nature biomedical engineering

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41551-018-0344-5

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Mechanical activation of noncoding-RNA-mediated regulation of disease-associated phenotypes in human cardiomyocytes

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Supplemental Methods

iPSC Maintenance and Cardiac differentiation. iPSCs were cultured on Matrigel (Corning, cat # 354277)-coated well plates with mTESR-1 (StemCell Technologies, cat # 85870) human pluripotent stem cell culture medium. iPSCs were passaged with Versene (Gibco, cat # 15040066) to maintain pluripotency or Accutase (Innovative Cell Technologies, cat # AT104) to induce cardiac differentiation as previously described.[1] For cardiac differentiation, iPSCs were cultured on Matrigel-coated well plates with mTESR-1 human pluripotent stem cell culture medium to 90% confluence. On day 0, cells were then treated with 10-12 μM CHIR99021 (LC Laboratories, cat # C-6556) in RPMI 1640 media (Gibco, cat # 11875) and B27 supplement (Thermo Fisher, cat # 17504). On day 1, CHIR was removed and cells were cultured in RPMI 1640 media and B27 minus insulin supplement (Thermo Fisher, cat # A18956). On day 3, cells were changed to a combined media consisting of 0.5 mL of used media, 0.5 mL of RPMI media containing B27 minus insulin supplement, and 5 μM IWP2 (Tocris, cat # 3533). Cells were changed with RPMI media containing B27 minus insulin supplement on day 5, followed by media changes with RPMI media containing B27 supplement on days 7 and 9. On days 11-17, lactate media consisting of RPMI 1640 (Gibco, cat # 11879), 5 mM lactic acid (Acros Organics, cat # 18987), and 6 mM HEPES (Teknova, cat # H1030) was added to purify cardiomyocytes. Lastly, cells were then cultured in RPMI media containing B27 supplement until dissociation on day 30.

Flow Cytometry Analysis. On day 30, purified cardiomyocytes were disassociated with 0.25% Trypsin-EDTA (Gibco, cat # 25200) at 37 C and fixed in 3.7% formaldehyde for 20 minutes at 4 C. Cells were resuspended in 90% methanol at 4 C for 15 minutes followed by incubation with anti-cardiac troponin T (1:200, Thermo cat # MS295P) and anti-smooth muscle actin antibodies (1:200, Abcam cat # ab32575) for 1 hour at room temperature. Cells were then incubated with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (1:1000, Thermo cat # A-11001) and Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (1:1000, Thermo cat # A-11011) for 1 hour at room temperature before being analyzed via FACS (FACSCanto, Becton Dickinson). See Supplementary Figure S14 for the gating strategy used.

Atomic Force Microscopy. Hydrogel stiffness measurements were determined by atomic force microscopy (Figure 1B, MFP-3D Bio, Asylum Research) with a silicon nitride cantilever (NanoAndMore USA Corporation, cat # PNP-TR). Tip deflections were converted to indentation force for all samples using their respective tip spring constants and Hooke's Law. All AFM data was analyzed using the Igor 6.34A software to determine Young's Modulus as previously described based on a Hertz model.[2,3]

Immunofluorescence assays. Cells were fixed with 3.7% formaldehyde in PBS for 20 minutes at 4ºC and permeabilized using 1% w/v Triton X-100 for 15 minutes at room temperature. For validation of stem cell pluripotency, iPSCs were incubated in 2.5% donkey serum blocking buffer for 30 minutes at room temperature followed by incubation with anti-Sox2 (1:200, Abcam cat, # ab171380), anti-Nanog (1:20, Abgent, cat # AP1486c), and anti-Oct4 (1:200, Abcam, cat # ab27985) antibodies in 2.5% donkey serum for two hours at room temperature. Samples were then incubated with Alexa Fluor 488 conjugated donkey anti-mouse secondary antibody (1:1000, Thermo, cat # A-21202), Alexa Fluor 568 conjugated donkey anti-rabbit secondary antibody (1:1000, Thermo, cat # A-10042), and Alexa Fluor 647 conjugated donkey anti-goat secondary antibody (1:1000, Thermo, cat # A-21447) in 2.5% donkey serum for 45 minutes. Samples were lastly incubated in 4',6-Diamidino-2-Phenylindole (DAPI, 1:5000, Thermo, cat # D1306) in DH_2O for 3 minutes at room temperature, mounted with Fluoromount-G (Southern Biotech, cat # 0100-01), and imaged using a 10x objective on a Nikon Eclipse TI fluorescent microscope with a CARVII confocal attachment (Becton Dickenson) and Metamorph 7.6 software. For gap junction and sarcomere assembly and localization, cardiomyocytes were incubated with anti-α-actinin (1:250, Sigma A7811) and anti-connexin 43 (1:200, Sigma, cat # C6219) antibodies followed by Alexa Fluor 488 conjugated goat anti-mouse secondary antibody (1:1000, Thermo, cat # A-11001) and Alexa Fluor 568 conjugated goat anti-rabbit secondary antibody (1:1000, Thermo, cat # A-11011) and imaged using a 60x water objective.

To analyze sarcomere organization, a line was traced through randomly selected sarcomeres in ImageJ to obtain fluorescent intensity values. A fast Fourier transform (FFT) was then performed and the power spectra was normalized to 1. The height of the first non-zero frequency term was used to estimate organization as previously described[4,5]. Sarcomere length was calculated by

measuring the length between randomly selected sarcomeres in ImageJ. Values on dynamically stiffened or static stiff gels were normalized to those on static soft gels for each cell line in the same manner as described in the calcium analysis. Raw values are provided in Supplemental Table S2.

To ensure no DNA damage from UV exposure, cardiomyocytes were incubated with anti-p53 antibody (1:250, Thermo, cat # AHO0152) followed by Alexa Flour 568 goat anti-rabbit secondary antibody.

Scrape-loading dye transfer assay. The scrape-loading dye transfer (SL/DT) technique was performed as previously described[6]. On day 10, cells were washed with PBS, 1 mg/mL Lucifer Yellow (Biotium, cat # 80015) was added, and scrape-loaded using a surgical razor blade. To ensure that Lucifer Yellow movement was due to gap junctions, cells were also loaded with 1 mg/mL 10 kDa rhodamine dextran (Sigma-Aldrich, cat # R8881). After 6 minutes at room temperature, cells were washed with PBS and fixed in 3.7% formaldehyde for 10 minutes. Cells were imaged using a 20x objective. Analysis was performed by first using Image J to threshold the region over which the dye was transferred and then measuring the average distance over which the dye traveled.

Quantitative PCR. RNA was extracted by Trizol-chloroform extraction according to manufacturer's instructions (Thermo Scientific, cat # 15596) and treated with Ambion® DNA-free™ DNase Treatment (Thermo Scientific, cat # AM1906) per manufacturer's instructions to remove genomic DNA contamination. 2 µg of RNA was then reverse transcribed to cDNA using Super Script III Reverse Transcriptase (Thermo Scientific, cat # 18080093). Quantitative PCR was performed (45 cycles, 95°C for 15 seconds followed by 60°C for 1 min) using a 7900HT Fast Real-Time PCR System (Thermo Scientific, cat # 4329001) with the primer sets described in supplementary material Table S1, and the iQ SYBR Green Supermix (Bio-Rad Laboratories, cat # 1708880). Data were analyzed by calculating quantities of RNA based on a standard curve generated from a fibronectin plasmid.

Measurements of total ANRIL, short ANRIL isoforms, and long ANRIL isoforms were plotted where the values are normalized to N/N 1 values. To assess the effect of stiffening on mRNA expression, values on stiffened and stiff substrates were normalized to soft values in the same manner as described in the calcium analysis. p16 and connexin 43 values for stiffened and stiff substrates were normalized to soft values. Raw values are provided in the supplemental data section.

Phospho-kinase Antibody Array. A kinase screen capable of measuring relative phosphorylation of 43 different kinases was used to determine the effects of stiffening on cell behavior. Cells were grown on soft MeHA for 5 days before stiffening. Cells were lysed 30 minutes after stiffening and the kinase experiment was performed per manufacturer's instruction (R&D Systems, cat # ARY003B). 150 μg of protein was incubated for each membrane overnight at 4 ºC followed by incubation with the primary antibody solution for 2 hours at room temperature. Membranes were then incubated with streptavidin-HRP for 30 minutes followed by application of the chemiluminescent reagent mix. Membranes were exposed to film and imaged. Quantification of scanned film was performed by measuring pixel density in ImageJ. Values for stiffened conditions were normalized to those on soft.

