Supplemental Methods

Cell Isolation and Culture

Neonatal mouse ventricular cardiomyocyte isolation was performed in accordance with an Animal Use Protocol approved by the University of California, San Diego Institutional Animal Care and Use Committee. P1-P2 neonatal mice were sacrificed by decapitation, and the hearts excised and stored in a trypsin solution overnight at 4°C. Hearts were rinsed, then shaken at 37°C in collagenase and mixed well to digest. After arresting enzymatic activity with ice-cold media, cells were centrifuged and re-suspended in culture media, then incubated at 37°C for 90 minutes. Adherent fibroblasts were discarded, and the cardiomyocyte solution centrifuged and re-suspended for plating. Cells were plated in media based in high glucose DMEM without pyruvate, with 17% Medium 199, 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin and 50 U/ml streptomycin (Invitrogen). Cultures were maintained in a media variation with 5% horse serum and 1% fetal bovine serum, and switched to maintenance media without antibiotics at least 24 hours before optical mapping or patch clamp experiments.

Immunofluorescence Microscopy

Cells were fixed in -20°C methanol for 10 minutes, and blocked for 1 hour in 3% goat serum. ar comeric α-actinin (Sigma A7811, Invitrogen A11029) and connexin-43 (Sigma C6219, Dylight DI-1594) were labeled, and coverslips were mounted with mounting agent Slowfade Gold with DAPI (Life Technologies). Images of micropatterned cardiomyocyte morphology were taken on a Olympus IX81 FV1000 confocal microscope at 60x and 100x magnification (Supplemental Figure 1 and Supplemental Video) at the UCSD Light Microscopy Facility.

Cell Electron Microscopy

Cell electronmicrographs were performed on samples fixed after 3 days of culture, then prepared for electron microscopy by the Farquhar Lab Electron Microscopy Core, UCSD. Images were collected by Masa Hoshijima.

Supplemental Results

Micropatterned Neonatal Mouse Ventricular Myocytes are Confluent and Coupled, and Acute Stretch Increases Sarcomere Length

Confirming the strain applied by substrate stretch is transferred to the myocytes, sarcomere lengths measured in micropatterned mouse neonatal cardiomyocyte cultures increased from 1.8±0.02 μm to 2.0±0.02 μm with acute anisotropic substrate stretch. Immunofluorescent imaging of confluent micropatterned cultures 3 days after plating confirms longitudinal and transverse mechanoelectric coupling, with connexin-43 expression at cell-cell junctions (**Supplement Figure 1**).

LV Pressure Loading Smoothes Membrane Folds in Loaded Hearts

Electron microscopy revealed significant changes in gross membrane conformation as well as density and localization of caveolae with increased ventricular pressure load (**Supplement Fig. S2**). The ratio of membrane length to absolute length (ML/AL) was measured as a quantification of slack membrane folds (**Supplement Fig.s S2A** and **S2B**). In the pressure-loaded WT heart, the ML/AL ratio decreased from 1.140±0.019 in the unloaded state to 1.025±0.004 in the loaded state (N=20, P<0.001). In the WT heart

fixed after load had been applied and removed, the average ML/AL ratio was 1.162±0.022 (N=10, P=N.S. *vs.* the initial measurement). n MβCD-treated hearts, the average ML/AL ratio was 1.094±0.010 in the unloaded preparation (N=10, P=N.S. compared with WT) and was also significantly reduced in the loaded state (N=10, 1.046±0.009, P<0.01 *vs.* unloaded MβCD-treated). ML/AL was not significantly different between the unloaded Cav3 KO heart and the unloaded WT heart (N=10, 1.178±0.026, P=N.S.) but was significantly reduced in the pressure-loaded Cav3 KO heart (N=10, 1.051±0.008, P<0.001 *vs.* unloaded Cav3 KO).

