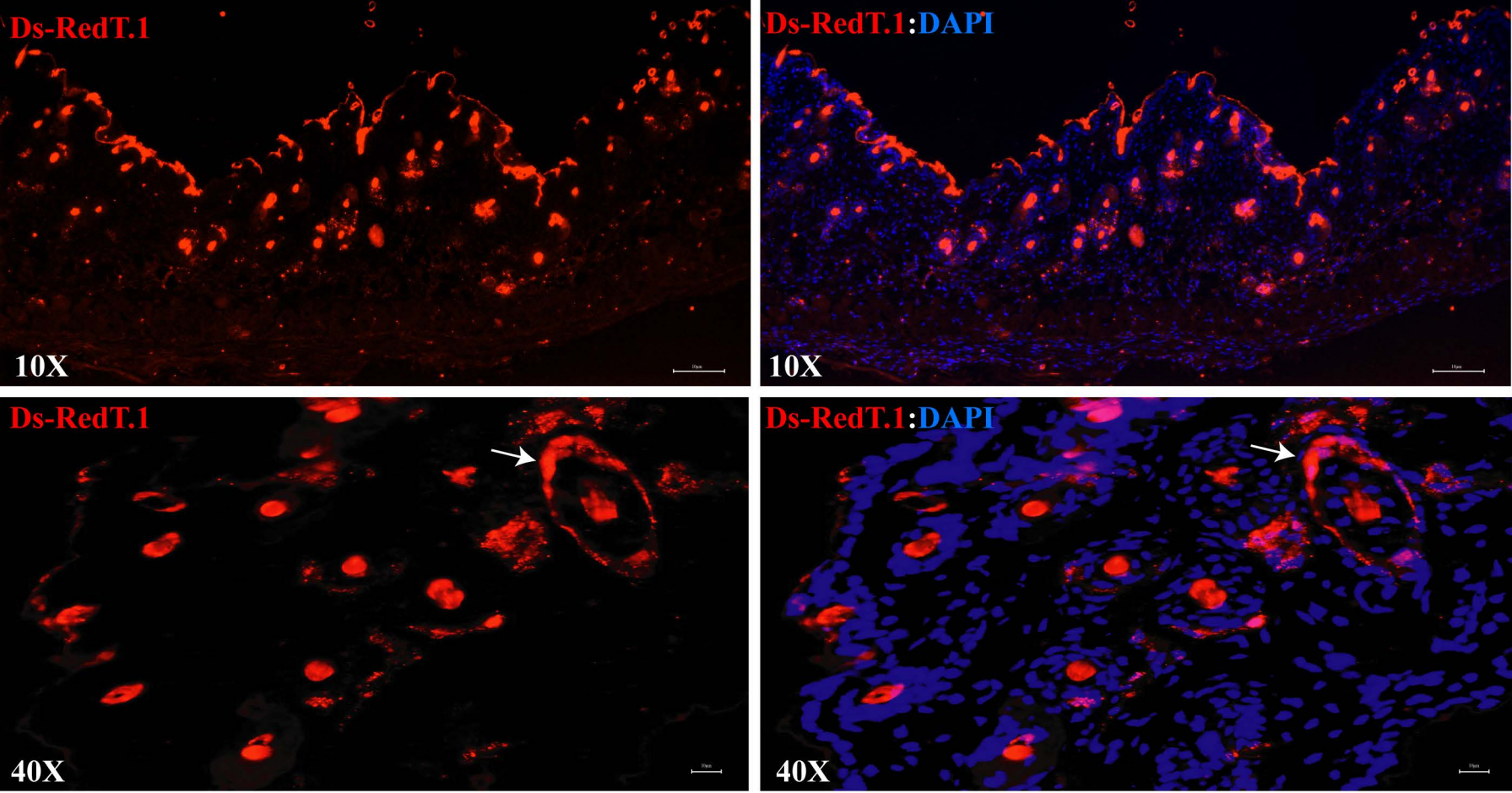
C. FACS plot with secondary antibodies only