Western Blot. For p-JNK and JNK analysis, cells were grown on MeHA substrates for 5 days before stiffening. Cells were lysed 30 minutes, 2 hours, and 6 hours post stiffening using RIPA buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Triton, 10% glycerol, 25 mM sodium deoxycholate, 0.1% SDS) containing Roche Complete Protease Inhibitor (Sigma-Aldrich, cat # 11697498001) and PhosSTOP (Sigma-Aldrich, cat # 4906845001). Lyastes were vortexed every 5 minutes for 30 minutes before centrifugation at 14000xg for 15 minutes. The supernatant was transferred to a clean tube and the protein concentrations of the samples were determined using a Pierce BCA Protein Assay kit (Thermo Scientific, cat # 23225), according to the manufacturer's instructions. 12 μg of protein from each sample was loaded into Bolt 4-12% Bis-Tris gels (Thermo, cat # NW04120BOX) and separated by electrophoresis in MES running buffer (50 mM MES, 50 mM Tris Base, 1 mM EDTA, 0.1% w/v SDS) under reducing and denaturing conditions before being transferred onto a nitrocellulose membrane using the iBlot 1 semi-dry transfer system (Invitrogen, cat # IB4010). Membranes were incubated with 5%

Seablock blocking buffer (Thermo, cat # 37527) in Tris buffered saline with tween (TBS-T, 150 mM NaCl, 15 mM Tris-HCl, 20 mM Tris Base, .1% Tween) for one hour followed by overnight incubation with either phosphorylated JNK (1:1000, Cell Signaling, cat # 4668) or total JNK (1:1000, Cell Signaling, cat # 9252) and Glyceraldehyde 3-phosphate dehydrogenase (1:7500 Abcam, cat # ab9484) or either phosphorylated connexin 43 (1:1000, Cell signaling, cat # 3511S) or total connexin 43 (1:2000, Sigma, cat # C6219) and Glyceraldehyde 3-phosphate dehydrogenase. Membranes were then incubated with Alexa Fluor 680 donkey anti-mouse (0.2 μg/mL, Thermo, cat # A10038) and Alexa Fluor 790 donkey anti-rabbit (0.2 μg/mL, Thermo, cat # A11374) for two hours. Blots were imaged using the Li-Cor Odyssey CLx imaging system (Li-Cor) and the integrated densities of bands were analyzed using the Li-Cor Image Studio Lite software.

For connexin analysis, cells were lysed on Day 10 as described above and 10 μg of protein were loaded into gels. To ensure phosphorylated connexin 43 antibody was working, human umbilical vein endothelial cells (Lonza) were treated with 250 nM phorbol 12-myristate 13-acetate (LC Laboratories, cat # P-1680) for 30 minutes. All values were normalized to soft values for each cell line.

JNK Signaling Analysis.

To activate JNK, cardiomyocytes were grown on soft hydrogels for 5 days before treatment with 100 ng/ml anisomycin (Cayman Chemical Company, cat # 11308) overnight. Cells were then fixed and stained for connexin 43 and α-actinin as described above. To inactivate JNK, R/R cells were treated with 10 μM SP600125 (Selleckchem, cat # S1460) after stiffening followed by calcium and immunofluorescent imaging on day 10.

Electrical stimulation of cardiomyocytes. Cardiomyocytes were field stimulated using a pair of platinum wires that were connected to MyoPacer Field Stimulator (IonOptix). Calcium handling was assessed as previously described.

Supplemental Figures

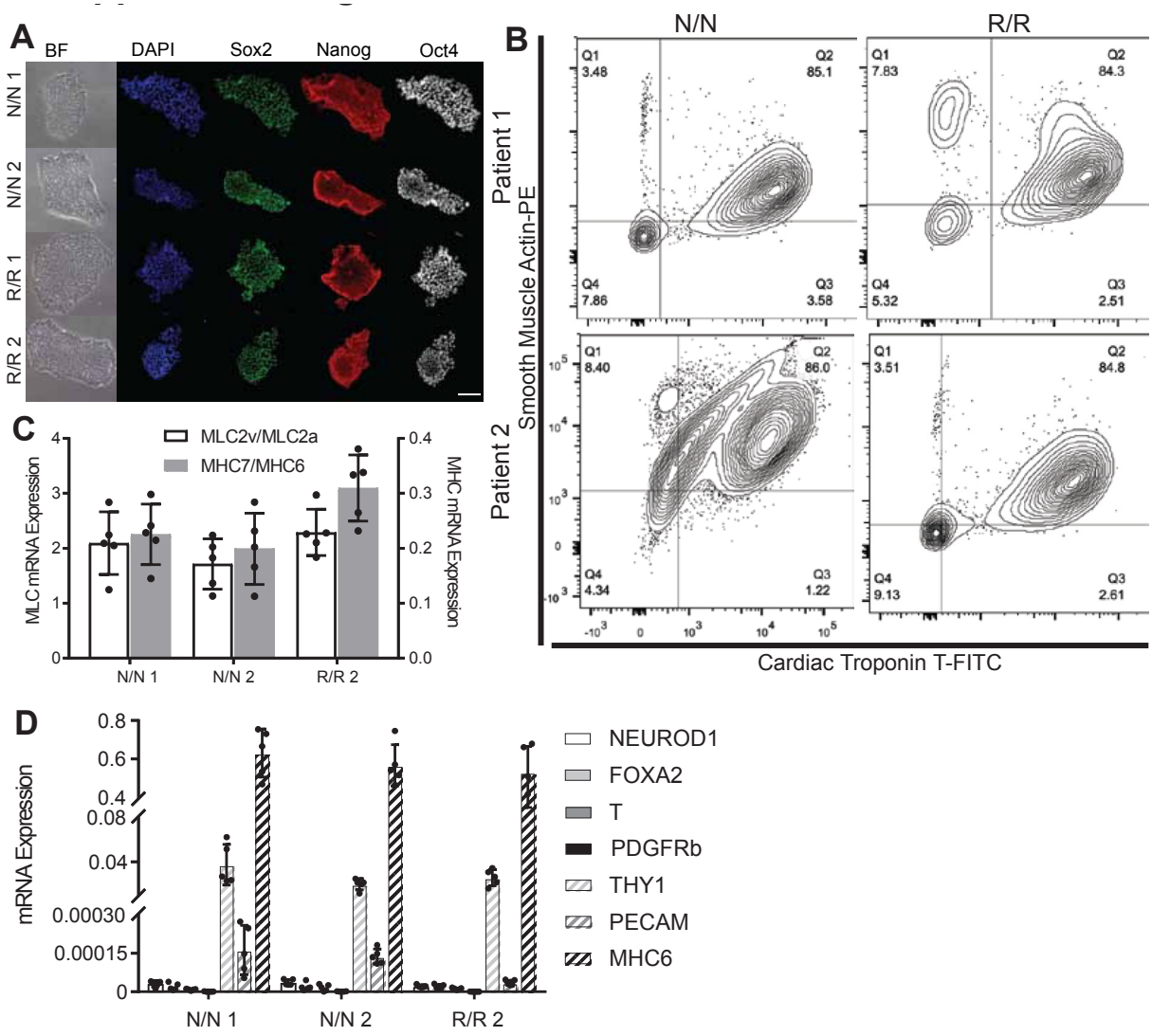

Figure S1: Cardiac Differentiation Efficiency and Cell Validation of iPSC-derived Cardiomyocytes. (A) Representative brightfield and immunofluorescent images of nuclei and Sox 2, Nanog, and Oct 4 positive stem cell colonies for the indicated genotypes and for multiple patients. Scale bar, 100 µm. Experiments were performed two independent times. **(B)** FACS analysis is shown for cardiac troponin T and smooth muscle actin expression in patient-derived iPSCs of the indicated genotypes after the cardiomyocyte differentiation protocol. ~85% cells were doubly positive for each patient line. Experiments were performed two independent times. **(C)** Ratio of isoform expressions of myosin light chain and myosin heavy chain were plotted (n

= 5 samples). Mean±s.d. with individual points overlaid. **(D)** mRNA expression for markers of contaminating cells were plotted (n = 5 samples). Myosin heavy chain 6 expression was plotted as a cardiac marker for comparison. All data was normalized to GAPDH. Mean±s.d. with individual points overlaid.