Supplemental Figures

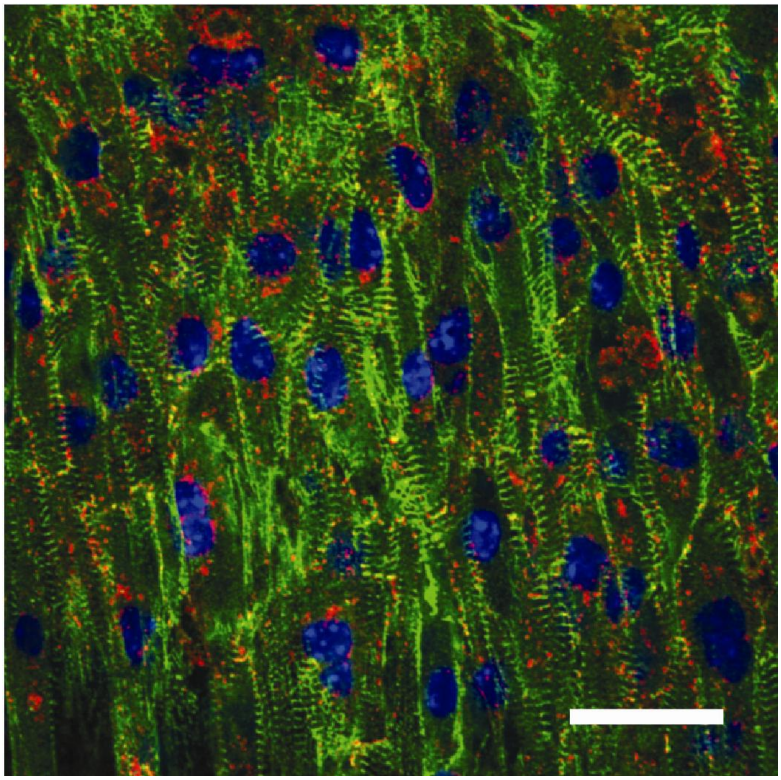


Figure S1: Immunofluorescently labeled NMVMs, cultured on micropatterned substrate for 3 days after isolation and imaged using a FV1000 microscope at 60x magnification. Green: α-actinin, red: connexin-43, blue: DAPI. Scale 30 μm.

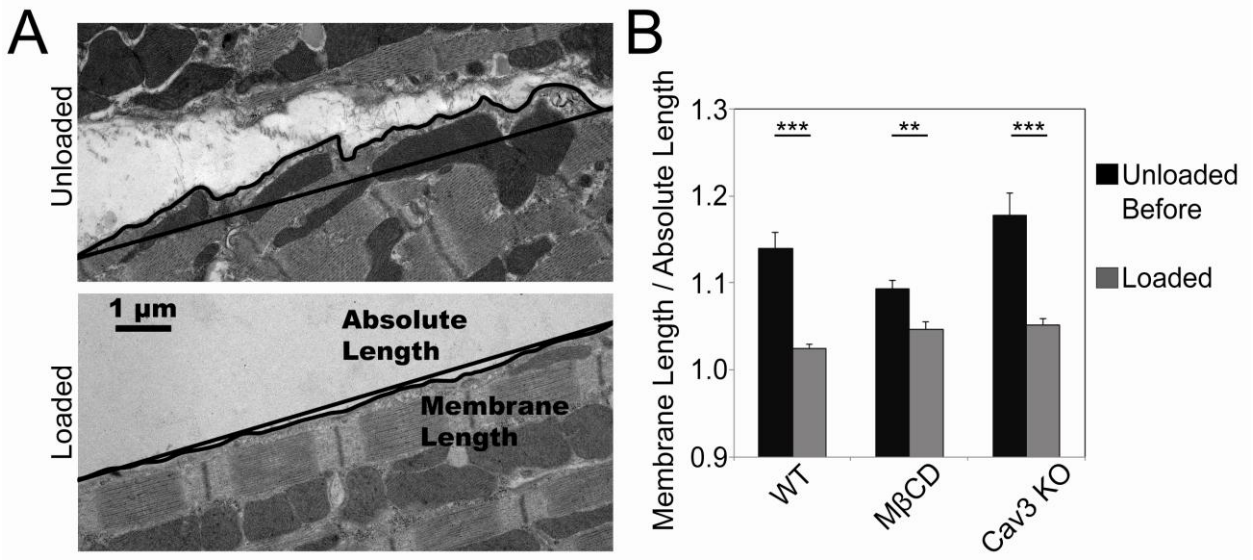


Figure S2: Stretch unfolds myocyte membrane in loaded hearts. (A) Excess folds in the sarcolemma of wild-type hearts are flattened during pressure load (lower micrograph) compared with the unloaded state (upper micrograph), as assessed by transmission electron microscopy (scale: 1 µm). (B) Membrane length-to-absolute length ratio was used to quantify slack membrane reduction in the loaded vs. unloaded WT ($P<0.001$), MβCD-treated ($P<0.01$), and Cav3 KO ($P<0.001$) hearts. (N=10 images per condition, comparison by unpaired t-test).