D. FACS analysis of non-myocyte cardiac cells against CSPG4 and CNTN2 antibodies, showing the majority of cells

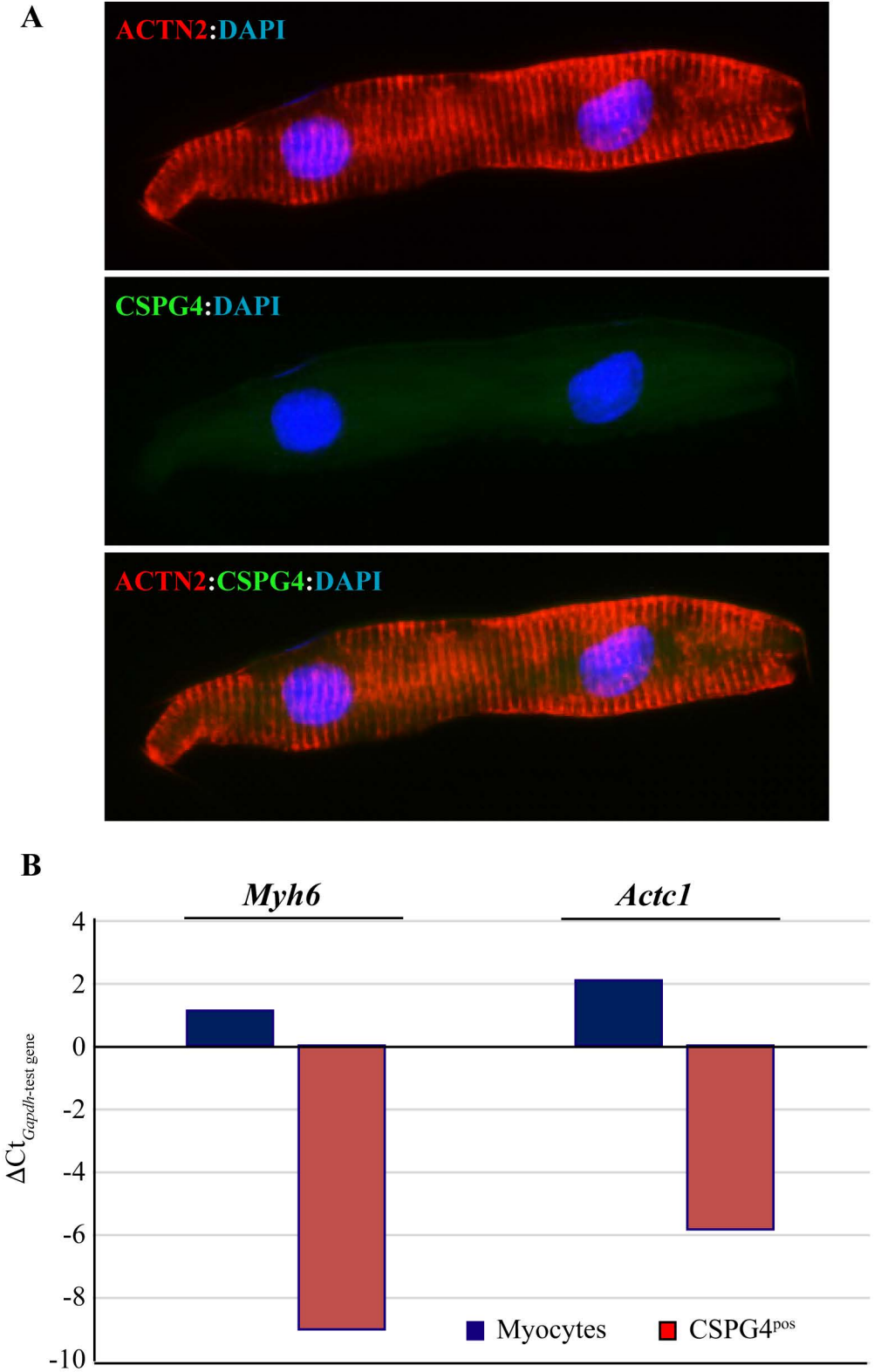


Online Figure II. Expression of CSPG4 in other cardiac cells and cardiac markers in CSPG4^{pos} cells:
A. Expression of CSPG4 in a subset of cardiac pericytes, identified by PDGRFB, the latter a marker for pericytes, in isolated non-myocyte cardiac cell fraction. Low (upper) and high (lower) magnification panels are shown. Approximately, 8.1±10.6% and 2.6±3.3% of cells stained positive for CSPG4 and PDGFRB, respectively. Only 9.8% of cells staining positive for CSPG4 also stained positive for PDGFRB.