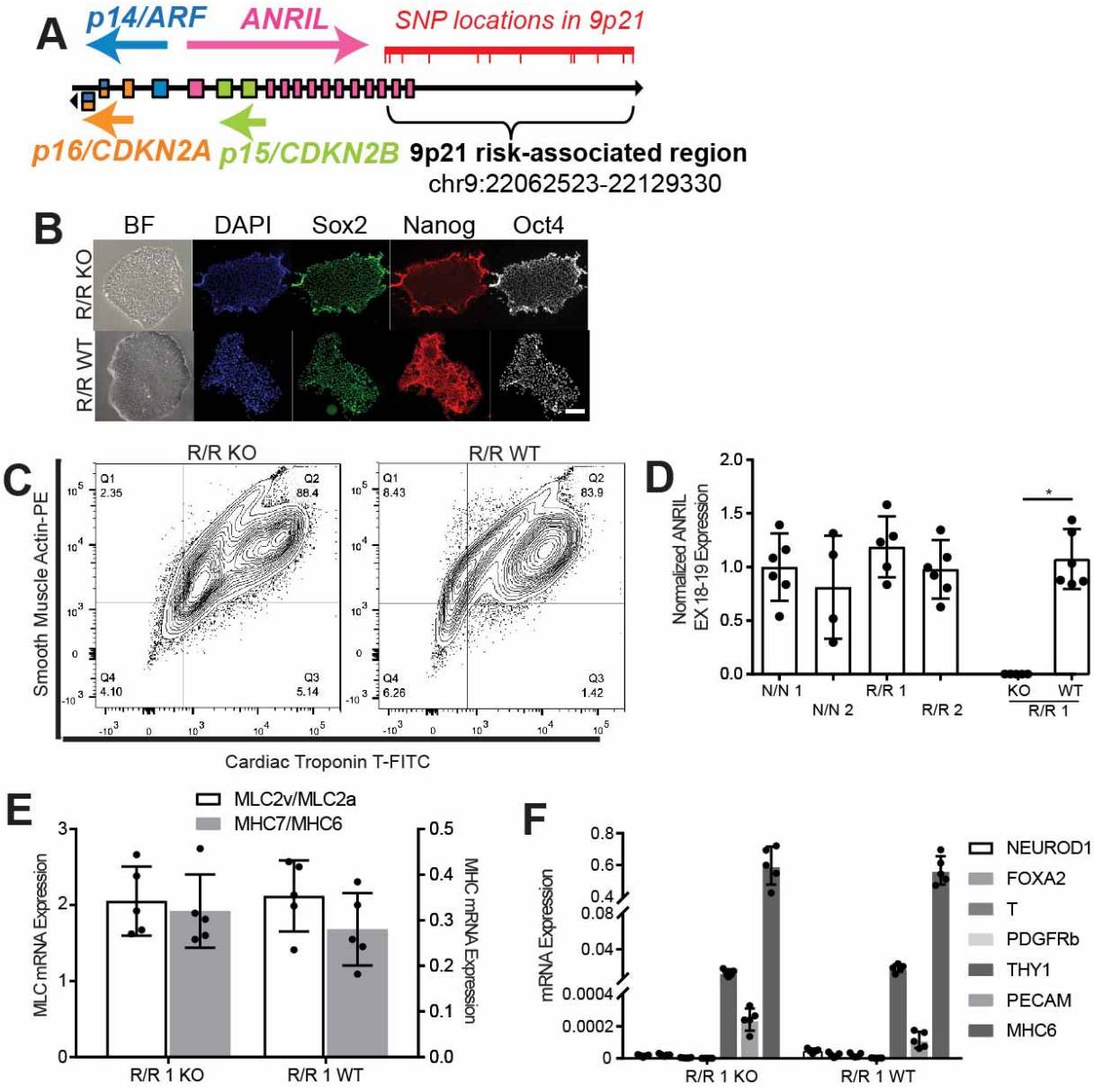

Figure S2: TALEN-mediated Deletion of the risk haplotype from R/R Patient 1 iPSCs. (A) Schematic of the 9p21 locus with locations of adjacent genes, ANRIL, and heart disease-associated SNPs. **(B)** Representative brightfield and immunofluorescent images of nuclei and Sox 2, Nanog, and Oct 4 positive stem cell colonies for the indicated cell lines. Scale bar, 100 µm. Experiments were performed two independent times. **(C)** FACS analysis of cardiac troponin T and smooth muscle actin positive cardiomyocytes. Percentage of doubly positive cells was approximately 85% for both cell lines. Experiments were performed two independent times. **(D)** mRNA transcript of exon 18-19 of ANRIL, demonstration deletion of the risk haplotype in

the R/R KO line in cells cultured on static soft hydrogels. *p < 0.00001 for R/R 1 KO vs WT; two-tailed Student's t-test. N/N 1, n = 6 samples; N/N 2, n = 4 samples; R/R 1, n = 5 samples; R/R 2, n = 6 samples; R/R 1 KO, n = 5 samples; R/R 1 WT, n = 6 samples. Mean±s.d. with individual points overlaid. **(E)** Ratio of isoform expressions of myosin light chain and myosin heavy chain were plotted (n = 5 samples). Mean±s.d. with individual points overlaid. **(F)** mRNA expression for contaminating cells were plotted (n = 5 samples). Myosin heavy chain 6 expression was plotted as a cardiac marker for comparison. Mean±s.d. with individual points overlaid.

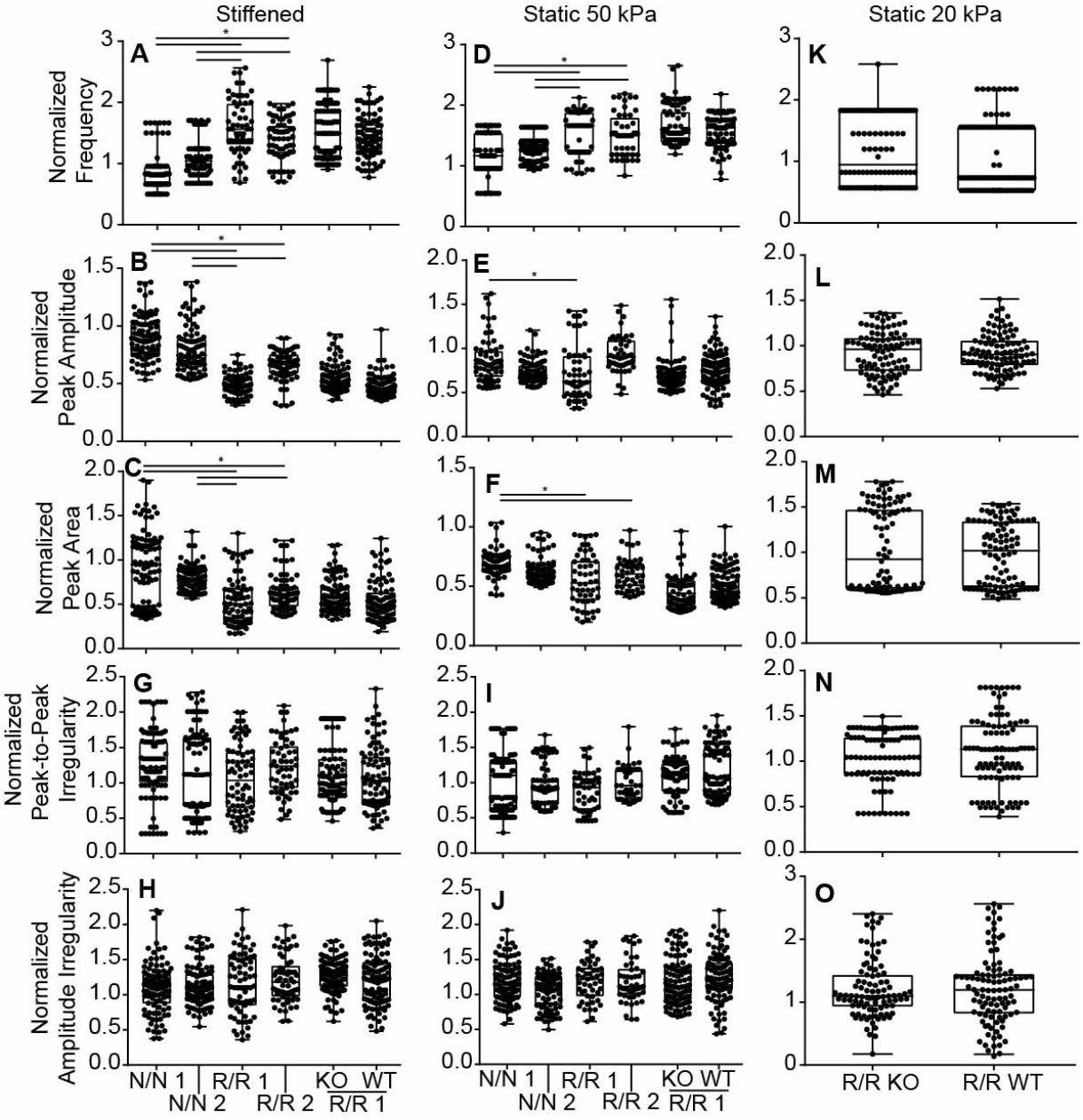

Figure S3: Ca^{2+} Handling in Response to Dynamic Stiffening or Stiff Substrates. (A) Frequency of spontaneous Ca^{2+} transients for cardiomyocytes on stiffened substrates was graphed. *p = 0.0001 for N/N vs R/R comparisons; one way ANOVA-Tukey. **(B)** Peak amplitude for cardiomyocytes on stiffened substrates was graphed. *p = 0.0001 for N/N vs R/R comparisons; one way ANOVA-Tukey. **(C)** Peak area for cardiomyocytes on stiffened substrates was graphed. *p = 0.0001 for N/N 1 vs R/R 1 and *p = 0.0001 for N/N 1 vs R/R 2, *p = 0.0001 for N/N 2 vs R/R 2, *p = 0.0037 for N/N 2 vs R/R 2; one way ANOVA-Tukey. N/N 1, n = 99

cells; N/N 2, n = 100 cells; R/R 1, n = 61 cells; R/R 2, n = 70 cells; R/R 1 KO, n = 100 cells; R/R 1 WT, n = 100 cells for panels A-C. **(D)** Frequency of spontaneous Ca^{2+} transients for cardiomyocytes on stiff substrates was graphed. *p = 0.0001 for N/N vs R/R comparisons; one way ANOVA-Tukey. **(E)** Peak amplitude for cardiomyocytes on stiff substrates was graphed. *p = 0.042 for N/N 1 vs R/R 1; one way ANOVA-Tukey. **(F)** Peak area for cardiomyocytes on stiff substrates was graphed. *p = 0.003 for N/N 1 vs R/R 1, *p = 0.006 for N/N 1 vs R/R 2; one way ANOVA-Tukey. N/N 1, n = 70 cells; N/N 2, n = 84 cells; R/R 1, n = 54 cells; R/R 2, n = 48 cells; R/R 1 KO, n = 100 cells; R/R 1 WT, n = 100 cells for panels D-F. (**G**) Peak-to-peak irregularity for cardiomyocytes on stiffened substrates was plotted. N/N 1, n = 100 cells; N/N 2, n = 94 cells; R/R 1, n = 74 cells; R/R 2, n = 59 cells; R/R 1 KO, n = 99 cells; R/R 1 WT, n = 91 cells. (**H**) Amplitude irregularity for cardiomyocytes on stiffened substrates was plotted. N/N 1, n = 99 cells; N/N 2, n = 100 cells; R/R 1, n = 69 cells; R/R 2, n = 61 cells; R/R 1 KO, n = 100 cells; R/R 1 WT, n = 100 cells. (**I**) Peak-to-peak irregularity for cardiomyocytes on stiff 50 kPa substrates was plotted. N/N 1, n = 100 cells; N/N 2, n = 100 cells; R/R 1, n = 49 cells; R/R 2, n = 48 cells; R/R 1 KO, n = 100 cells; R/R 1 WT, n = 100 cells. (**J**) Amplitude irregularity for cardiomyocytes on stiff 50 kPa substrates was plotted. N/N 1, n = 99 cells; N/N 2, n = 100 cells; R/R 1, n = 69 cells; R/R 2, n = 61 cells; R/R 1 KO, n = 100 cells; R/R 1 WT, n = 100 cells. (**K**) frequency of spontaneous Ca^{2+} transients, (**L**) peak amplitude, (**M**) peak area, (**N**) Peak-to-peak, and (**O**) amplitude irregularity for R/R KO and WT cardiomyocytes cultured on 20 kPa substrates were plotted (n = 100 cells). Values for stiffened and stiff substrates were normalized to those on soft substrates. Box plots show median, 25^{th} and 75^{th} percentiles, minimum, and maximum values for all panels.