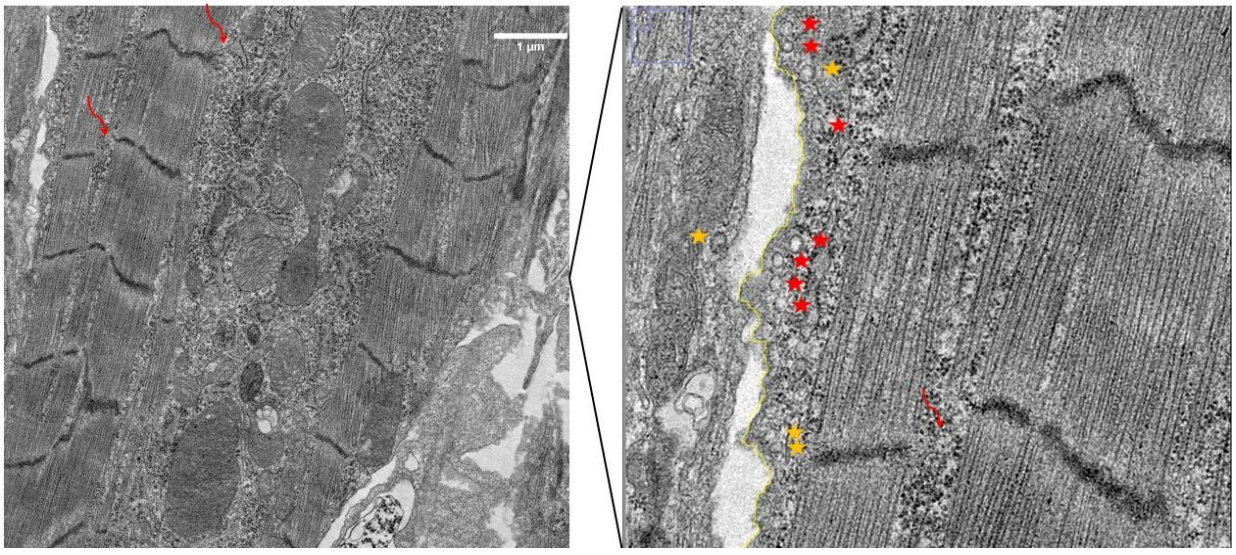


Figure S3: Electron micrograph of NMVM cultured on micropatterned PDMS for three days. Image enlarged on the right, with indications for sub-sarcolemmal (red stars) and integrated (yellow stars) caveolae, as well as structures resembling an immature t-tubule system (red arrows). Scale is 1 µm.