B. Absence of expression of CSPG4 in cardiac endothelial cells, identified by PECAM1, a known marker for the endothelial cells, in *Cspg4*:Ds-RedT.1 reporter mice. Ds-Red.T1 serves as a reporter for CSPG4^{pos} cells.



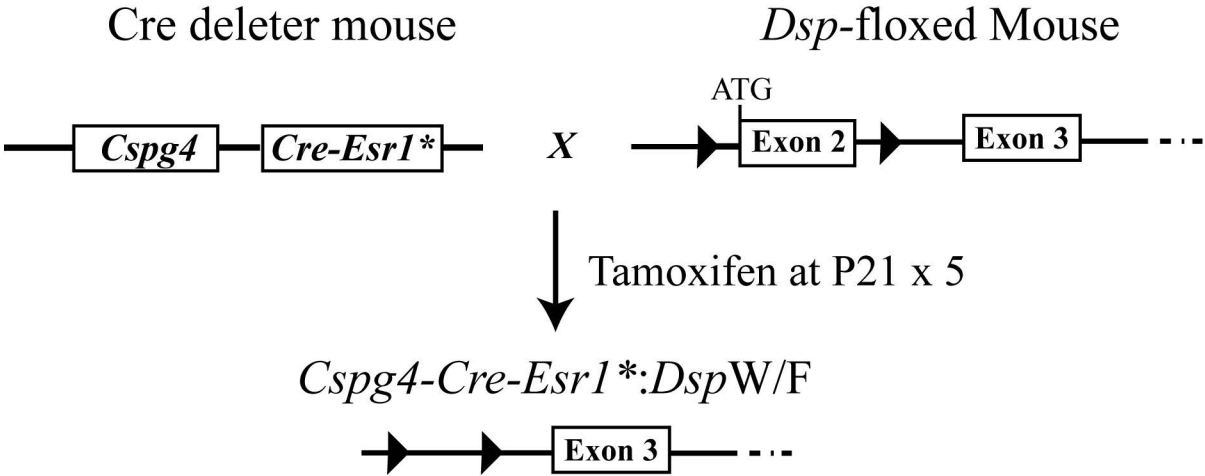
Online Figure III. This skin sections prepared from *Cspg4:Ds-RedT.1* mouse showing expression of Ds-RedT.1 reporter protein, as a surrogate for the expression of CSPG4 protein, in epidermal skin and around hair follicles, the latter shown by an arrow.
The upper and lower panels show low and high magnification fields, respectively. (hair shafts are autoflourescent)