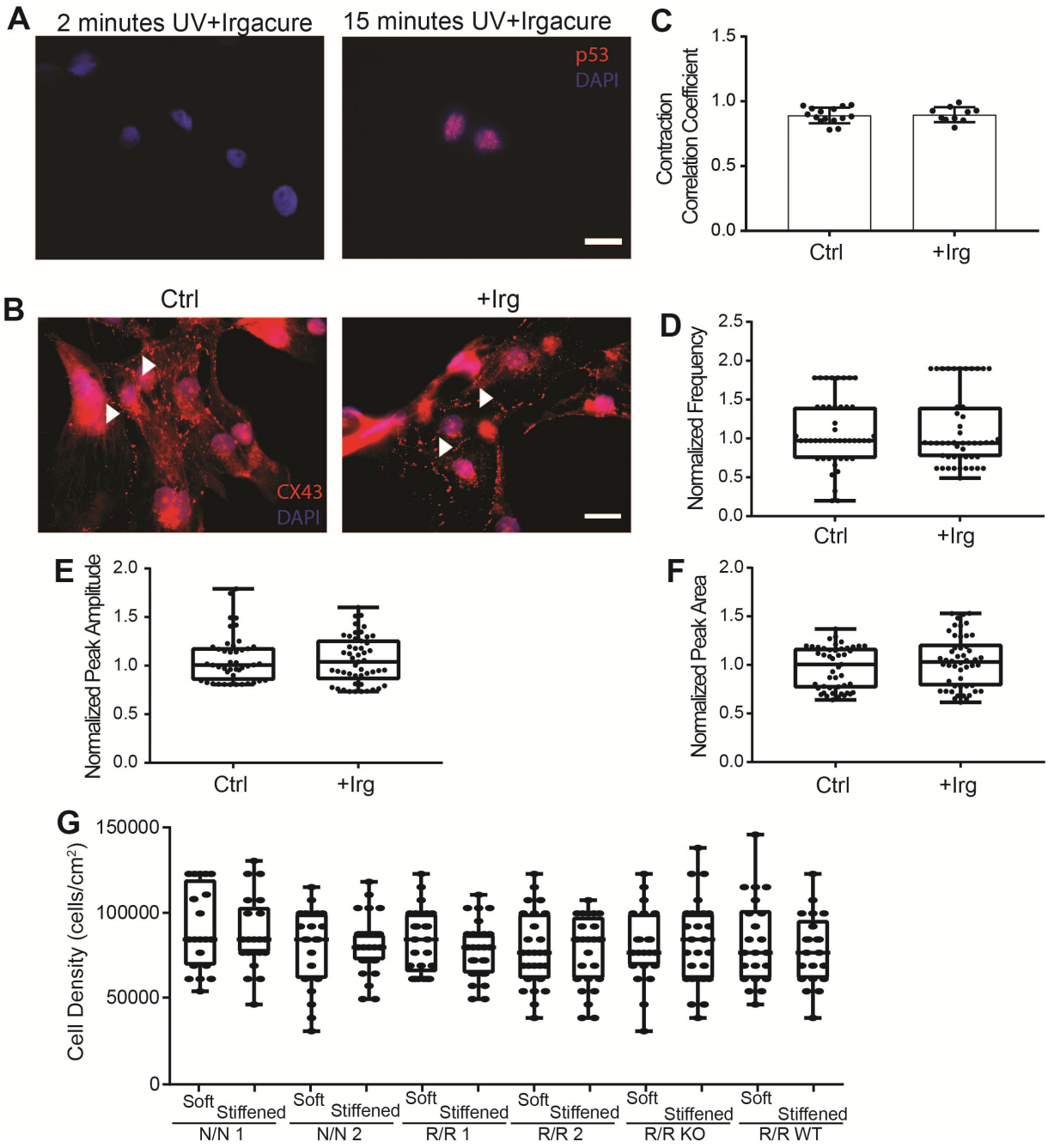

Figure S4: p53 and Connexin 43 Expression After UV Exposure and Cell Density Before and After Stiffening. Immunofluorescent staining is shown for (**A**) p53 pSer392 (red) and DAPI (blue) as well as for (**B**) connexin 43 (red) and DAPI (blue) after 2 and 15 minutes UV exposure. Scale bar, 10 µm. Experiments were performed three independent times. (**C**) Contraction correlation coefficient for R/R cardiomyocytes grown on polyacrylamide gels and either exposed to free radicals or not. Control, n = 15 videos; +Irgacure, n = 10 videos. (**D**) Frequency of

spontaneous Ca^{2+} transients was plotted. Control, n = 47 cells; +Irgacure, n = 50 cells. (**E**) Peak amplitude was plotted. Control, n = 44 cells; +Irgacure, n = 49 cells. (**F**) Peak area was plotted. Control, n = 47 cells; +Irgacure, n = 50 cells. Values for cells on gels exposed to free radicals were normalized to those that were not as indicated in panels C-E. (**G**) Cell density observed before and after stiffening MeHA hydrogels (n = 20-27 pictures). N/N 1, soft, n = 20 pictures, stiffened n = 21 pictures; N/N 2, soft, n = 22 pictures, stiffened n = 26 pictures; R/R 1, soft, n = 25 pictures, stiffened n = 27 pictures; R/R 2, soft, n = 27 pictures, stiffened n = 24 pictures. R/R 1 KO, soft, n = 22 pictures, stiffened n = 27 pictures; R/R 1 WT, soft, n = 22 pictures, stiffened n = 21 pictures. Mean±s.d. with individual points overlaid shown for panel C. Box plots show median, 25^{th} and 75^{th} percentiles, minimum, and maximum values for panel D-G.

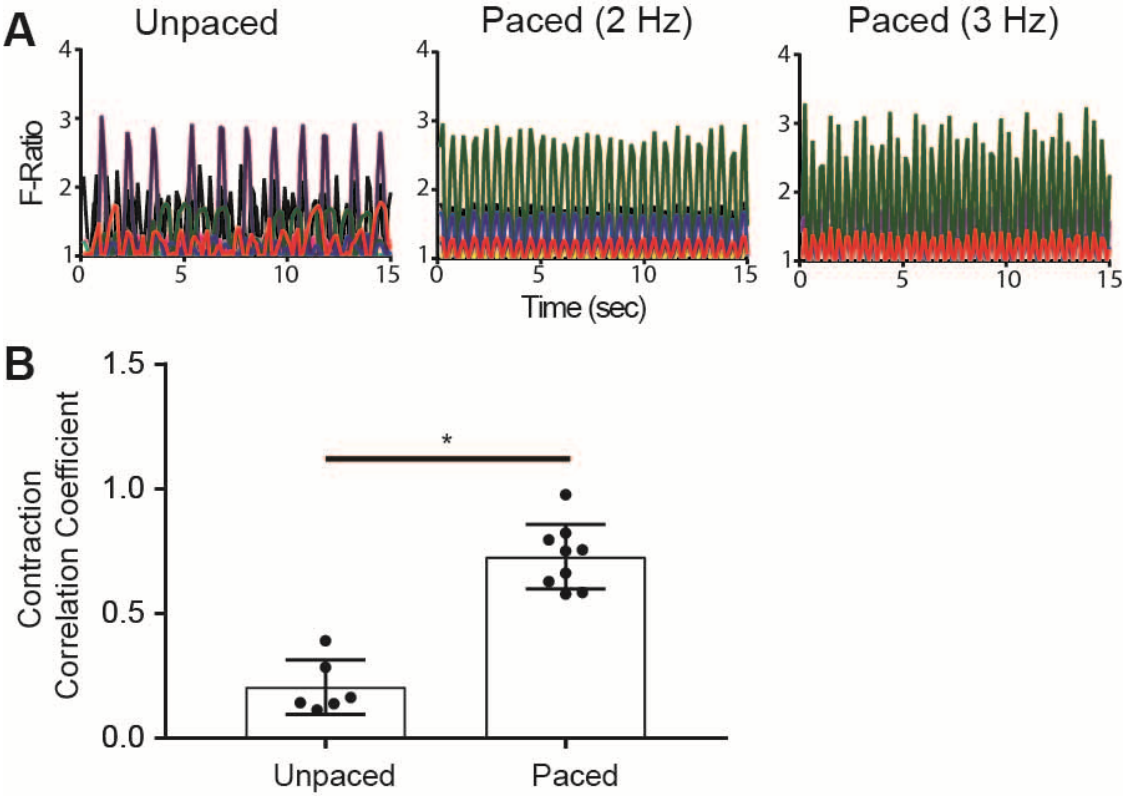

Figure S5: Electrical Pacing of R/R Cardiomyocytes. (A) Representative Ca^{2+} transients for R/R WT cardiomyocytes cultured on stiffened MeHA substrates and paced or not as indicated. Experiments were performed three independent times. (B) Contraction correlation coefficients are plotted for paced and unpaced cardiomyocytes. *p = 0.00002 based on two-tailed Student's t-test. Unpaced, n = 6 videos; Paced, n = 9 videos. Mean±s.d. with individual points overlaid.