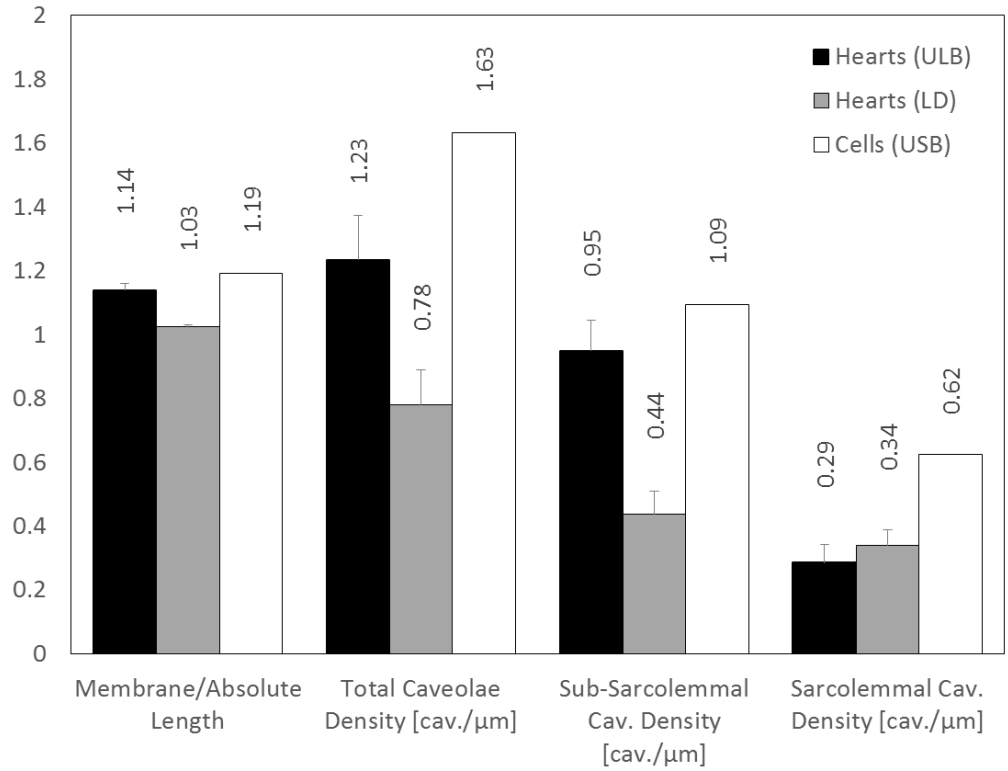


Figure S4: Micropatterned unstretched NMVM electronmicrograph measurements of membrane length per absolute length and caveolae density and distribution (N=1) are similar to those observed in unloaded adult mouse heart tissue (N=10 each, ULB and LD).

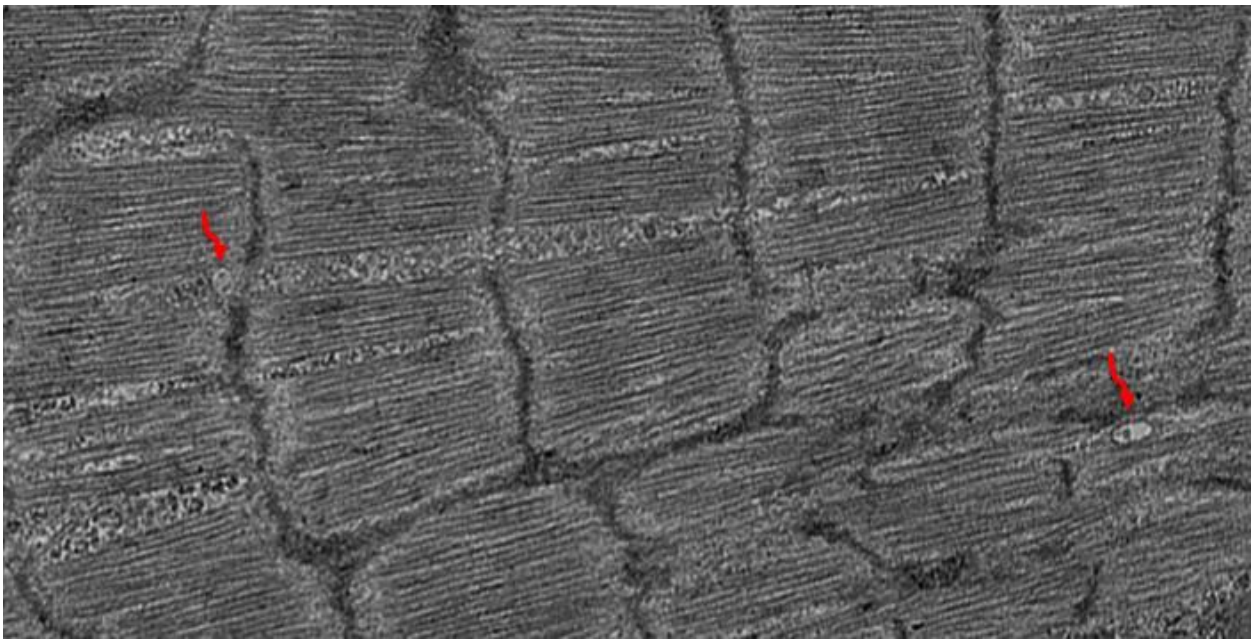
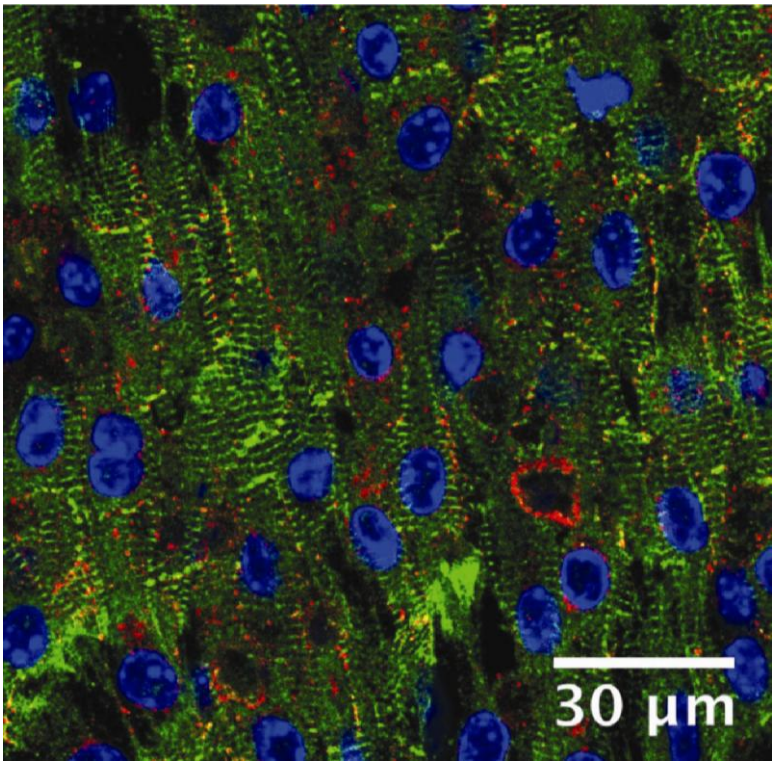