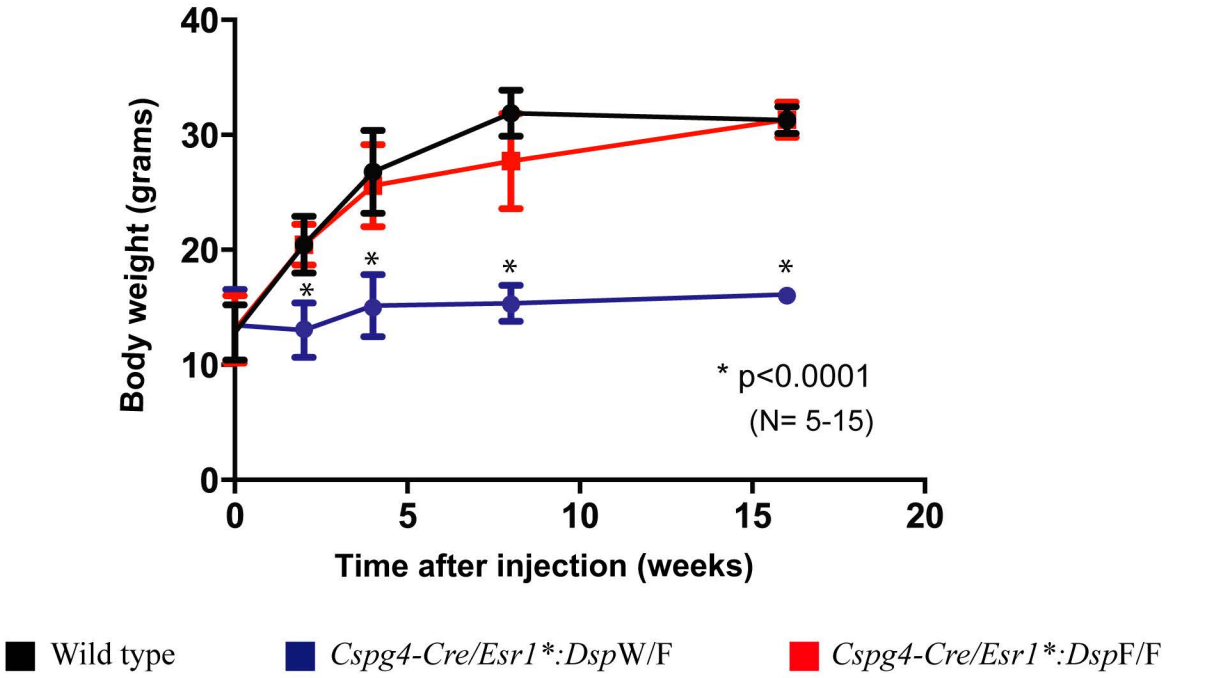
Online Figure IV.

A. Co-staining of isolated cardiac myocytes from a wild type mouse heart showing absence of expression of CSPG4 in cardiac myocytes.