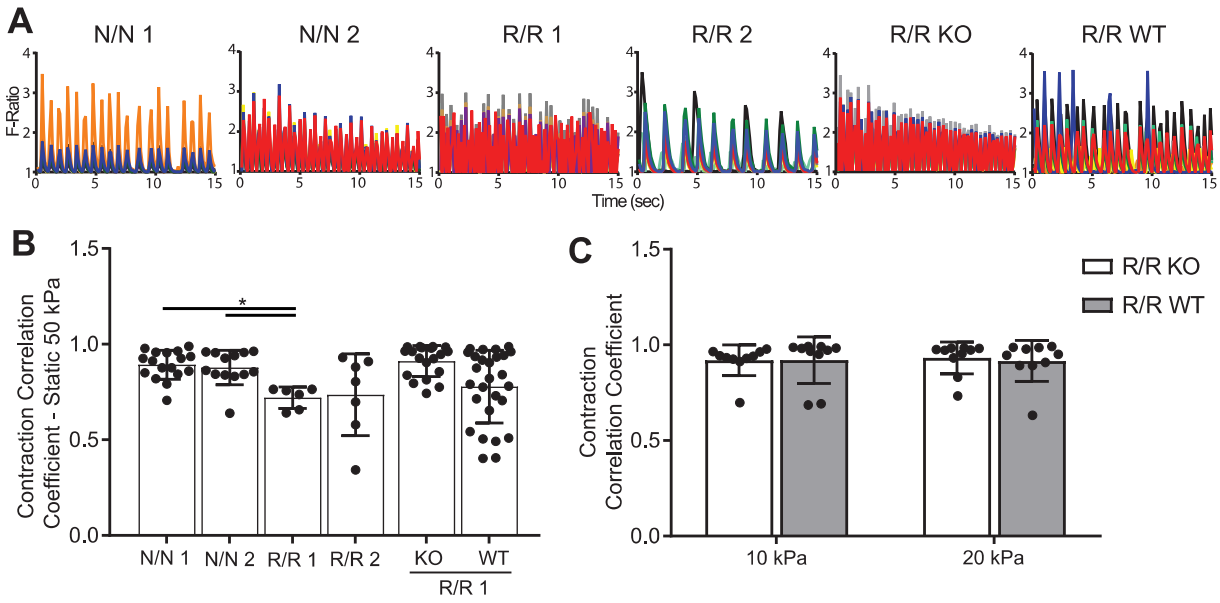

Figure S6: Synchronous Contractions on 20 and 50 kPa Substrates. (A) Representative spontaneous Ca^{2+} transients plotted as the fluorescent intensity F-ratio over a 10 second time interval are shown for cardiomyocytes cultured on stiff 50 kPa MeHA substrates. Experiments were performed three independent times. **(B)** Contraction correlation coefficient for R/R and N/N cardiomyocytes was graphed. *p = 0.0421 for N/N 1 vs R/R 1 and *p = 0.03 for N/N 2 vs R/R 1; one way ANOVA-Tukey. N/N 1, n = 17 videos; N/N 2, n = 13 videos; R/R 1, n = 6 videos; R/R 2, n = 7 videos; R/R 1 KO, n = 19 videos; R/R 1 WT, n = 27 videos. Mean±s.d. with individual points overlaid. **(C)** Contraction correlation coefficient (n = 10 videos) for R/R KO and WT cardiomyocytes cultured on 20 kPa gels were graphed. Mean±s.d. with individual points overlaid.

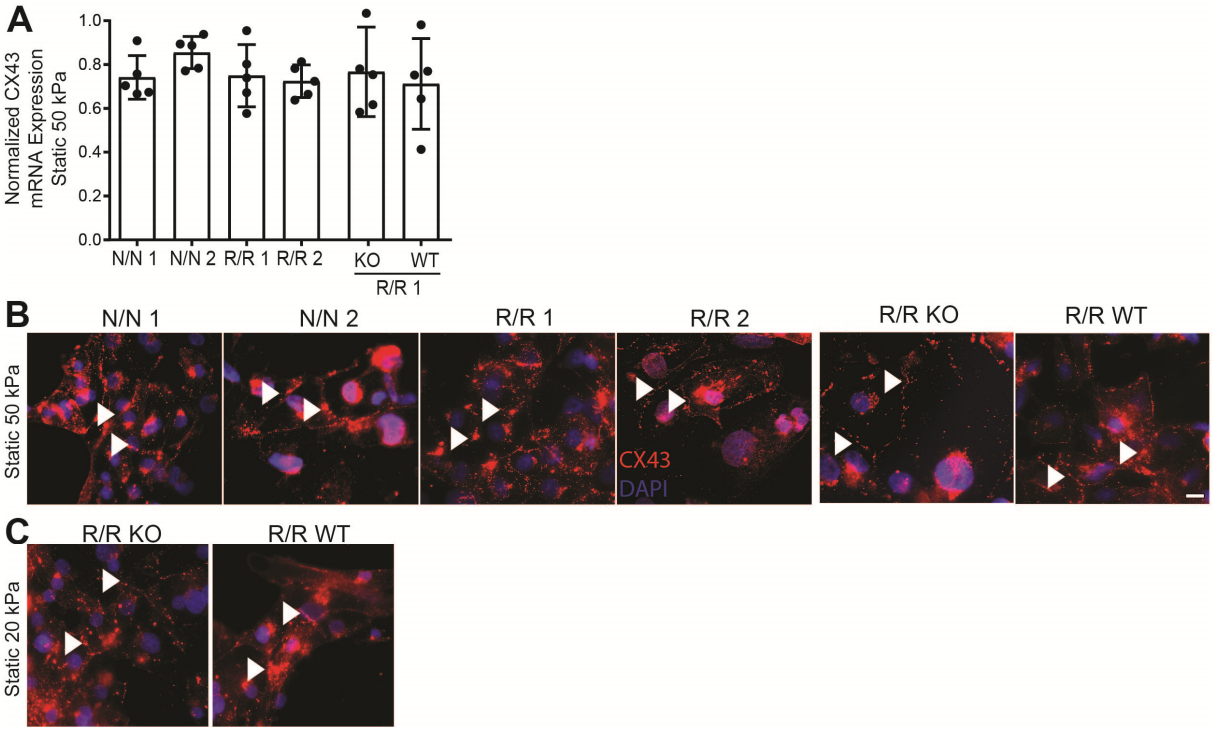

Figure S7: Connexin 43 Expression on Stiff Substrates. (A) mRNA transcript for cardiomyocytes cultured on stiff substrates was determined for connexin 43 (n = 5 samples) and plotted as a normalized to cardiomyocytes on soft substrates. Mean±s.d. with individual points overlaid. **(B)** Immunofluorescent staining for connexin 43 (red) and DAPI (blue) and for cardiomyocytes from the indicated patients and genotypes cultured on stiff 50 kPa MeHA substrates. Arrowheads indicate regions of functional connexin 43 expression. Scale bar, 10 μm. Experiments were performed three independent times. (**C**) Immunofluorescent staining for connexin 43 for R/R KO and WT cardiomyocytes cultured on 20 kPa substrates. Experiments were performed three independent times.