Figure S5: Electron micrograph of micropatterned cells, with arrows indicating structures resembling immature t-tubules.

Supplemental Video

Confocal stack of Cav3 KO NMVMs, fixed after three days in micropatterned culture and labeled identically to Figure S1. Imaged using a FV1000 microscope at 100x magnification. Single slice below:



Supplemental Tables

Table S1: Optical mapping data.

OPTICAL MAPPING																	
		Control				Gd3+				Cav3 KO				MβCD			
Hearts CVmax		ULB	LD	ULA	LD/ULB	ULB	LD	ULA	LD/ULB	ULB	LD	ULA	LD/ULB	ULB	LD	ULA	LD/ULB
[mm/s]	mean	653	529	678	0.81	458	340	478	0.73	591	572	604	0.97	691	692	684	1.00
	sem	16	21	17	0.05	45	55	69	0.09	21	30	47	0.04	16	42	18	0.05
	n	5				5				5				5			
		Control				GsMTx-4				Cav3 KO							
Cells CVmax		USB	ST	USA	ST/USB	USB	ST	USA	ST/USB	USB	ST	USA	ST/USB				
[mm/s]	mean	325	248	294	0.75	1017	840	922	0.81	503	553	549	1.14				
	sem	30	33	34	0.04	343	321	143	0.05	96	83	70	0.18				
	n	8				3				3							

Table S2: Electron microscopy, patch clamp, and lipophilic dye data.

ELECTRON MICROSCOPY									
		Control			Cav3 KO		MβCD		
		ULB	LD	ULA	ULB	LD	ULB	LD	
ML/AL									
	mean	1.14	1.03	1.16	1.18	1.05	1.09	1.05	
	sem	0.02	0.00	0.02	0.03	0.01	0.01	0.01	
n		10	10	10	10	10	10	10	
Total Caveolae									
[Cav./µm]	mean	1.23	0.78	1.27	0.12	0.10	0.42	0.17	
	sem	0.14	0.11	0.10	0.02	0.03	0.04	0.04	
Sub-Sarcolemmal Caveolae									
[Cav./µm]	mean	0.95	0.44	1.05	0.11	0.07	0.28	0.08	
	sem	0.09	0.07	0.09	0.01	0.02	0.03	0.02	
Sarcolemmal Caveolae									
[Cav./µm]	mean	0.29	0.34	0.22	0.01	0.04	0.14	0.08	
	sem	0.06	0.05	0.03	0.01	0.02	0.03	0.03	
n		10	10	10	10	10	10	10	
PATCH CLAMP									
		Control		GsMTx-4		Cav3 KO		MβCD	
Capacitance, Cm		USB	ST	USB	ST	USB	ST	USB	ST
[pF]	mean	21.9	43.4	16.0	39.9	13.7	16.3	12.1	24.9
	sem	2.9	8.2	2.1	4.4	1.5	2.9	0.8	3.9
Resistance, Rm									
[GΩ]	mean	1.0	2.3	1.9	1.6	1.2	1.6	1.7	1.2
	sem	0.3	0.8	0.2	0.6	0.4	0.8	0.2	0.2
Time Constant, Tm									
[ms]	mean	20.2	77.9	29.3	61.9	22.1	23.2	21.2	24.9
	sem	6.2	26.4	5.2	22.3	12.5	11.9	3.5	5.7
	n	15	15	11	8	13	12	18	22
LIPOPHILIC DYE									
		Control			Cav3 KO				
Fluo./Area		USB	ST	ST/USB	USB	ST	ST/USB		
[A.U.]	mean	302.52	556.32	1.84	1077.607	1060.779	0.98		
	sem	41.72	90.04	0.39	342.81	352.85	0.45		
	n	9	9	9	6	6	6		