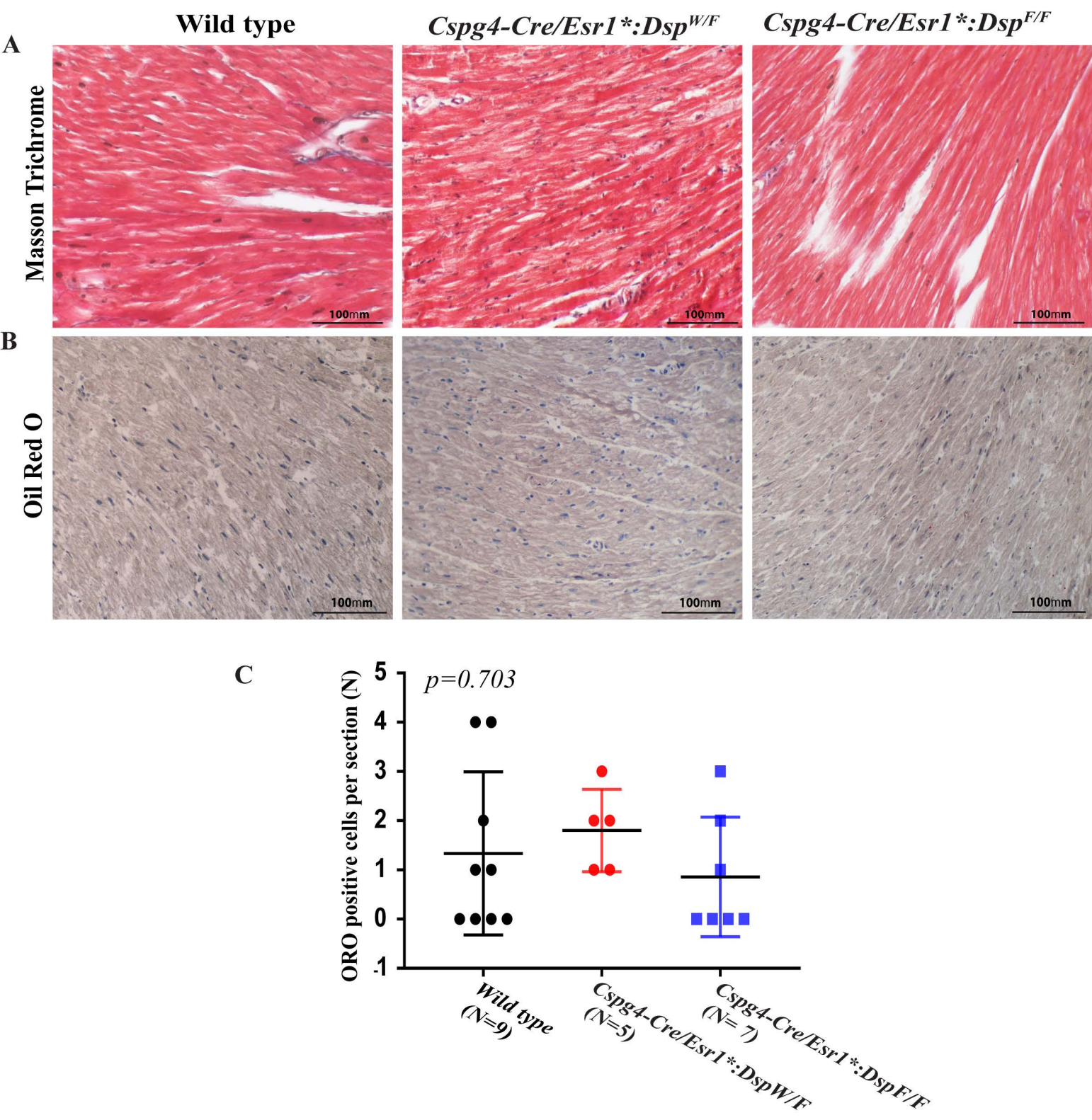
B. Extremely low levels of myosin heavy chain 6 (*Myh6*) and cardiac α actin (*Actc1*) transcripts, markers of cardiac myocytes in mouse cardiac CSPG4^{pos} cells. Ct values of the amplifications plots are depicted relative to the corresponding *Gapdh* Ct values.



Online Figure V.
Design of the constructs for the specific deletion of Dsp gene under transcriptional regulation of the Cspg4 locus.