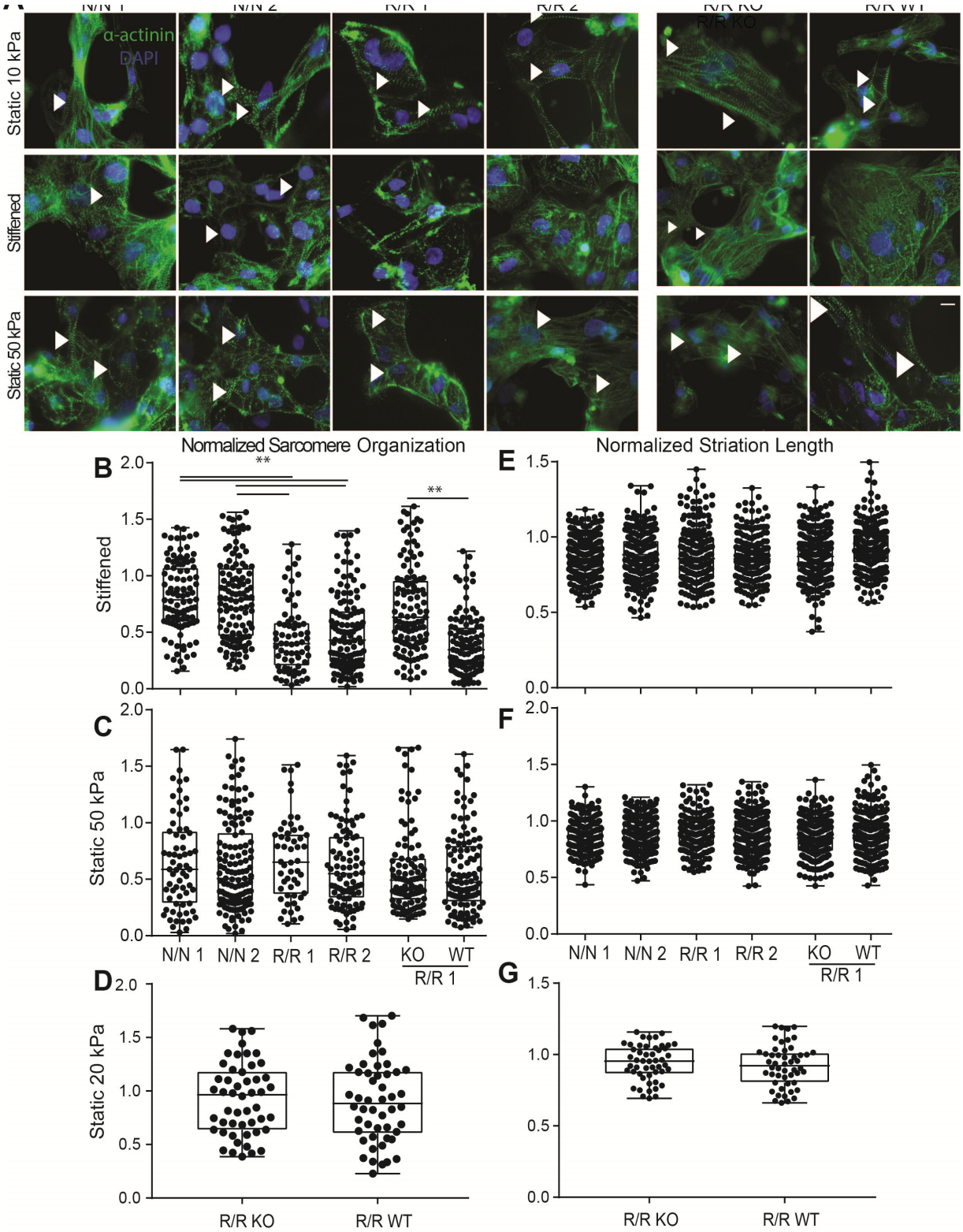

Figure S8: α-actinin Expression for Cardiomyocytes on the Different Substrates. (A) Immunofluorescent staining for α-actinin (green) and DAPI (blue) from the indicated patients

and genotypes cultured on soft, stiffened, and stiff MeHA substrates. Experiments were performed three independent times. **(B)** Sarcomere organization for cells on stiffened MeHA substrates was plotted. *$p < 0.0001$ for N/N vs R/R comparisons; one way ANOVA-Tukey. *$p < 0.0001$ for R/R 1 KO vs R/R 1 WT; two-tailed Student's t-test. N/N 1, n = 100 sarcomeres; N/N 2, n = 117 sarcomeres; R/R 1, n = 68 sarcomeres; R/R 2, n = 122 sarcomeres; R/R 1 KO, n = 115 sarcomeres; R/R 1 WT, n = 107 sarcomeres. **(C)** Sarcomere organization for cells on stiff 50 kPa MeHA substrates was plotted. N/N 1, n = 66 sarcomeres; N/N 2, n = 135 sarcomeres; R/R 1, n = 52 sarcomeres; R/R 2, n = 91 sarcomeres; R/R 1 KO, n = 97 sarcomeres; R/R 1 WT, n = 109 sarcomeres. **(D)** Sarcomere organization for cells on 20 kPa MeHA substrates was plotted (n = 50 sarcomeres). **(E)** Striation length for cells on stiffened MeHA substrates was plotted. N/N 1, n = 256 sarcomeres; N/N 2, n = 256 sarcomeres; R/R 1, n = 186 sarcomeres; R/R 2, n = 185 sarcomeres; R/R 1 KO, n = 248 sarcomeres; R/R 1 WT, n = 201 sarcomeres. **(F)** Striation length for cells on stiff 50 kPa MeHA substrates was plotted (n = 151-256 sarcomeres). N/N 1, n = 210 sarcomeres; N/N 2, n = 256 sarcomeres; R/R 1, n = 151 sarcomeres; R/R 2, n = 217 sarcomeres; R/R 1 KO, n = 230 sarcomeres; R/R 1 WT, n = 221 sarcomeres. **(G)** Striation length for cells on 20 kPa MeHA substrates was plotted (n = 50 sarcomeres).Values for stiffened and stiff substrates were normalized to those on soft substrates. Box plots show median, 25^{th} and 75^{th} percentiles, minimum, and maximum values for panels B-G.

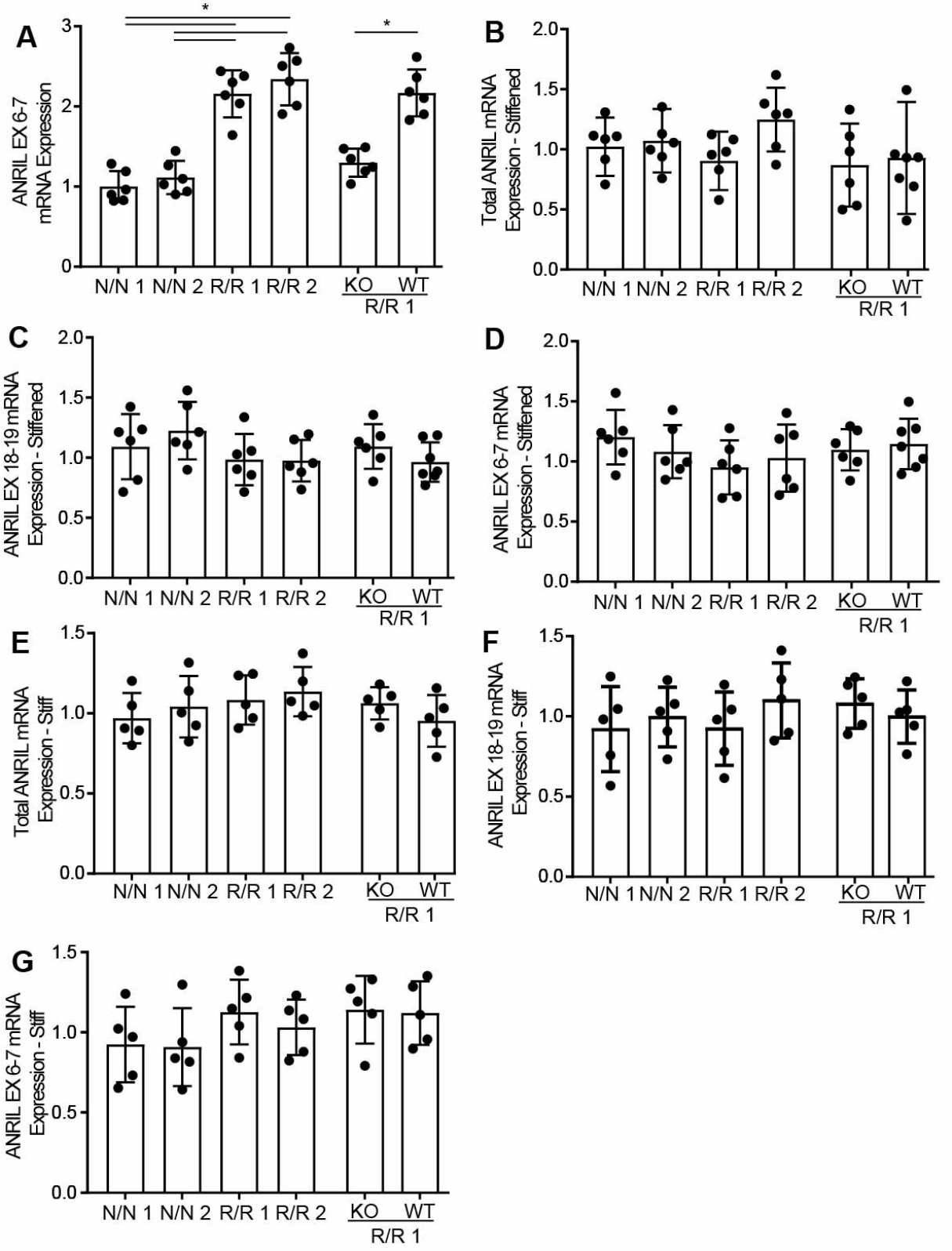
A
ANRIL EX 6-7 mRNA Expression
3
2
1
0
*
*
N/N 1
N/N 2
R/R 1
R/R 2
KO
WT
R/R 1
B
Total ANRIL mRNA Expression - Stiffened
2.0
1.5
1.0
0.5
0.0
C
ANRIL EX 18-19 mRNA Expression - Stiffened
D
ANRIL EX 6-7 mRNA Expression - Stiffened
E
Total ANRIL mRNA Expression - Stiff
F
ANRIL EX 18-19 mRNA Expression - Stiff
G
ANRIL EX 6-7 mRNA Expression - Stiff