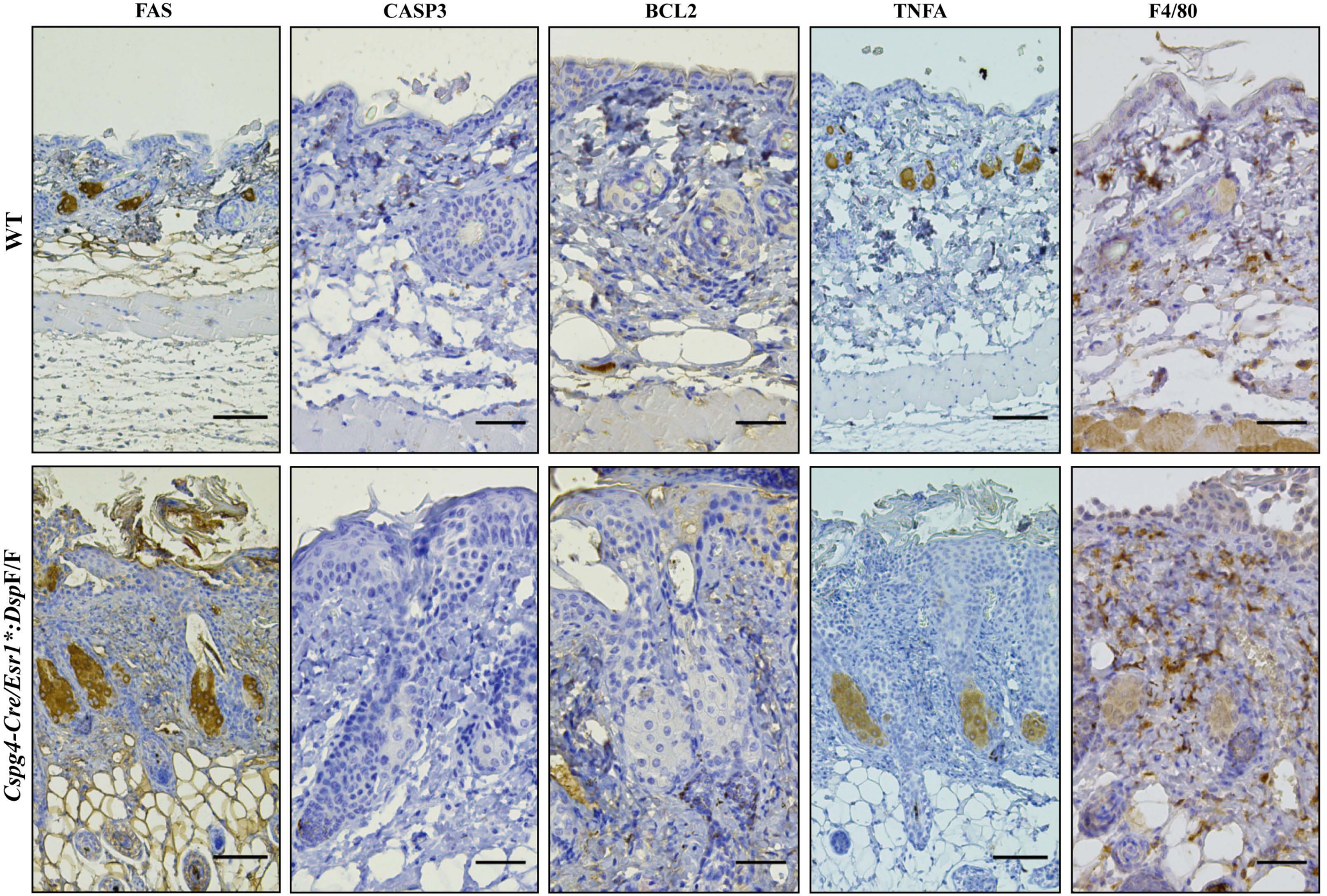
Online Figure VI. Growth retardation in the *Cspg4-Cre/Esr1*:Dsp*F/F mice. Body weight measured on a weekly basis was markedly less in the *Cspg4-Cre/Esr1*:Dsp*F/F as compared to WT or *Cspg4-Cre/Esr1*:Dsp*W/F mice.



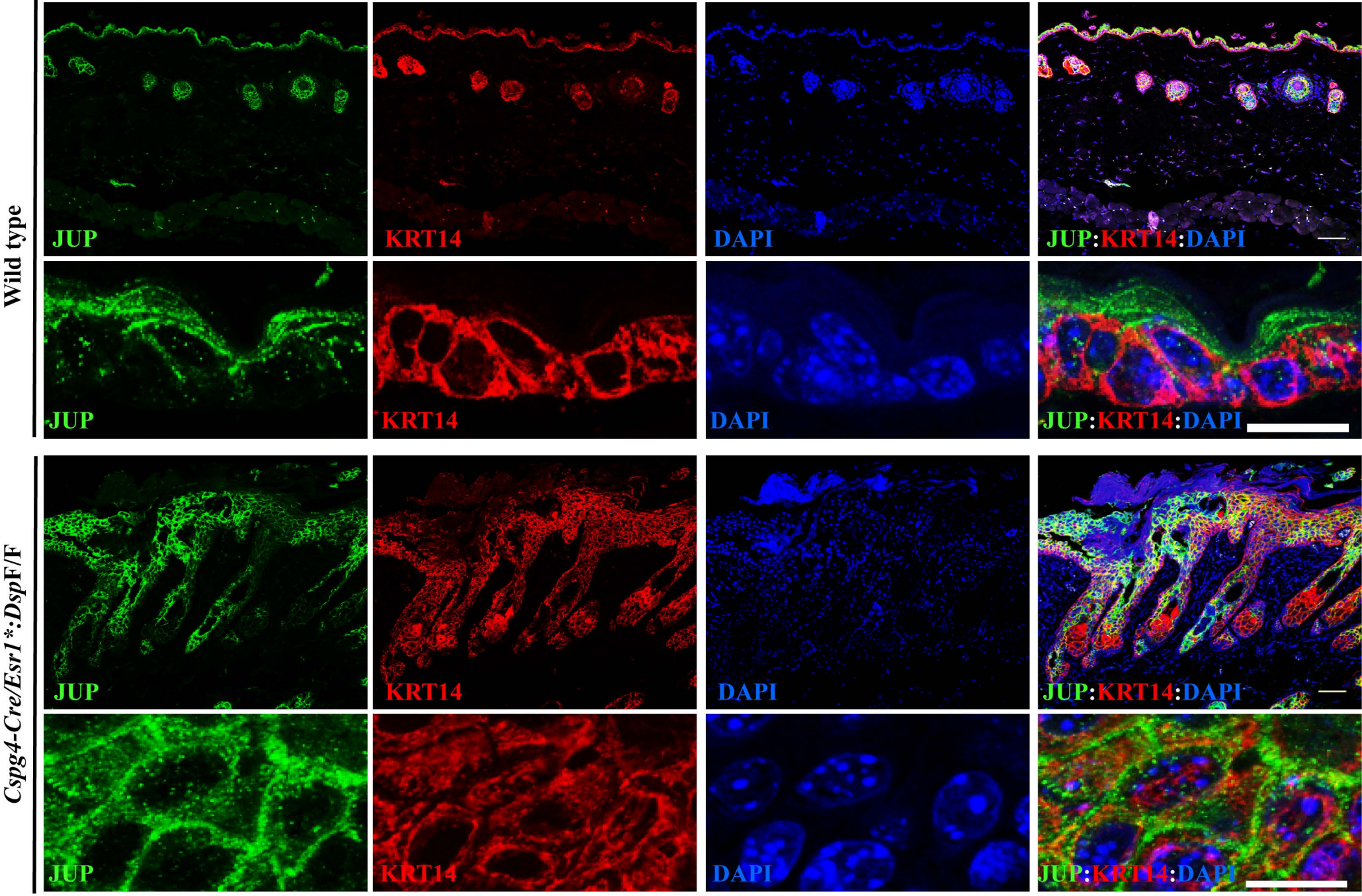
Online Figure VII. Fibro-adipocytes in the heart

Panel A shows Masson trichrome stained thin myocardial section in the wild type (WT), *Cspg4-Cre-Esr1*:Dsp*$^{W/F}$ and *Cspg4-Cre-Esr1*:Dsp*$^{F/F}$ showing no discernible fibrosis.

Panel B shows Oil red O stained thin myocardial section, and Panel C shows quantiative data showing no increased in the number of Oil red O positive cells in the Dsp-deficient mouse hearts.



Online Figure VIII. Immunostaining of skin sections from wild type (WT) and desmoplakin-deficient (*Cspg4-Cre/Esr1*:Dsp*F/F) mice for selected markers of sebocytes, apoptosis and inflammation, showing no differences in the expression levels of selected markers of apoptosis or inflammation between the two groups. Increased expression of FAS, a marker for sebocytes, is consistent with the hyperproliferative skin phenotype.



Online Figure IX. Co-immunostaining for keratin 14 (KRT14) and junction protein plakoglobin (JUP) in wild type (top two panels) and *Cspg4-Cre/Esr1*:Dsp*F/F (buttom two panels) mice.

Low (top) and high (lower) magnification panels in each genotype group representing individually stained thin sections for JUP, KRT and DAPI are shown along with the merged panels. JUP is co-localized with KRT14 in epidermal kertinocytes in the wild type as well as DSP-deficient mice. JUP and KRT14 show increased staining reflective of increased thickness of the epidermis in the *Cspg4-Cre/Esr1*:Dsp*F/F mice. Scale bar: 50 µm.