Figure S9: Short *ANRIL* isoform expression and *ANRIL* expression on substrates with different stiffness. (A) mRNA transcript of exon 6-7 of ANRIL, measuring short *ANRIL* isoform expression (n = 6 samples). *p = 0.0001 for N/N 1 vs R/R 1 and *p = 0.0001 for N/N 1 vs R/R 2, *p = 0.0001 for N/N 2 vs R/R 1 and *p = 0.0001 for N/N 2 vs R/R 2; one way ANOVA-Tukey. *p = 0.0002 based on two-tailed Student's t-test. mRNA transcript expression for (B) total *ANRIL* (N/N 1, n = 6 samples; N/N 2, n = 6 samples; R/R 1, n = 6 samples; R/R 2, n = 6 samples; R/R 1 KO, n = 6 samples; R/R 1 WT, n = 7 samples), (C) long *ANRIL* isoforms (N/N 1, n = 6 samples; N/N 2, n = 6 samples; R/R 1, n = 6 samples; R/R 2, n = 6 samples; R/R 1 KO, n = 6 samples; R/R 1 WT, n = 7 samples), and (D) short *ANRIL* isoforms (N/N 1, n = 6 samples; N/N 2, n = 6 samples; R/R 1, n = 6 samples; R/R 2, n = 6 samples; R/R 1 KO, n = 6 samples; R/R 1 WT, n = 7 samples) on dynamically stiffened hydrogels after normalization to expression on static soft hydrogels is plotted. mRNA transcript for (E) total *ANRIL* (n = 5 samples), (F) long *ANRIL* isoforms (n = 5 samples), and (G) short *ANRIL* isoforms (n = 5 samples) on static stiff hydrogels after normalization to expression on static soft hydrogels is plotted; note that no statistically significant differences were observed in panels B-G. Mean±s.d. with individual points overlaid for all graphs.

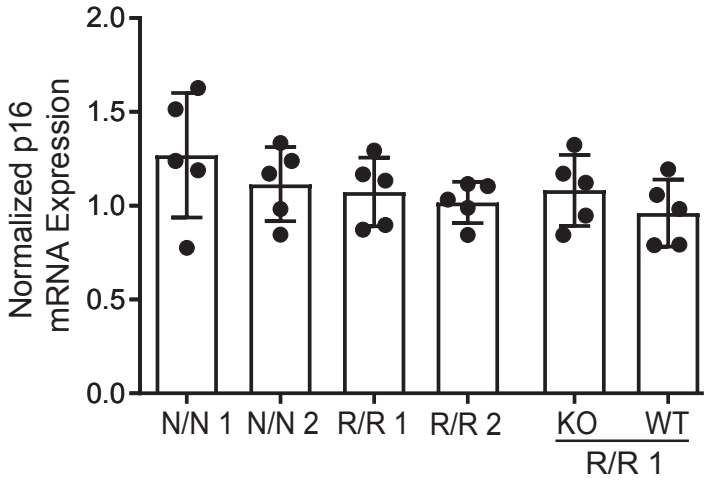

Figure S10: p16 Expression on Stiff Substrates. p16 mRNA transcript expression for cells on stiff substrates was plotted (n = 5 samples). Values were normalized to those on soft hydrogels. Mean±s.d. with individual points overlaid.

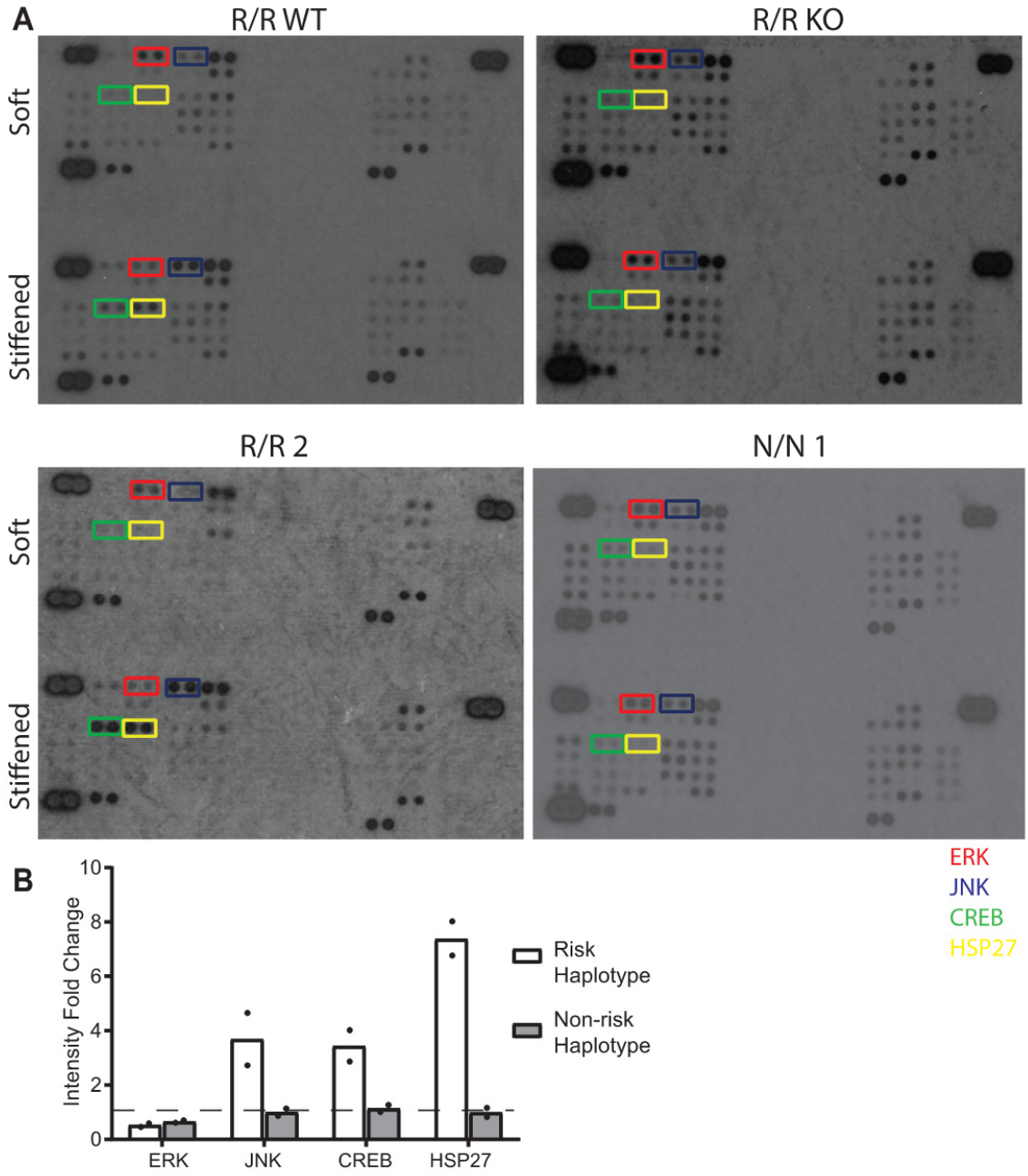

Figure S11: Phospho-kinase Array. (A) Kinase array demonstrating differences and similarities in phosphorylation changes in response to stiffening for R/R myocytes compared to N/N or R/R KO myocytes in the colored boxes. (B) Ratio of spot intensity for phosphorylated proteins in cells on stiffened matrix normalized to soft matrix (n = 2 arrays per haplotype). Indicated proteins represent phosphorylation events with the largest upregulation or downregulation, grouped based on the presence of the risk haplotype. Dashed line indicates a value of 1, i.e. no change as a function of stiffening. Individual points overlaid for panel B.

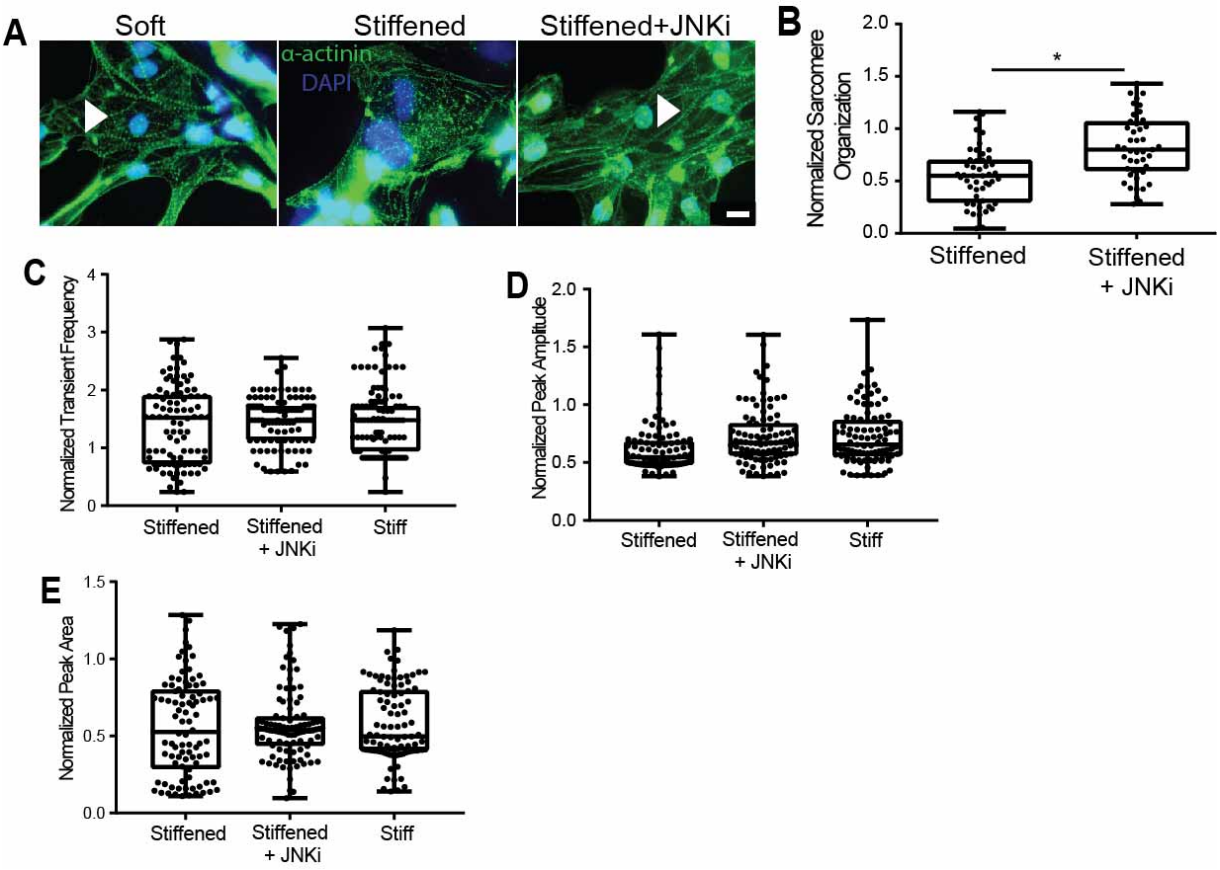

Figure S12: Effect of JNK Inhibition on α-actinin Organization and Ca^{2+} Handling. (A) Immunofluorescent staining for α-actinin (green) and DAPI (blue) for the given conditions. Experiments were performed three independent times. (B) Sarcomere organization was plotted. *p = 0.001; two-tailed Student's t-test. Stiffened, n = 49 sarcomeres; Stiffened+JNKi, n = 47 sarcomeres. (C) Frequency of spontaneous Ca^{2+} transients (Stiffened, n = 75 cells; Stiffened+JNKi, n = 82 cells; Stiff, n = 81 cells), (D) peak amplitude (Stiffened, n = 92 cells; Stiffened+JNKi, n = 93 cells; Stiff, n = 93 cells), and (E) peak area (Stiffened, n = 88 cells; Stiffened+JNKi, n = 98 cells; Stiff, n = 96 cells) were graphed. For all data that was normalized in these panels, values were divided by those on soft substrates to illustrate relative changes of JNK inhibition. Box plots show median, 25^{th} and 75^{th} percentiles, minimum, and maximum values for panels B-E.

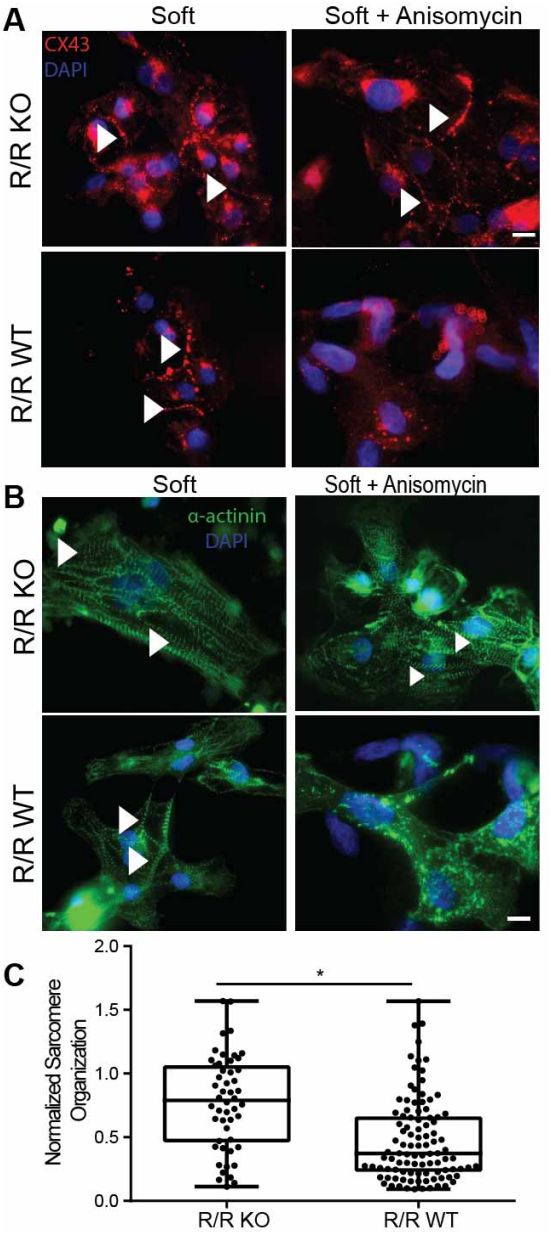

Figure S13: Connexin 43 and α-actinin Expression for R/R WT and KO Cardiomyocytes after Anisomycin Treatment. Immunofluorescent staining is shown for (A) connexin 43 (red) and (B) α-actinin (green) in R/R WT and KO cardiomyocytes; DAPI is shown in blue. Cells were selectively treated with anisomycin as indicated. Experiments were performed three independent times. (C) Sarcomere organization was plotted. *p = 0.00006; two-tailed Student's t-test. R/R KO, n = 50 sarcomeres; R/R WT, n = 100 sarcomeres. Box plot show median, 25th and 75th percentiles, minimum, and maximum values.

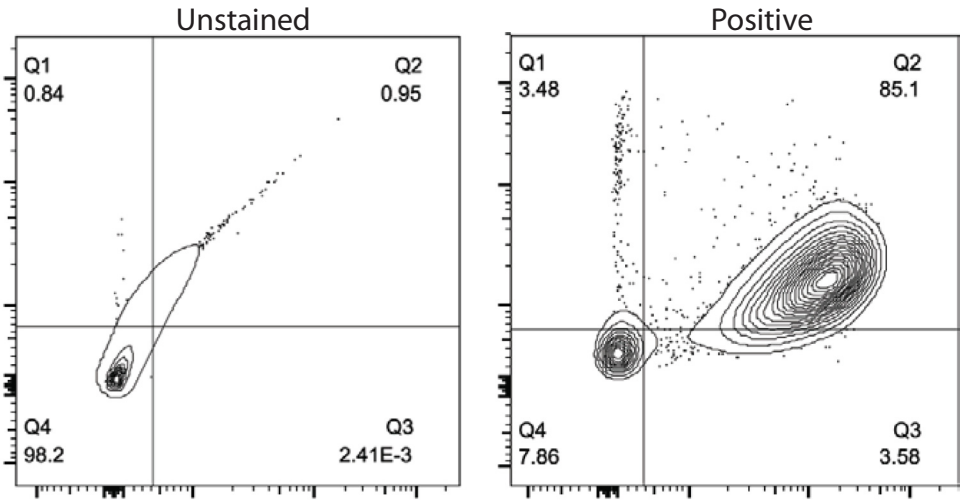

Figure S14: Gating Strategy Used for Flow Cytometry Analysis. Unstained control compared to positive control to demonstrate gating strategy.

Supplemental Tables

Dataset S1: (Separate file) Primers used for qPCR.

This table includes a list of primers for the indicated human genes.

Dataset S2: (Separate file) Raw data for figures where data was normalized.

This table has multiple tabs that indicate each figure's raw, un-normalized data. Each table notes the individual figure panel to which it corresponds.

Supplemental Movie Captions

Movie S1.

Representative calcium imaging of R/R cardiomyocytes cultured on soft hydrogels. Experiments were performed three independent times.

Movie S2.

Representative calcium imaging of N/N cardiomyocytes cultured on soft hydrogels. Experiments were performed three independent times.

Movie S3.

Representative calcium imaging of R/R cardiomyocytes cultured on stiffened hydrogels. Experiments were performed three independent times.

Movie S4.

Representative calcium imaging of N/N cardiomyocytes cultured on stiffened hydrogels. Experiments were performed three independent times.

Movie S5.

Representative calcium imaging of R/R KO cardiomyocytes cultured on soft hydrogels. Experiments were performed three independent times.

Movie S6.

Representative calcium imaging of R/R KO cardiomyocytes cultured on stiffened hydrogels. Experiments were performed three independent times.

REFERENCES

1. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent ste cells by modulating Wnt/beta-catenin signaling under fully defined conditions. *Nat. Protoc.* **8,** 162–175 (2013).
2. Kaushik, G., Fuhrmann, A., Cammarato, A. & Engler, A. J. In situ mechanical analysis of myofibrillar perturbation and aging on soft, bilayered Drosophila myocardium. *Biophys. J.* **101,** 2629–2637 (2011).
3. Hertz, H. Über die Berührung fester elastischer Körper. *J. für die reine und Angew. Math.* **92,** 156–171 (1881).
4. Hinson, J. T. *et al.* Titin Mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science.* **349,** 982–986 (2015).
5. Monteiro, A. *et al.* Deficient cMyBP-C protein expression during cardiomyocyte differentiation underlies human hypertrophic cardiomyopathy cellular phenotypes in disease specific human ES cell derived cardiomyocytes. *J. Mol. Cell. Cardiol.* **99,** 197–206 (2016).
6. El-Fouly, M., Trosko, J. E. & Chang, C. Scrape-loading and dye transfer. A rapid and simple technique to study gap junction intercellular communication. *Exp. Cell Res.* **168,** 422–430 (1987).